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Myocarditis

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Meet the editor

Dr. Daniela Cihakova is an Assistant Professor and Director of Immunologic Disorders Laboratory at Department of Pathology, Johns Hopkins University in Baltimore, Maryland, United States. Dr. Cihakova was born in the Czech Republic and received her MD and PhD from Charles University, Prague, Czech Republic. Her research focuses on the pathogenesis of autoimmune diseases. In particular, she has concentrated on studying myocarditis and dilated cardiomyopathy using a mouse model of myocarditis called experimental autoimmune myocarditis. With her colleagues, she has recently discovered that Il13 is protective in myocarditis and that IL17A drives the development of dilated cardiomyopathy. She also works on examining the role of monocyte and macrophages during myocarditis pathogenesis. Her other research interests include Sjogren's syndrome, congenital complete heart block, autoimmune polyglandular syndrome type 1 (APECED) and vasculitis.

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Preface

Myocarditis is among the most common causes of non-congenital, non-ischemic sudden death in otherwise normal, healthy young adults. Myocarditis is heterogeneous disease, most patients recover spontaneously, but some forms of myocarditis are very serious and could be fatal. Additionally, some myocarditis patients develop dilated cardiomyopathy. This book provides a compendium of studies from leading international experts on various aspects of myocarditis. It consists of 20 chapters in three sections.

In the first section, we have included chapters providing clinical perspectives on myocarditis. Here, readers can find comprehensive reviews summarizing the causes of myocarditis, its classification, diagnosis and treatment. The part also includes a chapter that focuses on perimyocarditis and its causes, presentation and clinical management. It also includes a comprehensive review of Chagas' chronic myocarditis, another important type of myocarditis. We have also included a chapter discussing myocarditis in HIV positive patients, with an emphasis on imaging techniques in diagnosis, and a chapter on supporting treatment for myocardial ischemia using angiopoietin-1.

The second part of the book is dedicated to the pathogenesis of myocarditis. The chapters in this part also discuss some clinical findings, but mostly focus on the underlying mechanism of the disease, using in-depth data from mouse models. Some of the chapters focus on host immune response. For example, one chapter reviews the role of pattern-recognition receptors in myocarditis, and another chapter examines the role of lymphocyte effectors in myocarditis pathogenesis. Other chapters in this part provide in-depth the myocarditis pathogenesis from the perspective of damage induced by an infectious agent and discuss the pathways and mechanisms activated during the viral infection. The second part also includes a chapter discussing the link between exposure to tobacco smoke and viral myocarditis.

Finally, the third part focuses on promising topics for future research and clinical care. It discusses new findings in disease pathogenesis that could lead to new directions for clinical diagnosis and treatment of myocarditis. First chapter discusses biomarkers of heart failure in myocarditis and dilated cardiomyopathy. Another chapter is dedicated to identifying proteins that could be used as diagnostic markers or therapeutic targets.

Next chapter discusses how electroanatomical mapping may guide endomyocardial biopsy and increase sensitivity of the biopsy. It has been long suspected that acute myocarditis or other acute cardiac injury can trigger autoimmune reaction against some heart antigens, one of the chapters of this section discuss ongoing clinical study that is addressing this question. The final chapter focuses on new perspectives for myocarditis therapy.

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Part 1

Myocarditis from Clinical Perspective

1

Clinical Presentation

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1. Introduction

Myocarditis is a clinical syndrome characterized by inflammation of myocardium. It can be produced by a myriad of etiologies including infectious, autoimmune, myocardial toxins, hypersensitivity reactions and physical agents. Virtually any infectious agent can produce myocardial inflammation and injury. Human myocarditis is most frequently caused by viral infection. Ongoing viral infection, myocardial destruction, and adverse remodeling can lead to persistent ventricular dysfunction and dilated cardiomyopathy. The modern molecular techniques have facilitated new insights into inflammatory autoimmune processes that affect the myocardium and ultimately result in acute or chronic dilated cardiomyopathy.

The clinical manifestations are highly variable, ranging from asymptomatic electrocardiographic or echocardiographic abnormalities to acute myocardial infarction-like syndrome, overt congestive heart failure, fulminant condition with new atrial or ventricular arrhythmias or profound cardiogenic shock and death. Myocarditis is occasionally the unrecognized culprit in cases of sudden cardiac death. Autopsy series have reported much higher rates of myocarditis than expected with overt clinical manifestation from different etiological agents. The prospective postmortem data have implicated myocarditis in sudden cardiac death of young adults at rates of 8.6 percent to 12 percent (Doolan et al., 2004; Fabre & Sheppard, 2005). Furthermore, it has been identified as a cause of dilated cardiomyopathy in 9 percent of cases in a large prospective series (Felker et al., 1999).

The clinical history in patients presented with myocarditis remains essential to envelop a wide variety of etiologies in the clinical scenarios, many of which are infectious (Brodison & Swann, 1998). In the past 10 years, however, viruses, including adenovirus, parvovirus B19, hepatitis C, and herpes virus 6, have emerged as significant pathogens (Mahrholdt et al., 2006). The geographical distribution can be of relevance for some forms of myocarditis. In selected countries, Chagas disease, Lyme myocarditis, acute rheumatic fever and disorders associated with advanced human immune deficiency virus infection are significant causes. Other important infrequent clinicopathologic variants in the etiological spectrum are systemic disorders like giant cell myocarditis, cardiac sarcoidosis and eosinophilic myocarditis. Additionally, drugs, vaccinations, toxins, physical agents like radiation, heat stroke and hypothermia can be the key point for some rare clinical diagnoses. The physical examination in patients with myocarditis might be normal, but more severe cases frequently evident for significant physical findings. Although histological findings remains the gold standard for establishing the diagnosis of myocarditis, low risk patients are often given a

presumptive diagnosis if imaging studies and a compatible clinical scenario suggest new onset cardiomyopathy. The clinical manifestations of suspected patients with myocarditis will be discussed in details.

2. Clinicopathological classification

Over the past two decades, a great deal of confusion may be traced to the changing diagnostic criteria, multifaceted classifications, and varying patterns of infectious disease. The details of classifications will be discussed in other chapter in this book.

The morphologic criteria for the diagnosis of myocarditis by means of endomyocardial biopsy interpretation was proposed by the Dallas criteria authors in 1986 who defined myocarditis as a process characterized by the presence of an inflammatory cell infiltration of the myocardium with necrosis and/or degeneration of myocytes that is not typical of the myocardial damage of ischemic heart disease. The inflammatory cells are typically lymphocytic but may also include eosinophilic, neutrophilic, giant cells, granulomatous, or mixed cellularity infiltration. The amount of inflammation and its distribution may be mild, moderate, or severe and focal, confluent, or diffuse, respectively. A retrospective study of 112 consecutive patients with biopsy-confirmed myocarditis demonstrated, 55 percent lymphocytic; 22 percent borderline (inflammatory cellular infiltrate with no evidence of myocyte necrosis); 10 percent granulomatous; 6 percent giant cell and 6 percent eosinophilic form of myocarditis (Magnani et al., 2006). The viral etiology of myocarditis is thought to be the primary causative agents in most cases. However, a direct causative relationship still less well established in many clinical occasions. The majority of these cases are classified as lymphocytic myocarditis.

The Dallas criteria considered to be the first attempt to develop standardized histopathological description of biopsy samples from patients presented with myocarditis (Aretz et al., 1987). However, the histopathology alone can be inadequate to identify the presence of active myocarditis. Some clinicians feel that the definition is too narrow owing to the limitation by variable interpretation, lack of clinical prognostic values, and low sensitivity (Baughman, 2006). A combination of histopathologic characteristics and clinical criteria has been proposed in 1991 (Lieberman et al., 1991) as an alternative scheme to be utilized in the diagnosis of myocarditis. Histologic evidence of myocarditis was demonstrated in 35 of 348 patients submitted to endomyocardial biopsy over 5 years. Analysis of the histologic findings and clinical course of these patients resulted in a clinicopathologic classification of myocarditis in which four clinical subgroups are identified.

2.1 Fulminant myocarditis

The fulminant myocarditis is less frequent form of presentation. Patients presents with acute heart failure and cardiogenic shock up to two weeks after a distinct viral prodrome. They have severe cardiovascular compromise and may require mechanical circulatory support. Multiple foci of active myocarditis are typical. The histopathologic finding does not match the clinical phenotypic severity. The ventricular dysfunction often normalizes if patients survive the acute illness or results in death (McCarthy et al., 2000). In one series, 14 of 147 patients (10.2 percent) with clinical myocarditis presented in a fulminant fashion, with the triad of hemodynamic compromise, rapid onset of symptoms (usually within 2 weeks), and fever (McCarthy et al., 2000). On follow up, 93 percent of the original cohorts were alive and

transplant free 11 years following initial biopsy, compared with only 45 percent in those with more classic forms of acute myocarditis.

2.2 Acute myocarditis

Classically, patients with acute myocarditis presents with a less distinct onset of illness with nonspecific symptoms related to the heart. Viral prodrome occurs between 20 and 80 percent of the cases, can be readily missed by the patient, and thus cannot be relied upon for diagnosis. They present with an established ventricular dysfunction and may respond to immunosuppressive therapy or their condition may progress to dilated cardiomyopathy. In a series of 245 patients with clinically suspected myocarditis, the most common symptoms include fatigue (82 percent); dyspnea on exertion (81 percent); arrhythmias (55 percent, both supraventricular and ventricular); palpitations (49 percent); and chest pain at rest (26 percent), (Kuhl et al., 2005). The presentation can mimic acute coronary syndromes in view of troponin release, ST segment elevation on electrocardiogram, and segmental wall motion abnormalities on echocardiogram.

2.3 Chronic active myocarditis

This group of patients with chronic active myocarditis represents the majority of older adult with myocarditis. They are also presents with a less distinct onset of illness and often insidious with symptoms compatible with moderate ventricular dysfunction such as fatigue and dyspnea. Affected patients may initially respond to immunosuppressive therapy but often have clinical and histologic relapses and develop ventricular dysfunction associated with chronic inflammatory changes, and mild to moderate fibrosis on histological study including giant cells.

2.4 Chronic persistent myocarditis

This group of patients with chronic persistent myocarditis, whom also presents with a less distinct onset of illness, is characterized by a persistent histological infiltrate, often with foci of myocyte necrosis but without ventricular dysfunction, despite other cardiovascular symptoms such as chest pain or palpitation.

The previously depicted four forms are still used to describe the clinical presentation and progression of myocarditis, particularly in the absence of ongoing histological evaluation. These categories may also provide some prognostic information and may suggest which patients can or cannot benefit from immunosuppressive therapy.

A new diagnostic criteria derived from limited data was proposed in 2009. The Lake Louise Consensus Criteria utilizes the cardiac magnetic resonance imaging (CMR) for the diagnosis of myocarditis (Friedrich et al., 2009). The CMR enhances the ability to detect myocardial inflammation through noninvasive means, as well as to improve diagnostic accuracy. In these criteria, four major domains are considered when making the diagnosis including, clinical presentation compatible with myocarditis, evidence of new or recent onset myocardial damage, increased T2 signal or delayed enhancement on CMR (compatible with myocardial edema and inflammation), and endomyocardial biopsy evidence of myocardial inflammation. The use of CMR appears suitable to identify patients with significant ongoing inflammation, which may be especially important for patients with recurrent or persisting symptoms and in patients with new onset heart failure. The awareness came out that the

recommendations proposed by these criteria are based on limited data and that not all centers will be able to apply all components of the suggested protocol.

3. Clinical manifestation

Myocarditis is synonymous with inflammatory cardiomyopathy which has a wide range of clinical presentation, from subtle to devastating clinical scenario that contributes to the difficulties in diagnosis and classification of this disorder. There are few population-based, epidemiologic studies which have defined the presenting symptoms of acute myocarditis; this is due to the absence of a safe and sensitive noninvasive test that can confirm the diagnosis. Worldwide, the true frequency of disease in its less severe forms, whether clinical or subclinical, across various age segments of the population are more difficult to appreciate. Table 1 summarized the most significant clinical manifestations and physical findings in patients presented with myocarditis. Typically myocarditis has a bimodal age distribution in the general population, with the acute presentation more commonly seen in young children and teenagers. In contrast, the presenting symptoms are more subtle and insidious, often with dilated cardiomyopathy and heart failure in the older adult population. Most studies of acute myocarditis reported a slight preponderance in male patients (Caforio et al., 2007). The male-to-female ratio is 1.5 to 1, which may be related to a protective effect of natural hormone variations on immune responses in women (Schwartz et al., 2004). The variable clinical manifestation of myocarditis in part reflects the variability in histological disease severity. Myocardial inflammation may be focal or diffuse, involving any or all cardiac chambers. Severe, diffuse myocarditis can result in a clinical manifestation of acute dilated cardiomyopathy.

Many patients with myocarditis present with a nonspecific illness characterized by fatigue, mild dyspnea, and myalgias. Most cases of viral myocarditis are subclinical; therefore, the patient infrequently seeks medical attention during acute illness. These subclinical cases may have transient electrocardiographic abnormalities. The reported antecedent infectious viral syndrome is highly variable, ranging from 10 percent to 80 percent of patients with viral myocarditis (Baboonian & Treasure, 1997; Feldman & McNamara, 2000; Imazio et al., 2008; Mason et al., 1995). The appearance of cardiac specific symptoms occurs primarily in the subacute virus clearing phase; therefore, patients commonly present two weeks after the acute viremia.

A few patients present acutely with fulminant congestive heart failure secondary to widespread myocardial involvement. Animal models have lead to a much greater understanding of the fulminant clinical course of myocarditis in which a rapid progression, severe ventricular dysfunction and cardiovascular collapse occurs (Ellis & Di Salvo, 2007). Fulminant myocarditis, manifested by severe hemodynamic compromise requiring high dose vasopressor support or mechanical circulatory support, was identified in 15 of 147 patients (10.2 percent) in a large prospective study (McCarthy et al., 2000). Fulminant cases were additionally characterized by a distinct viral prodrome, fever, and abrupt onset (generally <3 days) of advanced heart failure symptoms. These patients typically have severe global left ventricular dysfunction and minimally increased left ventricular end-diastolic dimensions. Of note, either borderline or active lymphocytic myocarditis can produce this dramatic clinical presentation. The histological features of chronic myocarditis are usually produced a more subtle clinical course. Adults may present with heart failure years after initial index event of myocarditis.

Clinical manifestations

1. Subclinical presentation (most cases of viral myocarditis)
2. Nonspecific symptoms (e.g fatigue, arthralgias and myalgias)
3. Clinical presentation, including :
   - Shortness of breath, orthopnea or paroxysmal nocturnal dyspnea
   - Pedal edema
   - Chest pain (concomitant pericarditis)
   - Palpitation (arrhythmias)
   - Presyncope or syncope (atrioventricular block)
   - Sudden cardiac death (dysrhythmic death)
   - Fever
   - Flu-like syndrome (e.g pharyngitis or tonsillitis)
   - Thromboembolic symptoms (systemic or pulmonary)

Physical findings

1. Normal or unremarkable findings
2. Relevant physical signs, including :
   - Tachypnea
   - Cyanosis
   - Elevated jugular venous pressure
   - Tachycardia
   - Signs of cardiovascular collapse and shock
   - Diffuse apex beat and laterally displaced (cardiomegaly)
   - Diminished intensity of first heart sound
   - Third and fourth heart sound summation gallops
   - Murmurs of mitral or tricuspid valves regurgitation
   - Pericardial friction rub and effusion (concomitant myopericarditis)
   - Bibasilar crackles
   - Hepatomegaly
   - Ascites
   - Peripheral edema

Table 1. The most significant clinical manifestations and physical findings in patient with myocarditis

The medical history may embrace a number of hints that merits an emphasis. Previous history of rheumatic heart disease or symptoms defined by Jones criteria e.g. fever or arthralgia can be a clue for the clinical diagnosis acute rheumatic fever. History of tick bite may correlate with suspected Lyme disease. Patients treated for neoplastic disorders with chemotherapeutic agents like doxorubicin may draw attention to anthracyclines-induced myocarditis. History of travel to Central or South America can be a clue for the diagnosis of Chagas disease. Additionally, giant-cell myocarditis should be considered in patients with acute dilated cardiomyopathy associated with thymoma, autoimmune disorders, ventricular tachycardia, or high-grade heart block. Furthermore, an unusual cause of myocarditis, such as cardiac sarcoidosis, should be suspected in patients who present with chronic heart

failure, dilated cardiomyopathy and new ventricular arrhythmias, or second-degree or third degree heart block or who do not have a response to standard care (Yazaki et al., 1998).
In the European Study of the Epidemiology and Treatment of Inflammatory Heart Disease, a 3055 patients with suspected acute or chronic myocarditis were screened, of them 72 percent had dyspnea, 32 percent had chest pain, and 18 percent had arrhythmias (Hufnagel et al., 2000). The most important clinical manifestations in patients with myocarditis are as followed:

3.1 Shortness of breath

Dyspnea on exertion and fatigue are common. A history of shortness of breath at rest, orthopnea, pedal edema or paroxysmal nocturnal dyspnea is suggestive of congestive heart failure. Dyspnea is due to left ventricular dysfunction causes elevated left ventricular diastolic pressure and low cardiac output.

3.2 Chest pain

Chest pain is usually associated with concomitant pericarditis. Chest discomfort is reported in one third of patients. The pain is most commonly described as a pleuritic, sharp, stabbing precordial pain. It may be substernal and squeezing and, therefore, difficult to distinguish from that typical of ischemic pain. However, myocarditis can be masquerading as an acute coronary syndrome both clinically and on the electrocardiogram, particularly in younger patients (Angelini et al., 2000). In one series of 34 patients with known normal coronary anatomy presenting with symptoms and electrocardiographic changes consistent with an acute coronary syndrome, 11 (32 percent) of the patients were found to have myocarditis on biopsy (Dec et al., 1992). Sarda et al., using myocardial indium111-labeled antimyosin antibody and rest thallium imaging, identified 35 of 45 patients (78 percent) who presented with acute chest pain, ischemic electrocardiographic abnormalities, and elevated cardiac biomarkers as having diffuse or focal myocarditis. However biopsy verification of actual myocarditis was not undertaken in this series. Complete recovery of left ventricular function occurred at six months in 81 percent of these patients (Sarda et al., 2001). Some presentations of myocarditis, especially those related to parvovirus B19, present like an acute lateral wall myocardial infarction. The ischemia associated with myocarditis may be due to localized inflammation or occasionally, due to coronary artery spasm (McCully et al,. 2005). It is essential for clinicians to consider acute myocarditis in younger patients who present with acute coronary syndromes when coronary risk factors are absent, electrocardiographic abnormalities extend beyond a single coronary artery territory or global rather than segmental left ventricular dysfunction is evident on echocardiography.

3.3 Palpitation, presyncope or syncope

Palpitation is a common presentation in patient with myocarditis. Presyncope or syncope in a patient with a presentation consistent with myocarditis may be a signal for high-grade atrioventricular block and risk for sudden death. Small focal inflammation in electrically sensitive areas may be the etiology of patients whose initial presentation is sudden death.

3.4 Fever

Fever with or without sweats and chills occur in 20 percent of patients presenting with myocarditis. A history of fever or flu-like syndrome in form of pharyngitis, tonsillitis, or

upper respiratory tract infection before admission occurs in 50 percent of patients (Imazio et al., 2008).

3.5 Other symptoms

Apart from the nonspecific symptoms recognized like malaise, myalgias and arthralgias, other extracardiac symptoms may identify infectious, toxic agents or autoimmune diseases affecting the heart and resulting in a myocarditis. A viral prodrome of fever, myalgias, and muscle tenderness may precede viral myocarditis, while a delayed hypersensitivity reaction may be first apparent from a cutaneous rash. Rash, fever, peripheral eosinophilia, or a temporal relation with recently initiated medications or the use of multiple medications suggest a possibility of hypersensitivity myocarditis.

The clinical diagnosis of myocarditis is challenging due to its varying presentation, nonspecific symptoms and physical findings. Accordingly, a high level of clinical suspicion is warranted and a presumptive diagnosis usually made based on patient demographics and the clinical course.

4. Physical examination

The physical examination of patient presented with myocarditis is frequently normal. Mild cases of patients with myocarditis may appear to have a simple viral syndrome. More acutely ill patients with acute myocarditis have the classic signs of circulatory impairment due to congestive heart failure. Patients may shows signs of fluid overload including elevated jugular venous pressure, bibasilar crackles, hepatomegaly, ascites and peripheral edema. More severe cases may show cardiovascular collapse and signs of shock. In addition to the signs of fluid overload, the physical examination may reveal direct evidence of cardiovascular signs in symptomatic patients. Tachypnea and tachycardia are common. Tachycardia is often out of proportion to fever. Cyanosis may occur as well. The apex beat may be diffuse and laterally displaced suggesting cardiomegaly. Heart auscultation may reveal diminished intensity of first heart sound. The third and occasionally fourth heart sound summation gallops may be noted with impaired ventricular function, particularly when biventricular acute myocardial involvement results in systemic and pulmonary congestion. If the right or left ventricular dilatation is severe, auscultation may reveal murmurs of mitral or tricuspid valves regurgitation. Table 1 summarized the most significant clinical manifestations and physical findings in patients presented with myocarditis.

A pericardial friction rub and effusion may become evident in some patients with diffuse inflammation as a result of myopericarditis. Pericardial tamponade was reported in very rare occasions. Pleural friction rub may develop as the inflammatory process involves surrounding structures. In cases where a dilated cardiomyopathy has developed, signs of peripheral or pulmonary thromboembolism may be encountered. Certain physical findings may imply a specific cause of myocarditis. Enlarged lymph nodes might suggest systemic sarcoidosis. A pruritic, maculopapular rash may suggest a hypersensitivity reaction, often to a drug or toxin. Acute rheumatic fever can present with the modified Jones criteria.

5. Electrocardiogram findings

Generally, the Electrocardiogram (ECG) is a sensitive means in myocarditis. However, its diagnostic value is limited by the low specificity and a wide diversity of changes which

observed during the course of disease. The ECG must be timely repeated, since minor abnormalities detected initially may become subsequently more apparent.
The ECG findings associated with myocarditis may include first, second or third degree atrioventricular block, intraventricular conduction delay (widened QRS complex), bundle branch or fascicle block, reduced R wave height, abnormal Q waves, ST-T segment changes or low voltage. In one report, either ST-segment elevation or T-wave inversion is present as the most sensitive ECG criterion in <50% of patients, even during the first weeks of the disease (Morgera et al., 1992). A gradual increase in the width of the QRS complex may be a sign of exacerbation of myocarditis. Frequent premature beats, supraventricular tachycardia and atrial fibrillation may arise as well. Arrhythmias such as sinus arrest, ventricular tachycardia, ventricular fibrillation or asystole may occur and threaten the life of patients with myocarditis. Hence, continuous ECG monitoring is crucial to detect potentially fatal arrhythmias.

6. Clinical manifestation of complications

Despite the fact that a substantial number of myocarditis are never coming to medical attention, a less frequent form of myocarditis is fulminant and leads rapidly to cardiovascular collapse and shock that required mechanical ventilation support. In contradiction, if these patients survive the first 3-4 weeks of illness they have almost complete recovery and far fewer long term complications compared with those patients with more indolent courses (Chau et al., 2006; Khabbaz et al., 2007). Generally, there are a number of well recognized complications that may encounter in the variety of clinical scenarios of patients with myocarditis.

6.1 Congestive heart failure

In many patients who develop heart failure, fatigue and decreased exercise capacity are the initial manifestations. However, diffuse, severe myocarditis, if rapid in evolution, can result in acute myocardial failure and cardiogenic shock. Signs of right ventricular failure include increased jugular venous pressure, hepatomegaly, and peripheral edema may supervene. The decline in right ventricular function "protects" the left side of the circulation so that signs of left ventricular failure may not be seen. If, however, there is predominant left ventricular involvement, the patient may present with the symptoms of pulmonary congestion including dyspnea, orthopnea, pulmonary crackles, and, in severe cases, acute pulmonary edema. Patients with persistent viral genome expression show limited recovery of left ventricular function, decreased stroke volume index and more stiffness of the ventricle with the resultant long-term morbidity of heart failure and a mortality of nearly 25 percent (Fuse et al., 2000).

6.2 Arrhythmias

A number of arrhythmias may be seen during the clinical course of myocarditis. Sinus tachycardia is more frequent than serious atrial or ventricular arrhythmias, while palpitations secondary to premature atrial or, more often, ventricular premature complexes are common. Ventricular arrhythmias and variable heart blocks are uncommon, but well recognized clinical presentations (Hosenpud et al., 1986; Marboe & Fenoglio et al., 1988). Persistent complex ventricular arrhythmias after apparent resolution of myocarditis were reported in children and young adults as well (Friedman et al., 1994).

Several series have examined the frequency of myocarditis among patients evaluated for life threatening ventricular arrhythmias that occurred in the absence of structural heart disease (Strain et al., 1983; Sugrue et al., 1984; Vignola et al., 1984). These patients tended to be young (younger than 50 years) and to have normal or near-normal left ventricular systolic function. The frequency of syncope or cardiac arrest as reported has ranged from 8 percent to 61 percent (Strain et al., 1983; Sugrue et al., 1984). Biopsy evidence of myocarditis among patients without structural heart disease has ranged from 8 percent to 50 percent. On the other hand, patients with ventricular arrhythmias due to lymphocytic or granulomatous myocarditis stay at higher risk. Sustained ventricular tachycardia or new heart block in the setting of rapidly progressive congestive heart failure suggests giant cell myocarditis. Granulomatous myocarditis has been associated more frequently with life threatening ventricular arrhythmias, syncope, and high-grade atrioventricular block requiring temporary or permanent ventricular pacing than has lymphocytic myocarditis (Davidoff et al., 1991; Fleming & Bailey, 1981; Sekiguchi et al., 1996). Furthermore, granulomatous myocarditis might be suspected in patients who present with apparently chronic dilated cardiomyopathy yet with new ventricular arrhythmias or heart block or who do not have a response to optimal care (Yazaki et al., 1998).

6.3 Sudden cardiac death

The risk of sudden dysrhythmic death in patients with myocarditis is increasingly appreciated in the current morbidity and mortality data. The discovery of myocarditis in 1 to 9 percent of routine postmortem examinations suggests that myocarditis is a major cause of sudden, unexpected death (Feldman & McNamara, 2000).

Although heart failure and cardiomyopathy are more common clinical presentations, patients with myocarditis may present with syncope or unexpected sudden cardiac death, presumably due to ventricular tachycardia or fibrillation (Drory et al., 1991; Eckart et al., 2004; Maron et al., 2003; Theleman et al., 2001). Myocarditis is a significant cause of sudden, unexpected death in adults younger than age 40 years and elite young athletes. In these presumably healthy individuals, autopsy findings have revealed myocarditis in up to 20 percent of cases (Wesslen et al., 1996). In an autopsy series of patients under age 40 who presented with sudden death in the absence of known heart disease, myocarditis was responsible for 22 percent of cases under age 30 and 11 percent in older subjects (Drory et al., 1991). In another autopsy study of sudden death occurring in 1866competitive athletes, myocarditis was present in 6 percent of the cardiovascular deaths (Maron et al., 2009). In one more series of autopsies in military recruits, myocarditis accounted for 20 percent of deaths due to identifiable structural cardiac abnormalities (Eckart et al., 2004).

6.4 Dilated cardiomyopathy

A substantial subset of symptomatic cases of postviral or lymphocytic myocarditis present with a syndrome of heart failure and dilated cardiomyopathy. A clinical and pathologic syndrome that is similar to dilated cardiomyopathy (DCM) may develop after resolution of viral myocarditis in animal models and biopsy proven myocarditis in human subjects (Gilbert & Mason, 1987). This has led to speculation that DCM may develop in some individuals as a result of subclinical viral myocarditis. Theoretically, an episode of myocarditis could initiate a variety of autoimmune reactions that injure the myocardium and ultimately result in the development of DCM. These abnormalities in immune

regulation and the variety of antimyocardial antibodies present in DCM are consistent with this hypothesis. Enteroviral RNA sequences may be found in heart biopsy samples in DCM but with a very variable frequency (0–30 percent), (Bowles et al., 1986; Giacca et al., 1994). Furthermore, analysis of human viruses other than enteroviruses suggests that adenoviruses, herpes, and cytomegalovirus can also cause myocarditis and potentially DCM, particularly in children and young subjects (Martin et al., 1994; Pauschinger et al., 1999).

In most acute cases of lymphocytic myocarditis, left ventricular function improves over one to six months with standard heart failure care. However a substantial minority will develop a persistent inflammation that leads to chronic cardiomyopathy. In the patients who develop chronic cardiomyopathy, the risk of heart transplantation and death is high. In a large review of 1230 cases of initially unexplained cardiomyopathy, 9 percent were thought to be due to myocarditis (Felker et al., 2000). A similar prevalence of 10 percent was noted in the Myocarditis Treatment Trial in which endomyocardial biopsy was performed in over 2200 patients with unexplained heart failure of less than two years duration (Mason et al., 1995).

6.5 Thromboembolism

Thromboembolism, arterial and venous, is more evident in patients with left ventricular dysfunction, and appears to be quite frequent complication in certain forms of myocarditis and cardiomyopathies. Additionally, the risk of thromboembolism from either tissue or thrombus from the biopsy site is higher in left ventricular biopsy. Right-sided thromboembolism can be due to thrombus from the venous access sheath especially with the internal jugular approach. The possibility of some small added diagnostic yield by taking biopsy samples of the left ventricle in addition to the right is outweighed by the small attendant risk of systemic embolism.

Thromboembolism is frequent in advanced Chagas disease, and its occurrence is probably underestimated (Bestetti, 2000; Samuel et al., 1983). At autopsy, 73 percent of patients have left or right ventricular mural thrombi, with evidence of pulmonary or systemic embolization in 60 percent (Arteaga-Fernandez et al., 1989). The apical aneurysm typical of Chagas disease is particularly prone to the formation of thrombi and is associated with a high incidence of thromboembolic events (Fernandes et al., 1987). Furthermore, there is a high incidence of thromboembolism in population with peripartum cardiomyopathy. Thrombi are the result of the hypercoagulable state of pregnancy and of stasis and turbulent flow in the dilated heart. Thrombi often form in patients with lower left ventricular ejection fraction (<35 percent), (Amos et al., 2006; Sliwa et al., 2006). Higher mortality rates have been reported to be due to thromboembolism as well (Ford et al., 2000).

6.6 Recurrent myocarditis

The clinical course of myocarditis in the majority of patients is self-limited and there is complete resolution of myocardial inflammation without further relapse or sequelae. However, the disease has been observed to recur in a similar scenario to initial presentation, which then may resolve spontaneously or be associated with heart failure, arrhythmias, or death. Chronic myocarditis may be considered to be one of the mechanisms of the process of recurrence. Recurrence was reported to in 10 to 25 percent of patients after apparent resolution of the initial illness (Daly et al., 1984; Dec et al., 1985). Recurrence of myocarditis

is well recognized in patients with acute rheumatic fever. It is also demonstrated in subsequent pregnancies after peripartum cardiomyopathy and recurrence should be suspected if ventricular function subsequently deteriorates (Dec et al., 1985). Women should be counseled to avoid pregnancy after a diagnosis of peripartum cardiomyopathy. Recurrence was also described in giant cell myocarditis in transplanted heart which responded to intensive immunosuppression. History of third time recurrences of active myocarditis proven by endomyocardial biopsy associated with complete atrioventricular block was described as well and viral studies showed no evidence of recent infection (Kanazawa et al., 2004). Another report present recurrent viral myocarditis and vaccine-associated myocarditis in a single patient with complete reversal of the cardiomyopathy and return to normal cardiac function (Makaryus et al., 2006). Moreover, some cases were observed to have recurrent myocarditis after tapering of immunosuppressive therapy and previous biopsy specimens showing healed myocarditis.
One report indicated that pericarditis on initial presentation may be associated with a higher rate of recurrence of myocarditis (Fowler et al., 1973). However, in reality, there are no reliable predictors that identify patients likely to have recurrence.

7. Manifestations of specific forms of myocarditis

Specific clinical forms of myocarditis of variable etiologies will be described below. Table 2 summarized some key clinical hints among specific forms of myocarditis that help with the clinical diagnosis

7.1 Viral myocarditis

Amongst the multiple infectious etiologies which have been implicated as the cause of clinically significant acute myocarditis, viral myocarditis being the most common and the enterovirus coxsackie B being the most significant. Numerous seroepidemiologic and molecular studies were linked coxsackievirus B to outbreaks of myocarditis occurred before the 1990s. The spectrum of viruses that were detected in endomyocardial biopsy samples shifted from coxsackievirus B to adenovirus in the late 1990s. In the last decade a number of reports implicate new viruses in the etiology of myocarditis and dilated cardiomyopathy. The parvovirus B19 was identified in patients with myocarditis in Germany (Kühl et al., 2005; Mahrholdt et al., 2006) and hepatitis C virus was reported in Japan (Matsumori 2005, 2006) as well.
Early studies suggested that cardiac involvement occurred in 3.5 to 5 percent of patients during outbreaks of coxsackievirus infection (Gerzen et al., 1972; Grist & Bell, 1969). Most cases of enteroviral myocarditis or pericarditis occur in children and young adults in whom more than two-thirds are male. In the majority of patients, active myocarditis remains unsuspected because the subclinical and self-limited pattern of presentation or the presence of myocarditis may infer only by the finding of transient electrocardiographic ST-T-wave abnormalities. In addition, subtle cardiac symptoms and signs may be overshadowed by the systemic manifestations of the underlying infection or disease process. Clinically, the patients give a history of a preceding upper respiratory febrile illness or a flu-like syndrome, and viral nasopharyngitis or tonsillitis may be evident. In the United States Myocarditis Treatment Trial, 89 percent of subjects reported a syndrome consistent with a viral prodrome (Mason et al., 1995). The patient may also have fever, myalgias, and muscle tenderness that is followed by chest pain, dyspnea or arrhythmias, and occasionally heart

failure. A pericardial friction rub is documented in half of cases, and the electrocardiogram shows ST segment elevations or ST- and T-wave abnormalities. Most adults recover completely and only a minority of cases progress to chronic dilated cardiomyopathy.

Clinical clues	Clinical diagnosis	Comments
Preceding upper respiratory febrile or flu-like illness (viral nasopharyngitis or tonsillitis)	Viral myocarditis	Often self-limited
Patients present with chronic heart failure, dilated cardiomyopathy and new arrhythmias or heart block with no response to standard care	Sarcoid myocarditis	Enlarged lymph nodes suggest systemic sarcoidosis
Cutaneous rash (pruritic, maculopapular), fever, peripheral eosinophilia or a temporal relation with recently initiated medications or the use of multiple medications	Hypersensitive/ eosinophilic myocarditis	
Patients treated anti-neoplastic chemotherapeutic agents	Anthracyclines-induced myocarditis	
History of travel to Central or South America, Systemic or pulmonary thromboembolism	Chagas disease	The apical aneurysm is typical in advanced disease
History of residence or travel through the endemic area; previous tick bites; prior or current erythema migrans lesions and coexistence of neurologic dysfunction	Lyme disease	Varying degrees of atrioventricular conduction block is common
Previous history of rheumatic heart disease or symptoms defined by Jones criteria e.g. erythema marginatum, polyarthralgia, chorea, subcutaneous nodules fever or arthralgia	Acute rheumatic fever	
Heart failure developing in the last month of pregnancy or within 5 months following delivery	Peripartum cardiomyopathy	Higher incidence of thromboembolism (hypercoagulable state of pregnancy). More often when left ventricular ejection fraction <35 %
Sustained ventricular tachycardia in rapidly progressive heart failure associated with thymoma, autoimmune disorders, or high-grade heart block	Giant-cell myocarditis	Syncope or sudden death develop due to ventricular arrhythmias or heart block

Table 2. Some key clinical hints among specific forms of myocarditis that help with the clinical diagnosis.

In addition to the coxsackievirus B, other members of the genus Enterovirus (coxsackievirus A, echovirus, and poliovirus) and many other viruses have also been associated less frequently with myocarditis; these viruses include influenza virus, Epstein–Barr virus, cytomegalovirus, human herpesvirus (Kindermann et al., 2008) and varicella-zoster virus. Myocarditis and pericarditis were reported in association with influenza virus infection during the 1918–1919 pandemic. Unusually, myocarditis has also been described as a complication of mumps in a severe but usually self-limited form. Molecular diagnostic assays have implicated mumps virus in some cases of endocardial fibroelastosis following myocarditis as well. In more recent study of 172 patients with a biopsy sample showing myocarditis, the most common viruses were parvovirus B19, 36.6 percent; enterovirus, 32.6 percent; co-infection with HHV-6 and parvovirus B19, 12.6 percent human herpesvirus 6 (HHV-6), 10.5 percent; adenovirus, 8.1 percent (Kuhl et al., 2005).
The novel influenza virus A (H1N1) pandemic began in Mexico in 2009 and rapidly spread worldwide. The cardiac complications of H1N1 infection were uncommonly reported. Sudden death as a result of myocarditis was a rare recognized complication in otherwise immunocompetent individuals, despite the absence of significant respiratory tract infection. A report from Japan described 10 patients presented with fulminant myocarditis which was confirmed by endomyocardial biopsy in 6 patients, 8 of the cases were rescued (Ukimura et al., 2009). Also, a documented influenza myocarditis was reported due to 2009 pandemic H1N1 virus occurred in a previously healthy adult (Haessler et al., 2010). Another reported fatal case of acute myocarditis in an immunocompetent young woman, the autopsy revealed a predominantly lymphocytic myocarditis (Gdynia et al., 2011). On the other hand, cases diagnosed with fulminant myocarditis were described in pediatric population with fatal outcomes within a 30-day of presentation (Bratincsák et al., 2010).
Though viral myocarditis is most often self-limited and without sequelae, fulminant condition with arrhythmias, heart failure occurs. Arrhythmias are common and are occasionally difficult to manage. Patients with fulminant myocarditis may require mechanical cardiopulmonary support or cardiac transplantation, but the majority survived and many demonstrate substantial recovery of ventricular function. Patients with myocarditis and pulmonary hypertension are at a particularly high risk of death. Deaths attributed to heart failure, tachyarrhythmias, and heart block has been reported and it seems prudent to monitor the electrocardiogram of patients with arrhythmias, especially during the acute illness. In some patients, myocarditis simulates acute myocardial infarction, with chest pain, electrocardiographic changes, and elevated serum levels of myocardial enzymes. Additionally, viral myocarditis are assumed to be the major causes of chronic dilated cardiomyopathy, some cases of myocarditis may recur as well, however the number of cases with acute myocarditis that progresses to chronic dilated cardiomyopathy remains anonymous.

7.2 Human immunodeficiency virus (HIV) myocarditis

The human immunodeficiency virus type I (HIV-1) infection that causes the acquired immunodeficiency syndrome (AIDS) has became a worldwide pandemic. It has been identified for more than 3 decades, during which time a number of factors may altered the nature of cardiac manifestation. Notably, the survival in adult with HIV infection and AIDS is prolonged as a result of earlier detection and the use of highly active antiretroviral therapy (HAART), (Hoover et al., 1993; Palella et al., 1998). On the other hand, disorders

such as hypertension, hyperglycemia, hyperlipidemia, lipodystrophy and coronary artery disease appeared to add further comorbidity to HIV infection (Fisher & Lipshultz, 2001; Friis-Møller et al., 2007; Tershakovec et al., 2004).

Human immunodeficiency virus is the most common cardiac pathologic finding at autopsy in HIV infected patients with prevalence as high as 70 percent (Anderson et al., 1988; Baroldi et al., 1988, Lewis, 1989). Myocarditis identified at autopsy or on endomyocardial biopsy in HIV-infected patients is most often nonspecific and manifested as focal, inflammatory lymphocytic infiltrates without myocyte necrosis. However, it is uncertain whether the myocarditis so frequently observed at autopsy is clinically relevant.

Myocarditis should be considered in any HIV-infected patient with dyspnea or cardiomegaly. It is most often present with signs and symptoms of congestive heart failure or asymptomatic left ventricular (LV) dysfunction established by echocardiography. Of note, the clinical features of concomitant noncardiac disorders may mask cardiac involvement and steer to inaccurate approach, since myocardial manifestations due of HIV infection may respond at least transiently to standard therapy. A prospective long-term clinical and echocardiographic follow-up study of asymptomatic HIV-positive patients showed a mean annual incidence of progression to dilated cardiomyopathy of 15.9 cases per 1,000 patients. The exact pathogenesis of myocarditis in the AIDS is unclear. Possible direct action of HIV on the myocardial tissue or an autoimmune process induced by HIV, possibly in association with other cardiotropic viruses. It is difficult to assess the clinical significance of viral infection of the myocardium in HIV infected patients. A histologic diagnosis of myocarditis was reported in 83 percent of patients with dilated cardiomyopathy. This significant proportion had focal, nonspecific lymphocytic myocarditis (Barbaro et al., 1998).

Dilated cardiomyopathy can be subclinical or may present with overt clinical findings. Cardiac involvement is often subclinical as echocardiographic studies have demonstrated LV dysfunction in 41 percent of asymptomatic HIV-positive individuals (Corallo et al., 1988). However, in the primary care setting, AIDS cardiac complications are unusual. One autopsy series demonstrated no cardiac disease in 115 consecutive autopsies of patients who died of AIDS-related complications (Lewis, 1989). In one series of 416 HIV-positive patients from Rwanda without a previous history of cardiovascular disease and not receiving HAART reported an echocardiographically evident dilated cardiomyopathy in 17.7 percent (Twagirumukiza et al., 2007). The overt clinical involvement is seen in 10 percent of HIV patients, and the most common clinically significant finding is a dilated cardiomyopathy associated with typical findings of congestive heart failure, namely edema and shortness of breath.

Apart from the clinical manifestation which may be seen as a direct consequence of HIV infection, a more likely, as a consequence of possible etiologies related to non-HIV cardiotrophic viral infection, postviral autoimmune mechanism, drug toxicity or neoplastic infiltration by Kaposi sarcoma or lymphoma.

Since the introduction of HAART regimens there has been a marked reduction in the incidence of myocarditis and opportunistic infections, which has led to a nearly 30 percent reduction in HIV-associated cardiomyopathy (Barbaro, 2005). Opportunistic infections include bacteria, fungi, protozoa, and viruses are the most frequent cause of morbidity and mortality in AIDS which is identified in 10 to 15 percent of cases (Hofman et al., 1993). However, symptomatic disease appears to be rare. Toxoplasma gondii is the most frequently documented infectious cause of myocarditis associated with AIDS. Myocardial

toxoplasmosis described in 1 to 16 percent autopsy series of patients dying of AIDS (Anderson et al., 1988; Baroldi et al., 1988; Matturri et al., 1990). Cytomegalovirus is another common opportunistic infection in patients with late stage AIDS that can cause myocarditis in selected patients (Barbaro et al., 1998; Niedt & Schinella, 1985). Other virus identified within the myocardium of HIV-infected or AIDS patients, either at antemortem endomyocardial biopsy or from autopsy material, have included Epstein-Barr and coxsackie B virus in adults (Barbaro et al., 1998; Dittrich et al., 1988) These viruses may be present as either primary infection or as coinfection and can occur with or without associated myocarditis and with or without associated LV dysfunction. Other infections like, myocardial tuberculosis appears to be rare (Miller-Catchpole et al., 1989). Fungal myocarditis is another unusual complication of disseminated infection that is identified most often at autopsy. Various fungal organisms identified in the myocardium at autopsy with associated myocarditis. Cardiac cryptococcus has been diagnosed in association with congestive heart failure and resolved after therapy (Kinney et al., 1989; Lafont et al., 1987; Lewis et al., 1985).

Other possible etiologies of LV dysfunction are drug toxicity from either abuse of illicit substances or iatrogenic disease from agents used in the therapy for AIDS. Alcohol, cocaine, or heroin may contribute to LV dysfunction in many cases (Brown et al., 1991; Peng et al., 1989; Soodini & Morgan, 2001). Therapeutic agents implicated as potential cardiac toxins include zidovudine (d'Amati et al., 1992; Herskowitz et al., 1992), interleukin-2 (Samlowski et al., 1989) and interferon alfa-2 (Deyton et al., 1989; Zimmerman et al., 1994). Neoplastic infiltration of the heart by Kaposi sarcoma is frequently seen at autopsy and usually associated with widespread disease in the terminal phases of AIDS (Silver et al., 1984). Non-Hodgkin lymphoma is also observed in this setting and also associated with widespread disease (Holladay et al., 1992).

7.3 Bacterial myocarditis

Nowadays, myocarditis of infectious etiology caused by non-viral agents is less frequent worldwide. Bacterial involvement of the heart is uncommon, but when it does occur, it is usually as a complication of endocarditis. Various bacteria include (*Corynebacterium diphtheriae, Streptococcus pyogenes, Staphylococcus aureus, Haemophilus pneumoniae, Salmonella* spp., *Neisseria gonorrhoeae, Leptospira, Borrelia burgdorferi, Treponema pallidum, Brucella, Mycobacterium tuberculosis,* Actinomyces*, Chlamydia* spp., *Coxiella brunetti, Mycoplasma pneumoniae* and *Rickettsia* spp). Bacteria like streptococcal and staphylococcal species and *Bartonella, Brucella, Leptospira,* and *Salmonella* species can spread to the myocardium as a consequence of severe cases of endocarditis. Some forms of bacterial myocarditis will be discussed below.

7.3.1 Diphtheritic myocarditis

Worldwide, the most common bacterial cause of myocarditis is diphtheria. As early as 1806, a relationship between infection (diphtheria) and chronic heart disease was postulated, but it was not until the 1970s, with the advent of endomyocardial biopsy, that the diagnosis of myocarditis could be established during life.

The risk of developing cardiac toxicity is proportional to the severity of local infection. *Corynebacterium diphtheriae* produce toxins that inhibit protein synthesis that can cause myocarditis and leads to a dilated, flabby, hypocontractile heart. The manifestations of

diphtheritic myocarditis include various dysrhythmias, conduction disturbances, and dilated cardiomyopathy. Cardiomegaly and severe congestive heart failure typically appear after the first week of illness. However, clinically evident cardiac manifestation like dyspnea, decreased heart sounds, gallop rhythm or cardiac dilatation is much less common, occurring in 10 to 25 percent of all patients with diphtheria (Morgan et al., 1963).
Myocarditis occurred in 22 percent of 656 hospitalized patients with diphtheria in the Kyrgyz Republic in 1995; 7 percent of patients with myocarditis and 2 percent of patients without myocarditis died (Kadirova et al., 2000). Myocarditis as evidenced by electrocardiographic changes such as ST-T wave changes, QTc prolongation, and/or first-degree heart block can be detected in as many as two-thirds of cases, often occurring when local respiratory symptoms are improving (Boyer & Weinstein, 1963; Lumio et al., 1948). The conduction system is frequently involved. The complete heart block result from diphtheritic myocarditis was almost always fatal before temporary cardiac pacemakers were developed. Diphtheritic myocarditis considered the most serious complication and remains the major cause of mortality (Kneen et al., 2004). The death rate is highest during the first week of illness particularly among patients with bull-neck diphtheria and among patients with myocarditis who develop ventricular tachycardia, atrial fibrillation, or complete heart block.

7.3.2 Lyme myocarditis

Lyme disease is an inflammatory disease caused by infection with the spirochete *Borrelia burgdorferi*. In United States, carditis occurs in approximately 5 percent, while, it is less frequent in Europe, affecting approximately 0.3 to 4.0 percent of untreated adults (Cox et al., 1991). This difference may be related to infection by different organisms.
A careful history should address risk factors or possible evidence of *B. burgdorferi* infection particularly in the presence of atrioventricular conduction abnormalities (McAlister et al., 1989). These include history of residence or travel through an endemic area; previous tick bites; prior or current erythema migrans lesions and coexistence of objective or subjective neurologic dysfunction compatible with neurologic Lyme disease. Cardiac Lyme disease occurs during the early disseminated phase of the disease, usually within weeks to a few months after infection (Fish, 2008). In a patient with suspected Lyme disease after a tick bite, the possibility of coinfection with Ehrlichia (ehrilichiosis) and Babesia (babesiosis) should be considered as both can also cause myocarditis.
There is a male predominance of approximately 3:1 in cardiac Lyme disease (Vlay, 1993). Patients with cardiac involvement may be asymptomatic and clinically inapparent. However, some patients develop symptomatic myocarditis with cardiac muscle dysfunction and/or associated pericarditis (Vlay et al., 1991) (Lorcerie et al., 1987). Symptoms mainly include palpitations, shortness of breath, chest pain, presyncope or syncope. In a review of 84 patients with Lyme carditis, the United States Centers for Disease Control and Prevention reported palpitations in 69 percent, conduction abnormalities in 19 percent, myocarditis in 10 percent and left ventricular failure 5 percent (Ciesielski et al., 1989). Endomyocardial biopsy samples resemble idiopathic lymphocytic myocarditis, and rarely the spirochetal organisms are identified (Cox et al., 1991; McAlister et al., 1989; Stanek et al., 1990).
Atrioventricular conduction block of varying degrees are the most common manifestation of Lyme carditis. In some patients, heart block is the first and only manifestation of Lyme disease (Kimball et al., 1989). Patients may present with first degree heart block but can progress to second degree or complete heart block over a short period of time (Peeters et al., 1990). One review of 52 patients with Lyme carditis found that 87 percent had

atrioventricular block, which was usually symptomatic (McAlister et al., 1989). Wenckebach periodicity occurred in 40 percent and complete atrioventricular block in 50 percent; other findings include bundle branch and fascicular blocks, which are rare. In another report, 38 percent of patients with Lyme carditis required a temporary pacemaker (Goldings & Jericho, 1986). Patients with a PR interval greater than 300 milliseconds carry a highest risk for progression to complete heart block, which may develop rapidly (Steere et al., 1980). Complete heart block caused by Lyme disease typically resolves within one week, and more minor conduction disturbances within six weeks (Fish, 2008; McAlister et al., 1989). More reports showed heart block usually persists for 3 to 42 days and often resolves spontaneously (Costello et al., 2009; Cox et al., 1991; Steere et al., 1980; van der Linde et al., 1989). In Europe, scattered case reports have suggested that *B. burgdorferi* may, in isolated cases, be a cause of chronic cardiomyopathy (Bartůnek et al., 2006; Palecek et al., 2010). This has not been shown in the United States. A small Dutch series evaluated 42 patients with dilated cardiomyopathy (Vlay et al., 1991). Nine were seropositive for anti-B. burgdorferi; six recovered fully, two had a partial response, and one showed no improvement.

7.3.3 Salmonella myocarditis

Typhoid fever is a life-threatening illness rarely complicated by myocarditis. Salmonella myocarditis may produce variable clinical manifestations from latent to severe clinical forms, such as acute congestive heart failure or sudden cardiac death (Al-Aqeedi et al., 2009; Burt et al., 1990). Postmortem studies suggest that myocarditis is a major cause of sudden unexpected death in young adults and may account for 20 percent of cases (Feldman & McNamara, 2000).

7.3.4 Yersinia myocarditis

Myocarditis sometimes occurs as a complication of Yersinia. Clinical evidence of Campylobacter-associated myocarditis described in association with *Campylobacter* spp. enteritis (Kotilainen et al., 2006). Mild, self-limited myocarditis accompanies 10 percent of cases of Yersinia induced arthritis and can occur independently. Typical manifestations include cardiac murmurs and transient electrocardiographic abnormalities, such as prolongation of the PR interval and nonspecific ST-segment and T wave changes. The syndrome of Yersinia-induced arthritis and carditis can be confused with acute rheumatic fever.

7.3.5 Legionella myocarditis

Myocardial involvement is a rare manifestation of Legionella infection, though, the most common extrapulmonary site of Legionnaires' disease is the heart. Numerous reports have described myocarditis, pericarditis, postcardiotomy syndrome, and prosthetic valve endocarditis (Antonarakis et al., 2006; Lowry & Tompkins, 1993; Tompkins et al., 1988). Most cases have been hospital acquired. Legionella carditis in the adult population is invariably seen in association of pneumonia, however, isolated Legionella myocardial involvement without associated pneumonia have been reported (Burke et al., 2009).

7.3.6 Mycoplasma myocarditis

Cardiac abnormalities rarely reported in conjunction with *Mycoplasma pneumoniae* infection including myocarditis and pericarditis (Martin & Bates, 1991; Paz & Potasman, 2002).

Myocarditis has been described in rare autopsy reports as well. Cardiac manifestations include rhythm disturbances, congestive heart failure, chest pain, and conduction abnormalities on the electrocardiogram.

7.3.7 Q fever myocarditis

Myocarditis, though uncommon, may be a particularly severe manifestation of Q fever. A study of 1070 patients with acute Q fever from southern France, 1 percent had pericarditis, and 1 percent had myocarditis. In other series of 1276 patients with Q fever over a 15-year period, only eight developed myocarditis but two were among only 12 total patients with Q fever who died (Fournier et al., 2001). Q fever may also cause endocarditis which usually occurs in patients with previous valvular damage or immunocompromise particularly a bicuspid aortic valve or a prosthetic valve.

7.3.8 Chlamydial myocarditis

Chlamydial infection also has been reported in association with clinical manifestations of myocarditis (Mavrogeni et al., 2008).

7.3.9 Relapsing fever myocarditis

Relapsing fever is an arthropod-borne infection characterized by recurrent episodes of fever, caused by spirochetes of the genus Borrelia. The first episode of illness tends to be the most severe. Myocarditis appears to be common in both louse-borne and tick-borne relapsing fever. Clinical and electrocardiographic evidence of myocarditis and myocardial dysfunction includes a prolonged QTc interval, commonly a galloping third heart sound, elevated central venous pressure, arterial hypotension, and rare pulmonary congestion. Heart involvement has been prominent in fatal cases (Wengrower et al., 1984).

7.4 Acute rheumatic fever

Acute rheumatic fever (ARF) is a nonsuppurative complication of group A streptococcus pharyngitis that occurs two to four weeks following infection and arises as an autoimmune response to extracellular or somatic bacterial antigens that share similar epitopes in human tissue. Rheumatic fever remains one of the most important cardiovascular diseases that cause significant cardiac morbidity and mortality in developing countries (Hutchison, 1998). In developed countries, ARF is generally preceded by pharyngitis but not skin infection (Guidelines for the diagnosis of rheumatic fever, JAMA 1992). However, data from endemic regions with ARF and rheumatic heart disease suggest a less clear association (McDonald et al., 2004, 2006; Noel et al., 2005).

Acute rheumatic fever occurs most frequently in children from 5 to 15 years of age. The incidence of rheumatic heart disease in patients with a history of ARF is variable; in general, valvular damage manifesting as a murmur later in life is likely to occur in about 50 percent of patients with evidence of carditis at initial presentation (Albert et al., 1995; Meira et al., 2005). The myocardial lesions consist of nonspecific lymphocytic myocarditis and Aschoff nodules. The latter are pathognomonic of ARF. Myocarditis is often indicated by cardiomegaly and/or congestive heart failure (CHF) particularly in the absence of a significant pericardial effusion. The presence of valvulitis is established clinically by auscultatory findings. Although CHF in rheumatic fever patients traditionally has been ascribed to severe myocardial inflammation, endomycardial biopsy in patients with

rheumatic carditis does not show significant evidence of myocyte damage (Narula et al., 1993). In addition, echocardiographic left ventricular ejection fraction and indices of myocardial contractility remain normal in patients with rheumatic carditis even in the presence of CHF (Essop et al., 1993). Further, CHF occurs only in the presence of hemodynamically significant valvular lesions.
The diagnosis of ARF is established largely on clinical grounds. The clinical manifestations initially described by Jones (Jones, 1944). Subsequently revised and lastly the established guidelines for the diagnosis of rheumatic fever reviewed by the American Heart Association Working Group in 2002 (Ferrieri, 2002). The five major manifestations include migratory arthritis, carditis and valvulitis, central nervous system involvement (eg, Sydenham chorea), erythema marginatum and subcutaneous nodules. Whereas, the four minor manifestations include, arthralgia, fever, elevated acute phase reactants (erythrocyte sedimentation rate, C-reactive protein) and prolonged PR interval. The probability of ARF is high in the setting of group A streptococcal infection followed by two major manifestations or one major and two minor manifestations. Strict adherence to the Jones criteria in areas of high prevalence may result in under detection of the disease. This was illustrated in a report of 555 cases of confirmed ARF among Australian aboriginals in whom monoarthritis and low-grade fever were important manifestations (Carapetis & Currie, 2001).

7.5 Chagas myocarditis

Chagas disease is a protozoan infection due to *Trypanosoma cruzi*; transmitted by an insect vector, produces an extensive myocarditis that typically becomes evident years after the initial infection. It is a major public health problem in endemic areas and in immigrants from rural Central or South America. Chagas myocarditis is by far the most common form of cardiomyopathy in Latin American countries (Schofield & Dias, 1991). Chagas disease consists of acute and chronic phases. During the chronic phase, many patients present the indeterminate form. The latter describes patients who have positive serology, but no symptoms, physical signs, or laboratory evidence of organ involvement (Dias, 1989).

7.5.1 Acute phase

The first signs of acute Chagas' disease develop at least 1 week after contact with the infected vector. Local skin indurated erythema and swelling produces the typical portal of entry lesions at the skin known as chagomas accompanied by local lymphadenopathy. The conjunctiva portal of entry may result in a unilateral painless periorbital edema and swelling of the palpebrae (Romana's sign). Infection can also occur through blood transfusion, congenital transmission, and, much less often, organ transplantation, laboratory accident, breast feeding, and oral contamination (Benchimol Barbosa, 2006). Although heart transplantation for Chagas cardiomyopathy has been successfully performed, reactivation of *Trypanosoma cruzi* is common. These initial local signs may be followed by malaise, fever sweating, myalgias anorexia, and a morbilliform rash may also appear. Generalized lymphadenopathy and hepatosplenomegaly may develop. Cardiac failure occur secondary to myocarditis; cardiac involvement is present in over 90 percent of those in whom the diagnosis is made (Pinto et al., 2004). The frequency and severity of myocarditis are inversely proportional to age (Dias & Kloetzel, 1968). The acute symptoms resolve spontaneously in virtually all patients, who then enter the asymptomatic or indeterminate phase of chronic T. cruzi infection. The electrocardiogram normalized in over 90 percent of patients after one year.

The indeterminate form usually lasts 10 to 30 years and only approximately 30 percent of the patients develop overt cardiac disease. Most patients remain asymptomatic throughout life. The natural history of this phase of disease is characterized by subtle degree of cardiac involvement and gradual appearance of clinical or electrocardiographic markers of cardiac involvement, which signals the onset of the chronic phase. In one review, progression from indeterminate to the full-blown clinical form in the chronic phase occurred at approximately 2 percent per year (Dias, 1989). In another report, 38.3 percent of patients with positive serology but without symptoms developed chagasic cardiomyopathy over a 10-year period (Coura et al., 1985). About 50 percent of patients remain with the indeterminate form indefinitely (Marin-Neto et al., 2003).

7.5.2 Chronic phase

The chronic form is characterized by dilatation of several cardiac chambers, fibrosis and thinning of the ventricular wall, aneurysm formation (especially at the left ventricular apex), and mural thrombi.

Chronic progressive heart failure is the rule and is associated with poor survival. Mortality associated with the chronic phase almost exclusively due to cardiovascular involvement. The cause of death is sudden cardiac death in 55 to 65 percent, progressive heart failure in 25 to 30 percent, and stroke in 10 to 15 percent (Rassi et al., 2001). Symptoms and physical signs at this stage of the disease arise from three basic syndromes that often coexist in the same patient, heart failure, cardiac dysrhythmia, and thromboembolism (systemic and pulmonary). Heart failure in Chagas heart disease is usually biventricular and commonly presented with fatigue. However, right-sided failure manifested with increased jugular venous pressure, peripheral edema, ascites, and hepatomegaly is characteristically more pronounced than those of left-sided failure manifested with dyspnea and pulmonary rales. Both systolic and diastolic dysfunction can occur (Sousa et al., 1988). Cardiac examination typically reveals the findings of murmurs of mitral and tricuspid regurgitation, wide splitting of the second heart sound due to right bundle branch block and prominent diffuse apical thrust.

Cardiac arrhythmias may cause palpitation, lightheadedness, dizziness, or syncope. Autonomic dysfunction results in marked abnormalities in the heart rate changes. Chest pain is common symptom and usually atypical in Chagas heart disease. It may mimic angina due to abnormal coronary vasomotion postulated as underlying mechanism (Marin-Neto et al., 1992). Sudden cardiac death accounts for 55 to 65 percent of deaths in CD; the real frequency of this complication is probably underestimated, particularly in rural areas (Rassi et al., 2001). Sudden cardiac arrest can occur even in previously asymptomatic patients (Rassi Júnior et al., 1995). However, most patients have severe underlying heart disease, including ventricular aneurysms at multiple sites (posterior-lateral, inferior basal, or apical), which is a characteristic finding in Chagas heart disease (Rassi Júnior et al., 1995). Sudden death is usually precipitated by exercise, and can be caused by VT or fibrillation, asystole, or complete AV block (Mendoza et al., 1986). The electrocardiogram is abnormal in most patients with cardiac involvement and typically shows right bundle branch block, left anterior hemiblock and diffuse ST-T changes, which may progress to complete atrioventricular block.Ventricular arrhythmia may also be seen in form of premature beats that may be multiform and runs of nonsustained ventricular tachycardia. The severity of ventricular arrhythmias tends to correlate with the degree of LV dysfunction. Other changes like, abnormal Q waves, various degrees of atrioventricular block, QT interval prolongation

and variation in the QT interval (QT dispersion) are frequent findings (Salles et al., 2003).Virtually all types of atrial and ventricular arrhythmias occur frequently, atrial fibrillation and low QRS voltage may be observed in advanced disease.
A potentially serious complication of chronic Chagas heart disease is thromboembolism. In a review of 1345 autopsies, cardiac thrombus or thromboemboli reported in 44 percent; both right and left cardiac chambers equally affected (Samuel et al., 1983). Although thromboembolic phenomena were more common in the systemic circulation, pulmonary embolism accounted for 14 percent of deaths. Cardioembolism appears to be an important cause of acute ischemic stroke. One series of 94 patients with Chagas disease in Brazil reported higher rate of cardioembolism (56 versus 9 percent) as compared to control group (Carod-Artal et al., 2005). Stroke was also reported significantly more frequent in patients who had Chagas disease related cardiomyopathy compared with patients who had other cardiomyopathies (15.0 versus 6.3 percent), (Oliveira-Filho et al., 2005). Echocardiography or contrast ventriculography may reveal a left ventricular apical aneurysm, regional wall motion abnormalities,or diffuse cardiomyopathy. The cause of death is either intractable CHF or an arrhythmia, with a minority of patients dying from embolic phenomena.

7.6 Fungal myocarditis

The incidence of invasive fungal disease has dramatically increased over the past few decades corresponding to the rising number of immunocompromised patients. Cardiac fungal infection, especially myocarditis, may be difficult to recognize clinically and may in itself produce a fatal outcome. Myocardial involvement frequently occurs in disseminated fungal infection in which multiple organs often affected. Conditions that appear predisposing to fungal infection are human immunodeficiency virus infection, medication like, corticosteroids, antineoplastic agents or broad-spectrum antibiotics, alone or in combination with invasive medical procedures (Nosanchuk, 2002). Candida was the most frequently observed organism, while Aspergillus was the second most frequent fungus to involve the heart. Rarely Cryptococcus identified as a cause of myocarditis as well.

7.7 Eosinophilic and hypersensitivity myocarditis

The association between eosinophilia (eosinophil count >500/mm3) and heart disease was first identified by Loeffler (Loeffler, 1936). A specific eosinophilic form of myocarditis identified following drug-induced hypersensitivity reactions and systemic hypereosinophilic syndromes (Taliercio et al., 1985).
Eosinophilic myocarditis is characterized by a predominantly mature eosinophils infiltration of the myocardium and other organ systems. It occurs in association with systemic diseases such as hypereosinophilic syndrome, Churg-Strauss syndrome and Löffler's endomyocardial fibrosis. Also it may occur in association with cancer, parasitic, helminthic or protozoal infections such as Chagas disease, toxoplasmosis, schistosomiasis, trichinosis, hyatid cysts and visceral larval migrans (Corradi et al., 2004; Corssmit et al., 1999; Spodick, 1997). Eosinophilic myocarditis has been reported after vaccination for several diseases, including smallpox (Arness et al., 2004; Barton et al., 2008). Acute eosinophilic necrotizing myocarditis is a rare aggressive form of eosinophilic myocarditis and may represent an extreme form of hypersensitivity myocarditis which characterized by acute onset and rapidly results in cardiovascular deterioration and circulatory collapse carried high mortality rates (Cooper & Zehr, 2005). The clinical manifestations of eosinophilic myocarditis may include right and left congestive heart failure, endocardial and valvular

fibrosis leading to regurgitation, and formation of endocardial thrombi. Clinical awareness warranted when presentation may mimics acute myocardial infarction, with ischemic chest pain and ST-segment elevation on electrocardiography (Galiuto et al., 1997).

Hypersensitivity myocarditis is a form of eosinophilic myocarditis due to autoimmune reaction affecting the heart muscle, often induced by drug. It is often first discovered at postmortem examination. In one series, the prevalence of clinically undetected hypersensitivity myocarditis in explanted hearts ranged from 2.4 to 7 percent (Wu et al., 2002).

Numerous drugs have been implicated in hypersensitivity myocarditis including, antibiotics (Burke et al., 1991) like penicillins, cephalosporins and sulfonamides; antipsychotics (Killian et al., 1999) like clozapine and tricyclic antidepressants (Ansari et al., 2003; Burke et al., 1991; Kounis et al., 1989); others like methyldopa, hydrochlorothiazide, furosemide, tetracycline, azithromycin, aminophylline, phenytoin and benzodiazepines (Ben m'rad et al., 2009; Pursnani et al., 2009; Taliercio et al., 1985). Hypersensitivity myocarditis not constantly develop early in the course of medication. Patients taking the antipsychotic agent clozapine have been reported to develop myocarditis more than two years after the drug was started (Haas et al., 2007). Prolonged continuous infusion of dobutamine has also been associated with hypersensitivity myocarditis which has been reported in 2.4 to 23 percent (Spear, 1995; Takkenberg et al., 2004). Cocaine also rarely produce a hypersensitivity myocarditis, unlike the hypereosinophilic syndrome, peripheral eosinophilia is typically absent (Isner & Chokshi, 1991).

Clinically, the presentation often heralded by fever, peripheral eosinophilia and a drug rash that occurs days to weeks after administration of a previously well-tolerated agent. Electrocardiographic abnormalities show nonspecific ST segment changes or infarct patterns (Fenoglio et al., 1981). Myocardial involvement varies but usually does not result in fulminant heart failure or hemodynamic collapse. However, some patients present with sudden death or rapidly progressive heart failure (Burke et al., 1991; Galiuto et al., 1997).

Eosinophilic myocarditis can be a manifestation of eosinophilia-myalgia syndrome, which is a multisystem disease caused by ingestion of contaminants in L-tryptophan containing products (Belongia et al., 1990), characterized by peripheral eosinophilia and generalized disabling myalgias (Martin et al., 1990). Eosinophils, lymphocytes, macrophages, and fibroblasts accumulate in the affected tissues, but their role in pathogenesis is unclear. The disease is frequently evolves into a chronic course but can be fatal in up to 5% of patients.

7.8 Giant cell myocarditis

Idiopathic giant cell myocarditis is a rare inflammatory disease that often affects previously healthy young adults and frequently fatal type of myocarditis (Cooper et al., 2008). The pathogenesis of this disorder is not known. It is identified by the presence of multinucleated giant cells associated with eosinophils and myocyte destruction in the absence of granulomas on endomyocardial biopsy. It is thought to be primarily autoimmune in nature because of the reported comorbidity with a variety of autoimmune disorders (Cooper et al., 1997), thymoma, (Kilgallen et al., 1998) and drug hypersensitivity (Daniels et al., 2000).

Idiopathic giant cell myocarditis is usually a fulminant form of myocarditis and characterised by a history of rapid progression of severe heart failure associated with refractory sustained ventricular arrhythmias. Giant-cell myocarditis is sometimes distinguished from the much more common postviral myocarditis by the presence of

ventricular tachycardia, heart block, and a downhill clinical course, despite optimal clinical care. In the series of 63 patients with giant cell myocarditis enrolled in the multicenter Giant Cell Myocarditis Treatment Trial, 75 percent identified with heart failure symptoms as the primary presentation, 14 percent with ventricular arrhythmia and heart block in 5 percent (Cooper et al., 1997). Most patients will require cardiac transplantation, the median survival from the onset of symptoms of less than 6 months and has an 89 percent rate of death or transplantation. This represents a significantly worse outcome compared to lymphocytic or viral myocarditis. Despite a 25 percent incidence of post-transplantation recurrence of giant cell myocarditis detected by biopsy, the 5-year survival after transplantation is about 71 percent which is comparable to survival after transplantation for cardiomyopathy.

7.9 Systemic lupus erythematosus myocarditis

Acute myocarditis is an uncommon manifestation of systemic lupus erythematosus (SLE), with a prevalence of 8 to 25 percent in different studies (Apte et al., 2008; Mandell, 1987). Myocarditis frequently asymptomatic but less often may accompany other manifestations of acute SLE. In particular pericarditis, commonly occur in about two-thirds of patients, and generally follows a benign course, however tamponade or constriction may occur infrequently.

Myocarditis generally parallels the activity of the disease and, although common histologically, rarely results in clinical heart failure unless associated with hypertension. African American ethnicity is associated with a higher risk of myocarditis compared with Hispanic and Caucasian ethnicity (Apte et al., 2008). Myocarditis should be suspected if there is resting tachycardia disproportionate to body temperature, ST and T wave electrocardiographic abnormalities and unexplained cardiomegaly. The cardiomegaly may be associated with symptoms and signs of heart failure, conduction abnormalities or arrhythmias (Moder et al., 1999). Patients with SLE are at increased risk for myocardial ischemia due to accelerated atherosclerosis or coronary arteritis. Endocardial involvement with fibrinous endocarditis (Libman & Sachs, 1924) is another serious manifestation can lead to valvular insufficiencies or embolic events. Likewise patients with the antiphospholipid syndrome have a higher incidence of valvular disease, a variety of thrombotic disorders, myocardial infarction, pulmonary hypertension, and cardiomyopathy. Myocardial biopsy reveal mononuclear cells infiltration distinguish active myocarditis from fibrosis and other causes of cardiomyopathy (Schattner & Liang, 2003) or rarely cardiotoxicity induced by hydroxychloroquine (Keating et al., 2005). Inflammation may lead to fibrosis that may be manifested clinically as dilated cardiomyopathy.

7.10 Sarcoid myocarditis

It is a granulomatous form of myocarditis. The clinical evidence of myocardial involvement is present in approximately 5 percent of patients with sarcoidosis. However, an autopsy series have reported higher rates of about 25 percent of subclinical cardiac involvement (Chapelon-Abric et al., 2004; Kim et al., 2009; Thomsen & Eriksson et al., 1999). The clinical manifestations of cardiac sarcoidosis are largely nonspecific and may precede, follow, or occur concurrently with involvement of other organs. Sarcoid heart disease should be considered in the evaluation of an otherwise healthy young or middle aged person with cardiac symptoms or in a patient with known sarcoidosis who develops arrhythmias, conduction disease, or heart failure. Patients who present with apparently chronic dilated

cardiomyopathy yet with new ventricular arrhythmias or second-degree or third degree heart block or who do not have a response to optimal care are more likely to have cardiac sarcoidosis (Yazaki et al., 1998). Cardiac symptoms were reported in 101 patients, when cardiac sarcoidosis was diagnosed in 84 percent compared to 4 percent in asymptomatic patients (Smedema et al., 2005). Endomyocardial biopsy shows characteristic noncaseating granulomas. However, the diagnosis can also be inferred if there is a tissue diagnosis of sarcoidosis from an extracardiac source in the presence of a cardiomyopathy of unknown origin.

The electrocardiographic abnormalities found in nearly 70 percent of patients with sarcoidosis (Chapelon-Abric et al., 2004). Cardiac involvement with sarcoidosis may produce clinical symptoms and electrocardiographic findings simulating myocardial infarction. Conduction abnormalities in form of first-degree heart block due to disease of the atrioventricular node or bundle of His, and various types of intraventricular conduction defects, are common among patients with cardiac sarcoidosis (Chapelon-Abric et al., 2004). These lesions may initially be silent, but can progress to complete heart block and cause syncope (Yoshida et al., 1997). Sustained or nonsustained ventricular tachycardia and ventricular premature beats are the second most common presentation of cardiac sarcoidosis; electrocardiography reveals ventricular arrhythmias in as many as 22 percent of patients with sarcoidosis (Sekiguchi et al., 1980). Supraventricular arrhythmias are infrequent. Sudden death due to ventricular tachyarrhythmias or conduction block accounts for 25 to 65 percent of deaths due to cardiac sarcoidosis, however, sudden death can occur in the absence of a previous cardiac event (Reuhl et al., 1997; Soejima & Yada, 2009; Yazaki et al., 2001). Both systolic and diastolic heart failure can occur. Left ventricular aneurysms develop in patients with extensive involvement of the myocardium. Mitral incompetence may occur with cardiac sarcoidosis due to associated systolic dysfunction and left ventricular dilation or due to papillary muscle involvement by sarcoid granulomas (Sato et al., 2008). Tricuspid regurgitation with atrioventricular block secondary to infiltration of tricuspid valves and conduction system by sarcoid granulomas has been reported as well (Goyal & Aragam, 2006). A left atrial granulomatous mass resembling myxoma has been reported too (Abrishami et al., 2004).

7.11 Peripartum cardiomyopathy

The syndrome is a rare disorder of pregnancy. It was recognized in 1937, as a distinct clinical entity (Gouley et al., 1937). Currently, the etiology of peripartum cardiomyopathy (PPCM) remains unclear. However, there is compelling data from animal and human studies suggesting that PPCM is actually a type of myocarditis arising from an infectious, autoimmune, or idiopathic etiology. The relationship between pregnancy and viral myocarditis was first published in 1968 (Farber & Glasgow, 1970). Endomyocardial biopsies in women with PPCM have demonstrated myocarditis in many patients. The highest incidence of myocarditis reported in PPCM was 76 percent (Midei et al., 1990), however much lower incidence was reported (8.8 percent), which found to be comparable to an age and sex matched control population undergoing transplantation for idiopathic dilated cardiomyopathy (9.1 percent), (Rizeq et al., 1994). Viral genomes of parvovirus B19, human herpes virus 6, Epstein–Barr virus, and human cytomegalovirus revealed in endomyocardial biopsy specimens from patients with PPCM (Bultmann et al., 2005). Other reported data linked with Chlamydial infection (Cenac et al., 2003).

Women present with heart failure during the peripartum period and become manifested in the last month of pregnancy or within 5 months of the delivery without apparent etiology for the heart failure can be found. The clinical scenario is challenging because many normal women in the last month of a normal pregnancy experience dyspnea, fatigue and pedal edema, symptoms can mimic early congestive cardiac failure. Physical examination can be significant for signs of right and left heart failure. Symptoms and signs that should raise the suspicion of heart failure include paroxysmal nocturnal dyspnea, chest pain, nocturnal cough, new regurgitant murmurs, pulmonary crackles, elevated jugular venous pressure and hepatomegaly. The electrocardiogram usually demonstrates a normal sinus or sinus tachycardia rhythm, but frequent ectopy and other atrial arrhythmias may also be present. Left ventricular hypertrophy, inverted T waves, Q waves, and nonspecific ST-T changes have also been reported (Brown & Bertolet, 1998). Recurrence in a subsequent pregnancy has been reported. However, significant improvement occurs in up to 50 percent of affected women; others are left with a progressive dilated cardiomyopathy.

8. Conclusion

The clinical signs and symptoms of myocarditis are highly variable. A thorough medical history with emphasis on possible causes is essential. A scrupulous awareness to ample clinical scenarios is essential for clinicians, particularly when the cases are lacking apparent etiologies or the presentations being similar to acute myocardial infarction, asymptomatic left ventricular systolic dysfunction, unexplained ventricular tachyarrhythmias or cardiogenic shock. Clinician need to be attentive when evidence of myocardial damage not attributable to epicardial coronary artery disease, primary valvular disease or noninflammatory causes. Usually, most cases of myocarditis are self limited and a spontaneous improvement occurs in a substantial number of patients with lymphocytic disease but is rarely, if ever, observed with granulomatous myocarditis. While routine diagnostic endomyocardial biopsy is not required in most cases of suspected acute myocarditis, the need for biopsy will depend upon the time course and severity of the clinical presentation.

Better understanding of the clinicopathologic aberration that characterize the diverse clinical scenarios and more comprehensive understanding of the natural history of the various subtypes of myocarditis should assist clinicians for better approach and subsequently plan more effective therapy in the future.

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2

Myocarditis in Childhood: An Update on Etiology, Diagnosis and Management

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Izmir

1. Introduction

Myocarditis is the term used to describe acute or chronic inflammation of the myocardium. For two decades, there has been increasing confrontations concerning the diagnosis, management and clinical outcome of the myocarditis. The cause of myocarditis frequently remains unknown. However, infections, systemic diseases, toxins and drugs have been reported being associated with inflammation of the myocardium (Kearney et al., 2001 & Mahrholdt H et al, 2006). The majority of cases are supposed to be due to infectious agents and it was recognized that any infectious agents could initiate myocarditis (Brodison & Swann, 2001). In North America and Europe, viral infection is the most common causes of myocarditis (Magnani & Dec, 2006). The true overall incidence of myocarditis remain obscure due to inconsistency of its definition and clinical manifestation in the paediatric population. Post-mortem study from Sweden reported incidence of myocarditis to be 1.06 % in 12.747 consecutive autopsies (Gravanis & Sternby, 1991). Since the clinical presentation of myocarditis is so variable, high index of suspicion is essential. Patients are asymptomatic and diagnosis is incidental in the majority of cases. The spectrum of disease ranges from nonspecific findings (chest pain, fever, myalgia, atrial or ventricular tachycardia) to acute heart failure and sudden death. Myocarditis as a cause of sudden death has been reported in up to 12% of young adults (Doolan et al., 2004). However, population based study from Finland (Kyto et al, 2007) documented that the incidence of fatal myocarditis (1.59 per 100000) was highest in infants under one year of age and incidence was lowest in young adults (5-24 years, 0.12-0.17/100000). Recently, Weber et al have also suggested that myocarditis is an infrequent, corresponding to approximately 2% of paediatric deaths (Weber et al., 2008).

Previous researches (Felker et al, 1999 & Lipshultz et al, 2003) strongly suggested that acute myocarditis may proceed to dilated cardiomyopathy. Diagnostic evaluation of a series of 1.278 patients (mean age: 50 years, range 15-87 years) with cardiomyopathy revealed that 9.8% of cases was diagnosed with myocarditis (Felker et al, 1999). In a paediatric study assessing the incidence of cardiomyopathy, viral myocarditis was found to be responsible for 27% of cases with dilated cardiomyopathy (Lipshultz et al, 2003). Moreover, in a prospective cohort study (Towbin et al, 2006), it has been shown that most common known cause of dilated cardiomyopathy is myocarditis (46%). Several mechanisms were postulated for progression of myocarditis to dilated cardiomyopathy, including direct viral injury,

autoimmune response of body through the effects of lymphocytes, natural killer cells, cytokines and apoptotic cell death (Kawai, 1999).

Diagnosis of acute myocarditis can be difficult owing to the lack of accepted and standardized criteria in addition to the nonspecific pattern of clinical presentation. The other issue hampered agreement on the most proper diagnostic criteria and documentation of cases, is broad diversity of aetiologies associated with myocarditis (Dec et al, 1985). At present, diagnosis has been made by use of pathological classification, commonly referred to as Dallas criteria (Aretz et al, 1987). The identification of inflammatory infiltrate with or without myocardial cell necrosis on conventionally stained myocardial tissue biopsy specimens is essential for histological diagnosis. On the basis of these criteria, myocarditis is described as active or borderline myocarditis in accordance with the presence or absence, respectively, of myocardial necrosis. The inflammatory infiltrate should be further identified as lymphocytic, eosinophilic or granulomatous (Figure 1). Sampling error, low sensitivity and specificity, discrepancy in expert interpretation remain limitations to the use of endomyocardial biopsy for diagnosis of acute myocarditis (Hauck et al, 1989 & Shanes et al, 1987). Inflammation in acute myocarditis may be focal, therefore it is challenging to biopsy the inflamed area of myocardium (Robinson et al, 2005). Additionally, it is invasive and potentially dangerous procedure, particularly in the paediatric patient (Checchia & Kulik, 2006). According to the some researchers (Parillo, 2001), this histopathological criteria could not be considered the gold standard for diagnosing acute myocarditis. Molecular pathological analyses, such as polymerase chain reaction (PCR) and in-situ hybridization allows rapid detection and documentation of the viral genetic material in the myocardium (Angelini et al, 2002 & Bock et al, 2010). In 2008, it was reported that immunohistological signs of myocarditis has been associated with poor outcome in myocarditis (Kindermann et al, 2008).

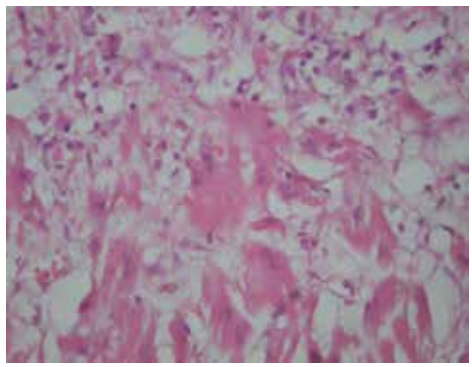

Fig. 1. The pathological diagnosis of viral myocarditis necessitates the manifestation of a inflammatory infiltrate associated with myocyte necrosis. The infiltrate consists of predominantly mononuclear cells (Stained by hematoxylin and eosin, magnification X 400; Text and image courtesy of Ragip Ortac, Gulden Diniz, Malik Ergin)

Therapy of myocarditis in children with inotropes and afterload reduction is usually sufficient. Although the long-term sequels are rare, dilated cardiomyopathy and sudden cardiac death may develop in clinical course. Extracorporeal membrane oxygenation and mechanical ventilations are other options for severe cases (Vashist & Singh, 2009). Newer therapeutic strategies such as intravenous immunoglobulin and immunosuppressive agents

have been investigated with the improvements in understanding pathogenesis of the myocarditis (Drucker et al, 1994 & Camargo et al 2009). It is the aim of this review to give a brief and complete discussion of pathogenesis, diagnosis and management of myocarditis.

2. Etiology

The vast majority of myocarditis in the developed countries result from viral infections (Table 1). The causes other than infections are autoimmune-systemic diseases, toxins and hypersensitivity to drugs (Brodison & Swann, 2001). Enteroviruses (particularly Coxsackie) and adenovirus were recognized as the major cause of viral myocarditis (Baboonian& Treasure, 1997; Pauschinger et al, 1999). During the last decade, parvovirus B19 (PVB19) and human herpesvirus 6 (HHV6) have been described as new pathogens (Kuhl et al, 2003). Moreover, investigators from Germany found that PVB19 and HHV6 are the most common causes of biopsy-confirmed viral myocarditis (Kuhl et al, 2005 & Mahrholdt et al, 2006). An investigation analysing the potential role of PVB19 in the clinical setting of acute myocarditis revealed that PVB19 was the most common agent. Kuhl et al had also noticed that Dallas criteria was frequently negative in patients with positive PVB19 PCR and macrophages were augmented in virus positive cases (Kuhl et al, 2003). These findings support the postulation of Bowles et al that different viruses have various pathogenic mechanisms such as lymphocyte-dependent vs. macrophage-dependent (Bowles et al, 2005). Nevertheless, PVB19 DNA has also been revealed in the myocardium of healthy donors (Donosa et al, 2005), in hearts of adults with dilated cardiomyopathy (Lotze et al, 2004), and in hearts of the patients with lupus and amyloidosis (Kuethe et al, 2007), despite the number of subjects studies was small. From these results, the question arises whether PVB19 certainly cause the underlying heart disease or whether it is just spectator attending in the heart as a result of former infection which is usual in young adults or children. A study by Kuethe et al was conducted to investigate this question. They suggested that PVB19 displays lifelong persistence, identification of PVB19 DNA was not correlated with clinical symptoms and serological analysis should be standardized procedure for future studies considering prevalence of PVB19 (Kuethe et al, 2009).

Polymerase chain reaction (PCR) analyses of myocardium in children and adult patients have showed the existence of adenoviral genome in cases with myocarditis and dilated cardiomyopathy with a larger frequency than enterovirus (Pauschinger et al, 1999 & Bowles et al, 2003). Geographical variation in viral etiology is also remarkable that hepatitis C virus has been more commonly documented in Japanese patients and parvovirus B19 is more frequently detected by PCR in German population (Magnani & Dec, 2006). Matsumori et al found that hepatitis C virus infection is often found in cases with dilated cardiomyopathy and that hepatitis C virus have an crucial role in the pathogenesis of cardiomyopathy (Matsumori et al, 1995). It was also suggested that antiviral therapeutics against hepatitis C virus could be indicated in these cases. Other viruses linked with myocarditis include Epstein-Barr virus, cytomegalovirus, herpes simplex virus, influenza A-B and HIV (Magnani & Dec, 2006). Multiple infections with different viruses have also been detected in cases (approximately one quarter of all cases) with systolic left ventricular dysfunction (Kuhl et al, 2005). Influenza A and B may also involve a combined myocarditis risk, particularly in patients with pre-existing cardiovascular diseases (Friman et al, 1995). From the study (Bowles et al, 2003) conducted in 624 patients with myocarditis (116 neonates, 191 infants), it was concluded that most common amplified viral genomes in myocardial tissues

included are adenovirus, cytomegalovirus, parvovirus and influenza A (ordered in decreasing frequency). It has been known that HIV may cause myocarditis and dilated cardiomyopathy (Breuckman et al, 2005). Direct viral injury, antiretroviral agents, coinfections and inhibition of contractility through HIV glycoprotein type I 120 play a role in the pathogenesis of myocarditis and dilated cardiomyopathy (Chen et al, 2002). The introduction of highly active antiretroviral therapy (HAART) has significantly reduced the incidence of HIV related- myocarditis. On the other hand, in developing countries where the supply of HAART is limited, researchers have observed increase in prevalence of HIV associated cardiomyopathy (Pugliese et al, 2000).

Viral			
Coxsackie virus	Respiratory syncytial virus		
Adenovirus	Vaccinia (smallpox vaccine)		
Human herpes virus	HIV		
Parvovirus B19	Influenza A and B		
Hepatitis C virus	Cytomegalovirus		
Epstein-Barr virus			
Bacterial			
Borrelia Burgdorferi	Streptoccus pneumoniae		
Mycobacterial	Treponema pallidum		
Mycoplasma pneumonia	Neisseria Meningitides		
Corynobacterium diphtheria	Rickettsia sp.		
Hemophilus influenza	Vibrio cholerae		
Parasitic-Fungal-Protozoal			
Ascaris sp.	Schistosomiasis		
Echinococcus granulosus	Larva migrans		
Aspergillus sp.	Candida		
Taenia Solium	Coccidioides		
Cryptococcus	Histoplasma		
Toxoplasma gondium	Tryponosoma cruzi		
Immunologic			
Rheumatic fever	Polymyositis, rheumatic arthritis		
Chagas disease	Sarcoidosis		
Systemic lupus erythamatosus	Thyrotoxicosis		
Diabetes Mellitus	Wegener's Granulomatosis		
Ulcerative colitis	Scleroderma		
Hypersensitivity-Drugs			
Amitriptyline	Amphotericin B	Arsenic	Scorpion envenomation
Anthracyclines	Electric shock	Penicilline	
Digoxin	Phenytoin	Copper	
Dobutamine	Colchicine	Isoniazide	
Cephalosporins	Iron	Lead	

Table 1. Main etiologies observed in myocarditis.

During the pandemic of influenza A (H1N1), myocarditis was documented in four children (80 children with H1N1 influenza) within a 30-day period (Bratincsak et al, 2010). In their retrospective review, three children had fulminant myocarditis, 1 with fatal outcome and 2

required extracorporeal membrane oxygenation support. From these findings, they assumed that new H1N1 influenza A virus is more frequently associated with a severe form of myocarditis that formerly encountered influenza strains. A study from Spain have also emphasized the importance of myocarditis as a risk factor for mortality.

Numerous bacterial infections may cause myocarditis, involvement of myocardium may have insidious course (electrocardiography changes) or may present with significant signs and symptoms. Fulminant septicaemia may result in myocarditis with fatal course. Most common causes of myocarditis associated with bacteraemia included are meningococcus, streptococcus and Listeria (Brodison&Swann, 1998). Borrelia burgdorferi causes Lyme carditis with acute or chronic course. Recently, a study o 207 children with early disseminated Lyme disease conducted by Costello et al found that 33 children (16%) had mild to fulminant myocarditis, 14 of whom had advanced atrioventricular block (none required permanent pacemaker). Lyme disease may rarely present with cardiomyopathy (Costello et al, 2009).

Various drugs implicated in the development of myocarditis including; anthracyclins, cyclophosphamide, cisplatin, 5-fluorourasil, Lithium, aminophylline, catecholamines, antibiotics (penicillines,), phenytoine and trastuzumab (Ellis&Disalvo, 2007). Myocyte injury may occur by direct toxic effect on heart or by provoking hypersensitivity reactions. Hypersensitivity reaction may be indicated fever, sinus tachycardia, peripheral eosinophilia and a rash that follows days to weeks after administration formerly well-accepted agent. Actually, it is not the drugs that heralds the reaction, but its metabolites (haptens) in the cases of hypersensitivity myocarditis. The pathological findings are indistinguishable and are independent of the drug involved. The inflammatory infiltrate predominantly consists of eosinophils, and can be located in focal areas, or diffusely within the myocardium with slight or no sign of necrosis, or substitute fibrosis. If the grade of myocardial inflammation or necrosis is severe, arrhythmias or hemodynamic collapse may likely occur. Eosinophilic necrotising myocarditis is an extreme form of hypersensitivity myocarditis that promptly cause cardiovascular collapse. Eosinophilic myocarditis has been documented following administration dobutamine and vaccines (Tetanus, small pox).

Systemic diseases that are related with active myocarditis include connective tissue diseases such as systemic lupus erythematosus, mixed connective tissue disease, systemic sclerosis, Churg-Strauss syndrome; celiac disease and Whipple's disease. Protozoal, helmintic and parasitic infections may also present with eosinophilic myocarditis. Myocardial abnormalities in SLE is multifactorial with coronary vasculitis, valvulopathy, hypertension, immune injury and drugs are the major contributors. Myocardial abnormalities are common in autopsy patients. However, clinically apparent myocarditis occurs in < 10 % of cases suggesting that the subclinical form is more frequent (Magnani&Dec, 2006).

Further understanding the etiology of myocarditis will illicit more direct therapeutic approaches such as vaccine and antiviral agents. Although some antiviral agents as ribavirin, oseltamivir and acyclovir have had moderate effect on influenza, RSV pneumonitis and CMV disease, currently there is no specific therapy approved for Enteroviruses, parvovirus B 19 and adenovirus. But, other options may be worth considering. A historical example of a promising therapy for viral myocarditis and prevention of dilated cardiomyopathy is that of decline in the incidence of endocardial fibroelastosis in children after initiation of mumps virus vaccine. It has been known that endocardial fibroelastosis is associated with congestive heart failure and death. Evidence from myocardial samples of patients with EFE supported the hypothesis that it is sequela of

viral myocarditis, in particular of that due to mumps virus. Based on the chronology of the fading of the disease, Ni et al. suggested that it is likely that vaccination was responsible for the remarkable decrease in documented case of EFE. So it is logical to think that vaccination against parvovirus B19, echovirus and adenovirus could diminish cases of myocarditis and dilated cardiomyopathy.

3. Pathogenesis

Special attention to understanding the mechanism and pathogenesis of myocarditis have been increased since Gore and Saphir showed in 1947 that diphteric and rheumatic carditis separately comprised only 10% of a series of 1402 patients of myocarditis (Gore & Saphir, 1947). In 1970's, several investigators demonstrated the persistence of neutralizing antibodies to coxsackie B (CVB) in cases with cardiomyopathy than healthy subjects (Kawai, 1971, Kawai et al., 1978, Toshima et al., 1979). This finding supported the hypothesis of a viral cause underlying the pathogenesis of cardiomyopathy. Evidence from murine models helped understanding pathogenesis of myocarditis (Liu&Mason, 2001). Myocarditis in susceptible mice is characterized by 3 separate disease processes, direct viral or other infectious agent access to myocardium tissue rapidly evolves into the second phase (Figure 2). In second phase, also called autoimmune phase of disease, immunological activation is the main feature. During the last phase, signs of myocarditis usually disappear and the damaged myocytes are substituted by diffuse fibrosis. Misdiagnosis and inappropriate therapy are especially possible at the time of transitional period among the 3 phases. In the case of reinfection and autoimmune recurrence, confusion can be compounded.

3.1 Viremia

Currently, there are two models of coxsackie B virus 3 induced myocarditis. The first one provokes acute viral myocarditis with a significant damage to myocytes and sudden death of animals within a week of infection (Fuse et al., 2005). In the second model, some degree of mice seemed to advance acute viral myocarditis following an infection with a cardiotropic strain of CVB3 (Fairwather&Rose, 2007). There also models with CMV, HIV and adenovirus (O'Donoughe et al., 1990, Salone et al., 2003, Beischel et al., 2004). During the time of active viremia, cardiotropic RNA virus (Coxsackie B) is taken into cells by receptor mediated endocytosis and are directly translated interior to the cells to produce protein (Huber, 1993). The virus enters the cell by endothelial receptors, particularly coxsackie-adenovirus receptor (CAR). Additionally, coxsackie B1, B3 and B5 uses decay accelerating factor (Shafren et al., 1995); adenoviruses uses αv integrin as coreceptors for viral entry (Wickham et al., 1994). It has been shown that CAR is highly expressed in the heart and brain, peaking in the perinatal period with subsequently declining with age and it is identified on the entire surface of the myocardium (Kashimura et al., 2004). Therefore, one can explain the susceptibility of neonates and infants to coxsackie B 3 myocarditis on the basis of expression level and the location of CAR. Titers of viral antigens in the myocardium is highest on the fourth day of inoculation of virus (Tomioka et al., 1986). No neutralizing antibodies to the virus were present until day 4. The antibody titers elevate promptly on day 8 and 10 reach the highest level on day 14 (Kawai, 1999). The emergence of a rising antibody titers is closely linked to elimination of the virus from the myocardium.

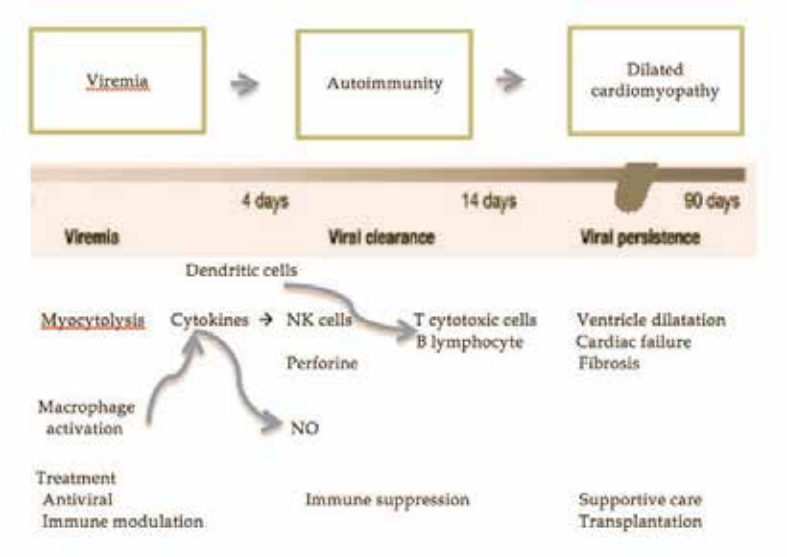

Fig. 2. Schematic drawings of pathophysiological processes of viral myocarditis. NO: Nitric oxide, NK: Natural killer cells.

3.2 Autoimmunity

First phase terminates with the stimulation of the host immune response that weakens viral proliferation but may also augment viral entry. Under ideal circumstances, immune system should normalize to a resting state once viral proliferation is limited. However, if host immune system stimulation persists unrestricted even with the elimination of the virus, autoimmune disease may develop, activating the second phase. This phase is distinguished by inflammatory cellular infiltration with natural killer cells and macrophages, then consequent expression of proinflammatory cytokines, especially interleukin-1, interleukin-2, TNF and interferon γ (Kawai, 1999, Matsumori et al., 1994). It has been shown that TNF triggers endothelial cells, recruits further inflammatory cells, more enhances cytokine production and has direct negative inotropic effects (Feldman&McNamara, 2000). Cytokines can also induce macrophages to express inducible nitric oxide synthase (NOS) in heart cells (Zaragoza et al., 1998). The role of NO in myocarditis is complicated. NO can reduce viral replication, and peroxynitrate production has strong antiviral effects (Zaragoza et al., 1997). Mice deficient in NOS were found to have greater viral titers and more widespread myocyte injury (Padalko et al., 2004). Alternatively, myosin induced autoimmune myocarditis animal model showed us that NOS expression in myocytes and macrophages is related with more severe inflammation, where NOS inhibitors can have potential to reduce myocarditis severity (Zaragoza et al., 1998, Mikami et al., 1997). Furthermore, improvement in myocarditis of the mouse model has been demonstrated by blocking IL-1b or TNF-a at the onset of the disease (Fairweather et al., 2004). Cihakova et al. also showed that the severity of CVB3 induced myocarditis as well as myosin-induced myocarditis is associated with the levels of IL-1b and IL-18 in the myocardium (Cihakova et al., 2008). T cells are activated in viral myocarditis by classical cell-mediated immunity. Viral peptide fragments are processed in the Golgi apparatus of the myocyte and presented to the cell. These prepared T-cells are capable of identifying the viral antigen and destroy the infected myocyte by

means of cytokine and perforine secretion (Ayach et al., 2003). But, persistent excited stimulation of the T cells is eventually harmful to the host, due to both direct T-cell mediated and cytokine-mediated cell damage diminish the number of contractile elements.
Continuous T-cell activation is induced through antigens intrinsic to the myocardium that share molecular mimicry with viral peptides. The virus may also prompt a TH2 reaction, stimulating more CD8 killer cells in the process. It can be explained partially by the presence of evidence that CD4/CD8 or p56lck knockout animals have a much better survival following coxsackie infection (Liu et al., 1995). Recently, it has been shown that CD4+ Th cell subset, referred as Th17 cells, are involved in several inflammatory diseases, including experimental autoimmune myocarditis and collagen induced arthritis (Aggarwal et al., 2003, Chen et al., 2006). In addition to the proinflammatory effects, evidence from previous works suggested that Th17 cells may facilitate the production of autoantibodies in the development of acute viral myocarditis (Yuan et al., 2009). In experimental study published last year (Yuan et al., 2010), they showed that IL-17 produced by Th17 may take part in the regulation of the equilibrium between antiviral immunity and autoimmunity in CAVB3-induce acute viral myocarditis and IL-17 will be a new therapeutic goal for viral myocarditis in future (Milenkovic et al., 2010).
Although the activation of CD4 cells also leads to B-cell clonal expansion and antibody production, antibodies might not be the critical initiating factor that directs the advancing of the myocarditis. The severity of myocarditis was also found to be dependent on the responding T-cell subset in T-cell knockout mice (Opavsky et al., 1999). But, it has been shown that antibodies are a significant modifier of the disease phenotype. In a study conducted in 1982, it was found that among 30% of cases with suspected myocarditis, as well as in 18/19 patients with proven viral infection due to coxsackie, influenza A or mumps virus (Maisch et al., 1982). They also showed that antimyolemmal antibodies was correlated with the degree of in vitro induced cytolysis of rat myocytes.
Adenoviral myocarditis differs from coxsackie virus in the setting of pathogenesis (Hayder&Müllbacher, 1996). The amount of CD2, CD3 and CD45ROt lymphocytes detected in the adenovirus-infected cases was decreased compared with those patients who had myocarditis with other pathogens (Pauschinger et al., 1999). It was also demonstrated that 71% of PCR-positive adenoviral did not have inflammation histologically (Martin et al., 1994). One of the strategies of adenovirus for modulating immune response is that interaction of adenoviral encoded proteins with host immune components. These proteins may protect cells from tumor necrosis factor mediated lysis, as well as downregulation of major histocompatibility complex class I antigen expression. On the other side, adenoviral E1a encoded proteins are able to encourage the induction of apoptosis and inhibition of interleukin-6 (IL-6) expression (Davison et al., 2003). Besides this, it restricts IL-6 signal transduction pathways. These functions of E1A may be relevant to the occurrence of dilated cardiomyopathy. HIV has been identified within myocytes and is related with interference of myocyte integrity and replacement of endocardial fibrosis. Therefore, it was suggested that HIV myocarditis may share similar pathogenic mechanism of those of coxsackie (d'Amati et al., 2001).
It should be underlined that the natural killer (NK) cells is also important in the pathogenesis of myocardial inflammation. These finding corroborate those observed in a prior animal study, in which animals depleted of their NK cells previous to infection with coxsackievirus develop a more severe myocarditis (Godeny et al., 1987). The NK cells particularly reduce the nonenveloped virus infection by destroying the infected cells.

3.3 Dilated cardiomyopathy

Several remodelling mechanisms leading to dilated cardiomyopathy may be particular to myocarditis. The association of myocarditis to dilated cardiomyopathy has been moderately elucidated by molecular techniques. Badorff and Knowlton, demonstrated that dystrophin is cellular target for coxsackie B3 viral protease (Badorrff&Knowlton, 1999). It may provide one of the molecular mechanisms clarifying the significant ventricular dilatation that may develop immediately following viral infection. Furthermore, it was found that dystrophin deficiency augments host vulnerability to coxsackie virus infection (Xiong et al., 2002). This conclusion may result from the findings that more efficient liberation of the virus from the infected myocytes and is related with an increase in virus-mediated cytopathic effects. Proteases from other viruses (adenovirus and HIV) may also cleave cytoskeletal proteins (Chen et al., 1999, Shoeman et al., 1993).

Identification of viral RNA at early, intermediate and late stages of myocarditis has been demonstrated in animal models. That persistent myocyte viral gene expression may be a cause of progressive dilated cardiomyopathy. Some findings propose that the persisting viral RNA seems to be capable of replication. In 172 patients with biopsy-confirmed viral infection, persistent viral genome of enterovirus, parvovirus B19, and HHV-6 was found to be associated with on-going impairment in ejection fraction (Kuhl et al., 2005); But, in the lack of measurable virus titers, it appears likely that the replication can be done in a limited or transformed manner (Klingel et al., 1992). All the more so, such replication might produce novel antigenic non-infectious or defective interfering viral elements, sufficient to cause evolving myocardial injury (Kawai, 1999). Cytokines possibly will participate in the development of dilated cardiomyopathy (Ono et al., 1998). During the second phase, they stimulate the matrix metalloproteinases, such as elastase, colleganases and gelatinase. Moreover, various reports suggest that several different viruses perform as a trigger for apoptosis. In addition to an immune-mediated mechanism activated by viral infection and persistent viral RNA in the myocardium, apoptosis may provide the third mechanism to elucidate the development of dilated cardiomyopathy.

3.4 Host factors

It is not fully understood which factors may define susceptibility to viral myocarditis and the development of cardiomyopathy. There are still remaining issues to be answered as why is it that who are in proximity to each other may be infected with the identical virus, but all do not develop myocardial injury? Why do certain infected cases continue to develop mild versus severe myocarditis or cardiomyopathy. But, up to date, the presence of genetic and environmental factors have been documented that influence particularly to viral myocarditis. Risk factors associated with severe myocarditis include age, viral variant, exercise, mouse strain and sex (Woodruff, 1980). Biochemical alterations such as selenium deficiency, vitamin E deficiency (Beck et al., 2003) and mercury exposure (Illback et al., 1996) have been documented to increase the viral virulence. Host genetic configuration not only influences the pathogenic mechanism of disease but also affects the severity of myocarditis. HLA-DQ locus and CD45 polymorphisms were to found to be essential determinants for early viral infection (Tchilian et al., 2006). Several investigations documented the significant association between dilated cardiomyopathy and MHC class II antigens, primarily HLA DR4 (Carlquist et al., 1991, Limas&Limas, 1989). In the study published in the Annals of Human Genetics, it is documented that HLA-DQA1* 0501 and DQB1*0303 are related to genetic susceptibility to idiopathic dilated cardiomyopathy (IDC), while DQA1* 0201, DQB1*

0502 and DQB1* 0504 present protection from IDC (Liu et al., 2005). However, associations of MHC class II alleles with dilated cardiomyopathy are possibly affected by ethnicity, sex, age and geographical variations. Besides the MHC haplotype, non- MHC genes should be considered (Neu et al., 1987). Two non-MHC loci on murine chromosomes 1 and 6, referred as Eam1 and Eam2, respectively, might influence autoimmune myocarditis (Guler et al., 2005). These loci intersect with loci implicated in other autoimmune diseases, such as lupus and diabetes, might give a clue that various autoimmune disease could be controlled by related genetic mechanisms. Initial antiviral response by the host has been recognized to be mediated at least in part by Toll-like receptors. TLR3 was found to play a significant role in the host innate immune response to infection with several cardiotropic viruses. Recent data suggests that variations in TLR3 alter the innate immune response and might change host susceptibility to increased cardiovascular pathology (Gorbea et al., 2010).

4. Clinical features

As stated previously, clinical picture of myocarditis is extremely variable, ranging from asymptomatic ECG abnormalities to heart failure (Dec et al., 1985, Bowles et al., 2003). The term ''*Acute* fulminant myocarditis'' is used for cases with severe congestive heart failure or cardiogenic shock (Amabile et al., 2006). Age of child influences clinical presentation of myocarditis (Dec et al., 1985). Viral prodrome of flu-like illness, respiratory symptoms or gastroenteritis may precede symptoms of heart failure. Neonates and infants present with poor feeding, irritability or listlessness, diaphoresis, apnea and episodic pallor. Usual symptoms of congestive heart failure, as well as mild cyanosis and pallor are observed on physical examination. It should be emphasized that neonates and affected younger infants may have intrauterine myocarditis with chronic course (Bowles et al., 2003). For very young infants acquiring myocarditis in the peripartal period, the prognosis is very poor, with more than 90% of children dying. Previous studies have implicated the myocarditis as the cause of sudden death (Friedman et al., 1998, Bowles et al., 2003). In the retrospective study of Krous et al., they evaluated the infants who died of sudden death infant syndrome in a safe sleep environment, accidental suffocation or myocarditis were assessed, and they have noticed the manifestation of scattered inflammatory cells and necrotic myocyte were noticed (Krous et al., 2009). From this observation, they suggested that few scattered inflammation and necrotic myocyte were normal finding in the developing heart exposed to new environmental pathogens. However, degree of cardiac infiltration was found to be greater in infants who died of myocarditis. In German study published in 2004, researchers found that viral myocardial affection is the cause of death in cases with SIDS (Dettmeyer et al., 2004). Besides, it was proposed by authors that PVB19 seems to play a more significant role than presumed so far.

Recent history of viral disease 10 to 14 days preceding presentation typically occurs in older children and adolescents (Friedman et al., 1998). Nonspecific gastrointestinal and respiratory complaints are more common than chest pain (Vashist&Singh, 2009). Jugular venous distension and pulmonary rales may be seen, and the resting tachycardia may be obvious, unlike in neonates. Since symptoms of myocarditis vary considerably in children, diagnosis can be challenging. Durani and colleagues, documented that most patients present with complaints of shortness of breath having tachypnea at presentation (Durani et al., 2009). Vomiting (48%) and poor feeding (40%) are also commonly seen in myocarditis. The authors also observed that the diagnosis of myocarditis was missed on the first presentation

to a physician in 83% of cases. In a retrospective review, of 31 children with probable and definite myocarditis, 57% were initially diagnosed as suffering pneumonia or asthma (Freedman et al., 2007). One point that deserves attention in these studies is the absence of other signs of congestive heart failure in the majority of cases with myocarditis. Only 50% of children had hepatomegaly and 34% had abnormal chest radiography. The electrocardiographic changes include sinus tachycardia with low voltage QRS complexes, inverted T waves typically occur in the clinical setting of myocarditis (Durani et al., 2009). Wide Q waves and ST segment changes as a pattern of myocardial infarction also may be observed. Supraventricular tachycardia, ventricular tachycardia or atrial fibrillation, as well as atrioventricular block may occur (Friedman et al., 1994). Of note, the sensitivity of electrocardiography in myocarditis is only 47% (Morgera et al., 1992). However, Freedman and colleagues found that sensitivity of electrocardiography as a screening test was 93% (Freedman et al., 2007). Additional interesting finding that is worth to mentioning that the presence of axis deviation (%37) in children with myocarditis. Although ventricular tachycardia is a rare initial manifestation of myocarditis, it may often develops in long-term follow up and may occasionally result in sudden death (Drory et al., 1991).

Myocarditis imitating an acute coronary syndrome has also been defined. Viral genomes were demonstrated in 71% of adult cases with normal coronary anatomy, clinically mimicking myocardial infarction. Parvovirus B19 was most common agent identified in this study (Kuhl et al., 2003). ECG criteria (wide Q waves in I, avL, V5 and V6, ST segment change > 2 mm, ventricular arrhythmias) was described for the diagnosis of myocardial infarction in children (Towbin et al., 1992). However, similar ECG findings can occur in myocarditis (Durani et al., 2009). Most common ECG findings in adult patients with myocarditis include ST segment elevation(55%), T wave inversion (27%), ST segment depression (27%) and pathological Q waves (%18) (Dec et al., 1992, Angelini et al., 2000). Myocardial infarction and myocarditis in neonates overlap and mimic each other (deVetten et al., 2011). Despite angiographically normal coronary anatomy, global or segmental wall motion abnormalities are commonly obvious (Angelini et al., 2000). Physicians should always bear in mind the possibility of acute myocarditis in younger cases who present with acute coronary syndromes when coronary risk factors are lacking, global rather than segmental left ventricular dysfunction is evident on echocardiography or ECG abnormalities encompass beyond a single coronary artery zone (Magnani&Dec, 2006).

5. Diagnosis

Although the misdiagnosis of the myocarditis is common, several diagnostic methods can aid physicians in making diagnosis of the myocarditis.

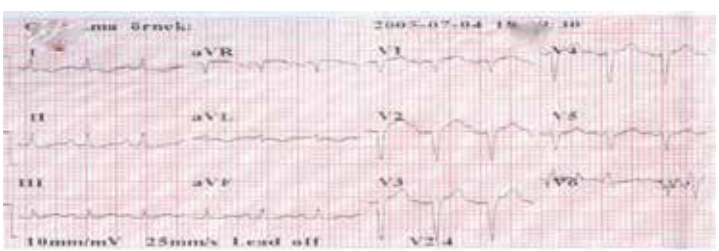

Fig. 3. 12 lead ECG in children with myocarditis. Negative T wave in DI, aVL, ST-T changes were noted in precordial leads.

5.1 Electrocardiography and chest radiography

Chest radiography and electrocardiography (ECG) can be used as first line diagnostic modality (Figure 3). Most common ECG changes are sinus tachycardia, axis deviation, ventricular hypertrophy and ST-T wave changes (Freedman et al., 2007). Moreover, evidence from previous studies suggests that the presence of northwest axis deviation, new left bundle branch block and abnormal QRS complexes is correlated with higher rates of transplantation or death (Magnani et al., 2006, Morgera et al.,1992, Nakashima et al., 1998, Greenwood et al. 1976). A recent adult study have shown that QRS prolongation is an independent predictor for transplantation or death in patients with suspected myocarditis (Ukena et al., 2011). In the majority of cases of myocarditis (up to 90%), abnormal chest radiography was documented (Durani et al., 2009, Freedman et al., 2007). Most common chest radiography finding is cardiomegaly, followed by pulmonary edema and pulmonary infiltrate (Figure 4).

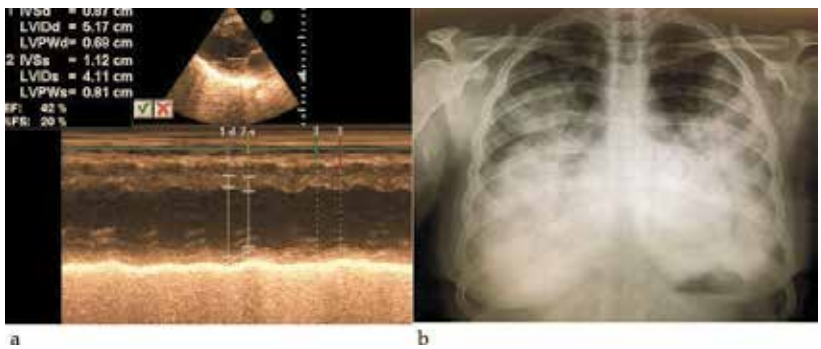

Fig. 4. M-mode echocardiography (a) and chest x-ray (b) of an adolescent girl with presumed viral myocarditis. M-mode echocardiography demonstrating systolic dysfunction with flattened interventricular septum. In chest radiography, there was prominent diffuse vascular congestion that is compatible with pulmonary edema. 13 year old girl admitted our hospital with dyspnea and tachycardia. She had a history of preceding viral upper respiratory infection. The patient intubated and connected to mechanical ventilation on day one of emergency room admission. She died within 72 hours after initial presentation.

5.2 Laboratory finding

General markers for inflammation such as erythrocyte sedimentation rate and C-reactive protein in serum are commonly elevated. However, their usage in diagnosis of myocarditis is limited. Freedman et al. demonstrated that the most sensitive marker for myocarditis was an increased aspartate transaminase (AST). AST elevation was found in 85% of probable and definite cases of myocarditis (Freedman et al., 2007). On the other hand, C-reactive protein and erythrocyte sedimentation rate have been elevated in cases of myocarditis with a range of 27 to 56%. Cardiac troponin t (cTnT) has also been investigated as a diagnostic marker for acute myocarditis since 1990's. cTnT, a contractile protein unique to cardiac muscle, is vastly concentrated in the myocytes and will be released into the blood within hours after heart muscle injury. Following myocardial cell necrosis an increased concentration of cTnT is noticable in blood for more than a week. Cardiac troponin T measurements are especially useful in clinical settings in which traditional enzyme determinations fail to diagnose myocardial cell damage effectively. Likewise, cTnT is not reliably corresponded to increases

in blood of cardiac enzymes or myoglobin in all cases of Wolff-Parkinson-White syndrome undergoing radiofrequency ablation. Cardiac troponin I, subunit of thin filament of contractile element of the myocardium, has high specificity (89%) and low sensitivity (34%) in adult patients with acute myocarditis, whereas cTnT has been documented to have a specificity of 83% and sensitivity of 71% in children. Moreover, higher levels of cTnT have been demonstrated to be a prognostic marker for poor outcome in adults presenting with acute myocarditis. Elevated levels of interleukin-10 (IL-10) and TNF appears to be predictor of fulminant myocarditis. Besides this, increase of serum Fas and Fas ligand levels, as well as immunohistological signs of inflammation (CD3 and/or CD68) on initial presentation are associated with fatal outcome in patients with acute myocarditis.

5.3 Echocardiography

Echocardiographic features of myocarditis are nonspecific. Patterns of echocardiography in myocarditis could mimic hypertrophic, dilated or right ventricular cardiomyopathy and as well as ischemic heart disease (Checcia&Kulik, 2006). Echocardiography can be used for assessing wall thickness, cardiac chamber size together with systolic and diastolic functions. Right ventricular dysfunction is relatively unusual. However, right ventricular dysfunction was found to be predictor of adverse outcome in patients with active myocarditis (Mendes et al., 1994). Left ventricular diastolic dysfunction with a restrictive pattern is also observed in most cases of myocarditis. Left ventricular wall thickening was found to be highest on days 1-3 after onset of acute myocarditis. It has also been noted that left ventricular thickening was more marked in the fulminant myocarditis (Felker et al., 2006). On the contrary to adult patients, echocardiographic findings of pediatric patients revealed that relatively thicker posterior wall was correlated with better prognosis and recovery (Carvalho et al., 1996). Segmental wall motion abnormalities are relatively frequent, but global hypokinesis is prevalent. Pericardial effusion commonly occurs. The presence of thrombi in ventricle has also been documented in up to 25% of cases (Daly et al., 1983).

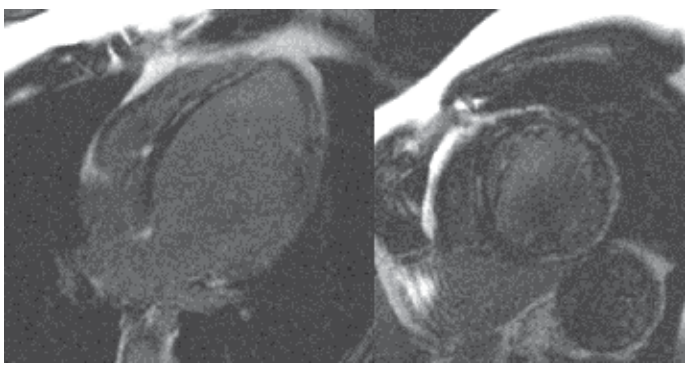

Fig. 5. Cardiac magnetic resonance imaging of acute myocarditis in a adolescent. In four chamber and short axis view, subepicardial late enhancement are noted. Text and image courtesy of Alper Yuksel, Yigit Goktay.

5.4 Magnetic resonance imaging

Current practice has focused on the use of cardiac magnetic resonance imaging (CMR) for the diagnosis of acute myocarditis (Gutberlet et al., 2008, Friedrich et al., 1998). CMR with a

unique potential for tissue characterization, particularly with the utilization of T1 and T2 weighted images, can assess 3 markers of tissue injury, which is, hyperemia and capillary leakage, necrosis and fibrosis and intracellular and interstitial edema (Friedrich et al., 2009). CMR visualizes the entire myocardium, recognizing borders of inflammation from later modeling. Thus, it can be used to monitor lesions and be used to show the execution of endomyocardial biopsy, as well as it may be useful in the quantification of the magnitude of damage (Danti et al., 2009, Mahroldt et al., 2004). Goitein et al. demonstrated that cardiac MRI have a larger impact than echocardiography in verifying the existence of myocarditis and evaluating the extent of disease (Goitein et al., 2009). It has been shown that echocardiography is useful in revealing wall motion abnormalities, whereas cardiac MRI could actually identify the often subtle patchy myocardial inflammation (Friedrich et al., 1998). Gadolinium is used as a contrast agent due to ability to penetrate cells whose membranes ruptured and allows contrast agent to diffuse into the cells (Weinmann et al., 1984). Myocardial blood flow and edema, that is likely to be increased in tissues which are inflamed, could augment signal enhancement in MRI. But, cardiac MRI features can be missed on the first pass perfusion (Skouri et al., 2006). Delayed enhancement MRI permits visualization of necrotic and fibrotic myocardium (Friedrich et al., 2009). The observations obtained from the studies using contrast media-enhanced cardiac MRI indicate that pattern of myocarditic lesions occur predominantly in the lateral free wall and get localized to the subepicardial or intramyocardial regions (Mahroldt et al., 2004, Friedrich et al., 1998). The finding of lateral free wall involvement (subepicardial region) partially explain why some young patients with acute myocarditis can present with only ST elevation on ECG (Figure 5). Postmortem studies also showed that lateral wall was the preferred location in myocarditis (Theleman et al., 2001, Shirani et al., 1993). Subendocardial region involvement pattern which is typical for myocardial infarction was never seen in patients with acute myocarditis (Mahroldt et al., 2004). Mahroldt et al. also demonstrated that in the right ventricle half of septum, that is common location of EMB, had relatively low density of inflammatory cells. Apart from lateral free wall pattern, Marhold et al showed that HHV6 myocarditis had pattern that was located in midwall area of the interventricular septum. Pericardial effusion has also been reported in 32 to 57% of cases with myocarditis (Friedrich et al., 2009). Its presence, although not specific for myocarditis, is a supportive evidence for active inflammation.

Recently, International Consensus Group on Cardiovascular Magnetic Resonance suggested the diagnostic criteria, known as ''Lake Louis Consensus Criteria'' (Friedrich et al., 2009). Cardiac MRI should be made in the setting of clinically suspected myocarditis according to these criteria. It was also stated that maximum diagnostic accuracy can be accomplished with the presence of any two or more of the following criteria: Regional or global myocarditis signal increases in T2 weighted images, increased global myocardial early gadolinium enhancement ratio between myocardium and skeletal muscle (T1 weighted images) or presence of at least one focal lesion with nonischemic regional distribution (late gadolinium enhancement).

In a retrospective study published in 2009, researchers found that myocarditis in children is characterized mainly by subepicardial and transmural enhancement. Global hypokinesia, left ventricular dilatation, ejection fraction less than 30% and transmural myocardial involvement were discovered to be associated with poor outcome (Vashist et al., 2009).

5.5 Biopsy

Despite its limitations, EMB is the gold standard for diagnosis of myocarditis. Together with simultaneous PCR and immunohistology, rapid detection of the viral genome is possible (Checcia&Kulik, 2006). The Dallas criteria have regulated the definition of myocarditis (Aretz et al., 1987). Active myocarditis is considered if light microscopy shows infiltrating lymphocytes and cytolysis. One of the potential advantage of this procedure it may aid physician in determining the management of myocarditis. Children with viral myocarditis may benefit from therapy with immune suppression while patients with cardiomyopathy may not (Liu et al., 2001). Several issues have to be considered before making a decision about biopsy. As stated earlier, some complications such as pneumothorax, dysrhythmia, perforation and death, may occur during the procedure, and it can be hazardous for particularly pediatric patients (Pophal et al., 1999). Limited sensitivity of EMB that is related with sampling error should also be evaluated (Hauck et al., 1989). Substantial controversy exist with respect to diagnostic criteria for examining tissue specimens. Poor interobserver variability may limit the utility of Dallas criteria (Shanes et al., 1987). A scientific statement from the American Heart Association, The American College of Cardiology and European Society of Cardiology published in 2007, has evaluated the role of EMB in myocarditis (Cooper et al., 2007). Various clinical scenarios have been described. Of these only two have received class I recommendation for EMB (Table). In a retrospective review analyzing the morbidity and mortality of EMB in children, highest risk was found in children with suspected myocarditis on inotropic support (Pophal et al., 1999). Authors also found that risk of biopsy in small children (< 10 kg) or sick infants was extreme. Compared with established risk of EMB in adults, there is an increased risk in children. Thus, careful risk-benefit analysis should be therefore undertaken for each patient.

6. Treatment

In spite of the significant progress in understanding the mechanisms of myocarditis pathogenesis in last two decades, advances in treatment strategies are still limited and the supportive care is the principal therapy. Most patients with acute myocarditis presenting with dilated cardiomyopathy respond favorably to standard anticongestive therapy including afterload reduction, diuretics, angiotensin converting enzyme inhibitors and the introduction of β blockers such as carvedilol or metoprolol succinate once the acute phase is controlled. Various experimental studies with β adrenoreceptor inhibitors or agonists showed different effects in acute myocarditis. Treatment with propranolol in mice infected with encephalomyocarditis virus (EMCV) reduced the severity of myocarditis and mortality (Wang et al., 2005). On the other hand, carvedilol, non-selective β blocker, improved the survival and decreased the virus replication of mice infected with EMCV through the enhancement of IL-12 and IFN-γ production, whereas metoprolol had no effect on this murine model (Nishio et al., 2003). Despite the lack of extensive studies in pediatric patients, administration of carvedilol has been found to be associated with improvement of left ventricle function and clinical symptoms and normalization of antioxidant enzyme activity (Bajcetic et al., 2008). Similar to effects of β blockers, ACE inhibitors and angiotensin receptor blockers have been documented to lessen viral myocardial injury in murine models (Yamamoto et al., 2003). It is also proposed that early introduction of beta-blockers and ACE inhibitors might prevent the remodelling that advances to dilated cardiomyopathy (Ellis&DiSalvo, 2007). Phosphodiesterase inhibitors such as milrinone, if well tolerated, can

be really helpful. A recent multi-institutional analysis revealed that milrinone was used most often for vasoactive support in children (Klugman et al., 2009). Anticoagulants should be considered if ejection fraction is severely decreased or in the setting of atrial arrhythmia (Gunthard et al., 2009). Digoxin should be used in low dose and with caution in patients with viral myocarditis since high dose digoxin was proven to increase mortality in animals with EMVC induced myocarditis as well as elevate intracardiac production of cytokines (Matsumori et al., 1999). Ventilation and oxygenation could be best achieved with continuous positive airway pressure (CPAP) or other non-invasive methods. CPAP, unloads inspiratory muscles and leads to decreased left ventricular afterload without compromising cardiac index via increasing intrathoracic pressure. Medications used for intubation can cause hypotension and acute cardiovascular collapse, thus CPAP also avoids this and is an outstanding adjunctive therapy for cardiac failure and myocarditis (Bradley et al., 1992, Naughton et al., 1995).

Extracorporeal membrane oxygenation support and ventricular assist device might be particularly useful for patients with fulminant myocarditis. Extracorporeal membrane oxygenation may also be considered in those who are in the recovery phase from acute myocarditis (Sezai et al., 2007). For patients with cardiogenic shock because of the acute myocarditis who worsen despite ideal medical therapy, extracorporeal membrane oxygenation and ventricular assist device may help as a bridge to transplant (Moloney et al., 2005). The full mobilization, survival rates up to 90% for fulminant myocarditis and decreased anticoagulation make these pulsatile ventricular assist device systems as the alternative choice of therapy for children (Patopov et al., 2007). Cardiac transplantation is reserved only for patients who are intractable with medical management and mechanical circulatory support. Almost half of the annual cardiac transplantation cases are performed for idiopathic dilated cardiomyopathy, at least 10% of which represent as myocarditis (Ellis&DiSalvo, 2007).

6.1 Immune therapy

It is well known that the long term morbidity and mortality following viral myocarditis seem to be dependent on cellular and humoral immunity abnormalities. Therefore, many investigations have been conducted to search the use of immunosuppressants and immune-modulator agents for treatment of acute myocarditis and dilated cardiomyopathy. However, debate still persists on whether immune therapy for acute myocarditis is useful or not. Initial adult studies investigating the effect of prednisone with or without azathioprine and cyclosporine demonstrated a slight improvement in left ventricular function. But, this improvement was temporary (Mason et al., 1995, Parillo et al., 1989). In a study conducted by Parillo et al., patients were grouped as reactive or nonreactive on the basis of histopathology, immunoglobulin deposition on EMB, an increased erythrocyte sedimentation rate or a positive gallium scan. At three months, reactive patients who were treated with prednisone (60 mg daily) had a statistically significant increase in ejection fraction compared with controls. After six months, improvement seen earlier was no longer present. In 1995, the Myocarditis Treatment Trial failed to show neither an improvement in left ventricle ejection fraction at 28 weeks nor an improvement in survival up to 4.3 years (Mason et al., 1995). Although a few uncontrolled studies showed benefit with several immune suppressive agents, meta analysis of adult studies did not confirm a significant favourable effect of immunosuppression (Garg et al., 1998, Maisch et al., 1998). There were also investigations to evaluate the results of immune suppressive regime in children with

acute myocarditis (Chan et al., 1991, Camargo et al., 1995). However, studies in children are inadequate and yet, no randomized controlled trials are present. In a study (Camargo et al., 1995) conducted among 68 children with severe dilated cardiomyopathy, patients were classified into either conventional treatment or given one of three immune suppressive agents, prednisolone, prednisolone plus azathioprine and prednisolone with cyclosporine. Children taking immunosuppression treatment with a second agent, demonstrated enhanced hemodynamic parameters, as well as histological improvement in inflammation. A meta analysis (Hia et al., 2004) assessing the impact of immunosuppression on the outcome of acute myocarditis in children was published in 2004. Better outcome was observed among children who received immunosuppressive therapy. On the other hand, the findings were not statistically significant. Randomized large controlled studies are needed to conclude that immunosuppressive therapy is beneficial for outcome of children with acute myocarditis. On the contrary, one trial published in 1997, demonstrated that survival was improved with the treatment of cyclosporine and corticosteroids in patients with giant cell myocarditis (Cooper et al., 1997). A different approach has also been investigated, in which Wojnicz et al. used HLA expression on endomyocardial specimens to classify inflammatory cohort (Wojnicz et al., 2001). Of 202 patients with dilated cardiomyopathy, 84 patients with increased HLA expression were randomized to receive either placebo or immunosuppression for 3 months. After 2 years, significant improvement in ejection fraction and end diastolic diameter were noted only among the immunosuppressive group.

It was suggested that intravenous immunoglobulin (IVIG) may be an useful therapy for acute myocarditis due to its both antiviral and immunomodulating effects. Previously, it has been shown that IVIG may be used in several autoimmune disorders, including idiopathic thrombocytopenic purpura, systemic vasculitis and Kawasaki disease (Rosen et al., 1993, Wolf et al., 1996). Up to date, there are no randomized controlled studies evaluating the use of IVIG to treat the children with acute myocarditis. A systemic review conducted by Robinson et al., evaluated the use of intravenous immunoglobulin therapy in acute myocarditis in both adults and children (Robinson et al., 2005). They determined that intravenous immunoglobulin might be useful in the presence of ongoing or active infection which may be causing obstinate cardiac failure. In a study conducted in children with presumed viral myocarditis, high dose IVIG treatment was found to be associated with improved recovery of left ventricular function and with a tendency of better survival (Drucker et al., 1994). In adults, the results of a randomized clinical trail suggested that for patients with recent onset dilated cardiomyopathy, IVIG did not enhance an improvement in ejection fraction (McNamara et al., 2001). However, in this cohort, ejection fraction was increased considerably during follow-up and short term prognosis remained favourable. Despite the presence of several case reports indicating that adults treated with intravenous immunoglobulin reveal better cardiac function, Cochrane review of IVIG administration in myocarditis and dilated cardiomyopathy demonstrated no benefit in adults (McNamara et al., 1997, Tedeschi et al., 2002). On the other hand, little is known about the exact mechanisms responsible for potential benefits of IVIG in the therapy of patients with acute myocarditis. Several studies both in the clinical setting and experimental models propose that immunoglobulin may reduce inflammatory cytokines that have direct negative inotropic effects and decrease the oxidative stress (Kishimoto et al., 2003).

Numerous cases with myocarditis recover spontaneously. It is hard to know if the noted improvement is a consequence of therapy with IVIG or immunosuppression versus natural

course of the disease. Therefore, studies assessing immunomodulation and immune suppressive agents were problematic to decode into an applicable, routine treatment for children and adults with acute myocarditis. One should also consider that initiating agents for acute myocarditis and following clinical course may change from time to time and by geographic site. Although such a controversy remains to be settled, IVIG may be used only in selected pediatric patients with acute myocarditis.

6.2 Antiviral treatment & vaccines

While viral infection is the most frequent cause of myocarditis, it might be possible to think that vaccines and antiviral agents might be helpful in the treatment of myocarditis. It is obvious that studies using polymerase chain reaction identified viral genomes in patients with acute myocarditis (Bowles et al., 2003). Bu, there are a few studies which demonstrated that requirement for transplantation and mortality was not dependent on the presence of viral genome (Kindermann et al., 2008, Kuhl et al., 2005). So, many investigators suggested that the presence of viral antigens or nucleotides in the myocardium alone is not satisfactory to prove that the virus is the cause of myocarditis (Matsumori et al., 2007). Since the diagnosis of viral myocarditis is frequently challenging and the diagnostic approaches have not been established or standardised, the number of clinical trials for virus proven myocarditis is limited. For that reason, in order to investigate therapeutic and preventative methods for myocarditis, various animal models have been developed. Several promising new agents including peroxisome proliferator activated gamma receptor activator, rapamycine, pycogenol, SUNC8079 and mycophenol mofetil have been studied in murine models of myocarditis during the last decade (Komiyoshi et al., 2005, Ellis&DiSalvo, 2007, Matsumori, 2007). It has been demonstrated that these agents decrease the severity of myocarditis and improve cardiac function, blocks activation of NF-κ, blocks mRNA expression of key cytokines (IL-1, IL-6 and TNF) and stabilizes mast cell (Matsumori, 2007). Synergistic effect of IFN- α and ribavirin has been demonstrated against both EMCV and coxsackie virus infection (Okada et al., 1992, Matsumori, 2007). IFN- β has reported to be effective in studies including small number of patients with left ventricular dysfunction whose biopsy specimens were positive for adenovirus or enterovirus (Kuhl et al., 2003).

Although various strategies for the prevention of acute myocarditis have been studied in murine models, up to now, there have been no vaccination trial in humans. Vaccination against mumps, rubella, poliomyelitis, measles and influenza has made myocarditis consequent to these infections quite rare and increases the arguments on whether vaccination against other cardiotropic viruses might prevent myocarditis in the future. A classical example in this regard was supported by the study of EFE described previously (Ni et al., 1997). The mumps virus vaccine has entirely eliminated this form of dilated cardiomyopathy. It is unlikely that antiviral vaccines to battle this disease will be improved in the near future due to low incidence of the disease.

6.3 Physical activity

Recommendations concerning physical activity affirm that all patients with presumed or definite myocarditis discontinue competitive sports and undergo a prudent convalescence period around six months after the onset of clinical manifestations. Athletes may return to sports activity if LV function, dimensions and wall motions return to normal, markers of inflammation in blood have resolved, 12-lead ECG has normalized and clinically relevant arrhythmias are absent on Holter ECG or graded exercise testing (Maron et al., 2005).

7. Outcome

Prognosis of myocarditis is as changed as its clinical presentations. Although the fewer data are available on the natural history of myocarditis in children, it is proposed that the outcomes in pediatric patients presenting with acute heart failure secondary to acute myocarditis tends to be more positive than the prognosis with dilated cardiomyopathy (Drucker et al., 1994, Lee et al., 1999). In a retrospective analysis of 36 children with histologically proven lymphocytic myocarditis (Lee et al., 1999), excellent outcomes have been demonstrated in children with myocarditis, especially those surviving 72 hours after presentation. Gagliardi and colleagues, classified 114 children into three groups as acute myocarditis, borderline myocarditis and non-inflammatory cardiomyopathy according to histological analysis (Gagliardi et al., 2004). Best survival rate (97%) was found in acute myocarditis group. They suggested that this high long term survival rate of this cohort may be due to effect of short term immunosuppressive therapy. On the other hand, in a multi-center study including children and adults, difference in outcomes between age groups was noted (Bowles et al., 2003). Survival rate for neonates and infants (33 and 45%, respectively) were significantly lower than the other groups. Survival rate was noted to be greatest in adolescent age group. A retrospective study involving 28 children with acute myocarditis, analysed the predictors of outcome. It was observed that ejection fraction < 30%, shortening fraction < 15%, left ventricle dilatation and moderate to severe mitral regurgitation at admission were associated with poor outcome (Kuhn et al., 2004). However, it was understood from the findings of adult trials that syncope, right ventricle dysfunction, elevated pulmonary artery pressure and advanced New York Heart Association functional class were predictors of increased probability of death or requirement for transplantation (Mendes et al, 1994, McCarthy et al., 2000, Magnani&Dec, 2006, Kindermann et al., 2008). Histological classification and severity of symptoms may also give a clue about prognosis. Giant cell myocarditis has a chance of 89% of death or transplantation. Surprisingly, acute fulminant myocarditis may have a better prognosis (Ellis & DiSalvo, 2007).

In general, transplantation is needed in 1-8% of patients with acute myocarditis (Ellis & DiSalvo, 2007). In spite of severe disease at presentation, there is a probability of improvement. Patients should not be listed promptly unless recovery is believed extremely unlikely despite judicious management.

8. Conclusion

Myocarditis in children is challenging given a variety of clinical manifestations that may share common pediatric illnesses such as respiratory infections and gastrointestinal disorders. A high index suspicion is so vital in the diagnosis. With the introduction of new additional diagnostic modalities including cardiac magnetic resonance imaging and biomarkers, cases will be identified easily in the future that would have been formerly missed. Myocarditis causes dilated cardiomyopathy in a significant portion of children. Prognosis for cases with acute viral myocarditis is much better than cases with established cases of dilated cardiomyopathy. Therefore, prompt diagnosis and early effective supportive care are crucial. Even if, much improvement has been achieved in pathogenesis, diagnosis and treatment of myocarditis, many questions remain to be answered and indicate the necessity for additional investigations.

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3

Acute Myocarditis in Emergency Medicine

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1. Introduction

Emergency doctors provide primary care to many patients with an acute-onset condition in the emergency room (ER) every day. We occasionally must address circulatory failure in patients that ranges from "acutely developing" to "severe." Usually, it is not difficult to diagnose or to choose the proper therapeutic procedures, because most of these cases are caused by heart diseases, such as ischemic heart disease or an arrhythmia. However, in some cases the causes of circulatory failure cannot be determined immediately.

It is known that cardiomyopathies account for some of those undiagnosed cases. Classically, cardiomyopathies have been regarded as idiopathic myocardial diseases because of the difficulty of detecting their etiology or the mechanisms causing the problem. Recent developments in biochemical technology have provided the option of approaching these unknown mechanisms using genetic analyses (Richardson et al., 1996). Cardiomyopathies are presently classified into several groups, defined by the cause, tissue type, and clinical course. In 2006, a committee of the American Heart Association (AHA) advocated new criteria for cardiomyopathies. With those criteria, primary cardiomyopathies are classified into a genetic type, a mixed type, and an acquired type (Maron et al., 2006).

We encounter several types of cardiomyopathy in the ER. Immediate adequate primary treatment of these cases must be prudent because circulatory insufficiency can rapidly progress to cardiac arrest.

In most cases, myocarditis is caused by an inflammatory response and is classified as an acquired type of primary cardiomyopathy. It is also categorized as either acute or chronic. The comprehensive concept of chronic myocarditis has not yet been established because cases of chronic myocarditis have not been sufficiently reported. For most cases of chronic myocarditis, the causes and developmental mechanisms remain unclear.

We have only practical guidelines for treating chronic myocarditis in Japan (JCS, 1996). These patients usually have long-term therapeutic histories. Therefore, the patients' own doctors who are familiar with their clinical histories should be responsible for treatment in the ER. On the other hand, most cases of acute myocarditis are of sudden onset, and severe cases are taken to the ER by the emergency medical service. Doctors in the ER must then be responsible for the primary treatment of these patients. Because there is the risk of sudden development of circulatory insufficiency with acute myocarditis, immediate and adequate initial treatment is necessary for survival.

In this chapter, we explain the causes, mechanisms as presently known, classification, and clinical course of myocarditis. Additionally, we outline the recommended treatment for the acute phase of myocarditis.

2. Epidemiology and prognosis

The clinical course of myocarditis varies. The symptoms range widely, from slight to severe, and it is difficult to investigate myocarditis epidemiologically. In 2002 in Japan, a large-scale investigation for cardiomyopathy was performed and reported that cardiomyopathy was found in 21.3 per 100,000 population (Miura et al., 2002). However, the subjects of this investigation were consulted patients with data provided by hospitals. It is thought that actual morbidity is higher, because there are many non-consulted persons with occult cardiomyopathy, such as early stage dilated cardiomyopathy or hypertrophic cardiomyopathy with no symptoms. Therefore, we still cannot grasp the exact morbidity of myocarditis in Japan. Okada et al. (1989) reported that among 10,000 autopsied cases during 1958–1978 there were 115 cases of myocarditis. In another report, occult myocarditis was found in 0.6% of autopsied cases in which cardiac disease had not detected while the patients were alive (Feely et al., 2000). Therefore, it should be recognized that myocarditis is not clinically infrequent and includes a mild type that displays no symptoms. The mortality rate for myocarditis has not been established. An investigative committee of the Ministry of Public Welfare and Labor in Japan reported in 1986 that 13 (4.7%) of 274 patients died within 1 month in a small-scale investigation targeting acute-phase myocarditis (Kawamura et al., 1986). Among those cases, the causes of death were cardiogenic shock in six cases (46%), congestive heart failure in five cases (38%), and complete atrioventricular block in two cases (15%). Intensive treatment for severe cardiac dysfunction and fatal arrhythmias are important to reduce mortality during the acute phase of severe myocarditis.

3. Cause of myocarditis and classification

Myocarditis is classified by cause, clinical type, and pathological picture. In clinical medicine, myocarditis presenting as a sudden development should be attended to because any delay of treatment presents a risk of fatal circulatory failure. It should be recognized that there is different prognosis depending on the clinical type of myocarditis. The classification of myocarditis is shown in Table 1.

3.1 Causes

Several causes or mechanisms of the development of myocarditis have been detected, among which infection is the most the frequent and important. In earlier years, myocarditis was often caused by rheumatic fever and diphtheria. More recently, in advanced nations it is mostly caused by viral infections. Bowles et al. (1986) reported that Coxsackie B virus was detected in patients with myocarditis using a dot blot hybridization method. Coxsackie virus, an enterovirus belonging to the picornavirus family, was extracted from patients with polio in 1982 and is particularly compatible with myocardium (Clements, 1993). Half of group A types and all of group B types of the Coxsackie virus cause myocarditis accompanied by symptoms of the common cold.

Presently, it is possible to detect many viruses with polymerase chain reaction or in situ hybridization. Therefore, we can detect myocarditis due to viral infection in most cases. In

Causes	Histological classification	Clinical classification
Infection	Lymphocytic myocarditis	Acute type
Virus	Giant cell myocarditis	Fulminant type
Bacterium	Eosinophilic myocarditis	Chronic type (including persistent symptoms type and unremarkable symptoms type)
Fungus	Granulomatous myocarditis	
Rickettsia		
Spirochete		
Parasite		
Others		
Drugs, chemical materials		
Allergy, autoimmunity		
Collagen disease		
Kawasaki disease		
Sarcoidosis		
Radiation		
Heat stroke		
Unknown (idiopathic)		

Table 1. classification of myocarditis. modified from the guideline for the diagnosis and treatment of myocarditis (JCS, 2005).

1. Nonsteroidal anti-inflammatory drugs
Indomethacin, oxyphenbutazone, phenylbutazone
2. Psychotropic drugs
(Tricyclic) antidepressant: imipramine, clomipramine, amitriptyline
Antimanic: lithium carbonate, lithium oxalic acid
Antiepileptic: phenytoin, carbamazepine
3. Diuretic: acetazolamide, hydrochlorothiazide, spironolactone, chlorthalidone
4. Depressor: methyldopa
5. Anticancer drug: adriamycin, daunorubicin, mitoxantrone
6. Antibiotics
Amphotericin B, penicillin, ampicillin, tetracycline
Chloramphenicol, streptomycin
7. Sulfaminum: sulfadiazine, sulfisoxazole
8. Antiphthisic: isoniazid (INH), para-aminosalicylic acid (PAS)
9. Biological agents: tetanus toxoid, α-interferon, interleukin 2 (IL-2)
10. Antidiabetic: sulfonylurea
11. Others: catecholamines, cocaine, amphetamine, arsenic

Table 2. Drugs that cause myocarditis.

recent reports, adenovirus, sharing the same receptors as the Coxsackie virus, parvovirus B19, human herpes virus 6, and hepatitis C virus, are known to cause myocarditis (Okabe et al., 1997).

Myocarditis does not always develop in patients infected by a virus compatible with the myocardium (e.g., the Coxsackie virus). In such cases, symptoms of myocarditis are unremarkable or slight, and the mechanism of development of myocarditis remains unknown. Based on the results in experimental animal models, Cupta et al. (2008) suggested a developmental pattern of myocardial damage by an infective virus and the host's reactions as follows: (1) initial infection of virus in myocardial cells; (2) innate immune reaction; (3) defense by adaptive immunity; and (4) recovery of inflammatory reaction.

Several kinds of drugs, external physical stimulations such as exposure to irradiation or excessive heat, metabolic disorders, pregnancy, collagen diseases, sarcoidosis, and Kawasaki disease are important causes of myocarditis. It should be well noted that drug-induced myocarditis, particular in Japan, Europe, and the United States, is caused by many of the drugs that are administered or prescribed to patients in advanced countries. Drugs that cause myocarditis are shown in Table 2.

3.2 Histological classification

Histologically, myocarditis is classified as lymphocytic myocarditis, giant cell myocarditis, eosinophilic leukocyte myocarditis, and granuloma myocarditis. Most cases of lymphocytic myocarditis are caused by infection. Other types are due to materials toxic to the myocardium, a drug allergy, or an autoimmune reaction.

3.2.1 Lymphocytic myocarditis

Lymphocytic myocarditis is the most frequent histological type, and most cases are caused by a viral infection. Kodama et al. found that 85% of patients with myocarditis were diagnosed with the lymphocytic variety (Kodama et al., 2001). The primary symptoms—fever and pharyngeal pain—are not specific for myocarditis during the early phase, but symptoms of circulatory failure, such as edema, are specific and are observed during the developed phase. The severity of circulatory failure ranges widely from slight to fulminant.

3.2.2 Giant cell myocarditis

Giant cell myocarditis is encountered infrequently. Clinically, it has a poor prognosis because it can easily progress to fulminant myocarditis. After the onset, circulatory failure develops suddenly and in many cases rapidly progresses to a fatal state. It is suggested that an allergic reaction or an autoimmune reaction participates in this development. Infiltration by inflammatory cells and multinucleated cells are observed in the myocardium (Davidoff et al., 1991). Cardiac function markedly deteriorates with massive necrosis of the myocardium. Identification of multinucleated cells is necessary for a definitive diagnosis. It is difficult to decide on the timing of a biopsy because multinucleated cells are seen only during the acute phase. In patients presenting with a chronic course, cardiac sarcoidosis must be excluded (Shield et al., 2002).

3.2.3 Eosinophilic myocarditis

The clinical symptoms of eosinophilic myocarditis—fever, pharyngeal pain, cough—during the early phase are similar to those seen with myocarditis due to a viral infection. The

differential diagnosis between viral myocarditis and eosinophilic myocarditis is often impossible because the eosinophil count is normal during the early phase of eosinophilic myocarditis (Morimoto et al., 2003). White blood cells (WBCs) gradually increase with the development of an inflammatory reaction; and materials toxic to the myocardium released from eosinophilic leukocytes cause severe contraction of the myocardium as the eosinophil count exceeds 500/nm^3.

In most cases, the increase in eosinophils is detected in a peripheral blood examination. However, some patients who suffer from circulatory insufficiency exhibit a delayed increase in eosinophils (Gets et al., 1991). Therefore, we should perform more frequent eosinophil counts during the acute phase. Infiltration of eosinophilic leukocytes, degranulation, and destruction of myocardium are observed in the biopsy specimen.

The main causes are an allergic reaction, drugs, and parasitic infection. Most cases, however, are treated as idiopathic myocarditis (Forrester et al., 1976). It has been reported that the mortality rate among patients with acute-phase eosinophilic myocarditis is approximately 7% (Morimot et al., 2003), (Mori et al., 2004).

3.3 Clinical classification

Myocarditis is clinically classified as acute or chronic. Since it is possible to detect the exact onset of acute myocarditis, it is easier to estimate the circulatory parameters for diagnosis and establish the strategy for treatment of acute myocarditis than for chronic myocarditis. In some cases of acute myocarditis, we treat it as fulminant myocarditis that has developed suddenly and may progress to severe circulatory failure during the acute phase (Aoyama et al., 2002). The worldwide morbidity and mortality rates associated with fulminant myocarditis are not yet clear. Cupta et al. (2008) reported that the frequency of fulminant myocarditis is 10% among all cases of acute myocarditis in the United States.

The global concept of chronic myocarditis has not been established, although several studies have suggested that viral infection (Fujioka et al., 2000) or autoimmunity (Lauer et al., 2000) play a role in its development. There are clinically two types of chronic myocarditis, and they have different clinical courses. One type has persistent, continuous symptoms, and the other has unremarkable symptoms (JCS, 1996).

In an investigation of clinical types of myocarditis in 48 patients reported by Kodama et al. (2001), nine were the acute type, 21 were the fulminant type, three were the chronic persistent symptom type, and 15 were the chronic unremarkable symptom type. The mortality rates during the first admission were 22% for the acute type, 43% for the fulminant type, 33% for the chronic persistent symptom type, and 40% for the chronic unremarkable symptom type. The long-term prognosis for patients who recovered from their cardiac dysfunction during the early phase was good. However, the long-term prognosis for patients who were diagnosed with the chronic, unremarkable symptom type was not good because they developed irreversible circulatory dysfunction, such as dilated cardiomyopathy.

4. Clinical symptoms and diagnosis

4.1 Clinical symptoms and physical symptoms

Clinically, symptoms range from slight problems—fever, pharyngeal pain, cough, vomiting, diarrhea, and arthropathies, as observed with the common cold—to severe circulatory failure. Therefore, we cannot list any symptoms characteristic of acute-phase myocarditis. In

cases of established cardiac dysfunction, patients suffer from dyspnea, edema, cyanosis, palpitations due to hypoxia or arrhythmia, and other severe symptoms such as loss of consciousness and cramping. Whenever we treat acute-onset patients who present with symptoms of cardiac dysfunction in the ER, we maintain the suspicion that they are in the pre-developmental phase of severe myocarditis.

Among the physical symptoms of myocarditis, dysfunction of the heart's conduction system, which occurs in 60%–80% of patients with myocarditis, is critical. Particularly notable is the 30% incidence of severe bradycardia requiring temporary pacing in those with fulminant myocarditis (Kawamura et al., 1986). A galloping rhythm, a heart murmur caused by backflow in the atrioventricular valves, and pulmonary moist rales are found in patients who are developing heart failure. Pericardial and pleural stridulation can be auscultated in patients with pericarditis or pleuritis. Furthermore, pericardial effusion or cardiac tamponade is observed in some cases of developmental pericarditis.

4.2 Blood examinations

Inflammatory reactions such as increased WBCs, an increased erythrocyte sedimentation rate (ESR), or an increased C-reactive protein (CRP) level are detected in blood examinations during the early phase. Additionally, creatine kinase MB (CPK-MB) and cardiac troponin T assays, which are elevated in the presence of myocardial damage, are useful. Troponin T sensitivity is particularly high, and the severity of the myocardial damage can be estimated by a fixed quantity analysis (Lauer et al., 1997). Troponin T continuously increases with development of cardiac dysfunction and maintains a high peak value in those with fulminant myocarditis.

4.3 Chest radiography

Cardiac dilatation is present in 70% of chest radiography examinations, and pulmonary congestion or pleural effusion is often present in patients with severe heart failure. It should be noted that cardiac dilatation and pulmonary congestion are not remarkable in some cases of myocarditis, which causes mainly right ventricular failure (McFalls and van Suylen, 1993).

4.4 Electrocardiography

Various abnormal changes are revealed by electrocardiography (ECG), although none of the changes are specific for myocarditis. ECG is not an invasive procedure, and it has the benefit of simplicity of performance. The sensitivity is high, and any changes on the ECG tracing probably enhance with the development of myocarditis even if only slight changes are detected during the early phase. ECG should be repeated in the case of suspected myocarditis. Limited elevation of ST-T, mainly observed with acute myocardial infarction (AMI), is present in some patient with myocarditis. Elevation of ST-T in all leads is present in cases complicated by pericarditis. Bundle branch block or atrioventricular block is present in cases complicated by dysfunction of the heart's conduction system. It should be noted that the change to a wide QRS complex on the ECG tracing suggests the development of cardiac dysfunction. Because there is the risk of sudden changes with fatal ventricular tachycardia (VT) or ventricular fibrillation (VF) in the case of frequent arrhythmias, continuous ECG monitoring is necessary.

4.5 Echocardiography

Temporal thickening of the ventricular wall and deterioration of ventricular wall motion are present at inflammatory sites. All-round centripetal thickening, diffuse deterioration of wall motion, and stenosis of the intracardiac space are present in typical cases (Hiramatsu et al., 2001). In the cases of severe circulatory failure or fulminant myocarditis, multiple left ventricular thrombi are frequently caused by the deterioration of all-round wall motion. Continuous wall motion dysfunction causes diffuse thinning of the wall and/or ventricular aneurysms, and dilatation of the left ventricle develops. Ultimately, there is no morphological difference from dilated cardiomyopathy. Diagnosis by exclusion of ischemic heart disease is necessary in patients with wall thickening or wall motion deterioration.

4.6 Cardiac magnetic resonance

Cardiac magnetic resonance (CMR) is a noninvasive, useful examination for myocarditis (JCS, 2011). It can be used to estimate the morphological changes in the ventricles, contractive function, perfusion in the myocardium, and histological characteristics in one performance. In myocarditis, hyperemia and capillary leakage in the cardiac microcirculation are caused by an inflammatory reaction. The site of inflammation in the myocardium has high signal intensity on T1-weighted magnetic resonance imaging (MRI) several minutes after gadolinium contrast enhancement during the acute phase. It is suggested that the changes of microcirculation caused by an inflammatory reaction can be directly visualized with CMR (Friedrich et al., 1998). In many cases, widespread edema in the myocardium is caused during the acute phase. High signal intensity on T2-weighted images is present in 36% of myocarditis diagnosed by the Dallas criteria (Aretz et al., 1987), (Mahrholdt et al., 2004). In those patients, follow-up CMR, performed a year later, shows reduced left ventricular capacity (Zagrosesek et al., 2008). We can predict the histological changes and prognosis for cardiac function using CMR.

4.7 Radioisotope examination

Gallium-67 (^{67}Ga) is specific for infiltration of large monocytes, but it does not have high sensitivity (O'Connel et al., 1984). Pyrophosphate scintigraphy using technetium-99m (^{99m}Tc) is sensitive and accumulates at the inflammatory site in the myocardium (Morguet et al., 1994).

4.8 Cardiac catheterization and endomyocardial biopsy

We perform cardiac catheterization for the differential diagnosis during the acute phase if the patient's circulatory condition can tolerate it. We first exclude significant coronary stenosis by coronary angiography and then perform endomyocardial biopsy (Sekiguchi et al., 1980). The endomyocardial biopsy is now the most important and reliable technique for a definitive diagnosis. However, we often cannot obtain samples of the lesion site because the inflammatory reaction in the myocardium occurs inhomogeneously in most cases (Baughman, 2006). Cooper et al. (2007) reported that cardiac tamponade or ventricular perforation occurs at the time of sampling with a 0.1%–0.5% frequency.

4.9 Detection of viruses

In cases of suspected viral myocarditis, we measure the antibody titer using paired sera collected at a more than a 2-week interval. The reliable positive ratio is only 10%, and the

capability to detect infected organs is not available. A definitive diagnosis is possible if we can directly detect the original viruses using a polymerase chain reaction or in situ hybridization. However, these techniques are not yet approved as standard examinations because their results vary widely depending on the institution in which they are performed.

4.10 Other diagnostic factors

In cases of suspected drug-induced myocarditis, we narrow down the list of causative drugs by detailed interviews with the patient regarding his or her clinical history. We can then identify the causative drug by a drug-induced lymphocyte stimulation test. Soluble Fas and Fas ligand (Fuse et al., 2000), interleukin-10 (Nishii et al., 2004), and tenascin-C (Imanaka-Yoshida et al., 2002) may be used in upcoming tests for diagnosing myocarditis.
Guidelines for diagnosis are presented in Table 3. The basic concept is to exclude ischemic heart disease and confirm an active lesion site by endomyocardial biopsy. It is currently impossible to detect the cause in most cases. Clinically, it is best to provide the primary care that is given to patients suspected of having myocarditis caused by a viral infection.

5. Development and strategy for treatment during the acute phase

Generally, the clinical conditions of patients are similar in many cases of myocarditis. A toxic protein produced by the infecting virus destroys the myocardial dystrophin complex within several days after onset of the myocarditis. It has been noted that this mechanism causes severe myocardial dysfunction accompanied by widespread myocardial cell death (Bandorff et al., 1999). Silver and Kowaldzuk (1989) reported that viral infection directly causes widespread myocardial ischemia by microvascular spasm.
After the viral infection is established, the immune response produces inflammatory cytokines in large quantities (Fairweather et al., 2005). This cytokine network originally plays a role in prophylaxis against the viral infection. Inflammatory cytokines such as interleukins 1 and 2 and tumor necrosis factor-α eliminate infected viruses by activating macrophages, lymphocytes, and endothelial cells. However, excessive cytokine release damages myocardial cells and causes myocardial dysfunction (Kawai, 1999). Additionally, inducible nitric oxide (NO) synthase (iNOS) induced by activated macrophages acts to encourage NO to eliminate infected viruses. It has been reported that excessive release of NO strongly damages myocardial cells (Mikami et al., 1996).
Infiltration by inflammatory cells, including T cells and natural killer cells, peaks 7–14 days after viral infection and causes widespread necrosis of myocardial cells (Seko et al., 1993). As already noted, infected virus and released inflammatory cytokines are the main mechanisms in the development of myocardial dysfunction during the acute phase. Severe deciduation of myocardial cells causes pump failure, which progresses to fatal circulatory collapse.
It is important to remember that cardiac dysfunction associated with myocarditis is reversible in many cases. Full recovery of cardiopulmonary function can be expected if the patient's life support is adequately performed. Therefore, we compress the strategy for treatment into three stages to give patients suffering from myocarditis the best chance for survival.
In most cases, the first strategic issue is intervention regarding the cause. Unfortunately, it is impossible to provide antibiotic therapy because effective antiviral drugs to address viral myocarditis have not been developed. There is a risk of further viral propagation when

1. Primary symptoms (nonspecific symptoms in most cases)
- Symptoms that appear with the common cold (fever, headache, cough, pharyngeal pain)
- Digestive symptoms (nausea, vomiting, diarrhea, abdominal pain)
- Others (eruption, arthralgia, myalgia)
  - *Note*: Some patients are found in sudden cardiac arrest

2. Physical findings
- Tachycardia, bradycardia, arrhythmia, weak heart sounds
- Galloping rhythm (III, IV sounds), pericardial friction murmur
- Systolic murmur

3. Abnormality of ECG: various changes
- Atrioventricular block, wide QRS complex, reduction of height in R wave, abnormal Q wave
- Change of ST-T level, low-voltage wave, frequent premature contractions
- Supraventricular tachycardia, atrial fibrillation, sinus arrest
- Ventricular tachycardia, ventricular fibrillation, asystole

4. Echocardiography
- Focal or diffuse thickening of the ventricular wall
- Focal or diffuse deterioration of ventricular wall motion
- Stenosis of intracardiac space
- Pericardial effusion

5. Blood examinations
- Detection of creatine kinase MB (CPK-MB)
- Detection troponin T
- Inflammatory reaction (increased WBCs, CRP level)
  - *Note*: Troponin T is sensitive using whole blood during the acute phase

6. Items 2–5 (above) change within several hours. Therefore, continuous observation is necessary. Bradycardia, wide QRS complex, frequent premature contractions, enhanced thickening of the ventricular wall and deterioration of ventricular wall motion, and continuous high troponin T levels are dangerous symptoms of fatal circulatory crisis.
7. A differential diagnosis of acute myocardial infarction (AMI) is necessary.
8. Endocardial biopsy provides a definitive diagnosis, but AMI cannot be excluded if tissue images are not obtained.

- Diagnostic criteria in tissue image:
- Infiltration of large and small monocytes
- Rupture, fusion, or disappearance of myocardial cells
- Edema or fibrotic changes in interstitial tissue

9. More than four-fold change of viral antibody titer in paired sera is adequate for viral detection. Polymerase chain reaction is effective for diagnosing a viral infection. Additionally, virus isolation or detection of viral antigen from a throat swab, urine, feces, blood, or particularly pericardial effusion or myocardial tissue are direct evidence of the diagnosis.

Table 3. Guidelines for diagnosing myocarditis. Modified from the guideline for diagnosis and treatment of myocarditis (JCS, 2005).

administering anti-inflammatory drugs such as immunosuppressants and steroids. On the other hand, we can expect to reverse cardiac dysfunction by initially giving anti-inflammatory drugs because it has been reported that allergic and autoimmune reactions strongly participate in the development of the giant cell myocarditis and eosinophilic myocarditis, both unusual forms. Therefore, early and adequate ascertainment of the cause is the maximum priority. Presently, the performance of myocardial biopsy is limited. Steroids should not be selected as a first choice even if the patient is in a shock state.
The second issue is to provide cardiopulmonary support during continuing fatal circulatory failure. During the acute phase of severe myocarditis, there is the risk of cardiogenic shock, complete atrioventricular block, fatal arrhythmia, and/or sudden cardiac arrest at any time. Therefore, most patients require intensive care. Drug therapy for myocarditis is no different from that for usual heart failure. Catecholamines, a diuretic, or both are administered following Forrester's classification. Mechanical cardiopulmonary life support should be immediately introduced when the circulatory insufficiency cannot be reversed with drugs. Full recovery of cardiac function is expected by advanced life support within several days in patients with acute myocarditis.
The third issue is to control the inflammatory reaction. If the actions of excessive inflammatory cytokines and NO are reduced, cardiac dysfunction is expected to be reversed during the acute phase. Although treatment using high doses of steroid, high doses of γ-globulin, and plasma exchange have been tried and evaluated, we have no evidence of their effectiveness.

5.1 Cardiopulmonary support

Immediate cardiac resuscitative support must be provided if the patients with fulminant myocarditis are to survive. Delay of treatment results in fatal circulatory collapse. Although the short-term mortality rate for fulminant myocarditis is generally high, the long-term functional prognosis for patients who survive the acute circulatory crisis is good compared with that for dilated cardiomyopathy (McCarthy et al., 2000).
The Scientific Committee of the Japanese Circulation performed a retrospective follow-up survey on severe fulminant myocarditis (Aoyama et al., 2002). The cases of 52 patients who required percutaneous cardiopulmonary support (PCPS) because of severe circulatory failure were investigated. The mortality rate for the acute phase was 40.4% (21/52). Among the 31 surviving patients, 30 (96.8%) had fully recovered from their cardiac dysfunction. McCarthy et al. (2000) noted that cardiac function had been maintained in good condition for a long time in more than 90% of patients who had recovered from fulminant myocarditis. Therefore, the "bridge" treatment of using mechanical cardiopulmonary support to avoid multiple organ dysfunction caused by hypoperfusion is an important treatment strategy during the acute phase of fulminant myocarditis. Patients who have survived on mechanical support, such as percutaneous cardiopulmonary support or a ventricular assist system, have also been reported (Chen et al, 2005), (Topkara et al., 2006).

5.1.1 Percutaneous cardiopulmonary support

A guideline for the use of percutaneous cardiopulmonary support (PCPS) has been formulated in Japan (Fig. 1) (Aoyama et al., 2002). In suspected cases of low cardiac output due to pump dysfunction, we may apply PCPS in accordance with continuous monitoring of the circulatory condition. Important clinical parameters to examine when making the decision of whether to use PCS include the urinary volume, SvO_2 (< 60%), development of

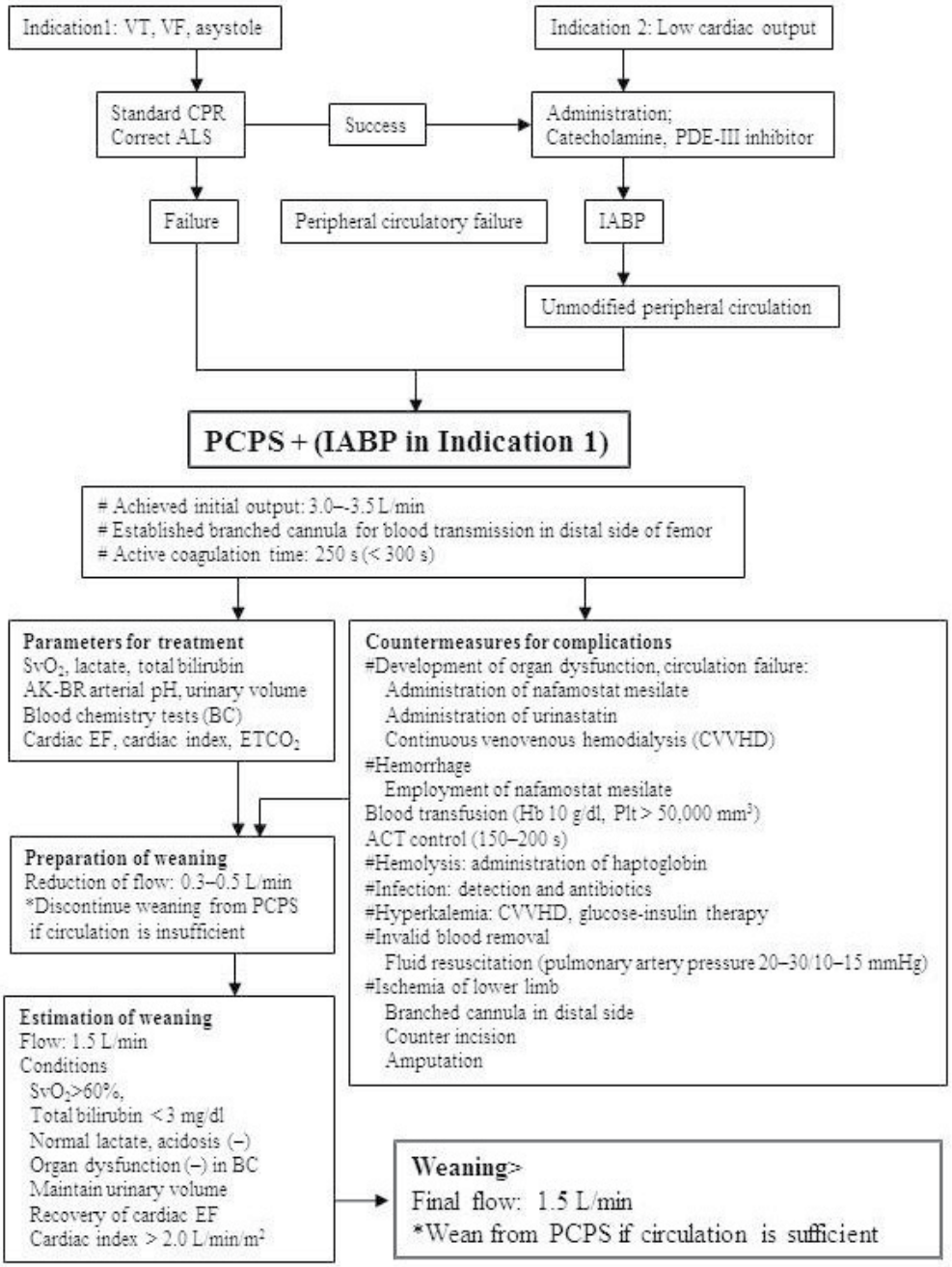

Fig. 1. Modified algorithm of PCPS for fulminant myocarditis (Aoyama et al, 2002).

metabolic acidosis, and multiple organ dysfunction suggested by blood chemistry examinations. The initial flow of PCPS should be established at 3.0–3.5 L/min. Concomitant use of intra-aortic balloon pumping (IABP) provides the benefits of reduced afterload,

improved peripheral circulation by pulsatile flow, and backup support at the completion of PCPS. It is recommended that a branched cannula be established in the distal side of the femoral artery for blood transfusion because there is a risk of ischemia in the lower limb.
Unfortunately, we occasionally experience patients in severe situations in whom sufficient organ perfusion cannot be provided even if PCPS is fully operative. Aoyama et al. (2002) reported that 40% of patients supported by PCPS died during the acute phase. Among them, multiple organ dysfunction due to hypoperfusion was found in 25% and ischemia in the lower limb in 23%. Because the prognosis of fulminant myocarditis depends on the outcome of radical treatment of the circulation, we discuss early exchange via a ventricular assist system when sufficient organ perfusion cannot be provided by PCPS.
Case report: We treated a patient with severe fulminant myocarditis who survived owing to emergency PCPS. The young woman consulted her family doctor because of fever and was prescribed anti-inflammatory drugs with a diagnosis of pharyngitis. She was admitted to our hospital because of sudden severe circulatory failure that required high-flow oxygenation and high doses of catecholamine. Blood examination showed an elevation in troponin I, CPK, and other cardiac enzymes. Chest radiography showed marked cardiac dilatation and pleural effusion (Fig. 2). Echocardiography revealed pericardial effusion and deterioration of all-round wall motion of the left ventricle (Fig. 3). No significant coronary stenosis was detected by coronary angiography. We introduced emergency PCPS with IABP because her left ventricular ejection fraction was decreased by less than 20%. Her circulatory condition dramatically recovered, and she was weaned from PCPS on the 10th day. She obtained full recovery of her circulatory function. We could not detect any viruses when measuring the antibody titer using paired sera.

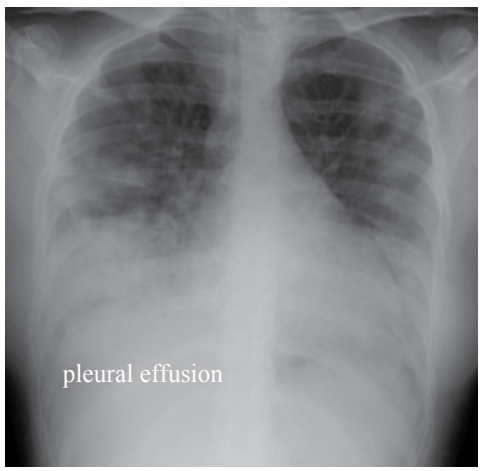

Fig. 2. Chest radiography shows marked cardiac dilatation and pleural effusion.

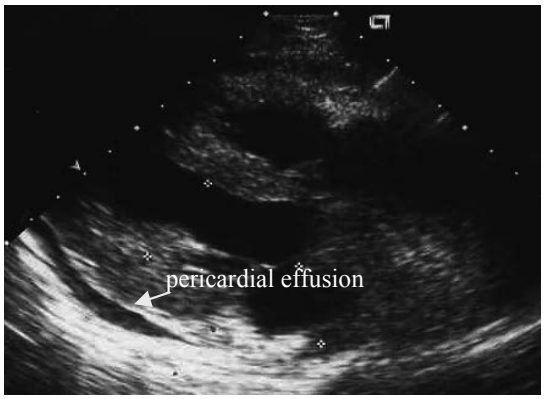

Fig. 3. Echocardiography shows pericardial effusion and deterioration of the all-round wall motion of the left ventricle.

5.1.2 Ventricular assist system

There is the risk of severe pulmonary congestion due to PCPS in cases of extreme deterioration of left ventricular function. In such cases, the patient should be switched to a ventricular assist system (VAS) before organ dysfunction develops. Grinda et al. (2004) reported that they introduced VAS in five cases of severe fulminant myocarditis and obtained good outcomes. Recently, the clinical effectiveness of modified VAS using an extracorporeally established centrifugal pump was advocated by John et al. (2007) in the United States. Modified VAS has combined the advantages of minimally invasive extracorporeal membrane oxygenation and high efficiency.

5.2 Immunoregulation therapy

Generally, the time limit for continuous mechanical circulatory support, including PCPS or VAS, is approximately 1 week. We withdraw the system even if recovery from circulatory failure is incomplete. In such cases, introduction of immunoregulation therapy is discussed. Many case reports have asserted the effectiveness of immunoregulation therapy. Although there is no established evidence, we believe that immunoregulation is acceptable in intractable cases because no radical treatments are presently available.

5.2.1 High-dose γ-globulin

The effectiveness of high-dose γ-globulin was reported by Takada et al. (1995). It is expected that γ-globulin counteracts the actions of the infective viruses and reduces suppression of cardiac function by inflammatory cytokines during the acute phase. γ-Globulin intensifies patients' immune competence, and therefore complications, such as an infection compromised by steroid administration, do not take hold. The mechanisms of high-dose administration have not been completely clarified, although several hypotheses have been suggested: (1) it functions as a neutralizing antibody; (2) it has an anti-inflammatory effect, reducing the release of inflammatory cytokines induced by a combination of the Fc part of γ-globulin and the suppressive Fc receptor of macrophages; and (3) it has the effect of anti-activation on activated complement (Rosen, 1993). However, it is not strongly recommended because there is no evidence that has been confirmed by large clinical trials. Finally, cardiac function has not recovered in several cases with its use.

5.2.2 High-dose steroids

The effectiveness of steroids as anti-inflammatory and immunosuppressant drugs has been widely accepted. Its effectiveness when treating patients with fulminant myocarditis has been also reported in many studies (Ino et al., 1995). However, administration of steroid was not proved effective in patients with lymphocytic myocarditis in a clinical trial (Mason et al., 1995). We would not hastily administer steroids to patients with acute-phase myocarditis with a suspected viral infection. On the other hand, it is expected that administration of steroids alleviates cardiac dysfunction in patients with giant cell myocarditis and eosinophilic myocarditis (Cooper et al., 1997). Particularly, high-dose steroids should be given prior to other treatments in patients with the fulminant type of giant cell myocarditis.

6. Conclusions

It is not easy to explain myocarditis concisely and clearly because there are varieties of causes, clinical types, clinical courses, and the severity of circulatory failure. Some patients present with common cold-like symptoms, whereas others require mechanical circulatory support in the ER because of a suddenly developing circulatory crisis. Information of the patient's background and clinical history is essential when deciding on a treatment plan for myocarditis in most cases, although we are sometimes unexpectedly confronted with emergency conditions regarding these patients in the ER.

Currently, the most troublesome issue in the course of treating myocarditis is when to perform a myocardial biopsy for a definitive diagnosis. Although noninvasive diagnostic methods such as CMR have been developed to reduce the risk of serious complications and physical strain on the patient, an effective diagnosis cannot be established during the acute phase. Regarding the treatment for myocarditis, we cannot presently exclude original causes in many cases and can only provide unpredictable "bridge" support, such as PCPS or VAS.

Many patients with myocarditis can survive if we remember the possibility of myocarditis in the differential diagnosis and provide immediate, adequate treatment. When we face illness of unknown origin in patients with a severe arrhythmia or circulatory failure, we should immediately assemble the medical staff and prepare cardiac support. Any delay in treatment can allow abrupt deterioration of the circulation. Additionally, we should establish a system of simultaneous processing of the histological diagnosis to decide on the propriety of immunoregulation therapy.

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4

Viral Myocarditis: Physiopathology and Diagnosis

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1. Introduction

In Europe and the USA, viral aetiologies largely prevail over other causes of myocarditis as previously indicated by recent data demonstrating the molecular detection of cardiotropic viruses in human cardiac biopsy samples (Kühl et al., 2005a). Clinical presentations of the myocarditis range from non-specific systemic symptoms (fever, myalgias, palpitations, or exertional dyspnea) to fulminant hemodynamic collapse (5 to 10 cases per million inhabitants and per year) and sudden cardiac death (Feldman & McNamara, 2000; Magnani & Dec, 2006).

Acute myocarditis remains a complex and challenging diagnosis in cardiology (Magnani & Dec, 2006). This cardiomyopathy is defined histologically by the Dallas criteria as an''inflammation of the myocardium'' associated with necrosis and an absence of ischaemia (Aretz et al., 1987; Cooper, 2009; Dennert et al., 2008; Felmann & McNamara, 2000). The use of the Dallas criteria in the diagnosis of myocarditis is associated with poor sensitivity and specificity, mainly because of the sampling error related to the often focal distribution of the specific histological lesions in cardiac tissue and because of the variability in pathological interpretation (Baughman, 2006; Mahrholdt et al., 2004). Moreover, the Dallas classification does not consider local quantification and differentiation of inflammatory cells and does not take into account viral infection and autoimmune regulation in cardiac tissues (Dennert et al., 2008). To improve the histological diagnosis, additional virological and immunological evaluations of cardiac tissues are required using immunohistochemical and PCR techniques, which allow identification and quantification of inflammatory cells and viral infection markers (Dennert et al., 2008). The diagnostic gold standard is endomyocardial biopsy with the histological Dallas criteria in association with new immunohistochemical and viral PCR analyses of cardiac tissues (Cooper et al., 2007). This new diagnostic approach can lead to better identification of the aetiological cause of the myocarditis and can improve the clinical management of viral myocarditis. This chapter chronicles the advances in understanding the physiopathology of viral acute and chronic myocarditis and in the development of new molecular techniques for an accurate and valuable virological diagnosis.

2. Human viral cardiac infection: proofs of concept and clinical relevance

Common human pathogenic DNA or RNA viruses can be responsible for acute or chronic endomyocardial tissue infection (Andréoletti et al., 1995; 2009; Kühl et al., 2005a). The detection of components (DNA or RNA genomes or viral proteins) of these viral agents by molecular techniques such as polymerase chain reaction (PCR) and/or immunohistochemical techniques demonstrating the viral protein expression in the cytoplasm of myocytes, was associated with an inflammation of the myocardium (acute myocarditis), arrhythmias, loss of contractility (Andréoletti et al., 2009; Cooper, 2009; Dennert et al., 2008). Moreover in serial of human cases, the presence of viral persistence was evidenced in the myocardium and associated with a left ventricular systolic dysfunction in relation to a reduction in the contractile function of the myocytes (Andréoletti et al., 2000; Badorff et al., 2001). Additional proofs of concept were that: (I) a longitudinal clinical investigation showed that the type of virus, the clinical presentation and the type of histological damage appeared to be related to the clinical course of the cardiac disease; (II) longitudinal clinical studies indicated that the immune clearance of the virus during or after the acute phase was correlated with an improvement of the left ventricular ejection fraction [LVEF] (Kühl et al. 2005b); (III) acute and chronic myocarditis as well as dilated cardiomyopathy (DCM) were reproduced in immunocompetent animal models following experimental viral infections (Andréoletti et al., 1997; Huber, 1993; Matsumori & Kawai, 1982). Altogether these clinical and experimental data supported the pathophysiological role of several human viruses in the genesis and the evolution of myocarditis and the DCM. Although up to 90% of people will be infected by at least one or more of these viruses in their life without getting their heart injured with associated clinical signs, only a few number will develop clinical symptoms. Therefore, it is highly suspected that a certain genetic background either related to immune alterations or to an improved susceptibility to viral cardiac infection (viral receptor or co-receptors polymorphisms) may be a prerequisite to develop clinical symptoms of myocarditis and/or progression to DCM following virus heart tissue infection. In addition, it has been shown that dystrophin mutations may make easier the development and progression of myocarditis and cardiac failure during coxsackievirus B3 infection, whereas dystrophin and/or sarcoglycan disruption by viral proteases account for myocardial injury (Andréoletti et al., 2007; Badorff et al., 2001; Lee et al., 2000). Human genetic studies of patients with myocarditis are rare and only 2 reports show an association between myocarditis and genetic factors such as HLA-DQ locus and CD45 polymorphism (Yajima & Knowlton, 2009). Future clinical investigations should therefore focus on the understanding of the underlying genetic susceptibility and related immune responses that explain why some patients are susceptible to develop clinical symptoms of acute myocarditis following viral infection, whereas other subjects clinically recover or progress to an 'idiopathic' DCM after the initial phase of the viral cardiac infection.

3. The pathophysiological mechanisms of viral myocarditis

The current understanding of the immunopathologcial phases of myocarditis derives largely from enterovirus-induced myocarditis in murine models (Andréoletti et al, 1997; Cooper et al., 2007; Liu & Mason, 2001; Yajima & Knowlton, 2009). During the enterovirus (virus Coxsackie B) natural course of infection, the data obtained from experimental murine

models allowed the identification of three distinct pathophysiological phases defined as acute, sub-acute and chronic myocarditis (Cooper, 2003; Feldman & McNamara, 2000; Yajima & Knowlton, 2009) (Figure 1).

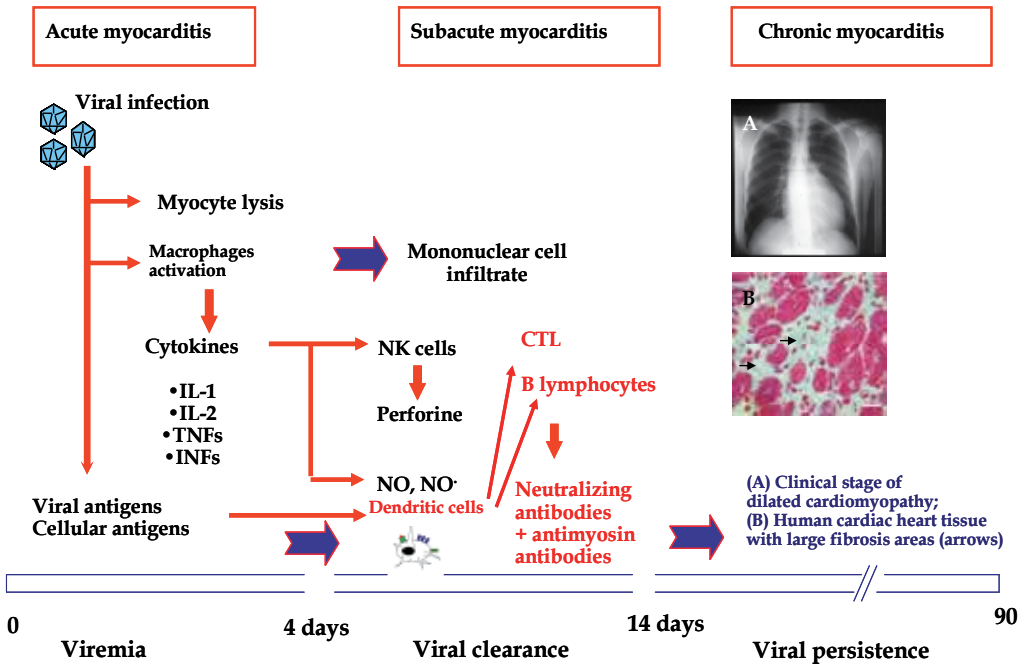

Fig. 1. Immunopathological phases of viral myocarditis.

3.1 Acute myocarditis during the initial Virus invasion

During the period of active viremia, cardiotropic RNA or DNA viruses interact with specific vascular endothelial cell receptors before to be translated in the endomyocardial target cells to produce viral proteins (Huber, 1993; Li et al., 2000) (Table 1). Mechanisms of viral entry into the host cell remains crucial and are of major interest because each of them is putative target for novel therapeutics. Before to interact specifically with the virus, expression of levels of the virus receptors for CVB3, PVB19, HVV6 (CAR/DAF, P-Antigen/ Alpha5-beta1 integrin and CD46, respectively) on endovascular endothelial cells of coronary vessels modulate the diffusion of these viral agents into the vessels and their subsequent translation into the endomyocardial target cells (Khül et al., 2005a; Yajima & Knowlton, 2009). Some inflammatory mechanisms as a primary persistent infection by HHV6 or PVB19 may modulate cell membrane receptors and /or immune suppression and preventing virus clearance; altogether these mechanisms could facilitate an endomyocardial super infection by a second viral agent as described in 27 % of virus positive DCM patients (Khül et al., 2005a) (Table 1).

Common cardiotropic Viruses	Detection Methods	Cardiovascular tissular Localization	Cellular Receptor / Coreceptor Involved	References
Adenovirus	EM	Cardiomyocytes, fibroblasts, endothelial cells	CAR / Integrins (αvβ3 and αvβ5)	Shi et al., 2009; Wickham et al., 1993
Enterovirus/ Coxsackievirus B	IHC, ISH	Cardiomyocytes	CAR / DAF	Shi et al., 2009; Coyne & Bergelson, 2006
Parvovirus B19	ISH, LCM + PCR	Endothelial cells, cardiomyocytes	Gb4Cer / Integrin (α5β1); Ku80 autoantigen	Bultmann et al., 2003; Bönsch et al., 2010
Human Herpes Virus 6	In vitro Infection	Endothelial cells	CD46	Caruso et al., 2002; Santoro et al., 1999
Cytomegalovirus	ISH	Cardiomyocytes	Integrins (α2β1, α6β1, and αvβ3)	Feire et al., 2004; Kyto et al., 2005
Epstein-Barr Virus	ISH	Lymphocytes B	CD21	Chimenti et al., 2004; Takano et al., 2008
Influenza Virus	ISH	Macrophages, Lymphocytes	ND	Cioc et al., 2002

EM indicated electron microscopy; IHC, immunohistochemistry ; ISH, in situ hybridrization; LCM + PCR, polymerase chain reaction using tissue samples isolated with laser capture microdissection; CAR, coxsackie and adenovirus receptor; DAF, decay accelerating factor ; CD, cluster of differentiation and ND, not determined

Table. 1. Cardiovascular tissular localization of common cardiotropic viruses and local suspected cellular receptors involved in the process of infection.

At the beginning of human viral infection, the first line of immune defense consists in the innate immune response (NKT cells, monocytes and macrophages). Cell-mediated immunity also plays an important role in viral clearing. Cytotoxic (CD8+) cells recognize degraded viral protein fragments that are presented by major histocompatibility-complex class I antigens on the myocyte surface (Seko et al., 1990) (Figure 1). The Toll-like receptors located at the surface or inside of dendritic cells (more particularly the Toll-like-receptors 4, 7, and 8 in the case of virus Coxsackie B infection) could recognize certain viral proteins or genomic and then activate intracellular signals (NF-kB pathways) responsible for a fast synthesis and secretion of pro-inflammatory cytokines as interferons and chemokines and also nitric oxide (Ayach et al., 2003; Kawai, 1999; Matsumori et al., 1994) (Figure 1). The pro-inflammatory cytokines then attract and activate many immune system cell lines to the site of the viral infection and they are directly responsible for cardiac myocytes lysis and also for lost of cardiomyocyte contractility and apoptosis cell-dead (Feldman & McNamara, 2000; Ventéo et al., 2010) (Figure 1).

3.2 Sub-acute myocarditis with an activation of the immune system and the development of autoimmunity

The activation of the immune system at the time of the viral invasion reaches to the initiation of specific cellular immunity response and to the immune shift towards a specific immune response. The viral particles are captured by antigen processing cells (APC) and degraded within the Golgi apparatus before being presented at the cell surface by the major histocompatibility complex class I (MHC class I) to the CD8+ lymphocytes. These primed T

cells capable to detect the viral antigen will destroy the infected cardiac cells through cytokines or perforines secretion (Kawai, 1999, Matsumori et al., 1994). In addition, some myocardic cellular antigens of the host present can share epitopic similarities (molecular mimicry) with viral antigens therefore inducing an autoimmune trait that can sustain the inflammatory response and therefore the chronic inflammation phases (Figure 1). This sub-acute phase is known to be linked to autoimmune responses as many patients produce auto-antibodies and auto-reactive T cells against heart proteins (Domenico & Gaetano, 2006; Feldman & McNamara, 2000) and the amplification of this phenomenon can lead to the destruction of cardiomyocytes. The mechanisms linking enteroviral infection with sub-acute myocarditis, relapses and post inflammatory heart failure could be mainly mediated by dendritic cells (DCs) that are specialized in antigen processing and presentation and most important in priming of T cells within lymph nodes. The EV-induced autoimmune myocarditis may require activation of these cells via CD40 with Toll like receptors (TLRs 3, 4, 7) or RNA helicases (RIG-I, MAD-5) co-stimulation (Kramer et al., 2008). Moreover in human infections, it is clear that human enteroviruses (HEVs) can escape from immunological system by decreasing the specific functions of immunity cell system (Oldstone, 2006). Recently it has been demonstrated that Enteroviruses could infect and consequently modify the maturation process and some specialized functions of DCs (Kramer et al., 2007). Therefore, Enteroviruses or some of its components could activate or decrease some DCs functions acting as modulators of innate response system or autoimmunity (Kramer et al., 2007).

3.3 Chronic myocarditis with a potential evolution to the dilated cardiomyopathy clinical stage

After the active virus replication resulting in acute and sub acute myocarditis phases, the pathological signs of myocarditis generally disappeared and the destroyed myocytes are replaced by diffuse fibrosis (Dec et al., 1985) (Figure 1). At this stage, a progressive heart bi-ventricular dilatation with a cardiac failure can be observed and has been related to cardiac persistent or chronic viral replication mechanisms (Andréoletti et al., 2000; Badorff et al., 1999) (Figure 1). This persistent or chronic viral infection could be related with various cardiac cell dysfunctions as impairment of Ca+ efflux in cardiomyocytes and of loss of cell contractility, apoptosis balance deregulation, cleavage of dystrophine, modulation of cellular signalling pathways or alteration of the extracellular matrix (Andréoletti et al., 2009; Dennert & McNamara, 2009; Kawai, 2009; Khül et al., 2008a).

4. The Virological mechanisms of cardiac infection

4.1 Acute viral myocarditis

Viruses generally infect human beings by fecal-oral (enteroviruses, *parvovirus* B19, *Herpesviruses*) or respiratory routes (enteroviruses, *influenza viruses A & B, parainfluenza viruses I II III*) and they usually perform a first phase of multiplication into the airway epithelial cells of the higher respiratory tract or tonsils; this can be followed by a potential rapid invasion of the lower respiratory tract as observed during influenza virus infection. After this initial and local replication phase, the viruses can diffuse by lymphatic way to the general circulation (viremic phase) allowing them to reach the cardiac tissues. In the heart, the virus infects and replicate actively into the cardiac myocytes but also into the cardiac fibroblasts that can play the role of reservoir cells for a persistent infection (Andréoletti et

al., 2009). During this active phase of replication, the development of the classical clinical signs of myocarditis is usually observed (Andréoletti et al., 2007; Magnani & Dec, 2006).

4.2 The chronic persistent viral Infection in heart tissues

4.2.1 The enterovirus model

After the acute myocarditis phase, persistence can be observed as described during human cardiac enterovirus (EV) infection. In some previous published studies, detection of viral genome has been demonstrated in patients with myocarditis and in patients with DCM, but it is unusual that replication competent virus can be isolated from the myocardium in patients with myocarditis (Andréoletti et al., 2009; Chapman & Kim, 2008a; Copper, 2009). The concept that EV endomyocardial persistent infections are the etiological cause of a subset of idiopathic DCM cases is supported by the detection of enterovirus genomic sequences and enteroviral capsid protein VP1 in up to 35 % of explanted heart tissues from end-stage DCM patients (Andreoletti et al. 2009; Li et al., 2000). In a previous published study, we observed that enteroviruses can persist with or without active viral replication in cardiac tissue of patients with end-stage dilated cardiomyopathy (Andréoletti et al., 2000). Enterovirus genome was detected in 25 of 70 patients with IDCM and, of these patients positive for genomic RNA, only 3 exhibited antigenomic RNA and VP1 antigen that demonstrated active viral replication, whereas 22 had latent infection characterized by the absence of antigenomic RNA associated with or not with VP1 antigen expression. No viral component was detected in control subjects (Andréoletti et al., 2000). These findings demonstrated that a small percentage of patients with end-stage chronic cardiac diseases had active enterovirus replication in their myocardium. Moreover, we demonstrated that enteroviral capsid protein VP1 was present in myocardial tissues from some patients with dilated cardiomyopathy and suggested that the pattern of VP1 detection may correlate with disease stage and severity. These data suggested that viral protein synthesis might be involved in persistent enterovirus infection in the pathogenesis of DCM (Li et al., 2000). It was observed that the ratio of positive- to negative-strand enteroviral RNA was greater with active virus replication than with persistent virus infection where a viral capsid protein synthesis activity was also evidenced in heart cardiac tissues (Andréoletti et al., 2000; Li et al., 2000, Chapman & Kim, 2008a). This slow viral replication could be explained by the existence of 5'NC genomic deleted viral forms that could be related to the development of slow replicating viral forms in heart tissues (Chapman et al., 2008b). As persistent expression of CVB proteins and 2Aprotease (2Apro) alone are sufficient for induction of cardiomyopathy in the mouse and as detection of HEV TD genomes in adult human heart disease is likely due to persistent HEV TD genomes, there is now a new hypothetical mechanism to link of acute viral myocarditis with postviral DCM (Chapman et al. 2008b).

4.2.2 The Parvovirus B19 model

Concerning the Parvovirus B19 virus, a persistent infection was detected in intra-cardiac endothelial cells of small arterioles and veins of patients with chronic cardiomyopathies by in situ hybridization and PCR techniques. This persistent PVB19 infection associated with a low viral replication may be associated with endothelial dysfunction, impairment of myocardial microcirculation, penetration of inflammatory cells and secondary myocyte necrosis. (Duechting et al., 2008). The molecular mechanisms responsible for the reactivation

of latent parvovirus B19 infection, the influence of immune activation triggering parvovirus B19 replication and chronic myocarditis, and immune-independent viral pathogenesis remains to be assessed (Bock et al., 2010).

4.3 The chronic latent viral cardiac infections

In cases of *herpesviruses* (HSV1, HSV-2 , HCMV, HHV-6) cardiac infection a latent phase can occur subsequently to the acute phase of infection as demonstrated in a mice model (Grodums & Zbitnew, 1976). During this HSV experimental latent infection, HSV-DNA can be identified as agents of a persistent heart infection in cardiomyocytes, fibroblasts or Schwann cells, which has be seen in unmyelitinated axons in murine heart tissues. In human subjects, CMVH was detected in cardiomyocytes and in cardiac fibroblasts of patients with histological proven myocarditis (Schönian et al., 1995). Moreover, it was not possible to detect viral mRNA coding for structural proteins known as late proteins but only mRNA coding for viral enzymatic proteins (early proteins) related to the regulation of viral replication or associated with the HCMV DNA replication (Lenzo et al., 2002) Whatever, the cellular sites as well as the mechanisms of latency and reactivation of the *herpesviruses* (EBV, HCMV and HHV6) in human heart tissues remain to be assessed (Andréoletti et al., 2009; Cooper, 2009; Dennert et al., 2008).

5. Viral causes for human acute or chronic myocarditis

Human Enteroviruses, (*picornaviridae*), specifically Coxsackie group B serotypes, Parvovirus B19, HHV6 of the B type and the adenovirus are the most frequently etiological viral agents implicated in the acute myocarditis of the child or the young adult (<35 year-old) (Andréoletti et al., 2009; Bowles et al., 2003; Feldman & McNamara, 2000; Kühl et al., 2005b; Magnani & Dec, 2006) (Table 2). Moreover HHV-1, Adenovirus, myxoviruses and also paramyxoviruses including respiratory syncytial virus (RSV), *influenza* and *parainfluenza* strains can also induce an acute infection of cardiac tissue as previously demonstrated in reported acute myocarditis cases developed in immunocompetent patients (Bowles et al., 2003; Dennert et al., 2008). Other viruses as EBV or CMV are also associated with this pathology after heart transplantation. HIV or HCV can be also etiological agents of myocarditis (Matsumori et al., 2006; Sudano et al., 2006) (Table 2).

Recent data showed that it was possible to detect viruses in 71% of the cases of acute myocarditis using molecular techniques for the virological analysis of cardiac biopsy samples (Table 2) (Kühl et al., 2003). Co-infections were found in more than 12% of the cases, generally associating HHV6 and Parvovirus B19. HHV6 seems to be an important cofactor of myocarditis due to Parvovirus B19. HHV6 may enhance the pathogenicity of Parvovirus B19 through alterations of the extracellular matrix and modulation of the expression levels of the PVB19) receptor (P-antigen) on endothelial cells facilitating infections of the coronary vascular endothelium (Table 2) (Kühl et al., 2005a).

Concerning chronic myocarditis, there is no clinical data from transverse or longitudinal studies indicating the incidence of various viral causes of cardiac infection. However, viral persistence in the myocardium was associated with ventricular dysfunction whereas viral genome clearance was related to the hemodynamic improvement (Kühl et al. 2005a, 2005b). As in the cases of acute myocarditis, recent studies showed the interest to test a broad panel of cardiotropic viruses at the DCM stage. Thus, in a case series of 245 patients with clinically suggested DCM, one or more viruses were detected in 67% of the cases (Table 2) (Kühl et al.,

Human cardiotropic Viruses	Acute myocarditis	Dilated Cardiomyopathy	References
Human enteroviruses	14 - 33 %	8 - 35%	Li et al. 2000; Andréoletti et al. 2000, Kühl et al. 2005a, 2005b
Parvovirus B19 (PrvB19)	<1 - 37%	51%	Kühl et al. 2005a, 2005b
Human Herpes Virus 6 (HHV6)	11%	6-22%	Kühl et al. 2005a, 2005b, Mahrhodt et al.2004
Adenovirus	8.1 - 23%	2 - 12%	Kühl et al. 2005a, 2005b, Bowles et al. 2003
Multiple infections (60% of cases = PrvB19+HHV6)	12%	27%	Kühl et al. 2005a, 2005b
Rare viral causes in immunocompetent patients* (non restrictive list)			
Cytomegalovirus (CMVH)	3%	0.8%	Kühl et al. 2005a, 2005b
Epstein Barr Virus (EBV)	<1%	2%	Kühl et al. 2005a, 2005b
Herpes Simplex Virus (HSV)	<1%	-	Kühl et al. 2005a, 2005b
Influenza viruses	<1 - 2%	-	Kühl et al. 2005a, 2005b
Hepatitis C	-	-	-
HIV	-	-	-

*(-) Not determined; * Prevalence determined in cases of solid organ transplantation.*

Table. 1. Prevalence of viruses detected by molecular biology-based techniques in cardiac tissue samples taken from patients with acute myocarditis or dilated cardiomyopathy.

2005a). Moreover in these patients, the absence of associated myocardial inflammation suggests that viral persistence can be responsible for a modulation of the immune response, which would be decreased from the beginning of the chronic phase of myocarditis (Kawai, 1999; Yajima & Knowlton, 2009).

6. Diagnosis of viral causes of myocarditis

Many common human viruses can be the etiological cause of acute or chronic myocarditis in children or young adult patients (Andréoletti et al., 2009). Because of the large number and serotypes of human viruses potentially responsible for cardiac infection the clinical interest

of viral serological assay remains of a limited interest in clinical practice (Mhafoud et al., 2011). A recent European study demonstrated that virus serology has no relevance for the diagnosis of myocardial infection in young adults; this study indicated that comparatively to the molecular analysis of endomyocardial biopsy tissues, the positive predictive value was 25% and that the negative predictive value was 49% (Mhafoud et al., 2011).Therefore the etiological diagnosis of viral myocarditis is based on the detection of the viruses or viral components (proteins or genomes) in peripheral blood samples at the time of viremia phase (clinically characterized by fever), but also at the entry and the excretion sites (throat, urine and stool samples), and in heart tissue samples that corresponds to the organ site of viral replication. Therefore, endomyocardial biopsy remains the gold standard for unequivocally establishing the histopathological and virological diagnosis of unexplained cardiomyopathies as acute or chronic myocarditis (Li et al., 2000; Mahrholdt et al., 2004). Its clinical impact on prognosis and treatment largely depends on establishing a rapid and standardized set of diagnostic methods including histopathological and virological analyses of endomyocardial tissue taken by endomyocardial biopsy (Aretz et al., 1987; Mahrholdt et al., 2004).

6.1 Endomyocardial biopsy (EMB)

As recommended by the Heart Failure Society of America and the Heart Failure Association of the European Society of Cardiology, the implementation of a right or left ventricular EMB is indicated in the case of acute symptoms of heart failure refractory to standard management, a substantial worsening of ejection fraction despite optimized pharmacological therapy, the development of hemodynamically significant arrhythmias, an heart failure with concurrent rash, fever, or peripheral eosinophilia, an history of collagen vascular disease such as systemic lupus, erythematosus, scleroderma, or polyarteritis nodosum, and a new-onset cardiomyopathy in the presence of known amyloidosis, sarcoidosis, or hemochromatosis when no obvious cause, in particular ischemic, could be established (Mahrholdt et al., 2004). EMB remains the gold standard for unequivocally establishing the diagnosis of unexplained cardiomyopathy Li et al., 2000). However, its sensitivity and its specificity are limited by the often-focal distribution of the specific histological lesions (Baughman, 2006).

Cardiac biopsy samples should be obtained in more than one area of the right ventricular septum and the number of samples should range from 5 to 10 of a volume from 1 to 2 mm^3. Five of these samples should be fixed in neutralized 10% formalin or 10% PFA; five should be flash-frozen or placed immediately conserved at –80°C to perform further classical or molecular virological techniques (Mahrholdt et al., 2004). Interestingly, it has been recently demonstrated that there were no differences in the number of positive left ventricular-EMB, right ventricular-EMB, or left ventricular- and right ventricular-EMB findings when related to the site of cardiovascular magnetic resonance– based late gadolinium enhancement. Preferential biopsy in regions showing late gadolinium enhancement on cardiovascular magnetic resonance does not increase the number of positive diagnoses of myocarditis. (Yilmaz et al., 2010).

6.2 Histopathological evaluation of cardiac biopsy samples

Histological evaluation of cardiac biopsies from patients with clinically suspected myocarditis is routinely done according to the Dallas criteria (Cooper, 2009). Since

myocarditis is a focal disease, 4 to 5 biopsy samples obtained in more than one area of the right ventricular septum should be analysed for light microscopic examination and immunohistochemical assays (Mahrholdt et al., 2004). For routine light microscopy examination, EMB tissue is embedded in paraffin and serial sections are obtained and stained with hematoxylin, eosin. Masson trichrome or Sirius red can be useful for a better evaluation of the fibrosis (Cooper, 2009). Additional slides have to be performed for subsequent immunohistochemical assays (Aretz et al., 1987; Mahrholdt et al., 2004).
To improve the histological diagnosis, additional immunological evaluation of cardiac tissues is required with immunohistochemical techniques allowing quantification, identification, and differentiation of inflammatory cells (Aretz et al., 1987). Criteria for immunohistological diagnosis in the EMB of inflammatory cardiomyopathy is specified quantitatively as >14 infiltrating leukocytes/mm², preferably T-lymphocytes or activated T-cells. If foci of T-lymphocytes are present, active myocarditis can be diagnosed due to the nature of the infiltrate even when the critical level of 14 leukocytes/mm² is not reached (Aretz et al., 1987). Subsequent biopsies will allow reliable follow-up of the myocarditis with a semi-quantitative evaluation of myocardial inflammation, necrosis and healing. If the levels of inflammation appear unchanged from the most recent previous cardiac biopsies, the term ongoing (or persistent) myocarditis will be used (Cooper, 2009).
Finally in cases of clinically suspected viral myocarditis or unexplained cardiomyopathy, Dallas criteria have to be associated with classical immunohistological assays for the identification of inflammatory cells and also for the detection of viral capsid or early or late antigens in EMBs (Andréoletti et al., 2009).

6.3 Classical virological analyses of samples taken from patients with myocarditis

The etiological diagnosis of viral myocarditis, specifically during the acute phase corresponding to the acute viral infection, is based on the detection of the viruses or viral components (proteins or genomes) in heart biopsy tissue samples and additionally to detection of viral genomes in peripheral biological samples as whole blood (viremia) , throat (classical entry site of viruses) and urine and stool samples. The direct virological techniques are now based on molecular biology (PCR, RT-PCR) and immunohistochemical assays for EMBs. By comparison, the contribution of viral serological assays to the clinical diagnosis of acute or chronic myocarditis is relatively poor and the serological assays are of interest only for a late and retrospective diagnosis of viral myocarditis (Dennert et al., 2009). However, Hepatitis C and B and HIV serologies should be systematic in a patients suffering from acute or chronic myocarditis (Aretz et al., 1987).
Using classical molecular techniques, the genome of enteroviruses, adenoviruses, human hepatitis viruses or *herpesviruses* can be detected in 40 to 70 % of the cardiac tissues of patients suffering from an histological–proven acute myocarditis (Table 1) (Badorff et al., 1999; Gravanis & Sternby, 1991). These molecular techniques must be performed on a pool of several EMBs taken, from different heart tissue areas that should be extracted together in order to optimize the efficiency of viral nucleic acid recovery rates (Mahrholdt et al., 2004). Some specialized laboratories use real-time PCR assays that allow a quantitative approach to estimate viral loads of the majority of cardiotropic viruses. However, no published data exist on the clinical value of real-time PCR viral loads and the determination of clinical thresholds that could be capable to differentiate a viral cardiac replicative infection from a persistent or latent viral endomyocardial infection. Only one published study reported a mean value of 500 copies of PVB19 genome per one microgram of extracted DNA as the

clinical threshold related to a significant endomyocardial inflammation (Bock et al., 2010) Only the strategy of associated classical RT-PCR and PCR assays or PCR-microarrays for the detection of all the most common cardiotropic viruses can provide an accurate diagnosis of viral myocarditis. However, sampling error in this focal cardiac disease and the frequent late timing of EMB can also hamper the clinical application of these molecular assays after disease onset. Finally, a positive RT-PCR or PCR result can provide an etiological diagnosis and have to be evaluated into the clinical context, whereas negative PCR results do not exclude a viral-related cardiac disease. Moreover, the only use of the molecular techniques cannot distinguish an active from a persistent viral cardiac infection. Therefore during the clinical course of myocarditis, the immunohistochemical detection of *enterovirus*, *adenovirus or parvovirus B19* capsid proteins or *herpesviruses* late proteins is necessary in order to differentiate a viral cardiac infection with replication activities from a persistent or latent cardiac infection (Matsumori et al, 2006; Sudano et al., 2006). In the present time, the combination of molecular and immunohistochemical assays on cardiac tissue samples provides a reliable diagnostic strategy for a complete diagnosis of a potential viral-induced acute or latent/persistent cardiac infections. The direct detection of viral genomes in association with the immunohistochemical detection of *viral* capsid or late proteins in cardiac tissues is crucial to characterize the phase of the viral infection (acute or persistent/chronic) and therefore to specifically adapt the therapeutic strategies (Andréoletti et al., 2009; Badorff et al., 1999; Gravanis & Sternby, 1991; Sudano et al., 2006).

6.4 Use of new techniques for the virological analyses of heart samples taken from patients with myocarditis or dilated cardiomyopathy

In the present time, classical real time PCR techniques allow a reliable qualitative detection of viral genomes in cardiac tissues of patients in cases of medical diagnosis of hypokinetic cardiomyopathies (Cooper, 2009) or in series of post mortem or explanted heart tissues (Andréoletti et al., 2000, Dennert et al., 2008; Cooper, 2009). The two critical points remain the number of endomyocardial biopsies (5 to 10) their anatomic origin as well the extraction phase that determine the quality of and the sensitivity of detection of the DNA or RNA viral genomic sequences (Cooper, 2009). The standardization of the extraction phase is now possible by the use of semi-automated or totally automated use of standardized procedure using certified available systems (Renois et al.,2010). A pool of several EMBs taken, from different heart tissue areas have to be extracted together in order to optimize the efficiency of viral nucleic acid recovery rates (Copper, 2009; Dennert et al., 2008). Combination of such automated extraction procedures with monoplex or multiplex classical real time RT PCR techniques can allow an international standardization of the molecular qualitative or quantitative detection of DNA or RNA cardiotropic viruses in human endomyocardial biopsies.

It is now possible to use new multiplex amplification assays followed by a microarray hybridization system allowing a simultaneous of 9 to 12 cardiotropic viruses (herpesviruses or human enteroviruses) in a single analysis. We experienced this system in our laboratory and we observed that multiplex PCR-microarray assay provided a consistently robust qualitative detection of at least 9 viruses in on reaction tube (HSV1, HSV2, CMVH, VZV, HHV6, HHV7, HHV8, enteroviruses species A & B) (unpublished data). We showed that there was a 100% concordance for positive virus detection results and that the theoretical limits of detection were as low as 10 genome copies for CVB3 Nancy per microgram of total RNA. Because the system used intergrated a internal extraction and ampliciation control, it

should be assumed that negative results indicate the absence of targeted viruses (Figure 2). The use of such systems could help to standardize the diagnostic detection of cardiotropic viruses in endomyocardial tissues and can be routinely used for a rapid viral detection and screening; however this system is only qualitative and cannot provide semi-quantitative or quantitative viral load values for each detected virus. Therefore in cases of a positive viral detection in cardiac tissues we currently use a second quantitative approach using standardized classical real time viral quantitative detection assays. The obtained results are standardized in viral genome copies per one ug of extracted DNA (Andréoletti et al., 2009, Bock et al., 2010) (Figure 2).

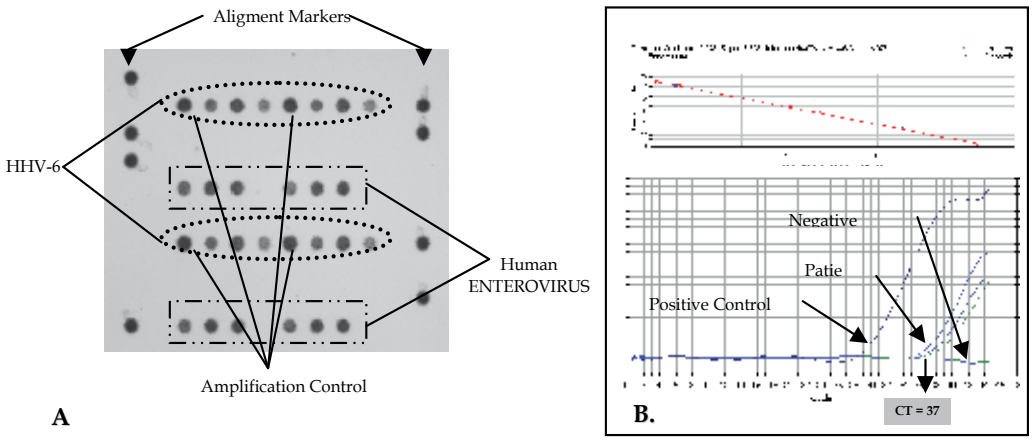

Fig. 2. Detection of a dual Human Enterovirus and HHV6 cardiac infection using a PCR-DNA microarray system in an adult patient with an idiopathic dilated cardiomyopathy. **A.** *PCR-DNA microarray system (CLART® Entherpex,Genomica, Madrid, Spain) allowing the detection and typing of 8 Human Herpes viruses and of the Human Enterovirus (HEVs) group. The study patient is co-infected by HHV-6 and Human Enterovirus (Poliovirus, Echovirus or Coxsackievirus).***B.** *Quantitative detection of HEVs in biopsy heart tissues from the same idiopathic DCM patient using classical real time PCR assay and confirming the HEV cardiac infection Amplification curves obtained for positive and negative controls and one HEV positive DCM patient X. Positive control corresponded to the CVB3 Nancy strain; Negative control corresponded to healthy heart tissue; CT, cycle threshold.*

A new strategy for the molecular detection of cardiac viral infections couples broad-range PCR amplification to electrospray ionization/ mass spectrometry analysis (PCR/ESI-MS). Previous versions of this method were known commercially as the Ibis T5000 and the current commercial hardware platform that conducts the MS analysis is now known as the Abbott PLEX-ID (Ecker et al., 2008). This technique uses primers designed to genomic regions highly conserved regions across viral domains of life. The method was initially developed for the identification of viruses, including previously unknown or unculturable viral agents in samples where multiple microbes may be present, primarily for biodefense applications (Ecker et al., 2008). It is now being developed for diagnosis of human cardiac infections by our team (Figure 3). Preliminary data obtain from 52 heart biopsy samples taken from 24 patients with idiopathic DCM showed a Kappa test correlation of 0.7 +/- 0.22 between PleX-ID detection system and 8 monoplex Q rt-PCR assays. Among the 24 study

patients, our findings indicated that EV (12.5%), PVB19 (12.5%) and dual EV-PVB19 (25%) infections were identified as major potential etiological causes of idiopathic DCM (personal data; not shown). Moreover this new system allowed a rapid semi-quantitative detection of EV associated with a genotyping identification of the EV strains (CVB3 or CVB5; personal data not shown) (Figure 3) .Finally this system allows a rapid and valuable quantitative detection and genotyping identification of common viruses in heart tissues and can be used in clinical practice for an accurate diagnostic of viral cardiac infection.

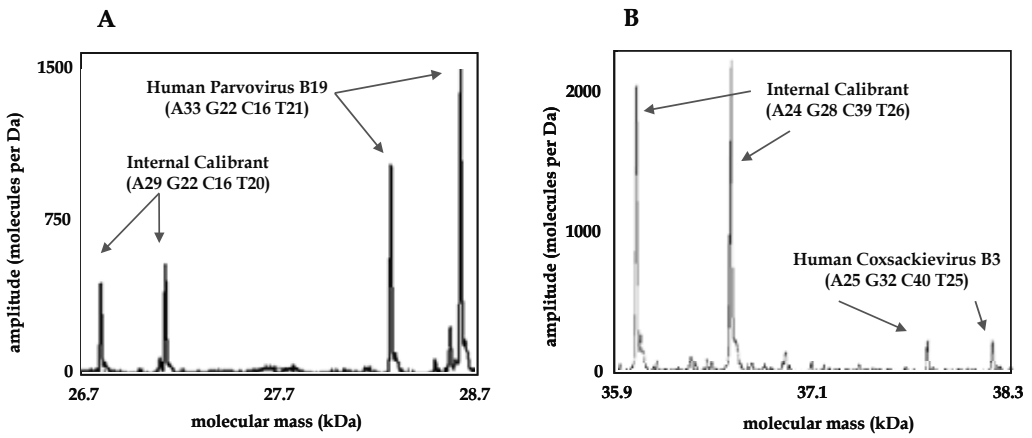

Fig. 3. Detection of parvovirus B19 (panel A) or coxsackievirus B3 (Panel B) in cardiac tissues of two patients (A and B) with idiopathic dilated cardiomyopathy using broad-range PCR amplification followed by ionisation and mass spectrometry analysis (personnal data 2011).

7. Conclusions and therapeutic perspectives

To improve histological diagnosis of viral myocarditis, additional virological evaluation of cardiac tissues is required with immunohistochemical and polymerase chain reaction (PCR) techniques allowing identification and quantification of viral infection markers. The diagnostic gold standard is endomyocardial biopsy (EMB) with the histological Dallas criteria, in association with new immunohistochemical and PCR analyses of cardiac tissues. These new viral diagnostic approaches can lead to better identification of the aetiology of myocarditis and can improve the clinical or therapeutic monitoring of viral causes of human myocarditis. Therapeutic strategies adapted specifically to the phase of the disease are currently under evaluation and may improve prognosis and clinical outcomes significantly. It might be more efficient to use positive immunomodulators (interleukins, interferon alpha, interferon gamma) alone or in combination with specific antiviral components such as ribavirin in the initial phase of the disease when viral replication activity can be detected in the cardiomyocytes (Külh et al., 2003). By contrast, immunosuppressive drugs would be more appropriate in the chronic phase of myocarditis, when no or low viral replication activities are detectable in cardiac tissues by immunohistochemistry assays.

Further specific strategies could consist to specifically block the entry of the virus in target cardiac cells, by preventing interaction of viruses with their cellular receptor and their

consequent signalling amplification systems, such as the tyrosine kinase p56lck, phosphatase CD45 and downstream ERK1/2 (Dennert et al., 2008). Preventing direct viral damage using antiviral therapy is another possible approach by use of specific viral drugs (anti-protease or inhibitors of RNA/DNA polymerases) or by use of general antiviral strategies (interfering RNA). Future randomized placebo-controlled trials should be based upon aetiological diagnosis (viral vs. other causes) and may provide novel treatment options and possibly a better prognosis for these selected patients.

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5

Perimyocarditis

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1. Introduction

Perimyocarditis is an acute inflammation of the pericardium and the underlying myocardium resulting in myocellular damage. It can simply be considered as acute pericarditis with elevated cardiac biomarkers. The coexistence of acute myocarditis and pericarditis is not uncommon since both are commonly caused by cardiotropic viruses. The 2 terms "perimyocarditis" and "myopericarditis" are used to describe the disease. While perimyocarditis implies predominant myocardial involvement and myopericarditis implies predominant pericardial involvement, both terms are used interchangeably without specific reference to the type of cardiac involvement. There is a wide spectrum of clinical presentations reflecting the extent of myocardial involvement ranging from asymptomatic cases with spontaneous recovery, to mild cases where symptoms are masked by the existing illness, to more severe cases complicated with heart failure necessitating inotropic support or even cardiac transplantation. Due to detrimental complications of acute myocarditis including left ventricular dysfunction and ventricular arrhythmias the diagnosis of myocarditis is more important and should be thoroughly looked for. Monitoring of the cardiac biomarkers is therefore mandatory in every patient presenting with clinical picture and electrocardiographic (EKG) evidence of acute pericarditis to exclude an underlying myocarditis. Acute pericarditis can present with ST segment elevation which can sometimes be focal rather than diffuse. In this scenario, chest pain associated with focal ST segment elevation and elevated cardiac biomarkers (if myocarditis co-existed) can mimic transmural myocardial infarction. Differentiation between both entities is of utmost importance to avert the un-necessary utilization of thrombolytic therapy which can be deleterious in perimyocarditis or to avoid missing a more serious diagnosis of ST segment elevation myocardial infarction (STEMI). After hospital discharge, patients should be followed for several weeks to exclude the development of heart failure or subclinical left ventricular dysfunction.

2. Etiology

Similar to myocarditis, perimyocarditis is most commonly of viral aetiology and less likely due to bacterial infection. Viral etiologies are predominately due to the coxsackie B virus,

however, other viruses have been incriminated including cytomegalovirus [1], parvovirus B 19 [2], Epestein-Barr virus [3], Rubella [4], influenza A virus [5] and during hepatitis A virus infection [6]. The most famous bacterial pathogens associated with perimyocarditis are Borrelia burgderferi [7] and Campylobacter jejuni [8]. Others include Mycoplasma pneumonia [9], Chlamydia pneumonia, [10], Brucella [11], Rickettsia Helvetica [12], Yersinia enterocolitica [13], rickettsial Q fever [14], Shigella boydii [15], Shigella sonnei [16], tuberculosis [17], following Streptococcal tonsillitis [18] and Meningococcal septicemia[19]. The protozoan Toxoplasma gondii has also been described as a cause [20]. Immunizations have also been linked with perimyocarditis. The Smallpox vaccine has received great attention especially after its reinstitution for military personnel in 2002 and the report of 50 cases of perimyocarditis [21, 22]. There are several reported cases of perimyocarditis that developed hours after Diphtheria-Tetanus-acellular Pertussis (DTaP) vaccination [23, 24]. Perimyocarditis has also been linked to the administration of certain drugs including meselazine used in the treatment of inflammatory bowel disease [25]. Table 1 represents a compilation of reported etiologies of perimyocarditis.

Etiology of perimyocarditis	
Viral pathogens	Coxsackie B virus Cytomegalovirus Parvovirus B19 Epestein barr virus Rubella Influenza A virus Hepatitis A virus
Bacterial pathogens	Borrelia burgderferi Campylobacter jejuni Mycoplasma pneumonia Chlamydia pneumonia Brucella Rickettsia Helvetica Yersinia enterocolitica Rickettsial q fever shigella boydii Shigella sonnei Tuberculosis Following streptococcal tonsiliitis Following meningococcal septicemia
Protozoa	Toxoplasma gondii
Immunizations	Smallpox vaccine Diphtheria-tetanus-acellular pertussis (DTaP) vaccination tetanus vaccination alone
Immunologic/Connective tissue disease	Sarcoidosis Rheumatoid arthritis Systemic lupus erythematosus Acute rheumatic fever
Drug induced	Meselazine Methydolpa Sulphonamide Cocaine

Table 1. Causes of perimyocarditis.

3. Clinical picture

Perimyocarditis has a wide spectrum of presentation with some cases being asymptomatic, some suffering from symptoms of the preceding viral illness and some presenting with acute heart failure and cardiogenic shock as in cases with fulminant myocarditis. 60% of the cases have constitutional symptoms including fever, arthralgia, malaise and chills. In 35% of the cases, there is chest pain which is usually mild, persistent, stitching, worsens with deep inspiration or coughing and radiates specifically to the trapezius ridge. Chest pain can sometimes be severe raising the suspicion of myocardial infarction which is always in the differential. Patients may also present with palpitations, syncope, Stokes-Adams attacks or sudden death due to arrhythmias including ventricular tachycardia and variable degrees of conduction abnormalities. Careful history taking is mandatory with specific reference to the patient's age, underlying medical problems including diabetes mellitus, hypertension, dyslipidemia, smoking history, positive family history of coronary artery disease and cocaine abuse that can place the patient at risk for myocardial ischemia. Clinical examination may be irrelevant with non-specific features as fever and tachycardia being the only positive clinical findings. Other clues in examination include a pericardial friction rub, however, only a minority of patients have pericardial rub on exam which tends to be transient and variable [26, 27]. A study of a cohort of patients with acute pericarditis confirmed poor sensitivity of a pericardial friction rub, which was found in only 35% of the cases [26]. Signs of decompensated heart failure (e.g. S3 gallop, elevated jugular venous pressure, lower limb edema and pulmonary congestion) can be detected in patients with fulminant myocarditis.

4. Cardiac biomarkers and other laboratory tests

Laboratory investigations in perimyocarditis can reveal elevated white blood cell count (WBC), erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) and Brain natriuretic peptide (BNP) levels. Cardiac biomarkers are also elevated due to myocarditis. The incidence of elevated cardiac troponin I in patients with viral or idiopathic acute pericarditis has been reported to be 32.2%; of these patients 23.7% had a troponin I level at admission higher than those seen in myocardial infarction [28]. Elevated cardiac biomarkers in pericardial disease are not unusual and further complicate the diagnosis, raising suspicion for alternative diagnoses including myocardial infarction [29]. A study by Machdo et. al. concluded that perimyocarditis has a higher cardiac mortality than pericarditis [30]. This illustrates the importance of checking cardiac markers in all patients presenting with pericarditis. Studies also showed that elevated troponin is more common than elevated CKMB [31, 32]. The sole increase in troponin without other cardiac markers might represent a mild degree of myocardial injury.

5. Electrocardiography

Because the pericardium is electrically inert, EKG changes found in patients with acute pericarditis are suggestive of an underlying myocardial involvement. The typical EKG evolution is seen in up to 60 % of cases of acute pericarditis [26]. EKG may reveal sinus tachycardia, diffuse ST segment elevation that is concave upwards involving any lead except aVR and V1. In pericarditis, T wave inversion occurs only after the elevated ST segment returns to baseline. ST-segment elevation associated with pericarditis should not

result in the reciprocal depressions in aVL that accompany inferior MI, although this may not apply in some cases of localized pericarditis [33]. The most specific EKG finding for acute pericarditis is PR segment depression (PR segment elevation in aVR) which is considered an early EKG marker in the evolution of acute pericarditis. PR segment depression is due to subepicardial atrial injury and is present in more than 60 % of the patients. Acute pericarditis causes characteristic EKG changes that typically evolve through 4 stages as demonstrated in table 2.

Stage 1	Occurs during the first few days of pericardial inflammation and may last up to 2 weeks	- Widespread concave upward ST segment elevation except in leads aVR and V1 - Ratio of ST segment elevation to the T-wave amplitude in lead V5 or V6 is ≥ 0.25 - PR segment depression - Absence of reciprocal ST changes
Stage 2	Occurs hours to days after initial symptoms and may last from days to several weeks	- ST segment returns to baseline - In the early phase, the J point returns to baseline while T waves are still upright and in the late phase T waves becomes flattened or even inverted
Stage 3	Begins between the second and third week and may continue for several weeks	- Diffuse T wave inversion
Stage 4	May last from days up to 3 months	- Resolution of T wave inversion and EKG returns back to baseline. - Rarely T wave in nversions may persist indefinitely (chronic pericarditis).

Table 2. The 4 Electrocardiographic stages of acute pericarditis.

Nevertheless, perimyocarditis can present with focal instead of diffuse ST segment elevation mimicking transmural myocardial infarction. This, in addition to the presence of chest pain and elevated cardiac biomarkers can make the differentiation of increasing difficulty. This is important because fatal complications can occur if thrombolytic therapy is administered for a patient with acute pericarditis, or if a diagnosis of transmural myocardial infarction is missed. Omar et al. demonstrated a similar scenario where an EKG of a patient presenting with chest pain revealed focal ST segment elevation (figure 1) and the cardiac biomarkers were elevated mimicking STEMI. [34] Careful history taking, EKG interpretation and urgent echocardiogram favored the diagnosis of acute perimyocarditis.
Previous studies have reported the use of thrombolytic therapy for what was later determined to be acute pericarditis [35, 36]. The utilization of urgent coronary angiography is not uncommon in patients with acute perimyocarditis. Salisbury and colleagues described the frequency of urgent coronary angiography in 238 patients with a final diagnosis of acute pericarditis to be 16.8 % [37].

6. Echocardiography

Echocardiography looking for pericardial effusion and regional wall motion abnormalities is mandatory to help in making the diagnosis and excluding other serious differentials. Imazio

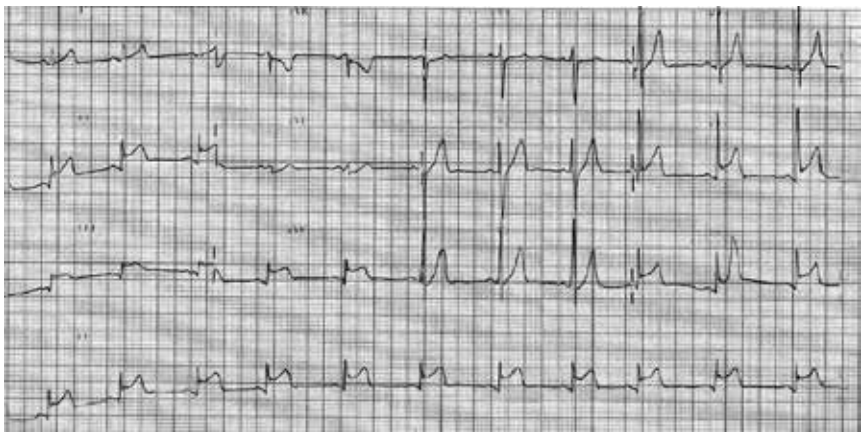

Fig. 1. Concave upward ST segment elevation in leads II, III, Avf, V5 and V6 in a patient with perimyocarditis. Notice the focal pattern of ST segment elevation (inferolateral leads) thereby mimicking transmural myocardial infarction. Adapted from Omar et. al. [34].

and collegues found that pericardial effusions are present in approximately 60% of cases of acute pericarditis, with 80% being mild, 10% being moderate, and 10% being severe [26]. Pericardial effusion was present in 38.1% of patients in the ST segment elevation group and 73.5% of the patients in the non ST segment elevation and was explained by the tendency of the larger pericardial effusions to decrease voltage including the magnitude of ST segment elevation [26]. The presence of regional wall motion abnormalities favors an ischemic process rather than acute pericarditis. Another potential echocardiographic finding in perimyocarditis is transient myocardial thickening (figure 2 and 3) which is due to interstitial edema and its presence likely predicts a poor prognosis as it has been associated with a fulminant course [38]. In a series of 25 patients with acute myocarditis who underwent echocardiogram and endomyocardial biopsy, a significant decrease in myocardial thickness was observed between the acute and the convalescent phase. The reduction of the edema shown by the biopsy was also significant [39].

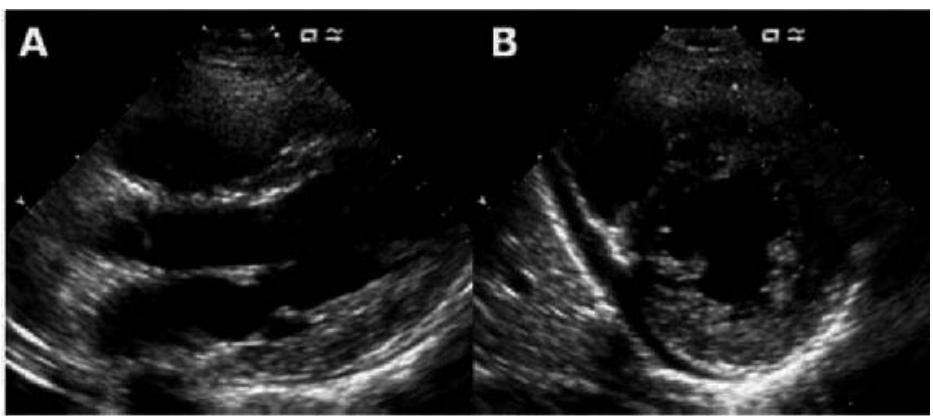

Fig. 2. Echocardiogram on admission. Parasternal long axis view (A) and short axis (B) in diastole. There is an asymmetrical thickening of the posterior wall involving the posterior papillary muscle, and slight pericardial effusion. Adapted from reference number 38 with permission.

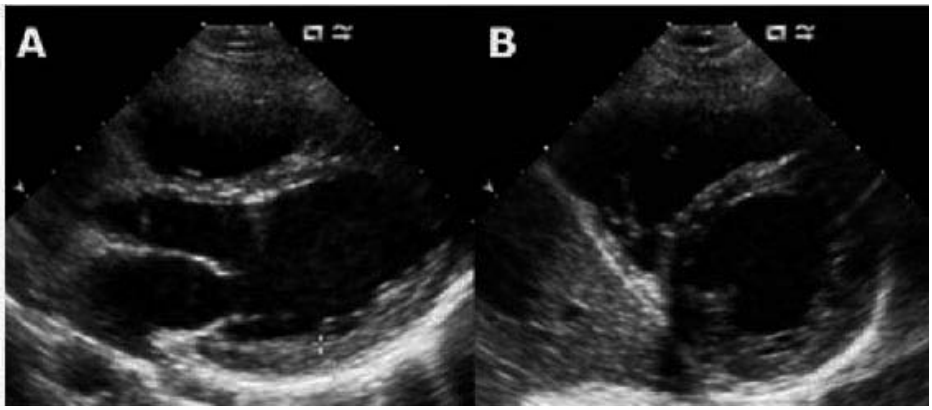

Fig. 3. Echocardiogram 5 days after admission. Parasternal long axis view (A) and short axis (B) in diastole. Normalization of the myocardial thickness in the posterior wall and posterior papillary and resolution of the pericardial effusion can be seen. Adapted from reference 38 with permission.

7. CT angiography

Another non-invasive diagnostic tool in patients presenting with chest pain, ST segment elevation and elevated cardiac biomarkers is the use of the 64-slice coronary CT angiography (CCTA) which is a reliable test that can be utilized in patients with low to intermediate pretest probability for coronary artery disease. Shturman et. al. used the 64-slice CCTA to rule out coronary artery disease in a case of perimyocarditis mimicking myocardial infarction [40]. This simple noninvasive test and can avert the unnecessary need for coronary angiography or thrombolytic therapy for a presumed STEMI. Recently, Computed tomographic angiography (CTA) "triple rule out" protocol has been utilized in the emergency department for patients presenting with acute chest pain to differentiate between pulmonary embolism, aortic dissection and acute coronary syndrome. Compared with the usual radiation dose of a standard 64-slice CCTA, the effective radiation dose of a "triple rule-out" scan is often increased by 50% which should limit its unrestricted use. Takakuwa et. al. reported the successful use of the "triple rule-out" scan in diagnosing acute perimyocarditis (figure 4) and excluding other serious etiologies as acute coronary syndrome, aortic dissection and pulmonary embolism [41].

8. Cardiac magnetic resonance imaging

In perimyocarditis, gadolinium contrast MRI is useful to confirm the diagnosis by detecting an area of delayed contrast-enhancement, to evaluate the severity of inflammation in the acute stage as well as to determine the extent of fibrosis in the pericardium and myocardium [42]. The normal pericardium is observed as an area of low intensity on T1-weighted images and T2-weighted images [43]. In acute pericarditis, the inflamed pericardium is thickened and appears as an area of medium to high intensity with delayed contrast-enhancement recognized in the swollen pericardium which extends to the subepicardial myocardium affected by myocarditis [42]. The delayed enhancement in cardiac magnetic resonance (CMR) is explained by the leaking of the contrast media into the interstitial space due to

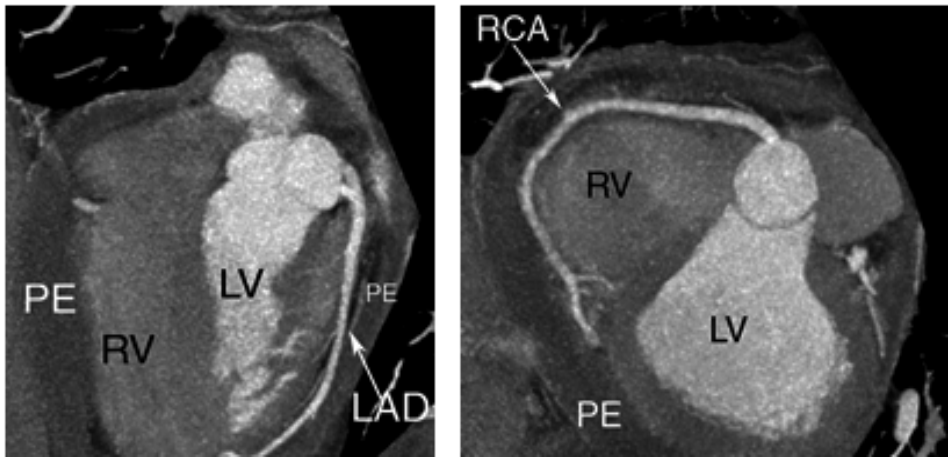

Fig. 4. Four chamber view (left). TRO study demonstrating mild-to-moderate pericardial effusion surrounding the heart. Left anterior oblique view (right) optimized to visualize the full length of the RCA. PE - pericardial effusion, RV - right ventricle, LV - left ventricle, LAD - left anterior descending coronary artery. Adapted with modification from reference number 41 with permission.

inflammation. As it stays out of the vessel, it cannot be washed away and is held for a longer time allowing it to be seen in the delayed enhancement images (figure 5) [44].

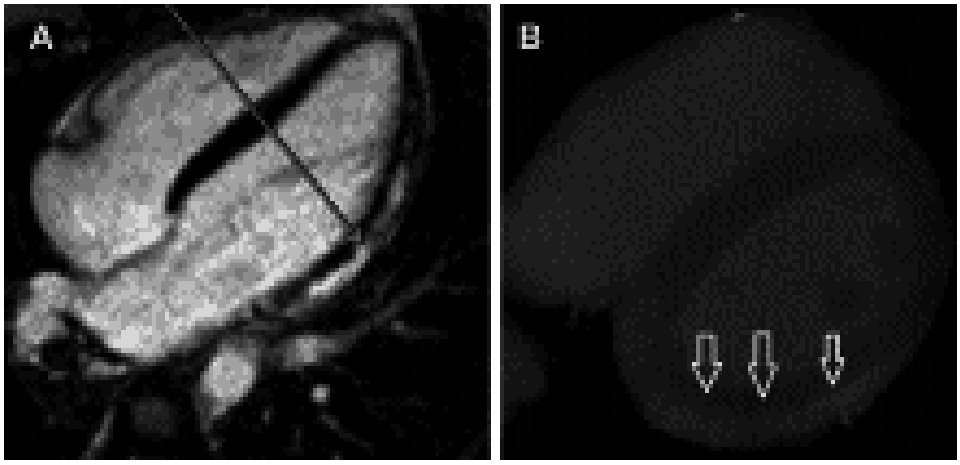

Fig. 5. Four chamber long-axis views. (A) Cardiac magnetic gadolinium delayed enhancement showing subepicardial hyperenhancement (arrow). (B) Cardiac CT delayed enhancement (arrows). Adapted from reference 44 with permission from Elsevier.

Cardiac magnetic resonance can also be a tool to differentiate between acute perimyocarditis and myocardial infarction. In acute myocarditis, myocardial late gadolinium enhancement is present in up to 88% of cases [45,46] which characteristically has patchy distribution not conforming to any particular coronary artery territory and is usually in the subepicardial and not the subendocardial layer [47] differentiating it from myocardial infarction.

9. Endomyocardial biopsy

Myocarditis may be focal or diffuse affecting any or all cardiac chambers. Although biopsy is the gold standard for the diagnosis, it is of limited utility especially in acute myocarditis, because of the patchy nature of active inflammation. In a series of over 2,000 patients with clinically suspected myocarditis, endomyocardial biopsy was only positive in 10% of the cases [48-50]. Given the potential risks of biopsy and the limited value it offers, its use should be limited to the patients with left ventricular dysfunction unresponsive to conventional therapy [51].

The various histologic patterns of myocarditis include either lymphocytic (including viral and autoimmune forms), eosinophilic (in which hypersensitivity myocarditis is the most common, followed by cases of hypereosinophilic syndrome), granulomatous (sarcoid and giant cell myocarditis), neutrophilic (bacterial, fungal, and early forms of viral myocarditis), and reperfusion type/contraction band necrosis (present in catecholamine-induced injury and reperfusion injury). Figure 6 represents the microscopic picture of acute perimyocarditis after diphtheria-tetanus vaccination.

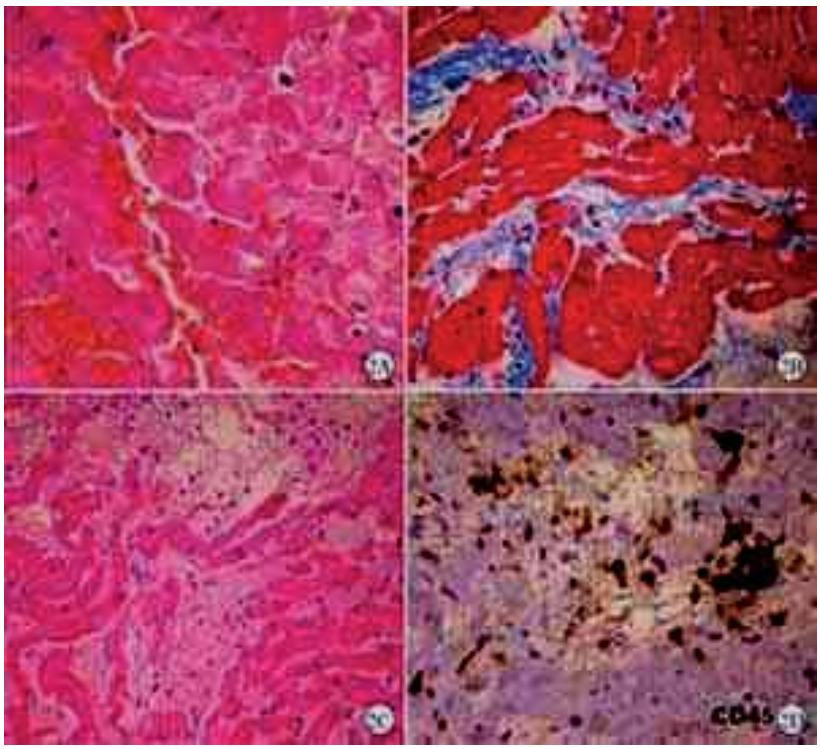

Fig. 6. Right ventricular endomyocardial biopsy. A . Haematoxylin-eosin, original magnification 200x: Diffuse interstitial oedema with scattered inflammatory cells. B. Immunohistochemical staining with anti-CD45 antibody, original magnification 400x: Lymphocyte inflammatory infiltrate associated with myocyte damage. C. Mallory triple stain, original magnification 200x: minimal interstitial fibrosis mixed with interstitial oedema. D. Haematoxylin-eosin, original magnification 200x: One focus of interstitial haemorrhage. Adapted from Journal of Chinese Clinical Medicne;2008,9;Vol.3,No.9. [24]

10. Management

As earlier described, perimyocarditis is a combination of both pericardial inflammation and myocardial damage. Treatment should therefore target both pathologies. Because myocarditis is a more serious diagnosis owing to the potential of serious ventricular arrhythmias and heart failure, the diagnosis of myocarditis is deemed more important. Acute pericarditis usually runs a smooth and benign course after empiric treatment with NSAID and routine hospitalization in most cases is not necessary. Perimyocarditis on the other hand has higher incidence of complications and is one of the indications for hospitalization. Checking the levels of cardiac biomarkers and echocardiography is therefore mandatory in any case of acute pericarditis. Imazio et. al. identified certain poor prognostic predictors that are more frequently associated with an increased risk of short term complications and therefore an indication for hospitalization [26]. Table 3 lists various indications for hospitalization of patients presenting with acute pericarditis

Indications for Hospitalization of Patients with Acute Pericarditis
1. Anticoagulation therapy
2. Body temperature greater than 100.4° F (38° C)
3. Echocardiographic findings of a large pericardial effusion
4. Findings of cardiac tamponade (i.e., hypotension and neck vein distention)
5. History of trauma and compromised immune system
6. Myopericarditis
7. Troponin I elevation

Table 3. Indications for Hospitalization of Patients with Acute Pericarditis. (adapted from reference 26)

There are certain scenarios when perimyocarditis present with focal EKG signs suggestive of STEMI. This can be challenging especially in developing countries where thrombolytic therapy is the mainstay of management of STEMI. In patients with acute pericarditis, thrombolytic therapy can be detrimental because of the risk of cardiac tamponade [52, 53].
Although the use of anticoagulants in patients with acute pericarditis is deemed unfavorable, in their study on 274 consecutive cases of idiopathic or viral acute pericarditis, Imazio and colleagues concluded that neither the use of heparin, anticoagulants nor glycoprotein IIb/IIIa inhibitors is associated with an increased risk of cardiac tamponade. [54] Risk factors for complications in that study included the lack of complete response to aspirin or NSAID (OR = 14.6, 95% CI 6.1 to 35.1; P = 0.001), or corticosteroid use (OR = 3.0, 95% CI 1.1 to 8.9; P = 0.048).
The mainstay of therapy for acute pericarditis is NSAID (class 1 recommendation in 2004 ESC guidelines). The goal of NSAID is to reduce pain and inflammation. Ibuprofen might be preferred because of its rare side effects, favorable impact on coronary artery blood flow and large dose range from 1200 to 1800 mg daily [55]. Aspirin can also be used in anti-inflammatory doses (up to 800 mg every 6 hours). Dose tapering is preferred to avoid recurrence. Gastric protection is mandatory and should be commenced in all patients. In perimyocarditis, NSAID should be used cautiously because in animal models they were shown to enhance the myocarditic process and may increase mortality [56-58]. Lower anti-inflammatory doses should therefore be considered whenever possible in perimyocarditis and its main use is to control symptoms. Failure to respond to NSAID within one week

(indicated by persistence of fever, new pericardial effusion, or continuing chest pain) indicates that a cause other than viral is responsible and should be searched for. Although colchicines can be used alone or in conjunction with NSAID in treatment and prevention of recurrent pericarditis, there is lack of data regarding its benefit in perimyocarditis [59].
The use of corticosteroids was found to be an independent risk factor for recurrence of acute pericarditis [60, 61] because of their ability to promote viral replication [62, 63]. Its use should therefore be restricted for those with autoimmune disease or in cases refractory to NSAID and colchicine and a specific cause has not been found. Despite the long list of pathogens, in most cases, a specific etiology for acute perimyocarditis can't be determined. In instances where an underlying treatable cause is confirmed, treatment of the target organism should be commenced. After hospital discharge, patient should be followed for several weeks to rule out the development of heart failure or subclinical left ventricular dysfunction. All patients should be advised to avoid strenuous exercise during the recovery phase (4-6 weeks) which can increase the risk of ventricular arrhythmias.

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6

Pathogenesis and Pathology of Chagas' Chronic Myocarditis

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1. Introduction

Chronic chagasic cardiomyopathy (CCC) is the most serious manifestation of the chronic phase of Chagas' disease and constitutes the most common type of chronic myocarditis in the world (Guerri-Guttenberg, et al., 2008, Milei, et al., 1996a, Milei, et al., 2009, Milei, et al., 1992a, Storino, et al., 1992). Chagas' disease, a chronic illness caused by the flagellate parasite *Trypanosoma cruzi* (*T. cruzi*), was first described in 1909 by the Brazilian physician Carlos Chagas (Chagas C, 1909). The insect vectors of the disease are present throughout most of South and Central America, and their zone of distribution extends across the southern United States (Rassi, et al., 2010). It was estimated by year 2000, that in endemic areas 40 million people were considered to be at risk of infection, being 20 million already infected. Every year near 200,000 new cases are expected to happen, and 21,000 deaths per year occur (WHO, 2005).

Although always considered to be confined to Latin America, due to migratory movements from endemic countries to Europe and North America, Chagas' disease is being detected more frequently in developed countries. Europe is estimated to have from 24,001 to 38,708 (lower or upper limit of estimate, respectively) immigrants with *T. cruzi* infection (Guerri-Guttenberg, et al., 2008). In the United States, six autochthonous cases, five transfusion related cases and five transplant related cases have been reported, but migratory movements still remain the main source of Chagas' disease. It has been estimated that around 89,221 to 693,302 infected Latin Americans migrated to the United States in the period 1981 to 2005 (Milei, et al., 2009).

Two phases of the disease can be distinguished: (1) acute phase, with transiently high concentration of parasites in tissue and blood, nonspecific symptoms, and a 5% myocarditis incidence, lasting 4 – 8 weeks; and (2) chronic phase, lasting lifelong. Chronic phase can be presented as indeterminate form, characterized by lack of symptoms and normal ECG and normal radiographic examination of the chest, esophagus and colon. Approximately 60 – 70% of patients remain in this form for the rest of their lives. Only 20-40% of infected individuals, 10-30 years after the original acute infection, will develop cardiac, digestive or mixed form of the disease, characterized by the appearance of megavicera (dilated cardiomyopathy, megaesophagus and/or megacolon). It poses a substantial public health burden due to high morbidity and mortality (Milei, et al., 2009, Rassi, et al., 2000, Rassi, et al., 2010).

CCC is manifested by a chronic, diffuse, progressive fibrosing myocarditis that involves not only the working myocardium but also the atrioventricular (AV) conduction system, autonomic nervous system and microcirculation (Andrade, 1985, Marin-Neto, et al., 2007, Milei, et al., 1991b). This leads to cardiomegaly, cardiac failure, arrhythmias, thromboembolism, and death (Milei, et al., 1991b). Colon and esophagus are also commonly affected by Chagas' disease, being megacolon with constipation and megaesofagus with achalasia also features of the disease (Rassi, et al., 2010).

2. Pathogenesis of Chagas' myocarditis

Milei et al. proposed a combined theory that could explain the pathogenic mechanism in chronic chagasic myocarditis (Milei, et al., 1996a, Storino & Milei, 1994). This theory is based on three ingredients: the parasite, host immune system and fibrosis. These ingredients are proposed as being the primary causative agents of damage on myocardial tissue, conduction system, autonomic ganglia and nerves and microvasculature.

2.1 First ingredient: the parasite

The role of *T. cruzi* in the chronic phase has been previously underestimated due to the fact that its presence was believed to be scarce and unrelated to the inflammatory infiltrate present at this stage. Nowadays, the involvement of the parasite in the chronic phase has been well documented. Using dissimilar methods, different authors demonstrated either the persistence of *T. cruzi* or parasite antigens in mice (Younées-Chennoufi, et al., 1988), the parasite DNA sequence amplified by the polymerase chain reaction (PCR) (Jones, et al., 1993, Schijman, et al., 2004), *T. cruzi* antigens from inflammatory lesions in human chagasic cardiomyopathy (Higuchi, Brito et al. 1993), or the immunohistochemical finding of the parasite in endomyocardial biopsies with PCR confirmation (Añez, Carrasco et al. 1999). This would suggest a direct role for the parasite in the perpetuation of myocardial inflammation. In other words, the antigen stimulation would persist throughout the chronic stage, even though the parasites are not morphologically detectable by light microscopy (Andrade 1992).

The role of parasitemia is more controversial. High parasitemia correlated with severity of disease in one report (Basquiera, et al., 2003), but showed no association in another (Castro C., et al., 2005).

Interestingly, it has been observed that immunosuppression reactivates rather than ameliorates the disease, as seen in patients receiving immunosuppressive therapy to prevent transplant rejection and in AIDS patients. Accordingly, many experimental models where strains of genetically manipulated mice lacking various immune functions showed increased susceptibility to develop the disease (Tarleton & Zhang, 1999).

2.1.1 Life cycle of *Trypanosoma cruzi*

When a reduviid bug feeds from an infected mammal, it takes up circulating trypomastigotes, which reach then the bug's gut. There, they differentiate to amastigotes, which proliferate and start to differentiate into epimastigotes. In this process, when amastigote is still sphere-shaped but has developed its flagellum, some authors call this stage spheromastigotes. Then, it elongates its cell body and flagellum, taking the classical epimastigote shape. At this stage, the parasite undergoes metacyclogenesis, differentiating in metacyclic trypomastigotes, the infective form for mammals. When the bug feeds again,

it excretes trypomastigotes with feces, which in turn reach blood torrent through bug's wound. Trypomastigotes can infect a wide variety of host cells, within them it differentiate into amastigotes and proliferate. Then, they can differentiate into trypomastigotes again, reach circulation and infect new cells. If an uninfected bug feeds from the animal in the moment of parasitemia, cycle starts again (Tyler & Engman, 2001).

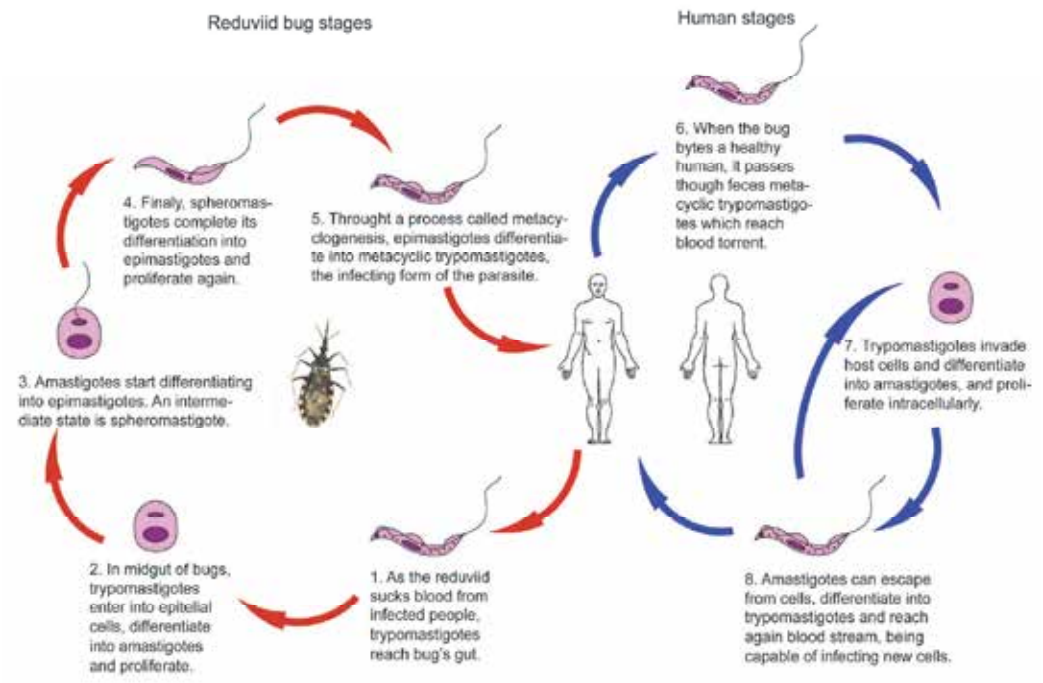

Fig. 1. Life cycle of *Trypanosoma cruzi*.

2.1.2 Genetic variability of *Trypanosoma cruzi* and its relation to its pathogenesis

The genetics of *T. cruzi* caught the attention of researchers in late 80' and early 90'. First studies on variability were performed analyzing electrophoretic variants on cellular enzymes. The groups resulting were called zymodemes and were named Z1, Z2, Z3. Only Z2 was associated with domestic transmission cycle.

The development of PCR based techniques, allowed the study of new variant regions and the characterization of multiple variants of a great number of genes. All these variants showed significant correlation with each other, suggesting the existence of two subtypes of *T. cruzi* based on these data (Macedo, et al., 2004). Moreover, *T. cruzi II* which is clearly linked to human pathology, being *T. cruzi I* mainly related to infection of wild sylvatic mammals. Even, applying LSSP-PCR to the study of the variable region of kinetoplast minicircle from *T. cruzi* provided evidence of a differential tissue distribution of genetically diverse *T. cruzi* populations in chronic Chagas' disease, suggesting that the genetic variability of the parasite is one of the determining factors of the clinical form of the disease (Vago, et al., 2000).

2.1.3 Cell host invasion and intracellular survival by *Trypanosoma cruzi*

Once *T. cruzi* reaches blood torrent, it invades a great variety of cells in the host. When parasiting non phagocytic cells, *T. cruzi* uses some surface glycoproteins to attach to cell: gp82, gp30 and gp35/50. All three glycoproteins are known to induce calcium mobilization from intracellular reservoirs. Gp82 is linked to the phospholipase C (PLC) and inositol 1,4,5 – triphosphate (IP3). Gp 35/50 is associated to increasing intracellular levels of cyclic AMP. On the other side, cruzipain, a protein known to be secreted by *T. cruzi*, acts on kininogen and produces bradykinin, which binds to its receptor, further increasing intracellular calcium. Increased intracellular calcium produces modifications in cytoskeleton that lead to parasite endocytosis (Yoshida & Cortez, 2008).

In the parasitoforous vacuole, mainly by the action of gp85/TS a glycoprotein with trans-sialidase action, and TcTox, a protease, the parasite degrades the membrane of the vacuole, escapes from it and proliferates within the cell (Alves & Colli, 2007).

2.1.4 Molecular mimicry

The induction of autoimmunity by similarities between *T. cruzi* and host epitopes has been long proposed as a mechanism that leads to tissue damage in the chronic phase of the disease. Both humoral and cellular autoimmune responses have been described, but we will discuss them in more detail in the section of immune system. The real importance of molecular mimicry in the pathogenesis of chagasic myocarditis is still a matter of debate (Girones, et al., 2005).

Although it seems that in some cases this mechanism triggers autoimmunity, in many others, autoimmunity seems to be an epiphenomenon of cellular destruction, with exposition of intracellular epitopes not normally exposed to the immune system. This, in turn may activate autoreactive lymphocytes leading to the appearance of autoantibodies that are not the cause of damage, rather a consequence (Girones, et al., 2005).

The most important cross reacting epitopes of *T. cruzi* and the correspondent epitopes in humans are listed in table 1, as well as the kind of immune response they elicit.

2.2 Second ingredient: host immune system

When the three ingredients theory was first proposed (Storino and Milei 1994, Milei, et al. 1996), second ingredients were mainly T lymphocytes and macrophages. In the subsequent years some evidence grew about the participation of humoral immune system through autoantibodies in the pathogenesis. As a consequence, the whole immune system of the host is now considered as the second ingredient.

As described earlier, mononuclear cells persist in the chronic stage of the disease, contributing to the inflammation through its products of secretion or through its own cytotoxicity (suppressor T cells) and cytolytic action (macrophages) (Storino & Milei, 1994).

As previously stated, molecular mimicry may be the main explanation of autoimmunity, triggering both cellular and humoral autoreactivity (Girones, et al., 2005). Figure 2 summarizes the most important immune events in CCC pathogenesis.

2.2.1 Innate immunity

In recent years innate immunity came to the attention of researchers of Chagas' disease pathogenesis. The role of NK cells has been particularly studied in early and late indeterminate forms of the disease and in CCC patients. In early indeterminate patients,

Parasite antigen	Human Antigen	Immune reaction
B13	Cardiac myosin heavy chain	Autoantibodies Autoreactive T cells
R13 (ribosomal protein)	Ribosomal protein β_1-adrenergic receptor M_2-muscarinic receptor 38-kDa heart antigen	Autoantibodies
Ribosomal protein PO	β_1-adrenergic receptor	Autoantibodies
FL-160	47-kDA neuron protein	Autoantibodies
Shed acute-phase antigen (SAPA)	Cha antigen	Autoreactive T cells
TENU2845/36 kDa	Cha antigen	Autoantibodies
Calcireticulin	Calcireticulin	Autoantibodies Autoreactive T cells
Galactosyl-cerebrosides	Galactosyl-cerebrosides	Autoantibodies
Unknown	Neurons, liver, kidney, testis	Autoantibodies
Sulphated glycolipids	Neurons	Autoantibodies
150-kDa protein	Smooth and striated muscle	Autoantibodies
Cruzipain	Cardiac myosin heavy chain M_2-muscarinic receptor	Autoantibodies
Microsomal fraction	Heart and skeletal muscle	Autoantibodies
Cytoskeleton	95-kDa myosin tail	Autoantibodies
SRA	Skeletal muscle Ca^{2+} dependent SRA	Autoantibodies
MAP	MAP (brain)	Autoantibodies
Soluble extract	Myelin basic protein	Autoantibodies Autoreactive T cells
55-kDa membrane protein	28-kDa Lymphocyte membrane protein	Autoantibodies

Table 1. Examples of cross-reacting epitopes (Girones, et al., 2005, Marin-Neto, et al., 2007).

compared to non infected people, increased values of pre-natural killer (NK)-cells ($CD3^-$ $CD16^+$ $CD56^-$), and higher values of proinflammatory monocytes ($CD14^+$ $CD16^+$ $HLA\text{-}DR^{++}$) were found. The higher values of activated B lymphocytes ($CD19^+$ $CD23^+$) contrasted with impaired T cell activation, indicated by lower values of $CD4^+$ $CD38^+$ and $CD4^+$ $HLA\text{-}DR^+$ lymphocytes, a lower frequency of $CD8^+$ $CD38^+$ and $CD8^+$ $HLA\text{-}DR^+$ cells; a decreased frequency of $CD4^+$ $CD25^{HIGH}$ regulatory T cells was also observed. All these data suggest a rather proinflammatory profile (Vitelli-Avelar, et al., 2006). This profile may be useful to limit parasitemia and confine infection to tissues. In fact, it has been demonstrated that NK cells are important in defense against the spread of parasitic infection (Brener & Gazzinelli, 1997), and are an important source of INF-γ, a key cytokine to activate macrophages and help with parasite clearance (Camargo, et al., 1997).

In late indeterminate form, $CD3^-CD16^+CD56^+$ and $CD3^-CD16^+CD56^{DIM}$ NK cells are increased but are in normal range in CCC patients, suggesting a protective role for them (Vitelli-Avelar, et al., 2005). NK cells showing $CD56^{DIM}$ may play a role in the down

modulation of cytotoxic deleterious T $CD8^+$ response reported in CCC patients (Sathler-Avelar, et al., 2009).
Monocytes display different cytokine profile. In indeterminate patients they produce more IL-10 (Gomes, et al., 2003) while in CCC patients they produce more TNF-α (Vitelli-Avelar, et al., 2008), leading to a proinflammatory profile that could be responsible for chronic myocarditis.
Toll-like receptors (TLR) are also implied in the response to acute infection with *T. cruzi*. TLR-2 has been shown to recognize GPI surface molecules from the parasite. In vitro and in vivo studies have demonstrated that macrophages stimulated with GPIs through TLR-2/CD14 receptors produce NO, TNF-α and IL-12 (Campos & Gazzinelli, 2004).

2.2.2 Cellular adaptative immunity

The role of immune cells in the pathogenesis of Chagas' heart disease has been the dominant hypothesis for many years. The paucity of parasite cells in the inflamed myocardium and the presence throughout the evolution of the disease of macrophages and lymphocytes in patched infiltrates lead to this hypothesis. As early as in 1929, Magariños Torres, observing those infiltrates postulated an "allergic" mechanism for CCC. Further, Mazza and Jörg followed this thought and supported the "allergic" theory (Storino & Milei, 1994).
The study of circulating lymphocytes in peripheral blood of chagasic patients showed an increase in the percentages and actual numbers of double-positive cells of the phenotype CD3+/HLA-DR+, as well as decrease in the percentage of CD45RA+/CD4+ and CD45RA+/CD8+ T cells, indicating greater numbers of activated T cells circulating. Consistent parallel increases were seen also in the B lymphocyte subset which stained double-positive for CD19/CD5 (Dutra W. O., et al., 1994). These results were similar for both indeterminate and CCC patients. Moreover, activated T cells lacking the co-stimulatory molecule CD28 are increased in chagasic patients (Menezes, et al., 2004) and express high levels of HLA-DR molecules (Dutra, et al., 2000). Some interesting differences were demonstrated between indeterminate and CCC patients. $CD28^-$ T cells in indeterminate patients showed expression of CTLA-4, which recognizes the same ligands as CD28, but instead of inducing cell activation it causes down modulation of T cells. On the contrary, T cells in CCC patients do not up-regulate CTLA-4 (Souza P. E. A., et al., 2007). It is particularly interesting that CD8+CD28- cells are increased in CCC patients compared to indeterminate patients, and that these cells do not require co-stimulation to exert their cytotoxic functions. More strikingly, CD4+CD28- cells behave differently in indeterminate and CCC patients. In the formers, they are closely related to IL-10 levels, while in CCC patients they correlate with INF-γ levels (Menezes, et al., 2004).
Another interesting difference has been found in cellular response between indeterminate and CCC patients. CD4+ cells from CCC patients had an increased expression of Vβ5+-TCR, not found in indeterminate patients. When CCC patients mononuclear cells from peripheral blood were cultured in the presence of trypomastigotes antigens, a selective expansion of CD4+ Vβ5+ cells was obtained; while when cultured in the presence of epimastigotes antigens, an expansion of CD8+ Vβ5+ cells was also noted. These findings could not be repeated in indeterminate patients. Trypomastigote stimulation led to the expansion of CD4+ Vβ17+ in indeterminate patients, not seen in CCC patients. This suggests that CCC patients and indeterminate patients respond to different antigen repertoires (Costa, et al., 2000).

Monocytes from indeterminate patients, when infected *in vitro* with *T. cruzi*, express low levels of HLA-DR and high levels of CD80, a ligand for CTLA-4 (Souza P. E., et al., 2004). The interaction of these monocytes with CTLA-4$^+$ T cells leads to the expression of IL-10, a cytokine known to down-modulate inflammatory responses (Gomes, et al., 2003). This is not observed in CCC patients. CD28$^-$ T cells, not expressing CTLA-4, express TNF-α and INF-γ (Menezes, et al., 2004).

In the same direction, CD4$^-$CD8$^-$ γδ T cells are found to be increased in indeterminate patients compared with CCC ones. These cells are also linked to the production of IL-10 and a down modulator effect on inflammation (Villani, et al., 2010).

Cells infiltrating myocardium have also been studied. As demonstrated with immunostaining of endomyocardial biopsies by our group, leukocytes infiltrating myocardium in Chagas' disease were approximately 50% macrophages, and 50% lymphocytes, mainly T lymphocytes (Milei, et al., 1992b). Further immunohistochemical characterization of these cells with CD45R for lymphocytes, CD20 and lambda and kappa light chains for B lymphocytes, CD45R0 for T lymphocytes and CD68 for macrophages, confirmed these findings (Milei, et al., 1996a).

Autoreactive T cells have caught the attention of many investigators. In experimental models, CD4$^+$ T cells from infected mice showed a proliferative response to the exposition to human cardiac myosin heavy chain and to *T. cruzi* B13 protein. They also arrested the beating of fetal heart cells and, more importantly, induced myocarditis in immunized mice and promoted rejection of transplanted normal hearts in the absence of *T. cruzi* (Ribeiro-Dos-Santos, et al., 2001). Also, it has been described that T cells infiltrating the myocardium of chagasic patients cross react with human cardiac myosin heavy chain and to *T. cruzi* B13 protein and express high levels of INF-γ and low levels of IL-4, switching to a Th1 profile (Cunha-Neto Edecio & Kalilf, 2001).

In recent years, Treg cells have come to attention in relation to Chagas' disease pathogenesis. These cells are characterized by the expression of CD4, CD25 and FOXP3 (Ziegler & Buckner, 2009). Treg cells are increased in indeterminate patients compared to CCC, which correlates negatively with levels of activated CD8$^+$ (Vitelli-Avelar, et al., 2005).

A second subset of T CD4+ cells, recently described, the Th17 cells, resulted important in Chagas' disease pathogenesis. These cells, mainly linked to autoimmune pathology, are characterized by the expression of CD4+, RORγt, and secrete IL-17 (Di Jin, et al., 2008). They were increased in a murine model of acute myocarditis induced by *T. cruzi* infection, as well as by immunization with heat-killed *T. cruzi* antigens (Bonney, et al., 2011). Both cell subsets seem to be related, as they require TGF-β to differentiate. In the presence of proinflammatory cytokines, differentiation to Th17 cell prevails and a pro-autoimmune profile develops (Ziegler & Buckner, 2009).

An additional mechanism is the bystander activation. This is the activation of autoreactive lymphocytes by antigen presenting cells in a proinflammatory environment (Fujinami, et al., 2006). This kind of autoreactive T cells activation has been described in Chagas'disease (Fedoseyeva, et al., 1999).

2.2.3 Humoral adaptative immunity

The importance of humoral immunity in controlling *T. cruzi* acute infection has been clearly established. Mice lacking B lymphocytes rapidly succumb to infection (Kumar & Tarleton, 1998). But the fact that attracted most attention is the production of autoantibodies.

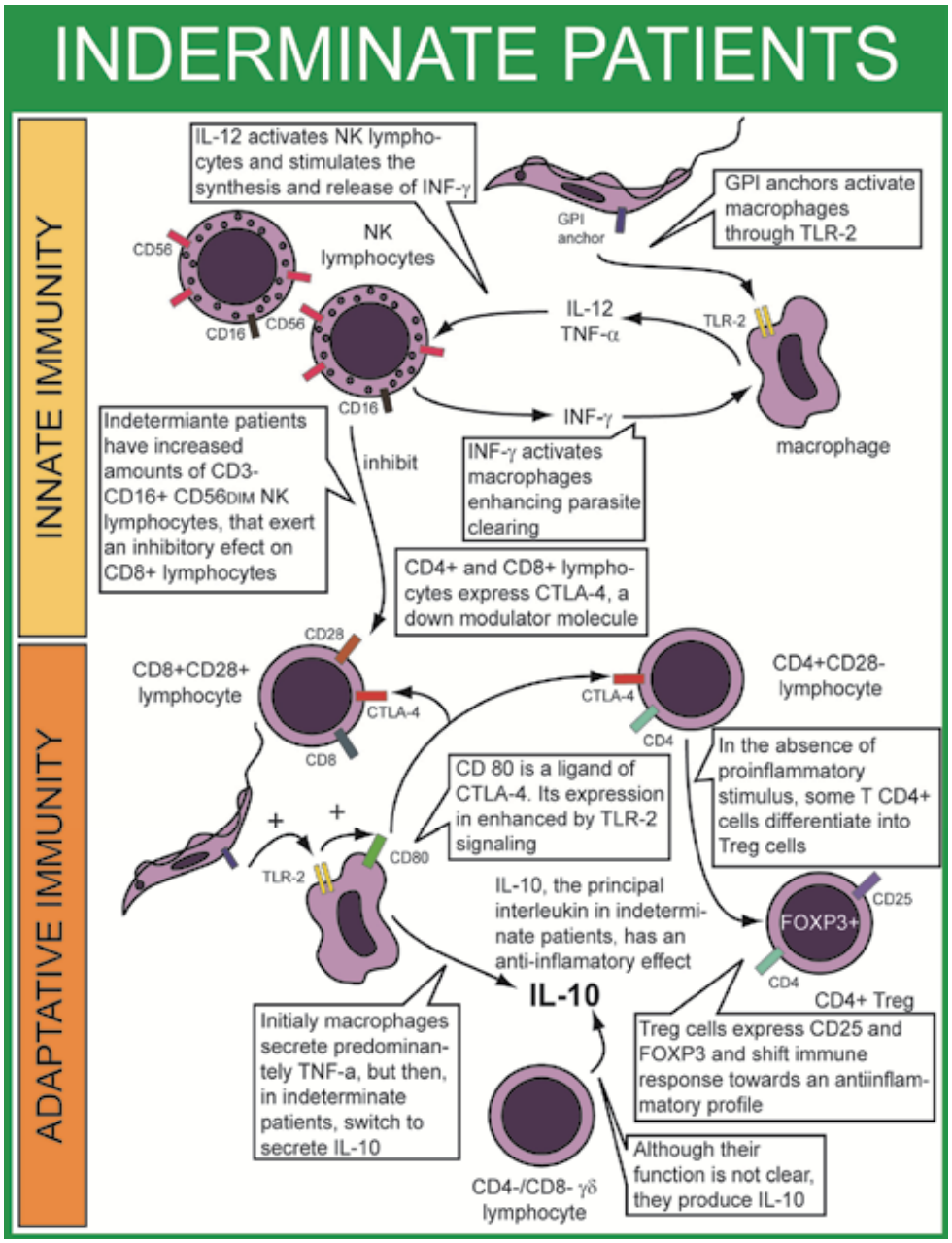

Fig. 2A. The immune pathogenesis of Chagas disease in indeterminate patients. The presence on numerous down regulating mechanisms shift the response towards an anti-inflammatory profile.

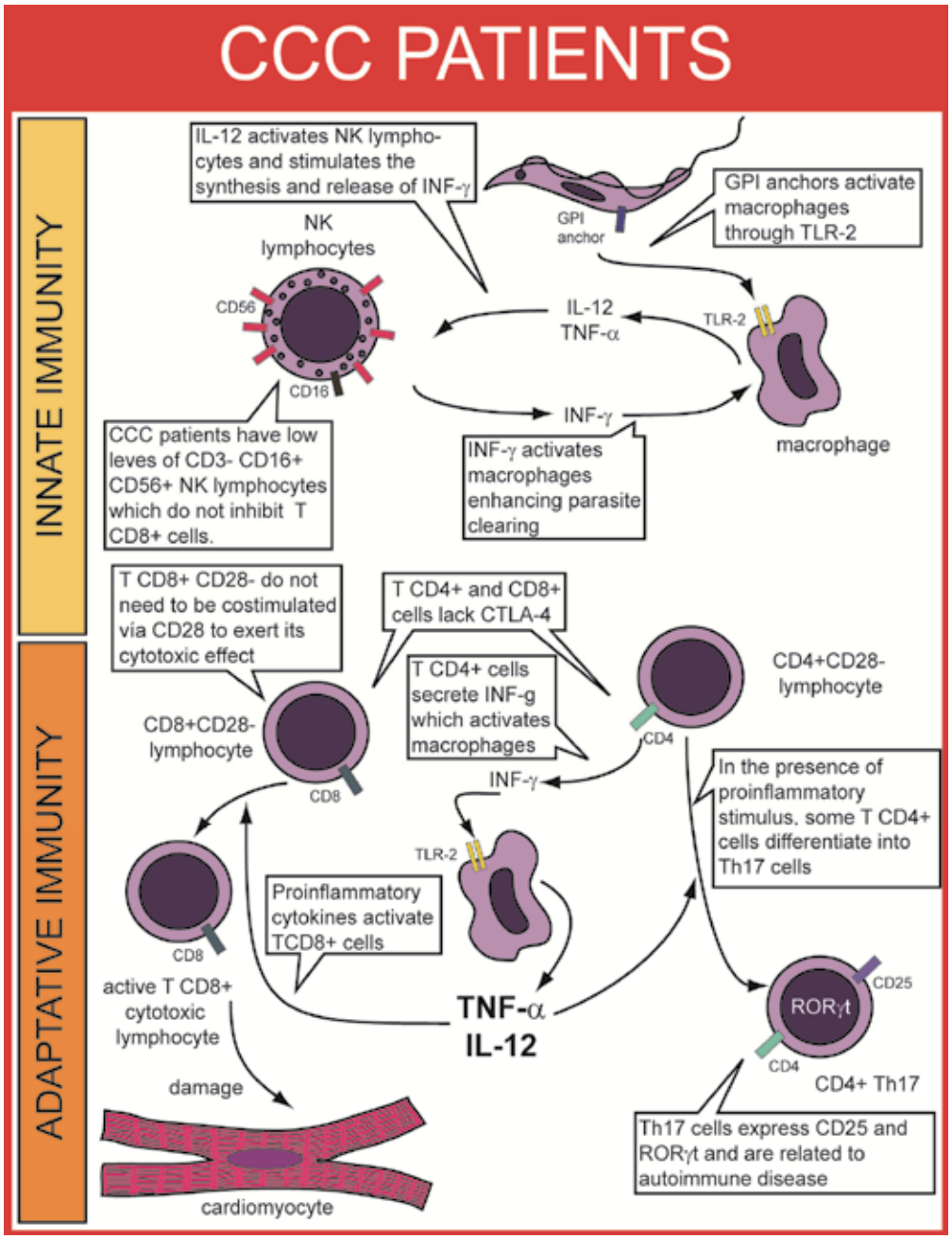

Fig. 2B. The immune pathogenesis of Chagas disease in CCC patients. Cells evolve towards a proinflammatory profile, with development of autoimmunity.

The first autoantibody to be described was one that reacted to endocardium, blood vessels and interstitium of skeletal muscle (EVI) (Cossio, et al., 1974), but was the same group of investigators who recognized the heterophil nature of the antibody and realized that had no pathogenic role (Khoury, et al., 1983).

Another autoantibody, studied by our group, was anti-laminin antibody (Sanchez, Milei et al. 1993, (Milei, et al., 1993). These antibodies were shown to react against *T. cruzi* amastigotes and trypomastigotes and human laminin (Szarfman, et al., 1982) and deposition of this antibody in marked thickened basement membranes of myocytes, endothelial cells, and vascular smooth muscle cells was shown by us with light microscopy, electron microscopy and immunohistochemical techniques in endomyocardial biopsies of chagasic patients (Sanchez, et al., 1993) but then we found that only 50% of patients had the antibody on their sera and no correlation with disease severity could be established (Milei, et al., 1993).

Anti-myosin antibodies were postulated by some authors to be generated through molecular mimicry with two *T. cruzi* antigens: B13 protein (Gruber & Zingales, 1993) and cruzipain (Giordanengo Laura, et al., 2000a, Giordanengo Laura, et al., 2000b). Although cruzipain antibodies mainly react to skeletal muscle myosin, they can cause conduction disturbances when transferred to uninfected mice and, when transferred to pregnant animals, they caused conduction disturbances in pups (Giordanengo Laura, et al., 2000b). On the other hand, immunossuppresed mice did not mount any humoral response when immunized with myosin but still develop myocarditis (Neu, et al., 1990). This fact made some authors doubt on the molecular mimicry hypothesis and rather consider antibodies to myosin a consequence of myocyte damage (Kierszenbaum, 2003).

Antibodies that react with muscarinic receptors were intensely studied. In early 1990's IgG from chagasic patients was observed to bind to muscarinic M2 receptors and activate them (Sterin-Borda L, et al., 1991). These anti-muscarinic antibodies were found to increase intracellular cGMP and decrease cAMP (Goin J., et al., 1997) and were positively related to the presence of dysautonomia (Goin J. C., et al., 1994). These antibodies also caused accumulation of inositol phosphate and nitric oxide synthase stimulation, with a negative inotropic effect on myocardium (Sterin-Borda Leonor, et al., 1997). As mentioned before, anti-muscarinic autoantibodies are positively related to the presence of dysautonomia (Goin J. C., et al., 1994), the presence of achalasia in chagasic patients (Goin J. C., et al., 1999), sinus node dysfunction (Altschuller, et al., 2007), but are not related with the degree of myocardial dysfunction (Altschuller, et al., 2007, Talvani Andre, et al., 2006), nor with the presence of brain lesions (Py, et al., 2009). In fact, patients with cardiomyopathy and left ventricular dysfunction but without autonomic dysfunction show low levels of anti-muscarinic antibodies (Sterin-Borda Leonor & Borda, 2000).

Antibodies against β_1-adrenergic receptors were also deeply studied. Described in early 1980's (Borda E., et al., 1984) these antibodies increased cAMP in mouse atrial fibers, increasing the release of PGE_2 and TXB_2 causing diminished contractility (Gorelik, et al., 1990). Increased cAMP activates PKA and then increases the intracellular calcium concentration. This causes in turn inhibition of the Na^+/K^+-ATPase and stimulates Ca^{2+}-ATPase activity leading to intracellular depletion of K^+ and further increase in Ca^{2+}. These alteration alter contractility and electric impulse generation and conduction (Borda E. S. & Sterin-Borda, 1996). Antiadrenergic autoantibodies titers could not be related to the severity of left ventricular dysfunction (Talvani Andre, et al., 2006) and patients with overt cardiomyopathy but without autonomic dysfunction show low levels of these antibodies (Sterin-Borda Leonor & Borda, 2000). Antibodies against β_2-adrenergic receptors have also been described but are mainly related to megacolon (Wallukat, et al., 2010).

Autoantibody	Hypothetic pathogenic role	Reference
Anti-Cerebroside	Probably related to neurologial symptoms	(Avila & Rojas, 1990)
Anti-Gal	Apparently protective	(Gazzinelli, 1991)
Anti-Brain Microtubules	Unknown	(Kerner, et al., 1991)
Anti-Ribosome	Unknown	(Levitus, et al., 1991, Skeiky, et al., 1992)
Anti- UsnRNPs	Unkwnown	(Bach-Elias, et al., 1998)
Anti-Sulfatides	May cause myocarditis and induce arrhythmias	(Garcia, et al., 1998)
Anti-Galectin-1	Increased in CCC patients	(Giordanengo L., et al., 2001)
Anti-Cha R3	Specific of CCC	(Girones, et al., 2001a)
Anti-Desmoglein-1	Related to Penphigus foliaceum	(Diaz, et al., 2004)
Anticardiolipin	Unknown	(Pereira De Godoy, et al., 2005)
Anti- TrkA, TrkB and TrkC	Prevents apoptosis of neurons and helps cellular invasion	(Lu, et al., 2010)
Anti-MBP	Related to gastrointestinal form	(Oliveira E. C., et al., 2009)

Table 2. Less studied autoantibodies in Chagas' disease.

Antibodies against AV node and sinus auricular node tissues have been studied as markers of CCC. When compared in chronic chagasic cardiopathy patients, non-chagasic cardiopathy patients, indeterminate chagasic subjects and healthy blood donors as controls, they more frequently found in chronic chagasic cardiopathy, but not enough to be good markers for chagasic cardiopathy group. Besides, no clear association with complex rhythm or conduction alterations was found (Arce-Fonseca, et al., 2005).
Many other autoantibodies have been described (table 2) but are not so widely studied and their role in pathogenesis of chagasic myocarditis is not clear.

2.2.4 Genetic factors

Human leukocyte antigens (HLA) have shown some relation to the development of CCC. HLA-B40 and Cw3 combination was protective for CCC (Llop, et al., 1991), as resulted DRB1*14, DQB1*0303 (Fernandez-Mestre, et al., 1998), HLA-DQB1*06 (Deghaide, et al., 1998) and HLA-A68 (Cruz-Robles, et al., 2004). On the other hand, HLA-C*03 (Layrisse, et al., 2000), DRB1*1503 (Garcia Borras, et al., 2009), DRB1*01, DRB1*08, DQB1*0501 (Fernandez-Mestre, et al., 1998) and HLA-DR16 alelles (Cruz-Robles, et al., 2004) were positively related to the development of CCC.
A number of other genes related to immune system have been studied in order to determine their relation to a predisposition to develop CCC. In table 3 we list those positively related to the appearance of CCC (Cunha-Neto E., et al., 2009).

Gene	**Polymorphism**
CCL2/MCPI	- 2518
CCR5	+ 53029
TNF-α	- 308
LTA	+ 80, + 252
BAT-1	- 22, - 348
NFkBIL-1	- 62, - 262
IL-1B	- 31, + 3954, + 5810
IL-10	- 1082
IL-12B	+ 1188
MAL/TRIAP	S180L

Table 3. Genetic polymorphisms related to CCC. Adapted from (Cunha-Neto E., et al., 2009).

2.2.5 The cytokines and chemokines

Although proinflammatory cytokines seem to be necessary for controlling parasitemia during acute phase of the disease (Cunha-Neto E., et al., 2009), CCC patients display a rather proinflammatory cytokine while indeterminate patients display a down modulator one. CCC patients had higher levels of TNF-α and CCL2 than indeterminate patients (Ferreira, et al., 2003, Talvani A., et al., 2004). Infiltrating macrophages from CCC patients expressed INF-γ, TNF-α and IL-6 but showed low levels of IL-2, IL-4 and IL-10 (Abel, et al., 2001, Reis D. D., et al., 1993, Reis M. M., et al., 1997). Also CCR5, CXCR3 and CCR7 and their ligands were increased in hearts of CCC patients, as well as monocytes expressing CXCR3, CCR5, CXCL9 and CCL5 (Cunha-Neto E., et al., 2009). It has been shown that INF-γ and CCL2 induceed myocytes to secrete arial natriuretic factor and caused hypertrophy (Cunha-Neto E., et al., 2005), and IL-18 and CCR7 ligands, which are increased in CCC, caused cardiomyocyte hypertrophy and fibrosis (Reddy, et al., 2008, Riol-Blanco, et al., 2005, Sakai, et al., 2006).

2.3 The third ingredient: fibrosis

Fibrosis is one of the most striking characteristics of CCC. In our patients with CCC in endomyocardial biopsies, fibrosis had replaced between 8.2 and 49% of contractile myocardium, with only one patient having less than 10% (Milei, et al., 1992b). While in autopsies, fibrosis was more extensive reaching in the conduction system than in the contracting myocardium (51.5 ± 18% vs 43.4 ± 8%, $p < 0.05$) (Milei, et al., 1996a).

The deposition of laminin in extracellular and basement membranes has been implicated in the pathogenesis of inflammatory process, as laminin is able to bind proinflammatory citokines (Savino, et al., 2007). The inflammatory infiltrate in CCC was related to the production of citokines such as INF-γ, TNF-α, IL-18, CCL2 and CCL21, that may have modulator actions on fibrotic process (Cunha-Neto E., et al., 2009).

3. A combined theory that could explain the pathogenic mechanism in chronic chagasic myocarditis

With the perpetuation of inflammation, necrosis and scarring fibrosis, damage to all histological components of myocardium occurs. Damage to contracting myocardial fibers

determines contractile failure as well as electrophysiological disturbances. Conduction system, nervous autonomic system and microvasculature are also damaged and as a consequence they cause further damage to contractile myocardium and produce electrical instability. Figure 3 illustrates with a flow chart the interactive network of different elements in the pathogenesis of CCC.

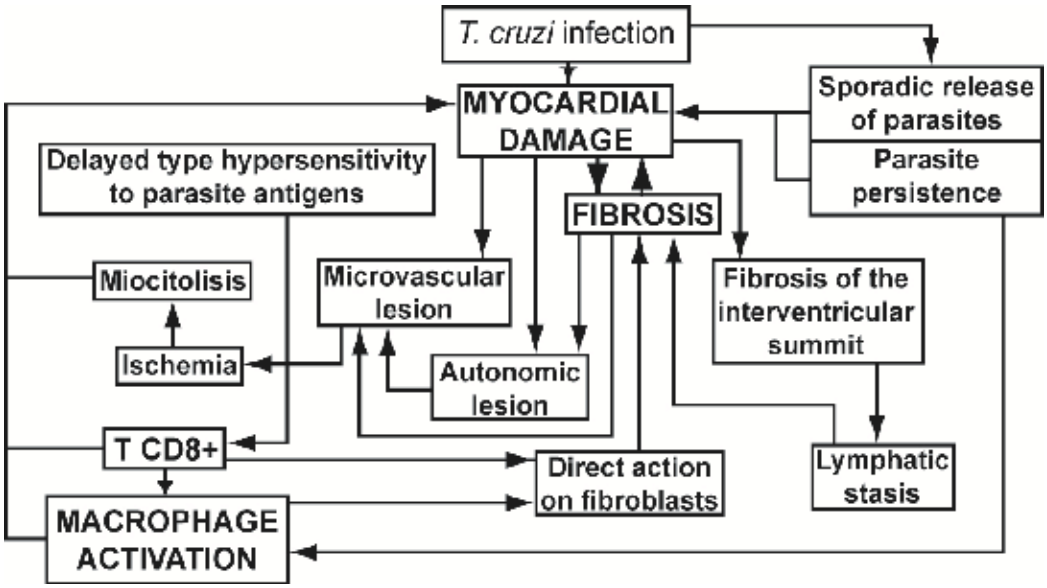

Fig. 3. Schematic representation of the integrated theory of multiple factors that determine myocardial damage in CCC.

4. Pathophysiological consequences of organ damage

4.1 Dysautonomia

As early as 1922 Carlos Chagas noted that the chronotropic response to atropine was altered in chagasic patients (Chagas C. & Vilella, 1922), but it was not until late 1950's that Köberle published his works showing impressive neuronal depopulation in microscopic sections obtained from the intercaval atrial strip in chagasic patients using a standardized technique of cardiac intramural neuronal counting developed by himself (Köberle, 1956a, 1956b). These findings led to the "neurogenic hypothesis" (Köberle, 1959), which explained all megas in Chagas' disease as a consequence of neuronal depletion.

Although many other authors claimed to have confirmed this finding (Mott & Hagstrom, 1965, Oliveira J. S., 1985), other authors called to attention about the criteria used to diagnose neuronal depletion because of the great variability in the number of neurons in autonomic ganglia (Rossi L., et al., 1994) and they also remarked that the only right criterion to establish neuronal depletion is the presence of proliferation of satellite cells, with the formation of Terplan's nodules, a characteristic lesion described as proliferating satellite cells which replace degenerating neurons, forming nodular structures. These lesions, once considered patognomonic, can be found in other cardiomyopathies (Rossi L., et al., 1994). The same author could not confirm the loss of neurons or denervation in CCC (Rossi L., 1988). Finally, it was demonstrated that, using Terplan's nodules as diagnostic criterion,

CCC patients with heart failure had more neuronal depletion than patients with dilated cardiomyopathy of other causes (Oliveira J. S., 1985). In our experience the neuroganglionic involvement was variable in autopsies of chagasic hearts (Milei, et al., 1991b).
According to pioneer neurogenic hypothesis (Köberle, 1959), early and irreversible damage to the parasympathetic system during acute phase of the disease causes a cathecolaminergic cardiomyopathy, but this point of view has been debated and evidence is contradictory. Functional test performed in CCC patients demonstrated impaired parasympathetic heart rate regulation (metaraminol, phenylephrine and atropine intravenous injections, facial immersion, Valsalva maneuver, head-up and head-down tilt tests, respiratory sinus arrhythmia, handgrip, graded dynamic exercise, and spectral analysis of Holter recordings) (Amorim, et al., 1968, Amorim, et al., 1973, Gallo, et al., 1975, Guzzetti, et al., 1991, Junqueira Junior, et al., 1985, Manço, et al., 1969, Marin-Neto, et al., 1975, Sousa, et al., 1987). However, a careful analysis of these data showed that many patients had normal autonomic function and most patients had heart failure, that could explain autonomic dysfunction *per se* (Davila, et al., 1998).
On the other hand, the study of indeterminate patients has shown conflicting results. While some authors could demonstrate impaired autonomic function (Molina, et al., 2006, Vasconcelos & Junqueira, 2009) others could demonstrate that autonomic function was normal in patients without myocardial damage and that abnormalities in autonomic dysfunction was proportional to heart dysfunction, leading these authors to propose that these abnormalities arise as a compensating mechanism for the progressive left ventricular dilatation (Davila, et al., 1991, Davila Spinetti, et al., 1999). These findings led to a new "neurogenic theory", which considers autonomic dysfunction as secondary to ventricular dilatation and hemodynamic alterations, but once installed, acts synergistically with parasitism and inflammation to cause further myocardial damage (Davila, et al., 2004).

4.2 Microvascular damage

Microcirculation abnormalities in CCC have been firstly pointed out by Jorg as an angiographic anarchy due to capillary loss (Jörg, 1974) and furtherly demonstrated in experimental models as well as in clinical practice (Rossi M. A., et al., 2010).
Many investigators have found abnormal myocardial perfusion using isonitrile-99m-technetium (Castro R., et al., 1988) and thallium-201 (Hagar & Rahimtoola, 1991, Marin-Neto, et al., 1992) scintigraphy in chagasic patients with normal epicardial coronary arteries. Furthermore, the progression of left ventricular systolic dysfunction is associated with both, the presence of reversible perfusion defects and the increase in perfusion defects at rest (Hiss, et al., 2009, Schwartz & Wexler, 2009). Anatomopathological studies in humans also provided evidence of microvascular damage in CCC. In late 1950's first reports showing collapse of arterioles and intimal proliferation (Torres, 1960) caught the attention of investigators. Also, microthrombi have been described (Rossi M. A., et al., 1984). As said, in endomyocardial biopsies thickening of capillary basement membranes was also found (Milei, et al., 1992b).
Additional evidence of microvascular damage was obtained from experimental models. Vascular constriction, microaneurysms, dilatation and proliferation of microvessels has been demonstrated (Factor & Sonnenblick, 1982, Morris, et al., 1989, Tanowitz H. B., et al., 1996, Tanowitz Herbert B., et al., 1992b).
Many factors have been advocated in the genesis of these lesions. First, the parasite itself. It was shown that *T. cruzi* produces a neuraminidase that removes sialic acid from de surface

of endothelial cells. This results in thrombin binding and platelet aggregation (Libby, et al., 1986). *T. cruzi* also produces tromboxane A_2 (TXA_2), specially during amastigote state (Ashton, et al., 2007), also favouring platelet aggregation and vascular spasm. Direct parasitism of endothelial cells by *T. cruzi* has also been demonstrated, and this causes the activation of the NF-κB pathway increasing the expression af adhesion molecules (Huang, et al., 1999a), and secreting proinflammatory citokines (Tanowitz Herbert B., et al., 1992a) and iNOS (Huang, et al., 1999b).

Endothelin-1 (ET-1) is another proposed pathogenic element. Elevated levels of mRNA for preproendothelin-1, endothelin converting enzyme and endothelin-1 were observed in the infected myocardium (Petkova Stefka B., et al., 2000), and elevated levels of ET-1 have been found in CCC patients (Salomone, et al., 2001). Mitogen-activated protein kinases and the transcription factor activator-protein-1 regulate the expression of endothelin-1, and both are shown to be increased in myocardium, interstitial cells and vascular and endocardial endothelial cells (Petkova S. B., et al., 2001). Besides, treatment with phosphoramidon, an inhibitor of endothelin converting enzyme, decreases heart size and severity of lesions in an experimental model of Chagas' disease (Jelicks, et al., 2002).

Inflammation also produces dysfunction of endothelial cells. Macrophages secrete TXA_2 and platelet activating factor (PAF) act on endothelium causing vasoconstriction (Rossi M. A. & Carobrez, 1985). Endothelial cells infected *in vitro* with *T. cruzi* lose their antithrombotic properties in response to interleukin 1 β (IL-1β) (Bevilacqua, et al., 1984, Nachman, et al., 1986).

It is remarkable that, although the data presented, endothelial function seems to be normal in CCC patients without heart failure, as measured by increases in blood flow in response to acetylcholine and sodium nitroprusside (Consolim-Colombo, et al., 2004). Further, in our concept, microvascular damage found in CCC, seems to be secondary to fibrosis and distortion of myocardial fiber arrangement by necrosis and chronic infiltrates, but as once established, may contribute to the perpetuation of myocardial damage.

5. Pathology

Pathological findings are described mostly according to our own findings.

5.1 Macroscopic features

The most striking characteristic of CCC is enlargement of heart with variable degrees of dilatation of chambers (Andrade, 1985) (Figure 4A). In autopsy series, hearts were overweighted (Andrade, 1985, Baroldi, et al., 1997, Bestetti, et al., 1993, Lopes, et al., 1981) compared with indeterminate chagasic patients and non-chagasic subjects. Marked cardiomegalies reached up to 500 grams. Right ventricle (RV) and atrium (RA) were generally more compromised than left chambers, being RV the most dilated in one paper (Laranja, et al., 1956) but RA was in other (Andrade, 1985).

A second remarkable feature is the thinning of the left ventricular apical wall, resulting in apical aneurysm, a very characteristic lesion in CCC (Figure 4B) (Moia, et al., 1955).

Other lesions described are flattening of the papillary muscles and a marked subendocardial sclerosis, parietal and/or aneurismal thrombosis and fibrotic plaques in pericardium (Milei, et al., 1996a, Milei, et al., 1991b, Storino & Milei, 1994).

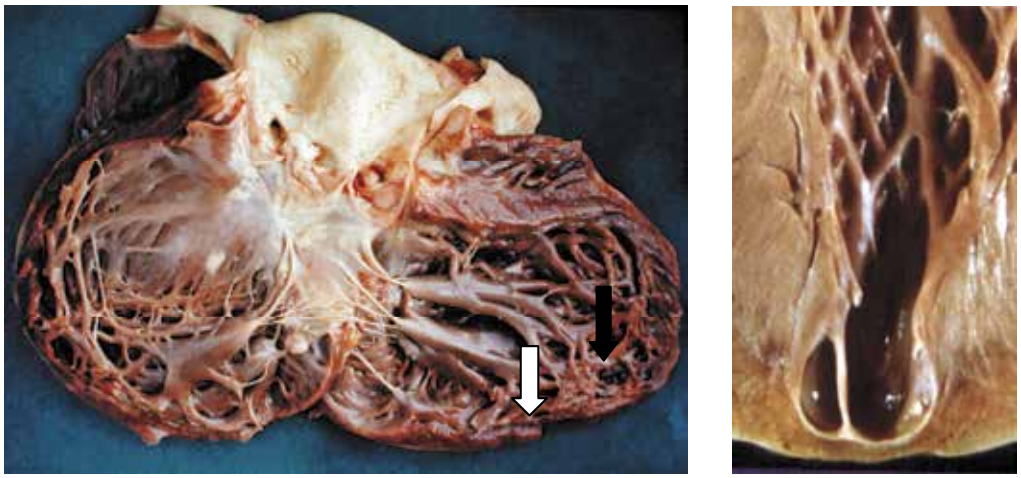

Fig. 4. A. High grade heart dilatation. Thining of the apical wall of the left ventricle (white arrow) and cavitary thrombus (black arrow). B. Characteristic apical aneurysm. A from Milei, et al., 1996b, B from Milei, et al., 2008.

5.2 Histological features

Microscopically, myocardial lesions consisted of a chronic inflammatory process with fibrotic scars and extensive mononuclear infiltrates. Such infiltrates were more prominent in the working myocardium and in the specialized cells of the left branch of the His bundle than in the AV node and in the right hisian branch, showing a microfocal disposition (Figure 5A). The percentage of fibrosis was variable and ranged between 8.2 to 49% (Milei, et al., 1996a, Milei, et al., 1992b) (Figure 5B).

Extensive myocytolysis and spotty contraction band necrosis were observed. Cell hypertrophy in the apparently preserved myocytes was revealed by hypertrophic bizarre nuclei. Dilated lymphatic channels widespread in the ventricular septum and in the AV node, His bundle, and in the root of the right and left bundles branches were observed. In the case of apical aneurysm of the left ventricle, dilated lymphatic were distributed subepicardically (Milei, et al., 1996a).

The serial sectioning of the conducting system showed prominent lesions. Sino-atrial node presented mononuclear infiltrates, necrosis of specialized fibers, and intense fibrosis (Milei, et al., 1991b). In the remaining specialized system lesions consisted of mild to moderate diffuse fibrosis of the AV node and of the penetrating and branching portions of the His bundle, complete destruction of the proximal segments of the right and left bundles branch by varying degrees of replacement by dense collagen tissue (Figure 5A). The remaining specialized fibers presented atrophy and mild fatty infiltration and were surrounded in most cases by infiltrates consisting mainly of lymphocytes and macrophages. The subendocardial Purkinje fibers were usually damaged by chronic inflammation and fibrosis (Milei, et al., 1991b) (Figure 5B). These vast fibrosis in the conduction system (Figure 5C) showed severe conduction alterations in electrocardiograms, although curiously in one revision, there were needed sophisticated electrophysiological studies to demonstrate electrical abnormalities in these patients (Andrade, et al., 1988)

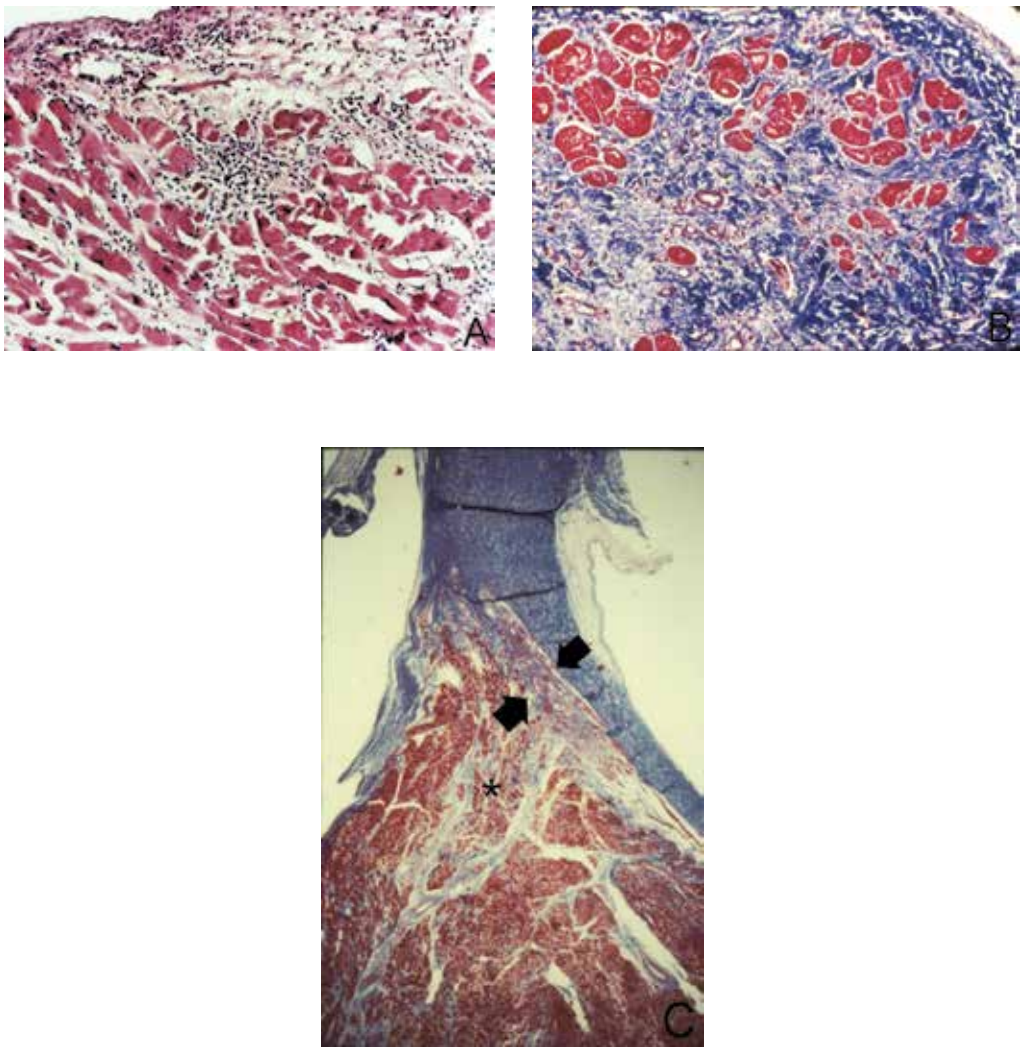

Fig. 5. A. Extensive mononuclear infiltrates, myocytes loss, and subendocardial fibrosis. Hematoxylin and eosin stain, X25. B. Atrophic myocardial fibres (red) separated by thick bands of fibrous tissue (blue). Mallory trichrome, X 25. C. Bifurcating His bundle showing severe fibrosis at the left branch (between arrows). The right branch (asterisk) is intramyocardial and surrounded by connective tissue. Mallory trichrome, X25. A and C from Milei, 1996a. B from Milei, 2008.

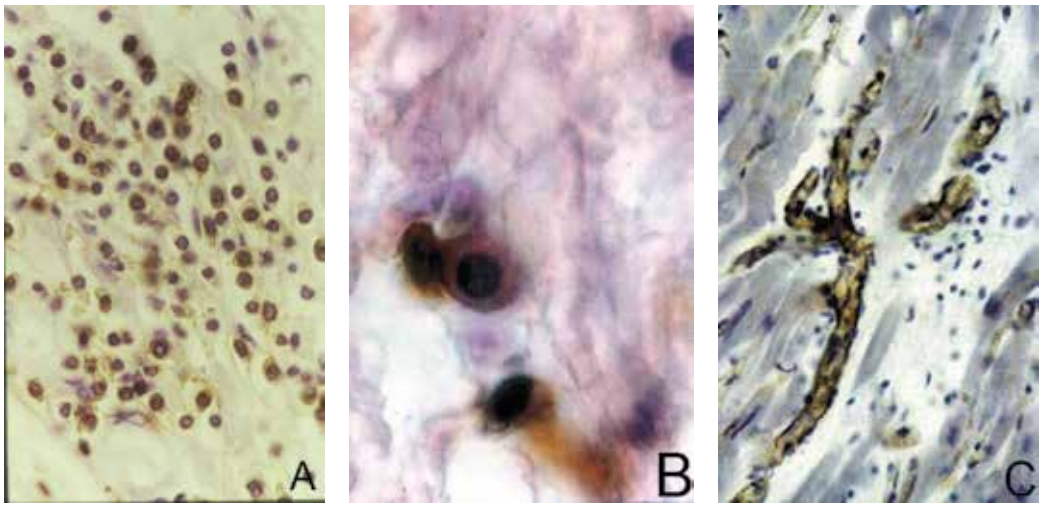

Fig. 6. A. Detail of the left bundle of His is shown. Immunostaining for T lymphocyte. Positive cells express CD45R0 antigen (brown); specialized myocardial cells have almost disappeared. Extensive mononuclear infiltrate, the majority of them being T lymphocytes. X20. B. Double immunostaining for the simultaneous demonstration of T lymphocytes (CD45RO) and macrophages (CD68). T lymphocytes (brown) in close contact with a macrophage (pinky cytoplasm). X1000. C. Immunostaining to show endothelial cells. Capillaries and small vessels are clearly showed by the expression of CD31. Vessels are midly or moderately disorted because of the surrounding fibrosis. X100. From Milei, 1996a.

In our studies in endomyocardial biopsies, infiltrates were approximately 50% lymphocytes and 50% macrophages. Almost 80% of lymphocytic population were T lymphocytes, being only 20% B lymphocytes. Eosinophils were scarce in infiltrates reaching 5%, and were associated with areas of necrotic myocardium. Mast cells also were scarce or absent in specialized and in contracting myocardium. (Milei, et al., 1996a, Milei, et al., 1992b)
Histological study of aneurisms showed a thinned wall 2-4 mm, with sclerotic plaques of thickened endocardium of up to 92% of total tissue and extensive mononuclear chronic inflammatory infiltrates and widespread fibrosis in myocardium. Myocytes were organized in thin bands or atrophic units surrounded by fibrotic tissue (Figure 5B) (Milei, et al., 1991a).
Autonomic ganglia showed above described Terplan's nodules, with satellite cell proliferation replacing degenerated autonomic neurons. As stated, these lesions, once considered patognomonic, can be found in other cardiomyopathies (Rossi L., et al., 1994).

5.3 Immunohistochemical findings

Immunophenotyping of infiltrates allowed a better characterization of the cells participating in the inflammatory infiltrates, mainly macrophages ($CD68^+$) and lymphocytes ($CD45R^+$). In our works 26.5% percent of them were T lymphocytes ($CD45R^+$, $CD45R0^{+)}$ and 10.5% were B lymphocytes ($CD20^+$, light chains kappa and/or lambda$^+$) (Figure 6A). Thirty percent of the infiltrate was composed of macrophages ($CD68^+$). The remaining infiltrate was composed of mononuclear cells resembling macrophages and CLA-negative mononuclear cells. Contacts between CD68 positive cells and T lymphocytes were frequently found

(Figure 6B). CD31 antibodies clearly pointed out normal endothelial cells, in either normal or damaged vessels (Figure 6C) (Milei, et al., 1996a).

5.4 Ultrastructural features

Myocardial fibers showed nuclear enlargement, nuclear membrane invaginations, lipofuscin deposits, myofibrils derangements and loss, swelling, mitochondrial atrophy, dilatation of sarcotubular system, and interstitial fibrosis (Carrasco, et al., 1982, Palacios-Prü, et al., 1982). These findings have been confirmed by our group in endomyocardial biopsies (Ferrans, et al., 1988, Milei, et al., 1992b). Platelet thrombi can be demonstrated within capillaries (Figure 7B).

Other striking alteration in these specimens was the thickening of the basement membranes of cardiac myocytes (Figures 7A, 7C), endothelium Figure 7C) and vascular smooth muscle up to 20 times their normal thickness of 500 Å (Ferrans, et al., 1988). The thickened basement membranes appeared structurally homogeneous, without being multilayered or subdivided into a lamina rara and a lamina densa. They were of relatively low electron density, had a finely fibrillar appearance at high magnification and measured up to 1 μm in thickness. Using gold-conjugated antibodies, we could demonstrate the presence of laminin in the thickened basal membranes of myocytes and endothelium (Sanchez, et al., 1993).

Regarding the ultrastructure of aneurysms resected from chagasic patients we observed, hypertrophy of myocytes, with swelling, partial or complete loss of myofibrils, swelling of mitochondria, disruption of mitochondrial cristae, lipofuscin granules, and intact sarcolemmas . Basement membranes were thickened, as previously described (Milei, et al., 1991a)

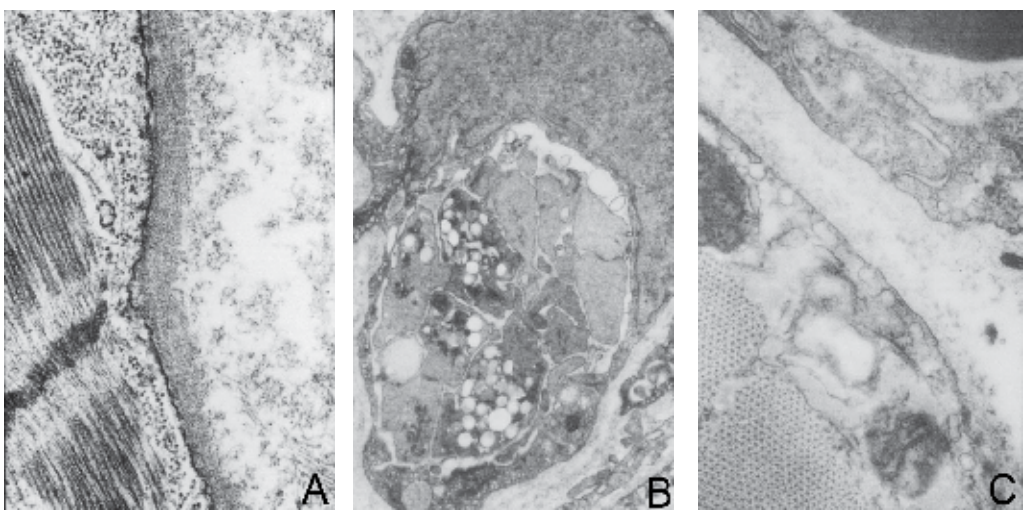

Fig. 7. A. Myocardial fibre with thickened basement membrane. B. Platelet thrombus within a capillary. C. Thickened basement membranes in a myocardial fibre and a capillary. From Milei, et al., 2008.

6. Conclusions

As shown across the sections of this chapter, the numerous hypothesis about pathogenic pathways of CCC have supporting data and pitfalls. Finally all proposals interact with each other, giving us the idea that none of these theories explains the very complex development of CCC by itself. Rather, it seems more feasible that all these hypothesis conform a network of damaging elements, and that all ingredients cause and/or enhances each other. The triggering factor is obviously the interaction between parasite and host's immune system. Cell parasitism, the inflammatory process and consequent necrosis and fibrosis cause damage to contracting myocardium, autonomic system, conduction system and microcirculation. Autonomic damage causes impaired regulation of microvasculature and further alterations in blood flow. Ischemia causes more myocardial damage. Necrosis exposes intracellular epitopes and causes autoantibodies production with more necrosis, fibrosis and so on. It seems that, if adequate down modulator immune mechanisms work properly, this vicious circle stops and patients do not develop cardiomyopathy, rather they remain in the indeterminate form lifelong.

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7

Myocarditis in HIV Positive Patients

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1. Introduction

Myocarditis is an acute or chronic inflammatory process that affects the myocardium in response to the action of various infectious, chemical or physical agents. In most patients the disease is self-limiting. The natural course of myocarditis varies greatly, ranging from an asymptomatic state secondary to local inflammation, through development of dilated cardiomyopathy with a variable course, to fatal heart failure due to disseminated myocarditis [Subinas et al., 2005].

In patients infected with human immunodeficiency virus (HIV) cardiovascular abnormalities are frequent but clinically discrete. Cardiologists and physicians throughout the world are increasingly reporting cardiac muscle disease in association with HIV. With current advances in HIV and acquired immunodeficiency syndrome (AIDS) management and increased survival, cardiac manifestations of HIV disease including HIV related myocardial disease will become more important and will be encountered more frequently. Because cardiac complications in HIV positive patients are often clinically inapparent, periodic screening of these patients is recommended, especially in those with low CD4 counts or receiving treatment with cardiotoxic drugs. The heart may be a marker of the HIV infected patient's overall health, and a decline in cardiac function should trigger more comprehensive evaluation. As the role of infection and inflammation in many other cardiovascular diseases is now recognized, identification of the molecular mechanisms of HIV related myocaditis might have broader implications for a wide range of patients [Azis F et al., 2010].

The diagnosis of myocarditis in HIV positive patients during an acute episode may prove difficult, due to the lack of diagnostic techniques with acceptable degrees of specificity and sensitivity. For these patients although endomyocardial biopsy is still considered the diagnostic gold standard, the developments of new imaging techniques, such as cardiac magnetic resonance imaging (CMR), and nuclear imaging by antimyosin scintigraphy have contributed greatly to the diagnosis of myocarditis.

2. Myocarditis in HIV positive patients

HIV infection and AIDS have a well-recognized association with myocarditis and dilated cardiomyopathy [Azis F et al., 2010]. This increased predisposition is multifactorial and may include the direct effects of HIV itself, co-infection by opportunistic organisms, toxic effects of commonly used medications or illicit drugs, and nutritional deficiencies [Azis F et al., 2010]. Further, autoimmunity can be an important contributor to the pathogenesis of cardiomyopathy in these patients as many studies demonstrated the presence of cardiac-

specific antibodies in HIV positive patients when compared with HIV negative controls [Currie & Boon 2003, Currie et al., 1998]. Thus, although the precise mechanisms are poorly understood, alterations in the immune system likely play an important role in the pathogenesis of heart muscle disease in HIV-infected patients. The prevalence of myocarditis in HIV infected patients has been difficult to establish with estimates ranging from 6% [Barbaro et al., 1998b] to 52% [Levy et al., 1989]. In other studies about 10 percent of people with HIV develop myocarditis, either because HIV directly invades the heart muscle or because the patient's weakened immune system makes the heart muscle more susceptible to attack by other infectious agents, especially toxoplasmosis [Grange et al., 1990, Matturi et al., 1990].

HIV deserves special mention because it seems to function differently than other viruses. HIV-1 glycoprotein 120 can directly disrupt cardiac contractility without any inflammatory response [Currie & Boon, 2003]. This may explain why HIV genomes can be amplified from patients without histologic signs of inflammation. Myocarditis is the most commonly cardiac abnormality found on biopsy tissue, present in some degree, in more than 50% of HIV patients [Howes et al., 2010]. In addition, in patients who are infected with HIV, T-cell – mediated immune suppression increases the risk of contracting myocarditis due to other infectious causes [Howes et al., 2010].

2.1 Etiology

The actual pathogenesis of cardiac injury in HIV infection is not clear. It is however generally agreed that several factors come into play either singly or in combination to produce cardiac pathology [Aziz 2010]. There is a wide range of hypotheses regarding the pathogenesis of HIV associated heart muscle disease. These include myocardial invasion with HIV itself, opportunistic infections, viral infections, and autoimmune response to viral infection, drug-related cardiac toxicity, nutritional deficiencies, endothelial dysfunction, autonomic dysfunction, and prolonged immunosuppression. Zidovudine (AZT), an antiretroviral drug used in treatment of HIV, has also been associated with myocarditis [Herskowitz et al., 1992a].

Opportunistic infections are common complications of AIDS and the most frequent cause of morbidity and mortality. However, relatively few pathogens have been isolated from the myocardium of AIDS patients [Olson 2003]. Myocardial involvement is usually associated with disseminated disease and multiple foci of infection. Typically, infectious organisms are identified in patients dying of noncardiac causes, and the findings of myocardial abnormalities are regarded as incidental. Opportunistic pathogens represent diverse causes of infectious disease, including bacteria, fungi, protozoa, and viruses [Olson 2003].

Toxoplasma gondii (T gondii) is the most frequently documented infectious cause of myocarditis associated with AIDS, and the heart is the second most common site of infection after the brain. Autopsy series have described T gondii myocarditis (myocardial toxoplasmosis) in 1% to 16% of patients dying of AIDS [Baroldi et al., 1988, Anderson et al., 1988, Matturi et al., 1990]. Evidence of myocardial toxoplasmosis includes trophozoites or pseudocysts in myocardial fibers. A minority of cases has associated myocarditis with focal areas of necrosis and lymphocytic infiltrates [Jautzke 1993]. Antemortem diagnosis of toxoplasma myocarditis associated with left ventricular (LV) dysfunction has been described, including its successful treatment [Grange et al., 1990, Albrecht et al., 1994].

Pericardial tuberculosis has been reported in association with AIDS, typically in the setting of widespread disease. However, myocardial tuberculosis appears rare [Miller-Catchpole et al., 1989, Kinney et al., 1989].
Fungal myocarditis is an unusual complication of disseminated infection that is identified most often at autopsy [Olson 2003]. Various fungal organisms identified in the myocardium at autopsy with associated myocarditis have included *Aspergillus fumigatus*, *Candida albicans*, *Histoplasma capsulatum*, *Coccidioides immitis*, and *Cryptococcus neoformans*. Cardiac cryptococcus has been diagnosed in association with congestive heart failure and shown to resolve after therapy with amphotericin B and flucytosine [Kinney et al., 1989, Lewis et al., 1985, Lafont et al., 1987].
Several viruses have been implicated in myocarditis associated with AIDS. Cytomegalovirus (CMV) is a common opportunistic pathogen in AIDS, but it is associated less frequently with myocarditis [Michaels et al., 1997]. When inclusion bodies are the criterion for the detection and diagnosis of solid organ involvement by CMV, the rate of infection is underestimated compared to in situ DNA hybridization techniques [Wu et al., 1992, Myerson et al., 1984].
Other viruses identified by culture or polymerase chain reaction (PCR) within the myocardium of HIV-infected or AIDS patients, either at antemortem endomyocardial biopsy or from autopsy material, have included Epstein-Barr and coxsackie B virus in adults [Barbaro et al., 1988a] and adenovirus in children [Bowles et al., 1999]. These viruses may be present as either primary infection or as coinfection and can occur with or without associated myocarditis and with or without associated LV dysfunction.

2.2 Pathogenesis

The first clinical report to suggest a relationship between nonspecific myocarditis and dilated cardiomyopathy in AIDS patients appeared in 1986 [Cohen et al., 1986]; 3 patients had clinical, echocardiographic, and pathologic findings of dilated cardiomyopathy and 2 of the patients had focal lymphocytic infiltration associated with myocyte necrosis. Subsequent reports suggested an association between focal nonspecific myocarditis at autopsy and clinical cardiomyopathy [Barbaro et al., 1998a, Reilly et al., 1988]. Numerous hypotheses have been suggested to account for the etiology of nonspecific myocarditis and cardiomyopathy observed in HIV-infected patients, including direct HIV-1 infection of myocardial cells or coinfection with other cardiotropic viruses, [Olson 2003] cytokine cardiotoxicity [Suffredini et al., 1989, Lahdevirta et al., 1988, Levine et al., 1990], postviral cardiac autoimmunity [Herskowitz et al., 1989, 1993], nutritional deficiencies [Olson 2003], and cardiotoxicity due to illicit drugs [Olson 2003] or pharmacologic agents [Herskowitz et al., 1992a, Olson 2003].
The histologic findings of a monoclonal or oligoclonal inflammatory cellular infiltrate suggest a viral or autoimmune cause for the myocarditis associated with HIV infection. Myocarditis is more likely in individuals with more profound immunosuppression because CD4 < 400 cells/μL is more frequently observed in patients with dilated cardiomyopathy [Barbaro et al., 1998b]. In this same series, inflammatory myocardial cellular infiltrates were predominantly CD3 lymphocytes in 12 patients and CD8 lymphocytes in 64 patients. A separate report [Herskowitz et al., 1992b] described 35 HIV-infected patients with global LV dysfunction who underwent endomyocardial biopsy in which active or borderline myocarditis was observed in 55% of patients. For those patients with biopsy-proven myocarditis, mean LV ejection fraction was 28% whereas for patients without myocarditis it was 48%. The cellular infiltrate was primarily composed of CD8 T lymphocytes.

Although it has been suspected that myocarditis and cardiomyopathy associated with HIV-1 infection may be caused by direct viral infection of myocytes, definite evidence for this is lacking. Difficulty in demonstration of a link among HIV-1 infection, myocarditis, and cardiomyopathy in AIDS is related, in part, to lack of a suitable in vivo model of the disease. Because of the limited host range of HIV-1 and the difficulty in handling nonhuman primates infected with simian immunodeficiency virus-1, little investigation has been reported of myocarditis or cardiomyopathy associated with AIDS in animal models [Lewis et al., 2000].

What is the potential for direct myocardial infection? HIV-1 invades T cells by attachment to a CD4 surface-membrane receptor. However, there are no CD4 receptors on myocyte surface membranes. It is possible that the virus gains access to myocytes by other mechanisms, although the evidence to support this concept is limited. It is also possible that injury to myocytes may facilitate entry of the HIV virion; Epstein-Barr virus (EBV) promotes entry of HIV into CD4 receptor negative cells, with subsequent replication [Olson 2003].

The presence of viral genomic material within the myocytes of HIV-infected patients with myocarditis and cardiomyopathy does not definitely establish viral infection as causal. Furthermore, the significance of the finding of viral transcripts within cells is uncertain because patients may or may not have LV dysfunction [Herskowitz et al., 1993]. HIV-1 genomic material reportedly was detected within the genome of myocardial cells, although typically the findings have been sparse and may not have represented myocyte infection because the HIV nucleic acid sequence may actually have been located in endothelial cells or macrophages [Grody et al., 1990, Lipshultz et al., 1990, Flomenbaum et al., 1989, Cenacchi et al., 1990].

In one study, in 58 of 63 patients with AIDS, LV dysfunction, and biopsy-proven nonspecific lymphocytic myocarditis, a positive hybridization signal was observed but staining was weak and affected myocytes were generally not surrounded by inflammatory cells [Barbaro et al., 1998a].

In adults with AIDS-associated myocarditis, non-HIV viruses or viral genomic material identified in myocardial tissue has included CMV, Coxsackie virus group B, and EBV [Barbaro et al., 1998b, Wu et al., 1992]. In an autopsy study of 32 children who died with advanced HIV disease, including 23 with histologic evidence of myocarditis, viral sequences detected by polymerase chain reaction included adenovirus in 6, CMV in 3, and both adenovirus and CMV in 2. No other viruses were detected by polymerase chain reaction, including HIV [Bowles et al., 1999].

A high proportion of HIV-seropositive patients with LV dysfunction have evidence of latent infection of myocytes with CMV immediate-early genes [Wu et al., 1992]. Although observation of the intranuclear inclusions of active, lytic CMV infection is unusual, it has been suggested that latent viral infection may promote enhanced major histocompatibility complex expression, thereby provoking immune-mediated injury typical of animal models of myocarditis [Wu et al., 1992].

Immune-mediated mechanisms other than direct myocardial viral infection may account for the cellular infiltrates and cardiomyopathy observed in AIDS patients. Studies performed by Herskowitz demonstrated circulating autoantibodies in 4 of 6 AIDS patients with cardiomyopathy, whereas AIDS patients without cardiomyopathy did not have these antibodies [Herskowitz et al., 1989]. In the patients with autoantibodies, antimyosin antibodies were identified. In these same individuals, no evidence of HIV-1 or other viruses was identified from myocardial biopsy specimens evaluated by in situ hybridization,

lending support to an autoimmune mechanism of disease. A study by Gu et al. [Gu et al., 1992] used monoclonal antibodies to HIV core proteins that reacted with myocyte antigens in 38 of 42 AIDS patients (and 11 of 28 non-AIDS patients), suggesting antibodies occurring in AIDS patients may react with antigenic epitopes of myocytes, thereby promoting autoimmune-mediated heart muscle disease.

Multiple cytokines are suspected of having a role in the mediation of myocardial inflammation, myocyte necrosis and ventricular dysfunction in myocarditis, although specifics are incompletely understood in human disease [Liu et al., 2001]. The mononuclear cells characteristic of lymphocytic myocarditis, including the focal nonspecific myocarditis of AIDS, are a likely source of cytokines that promote inflammation and maintenance of immune response, which may lead to impaired contractile function and fibrosis. Matsumori et al. 1994 showed that patients with myocarditis have markedly increased concentrations of cytokines, including tumor necrosis factor alfa (TNF-α) and interleukin -1 and -6. In animal models of myocarditis, similar profiles of cytokine activation have been described and have been demonstrated directly in cardiac tissue [Yamada et al., 1994]. TNF-α has been demonstrated to be a negative inotrope [Suffredini et al., 1989] and is increased in patients with congestive heart failure [Sharma et al., 2000], AIDS [Odeh et al., 1990, Yamamoto et al., 1995], and myocarditis [Matsumori et al., 1994]. HIV may cause myocyte injury by an "innocent bystander destruction" mechanism, as may occur in AIDS-associated encephalitis [Ho et al., 1987]. However, whether these mechanisms operate in the myocarditis and dilated cardiomyopathy of AIDS is unknown.

Three histological patterns of myocarditis have been described in patients with AIDS:

- lymphocytic infiltration with myocyte necrosis [Anderson et al., 1988], which meets the Dallas criteria;
- lymphocytic infiltration without inflammation [Lewis et al., 1992];
- myocyte damage without evidence of inflammatory infiltrate [Lafontet al., 1987].

2.3 Clinical manifestation

Clinical presentation of HIV associated myocarditis in symptomatic patients is generally similar to myocarditis due to other causes. The absence of symptoms and signs of heart disease does not however exclude cardiac involvement, as occurrence of sub-clinical cardiac abnormalities with possible fatal consequences in this population has been described [Kaminski et al., 1990]. Diagnosis requires the possibility of cardiac involvement to be constantly in mind and symptoms associated with myocarditis are varied, and relate either to the actual inflammation of the myocardium, or the weakness of the heart muscle that is secondary to the inflammation. Signs and symptoms of myocarditis include: **chest pain** (often described as "stabbing" in character); **congestive heart failure** (leading to edema, breathlessness and hepatic congestion); **palpitations** (due to arrhythmias); **sudden death** (in young adults, myocarditis causes up to 20% of all cases of sudden death); **fever** (especially when infectious); symptoms in infants and toddlers tend to be more non-specific with generalized malaise, poor appetite, abdominal pain, chronic cough. Later stages of the illness will present with respiratory symptoms with increased work of breathing and is often mistaken for asthma.

Since myocarditis is often due to a viral illness, many patients give a history of symptoms consistent with a recent viral infection, including fever, rash, diarrhea, joint pains, and frequent fatigue.

Myocarditis is often associated with pericarditis, and many patients present with signs (pericardial friction rub) and symptoms that suggest concurrent myocarditis and pericarditis.

2.4 Diagnostic

Myocarditis refers to an underlying process that causes inflammation and injury of the heart. It does not refer to inflammation of the heart as a consequence of some other insult. Many secondary causes, such as a heart attack, can lead to inflammation of the myocardium and therefore the diagnosis of myocarditis cannot be made by evidence of inflammation of the myocardium alone.

Myocardial inflammation can be suspected on the basis of electrocardiographic (ECG) results, elevated C-reactive protein (CRP) and/or erythrocyte sedimentation rate (ESR) and increased IgM (serology) against viruses known to affect the myocardium. Markers of myocardial damage (troponin or creatine kinase cardiac isoenzymes) are elevated.

The difficulty in diagnosing myocarditis lies in the known absence of specificity and sensitivity of the various diagnostic techniques used. Systematic biochemical measurements are not diagnostic and an increase in cardiotropic virus antibodies only reflects the response to a recent viral infection, but does not indicate active myocarditis. Endomyocardial biopsy, considered to be the diagnostic gold standard, is associated with a not inconsiderable risk of injury, as well as with sampling errors due to the focal involvement of the myocardium, which therefore reduces its diagnostic sensitivity. Radioactive isotope studies, widely used for the diagnosis of myocarditis, are limited by their low specificity, the exposure to radiation, and their cost [Subinas et al. 2005].

2.4.1 Physical examination

Physical findings of myocarditis can range from a normal examination, through all classes of congestive heart failure (CHF) to cardiovascular collapse and shock. Patients with mild cases of myocarditis have a nontoxic appearance and simply may appear to have a viral syndrome. Tachypnea and tachycardia are common. Tachycardia is often out of proportion to fever [Howes 2010].

More acutely ill patients have signs of circulatory impairment due to left ventricular failure. A widely inflamed heart shows the classic signs of ventricular dysfunction including the following: jugular venous distention, bibasilar crackles, ascites and peripheral edema.

Third heart sound (S_3) or a summation gallop may be noted with significant biventricular involvement. Intensity of the first heart sound (S_1) may be diminished. S_3 generally occur between 0.12 and 0.24 second after the aortic component of the second heart sound. Clinically, the third heart sound (S_3 gallop) may be a physiologic sound in children and young adults. It may be produced by factors that generate increased rate or volume of flow with high cardiac output or by conditions associated with cardiac dilatation and altered ventricular compliance, as in CHF [Smiterman TC and Willerson JT, 2007].

Cyanosis may occur. Murmurs of mitral or tricuspid regurgitation may be present due to ventricular dilation [Howes 2010].

In cases where a dilated cardiomyopathy has developed, signs of peripheral or pulmonary thromboembolism may be found [Howes 2010].

Diffuse inflammation may develop leading to pericardial effusion, without tamponade, and pericardial and pleural friction rub as the inflammatory process involves surrounding structures [Howes 2010].

2.4.2 Invasive techniques

Cardiac angiography: This is often indicated to rule out coronary ischemia as a cause of new-onset heart failure, especially when clinical presentation mimics acute myocardial infarction. It usually shows high filling pressures and reduced cardiac outputs [Tang et al., 2009].

The gold standard is still **biopsy of the myocardium**, generally done in the setting of angiography. A small tissue sample of the endocardium and myocardium is taken, and investigated by a pathologist by light microscopy and—if necessary—immunochemistry and special staining methods [Cunningham et al., 2006]. Histopathological features are: a myocardial interstitium with abundant edema and inflammatory infiltrate, rich in lymphocytes and macrophages. Focal destruction of myocytes explains the myocardial pump failure. The need for routine myocardial biopsy in patients with HIV is controversial and associated risks are significant – sensitivity is low, especially in patchy lesions, and beyond research protocols, its use is limited to patients with extensive cardiac damage with no identifiable cause [Wu et al., 1990].

Myocarditis identified at autopsy or on endomyocardial biopsy in HIV-infected patients is most often nonspecific and manifested as focal, inflammatory lymphocytic infiltrates without myocyte necrosis. Other reported histopathologic findings include lymphocytic infiltration with myocyte necrosis fulfilling the Dallas criteria or myocyte damage without associated cellular inflammatory infiltrate [Anderson et al., 1988, Barbaro et al., 1998]. The autopsy finding of focal myocarditis in many patients who die of AIDS-related complications, but have no known premortem heart disease, suggests that focal lymphocytic infiltration may have no clinical significance. By comparison, diffuse lymphocytic myocarditis meeting the Dallas criteria appears rare [Anderson et al., 1988].

The prevalence of nonspecific myocarditis is related to the stage of HIV infection and the presence of structural heart disease. In one study of HIV-infected patients with a pre-mortem diagnosis of dilated cardiomyopathy, histologic findings consistent with lymphocytic myocarditis by the Dallas criteria were identified in 63 of 76 patients (83%) [Barbaro et al., 1998].

2.4.3 Non-invasive techniques

Electrocardiography (ECG) is a useful screening tool in patients with HIV infection, and ECG changes may precede echocardiographic abnormalities. Patients with abnormal ECG patterns should be further investigated [Tang et al., 2009].

Electrocardiography is often nonspecific (eg, sinus tachycardia, nonspecific ST or T-wave changes). Occasionally, heart block (atrioventricular block or intraventricular conduction delay), ventricular arrhythmia, or injury patterns with ST- or T-wave changes mimicking myocardial ischemia or pericarditis (pseudoinfarction pattern) may indicate poorer prognosis [Gorgels 2007].

A chest X- ray can offer data about the size and shape of hart, as well as identification of fluid in or around the heart that might indicate heart failure [Round 2007].

Echocardiography has been shown to be extremely useful for the diagnosis and monitoring of HIV associated myocardial disease. Echocardiography is performed to exclude other causes of heart failure (eg, valvular, amyloidosis, congenital) and to evaluate the degree of cardiac dysfunction (usually diffuse hypokinesis and diastolic dysfunction). It also may allow gross localization of the extent of inflammation (ie, wall motion abnormalities, wall thickening, and pericardial effusion). In addition, echocardiography may distinguish

between fulminant and acute myocarditis by identifying near-normal left ventricular diastolic dimensions and increased septal thickness in fulminant myocarditis (versus increased left ventricular diastolic dimensions and normal septal thickness in acute myocarditis), with marked improvement in systolic function in time [Tang et al., 2009].

De Castro et al., in 1994 performed a study of 136 HIV-infected patients without clinical, electrocardiographic or echocardiographic evidence of cardiovascular dysfunction on admission who were prospectively studied with serial echocardiograms; 93 of these patients had AIDS. During a mean follow-up period of 415 days, seven patients, all in the AIDS subgroup, developed clinical and echocardiographic findings of acute global left ventricular dysfunction; six of these seven patients died of congestive heart failure. Necropsy findings in five of these patients revealed acute lymphocytic myocarditis in three, cryptococcal myocarditis in one, and interstitial edema and fibrosis in one.

Cardiac computed tomography (CT) can have a role in the management of the undifferentiated heart failure patient, principally in excluding the presence of significant obstructive epicardial disease using CT angiography. Current generation 64-slice scanners demonstrate excellent diagnostic accuracy for both proximal coronary vessels and smaller distal vessels [Leber et al., 2005, Raff et al., 2005, Fine et al., 2006]. These recent studies especially demonstrate a high (greater than 95%) negative predictive value for the exclusion of significant epicardial stenosis.

Hence, although it has not been prospectively evaluated in the newly diagnosed heart failure population, the data would indicate that this modality can be used to stratify the patient with heart failure into an ischemic or non-ischemic etiology group.

Cardiac MRI (CMR) shows the accumulation of contrast in the myocardium as a consequence of the breakdown of the myocyte membrane resulting from the inflammatory process. The uptake of contrast usually has a characteristic patchy pattern for about the first 2 weeks after the acute event, later becoming progressively more disseminated. [Friedrich 1998] Moreover, this pattern of contrast uptake is easily distinguished from the subendocardial pattern of uptake seen in acute myocardial infarction.

CMR with contrast in association with cine-MRI is a useful tool for the diagnosis of myocarditis and provides an alternative to endomyocardial biopsy.

The availability of this diagnostic technique in the context of an acute episode might obviate the use of other, invasive diagnostic techniques which are not exempt from associated disease.

Roditi et al., in 2000 evaluated 20 patients with T1 spin-echo cine MR angiography and gadolinium-enhanced spin-echo imaging. Focal myocardial enhancement was associated with regional wall motion abnormalities in 10 of the 12 patients with suspected or proven myocarditis. The authors concluded that focal myocardial enhancement combined with regional wall motion abnormalities (hypokinesis, akinesis, or dyskinesis) strongly supported a diagnosis of myocarditis.

A combined CMR approach using T2-weighted imaging and contrast-enhanced T1-weighted images yields high diagnostic accuracy and thus, is a useful tool in the diagnosis and assessment of patients with suspected acute myocarditis [Abdel-Aty et al., 2005]. Friedrich et al. in 1998 were the first to propose CMR for the noninvasive diagnosis of acute myocarditis. Using T1-weighted images, they found that the myocardium in patients with suspected myocarditis has greater signal intensity relative to skeletal muscle [Friedrich et al., 1998]. T2-weighted images early after symptom onset can show focal increases of subepicardial and mid-wall myocardial signal, defining areas of myocardial edema [Abdel-

Aty et al., 2005]. Late gadolinium enhancement (LGE) - CMR has been shown to have additional value in the detection of active myocarditis as defined by histopathology [Mahrholdt et al., 2004].
LGE in the setting of myocarditis has a "nonischemic" pattern, typically affecting the subepicardium and the midmyocardial wall. This focal enhancement becomes diffuse over a period of days to weeks, then decreases during healing and may become invisible after recovery [Mahrholdt et al., 2004]. Alternatively, large areas of scarring might still be visible after healing, causing distinctive enhancing linear mid-wall striae. CMR-guided endomyocardial biopsy can result in a greater yield of positive findings than routine right ventricular biopsy [Mahrholdt et al., 2004].
This technique has not yet been fully evaluating in asymptomatic HIV infected subjects to establish the prevalence of unrecognized myocarditis.
Nuclear imaging: Antimyosin scintigraphy (using antimyosin antibody injections) can identify myocardial inflammation with high sensitivity (91-100%) and negative predictive power (93-100%) but has low specificity (31-44%) and low positive predictive power (28-33%). In contrast, gallium scanning is used to reflect severe myocardial cellular infiltration and has a good negative predictive value, although specificity is low [Tang et al., 2009].
In preliminary studies, a positive gallium scan improved the diagnostic yield of biopsy fourfold (baseline incidence of myocarditis - 8%; incidence associated with a positive scan – 36%). Gallium is an inflammatory avid isotope, whereas antimyosin antibodies are capable of labeling myocytes. Because histologic myocarditis consists of active inflammation in the presence of myocyte necrosis, indium 111 antimyosin antibodies may be useful in detecting this condition [O'Connell 1987].
Specific outcome data in HIV infected patients are missing.

2.5 Personal contribution

In Romania as in many other developing countries over the world cardiac MRI cannot be used widely for diagnosis. In the last several years within our cohort of adolescents and young adults HIV infected since their childhood we have noticed an increased number of patients with symptoms that suggest cardiac involvement. Dilative cardiomyopathy noticed more often in children infected by HIV was diagnosed especially postmortem at necropsy. As long as these patients present an increased rate of survival we are challenged to perform accurate diagnosis of cardiac involvement during their life.
During the last 2 years we have the opportunity to evaluate 10 patients with HIV and symptoms of cardiac involvement by performing: ECG, echocardiography, and nuclear imaging using technetium 99 (^{99}Tc). The 10 patients, 5 women and 5 men, were aged between 17 and 55 years. Echocardiography demonstrated in 4 cases normal left ventricular diastolic dimensions and small increases in septal thickness and in other 6 cases increased left ventricular diastolic dimensions and normal septal thickness. From the 4 patients with minimal echocardiography changes, nuclear imaging using technetium 99 showed no wall motions disorders and no changes in myocardial perfusions in 3 patients. In one patient we found no changes in echocardiography and ECG, while myocardial scintigraphy with ^{99}Tc showed changes in wall motility (akinesia) at rest and on stress and ischemic areas at the antero-septal wall and myocardial apex (4%), while at rest the affected area by myocardial scintigraphy was about 2%, as we can noticed in figure no. 1 [Cambrea et al., 2009]. From those 6 patients who presented changes on echocardiography, nuclear imaging with ^{99}Tc 2 patients demonstrated dilatative cardiomyopathy with no ischemic area, as shown in figure

no. 2. The other 4 patients presented with dilative cardiomyopathy and ischemic areas on stress, as demonstrated in figure no. 3. All 10 patients had received HAART including protease inhibitors for at least 5 years and significant changes in lipid profile.

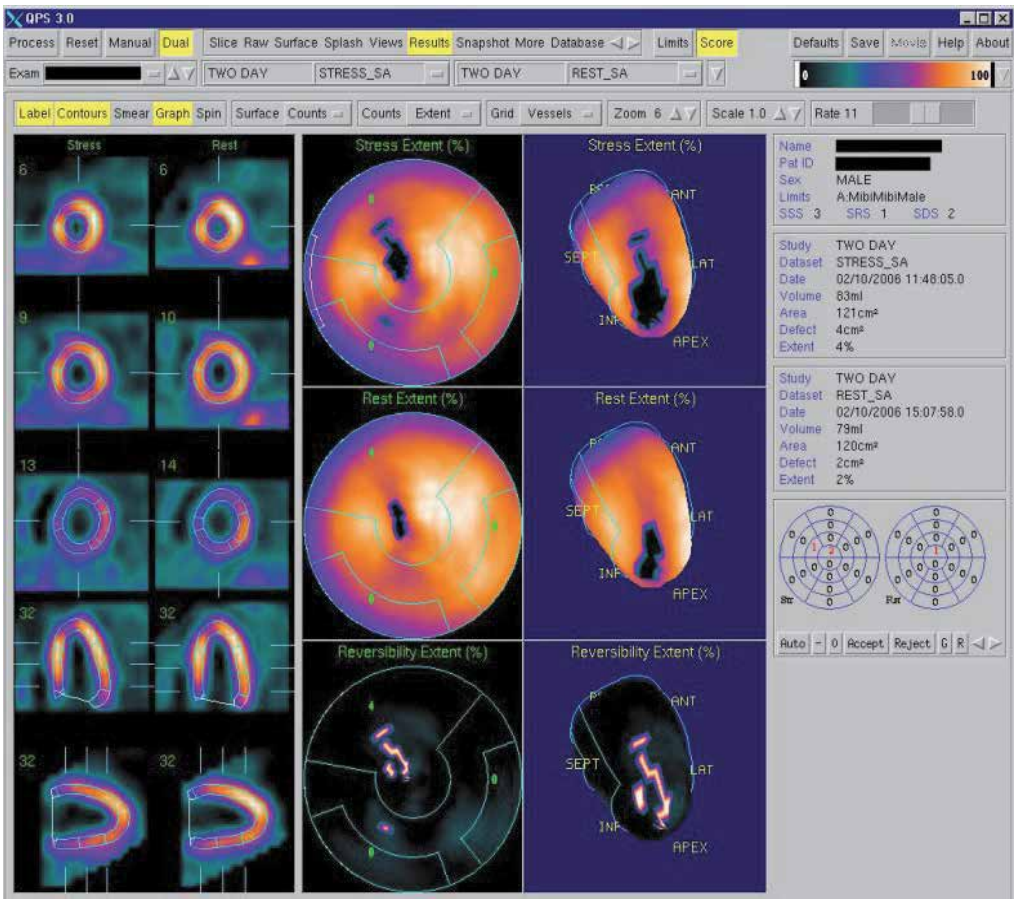

Fig. 1. Myocardic scintigraphy - ischemic areas at the antero-septal wall and myocardial apex.

2.6 Differential diagnosis of myocarditis in HIV

It is difficult to assess the clinical significance of viral infection of the myocardium in HIV-infected patients. AIDS or HIV-infected patients with myocarditis most often present with signs and symptoms of congestive heart failure or asymptomatic left ventricular dysfunction. The diagnosis of dilatative cardiomyopathy in this setting is best established by echocardiography. More specific diagnosis can be established by endomyocardial biopsy, as clinically indicated. However, in the vast majority of cases endomyocardial biopsy will not identify a specific cause that will modify therapy. In a minority of patients, biopsy may establish a treatable cause of myocarditis. Therefore, the clinician should consider the specifics of each case before making a recommendation regarding whether endomyocardial biopsy is necessary [Olson 2003].

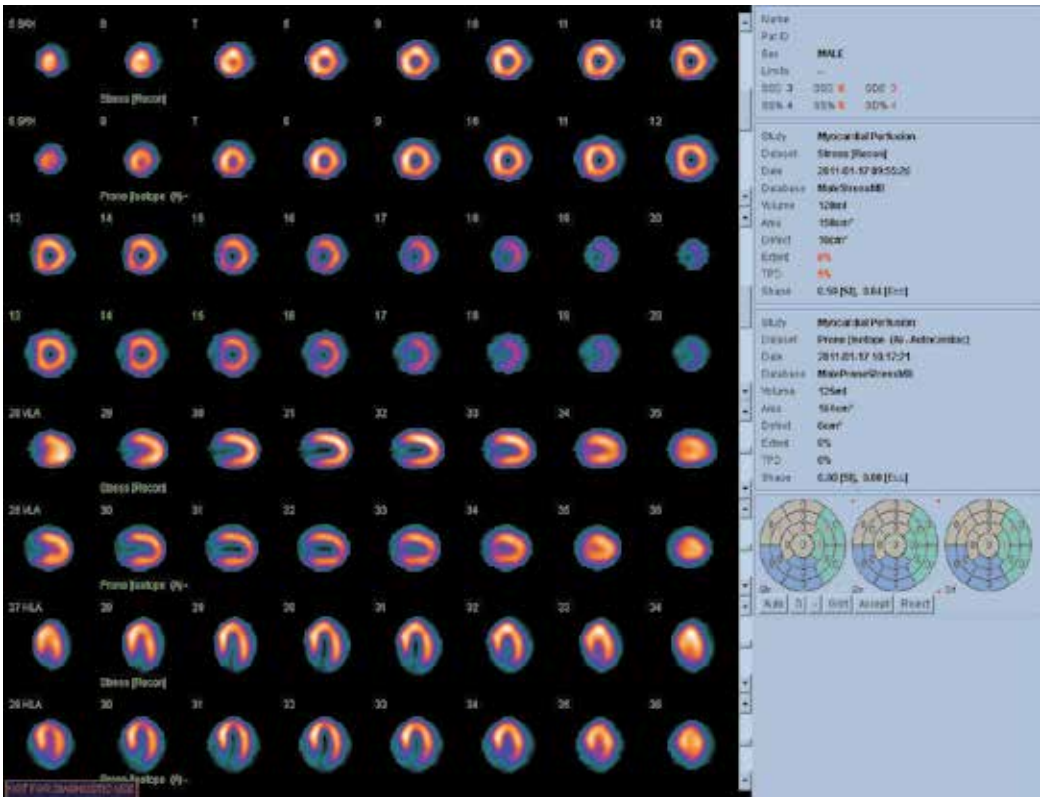

Fig. 2. Myocardic scintigraphy - dilatative cardiomyopathy with no ischemic area.

Aside from nonspecific or infectious myocarditis, the differential diagnosis of LV dysfunction in the AIDS patient includes drug toxicity from either abuse of illicit substances or iatrogenic disease from agents used in the therapy for AIDS. AIDS patients often take a great variety of prescription and nonprescription drugs and use illicit drugs. Alcohol, cocaine, or heroin may contribute to LV dysfunction in many cases [Virmani et al., 1988; Regan et al., 1990; Soodini et al., 1991]. Pharmacotherapy is also potentially associated with LV dysfunction in AIDS patients. Therapeutic agents implicated as potential cardiac toxins include zidovudine [Herskowitz et al., 1992b; d'Amati et al., 1992], and interferon alfa-2 [Deyton et al., 1989; Zimmerman et al., 1994].

If neoplastic infiltration is suspected as a cause of LV dysfunction, cardiac computed tomography or magnetic resonance imaging may be a useful adjunct to echocardiography for characterizing cardiac involvement. Neoplastic infiltration of the heart by Kaposi sarcoma is frequently seen at autopsy and usually associated with widespread disease in the terminal phases of AIDS [Silver et al., 1984]. Non-Hodgkin lymphoma is also observed in this setting and also associated with widespread disease [Holladay et al., 1992].

In addition to HIV-related cardiac conditions, differential diagnosis also includes non-HIV disease, because the latency of HIV disease may be long and patients are at risk for development of hypertensive heart disease, coronary artery disease, or other causes of left ventricular dysfunction [Olson 2003].

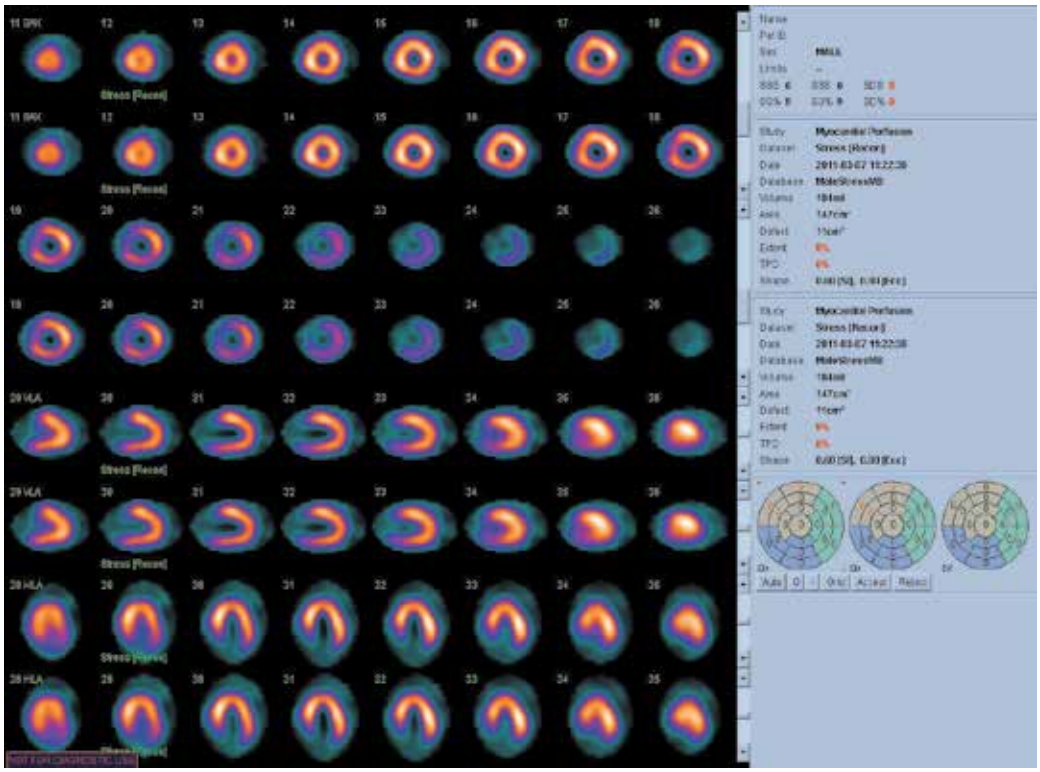

Fig. 3. Myocardic scintigraphy - dilative cardiomyopathy and ischemic areas on stress.

2.7 Treatment

Treatment for HIV related myocarditis is generally similar to that for non-HIV related myocarditis. Symptomatic treatment is the only form of therapy for HIV positive patients with myocarditis. In the acute phase, supportive therapy including bed rest is indicated. For symptomatic patients, digoxin and diuretics provide clinical improvement. For patients with moderate to severe dysfunction, cardiac function can be supported by use of inotropes such as Milrinone in the acute phase followed by oral therapy with ACE inhibitors (Captopril, Lisinopril) when tolerated. Patients who do not respond to conventional therapy are candidates for bridge therapy with left ventricular assist devices. Heart transplantation is reserved for patients who fail to improve with conventional therapy. Patients with HIV and myocarditis have enhanced sensitivity to digoxin and anticoagulation presents risks to patients with cerebral vasculopathy and possible aneurysm formation [Howes et al., 2010].

The use of immunosuppressive regimens in these patients is controversial and no convincing benefits have been reported other than with intravenous immunoglobulin [Lipshultz et al., 1995], whose efficacy may reflect inhibition of cardiac auto antibodies by competition with Fc receptors or dampened effects of cytokines and cellular growth factors.

The introduction of highly active antiretroviral therapy (HAART) regimens has substantially modified the course of HIV disease by lengthening survival and improving quality of life of HIV-infected patients [Zareba & Lipshultz 2003]. There is also good evidence that HAART significantly reduces the incidence of cardiovascular manifestations of HIV infection. By preventing opportunistic infections and reducing the incidence of

myocarditis, HAART regimens have reduced the prevalence of HIV-associated myocarditis to about 30% [Barbaro 2005]. One Italian study reported an almost 7-fold reduction of the prevalence of HIV-associated myocarditis from the pre-HAART era [Pugliese et al., 2000]. In that study there is no conclusive evidence that HAART reverses cardiomyopathy, but it does appear that by preventing profound immunosuppression and the development of AIDS, heart muscle remains healthier [Pugliese et al., 2000].

3. Conclusions

Cardiac dysfunction should be considered in the differential diagnosis of any HIV-infected patient with dyspnea or cardiomegaly. In the setting of AIDS or HIV infection, the diagnosis of dilated cardiomyopathy is established by echocardiography. A significant proportion, perhaps exceeding 80%, of patients with dilated cardiomyopathy may have focal, non-specific lymphocytic myocarditis [Barbaro et al., 1998a].

Although viruses, in general, are well established as a cause of acute myocarditis, a causal role for viruses in the pathogenesis of dilated cardiomyopathy has not been demonstrated conclusively, including HIV infection.

A low CD4 count is an excellent predictor of the presence of LV dysfunction. The risk of dilated cardiomyopathy may also be increased with a history of illicit drug use [Soodini et al., 2001].

Myocarditis due to HIV-1 myocyte infection does not seem to be the most likely cause of LV dysfunction in patients with AIDS. It is more likely that the cause of LV dysfunction and congestive heart failure in this setting is multifactorial, related to drug toxicity, non-HIV viral infection, poor nutrition, or cytokines. Another situation in HIV positive patients that can cause myocarditis with or without ischemia is dyslipidemia as a consequence of highly active antiretroviral therapy that included protease inhibitor for a long period of time.

The evaluation and management of HIV positive patients with myocarditis and specific dilated cardiomyopathies remains clinically challenging. Essential to the appropriate care of these patients is not only an understanding of the patient's cardiac morphology and function but also identification of pathologic and modifiable substrate.

The ultimate proof that the patient has myocarditis is provided by endomyocardial biopsy, but the patchy nature of the disease limits its diagnostic role [Karamitsos et al., 2009].

Computed tomography or magnetic resonance imaging may help but are not widely used for diagnosis. Gadolinium-enhanced magnetic resonance imaging is used for assessment of the extent of inflammation and cellular edema, although it is still nonspecific. Delayed-enhanced MRI has also been used to quantify the amount of scarring that occurred following acute myocarditis [Al-Mallah & Kwong 2009].

By virtue of its safety, high degree of accuracy and reproducibility, and multiparametric nature, cardiac MRI represents the principal imaging modality that potentially addresses each of these points of care for heart failure patients. However, coronary CT angiography can aid in ruling out epicardial coronary artery stenosis as the cause of LV dysfunction in selected patients presenting with congestive heart failure.

In addition to clinical examination and biological evaluation, in the absence of cardiac MRI, a combination of ultrasound and scintigraphic investigations of the heart can provide sufficient data to establish myocardial dysfunction with or without ischemia.

Because cardiac CT, CMR and cardiac scintigraphy were not widely used in patients with myocarditis and in HIV cases are only sporadic presentations, to identify particular aspects

of myocarditis in HIV positive patients is necessary to extend these new investigational noninvasive methods to a large number of patients.

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8

Exacerbation of Viral Myocarditis by Tobacco Smoke: The Catecholamine Hypothesis

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1. Introduction

1.1 Tobacco smoke exposure and cardiovascular disease

More than 1 in every 10 cardiovascular deaths in the world during the year 2000 were attributable to smoking, demonstrating that it is an important preventable cause of cardiovascular mortality (Ezzati et al., 2005). It has been clearly established that exposure to environmental tobacco smoke increases the risk of cardiovascular disease among persons who have never smoked (Steenland, 1992). The cardiovascular effects of secondhand smoke are nearly as large as those confronting the principal smoker (Barnoya & Glantz, 2005). Non-smokers living with smokers have about a 25% increase in risk of death from heart disease and are more likely to suffer a stroke. Exposure to secondhand smoke may increase the risk of heart disease by non-smokers as much as 60% (Whincup et al., 2004). In 2004, Ong & Glantz estimated that only about 69% of U.S. indoor workers were covered by a smoke-free workplace policy. Making all workplaces smoke free as been accomplished for the commercial aircraft industry (Repace, 2004) would in one year prevent about 1500 myocardial infarctions and 350 strokes, and result in nearly 49 million dollars in savings in direct medical costs. These estimates are supported by reports of reduced incidence of admissions for myocardial infarction associated with smoking bans (Sargent et al., 2004).

The cardiovascular effects of tobacco smoke exposure have been summarized in several excellent reviews (for example, see Barnoya and Glantz, 2005). Among the strongest pathophysiological correlates of tobacco smoking are accelerated endothelial dysfunction (Puranik & Celermajer, 2003) and acute clinical events, the latter being largely thrombotic (Ambrose & Barus, 2004). Recent studies indicate that increased oxidative stress is a potential mechanism for initiating cardiovascular dysfunction (Yang et al., 2004; Talukder et al, 2011). Tobacco smoke has also been demonstrated to increase inflammatory markers in patients exposed to secondhand smoke, including homocysteine, C-reactive protein, fibrinogen, and oxidized LDL cholesterol, (Panagiotakos et al., 2004) suggesting that an increased inflammatory response may contribute to accelerated atherosclerosis. Clearly, the incidence of ischemic coronary and peripheral vascular disease is increased in patients exposed to tobacco smoke, either as smokers or through exposure to secondhand tobacco smoke (Ambrose & Barus, 2004; Benowitz, 2003; Law & Wald, 2003; Burns, 2003; Leone et al., 2004). The cardiovascular effects of tobacco smoke exposure are summarized in Table l.

MECHANISMS OF CARDIAC AND VASCULAR INJURY FROM TOBACCO SMOKE EXPOSURE
Initiates inflammatory response
Enhanced platelet activation and thrombosis
Increased oxidative stress
Increased insulin resistance
Endothelial cell dysfunction
Accelerates atherosclerosis

Table 1. Cardiovascular effects of tobacco smoke exposure.

1.2 Tobacco smoke exposure and heart failure

National hospital discharge surveys estimate that 4.8 million Americans have heart failure, which has become an increasingly frequent reason for inpatient admission (Ho et al., 1993). One recent study estimated that among individuals aged 55 and older almost 1 in 3 will develop heart failure during their remaining lifespan. Heart failure is a fatal disease, with only 35% surviving 5 years after diagnosis (Bleumink et al., 2004). Coronary disease and diabetes mellitus along with hypertension are the leading causes of heart failure in the United States and it is therefore not surprising that tobacco smoke exposure has been identified as an independent risk factor for developing heart failure (Kannel et al., 1994; He et al., 2001). However, in at least 5% of patients, the cause of heart failure is initially unknown (Baldasseroni et al., 2002) and it has been estimated that myocarditis or inflammation of the heart muscle, may account for some 9% of these cases (Felker et al., 2000). Although estimates vary, these figures indicate that hundreds of thousands of Americans and millions of patients worldwide with heart failure may have myocarditis as a primary or exacerbating cause. The true incidence of myocarditis from any cause among the general population is unknown and most patients do not develop clinical manifestations of heart failure (Olinde & O'Connell, 1994). However, the Myocarditis Treatment Trial in which endomyocardial biopsy was performed in over 2,200 patients with unexplained heart failure of less than two years duration indicated a prevalence of 10%, implicating myocarditis as a potential cause of heart failure in hundreds of thousands of patients (Mason et al., 1995). Since only around 5% of patients systemically infected with a cardiotropic virus (Coxsakie β) may develop cardiac involvement, it is important to determine what factors may increase the risk of cardiac involvement (Grist & Bell, 1969; Gerzen et al., 1972; Rodeheffer & Gersh, 1996).

1.3 Catecholamines exacerbate myocarditis

Evidence from several different sources indicates that catecholamines may exacerbate viral myocarditis in animals and patients. A variety of sympathomimetic agents have been reported to induce or exacerbate myocarditis (Table 2). It is also important to note that nicotine has

been shown to increase catecholamine levels in the blood (Wong et al, 2007). The most direct evidence arises from carefully controlled studies of murine myocarditis indicting that hypercatecholaminergeic states, secondary to pheochromocytoma and during infusion with sympathomimetic drugs, can cause or significantly exacerbate myocarditis (Cho et al., 1987; Van Vliet et al., 1966; Bindoli et al., 1992; Kammermeier & Grobecker, 1995; Davila et al., 1995; Prichard et al., 1991; Brown & O'Connell, 1995; Siltanen et al., l982; Haft, 1974; Noda, 1970; Morin & Cote, 1972; Seta et al., 1997; Nash & Carter, 1967; Karch, 1987; Krentz et al., 2001; Rezkalla et al., 1988). Reports of a prospective study by Karch (Karch, 1987) showed significantly elevated levels of epinephrine and norepinephrine in a group of patients who presented with cardiac symptoms immediately after using cocaine. Moreover, sympatholytic agents and states may ameliorate the manifestations of myocarditis and decrease mortality, although this effect is controversial (Rezkalla et al., 1988; Anandasabapathy & Frishman, 1998; Mehes et al., 1966; Dunn & Vickers, 1994). We have shown a beneficial effect of beta blockade, ameliorating cocaine and catecholamine exacerbation of myocarditis in a murine model (Wang et al., 2005). It is provocative to consider that many of the interventions shown to ameliorate viral myocarditic pathgenicity, including calcium channel blockers, act predominantly to attenuate sympathomimetic effects on the heart (Dong et al., 1992; Hiraoka et al., 1996; Lowenstein et al., 1996; Wang et al., 1997; Keaney et al., 1996). Additional evidence includes the observation that, among commonly abused substances in the general population (alcohol, nicotine, caffeine, marijuana, and cocaine), cocaine has been most strongly associated with an increased incidence of myocarditis, suggesting that its unique sympathomimetic properties, not shared with these other agents, may be the causative factor. Moreover, in the clinical area, it has been accepted clinical practice for many years to restrict the activities of patients with myocarditis, primarily based on animal studies and circumstantial clinical evidence in man that exercise exacerbates the disease (Abelmann, 1966; Cabinian et al., 1990; Gatmaitan et al., 1970; Friman et al., 1983; Kiel et al., 1989; Tilles et al., 1964; Elson & Abelmann, 1965; Hosenpud et al., 1987). In addition to directly increasing the work of the heart, normal exercise is associated with a marked increase in circulating catecholamine levels (Cabinian et al., 1990; Gatmaitan et al., 1970; Friman et al., 1983; Kiel et al., 1989). While the cardiac effects of sympathomimetic drugs and interventions using sympatholytic agents require additional testing in animal models of myocarditis and in man, the available evidence points to catecholaminergic effects in the development of myocarditis.

1.4 Adrenergic activation by cigarette smoke

It has been estimated that there are over 4,000 chemical constituents in cigarette smoke. Of these, about 400 have been measured or estimated in mainstream or sidestream smoke; of the 400, a significant amount of toxicology data exists for less than 100 (Fowles & Bates, 2000). Nicotine is the most comprehensively studied constituent of cigarette smoke, and has been shown to act primarily on nicotinic acetylcholine receptors in autonomic ganglia and in the brain (Benowitz, 2008; Mobascher & Winterer, 2008). This results in activation of the sympathetic nervous system, increasing the release of epinephrine (EPI) and norepinephrine (NE) from adrenergic nerve endings and the adrenal medulla, both systemically and into the local milieu of adrenergically innervated organs, including the heart (Haass & Kübler, 1997; Adamopoulos, et al., 2008; Shinozaki et al., 2008). Cigarette smoke also contains monoamine oxidase inhibitors (Cooper & Magwere, 2008; Fowles & Bates, 2000; Herraiz et al., 2005). Cigarette smoke has been shown to increase norepinephrine and epinephrine levels with

SYMPATHOMIMETIC AGENTS WITH THE POTENTIAL TO CAUSE MYOCARDITIS	
Catecholamines	(Wang et al, 2005)
Ephedrine	(Naik & Freudenberger, 2004)
Cocaine	(Wang et al, 2002a)
Amphetamine	(Smith et al, 1976)
Terbutaline	(Sykes et al, 1991)
Clozapine	(Wang et al, 2008)
Methylxanthines	(Nino et al, 1987)

Table 2. Sympathomimetic agents that may initiate or exacerbate myocarditis.

associated increases in heart rate, blood pressure and coronary vasoconstriction (Haass & Köbler, 1997, Adamopoulos et al., 2008, Shinozaki et al., 2008). Nicotine may also potentiate catecholamine actions, perhaps via effects on nitric oxide synthesis or release (Haass & Köbler, 1997). The adrenergic actions of nicotine can be attenuated or blocked by administration of beta-adrenergic antagonists, although it has been proposed that the drug may act by additional mechanisms to activate the beta-adrenergic pathway (Haas & Köbler, 1997; Sofuoglu et al., 2006; Marano et al., 1999, Wang et al., 2005).

2. Experimental evidence

2.1 Overview

Our previous studies indicated that catecholamines exacerbate the severity of viral myocarditis in a murine model (Soodini & Morgan, 2001; Wang & Morgan, 2003; Wang et al., 2002a, 2002b, 2005). We performed studies with a variety of agents known to enhance the adrenergic tone, all of which exacerbated myocarditis in our viral model; in contrast, treatment with propranolol ameliorated the severity of myocarditis. Tobacco smoke has also been demonstrated to increase adrenergic activation in animals and patients, in part due to its content of nicotine (Cavarra et al., 2001; Pomerleau, 1992; Su, 1982) which in turn may exacerbate the severity of viral myocarditis in a murine model through adrenergically mediated mechanisms (Bae et al., 2010). As a more generalized hypothesis, exposure to tobacco smoke may be an important and as yet underappreciated cause of heart failure in patients receiving treatments with pro-inflammatory drugs (i.e. clozapine) or who are exposed to pathogens (i.e. viruses) or physical factors (i.e. radiotherapy) that can produce a level of myocarditis susceptible to exacerbation.

Sympathomimetic agents have the potential to produce toxic effects on the heart through a variety of mechanisms that are summarized in Figure 1. Note the overlap with mechanisms

attributed to tobacco smoke exposure in Table 1, most notably with regard to myocarditis, their effects on inflammation, immune function, and factors associated with cardiac and vascular dysfunction. Although multiple factors undoubtedly contribute to the exacerbation of myocarditis we have observed with tobacco smoke exposure, it is reasonable to propose that catecholamine-related effects play a central role.

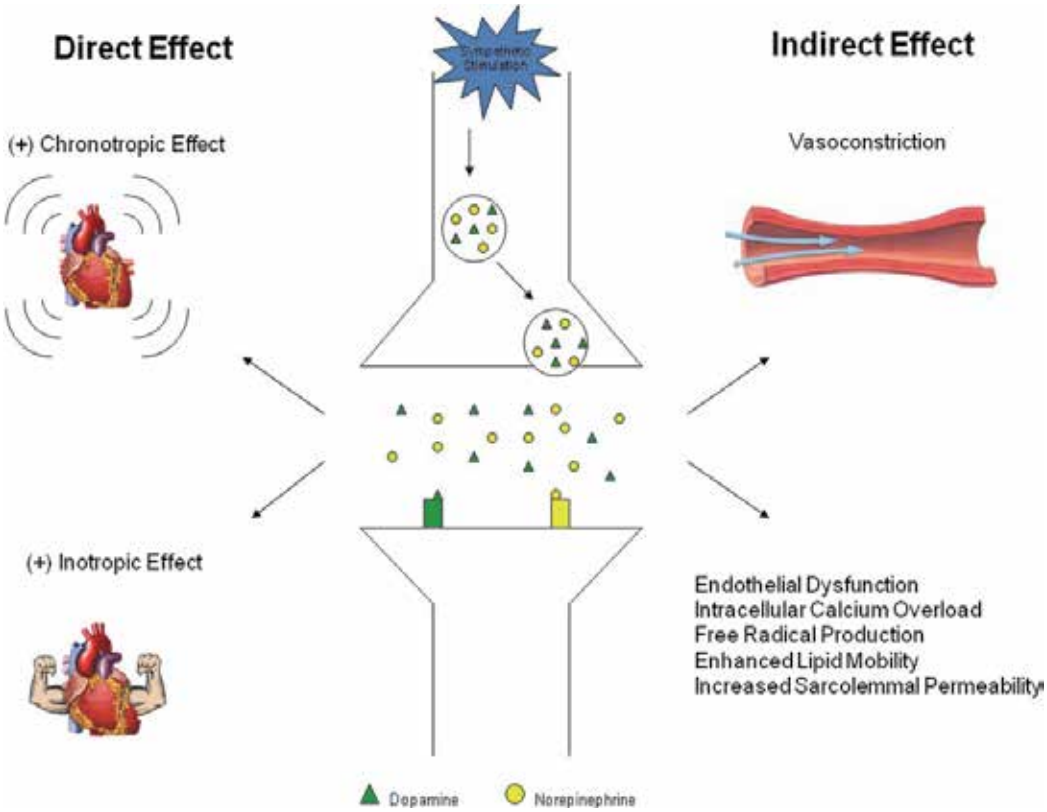

Fig. 1. Direct and indirect effects of catecholamines and related sympathomimetic agents on the heart.

2.2 Propranolol ameliorates and epinephrine exacerbates progression of acute and chronic viral myocarditis

Recent studies point to important interactions between proinflammatory cytokines and neurohumoral mediators in heart failure. We investigated the influence of the β-adrenergic system on cytokines and neurohumoral factors and the sequelae of viral myocarditis (Wang et al., 2005). In an experimental model with virus-infected BALB/c mice, we studied the acute and chronic effects of epinephrine and propranolol on myocardial morphology, cytokine gene expression, and survival. BALB/c mice were inoculated with the encephalomyocarditis virus (EMCV) or sham inoculated with saline and followed for 30 days. Epinephrine increased the severity of inflammatory cell infiltration and myocardial necrosis induced by EMCV-inoculated mice. Survival rate after 30 days was reduced to 40% in epinephrine-treated EMCV-inoculated mice compared with 70% in untreated EMCV-inoculated mice ($P<0.05$). Treatment with the β-blocker propranolol significantly decreased

mortality, myocardial necrosis and infiltration of inflammatory cells in EMCV-inoculated mice. Propranolol also suppressed gene expression of the cytokines TNF-α, IL-6 and IL-10 involved in inflammation. A single dose of epinephrine 120 days after EMCV inoculation caused death in 70% of infected mice; propranolol significantly reduced the incidence of death to 33%. These results indicate that acute and chronic stages of viral myocarditis are modulated by the β-adrenergic system and its interactions with proinflammatory factors.

2.3 Cocaine enhances myocarditis induced by encephalomyocarditis virus in murine models

This study (Wang et al., 2002a) was designed to investigate whether cocaine can exacerbate viral myocarditis and increase its incidence. Clinical evidence suggests that cocaine abuse increases the incidence of myocarditis. However, it had not been directly demonstrated that cocaine exposure enhances murine myocarditis. BALB/c mice were divided into eight groups saline control, encephalomyocarditis virus (EMCV), 10 mg/kg cocaine (Coc-10), 30 mg/kg cocaine (Coc-30), 50 mg/kg cocaine (Coc-30), EMCV+Coc-50. After inoculation with EMCV, the mice were daily treated with either cocaine or saline for 90 days. Mice were euthanized at different days after EMCV inoculation. Mortality was recorded and myocarditis severity was evaluated. The mortality of the myocarditis mice treated with cocaine increased significantly from 22% (EMCV) to 25.7% (Coc-10+ EMCV), 41.4% 9 (Coc-30+EMCV) and 51.4% (Coc-50+EMCV) ($P<0.05$) respectively. The incidence and severity of inflammatory cell infiltration and myocardial lesions was higher in infected mice exposed to cocaine. Cocaine administered only before infection did not exacerbate myocarditis. Norepinephrine assay showed that cocaine exposure significantly increased myocardial norepinephrine concentration, but this increase was partially inhibited in infected animals. Adrenalectomy abolished the effect of cocaine on mortality. Furthermore, propranolol, a β-blocker, significantly decreased the enhancing effects of cocaine on myocarditis mice. In conclusion, cocaine increases the severity and mortality of viral myocarditis in mice. Increased catecholamines may be a major factor in this effect.

A variety of viruses have been reported as causative agents of myocarditis in man, including Coxsackie, echo and influenza viruses, cytomegalovirus, poliomyelitis virus, Epstein Barr virus, herpes simplex virus, adenovirus and several others (Rodeheffer & Gersh, 2001; Ensley et al., 1995; Abelmann, 1966). Our laboratory has demonstrated that cocaine can exacerbate viral myocarditis in a murine model (Wang et al., 2002a). Possible mechanisms of such an interaction include, (a) cocaine-induced damage to the endothelial/endocardial cells in the extracellular matrix or to the myocytes themselves, thereby reducing structural and immunological barriers to cellular penetration of the virus and increasing the myocardial and vascular permeability and infectivity of viral particles. Evidence supporting "possibility (a)" includes reports suggesting that cocaine can damage the endothelial lining of cells after even a single exposure, thereby accelerating atherosclerosis in animal models (Egashira et al., 1991; Bacharach et al., 1992). Cocaine has also been reported to increase natural killer cell activity (Van Dyke et al., 1986). Both lymphocytic and eosinophilic myocarditis have been reported in cocaine abusers (Virmani et al., 1988; Tazelaar et al., 1987) and heart failure is a common finding (Weiner et al., 1986; Duell, 1987). Alternatively, "possibility (b)", cocaine may exacerbate viral myocarditis by permitting enhanced viral replication of the viral agent once it has penetrated the cell membrane. Such an effect may occur through a direct or catecholamine-mediated alteration in the cellular milieu that in turn could alter viral transcription and replication. Enhanced viral replication could occur through a change in

cellular pH, shift in osmolarity, or likely depletion of high-energy stores necessary for protective proteolytic enzyme activity. Of course, it is likely that the effects of cocaine on the animal or a patient with myocarditis are complex and involve several mechanisms or conditioning factors, including drug diluents with intrinsic pharmacological activity or sensitizing effects (Ensing, 1985; Wolf and Blum, 1983). However, the observation that exacerbation of myocarditis seems to occur with cocaine but not other commonly abused drugs without prominent cardiac effects, including heroin and phencyclidine, suggests that cocaine may have a unique combination of properties that make its use in patients exposed or infected with viral pathogens likely to enhance development of myocarditis and its sequelae. Our data indicate that the unique ability of cocaine to increase local release and circulating levels of catecholamines is the primary factor responsible for exacerbation of myocarditis (Ensing, 1985).

2.4 Clozapine-induced myocarditis: role of catecholamines in a murine model

Clozapine, an atypical neuroleptic agent, is very effective in the treatment of resistant schizophrenia. However, cardiotoxicity of clozapine, particularly in young patients, has raised concerns about its its safety and smoking may have a significant effect on serum concentrations of clozapine (Seppala et al., 1999). A particularly high incidence of myocarditis has been reported among patients treated with atypical neuroleptic drugs, including clozapine and risperidone. Clozapine has been shown to markedly increase circulating levels of catecholamines (Coulter et al., 2001; Elman et al., 2002). Increased catecholamines have been postulated to trigger an inflammatory response resulting in myocarditis, dilated cardiomyopathy, and death, although this has not yet been thoroughly studied. Here (Wang et al., 2008), we used the mouse to study whether clozapine administration could caused adverse myocarditis associated with an increase in catecholamines. Male BALB/c mice, age ~6 weeks, were administered 5, 10 or 25 mg/kg clozapine daily for 7 and 14 days; one group was administered 25 mg/kg clozapine plus 2 mg/kg propranolol for 14 days. Saline-treated mice served as controls. Heart sections were stained with hematoxylin and eosin for histopathological examination. Plasma catecholamines were measured with HPLC. Myocardial TNF-α concentrations were determined by ELISA. Histopathologic examination of clozapine-treated mice showed a significant dose-related increase in myocardial inflammation that correlated with plasma catecholamine levels and release of TNF-α. Propranolol significantly attenuated these effects. A hypercatecholaminergic state induced by clozapine could explain the occurrence of myocarditis in some patients. Our data suggest that β-adrenergic blocking agents may be effective in reducing the incidence and severity of clozapine-induced myocarditis.

3. Tobacco smoke

3.1 Exacerbation of viral myocarditis by tobacco smoke as a cause of heart failure

In this study (Bae et al. 2010), we determined whether exposure to tobacco smoke will exacerbate the severity of viral myocarditis in mice. Viral myocarditis was generated n 4-week old male BALB/c mice by i.ρ. injection of encephalomyocarditis virus (EMCV). Mice were exposed to cigarette smoke for 90 minutes/day 5x/week. Four groups were studied: 1) Control (C, no smoke and no virus), 2) Smoke (S) only, and 3) Virus only (V), and 4) Pre-exposure to smoke for l week prior to virus injection (S+V). We observed an over 2-fold

increase in mortality among mice that were pre-exposed to smoke compared to the virus alone. Tobacco smoke alone did not affect mortality. There was a significant increase in virus load among hearts from mice exposed to S+V compared to V alone.

In this study, we also investigated the rate of apoptosis 5 days after i.p. injection of virus. Viral exposure alone significantly increased the number of apoptotic cells. The number of apoptotic cells was increased further by smoke exposure prior to viral injection. Viral injection increased the translocation of apoptosis-inducing factor from mitochondria, a hallmark of caspace-independent apoptosis activation. Exposure to tobacco smoke exacerbated these effects without changing the total expression of apoptosis-inducing factor suggesting activation of caspace-independent apoptotic pathways as well.

Apoptosis has been shown to play an important role in human and animal heart failure (Kang et al., 2000; Kang & Izumo, 2003). Other investigators have demonstrated EMCV-induced apoptosis in the hearts of mice and pigs (for example, see Mizutani et al., 1996 and Brewer et al., 2001). Activation of caspaces may be a critical facilitator of viral infection in cardiomyocytes (DeBiasi et al., 2004, Kyoto et al., 2004). In fact, DeBiasi et al., have shown that inhibition of caspaces effectively blocks virus-induced apoptosis in vitro, although caspace-independent factors also appeared to be involved. Apoptosis-inducing factor release from mitochondria is one important caspace-independent factor, and appears to play an important role in EMCV-infection related apoptosis, as shown in our study. These data were later to show that viral infection induced a significant increase in apoptosis, through caspace-independent apoptosis, and that preexposure to tobacco smoke exacerbated this effect.

Several studies have shown a relationship between myocyte apoptosis and increased sympathetic activity in patients with underlying heart disease, most commonly heart failure (Singh et al., 2001). Communal et al., 1998 showed that over-stimulation by norepinephrine produced apoptosis in ventricular myocytes of adult rats and blocking the beta-receptor decreased apoptosis. These data are consistent with our hypotheses that catecholamines are a major factor inducing inflammation and cell death in tobacco smoke exposed animals.

4. Conclusions

Evidence from our laboratory and elsewhere (summarized above) indicates that both mainstream and secondhand tobacco smoke exposure can exacerbate viral myocarditis. Induction of a systemic inflammatory response appears to be a central initiating mechanism responsible for myocarditis, an effect that can be mediated by a cardiotropic viral infection itself and exacerbated by additional factors, most notably, catecholamines and other sympathomimetic agents, (Figure 2).

There appears to be an additive or synergistic effect of factors producing inflammation, which in turn can enhance viral load in the myocytes and exacerbate the extent and severity of myocarditis. Severe cardiac dysfunction and clinical heart failure may result. Our data suggest that disruption of gap junctions plays an important role in this regard (Figure 1). Moreover, to the extent that catecholamines are responsible, beta-adrenergic antagonists can attenuate the effect. Although the results presented in this review are consistent with a primary pathogenic role of catecholamines in the exacerbation of myocarditis by tobacco smoke, they do not exclude other factors that may operate through different mechanisms.

Additional animal and human studies will be necessary to further elucidate the several factors that may be involved. However, taken together, the available data indicate that tobacco smoke can exacerbate myocarditis which in turn may result in irreversible cardiac dysfunction and failure. Even in the absence of additional data, these results provide another example of the adverse effects of tobacco smoke and strengthen the argument for minimizing exposure to this agent in our environment.

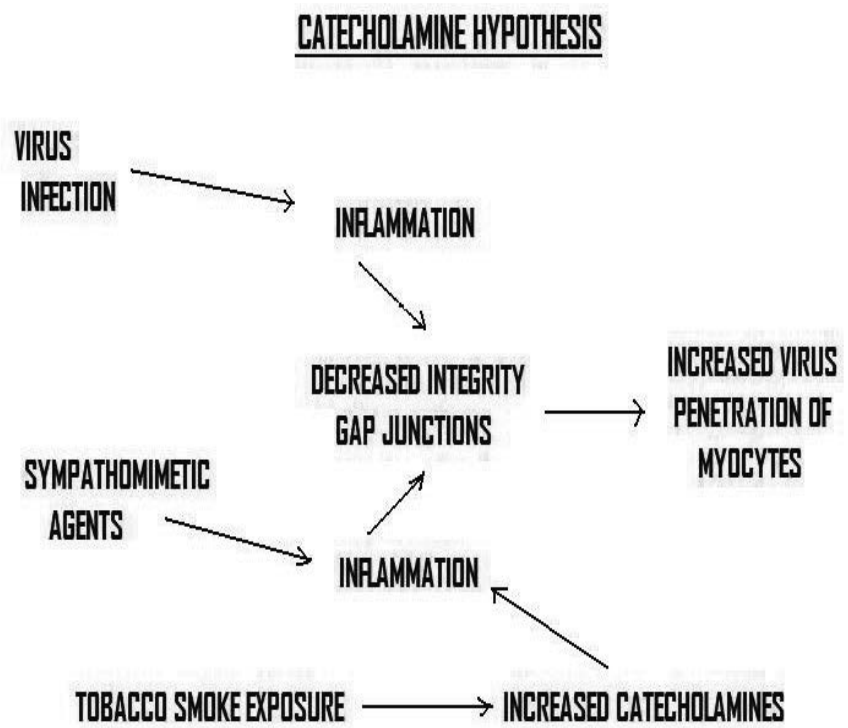

Fig. 2. Proposed primary mechanism for exacerbation of viral myocarditis by tobacco smoke exposure: the catecholamine hypothesis.

5. Acknowledgement

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9

Angiopoietin-1 for Myocardial Angiogenesis

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1. Introduction

In response to ischemic damage, the heart undergoes vicious process of remodeling wherein the damaged myocardium is replaced by scar tissue and as compensatory mechanism, its existing collateral vessels and neovascularization with concomitant changes in cell recruitment, multiplication and cytokine/growth factor action. Angiogenesis is a complex process which involves an interplay between multiple pro- and anti-angiogenic factors and a harmonized interaction between endothelial progenitor cells, smooth muscle cells, pericytes and supportive environment. Besides Vegf/ Vegf receptor system, angiopoietin family of pro-angiogenic growth factors in conjunction with their receptor system are critical for vascular protection, remodeling, proliferation and maturation beside preservation of the integrity of newly formed vascular structures for functional activity (Thurston et al. 2000; Saharinen et al. 2005; Brindle, Saharinen et al. 2006).

An outside intervention to support the inefficient intrinsic myocardial repair processes by administration of stem/ progenitor cells has emerged as a promising strategy for the treatment of ischemic heart diseases. The transplanted stem cells have shown both myogenic as well as vasculogenic differentiation potential and participate in the myocardial regeneration *via* angiomyogenesis (Chen et al. 2010; Uemura et al. 2006; Eguchi et al. 2007). In addition to differentiation, stem cells can also ameliorate inflammation, migrate to ischemic regions and secrete bioactive molecules as a part of their paracrine activity and significantly contribute myocardial protection and angiogenesis. Alternatively, multimodal therapeutic strategies have also been adopted to accentuate the angiomyogenic potential of stem cells. This includes preconditioning of stem cells with growth factor treatment, their genetic modification with plasmids encoding for various angiogenic growth factors and concomitant administration of recombinant angiogenic growth factor proteins (Jiang et al. 2006; Haider et al. 2008; Kim et al. 2009; Lu et al. 2009). Such multimodal treatment strategies have elicited beneficial effects in terms of improving stem cell survival and enhancing their paracrine behavior besides stimulation of angiogenesis through direct recruitment, proliferation and maturation of precursor cells such as endothelial progenitor cells, mesenchymal stem cells and monocytes to the ischemic heart (Banai et al. 1994; Hiasa et al. 2004; Elmadbouh et al. 2007; Haider et al. 2008). We discuss here the biological regulation of angiopoietin-1 expression, its interaction with specific receptor system and the advantages of transgenic over expression of angiopoietin-1 either alone or in combination

with Vegf to support angiogenesis as a therapeutic option for the treatment of ischemic heart disease.

2. Angiopoietin-1

2.1 Angiopoietin-1 and Tie2 ligand/receptor interaction in angiogenesis

The angiopoietin family of proteins consists of four members, all of which interact with the endothelial receptor tyrosine kinase, (tunica intima endothelial kinase 2, Tie2) (Thomas & Augustin 2009). Whereas two of these factors, angiopoietin-1 and angiopoietin-4, are constitutive agonists and Tie2 receptor activators (Davis et al. 1996), angiopoietin-2 and angiopoietin-3 have different effector functions and may activate or antagonize angiopoietin-1 induced Tie2 phosphorylation (Suri et al. 1996; Valenzuela et al. 1999; Fiedler et al. 2003). The essential role of angiopoietin-1 in the expansion and stabilization of newly formed vessels has been widely demonstrated (Suri et al. 1998). The process of vasculogenesis consists of differentiation, proliferation, and coalescence of vascular endothelial cells to establish a primitive vascular network in the early stage. This is followed by maturation of the neovasculature through the process of angiogenic remodeling that involves sprouting, branching, pruning, differential growth of vessels, and the recruitment of supporting cells (Suri et al. 1998; Hattori et al. 2001).

Both angiopoietin-1 and angiopoietin-2 have discrete participation in the occurrence of angiogenic cascade wherein angiopoietin-2 accumulates at the leading edge of proliferating vessels and angiopoietin-1 shows diffused localization behind the leading edge (Suri et al. 1998). Based on this distinct pattern of expression, it is suggested that angiopoietin-2 negatively mediates Tie2 activation and destabilizes the vessels to make these responsive to other angiogenic growth factors including Vegf, Pdgf and Fgf. On the contrary, angiopoietin-1 activates Tie2 and triggers remodeling and stabilization of the newly formed vasculature which leads to its maturation (Suri et al. 1998; Huang et al. 2009). The antagonizing activity of angiopoietin-2 is imperative for normal vascular maturation and spatial configuration (Feng et al. 2009). Over expression of angiopoietin-1 results in increased number, size and branches of the blood vessels without affecting the association among endothelial cells and with no evidence of plasma leakage, edema, or erythrocyte extravasation unlike Vegf (Suri et al. 1998; Thurston et al. 1999; Thurston et al. 2000; Kim et al. 2002). Angiopoietin-1 also contributes to attenuation of inflammatory response by mediating anti-permeability effects to counter Vegf and tumor necrosis factor (TNF)-induced inflammatory molecules in the endothelial cells such as vascular adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and endothelin-1 which control cell-cell interaction, maintain vascular quiescence and prevent leakiness (Kim et al. 2002; Hughes et al. 2003; Jeon et al. 2003; McCarter et al. 2006).

Tie2 receptor, a member of receptor tyrosine kinase family, consists of an extracellular domain, a trans-membrane domain and a split intracellular kinase domain (Takahara et al. 2004). This receptor is more specifically expressed on vascular endothelium in both quiescence and active states although it is also found on some other cell types such as smooth muscle cells, fibroblasts, mural cells, ganglion cells and carcinoma cells (Dumont et al. 1992; Takahara et al. 2004; Kosacka et al. 2005; Nakayama et al. 2005; Hamaguchi et al. 2006). Tie2 is highly conserved from zebra fish to mammals with the greatest amino acid homology occurring in the kinase domain, indicating the importance of its biological function (Lyons et al. 1998). Disrupting the function of Tie2 in transgenic mice was lethal

and resulted in early embryonic death due to failure of vascular branching and differentiation. Homozygous mutated embryos also displayed abnormalities in the development of the heart (Dumont et al. 1994; Sato et al. 1995). Lack of Tie2 also resulted in angiogenic defects in term of vessel branching and remodeling, and displayed defects in the developing vessels to have scarce peri-endothelial cells and thinner collagen-like fibers (Suri et al. 1996). Dysregulated expression of Tie2 have also been observed in several clinical diseases including venous malformations, intramuscular hemangiomas, pulmonary hypertension and infantile hemangiomas (Yu et al. 2001; Wang et al. 2004; Morris et al. 2005). On the contrary, overexpression of angiopoietin-1 in the skin of experimental animal models led to the formation of highly branched and larger vessels, and resulted in reduction of microvascular leakage (Suri et al. 1998; Thurston et al. 1999). Tie2 over expression in the skin caused psoriasis-like phenotype after birth and persisted throughout adulthood, and was featured by epidermal hyperplasia, accumulation of inflammatory cells and altered dermal angiogenesis (Voskas et al. 2005). These findings clearly suggested that a delicate level of Tie2 receptor was required for physiological functioning and any unregulated induction or loss of Tie2 resulted in potentially worsened effects. Despite considerable similarity with Tie1, experiments with Tie1- or Tie2-deficient mice have provided evidence of their distinct functions in response to different members of angiopoietin family (Seegar et al. 2011).

2.2 Angiopoietin-1, Tie2 receptor and intracellular signaling

It is interesting to note that angiopoietin-1 and angiopoietin-2 have different effects on vascular formation and development, however, they bind to Tie2 receptor with distinct kinetics of release following binding thus indicating that activation of Tie2 receptor is regulated independently by these two molecules. In fact, angiopoietin-2, a natural antagonist of angiopoietin-1 (Maisonpierre et al. 1997), binds to Tie2 receptor without its activation (Davis et al. 2003). Similarly, structural characteristics and distinguishable interaction with other molecules in the extracellular environment of the ligands may also essentially contribute to their counteractive properties (Kim et al. 2005). More recent studies have shown that the effects of angiopoietin-1 and angiopoietin-2 on the receptor tyrosine kinase Tie2 are differentially regulated at the endothelial cell surface (Hansen et al. 2010) and a critical balance is maintained between angiopoietin-1 and angiopoietin-2 expression by sonic hedgehog and fibroblast growth factor-2 during angiogenesis (Fujii & Kuwano, 2010). Phosphorylation of tyrosine residues of Tie2 occurs subsequent to binding with angiopoietin-1 and activates kinase domain of the receptor to initiate various downstream intracellular signaling cascades (Murray et al. 2001). The phosphorylation of tyrosine residues on the intracellular domain of Tie2 receptor interacts with the p85 subunit of PI3K *via* Src homology 2 or phosphotyrosine binding domain. These molecular changes result in activation of PI3K and its downstream Akt in the endothelial cells and ultimately lead to multiple responses such as cell survival, differentiation and chemotaxis (Witzenbichler et al. 1998; Fujikawa et al. 1999; Abdel-Malak et al. 2009; Bai et al. 2009). Although some studies have already demonstrated that angiopoietin-1 mediated activation of Tie2 does not cause mitogenesis of endothelial cells, the others have reported a pro-proliferative effect of angiopoietin-1 on vascular cells (Kanda et al. 2005; Abdel-Malak et al. 2009). These contradictions in the data may be explained on the basis of the observation that angiopoietin-1 may induce various effects on endothelial cells depending on the tissue type and conditional environments.

Angiopoietin-1 activity also involves Forkhead box O-1 (FOXO1) transcription factor which principally acts as a regulator of cell cycle and endothelial cell functions in vascular destabilization and remodeling (Kanda et al. 2005; Evans-Anderson et al. 2008). Angiopoietin-1 is known to inhibit the activity of FOXO1 *via* phosphorylation and promotes cell proliferation in the cultured endothelial cells by upregulation of cyclin D1 downstream of FOXO1 (Kanda et al. 2005; Huang & Tindall 2007). Phosphorylation of FOXO1 by Akt in the endothelial cells occurs at three conserved sites which results in the inhibition of FOXO1 by promoting its translocation from the nucleus to the cytoplasm (Daly et al. 2004; Huang & Tindall 2007). In addition to interaction with FOXO1, GATA3 which is highly expressed in human endothelial cells especially in the large vessels, plays a significant role in the expression of angiogenesis related genes and endothelial cell functions subsequent to stimulation with angiopoietin-1 (Song et al. 2009). Knock down of GATA3 significantly abrogated these effects of angiopoietin-1. Besides these transcription factors, angiopoietin-1 can activate MAPK in the cultured endothelial cells (Fujikawa et al. 1999; Kim et al. 2002; Zhu et al. 2002). Pharmacological inhibition of ERK1/2 abolishes Tie2 phosphorylation and its downstream signaling for morphogenesis of capillary endothelium and suppresses endothelial cell proliferation which are involved in angiogenesis (Kim et al. 2002). However, inhibition of ERK1/2 activity in endothelial cells does not effect angiopoietin-1-induced survival and migration (Fujikawa et al. 1999). Hence, it is suggested that MAPK activity as a consequence of Tie2 activation during angiogenesis is more important for endothelial sprouting and branching than for endothelial recruitment and maintenance. On the other hand, angiopoietin-1 mediated activation of p38 MAPK signaling promoted mural cell recruitment during angiogenesis (Zhu et al. 2003). Angiopoietin-1 also has the ability to directly bind to the monocytes without interacting with Tie2 and promote their transendothelial migration by directly activating PI3K for its role in inflammatory angiogenesis (Ahmad et al. 2010).

2.3 Angiopoietin-1, Tie2 receptor and extracellular response

Under physiological conditions, endothelial cells of the vasculature remain quiescent in the inner layer of vessels. However, during active vascular remodeling in response to pathological conditions like vascular occlusion, myocardial infarction or *de-novo* vascular formation, circulating endothelial progenitors and local endothelial cells migrate to the ischemic areas. More so, some of these cells penetrate and traverse to distant sites of the occluded vessels to participate in the repair process. Angiopoietin-2, which is mainly released by endothelial cells and localized at the site of vascular remodeling, functions as a Tie2 blocker and promotes the destabilization of pericytes from existing vessels and increases vascular permeability. This in turn allows the infiltration of proteases, cytokines and angiogenic cells to support robust angiogenic response. The blood vessels are thus formed by the complex contribution of an intricate network of smooth muscle layer that surrounds endothelial cells in arteries, arterioles and veins resulting from migration, proliferation and interaction of different cell types like pericytes, smooth muscle cells and fibroblasts (Asahara et al. 1999; Carmeliet 2000; Bentley et al. 2009). It is suggested that interaction between angiopoietin-1 and Tie2 also regulates cross-talk between endothelial cells and pericytes (Davis et al. 1996; Sundberg et al. 2002). Angiopoietin-1 is a pericyte derived signal that mediates maturation and quiescence of the microvascular endothelium (Armulik et al. 2005). In addition to its role as an effector for the secondary step of vascular formation, angiopoietin-1 can stimulate endothelial cell migration and induce angiogenesis

independent of its interaction with angiopoietin-2 or Vegf (Koblizek et al. 1998; Hayes et al. 1999; Babaei et al. 2003). The pro-angiogenic activity of angiopoietin-1 independent of Vegf involves phosphorylation of Tie2 and activation of PI3K/Akt signaling. These observations have been substantiated by *in-vivo* experimental evidence which showed that the angiogenic efficacy of angiopoietin-1 alone was comparable to that of Vegf stimulation (Babaei et al. 2003). More recent studies have shown that angiopoietin-1 also regulate the functions of hematopoietic stem cells in the bone marrow. Treatment of ckit+ cells with angiopoietin-1 helped the cells to maintain their functional activity *in vitro* on long term basis, however, with little influence on their colony forming potential (Gomei et al. 2010).

2.4 Angiopoietin-1, Tie2 and anti-apoptotic effect

Angiopoietin-1 is critical for cell survival and proliferation and functions *via* PI3K/Akt and MAPK/ERK signaling pathway (Daly et al. 2004; Kanda et al. 2005). ERK1/2 kinases have important role in regulation of apoptosis in various cells including endothelial cells wherein ERK1/2 have been consistently shown to mediate the anti-apoptotic effects of VEGF and angiopoietin-1 by targeting caspase-9, -3 and -7. Angiopoietin-1 also induces p38 MAPK phosphorylation as a part of its anti-apoptotic activity in the endothelial cells (Gratton et al. 2001; Harfouche et al. 2003). The pro-survival effects of angiopoietin-1 have been extensively studied in variety of cells against pro-apoptotic stimuli (Tuo et al. 2010; Lee et al. 2008; Liu et al. 2008; Bai et al. 2009). In most cases, angiopoietin-1 treatment phosphorylated Tie2 receptors leading to activation of Akt signaling. Treatment of neuronal progenitor cells with angiopoietin-1 protected the cells against oxygen-glucose deprivation induced apoptosis by activation of PI3K/Akt to inhibit pro-apoptotic signaling (Bai et al. 2009). Similarly, activation of pro-apoptotic signaling was reversed in myocardial endothelial cells in high glucose culture conditions upon treatment with angiopoietin-1 which incidentally also increased angiogenesis (Tuo et al. 2010). These salutary effects of angiopoietin-1 were however, antagonized and blunted by angiopoietin-2. Treatment with angiopoietin-1 protein or its transgenic expression in endothelial cells also induces some secondary mediators such as interleukin-8 through ERK1/2, SAPK/JNK, and PI3K pathways, which trigger c-Jun phosphorylation on Ser63 and Ser73 (Abdel-Malak et al. 2008). Interleukin-8 then acts in autocrine fashion to suppress apoptosis and facilitate cell proliferation and migration (Abdel-Malak et al. 2008).

3. Angiopoietin-1 gene delivery in combination with Vegf

Whereas Vegf is one of the most potent vasoactive growth factors which is involved in angiogenesis and regulates vascular permeability, angiopoietin-1 is also being recognized for its angiogenic potential besides its role as a vascular stability factor. Both of these growth factors are discretely produced in a succession during the development of mature blood vessels (Thurston, 2002). Angiopoietin-1 acts as a mitogen for endothelial cells and synergistically induces sprout formation with Vegf (Koblizek et al. 1998). The regulatory mechanism of angiopoietin-1 induced neovascularization involves pro-survival effects on the endothelial cells by activation of PI3K/Akt signaling and stabilization of nascent blood vessels to become leak resistant (Thurston et al. 2000).

Both Vegf and angiopoietin-1 have been extensively used for angiogenic protein or gene therapy to exploit complementarities between their functional relationship (Zhu et al. 2002; Cheng et al. 2007). Given a coordinated role of angiopoietin-1 and Vegf during both

physiologic and pathologic development of blood vessels, simultaneous use of the two growth factors have been reported for the treatment of tissue ischemia (Gale et al. 2002; Ye et al. 2007). The application of this combinatorial growth factor therapy approach is not only for induction of angiogenesis; it is also intended to involve circulating endothelial progenitor cells. These progenitors then home into the ischemic tissues in response to the concentration gradient for participation in the ongoing repair process of vasculogenesis (Kalka et al. 2000; Wang et al. 2006). We have already reported the feasibility of combining stem cell mobilization from bone marrow in combination with Vegf gene delivery to the infarcted heart to show that the mobilized stem cells homed into the heart and participated in myocardial angiogenesis (Wang et al. 2006). Although the use of recombinant growth factors has given encouraging results with both Vegf and angiopoietin-1, the very short biological half-life of these growth factors warrants alternative treatment strategies. A more recent study has reported covalent immobilization of Vegf and angiopoietin-1 onto three-dimensional porous collagen scaffolds using 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) chemistry to enhance the duration of the growth factors availability and effectiveness (Chiu & Radisic, 2011).

As an alternative to recombinant protein administration, transgenic expression of angiopoietin-1 and Vegf gene therapy is being assessed to achieve arteriogenesis and angiogenesis for the treatment of myocardial ischemia (Samuel et al. 2010; Siddiqui et al. 2003; Ye et al. 2007). The efficacy of Vegf and angiopoietin-1 gene delivery to the heart has been extensively studied to promote angiogenesis and improve regional blood flow et al. 2007; Haider, Ye et al. 2004). The authors reported the first bi-cistronic adenoviral vector encoding for Vegf and angiopoietin-1 for co-expression of the two angiogenic growth factors. The vector was used to genetically modify stem cells for overexpression of angiopoietin-1 and Vegf. Transplantation of genetically modified skeletal myoblasts demonstrated development of functionally mature blood vessels in the infarcted heart and in the hind limb ischemia model in rabbits (Niagara et al. 2004; Ye et al. 2007). These observations were in harmony with the previously published data suggesting enhanced perfusion accompanied by the development of stable and mature blood vessels with combined Vegf and angiopoietin-1 administration (Arsic et al. 2004; Gurunluoglu et al. 2002; Shyu et al. 2003). In a recently reported study, Tao *et al.* co-expressed Vegf/angiopoietin-1 using adeno-associated viral vectors (AAVs) expressing cardiac-specific and hypoxia-inducible Vegf and Ang1 into the porcine infarcted heart immediately after ligation of the left descending coronary artery (Tao et al. 2010). Vegf and Ang1 were predominantly expressed in the heart in the infarct and border of the infarct. Gated single-photon emission computed tomography showed improved cardiac function and myocardial perfusion at 8 weeks after vector injection which corresponded well with higher vascular density. They also observed higher level activation of Akt and Bcl-xL, less Caspase-3 and Bad, and reduced TUNEL positivity in angiopoietin-1/ Vegf treated animal hearts. These results showed that simultaneous expression of angiopoietin-1/ Vegf in the infarcted heart stimulated pro-survival pathways besides improved regional blood flow. Although the authors claimed to have observed significant change in the number of cycling cardiomyocytes subsequent to angiopoietin-1/ Vegf overexpression, this may be insufficient to replace the massive loss of the functioning cardiomyocytes in the infarcted heart. Although direct injection of angiopoietin-1 and Vegf growth factors has been shown to significantly improve the regional blood flow in the ischemic heart, this strategy if combined with stem cell therapy would be more effective in addressing the core issue of myocardial regeneration which requires neomyogenesis for replacement of the scar tissue.

4. Combining stem cell transplantation and Angiopoietin-1 delivery

Gene delivery strategy has developed over the years from direct plasmid injection to stem cell based ex-vivo delivery strategy. Stem cells are excellent carriers of therapeutic genes for delivery to the various body tissues and organs including the heart (Suzuki et al. 2001; Yau et al. 2007; Ye et al. 2007; Haider et al. 2008). Despite all the progress made, it remains to be defined whether cell based gene therapy can overcome several potential impediments such as poor transfection efficiency, unregulated transgene expression, low survival rate of transplanted cells into ischemic zones etc. On the same note, there are a number of parameters which require optimization including cell type, number of transfected cells to be transplanted, time of cell transplantation after infarction, and route of cell transplantation.

Angiopoietin-1 is one of the many angiogenic growth factors which have been extensively studied for pro-angiogenic activity in the ischemic tissues. We performed a comparative assessment of the methods to deliver angiopoietin-1 gene delivery for angiogenic repair of the infarcted heart using an experimental porcine heart model of chronic infarction (Ye et al. 2007). Our results showed that skeletal myoblast based delivery of angiopoietin-1 transgene was more effective as compared to the approach of direct injection of adenoviral vector encoding for angiopoietin-1. The genetically modified skeletal myoblasts carrying angiopoietin-1 transgene served as a reservoir of the transgene product and ensured localized release of angiopoietin-1 at the site of the cell graft without safety concerns associated with the use of direct injection of adenoviral vector (Ye et al. 2007). Besides we observed extensive survival of the transplanted skeletal myoblasts which underwent myogenic differentiation to repopulate the infarcted myocardium.

Given that the development of stable and functional blood vessels is regulated by a critical balance between several pro- and anti-angiogenic factors which also co-ordinate with various vasculogenic cells, we hypothesized that a single angiogenic factor may be insufficient to achieve the desired outcome. We therefore opted to combine angiopoietin-1 and Vegf for co-expression to achieve angiogenic synergism between the two growth factors (Ye et al. 2007). We developed a bicistronic adenoviral vector which encoded for human $Vegf_{165}$ and angiopoietin-1 driven by the same promoter. The vector was used to genetically modify human skeletal myoblasts which were later transplanted in a porcine heart model of coronary artery ligation. We observed excellent survival of the transplanted skeletal myoblasts for up to 12 weeks using transient immunosuppression. Immunohistological studies showed myogenic differentiation of the skeletal myoblasts and increased blood vessel density in the infarct as well as peri-infarct regions with highest maturation index in the animal heart treated with skeletal myoblasts co-expressing Vegf and angiopoietin-1. Regional blood flow, measured with fluorescent microspheres, was significantly improved which revealed the functional competence of the newly formed blood vessels. These findings signified the feasibility of multimodal therapeutic approach based on simultaneous delivery of angiopoietin-1 and Vegf combined with cell transplantation.

Although skeletal myoblasts showed excellent ability as transgene carriers, one of the major drawbacks is their failure to develop gap junctions with the host cardiomyocytes and arrhythmogenicity (Fouts et al. 2006). We therefore hypothesized that the use of bone marrow derived mesenchymal stem cells might be a better option. Mesenchymal stem cells have been extensively studied for their cardiac reparability and regenerative potential (Chen et al. 2010; Kim et al 2010.; Labovsky et al. 2010; Haider et al. 2009) besides having superior transgene carrying capability (Chen et al. 2010; Huang et al. 2010; Tang et al. 2010; Haider et

al. 2008). Besides, we also opted to replace Vegf with survival signaling molecule Akt to support survival of the genetically modified mesenchymal stem cells (Jiang et al. 2006). Our choice of transgene combination of angiopoietin-1 and Akt achieved maximum beneficial effects in terms of donor stem cell survival and angiomyogenic repair of the infarcted heart. More importantly, the therapeutic benefits in terms of cell graft survival, stability of newly formed blood vessels and global cardiac function were stable for up to 3 months (Shujia et al. 2008). A more recent study has used sendai viral vector for transduction of mesenchymal stem cells, however, it remains difficult to see the advantages of mesenchymal stem cells modified with sendai vector harboring human angiopoietin-1 gene (Piao, Wang et al.)

5. Conclusions

Genetic modification increases the therapeutic efficacy of stem cells by improving their survival, enhancement of paracrine activity and by supporting their angiomyogenic differentiation (Tang et al. 2004). A combined cell and gene therapy approach reverses the deteriorating function of the infarcted heart (Mangi et al. 2003; Matsumoto et al. 2005) and offers an extended and localized expression of the transgene product. From the clinical standpoint, the strength of stem cell therapy and gene therapy approaches lies in their combined application to achieve stable therapeutic benefits. The viability and persistence of the genetically modified stem cells and their derivative graft in the heart can be significantly enhanced by restoration of regional blood flow *via* biological bypass surgery which is achieved by neovascularization of the infarcted heart. The new emerging pro-angiogenic role of angiopoietin-1 independent of VEGF, in addition to its well recognized participation in the angiogenic cascade as a maturation factor, makes angiopoietin-1 as a growth factor of choice for ex-vivo stem cell based gene therapy which can be used independently or in combination with VEGF to support angiomyogenic recovery of the infarcted heart.

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Part 2

Pathogenesis of Myocarditis

10

Pattern-Recognition Receptors Sensing Viral Infection in Myocarditis and Inflammatory Heart Disease

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1. Introduction

Myocarditis is a potentially life-threatening heart disease affecting both children and adults which presents with a broad spectrum of clinical manifestations. Symptoms range from asymptomatic infection to a fulminant course that rapidly progresses to dilated cardiomyopathy (DCM) and heart failure. Viral infection of the heart is a major cause of acute myocarditis, leading to myocardial inflammation and tissue damage. Cellular infiltration in acute viral myocarditis may be caused by direct cytopathic effects of the virus, pathologic responses to persistent viral replication as well as autoimmunity triggered by the virus (Liu and Mason, 2001; Bowles and Vallejo, 2003; Linde et al., 2007; Rose et al., 2009; Rutschow et al., 2010). The cardiovirulence of the viral agent, together with the host´s genetic susceptibility and the variability in the innate and acquired immune system, appear to determine the extent of the inflammatory reaction, thereby predicting clinical outcome (Kindermann et al., 2008).

Recently, meaningful advances have been made in our understanding of cellular mechanisms that are aimed to suppress viral propagation in inflammatory heart disease. In the first line of defense against viral replication, the host´s innate immune system is activated by unspecific, albeit effective interactions of cellular receptors with distinct pathogen-derived ligands. A variety of pattern-recognition receptors have been shown to be implicated in the detection of invading microbial agents (Kawai and Akira, 2010; Takeuchi and Akira, 2010). Upon viral infection of the heart, different signal pathways initiate an inflammatory response and orchestrate the concerted anti-viral defense machinery in a complex manner. Toll-like and RIG-I-like receptors are essential for the recognition of pathogen-associated molecular patterns and their activation induces intracellular signalling pathways which lead to the production of pro-inflammatory cytokines, chemokines, and interferons (Bowles and Vallejo, 2003; Fairweather et al., 2005; Triantafilou et al., 2005; Frantz et al., 2007; Linde et al., 2007; Yajima and Knowlton, 2009; Kawai and Akira, 2010; Takeuchi and Akira, 2010; Yamamoto and Takeda, 2010; Zhu and Mohan, 2010). Vice verse, interferons appear to induce the expression of a subset of toll-like receptors (Khoo et al., 2011). Type I interferons execute anti-viral responses by modulating cell growth,

establishing an anti-viral state and influencing the activation of various immune cells. These potent anti-viral cytokines are best known for their role as innate immune mediators (Yajima and Knowlton, 2009). In the course of viral myocarditis, apoptotic signalling pathways are activated and modulate disease pathogenesis. However, cardiotropic viruses have evolved a battery of highly specific strategies to circumvent this innate protective response of the host and successfully replicate in cardiomyocytes (Versteeg and García-Sastre, 2010). Viral factors with or without homology to host proteins specifically target key components of the anti-viral defense machinery, some of which are transcription factors involved in establishing an anti-viral state.

In the present review we focus on the contribution of pattern-recognition receptors engaged in the early response cascade against cardiotropic viruses. In particular, we report on different signal transduction pathways emerging from membrane-associated and cytosolic receptors that inhibit viral dissemination and, furthermore, give a brief overview of the various adaptor molecules involved in these pathways. Not covered in this review are the diverse viral mechanisms for antagonizing the innate immune response and the precise cellular actions of cytokines in executing this anti-viral defense. Understanding the exact mechanisms by which viral components activate pattern-recognition receptors in the heart and modify gene expression profiles may help to improve novel therapeutic regimes for the treatment of viral myocarditis (Topkara et al., 2010).

2. Clinical course of myocarditis

Myocarditis is defined as a heterogeneous common disease pathway for myocardial inflammation resulting from a variety of infectious, immune and non-immune insults. The histopathologic hallmark of the disease is characterized by inflammatory infiltrates of the myocardium with concomitant necrosis or degeneration of adjacent cardiomyocytes in the absence of predominant ischemia. Despite this clear definition, the classification and diagnosis of myocarditis continue to present clinical problems. In recent years our understanding of the pathophysiology and natural clinical course of the disease has improved, probably because histological findings obtained from endomyocardial biopsies have routinely been combined with modern molecular biological and immunological tools in the detection of viral genomes persisting in the heart (Heidecker et al., 2011).

From epidemiological studies, it is known that viruses are the most frequent cause of myocarditis in Europe and North America although in rare cases bacteria, protozoa, and even fungi have also been implicated as infectious agents (Ellis and Di Salvo, 2007). Among the more common cardiotropic viruses are enteroviruses including Coxsackie virus, adenoviruses, parvovirus B19, hepatitis C virus, and human immunodeficiency virus (Kühl et al., 2005; Magnani and Dec, 2006; Mahrholdt et al., 2006; Noutsias et al., 2009; Rose, 2009; Pankuweit and Maisch, 2010). Other non-infectious etiologies of myocardial inflammation include hypersensitivity reactions against drugs, unexplained toxic reactions, and immunological syndromes including Churg-Strauss syndrome, giant cell myocarditis, systemic lupus erythematosus, sarcoidosis, Wegener´s granulomatosis, and others (Magnani and Dec, 2006; Ellis and Di Salvo, 2007; Rose, 2009). Although infections with cardiotropic viruses are believed to be the most common cause of acute lymphocytic myocarditis, a large number of patients have no identifiable source of the illness (Liu and Mason, 2001; Rose, 2009). Particularly, when the inflammatory process in the myocardium is moderate, the diagnosis frequently remains a challenge for clinicians. The diagnostic sensitivity for viral

myocarditis is comparably low, even when applying modern molecular biological techniques, probably because, due to sampling error, the diagnostic accuracy of endomyocardial biopsy remains a significant limitation (Magnani and Dec, 2006; Ellis and Di Salvo, 2007). The Dallas criteria, originally proposed in an effort to standardize the histopathologic diagnosis of myocarditis, have been shown to be of limited use for epidemiological investigations (Magnani and Dec, 2006; Ellis and Di Salvo, 2007). Thus, despite recent advances in the recognition of myocardial inflammation, the true incidence and prevalence of acute viral myocarditis is still unknown.

The spectrum of clinical manifestations of viral myocarditis is extensive, ranging from asymptomatic infection to fever, myalgias, palpitations, exertional dyspnea, and hemodynamic collapse. Fulminant myocarditis may lead to systolic failure, malignant ventricular arrhythmias and sudden cardiac death (Baughman, 2005; Magnani and Dec, 2006; Rose, 2009, Blauwet and Cooper, 2010). The cardiac symptoms may also be present in more chronic forms of DCM, therefore contributing much to the difficulties in establishing the correct diagnosis. The main reason for the clinical variability appears to lie in the characteristics of the viral agent, together with the host´s innate immune system and genetic susceptibility to infection. Given the diversity and insidious onset of clinical manifestations, endomyocardial biopsy in combination with other techniques such as immuno-histochemistry, serological analyses, PCR and *in situ* hydridization remains the gold standard for the detection of virus-induced myocardial inflammation (Magnani and Dec, 2006; Rose, 2009).

Although, in severely affected individuals, fulminant viral myocarditis can lead to rapid progressive heart failure, the disease usually resolves spontaneously without persisting ventricular dysfunction (Baughman, 2005; Magnani and Dec, 2006; Rose, 2009, Blauwet and Cooper, 2010). Continuing cardiovascular symptoms in the absence of ventricular compromise may indicate that chronic persistent myocarditis has developed, which is characterized by the maintenance of lymphocytic infiltrates combined with foci of myocyte necrosis. Whereas it has been well-established that chronic active myocarditis can induce heart failure, there is still a controversy with respect to the causal relationship of asymptomatic viral infections for the pathogenesis of idiopathic DCM (Yajima and Knowlton, 2009). Several studies have suggested an association between viral persistence in the myocardium and the development of DCM. However, the etiologic significance of viral genomes detected in endomyocardial biopsies from DCM patients is currently unknown (Bock et al., 2010).

3. Toll-like receptors in the inflamed heart

Despite a wealth of information regarding the symptomatology and clinical course of the disease, the complex pathophysiological mechanisms underlying inflammatory heart disease are only partially understood. Lessons learned from transgenic mouse models have shed some light on the essential role of endogenous receptors and transcriptional regulators engaged in early anti-viral response. From clinical and animal studies we know that the host´s innate immune system acts as the first line of defense against viral replication in a wide array of pathogenic viruses. The innate immune system, which senses pathogen invasion and primes antigen-specific adaptive immunity, has long been considered to be only non-specific and somewhat simpler than that of the adaptive system. However, recent findings on pattern-recognition receptors and their downstream signalling pathways have

led to a reconsideration of the role of innate immunity now seen as a highly potent defense apparatus against microbial pathogens which works in close cooperation with adaptive immunity.

Initial sensing of invading microorganisms by the innate immune system is mediated by pattern-recognition receptors (PRRs), which include toll-like receptors (TLRs), RIG-I-like-receptors, NOD-like receptors, and C-type lectin receptors (Akira et al., 2006; Bauer et al., 2009; Yajima and Knowlton, 2009; Kawai and Akira, 2010; Takeuchi and Akira, 2010). These four classes of germline-encoded PRR families are responsible for recognizing exogenous structures conserved among microbial species, which are called pathogen-associated molecular patterns (PAMPs). Currently, the paradigm of PRRs has changed, since it has been shown that PRRs also recognize endogenous molecules released from damaged cells, collectively termed damage-associated molecular patterns (DAMPs) (Piccinini and Midwood, 2010; Lamkanfi, 2011).

Among the PRRs, toll-like receptors and C-type lectin receptors are transmembrane glycoproteins, whereas retinoic acid-inducible gene (RIG)-I-like receptors and NOD-like receptors function as cytosolic PRRs (Figure 1). Toll-like receptors were first discovered in Drosophila as evolutionary ancient molecules that function as receptors for endogenous ligands such as proteolytically cleaved Spätzle protein, but later were found to be present also in the mammalian system where they respond to microbial components as well as endogenous ligands including heat shock proteins HSP60, HSP70, and gp96 (Medzhitov et al., 1997; Ohashi et al., 2000; Vabulas et al., 2002; Vabulas et al., 2002b; Kim et al., 2009; Arnot et al., 2010). Toll-like receptors are expressed on macrophages, dendritic cells, endothelial cells, and interestingly also on cardiac myocytes (Boyd et al., 2006). Structurally, they are characterized by a highly variable amino-terminal region containing a leucine-rich repeat (LRR) ectodomain, followed by a hydrophobic transmembrane region and a cytoplasmic toll/interleukin 1 receptor (TIR) homology domain, which mediates interaction between TLRs and downstream signalling molecules (Choe et al., 2005; Bell et al., 2006; Jin et al., 2007; Kang et al., 2009; Park et al., 2009). Ligand binding is mediated by the extracellular LRR ectodomain, which is composed of 19-25 tandem copies of the "xLxxLxLxx" motif (Jin and Lee, 2008). In humans, 10 members of the germline-encoded TLR family have been identified so far, TLR1-TLR9 being conserved in humans and mice. Due to a retroviral insertion, TLR10 is not functional in mice and the murine TLRs 11-13 are not present in humans (O'Neill, 2008; Kawai and Akira, 2010).

Ligand binding of PAMPs by TLRs occurs at the plasma membrane (TLR1, TLR2, TLR4-6) as well as in endolysosomal compartments and the endoplasmic reticulum (TLR3, TLR7-9) (Frantz et al., 2007. Kawai and Akira, 2010; Takeuchi and Akira, 2010; Yamamoto and Takeda, 2010). The difference in the downstream signalling cascades activated can be partly explained by the individual TLR molecule, which recognizes a specific subset of PAMPs and recruits different TIR domain-containing adaptors to the receptor. Ligands for TLRs include a broad range of various microbial components, such as bacterial lipoprotein moieties (TLR1-2, TLR6), lipopolysaccharide (TLR4), and flagellin protein (TLR5). Toll-like receptor 3, the first TLR family member to be implicated in the recognition of viral nucleic acids, binds to double-stranded RNA (dsRNA) molecules, which are produced as intermediates during the replication cycle of many viruses (Alexopoulou et al., 2001). TLR7 and TLR8 receptors recognize single-stranded RNA (ssRNA) and are expressed in a variety of immune cells, including dendritic cells, lymphocytes, monocytes, and NK cells (Bauer et al., 2008). Triantafilou and co-workers reported that inflammatory responses in human myocarditis

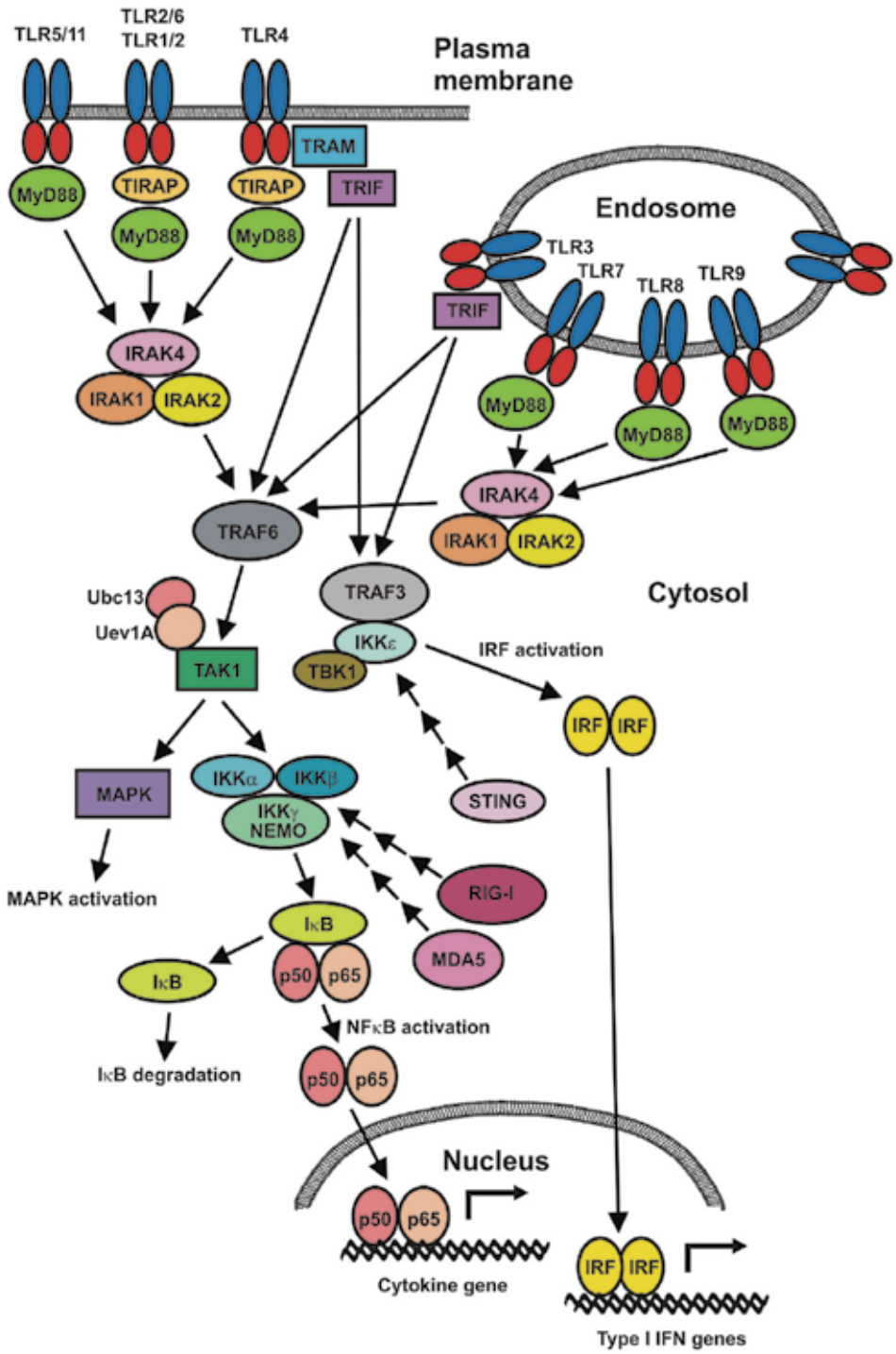

Fig. 1. Signalling pathways engaged in the detection of highly conserved, relatively invariant structural motifs of pathogens. Depicted are different pathways for the recognition

of microbial infection including toll-like receptor (TLR)-mediated MyD88-dependent and TRIF-dependent pathways as well as cytosolic sensors for foreign nucleic acid sequences (STING, RIG-I, and MDA5). For details on key receptors, their signalling adaptors and downstream mediators see text. **Abbreviations:** IKK; IκB kinase, IRAK; IL1-receptor-associated kinase, IRF; interferon-regulatory factor, MDA5; melanoma differentiation-associated gene 5, MyD88; myeloid differentiation primary response gene 88, NEMO; NF-κB essential modulator, NF-κB; nuclear factor-κB, RIG-I; retinoic acid-inducible gene-I, STING; stimulator of interferon genes, TAK1; transforming growth factor-β-activated kinase 1, TBK1; TANK-binding kinase 1, TIRAP; toll-interleukin 1 receptor (TIR) domain-containing adaptor protein, TLR; toll-like receptor, TRAF; tumour necrosis factor receptor-associated factor, TRIF; TIR-domain-containing adaptor protein inducing interferon-β (also known as TICAM), TRAM; TRIF-related adaptor molecule.

induced by Coxsackie virus B3 (CVB3) are mediated through TLR8 and to a lesser extent through TLR7 (Triantafilou et al., 2005). Elevated expression levels of TLR8 have been associated with heart failure and adverse clinical outcome in patients with enterovirus-associated dilated cardiomyopathy (Satoh et al., 2007). Toll-like receptor 9 functions as a sensor for unmethylated cytosine-phosphate-guanine (CpG) sequences in bacterial and viral DNA, which are rarely found in vertebrates (Barton et al., 2006; Guggemoos et al., 2008). Riad and colleagues demonstrated that, in the acute phase of CVB3-induced myocarditis, TLR9 knockout mice displayed improved LV function associated with reduced cardiac inflammation as compared to CVB3-infected wild-type mice. The cardioprotective effects due to TLR9 deficiency were associated with suppression of the TLR9 downstream pathway as indexed by reduced cardiac levels of the adapter protein MyD88 and the proinflammatory cytokine TNF-α (Riad et al., 2010).

In non-infected cells, TLR3 and TLR7-9 reside in the endoplasmic reticulum, whereas after uncoating and exposure to viral nucleic acids they traffic to the endosomal compartments, where they finally trigger a signal cascade resulting in the activation of the transcription factor NF-κB. The subcellular localization of the various TLR family members appears to be tightly regulated, probably because this avoids unbalanced activation to self-DNA in the absence of viral encounter. The diverse distributions of individual TLRs allow for the surveillance of different intracellular compartments, as viral entry usually occurs by receptor-mediated endocytosis and endosomal fusion or by direct fusion with the plasma membrane (Barton et al., 2006; Barton and Kagan, 2009).

Structural studies have revealed that the hydrophobic ligands of TLR1, TLR2, and TLR4 interact with internal protein pockets on the ectodomain, while hydrophilic dsRNA binds to the solvent-exposed surface of TLR3. Binding to cognate ligands induces homodimerization of the TLR ectodomains, whereas TLR2 forms heterodimers with TLR1 or TLR6 which interact with triacyl- and diacyl lipoproteins, respectively (Jin et al., 2007; Kang et al., 2009; Kawai and Akira, 2010). The membrane-adjacent carboxy-termini of the extracellular domains then converge and probably facilitate dimer formation of the cytoplasmic TIR domains to activate intracellular signalling.

Upon stimulation, the dimeric TLR molecules, except for TLR3, recruit a cytoplasmic adaptor called MyD88 (myeloid differentiation primary response gene 88), which is composed of a death domain (DD) in addition to a TIR domain (Muzio et al., 1997; Frantz et al., 2007; Kawai and Akira, 2010). CpG-DNA activates the TLR signaling pathway via MyD88 and TRAF6 (tumour necrosis factor receptor-associated factor 6), leading to

activation of kinases of the IκB kinase complex (Häcker et al., 2000). When infected with the spirochete *Borrelia burgdorferi*, mice deficient in MyD88 expression develop myocarditis and arthritis similar to the disease in wild-type mice (Liu et al., 2004). However, the pathogen burden was much higher in MyD88$^{-/-}$ mice than in wild-type mice, probably because degradation of the bacteria was critically impaired.

In response to stimulation with dsRNA, TLR3 recruits another adaptor molecule referred to as TRIF (TIR domain-containing adaptor protein inducing interferon-β, also known as TICAM), which associates with TRAF6 and RIP1 (receptor interacting protein 1) (Yamamoto et al., 2003). TRIF plays a critical role in MyD88-independent TLR3 signalling via TRAF6 and TANK-binding kinase (TBK)-1, leading to the activation of two distinct transcription factors, NF-κB and interferon-regulatory factor 3 (Sato et al., 2003). Hardarson and colleagues reported that TLR3 is an essential component of the innate stress response in encephalomyocarditis virus (EMCV)-induced cardiac injury (Hardarson et al., 2007). Mice lacking TLR3 expression were more susceptible to EMCV infection and had a significantly higher viral load in the heart, but lesser inflammatory changes of the myocardium as compared to control mice. TLR3-deficient mice had impaired proinflammatory cytokine and chemokine production in the heart, while expression of interferon-β was not impaired (Hardarson et al., 2007).

Satoh and colleagues reported that in 44 patients with myocarditis increased expression of TLR4 was associated with replication of enteroviral RNA and that these RNA levels were related to cardiac dysfunction (Satoh et al., 2003). In TLR4 signalling, the ligand-bound receptor utilizes MyD88 and TIRAP (toll-interleukin 1 receptor (TIR) domain-containing adaptor protein) for MyD88-dependent as well as TRIF and TRAM (TRIF-related adaptor molecule) for MyD88-independent pathways (Yamamoto et al., 2003b; Fitzgerald et al., 2003). The adaptor protein TRAM is required for activation of TRIF and recently a splice variant of TRAM called TAG (TRAM adaptor with GOLD domain) has been identified that acts as a negative regulator of TRIF-dependent signalling (Palsson-McDermott et al., 2009). TLR1, TLR2, TLR4, and TLR6 signalling requires, in addition, TIRAP which is important for bridging between the cytoplasmic TLR tail and MyD88 (Fitzgerald et al., 2001). Recently, it was shown that infection with Coxsackie virus group B serotype 3 (CVB3) resulted in cardiac remodelling, severe heart failure, and high mortality in TRIF-deficient mice, while wild-type mice showed only mild myocarditis and normal survival postinfection (Riad et al., 2011). Furthermore, virus control was markedly reduced in mice lacking TRIF expression and, interestingly, TRIF-deficient myocytes displayed a TLR4-dependent suppression of interferon-β. These findings suggest that TRIF confers cardioprotection against CVB3 infection.

The recruitment of these adaptors triggers a cascade of signalling events, which leads to the activation of the transcription factors NF-κB and interferon-regulatory factors (IRFs). These transcription factors ultimately induce the expression of various inflammatory cytokines, which execute important functions in anti-viral defence. The first step in the synthesis of cytokines leads to the activation of interleukin 1 receptor-associated kinase 4 (IRAK4), which functions as a serine/threonine kinase with an aminoterminal death domain (DD) (Suzuki et al., 2002; Suzuki et al., 2002b). Subsequently, IRAK1 and IRAK2 are phosphorylated by IRAK4 and, after dissociation of MyD88, a complex with TRAF3 and TRAF6 is formed (Kawagoe et al., 2007; Kawagoe et al., 2008; Lin et al., 2010). TRAF6 acts as an E3 ligase in conjugation with the E2 ubiquitin-conjugating enzymes Ubc13 and Uev1A and catalyzes the formation of a lysine63-linked polyubiquitin chain on target proteins, including TRAF6

itself, IRAK, and the NF-κB essential modulator (NEMO) (Deng et al., 2000). Transforming growth factor-β-activated kinase 1 (TAK1) is recruited and ubiquitinylated by TRAF6 (Wang et al., 2001). Subsequently, the IKK complex composed of IKKα, IKKβ and NEMO is formed which phosphorylates inhibitor of NF-κB (IκB) kinase-ß (IKKβ). The activated IKK complex then induces phosphorylation and subsequent degradation of IκB by the proteasome. Upon degradation of IκB, the freed NF-κB is no longer sequestered in the cytosol, but translocates into the nucleus, where it drives the expression of cytokine genes. Simultaneously, TAK1 activates the mitogen-activated protein kinase (MAPK) cascade leading to the activation of the transcription factor AP-1, which also targets gene expression of cytokine genes (Wang et al., 2001).

4. Cytosolic sensors for recognizing foreign nucleic acids

Members of the cytosolic RIG-I-like receptor (RLR) family act as cytosolic sensors for genomic RNA of dsRNA viruses and dsRNA intermediates that are generated during replication of ssRNA viruses (Yoneyama et al., 2004; Kato et al., 2005; Bowzard et al., 2009; Ranjan et al., 2009). Retinoic acid-induced protein I is composed of two amino-terminal caspase activation and recruitment domains (CARDs), a central DEAD box helicase/ATPase domain, and a carboxy-terminal regulatory domain (Yoneyama et al., 2004). The latter domain plays a critical role in the specific recognition of dsRNA and 5′-triphosphorylated ssRNA. Other members of the RIG-I family include MDA5 (melanoma differentiation-associated gene 5) and LGP2 (laboratory of genetics and physiology 2). RIG-I and MDA5 distinguish between different RNA viruses and contribute to the host´s anti-viral response through recognition of either 5′-triphosphorylated and uncapped ssRNA or dsRNA, species not found among endogenous self-RNA (Kato et al., 2006). Transgenic mice, deficient in RIG-I or MDA5 expression, are highly susceptible to infection with RNA viruses compared to control mice (Kato et al., 2006). The RIG-I homolog LGP2, which lacks the amino-terminal CARDs, potentiates viral RNA recognition by RIG-I and MDA5 through its ATPase domain and has been found to be essential for type I interferon production in response to picornaviridae infection (Satoh et al., 2010). Recently, it has been shown that also DNA-dependent RNA polymerase III is pivotal in sensing viral DNA in the cytoplasm (Ablasser et al., 2009; Chiu et al, 2009). AT-rich dsDNA can serve as a template for RNA polymerase III, which is transcribed enzymatically into dsRNA containing a 5'-triphosphorylated moiety. Activation of RIG-I by dsRNA ultimately induces production of type I interferon and activation of the transcription factor NF-κB.

Recently, it was reported that overexpression of STING (stimulator of interferon genes), a transmembrane protein found in the endoplasmic reticulum of numerous cells such as macrophages, dendritic, endothelial and epithelial cells, induces activation of NF-κB and IRF3 to stimulate type I interferon synthesis (Ishikawa et al., 2009; Barber, 2011). STING-knockout mice were susceptible to lethal infection after exposure to herpes simplex virus 1, suggesting that STING plays an important role in detecting foreign DNA.

Another cytosolic sensor for both bacterial and viral pathogens is AIM2 (absent in melanoma 2), which is essential for inflammasome activation in response to *Fransicella tularensis*, vaccinia virus, and mouse cytomegalovirus (Rathinam et al., 2010). AIM2 regulates caspase-1-dependent maturation of IL-1β and IL-18 and plays a role in natural killer cell-dependent production of interferon-γ, as has been shown for AIM2-deficient mice. However, the role of AIM2 in the pathogenesis of myocarditis has not been investigated so far.

PRRs	Ligand	Localization	Origin of the ligand	Selected references
TLR2	Lipoprotein	Plasma membrane	Bacteria, viruses	Following coronary artery ligation, *tlr*2$^{-/-}$ mice show reduced mortality and preserved left ventricular function as compared to wild-type mice (Shishido et al., 2003; Riad et al., 2008). Ischemia-reperfusion results in smaller infarct size (Favre et al., 2007). *Tlr*2$^{-/-}$ mice are protected from doxorubicin-induced cardiomyopathy (Nozaki et al., 2004).
TLR3	dsRNA	Endolysosome	Viruses	*Tlr*3$^{-/-}$ mice are more susceptible to EMCV infection and have a higher viral load, but lesser myocardial inflammation (Hardarson et al., 2007).
TLR4	Lipopoly-saccharide (LPS)	Plasma membrane	Bacteria, viruses	*Tlr*4$^{-/-}$ mice are protected from ischemia-reperfusion injury (Chong et al, 2004; Oyama et al., 2004; Kim et al., 2007) and LPS-induced mortality and cardiac dysfunction (Tavener et al., 2004; Nemoto et al, 2009). *Tlr*4$^{-/-}$ mice develop less-severe cardiac hypertrophy following pressure overload by aortic banding than wild-type mice (Ha et al., 2005).
TLR5	Flagellin	Plasma membrane	Bacteria	Exposure to the TLR5 ligand flagellin triggers cardiac innate immune responses that result in acute contractile dysfunction (Rolli et al., 2010).
TLR6	Diacyl lipoprotein	Plasma membrane	Bacteria, viruses	TLR6 Ser249Pro polymorphism has been associated with lower left ventricular thickness in hypertensive women (Sales et al., 2010).
TLR7 (TLR8)	ssRNA	Endolysosome	Bacteria, viruses	*Tlr*7$^{-/-}$ mice show markedly reduced myocardial cellular infiltration in experimental autoimmune myocarditis (Pagni et al., 2010). TLR8 expression is higher in DCM patients than in controls (Satoh et al., 2007).
TLR9	CpG-DNA	Endolysosome	Bacteria, viruses	Upon murine cytomegalovirus-induced myocarditis, *tlr*9$^{-/-}$ mice show higher severity of myocardial infiltration compared to wild-type (Pagni et al., 2010). Myocardial TLR9 expression is reduced in DCM patients (Ruppert et al., 2008).
RIG-I	Short dsRNA	Cytoplasm	Viruses	RIG-I mRNA is expressed at high levels in normal heart tissue (Ellis et al., 2002). Interferon-γ upregulates RIG-I in pericardial mesothelial cells, suggesting that RIG-I may be involved in the pathogenesis of pericarditis (Hatakeyama et al., 2007)

Table 1. Pattern recognition receptors (PRR) and their ligands engaged in myocarditis and inflammatory heart disease.

5. Concluding remarks

Beside direct effects of replicating viruses, unbalanced autoimmune responses associated with the production of auto-antibodies against cardiac tissue may also contribute to the progression of myocardial injury in inflammatory heart disease (Eriksson et al., 2003). The detection of auto-reactive antibodies directed against a number of different cardiac antigens is a prominent feature of all forms of persistent myocarditis and inflammatory cardiomyopathy, and there are reports demonstrating that the presence of anti-myosin autoantibodies is associated with deterioration of left ventricular function (Liu and Mason, 2001; Shishido et al., 2003; Dörner et al., 2005; Rose, 2009). In addition to the critical role of TLRs in mediating cardiac dysfunction in infectious conditions, emerging evidence suggests that the TLRs are also involved in modulating cardiomyocyte survival and ischemic myocardial injury (Chao, 2009; Riad et al., 2008). Despite significant progress in the identification of receptors triggering innate immune responses, further research is necessary to unravel the cooperative interactions between the innate and acquired immune system active in the protection of the heart against viral or autoimmune damage.

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11

Innate Lymphocyte Effectors (Natural Killer, Natural Killer T and γδ T Cells) in Infection and Myocarditis

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1. Introduction

Myocarditis is defined as an inflammation of myocardium where the infiltrating leukocytes are intimately associated with cardiomyocyte necrosis or drop-out (Liu, 2005; Woodruff, 1980). Cardiac damage may be minimal and self-limiting or may result in chronic fibrosis and cardiac dysfunction leading to death in children and young adults (Eckart et al., 2004; Fabre, 2006; Solberg et al., 2010). As discussed in other chapters of this book, infections with a highly diverse group of viruses, bacteria, fungi, and worms have been implicated in infectious myocarditis (Friman et al., 1995). Enteroviruses and adenoviruses are usually considered as the predominant viral etiological agents, and are associated with approximately 80% of clinical myocarditis where a viral infection is documented. However, virtually any virus infection may initiate myocarditis (Bowles et al., 2003; Woodruff, 1980). While seasonal influenza virus is only a minor etiological agent in myocarditis, evidence from the most recent influenza H1N1 pandemic (Vila de Muga et al., 2010; Wiegand et al., 2010; Zheng et al., 2010) suggests a higher incidence of both mortality and morbidity, and accounts for 5% of complications in infected children (Zheng et al., 2010).

Myocardial injury results either directly from replication and induction of death or dysfunction in infected cardiocytes, or from host responses to infection (Huber, 2010). Although anti-viral host responses (innate or adaptive) are intended to control and eliminate the infection, cytokines and by-products such as nitric oxide or oxygen free radicals may also damage adjacent uninfected cells (Szalay et al., 2006). Innate immunity is the initial host response to infection and usually occurs within hours or days of virus introduction. The major characteristic of the innate response, besides its rapidity, is that it is broadly reactive to multiple infectious agents. While it is highly unlikely that innate immunity can completely eliminate the infection, it can suppress microbial replication until the far more potent and highly specific adaptive immune response kicks in. The reason for this is quite simple, viruses replicate rapidly with, for example, one picornavirus infected cell in tissue culture producing up to a million progeny virions within 18-24 hrs. In vivo, such rapid and uncontrolled growth could result in extensive tissue injury or death of the organism prior to a useful adaptive immune response being established since during a primary immune response, production of meaningful numbers of virus-specific T cells could

take 7-10 days after virus inoculation. The best known innate immunity results from microbial products binding to and activating Toll-Like Receptors (TLR) or RNA helicases (RIG-I and MDA-5) which activate transcription factors (NFkB) leading to expression of cytokines (TNFα, IL-1β and IL-6) and nitric oxide (Hosoi et al., 2004; Michelsen et al., 2004); or interferon response factors (IRF3/7) leading to expression of type 1 interferons (IFNα/β) RANTES and IP-10 (O'Neill, 2004; Vogel et al., 2003). These roles for TLR are discussed elsewhere. This review will concentrate on lymphocytes belonging to the innate immune response and discuss their role in myocarditis. These lymphocytes include natural killer (NK), natural killer T (NKT) and γδ T cells.

2. Natural killer cells

Natural killer (NK) cells are capable of distinguishing between infected/transformed cells and uninfected/non-transformed cells and are able to kill the former using perforin or granzyme dependent mechanism (Topham & Hewitt, 2009). The best recognized mechanism for NK cell activation is through Type 1 interferons (IFNα/β). Type 1 interferons upregulate multiple interferon response factors (IRFs) and two of these IRFs are strongly implicated in NK cell proliferation and activation (Taniguchi et al., 2001). NK cell numbers are dramatically reduced in IRF2-/- mice (Lohoff et al., 2000). NK cell numbers are also reduced in IRF1-/- mice but in this case, the defect is not inherent in the NK cell progenitor since adoptive transfer of IRF1-/- bone marrow into wild-type mice results in NK cell proliferation (Ogasawara et al., 1998). Rather, IRF-1 appears to control IL-15 expression in bone marrow stromal cells and IL-15 promotes NK cell generation. Similarly, other cytokines including IL-2, IL-12 and IL-18 promote NK cell responses (Agaugue et al., 2008). In contrast to IRF1, IRF2 is inherently important in the NK cell progenitor since adoptive transfer of IRF2-/- bone marrow into wild-type recipients fails to generate NK cells. How NK cells recognize aberrant cells has received substantial study since these effectors are non-T cells, lack the T cell receptor and CD3, and do not undergo genetic recombination of recognition receptors (Biron et al., 1999; Orange et al., 2002). NK cells express substantial numbers of both activating and inhibiting receptors (reviewed in (Lanier, 2008)), and despite lacking classical T cell receptors, NK cells can recognize microbial molecules. Examples include NKp46 recognition of the influenza hemagglutinin protein (Mandelboim et al., 2001), Ly49H recognition of m157 (mCMV) (Arase et al., 2002), NKp44/NKp46 recognition of NDV hemagglutinin-neuraminidase (Jarahian et al., 2009), and Ly49P recognition of m04 (mCMV) (Kielczewska et al., 2009). NK activation receptors pair with ITAM-bearing DAP12, FcεRI-γ and CD3-ζ signaling molecules. Stimulation of the NK activating receptors leads to phosphorylation of the ITAM components and recruitment of Syk and ZAP-70. This results in actin cytoskeletal reorganization, which promotes secretion of preformed cytokines. The cytokines primarily produced by NK cells are IFNγ/TNFα, or perforin/granzyme. Activation also increases transcription of cytokine genes. In contrast, inhibitory NK receptors are either monomeric type 1 glycoproteins of the immunoglobulin superfamily [examples include: killer cell immunoglobulin-like receptors (KIRs) and leukocyte immunoglobulin-like receptors (LILRs)] or type II glycoproteins containing a C-type lectin—like scaffold [examples include: Ly49 and CD94-NKG2A]. Both types of receptor contain immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in their intracellular domains which, when activated, recruit tyrosine phosphatases that block the phosphorylation steps initiated by the NK activating receptors and thus inhibit NK cell functions (Lanier, 2008).

NK inhibitory receptors recognize major histocompatibility complex class I (MHC I) molecules which represents one mechanism by which NK cells distinguish between infected and normal cells, as many viruses attempt to evade the immune system through down-regulation of MHC molecules (Hewitt, 2003).

Evidence for a major role of NK cells in clinical myocarditis is rather weak. NK cells were not observed in heart tissue from 18 cases of biopsy proven myocarditis (Chow et al., 1989). Other studies have found either increased numbers of NK like cells in peripheral blood of dilated cardiomyopathy patients (Yokoyama, 1988) or diminution of NK cell activity in such patients (Maisch et al., 1985). Studies in patients with Chagas' disease find no alteration in NK cells early in the disease but an increase in these innate effectors occurs at later stages (Sathler-Avelar et al., 2003). One potential problem with clinical studies is that diagnosis of myocarditis or dilated cardiomyopathy is a relatively late event in the disease process and may be quite removed from the initiating acute infection (Woodruff, 1980). In fact, while viral genomic sequences can be detected in clinical heart biopsies for months and possibly for years, it is rare for infectious virus to be isolated from the hearts of myocarditis patients. Any role for NK cells may be over by the time human tissue is studied. The best evidence that NK cells might participate in viral myocarditis comes from mouse models. These studies indicate that NK cells are important in controlling coxsackievirus B infections in vivo (Gauntt et al., 1988; Gauntt et al., 1989; Vella & Festenstein, 1992) as depletion of these cells substantially increases virus titers in the heart or pancreas. The ability of NK cells to suppress virus infection may relate to their cytolytic activity to infected cardiocytes. Rapid elimination of infected cells before virus replication is complete would restrict the number of progeny virions produced and therefore limit the next cycle of infection. The second mechanism by which NK cells may help control virus infection is through either augmenting or accelerating the adaptive immune response to the virus. NK cells directly interact with both dendritic cells and activated T cells causing maturation of the dendritic cells and increased activation of the T cells (Zingoni et al., 2005). Interactions occur through up-regulation of OX40L on the NK cells and OX40 on activate CD4+ lymphocytes. Also, NK cells contain high concentration of pre-formed cytokines which can be rapidly released upon NK receptor engagement and these cytokines provide the environment necessary for optimal adaptive immunity development. As with the mouse model of CVB3 myocarditis, NK cells also control spread of Trypanosoma cruzi in the mouse model of Chagas' disease (Brener & Gazzinelli, 1997).

3. Natural killer T and γδ T cells

The other two major innate lymphocyte populations are natural killer T (NKT) and γδ T cells. NKT cells primarily recognize lipid antigens presented by CD1d molecules. The γδ T cells represent a more diverse population and in many cases, the antigen specificity of these cells is not known. However, as discussed below, the γδ T cells known to be involved in experimental viral myocarditis are also CD1d restricted. For this reason, description of the CD1 family of molecules is provided followed by discussion of the NKT and γδ T cells in innate immunity.

3.1 CD1 molecules and regulation of their expression

There are five distinct CD1 molecules, CD1a, CD1b, CD1c, CD1d and CD1e (Figure 1). Although these different molecules most likely arose from a single common ancestral gene

and are located as a cluster of genes on the same chromosome (De Libero & Mori, 2003), they share only approximately 30% homology and have distinct expression patterns and functional characteristics (Blumberg et al., 1995; Calabi et al., 1989; Kasmar et al., 2009). These proteins belong to a family of non-polymorphic, class I-like major histocompatibility complex (MHC) molecules (Boes et al., 2009). Humans express CD1a, CD1b, CD1c, CD1e (Group 1 CD1 molecules) and CD1d (Group 2 CD1 molecule). Mice express two isoforms of CD1d but lack any of the Group 1 CD1 molecules (Bradbury et al., 1990; Sugita et al., 1999). Other mammals express varying combinations of the different CD1 isoforms. For example, ruminants, such as cattle, express CD1a, three isoforms of CD1b and CD1e but lack either homologues of CD1c or CD1d (Van Rhijn et al., 2006). To date, all mammals have at least one CD1 molecule and a similar CD1-like molecule has been recently found in birds (Dvir et al., 2010). The wide distribution of CD1 expression among species underlines the importance of these molecules in immunity. A major difference between the non-classical CD1 molecules and the classical MHC I and MHC II molecules is that the latter molecules primarily present peptide antigens while CD1 molecules present amphipathic glycolipid (Kasmar et al., 2009; Kulkarni, 2010) and possibly hydrophobic peptide (Van Rhijn et al., 2009) antigens to T cells which provides a more comprehensive sampling of microbial products than the classical MHC molecules alone could provide. There are few CD1 genes (maximum of 12 but not all are present in all species) compared to the classical MHC molecules (>200), and CD1 proteins are highly conserved with few if any allelic variations. However, crystal structure analysis suggests that CD1 proteins have substantial flexibility and can conformationally change to present diverse microbial and self glycolipids (Zajonc et al., 2008).

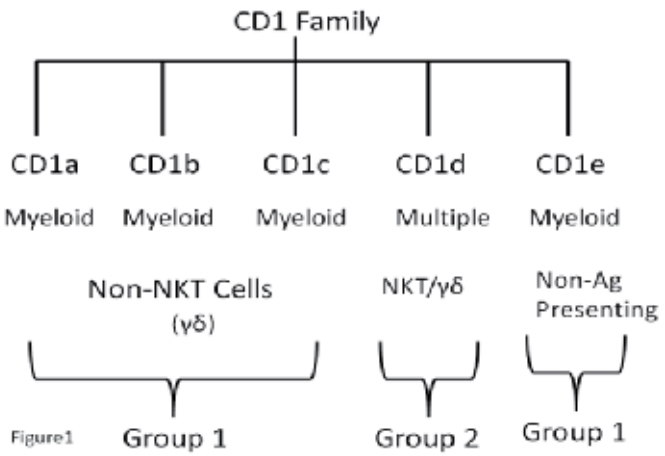

Fig. 1. CD1 family of non-classical MHC class I-like molecules.

There are five known members of the CD1 family divided into Group 1 and Group 2 molecules. All CD1 molecules present lipid antigens, unlike classical MHC molecules which primarily present peptide antigens. All CD1 genes derive from a common ancestral gene.
Unlike the other four members of the CD1 family, CD1e is only found as a soluble form in endosomes where it aids in trimming phosphatidylinositol for presentation by CD1b (de la Salle et al., 2005). CD1a, b, and c molecules are expressed on myeloid cells while CD1d is

expressed on these cells and additionally on non-hemopoietic cells including cardiac myocytes and endothelial cells (Blumberg et al., 1995; Exley et al., 2001; Huber et al., 2003) . CD1 molecules structurally resemble class I MHC molecules since they consist of a single polypeptide chain coded by the CD1 gene and are associated with β2 microglobulin. However, antigen presentation more closely resembles class II MHC molecules since antigen loading occurs in the endosome pathway and is TAP independent (Boes et al. , 2009; Brutkiewicz et al., 1995; Odyniec et al., 2004). The CD1 extracellular domain has a deep antigen binding groove containing two to four hydrophobic pockets into which the alkyl lipid tails of antigens are inserted leaving the glycosylated portion available for T cell recognition (Cheng et al., 2006; Zajonc et al., 2003; Zajonc et al., 2008). The cytoplasmic tails of CD1b, CD1c and CD1d contain a tyrosine motif which directs these molecules to the late endosome while the CD1a cytoplasmic tail lacks this motif and directs this molecule to the early endosome. The difference in trafficking of the CD1 molecules may reflect an evolutionary process since bacteria localize to different cellular organelles and expression of CD1 isoforms to distinct endosome compartments should promote maximal capture and presentation of microbial antigens to host immunity (De Libero & Mori, 2003). CD1b presents bacterial lipids including mycobacterial mycolic acids (Beckman et al., 1994), lipoarabinomannan (Sieling et al., 1995), glucose monomycolate (Moody, 2001), and self-glycosphingolipids such as GM1 ganglioside (Shamshiev et al., 2000). CD1a and CD1c present bacterial phospholipids (Beckman et al., 1996). CD1d presents a bacterial sphingolipid from *Sphingomonas* (Kinjo et al., 2005), alphaproteobacterium from *N. aromaticivorans* (Mattner et al., 2008), glycolipids from *B. burgdorferi* (Kinjo et al., 2006), and a self-sphingolipid isogloboside (Mattner et al., 2005). The sphingolipid α-galactosylceramide (αGalCer) isolated from marine sponges, is the classical CD1d ligand (Kawano et al., 1997), but CD1d has also been shown to present an α-galactosyl-diacylglycerol of *B. burgdorferi* (BbGL-II) (Kinjo et al., 2008; Kinjo et al., 2006). Evidence for CD1 presentation of viral antigens is sparse despite the fact that CD1-restricted T cells have been shown to respond in various viral infections including HIV, HSV, influenza and picornavirus (De Santo et al., 2008; Exley et al., 2001; Li & Xu, 2008; Yuan et al., 2006). Indeed, it would be highly unlikely that CD1 could directly present picornavirus molecules since these are non-enveloped viruses and should therefore lack any potential for glycolipid or lipopeptide antigens. Possible explanations for CD1-restricted immune responses to viruses exist. For example, infection may promote cellular lipidation of virus proteins (Van Rhijn et al., 2005) or infection may cause increased expression of endogenous glycolipid antigens (De Libero et al., 2005; Paget et al., 2007). Lysosomal α-galactosidase A is an enzyme which degrades endogenous lipid antigens (Darmoise et al., 2010). However, subsequent to many infections, α-galactosidase A activity can be severely curtailed leading to endogenous lipid accumulation. This means that CD1d dependent innate immunity may be directed to both exogenous and endogenous antigens during infections.

Endogenous glycosphingolipids binding to CD1 include GM1 ganglioside, sulfatide, galactosylceramide, and sphingomyelin (Darmoise et al., 2010; De Libero & Mori, 2003; Franchini et al., 2007; Hegde et al., 2010; Roy et al., 2008). The self-glycosphingolipids are not only important as self-antigens for T cell activation, but their presence may stabilize and promote CD1 expression on the cell surface (De Libero & Mori, 2003). Unlike microbial glycolipids which require processing in the endosomes, glycosphingolipids can directly bind to CD1 molecules expressed on the cell surface and can displace glycolipids already in these surface CD1 molecules (De Libero & Mori, 2003). Although endogenous

glycosphingolipids have been primarily viewed as the probable self antigen in CD1-dependent immunity, recent studies by Pei et al (Pei et al., 2010) demonstrated that cell lines incapable of glycosphingolipid biosynthesis were nonetheless capable of activating CD1-restricted cells. Thus, the types of self antigen capable of activating the CD1-dependent innate immune response are likely to be broader than originally thought.

Group 1 CD1 molecules are not expressed on monocytes in the blood and recent studies have shown that serum immunoglobulin and lipids suppress expression of these molecules (Leslie et al., 2008; Smed-Sorensen et al., 2008). However, once monocytes leave the circulation, Group 1 CD1 molecules can be induced by signaling through TLR2 (Roura-Mir et al., 2005), TLR2/TLR5 agonists, or cytokines (GM-CSF and IL-4) (Moody, 2006). CD1d is not up-regulated by GM-CSF and IL-4 (Exley et al., 2001; Sallusto & Lanzavecchia, 1994). CD1d is constitutively expressed in dendritic cells, monocytes and macrophage, but levels can be further increased subsequent to infection (Dougan et al. , 2007; Durante-Mangoni et al., 2004; Huber et al., 2003; Skold & Behar, 2003). Such up-regulation depends upon signaling through TLR and cytokines (IFNγ, IFNβ, TNFα) (Raghuraman et al., 2006; Skold et al., 2005). While microbial infections can up-regulate CD1 expression, they can also result in CD1 down-regulation (Donovan et al., 2007; Raftery et al., 2006). Viruses are well-known for their ability to evade immunity through multiple different mechanisms (Alcami & Koszinowski, 2000; Antoniou & Powis, 2008; Vossen et al., 2002). While most investigations of immune evasion by viruses center on the adaptive immune response, viruses also interfere with innate immunity. The HIV Nef protein binds to CD1d decreasing CD1d transport to the cell surface (Hage et al., 2005). Similarly, HSV, suppresses CD1 expression by interrupting the CD1 recycling pathway (Yuan et al., 2006). Karposi sarcoma-associated herpesvirus (KSHV) uses its modulator of immune recognition (MIR) proteins to ubiquitinize the cytoplastic tail of the CD1d molecule leading to its endocytosis (Sanchez et al., 2005). Activation of TLR7/8 blocks CD1 expression at the protein and mRNA levels (Assier et al., 2007). Finally, infection can change the endosomal processing of glycolipids which could restrict antigen availability to CD1 molecules.

Unlike Group 1 CD1 molecules, CD1d can be expressed on non-hemopoeitic cells (Huber et al., 2003; Monzon-Casanova et al. ; Sikder et al., 2009) CVB3 infection augments CD1d expression on macrophage, dendritic cells and T cells (Huber, 2006). The virus also causes de novo CD1d expression on non-hemopoietic cells (cardiac endothelial cells and myocytes), but only in non-hemopoietic cells actively replicating virus. Uninfected myocytes/endothelial cells immediately adjacent to infected cells remain CD1d negative (Huber et al., 2003). The requirement for active virus replication strongly suggests that TLR signal pathways such as TLR3 (recognizing single stranded RNA) or TLR7/8 (recognizing double stranded RNA) are necessary. However, virus replication alone is insufficient. Mice or cells infected with a non-pathogenic variant of CVB3, H310A1 (Knowlton et al., 1996), fail to up-regulate CD1d either on hemopoeitic or non-hemopoeitic cells (Huber et al., 2003; Huber & Sartini, 2005b). A major difference between the non-pathogenic and pathogenic (H3) variants of CVB3 is that the pathogenic virus is a potent inducer of TNFα. Further studies showed that TNFα and H310A1 infection up-regulated CD1d expression whereas either TNFα or H310A1 infection alone did not. In the mouse model of CVB3 induced myocarditis, CD1d is required for cardiac inflammation and injury. Mice lacking CD1d fail to develop myocarditis despite high levels of virus replication in the heart (Huber et al., 2003). Since CD1d is up-regulated on both hemopoeitic and non-hemopoeitic cells subsequent to CVB3 infection, a major question is where expression of this molecule is most

important in pathogenesis. CD1d-restricted effectors are cytolytic to CVB3 infected cardiocytes in vitro and expression of CD1d on infected cardiocytes in vivo may contribute directly to their death through cytolytic T lymphocyte activity. To address this question, bone marrow transplantation was performed between wild-type (CD1d+/+) and CD1d-/- mice where either the hemopoeitic cells were CD1d+ and the non-hemopoeitic cells (heart) was CD1d- or the opposite (Huber, 2006). These studies showed that CD1d expression on both hemopoeitic and non-hemopoeitic cells contributed to heart disease, although CD1d expression on hemopoeitic cells was of primary importance. There are no published studies showing the importance of CD1in clinical myocarditis. It is therefore not possible to evaluate the significance of CD1-dependent innate immunity in the human disease. However, based on the tight control of CD1 for pathogenesis in the experimental disease, the strong association between various microbial infections and clinical myocarditis, and the importance of CD1-restricted immunity in many different microbial infections; future investigation into a role for CD1 in this disease would be warranted.

3.2 Natural killer T cells and CD1-restricted γδ T cells

Many T cells respond to CD1 molecules (Barral & Brenner, 2007; Kaufmann, 1996) and express either T cell receptors (TCR) consisting of α/β or γ/δ polypeptide chains. Group 1 CD1-restricted αβ T cells are clonally diverse with fine antigen specificity, recognition of both self and foreign lipid antigens and either double negative (CD4-CD8-) or single positive (CD4+ or CD8+) (Barral & Brenner, 2007; Kaufmann, 1996; Vincent et al., 2005). The αβ T cell response is slow, similar to classical MHC αβ T cell responses indicating that these CD1-restricted effectors probably do not belong to the innate immune system. There are two major populations of γδ T cells in humans (Vδ1 and Vδ2) with Vδ2 cells primarily present in the circulation and Vδ1 cells primarily found in tissues and intestine (Das et al., 2004). Subsets of both γδ populations recognize antigens in context of non-classical MHC class I-like molecules including group 1 CD1(Rincon-Orozco et al., 2005; Russano et al., 2007). Activation of the group 1 CD1 restricted effectors requires IL-12, NKG2D activation on the effector and adhesion molecule interactions (LFA3/CD2, LFA1/ICAM1) in addition to TCR engagement. Since mice lack Group 1 CD1 molecules, this species does not have Group 1 CD1-restricted immunity. However, these effectors may function in humans.

T cells reacting to CD1d (Group 2 CD1) are also diverse. CD1d-restricted natural killer T (NKT) cells are designated as either invariant NKT (iNKT, also known as Type 1) or diverse NKT (also known as Type 2) cells (Barral & Brenner, 2007; Kronenberg, 2005; Ronchi & Falcone, 2008; Taniguchi et al., 2010). Type 1 iNKT cells have a TCR comprised of a single type of TCRα chain (Vα14Jα18 for mice and Vα24Jα18 for humans) and one of a limited number of distinct TCRβ chains resulting in limited clonal diversity. In contrast, Type 2 NKT cells use TCR comprised of diverse α and β chains. iNKT cells comprise between 2-40% of CD3+ cells in various tissues (Bendelac et al., 2007; Terabe & Berzofsky, 2008), have a constitutively activated phenotype, and rapidly secret large amounts of cytokines (IFN-γ, IL-4, IL-17, IL-5, and IL-13) upon activation due to the presence of pre-formed cytokine mRNA in the cells (Kronenberg, 2005; Michel, 2007; Olson et al., 2009; Stetson et al., 2003). Three mechanisms of iNKT cell activation have been described (Figure 2). Direct activation involves recognition of microbial antigens presented by CD1d on antigen presenting cells (TCR-mediated). In contrast, indirect activation either involves microbial stimulation of antigen presenting cells to release cytokines (IL-12 and Type 1 IFN) and presentation of

self/altered self lipid antigens on CD1d; or cytokines (IL-12 and IL-18) in the absence of CD1d antigen presentation (Brigl et al., 2003). Both inflammation and TLR activation can affect expression of enzymes involved in lipid metabolism (Khovidhunkit et al., 2004; Salio et al., 2007) and this may either increase total self lipid in endosomes or alter self lipids making them appear more foreign to the immune system. The mechanism of iNKT cell activation can impact the types of cytokines released with direct CD1d activation resulting in both Th1 (IFNγ) and Th2 (IL-4/IL-13) release while indirect activation causes predominantly Th1 (IFNγ) expression (Brigl et al., 2003). iNKT cells producing Th2 cytokines modulate NK cells to express TGFβ and TGFβ promotes T regulatory cell activation(Chen et al., 2009; Monteiro et al., 2010). Thus, depending upon the mode of iNKT cell activation, these effectors can be either pro- or anti-inflammatory. Type 2 NKT cells also can have a Th1 or Th2 phenotype with corresponding cytokine profiles, and therefore may have either potentiating or protective roles in infections and autoimmune diseases (Arrenberg et al., 2009). A number of reports indicate that Type 1 and Type 2 NKT cells are antagonistic to each other and form a regulatory network to control adaptive immunity. Most reports suggest an anti-inflammatory role for Type 2 NKT cells which can be protective in autoimmune diabetes in NOD mice (Duarte et al., 2004), experimental allergic encephalomyelitis (Jahng et al., 2004) and Con-A induced hepatitis (Halder et al., 2007). Furthermore, while type 1 NKT cells may increase tumor immunosurveillance, Type 2 NKT cells may suppress anti-tumor immunity (Ambrosino et al., 2007; Terabe & Berzofsky, 2008). Activation pathways for γδ T cells can also be diverse with both direct antigen presentation in MHC or MHC-like molecules or indirect with minimal or no antigen presentation (Kaufmann, 1996). Unlike NKT cells, γδ T cells may either react to CD1 itself in the absence of any antigen or to antigen without presentation by MHC/MHC-like molecule involvement. Although both human and mouse γδ T cells have been found to recognize antigens presented by Group 1 CD1 and other non-classical MHC antigens (Chien & Konigshofer, 2007; Cui et al., 2009; Spada et al., 2000; Van Kaer et al., 1991), only this laboratory has reported a subpopulations of γδ cells (Vγ4 TCR) recognizing CD1d (Huber et al., 2003).

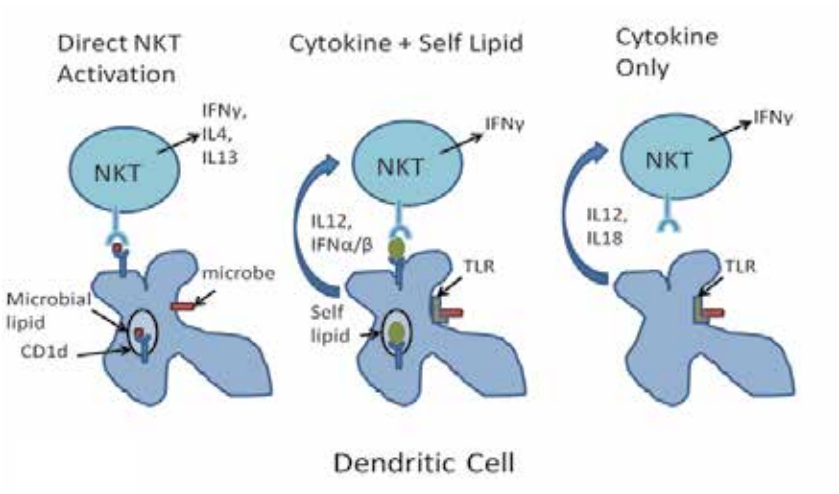

Fig. 2. Mechanisms for NKT cell activation.

There are three major mechanisms for activating NKT cells. Direct activation involves phagocytosis of microbes and binding of microbial lipids into the CD1d groove in the late endosome with transport of the CD1d-lipid complex to the antigen presenting cell surface. NKT cells activated through this pathway produce a broader range of Th1 and Th2 cytokines. A second pathway involves microbial stimulation of TLR on the antigen presenting cell which can both affect self-lipid expression/availability and stimulate cytokine expression from the antigen presenting cells. NKT cells stimulated by the recognition of self-lipid/CD1d and the cytokine milieu secrete primarily Th1 cytokines. The third mechanism is either not or substantial less dependent upon CD1d recognition by the NKT cells but NKT cell activation is primarily induced through cytokines alone.

3.3 NKT and γδ T Cells in myocarditis

Several cases of clinical cardiomyopathy have been reported where substantial numbers of γδ cells are in the inflammatory infiltrate (Eck et al., 1997; Takeda et al., 2008; Takeda et al., 2005). However, there is little direct evidence for a pathogenic role for these innate effectors in humans. As indicated above for NK cells, the lack of direct evidence for innate effectors in clinical myocarditis may simply reflect the fact that innate immunity should function early after infection and may disappear from the heart by the time that clinical symptoms are evident. In contrast to myocarditis in humans, substantial evidence implicates innate lymphocyte effectors in mouse models of coxsackievirus B3 (CVB3) and Borrelia burgdorferi (Lyme disease) myocarditis (Figure 3). As described above, mice lacking CD1d fail to develop myocarditis subsequent to CVB3-H3 (highly myocarditic variant of CVB3 (Knowlton et al., 1996)) infection despite high levels of virus replication in the heart (Huber et al., 2003). Infecting iNKT deficient mice with CVB3-H3 had no effect indicating that iNKT cells do not contribute to pathogenesis with this CVB3 variant. Surprisingly, CD1d deficient mice had significantly reduced numbers of activated γδ T cells belonging to the Vγ4 subset, and further analysis demonstrated that these Vγ4 cells are CD1d restricted as they killed CVB3-H3 infected CD1d+ but not infected CD1d- cardiac myocytes and cytotoxicity of the CD1d+ myocytes was blocked by anti-CD1d antibodies but not by antibodies to the classical MHC I and MHC II antigens (Huber, 2000; Huber et al., 2003). More importantly, activation of Vγ4 cells correlated to induction of CD4+IFNγ+ (Th1) virus-specific cells, which indicates that γδ cells might impact myocarditis through their effects on the antigen-specific, adaptive immune response (Huber & Sartini, 2005a; Huber et al., 2002). Previous studies had shown that heart-specific, autoimmune CD8+ cytolytic T lymphocytes are the primary immunopathogenic effector in CVB3 induced myocarditis (Guthrie et al., 1984; Henke et al., 1995; Huber & Lodge, 1984; Huber et al., 1988; Huber et al., 2002). These autoimmune CD8 cells kill uninfected cardiocytes through recognition of cardiac myosin epitopes (Huber & Gauntt, 2000) and can adoptively transfer myocarditis into uninfected recipients (Huber et al., 1987). However, the autoimmune CD8 T cell response is absolutely dependent on CD4+IFNγ+ cells (Huber et al., 2002). This is not surprising as many studies have shown that CD4+ Th1 cells promote CD8 T cell activation (Krawczyk et al., 2007; Serre et al., 2006). Although Vγ4 cells are required for generation of CD4+IFNγ+ cells, once the CD4+IFNγ+ cells exist, Vγ4+ cells are no longer necessary for autoimmune CD8 cell induction or myocarditis (Huber et al., 2002). The CVB3 model is not the only one showing that γδ T cells are required for immunopathogenic CD4 and CD8 T cell responses. Trypanosoma cruzi, the etiological agent in Chagas' disease, causes myocarditis with cardiac injury at least partially mediated by T cells and IFNγ (dos Santos et al., 2001; Marin-Neto et al., 2007; Ribeiro-Dos-

Santos et al., 2001; Soares et al., 2001). As with the CVB3 model, γδ cells are required for the pathogenic CD4 and CD8 responses in T. cruzi infections (Nomizo et al., 2006). However, unlike the CVB3 myocarditis model, the relevant γδ cell in T. cruzi infection expresses the Vγ1 T cell receptor. Why Vγ4 cells are operational in CVB3 disease while Vγ1 cells function in T. cruzi infection, is not currently known, but might reflect the difference between a virus and protozoa as the etiological agent. Nonetheless, the principle is the same: innate effectors control the activation of pathogenic antigen-specific adaptive immunity which subsequently causes cardiac damage.

The next question is how γδ cells control induction of adaptive immunity. Regulatory T cells (Tregs) are important negative immune modulators, constitute up to 10% of peripheral CD4+ T cells in naive mice and humans, and express CD25 (IL-2 receptor α chain) (Sakaguchi, 2005; Sakaguchi et al., 2008; Torgerson, 2006). There are several types of T regulatory cells which can basically be divided into natural (nTreg) and inducible (iTreg) populations. nTreg cells are generated in the thymus, and presumably arise from T cells with high affinity TCR for self antigens. nTreg cells are functionally mature when leaving the thymus and do not require antigen exposure peripherally to generate immunosuppressive activity. In contrast to nTreg cells, iTreg can be converted from effector T cell populations in the periphery subsequent to antigen challenge. The transcription factor, FoxP3, is usually associated with Treg cell development and transduction of exogenous FoxP3 into CD4+CD25- cells converts these cells into CD4+CD25+ Treg cells (Sakaguchi et al., 2008). Induction of FoxP3 expression and therefore, Treg cell activation depends upon the presence of TGFβ (Mantel & Schmidt-Weber, 2010). While FoxP3 is necessary for conversion of CD4+ cells to Treg cells, IL-2 is required for Treg cell maintenance/survival. Animals lacking either CD25 (IL-2R) or IL-2 develop lymphoproliferative and autoimmune diseases (Malek & Bayer, 2004) associated with a decrease in Treg cells. Three mechanisms have been proposed for Treg cell function (Sakaguchi et al., 2008). The first mechanism hypothesizes that Treg cells out-compete effector T cells for MHC-antigen complexes on antigen presenting cells which effectively blocks effector T cell activation. In the second mechanism, Treg cells directly interact with dendritic cells through CTLA4 which down-regulates required accessory molecule expression needed for successful antigen stimulation of effector T cells. Finally, Treg cell secretion of TGFβ or IL-10 may inhibit T cell differentiation (Ozdemir et al. , 2009; Ray et al., 2010).

CVB3 infection up-regulates CD1d on infected cardiac myocytes and myeloid cells. CD1d activates NKT and Vγ4 T cells which can either recognize CD1d on myocytes leading to myocyte death or interact with dendritic cells (DC) through CD1d to alter antigen presentation function of the dendritic cells or to produce cytokines. NKT cells may also activate NK cells. NK and NKT cells promote activation of Treg cells by promoting myeloid derived suppressor cell differentiation, while Vγ4 T cells have the opposite effect and either directly kill Treg cells through CD1d expressed on the Treg cell population or induce dendritic cell maturation and enhanced antigen presentation to adaptive immune (CD4, CD8) effectors. Treg cells inhibit activation of CD4+IFNγ+ (Th1) cells which are required for generation of cytolytic, autoimmune CD8 effector cells. The CD8 effector cells are the primary immunopathogenic mediator to cardiac injury in CVB3 induced myocarditis.

Treg cells prevent autoimmunity in myocarditis (Frisancho-Kiss et al., 2006; Huber et al., 2006; Wang, 2010). Distinct populations of innate effector T cells can have dramatically different effects on Treg cell responses. iNKT cells can promote Treg cell activation (Nowak et al., 2006; Roelofs-Haarhuis et al., 2004; Roelofs-Haarhuis et al., 2003). Under appropriate

stimulation (see Figure 2), iNKT cells produce IL-13, a Th2 cytokine, which can induce TGFβ production from CD11b+Gr-1+ myeloid derived suppressor cells. TGFβ promotes FoxP3 expression and Treg cell differentiation (Mantel & Schmidt-Weber, 2010). Indirectly, NKT cells activate NK cells (Carnaud et al., 1999) and subpopulations of NK cells suppress adaptive immunity either through their effects on dendritic cells or T cells (Flodstrom-Tullberg et al., 2009; Lunemann et al., 2009). CD1d and NKT cells are crucial to Treg cell development (Sonoda et al., 1999), and treating mice with αGalCer increases Treg cell activation (La Cava et al. , 2006) and suppresses autoimmune diabetes in NOD mice (Cardell, 2006; Ly et al., 2006). NKT cells secret high levels of TGFβ and IL-10 (Sonoda et al., 2001; Stein-Streilein et al., 2000) which alter dendritic cell (DC) cytokine (IL-10) and accessory molecule (CD40, CD80 and/or CD86) expression (Kumanogoh et al., 2001; McGuirk & Mills, 2002; Salomon et al., 2000) that favors T regulatory cell responses. (Bach et al., 2004; Chen et al., 2009). NKT cells are protective in Chagas' disease (Duthie & Kahn, 2006; Olson et al., 2009) and IFNγ expression by the NKT cells appears to be crucial to their protective effect. Similarly, treating CVB3 infected mice with αGalCer is protective, again indicating a role for NKT cells in preventing myocarditis (Wu et al., 2010). However, whether these NKT cells also suppress immunopathogenicity by enhancing Treg cell responses is not clear. γδ T cells appear to have the opposite effect on Treg cell responses. IL-23 activated γδ cells prevent conversion of effector T cells to iTreg cells (Petermann et al., 2010). Similarly γδ cells reduce IL-10 producing Treg cells in the lung in an asthma model γδ cells (Hahn et al., 2008). Vγ2Vδ2 cells prevent IL-2 induced expansion of CD4+CD25+FoxP3+ T (Gong et al., 2009). γδ T cells promote dendritic cell maturation and enhance antigen presentation. Also, these innate effectors suppress IL-2 expression which is needed for Treg cell maintenance. Recently, it has been shown that CVB3-H3 infection of mice deficient in γδ cells results in significant increases in Treg cells and accumulation of a population of CD1d+ Treg cells (Huber, 2009, 2010). These CD1d+ Treg cells are substantially more immunosuppressive to myocarditis than the CD1d- Treg cells on a per cell basis. These CD1d+ Treg cells are absent in mice containing γδ cells, and adoptive transfer of activated γδ cells into CVB3-H3 infected γδKO mice both restores myocarditis susceptibility and eliminates the CD1d+ Treg cell population. Direct co-culture of the activated γδ cells on CD1d+ and CD1d- Treg cell populations shows that the γδ effector cells are lytic to the CD1d+ Treg in a CD1d- and caspase-dependent manner. (Huber, 2010).

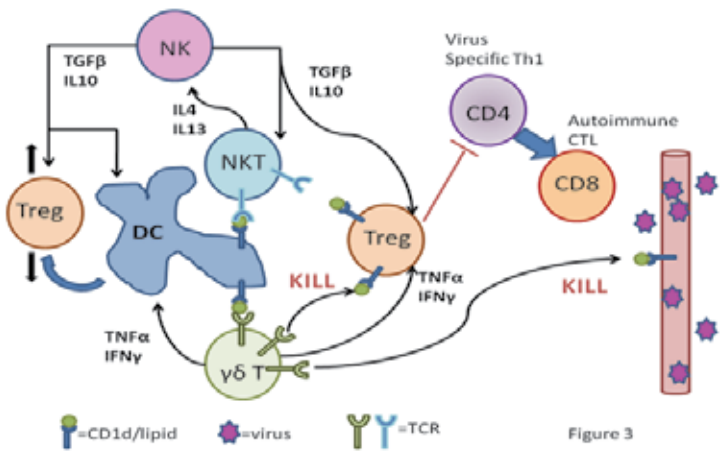

Fig. 3. Cross-talk between innate effectors in control of adaptive immunity.

4. Conclusions

Innate immunity is the rapid host response and occurs within hours of microbial infections. Although a major role for innate immunity is to help dampen microbe replication until the adaptive immune response is adequately developed for final microbial elimination, another major role for innate immunity is to control and direct the developing adaptive immune response. There is substantial cross-talk between innate lymphocyte effectors during myocarditis. Both NKT and a population of γδ cells recognize CD1d, a non-classical MHC class I-like molecule. However, evidence implies that while NKT cells are protective in myocarditis, γδ cells are pro-inflammatory and pathogenic. Interesting similarities have been found in the role of NKT and γδ cells in two different myocarditis mouse models: CVB3 and Trypanosoma cruzi induced myocarditis. The fact that similar immune processes of pathogenicity and protection appear to function in these two models provides circumstantial evidence that these innate effectors may have identical roles in other forms of myocarditis and also in clinical disease. To date, little evidence actually exists for innate effectors in clinical disease. However, the strong association between microbial infections and myocarditis in humans means that innate immunity should be important.

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12

The Key Players of Coxsackievirus-Induced Myocarditis

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1. Introduction

Myocarditis is a devastating cardiac disease causing death in children and young adults worldwide (Esfandiarei et al. 2008). The disease is clinically characterized by inflammation of the myocardium and degeneration of myocytes. Clinical symptoms of viral myocarditis range from flu-like and/or gastrointestinal illness to ventricular dysfunction ending commonly in heart failure. Acute disease is accompanied by multi-organ abnormalities and presents mostly in neonates and young children. Chronic disease occurs in one third of patients and is likely a consequence of autoimmune-mediated myocardial injury and viral persistence. As cardiac myocytes are destroyed by virus and/or self-induced cytopathic immune effects, excessive repair or fibrosis of myocardial tissue impairs disease progression rather than protects the tissue from further damage. Fibrosis or scar tissue, can lead to abnormal ventricular architecture that inevitably leads to the disruption of its function. At this stage of disease, chronic myocarditis progresses to dilated cardiomyopathy and can eventually lead to congestive heart failure (Esfandiarei et al. 2008).

The exact cause for myocarditis is still unknown though pathogen infections, hypersensitivity reactions, and systemic and autoimmune diseases are all likely contributing factors. It is suggested that acute viral myocarditis that progresses to chronic disease mirrors the clinical pathology observed with dilated cardiomyopathy patients and is likely the result of an inappropriate immune response after virus infection that leads to chronic inflammation and virus persistence (Escher et al. 2011).

Tracking the incidence of myocarditis is challenging. It is difficult to determine the potential disease burden to populations since there is a range in clinical symptoms associated with disease and endomyocardial biopsy to diagnose disease is rarely practised (Blauwet et al. 2010). Though mounting evidence of viral genomes recovered from chronic dilated cardiomyopathy patients provides some insight in to the potential burden of this devastating cardiac disease (Kuhl et al. 2005). Viral-induced myocarditis and dilated cardiomyopathy leads to a worse prognosis than other possible myocarditis/dilated cardiomyopathy etiological agents. Isolation of enteroviral RNA from endocardial biopsies of myocarditis and dilated cardiomyopathy patients renders these patients six times more susceptible to death after two years from diagnosis compared to virus-negative patients (Why et al. 1994). Sex is another contributing factor to disease susceptibility. Initially, it was

reported that men develop myocarditis twice as often as women and recent clinical work as supported this notion though the incidence is not quite as high as claimed in 1980 (Woodruff 1980; Mason et al. 1995; Kuhl et al. 2003; Cooper 2009).

Diagnosis and treatment of viral myocarditis is challenging due to the lack in specific clinical features and signature serological markers known for the acute phase of disease. The standard Dallas criteria that were typically used for diagnosis of myocarditis are not appropriate for determining viral or autoimmune myocarditis since they avoid the identification of inflammation or viral genome in the heart (Aretz 1987; Aretz et al. 1987). A more appropriate set of guidelines was established in 1995 by the WHO, with a classification of cardiomyopathies requiring endomyocardial biopsy, histological Dallas criteria, immunohistochemistry and viral PCR amongst the criteria for diagnosis (Richardson et al. 1996). Four stages of clinical disease in humans have been described since the implication of the 1995 WHO criteria: fulminant, subacute, chronic active and chronic persistent myocarditis. The first three stages involve mild to moderate dysfunction of the left ventricle, with the fourth, chronic persistent stage, characterized by normal ventricular function. Both chronic stages have ongoing inflammation with the development of scar tissue from myocardial damage. It is only during the chronic persistent stage that viral genome is detected from endomyocardial biopsy tissue (Lieberman et al. 1991; Olsen 1993). The incidence of viral infection, specifically coxsackievirus B infection, in human myocarditis, originates from seroepidemiologic and molecular studies between the 1950s and 1990s and from observations of viral genomes in cardiac tissue more prevalently in dilated cardiomyopathy patients compared to valvular or ischemic cardiomyopathies.

The true etiology and molecular pathogenesis responsible for viral myocarditis in humans remains unclear. Serological studies and endomyocardial biopsies from myocarditis patients have associated over 20 different viruses including coxsackieviruses, adenoviruses, cytomegaloviruses, parvoviruses, influenza viruses, and even human immunodeficiency viruses with the disease (Yajima et al. 2009; Blauwet et al. 2010). Of all the viruses implicated, it is the enteroviruses, specifically coxsackievirus B, which show the most likely contribution.

Coxsackievirus A and B are enteroviruses that are a part of the *Picornaviridae* family (Baboonian et al. 1997). They are human pathogens transmitted fecal-orally that cause enteric diseases (coxsackievirus A) as well as severe disease in the heart, pancreas, and central nervous system (coxsackievirus B) (Baboonian et al. 1997). Coxsackievirus A has 23 different serotypes, whereas coxsackievirus B has six. The six coxsackievirus B serotypes can instigate a variety of diseases including two very important autoimmune diseases: myocarditis and type I diabetes (Richer et al. 2009). It is also important to note that all coxsackievirus B serotypes are capable of triggering systemic disease in infants that can devastating lead to death (Esfandiarei et al. 2008). Relevant to myocarditis is the coxsackievirus B serotype 3 (coxsackievirus B3). Coxsackievirus B3 has been linked to approximately 30% of new dilated cardiomyopathy cases per anum though data establishing a direct link between coxsackievirus B3 pathogenesis and the onset of myocarditis in patients is lacking (Huber et al. 1998). The B3 serotype has a 7.4-kb single-stranded positive-sense RNA genome containing a VPg (3B) protein at the 5′ end. The 7-methyl guanosine-like cap influences replication and translation following virus entry (Flanegan et al. 1977). To gain entry in to a cell, coxsackieviruses interact with both coxsackievirus and adenovirus receptor (CAR) and decay accelerating factor (DAF) located both in the host cell membrane (Figure 1) (Pelletier et al. 1988).

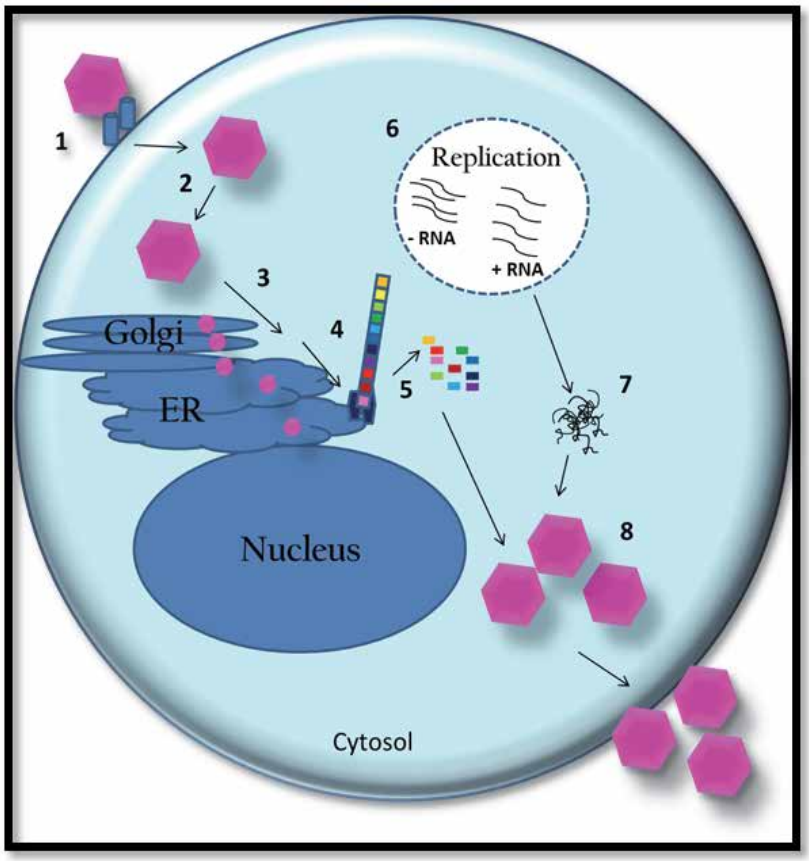

Fig. 1. Tentative coxsackievirus life cycle. The general host and virus components suggested to contribute to production of new coxksackievirus: 1) Viral entry through binding coxsackievirus and adenovirus receptor (CAR) and decay accelerating factor (DAF), 2) Internalization and transport of viral particles to the Golgi and endoplasmic reticulum (ER) 3) viral uncoating, 4) release of viral RNA, translation of RNA by ribosomes on the rough endoplasmic reticulum (ER) into viral polyprotein, 5) autocleavage of polyprotein into viral structural and functions proteins, 6) positive and negative strand RNA transcription to replicate viral genome, 7) release of viral genome in to the cytosol to encapsidate with structural proteins, 8) formation and release of viral progeny.

Once the virus enters the cytosol and uncoats, its positive-sense genome is released in the cytosol for translation and later, transcription. As a polyprotein comprised of the virus proteins VP4, VP3, VP2, VP, 2A, 2B, 2C, 3A, 3B, 3C, and 3D emerges from translation at the rough endoplasmic reticulum (ER), it is cleaved into its respective structural and functional proteins. The virally encoded $3D^{pol}$ is a RNA-dependent RNA polymerase that transcribes viral positive-sense RNA in to negative-sense RNA strands that serve as intermediates for the transcription of multiple positive-sense RNA strands needed for new progeny virions. After synthesis, the newly generated positive-RNA strands are packaged in new virus particles formed by the newly generated structural and functional virus proteins. The new progeny viruses are then released via plasma membrane by a mechanism likely mediated by viral

protein 2B (van Kuppeveld et al. 1997; Esfandiarei et al. 2008). Though many groups have identified key virus and host interactions throughout the coxsackievirus B life cycle, there still remains many stages unsolved. Nevertheless, the role of coxsackievirus B in the onset of myocarditis has been extensively studied thus far in animal models and cell culture systems. Here, we will discuss clinical and mouse studies that have investigated coxsackievirus-induced myocarditis and the role of key immune players in disease pathogenesis.

2. *In vivo* experimental systems

Mice provide a model system to distinguish characteristics of disease between autoimmune myocarditis and viral myocarditis. Mice are advantageous models because they share similar genetics to humans, they are cost-effective in handling and breeding, many transgenic strains are available and they are responsive to cardiotropic viruses (Cunningham 2001; Fairweather et al. 2001; Esfandiarei et al. 2008). Coxsackievirus B3 has been detected in 30-50% of dilated cardiomyopathy patients, providing support for a coxsackievirus B3-induced myocarditis mouse model (Escher et al. 2011). Following a single dose of coxsackievirus B3, acute myocarditis, encephalomeningitis, hepatitis, and even pancreatitis can ensue. Severe systemic pathogenicity observed with coxsackievirus B3 infection has also been related to the presence of sarcoma (Src) family kinase Lck ($p56^{lck}$) (Liu et al. 2000). With coxsackievirus B3 infection, mice tend to either develop chronic dilated cardiomyopathy mirroring clinical disease after recovering from viral infection or mice die from severe cytopathic effects, thus making coxsackievirus B3-induced myocarditis an excellent yet challenging model to study (Liu et al. 2000).

Studies with mice have demonstrated the significant contribution of the Th1 immune response to disease severity even though coxsackievirus B3 directly targets and destroys the myocardium. The contribution of a Th1 response to viral myocarditis pathogenesis is supported by studies modulating and inhibiting immune components and improving cardiac damage and function (Jiang et al. 2008).

Interestingly and in parallel with human myocarditis, male mice infected with coxsackievirus B3 experience acute myocarditis with greater severity compared to females. Greater disease severity in males has been linked to enhanced cardiac and splenic mast cell and macrophage TLR-4 expression at 12 hours post-infection (Frisancho-Kiss et al. 2006; Frisancho-Kiss et al. 2007). In males virally infected, signalling through cardiac TLR-4 enhances the production of cardiac IL-1β and IL-18 and promotes a Th1 skewed immune response (Fairweather et al. 2003). Also, male mice infected with coxsackievirus B3 harbour macrophages of a different phenotype and have more severe disease compared to infected female mice though viral replication is at similar levels in the heart (Frisancho-Kiss et al. 2006; Frisancho-Kiss et al. 2007). Moreover, genes relating to cholesterol metabolism in macrophages and to androgen receptor, which are known predictors for myocarditis, dilated cardiomyopathy and heart failure in male mice and humans, are upregulated considerably in spleens of male mice (Onyimba et al. 2011). This not only affords support for a gender difference in the susceptibility to viral-myocarditis, but critically implicates the innate response in disease severity. Furthermore, to bring male mice to the same immunological plane as female mice during myocarditis, Frisancho-Kiss et al removed the gonads from BALB/c mice and observed an increase in IL-4 production, the activation of macrophages and induction of regulatory T cells in the heart (Frisancho-Kiss et al. 2009).

Myocarditis can be induced in female mice with TNF-α treatment on days 1 and 3 post coxsackievirus B3 infection (Huber 2010). The lack of disease susceptibility in females is

attributed to low mRNA and protein levels of TNF-α and IL-Iβ as well as reduced CD1d expression on splenic lymphocytes. CD1d is an important non-classical major histocompatibility complex antigen that can be regulated by TNF-α to induce myocarditis susceptibility in female mice (Huber 2010).

Not only is susceptibility to developing myocarditis dictating by sex, but development of chronic disease depends on the mouse strain. A.BY/SnJ & SWR/J are susceptible mouse strains that can develop ongoing myocarditis, where viral RNA is detected within the myocardium. C57BL/6J & DBA/1J mice are resistant strains capable of eliminating virus just after the early acute phase of disease. Tomioka and colleagues investigated neutralizing antibodies and their role in virus-induced myocarditis and B-cell-mediated immunity using BALB/c mice (Esfandiarei et al. 2008). NK-deficient mice have been used to look at the role of natural killer (NK) cells in killing virus-infected cardiomyocytes (Godeny et al. 1986). Perforin knockout mice inoculated with coxsackievirus B3 have also been used to describe the interplay of virus infection and lymphocyte infiltration in the myocytes and their effect on disease outcome (Godeny et al. 1987). Interestingly, with coxsackievirus B3 infection in suckling, weaning and adolescent mice, coxsackievirus B3 replicates in the heart, pancreas, spleen, and brain and causes human disease-like symptoms. In fact, following IP injection, three distinct immunovirological phases of disease have been observed.

In mice, coxsackievirus B3 can induce two forms of inflammatory heart disease, acute only or acute and chronic (biphasic) autoimmune disease (Horwitz et al. 2000; Cunningham 2001; Fairweather et al. 2001). Interestingly, coxsackievirus B3 replication is mainly observed in the pancreas and to a lesser extent in the heart. In genetically susceptible mice, such as A/J2, Balb/c and NOD mice, chronic autoimmune myocarditis after coxsackievirus B3 infection is observed. Autoimmune myocarditis in mice presents as early as day 7 pi with inflammatory cell infiltration in the heart and the formation of multifocal inflammatory lesions. At this stage, autoantibodies (autoAbs) against heart antigens, like cardiac myosin (cardiac myosin), are seen. In NOD mice, isotype switching ensues after 2 to 3 weeks, with the autoAbs switching from IgM to IgG subclasses (Kaya et al. 2001; Kaya et al. 2002). It is particularly remarkable to note that the chronic autoimmune heart disease induced by coxsackievirus B3 in mice resembles inflammatory heart disease seen with myocarditis and dilated cardiomyopathy in humans. There are still many complications with the full characterization and study of chronic virus and/or autoimmune induced myocarditis in mice and humans. One such complication is the acute infection that precedes chronic disease.

3. Experimental autoimmune myocarditis

As described in the previous section, mouse models greatly aid the analysis of autoimmune diseases. To set apart the autoimmune phase of disease from acute infection an experimental induced autoimmune myocarditis model was developed (Blyszczuk et al. 2008). Experimental induced autoimmune myocarditis mimics the typical chronic phase of disease observed in genetically susceptible mice infected with coxsackievirus B and different stages of disease severity observed with experimental induced autoimmune myocarditis models are graded according to the extent of inflammatory infiltrates at the peak of inflammation. Autoimmune myocarditis can be induced by the injection of cardiac myosin with complete Freund's adjuvant and pertussis toxin. Mice injected with this combination of self- antigen and adjuvants are able to generate cardiac myosin-specific autoantibodies and present with

heart pathology similar to coxsackievirus B3-induced disease 3 weeks post-injection (Figure 2) (Kodama et al. 1992).

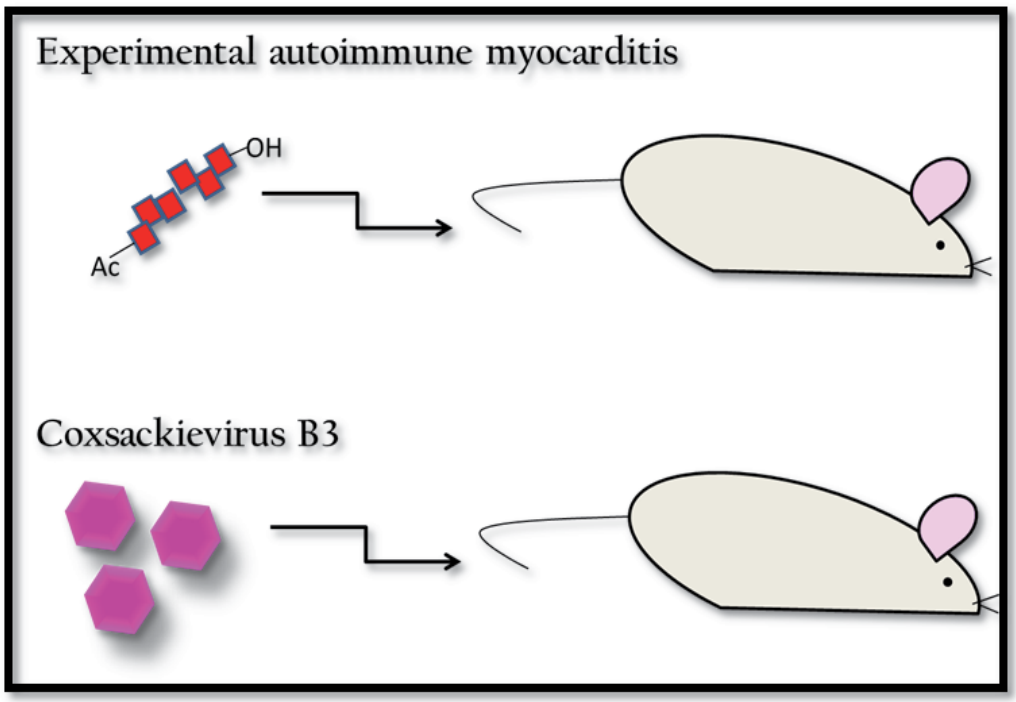

Fig. 2. Experimental autoimmune myocarditis versus coxsackievirus B3 induced myocarditis. Experimental autoimmune myocarditis involves the induction of autoimmunity by the injection of self-protein such as cardiac myosin or troponin I with adjuvants or without or injections of other cardiomyogenic peptides to induce chronic myocarditis as seen with coxsackievirus B3 infection in susceptible mice.

Neu et al suggest that autoimmune myocarditis is induced indirectly by viral infection and that one causative factor may be an autoimmune response to the cardiac myosin released or exposed after the virus-mediated myocyte damage (Neu et al. 1987). If this is the case, autoimmune disease should be induced by immunization with cardiac myosin alone and susceptibility to coxsackievirus B3-induced autoimmune myocarditis should be as likely as susceptibility to the myosin-induced autoimmune disease (Neu et al. 1987). Troponin I has also been used as an autoantigen for induced autoimmune myocarditis (Leuschner et al. 2009). Programmed cell death-1 receptor deficient mice develop cardiomyopathy with production of high-titered autoantibodies against cardiac troponin I (Okazaki et al. 2003). Cardiac troponin I induces a robust autoimmune response that encompasses both humoral and cellular responses that leads to severe inflammation and fibrosis in the myocardium of mice. Mice induced with cardiac troponin I have a genetic and sex biased susceptibility for myocardial inflammation compared to other autoimmune disease models (Leuschner et al. 2009). The key to reproducing ideal autoimmune disease conditions for studying myocarditis is to use a defined immunogen, ie cardiac myosin, troponin I etc. obtained from the same species as the study's model. Moreover, genetically defined inbred strains of susceptible and resistant mice allow

results to be reproducible and facilitate detailed analysis of the autoimmune disease cellular and molecular components (Neu et al. 1987).

Other species such as rats and Guinea pigs have also greatly contributed to our understanding of myocarditis. Immunization of different animal models with heart homogenates derived from various species has not been, unfortunately, successful at inducing myocarditis, where the results were often controversial (Neu et al. 1987). In most of those models, mild lesions appeared in the heart. However, out of the differing species models, 2 reports: the guinea pig model by Hosenpud et al and the murine model by Neu et al, showed enlargement of the heart with autoimmune myocarditis (Hosenpud et al. 1985; Neu et al. 1987). The authors also noted discoloration of cardiac surfaces, pericardial effusion and lethal clinical course that were conditions not previously observed in experimental induced autoimmune myocarditis. Kodama et al were also able to produce such pathological and clinical conditions using the Lewis rat model (Kodama et al. 1992) though the cardiac myosin immunization murine model seems to have had the greatest impact on our understanding of autoimmune myocarditis.

There are many ways in which autoimmune myocarditis can been induced in animal models. Immunization of susceptible mice with cardiac myosin or with a myocardiogenic peptide derived from the alpha cardiac heavy chain emulsified in complete Freund's adjuvant induces myocarditis in mice with a peak of inflammation in the heart around day 21. This inflammation observed with induced autoimmune disease is similar to that seen in the coxsackievirus B3 induced autoimmune myocarditis model during the chronic phase, however in this case, does not include the earlier complication of a pathogen infection. The immunization with cardiac myosin is associated with production of cardiac myosin-specific autoantibodies and cardiac myosin-specific T cells (Kodama et al. 1992; Godsel et al. 2001; Leuschner et al. 2009; Rose 2010). It has been demonstrated that the induction of disease by immunization with cardiac myosin can only be successful in genetically susceptible mice (Neu et al. 1987). We suggest that the complex genetics of the host therefore determines whether an infection will resolve or proceed to an adverse autoimmune outcome (Poffenberger et al. 2010). Identifying particular traits that favour susceptibility or resistance to an autoimmune incidence helps us understand how infectious diseases culminate in autoimmune disease. Other methods used to induce autoimmune myocarditis include administration of 2 early, critical proinflammatory cytokines, IL1b and TNF-α and injection of mice with myosin in combination with CFA and additional lipopolysaccharide. Some groups have reported the induction of autoimmune myocarditis using immunization with porcine cardiac myosin (Wang et al. 1999). Interestingly, Wittner et al. produced myosin autoantibodies and myocarditis in rabbits using immunization with bovine heart myosin (Wittner et al. 1983). Daniels et al reported the development of a recombinant model of experimental induced autoimmune myocarditis, induced by immunization with a 68kDa fragment of cardiac myosin referred to as Myo4 (Daniels et al. 2008). Myo4 induces severe autoimmune myocarditis in A/J mice 21 days post immunization. The immune response to Myo4 immunization is characterized by Th1 and Th17 features. Myo4-experimental induced autoimmune myocarditis has advantages over other models in terms of immunogen production and the ability to measure antigen-specific functional immunity in *ex vivo* assays. Myo4 and other immunoantigen experimental induced autoimmune myocarditis provide a means to investigate epitope spreading and the effect on disease pathology as well as prophylactic and therapeutic treatment studies aimed at developing therapeutics to alleviate acute and chronic autoimmune myocarditis (Daniels et al. 2008).

Experimental induced autoimmune myocarditis is mediated by a CD4+ T cell immune response. Homing of cardiac myosin-specific CD4+ T cells into the myocardium is the first pathologic event observed with experimental induced autoimmune myocarditis. Subsequently, neurohumoral factors such as cytokines and chemokines are released in the myocardium. This then recruits various bystander inflammatory cells to cross vascular endothelial cell walls and enter the myocardium. In effect, blocking the recruitment of inflammatory mediators to the myocardium may present as a critical target for myocarditis therapy (Tanaka et al. 2011). Experimental induced autoimmune myocarditis development is also typically associated with elevated titers of heart-specific autoantibodies, though in rats and susceptible mouse strains the response depends on the induction and expansion of heart specific, autoreactive CD4+ T cells. In BALB/c mice, CD4+ T cells belong to a specific subset of IL-17 producing helper cells, i.e. Th17 cells (Chang et al. 2008). With experimental induced autoimmune myocarditis, heart-infiltrating alpha-myosin-specific CD4+ Th17 cells are pathogenic and cause ongoing inflammation in the myocardium (Blyszczuk et al. 2008). Notably, autoimmune myocarditis induced by myosin can be reiterated in mice adoptively transferred with pathogenic CD4+ T lymphocytes (Sukumaran et al. 2011). In addition to T cells, passive administration of antimyosin monoclonal antibodies induces myocarditis in DBA/2 but not in BALB/c mice. DBA/2 mice are susceptible to passively induced disease due to the presence of myosin or a myosin like protein in their extracellular matrix. In addition to antibodies and T cells contributing to the pathogenesis of inflammatory myocardial lesions (Leuschner et al. 2009), injection of activated bone marrow (BM)-derived dendritic cells loaded with heart-specific self peptide can instigate disease pathogenesis (Kania et al. 2009).

Autoimmune myocarditis can be induced experimentally by several means as is the case for protection from induced disease. Amelioration of disease has been demonstrated with oral administration of specific antigens in several autoimmune models (Gonnella et al. 2009). Protection from experimental induced autoimmune myocarditis has also been observed with many studies from Godsel et al (Godsel et al. 2001). They successfully administered syngeneic splenocytes covalently coupled with ethylene carbodiimide (ECDI) by intravenous injections to prevent and treat a number of autoimmune diseases in animal models. Initial application of this approach in humans has been fortunately successful. Godsel et al essentially demonstrate in animal models that coupled-cell tolerance is an effective approach for the prevention of myocarditis and may present as a useful antigen-specific immunotherapy for treating myocarditis in humans (Godsel et al. 2001). Antigen-specific peripheral tolerance induction also represents a powerful tool for dissecting the mechanisms involved in cardiac autoimmunity. Although myosin-specific tolerization is commonly used to prevent experimental induced autoimmune myocarditis, it is also important to note that tissue homogenates are equally useful. This approach has yet to be tested in other models of myocarditis including coxsackievirus myocarditis (Godsel et al. 2001). Horwitz et al's investigation in to cardiac myosin tolerance and protection from experimental induced autoimmune myocarditis conflicted with results presented in the Godsel et al studies (Horwitz et al. 2005). Since cardiac myosin is a major autoantigen in virus-induced myocarditis the question was asked whether inhibition of this autoantibody response to cardiac myosin could prevent destructive autoimmunity. In NOD mice, cardiac myosin-specific antibodies develop following both coxsackievirus B3 infection and experimental induced autoimmune myocarditis induction. To ask whether the induction of peripheral tolerance to a single self-antigen could be used to prevent coxsackieviral-induced

autoimmune myocarditis, a disease likely directed at multiple self-antigens, NOD mice were tolerized to cardiac myosin using a covalently coupled antigen approach and subsequently challenged with coxsackievirus B3. Cardiac myosin tolerance to effectively prevent coxsackievirus B3-mediated autoimmune myocarditis was not observed, possibly reprimanding cardiac myosin as a single autoantigen player in viral-mediated disease (Horwitz et al. 2005).

Another possible player in viral-mediated disease as demonstrated with experimental induced autoimmune myocarditis may be multinucleated giant cells. Wang et al showed that the cardiac lesions in experimental induced autoimmune myocarditis are histologically similar to human myocarditis, with myocyte swelling, necrosis, and fibrosis that are accompanied by mononuclear cell infiltration consisting of granulocytes, macrophages, CD4+, CD8+ T cells, B cells and multinucleated giant cells (Wang et al. 1999; Blyszczuk et al. 2008; Daniels et al. 2008). Also, the histological features observed in myocarditis induced by cardiac myosin are very similar to the description of active giant-cell myocarditis in humans (Wang et al. 1999; Blyszczuk et al. 2008). Therefore, it has been inferred that macrophage-derived multinucleated giant cells, together with other inflammatory cells, are important mediators of myocyte destruction. The presence of a heterogeneous population of cellular components in the cardiac infiltrate also implies the existence of a complex, cytokine-rich microenvironment that may contribute to the pathogenesis of autoimmune myocarditis. TNF-α, IL-1β, IL-2, IFN-γ, IL-4 and IL-10 are cytokines that have been detected in culture supernatants of splenocytes derived from mice at the peak of myocardial disease. Their detected levels were higher in mice that developed myocarditis than mice immunized but not developing disease. Furthermore, it has been previously suggested that TNF-α contributes to myocarditis pathogenesis by either causing direct injury of cardiomyocytes or, together with IL1b, triggering the production of nitric oxide. The expression of E-selectin, VCAM-1 and ICAM-1 has also been found to be markedly increased in inflamed hearts during induced disease. The increased expression of these adhesion molecules on the vascular endothelium may contribute to the extravasation and accumulation of inflammatory cells observed in the myocardium (Wang et al. 1999; Blyszczuk et al. 2008).

There are limitations of using experimental induced autoimmune myocarditis as a model to separate the mechanisms of biphasic (acute infection and chronic) viral disease, where the model relies too heavily on the inoculation of a single antigen. There are undoubtedly other key immune players that contribute to the onset of autoimmune disease following virus infection with the innate immune system being one of the major contributors having a dual role: controlling and perpetuating disease. Despite the fact that the experimental induced autoimmune myocarditis models are rather artificial, they offer the advantage of studying disease pathogenesis and autoimmune mechanisms *in vivo* in the absence of an infective agent. Immunization with myosin peptide-loaded activated dendritic cells offers a useful tool to dissect the role of antigen-presenting cells (APCs) and effector cells while studying disease mechanisms. From the experimental induced autoimmune myocarditis model we can not only learn about autoimmune mechanisms contributing to disease development, but we can study the pathophysiology of inflammatory heart disease and design novel immunomodulating treatment strategies. In addition, the experimental induced autoimmune myocarditis model might offer a potential tool to improve the diagnostic accuracy of currently available and future imaging technologies (Blyszczuk et al. 2008).

Work from our lab using the experimental induced autoimmune myocarditis model has helped focus our attention to the role of innate immunity in response to an infectious agent

and as a driving force in the development of autoimmune disease. The innate immune system includes many key players like pathogen recognition receptors that recognize highly conserved pathogen-associated molecular patterns on microbial invaders. These receptors include the Toll-like receptors and expression of these receptors on antigen presenting cells such as macrophages and dendritic cells determine not only innate immunity but the subsequent adaptive immune response.

4. Key players in immunity

4.1 Pathogen recognition receptors

Cardiotropic viruses can be cytopathic, killing off host cells, yet their viral RNA is detected and tends to persist in cardiac muscle. Viral persistence in the myocardium can then lead to chronic inflammatory cardiomyopathy. Pathogens such as viruses are recognized by Toll-like receptors (TLRs) and other pattern recognition receptors of the innate immune response. The recognition of a pathogenic insult releases proinflammatory cytokines that serve as protectors from infection and perpetrators of chronic inflammatory disease (Lane et al. 1993; Kawai et al. 2006). Activation of Toll-like receptors by pathogen associated molecular patterns and the subsequent production of proinflammatory cytokines can lead to protection as well as the exacerbation of an autoimmune response.

Pathogen-associated molecular patterns from viruses like double-stranded RNA are sensed by Toll-like receptor 3 (TLR3) (Schnare et al. 2001; Pasare et al. 2003). Infections with coxsackievirus B in TLR3 knock out mice have demonstrated an important role for TLR3 in host defense. The innate antiviral response is mediated, at least in part, by nucleic acid-sensing receptors such as TLR3, retinoic acid inducible gene I (RIG-I), and melanoma differentiation-associated protein-5 (MDA-5). The activation of RIG-I/MDA5 receptor pathways is thought to evoke type I IFN responses. TLR3, which recognizes double stranded RNA, is critical in the antiviral immune response against coxsackievirus B3. TLR3-deficient mice are highly susceptible to coxsackievirus B3, where impaired antiviral responses and acute myocarditis ensue. Increased disease in TLR knock out mice is associated with decreased production of IL-12p40, IFNγ and IL-1β post infection. Mice deficient in the TLR3 adaptor protein, Trif, have a similar disease course as TLR3 knock out mice suggesting that the TLR3-Trif pathway is also important in the host response to coxsackievirus B3 infection. Research from Negishi et al reveals a critical cooperation between the RIG-I/MDA5-type I IFN and TLR3-type II IFN signaling axes for efficient innate antiviral immune responses (Negishi et al. 2008). Importantly, a rare TLR3 variant has been identified in patients diagnosed with enteroviral myocarditis (Gorbea et al. 2010). These patients also held a greatly increased incidence of a common polymorphism. Gorbea et al also demonstrated that induction of the TLR3 variant or the TLR3 possessing the common polymorphism with synthetic double stranded RNA hindered proper TLR3-mediated signaling. Also, with coxsackievirus B3 infected cell lines, mutated TLR3 impaired type I IFN signalling and production and failed to control viral replication (Gorbea et al. 2010). The Gorbea et al study thus suggests that individuals who possess these particular TLR3 variants may have an ineffective innate anti-enteroviral response that fails to clear the virus and in turn, elevates the associative risk for cardiac disease. Interestingly, human cardiac myosin pathogenic epitopes can directly stimulate other human Toll-like receptors such as Toll-like receptors 2 and 8 (TLR2, TLR8). Stimulation of these receptors allows for the production of proinflammatory cytokines from human monocytes. TLR8, found within

endosomes, detects single stranded RNA, such as the coxsackievirus B3 genome and instigates inflammation (Triantafilou et al. 2005; Zhang et al. 2009).

Signalling through another critical receptor, Toll-like receptor 4 (TLR4) also leads to the expression of proinflammatory cytokines, but has been implicated as a cardiomyopathy etiological factor. Satoh et al have suggested that myocardial expression of TLR4 is linked to coxsackievirus B3 replication in human cardiomyopathy and that TLR4 may be directly involved in the pathogenesis of disease (Satoh et al. 2004). Viral proteins have actually been found to co-localize with TLR4 in infected cardiac tissue. In coxsackievirus B infected mice, TLR4 deficiency reduces viral pathogenesis and the production of several cytokines including IL-1β and IL-18 (Pasare et al. 2003; Pasare et al. 2004).

Another major player in host defense is the critical adaptor protein for TLR signaling myeloid differentiation primary response gene (MyD88). MyD88 signaling has been associated with several aspects of the pathogenesis of chronic autoimmune myocarditis. MyD88 activates self-antigen presenting cells and promotes autoreactive CD4+ T-cell expansion in experimental induced autoimmune myocarditis. To determine the role of MyD88 in the progression of acute myocarditis to an end-stage heart failure, Blyszczuk et al used alpha-myosin heavy chain peptide (MyHC-alpha)-loaded activated dendritic cells (Blyszczuk et al. 2008). They induced myocarditis in wild-type and MyD88 knock out mice and observed comparable heart-infiltrating cell subsets and CD4+ T-cell responses. Injection of complete Freund's adjuvant or MyHC-alpha/complete Freund's adjuvant into diseased mice caused cardiac fibrosis, ventricular dilation, and disrupted heart function in wild-type but not MyD88 knock out mice (Pasare et al. 2003; Marty et al. 2006; Blyszczuk et al. 2008). The protection of MyD88 knock out mice from the induction of experimental induced autoimmune myocarditis is likely from the impairment of other key players of autoimmunity such as antigen presenting cells. The role of MyD88 in cardiac fibrosis has been demonstrated with chimeric mice, where the origin of fibroblasts that replace inflammatory infiltrates was determined to be from the bone marrow. MyD88 has thus been suggested to be critical for the development of cardiac fibrosis during progression to heart failure (Pasare et al. 2003; Marty et al. 2006). Fuse et al observed elevated MyD88 cardiac protein levels in the hearts of wild-type mice after exposure to coxsackievirus B3 and MyD88 knock out mice have a greater survival rate (86%) compared to wild type mice (35%) after coxsackievirus B3 exposure (Fuse et al. 2005). MyD88 is implicated not only in cardiac inflammation and mediating cytokine production, but is also associated with skewing the Th1/Th2 cytokine balance, increasing the expression of coxsackie-adenoviral receptor important for virus entry and viral titers after coxsackievirus B3 incidence. In the absence of MyD88, protection from virus infection and disease is observed and is suggested to be associated with IRF-3 and IFN-β activation (Fuse et al. 2005). From the above mentioned MyD88 work, it is fair to infer that MyD88 could be a useful target for preventative heart-specific autoimmunity and cardiomyopathy treatments (Marty et al. 2006). TLR signalling may be a major contributor to the initiation and progression of autoimmune myocarditis though there are many additional players such as the cells that express viral and self antigen sensors (antigen presenting cells) that remain poorly understood.

4.2 Antigen presenting cells (APCs)

Following viral infection, a cellular immune response is needed to completely clear the virus. However these same cells can drive chronic inflammation and autoimmune responses. Antigen presenting cells and other cell types critical in activating the cellular

response to viral infection such as CD4+ T cells, CD8+ T cells, γδ T cells, B cells, macrophages, mast cells, neutrophils, NK cells and DC cells are all detected in the hearts of mice post coxsackievirus B3 infection and with experimental induced autoimmune myocarditis induction (Afanasyeva et al. 2004; Cooper 2009; Kemball et al. 2010).

Antigen presenting cells play a pivotal role in the stimulation of acquired immunity and can be manipulated by cytokines and environmental factors. The manipulation of antigen presenting cells modulates the T cell response and results in changes in tolerance to specific antigens. Antigen presenting cells influence lymphocyte responses by 1) promoting helper T-cell 1 (Th1), Th2 or Th17 responses, 2) inducing peripheral tolerance, and 3) activating regulatory T cells (Chatenoud et al. 2005; Ait-Oufella et al. 2006; Blyszczuk et al. 2008). From autoimmunity studies it has been curiously determined that regulatory T cells and Th17 cells have opposing functions during autoimmunity (Langrish et al. 2005). Th17 cells are an important pro-inflammatory T cell lineage during heightened tissue inflammation and autoimmunity, whereas regulatory T cells cells function to suppress immune responses (Richer et al. 2008; Korn et al. 2009; Marchant et al. 2010; Wing et al. 2010; Zou et al. 2010). Antigen presenting cells trigger changes in regulatory T cells and other T cell populations, which alters disease outcome and may be a promising therapeutic avenue to further investigate in viral-induced autoimmune diseases such as viral myocarditis.

Damage to the myocardium and the onset of coxsackievirus B3-induced acute myocarditis in mice is attributable to many immune factors including activated antigen-specific T cell activity. Two signals are required for activating T cells: first through the T-cell receptor engaging with antigen loaded MHC on antigen presenting cells and next through costimulatory molecules on antigen presenting cells such as CD40 and B7. CD40L on T cells engages CD40 on antigen presenting cells activating them to secrete cytokines and express adhesion molecules. Signalling from both the T cell receptor and costimulatory molecules promotes the proliferation of the antigen-specific T cells and stimulates an anti-antigen immune response. Early work with CD40/CD40L revealed enhanced CD40 expression on cardiac myocytes of coxsackievirus B3-infected mice and reduced myocardial inflammation with anti-CD40L/B7-1 monoclonal antibody treatment (Seko et al. 1998). Increased expression of CD40 and the B7 family of costimulatory molecules has also been observed in myocardial tissue from patients with dilated cardiomyopathy and acute myocarditis (Seko et al. 1998). Recently, CD40-Ig treatment was used post-coxsackievirus B3 infection to block the interaction between CD40/CD40L in male Balb/c mice and notably, this treatment reduced inflammation and coxsackievirus B3 transcription. CD40-Ig treatment also skewed the Th1/Th2 response in favour of Th2 cytokines rather than Th1 (Bo et al. 2010). This work and studies with myocardial tissue from patients has important implications not only for the role of CD40, but also for potential therapeutic options that downregulate the inflammatory Th1 response in coxsackievirus B3-mediated acute myocarditis.

Dendritic cells are highly specialized antigen presenting cells that upon encountering a pathogen undergo maturation. This process involves antigen processing, upregulation of major histocompatibility class (MHC) class II molecules, induction of costimulatory activity and migration to lymph nodes, where they prime antigen-specific T cells. With their antigen processing capability, dendritic cells can trigger activation of autoreactive T cells (Eriksson et al. 2003). Dendritic cells may also be involved in both host defense and maintenance of peripheral tolerance. Dendritic cells may also play an important role in autoimmune myocarditis (Marty et al. 2006). Dendritic cells from infected susceptible mice produce lower levels of cytokines and chemokines, particularly IP-10, a chemokine

with cardioprotective properties. In the hearts of healthy wild type mice, tissue-resident dendritic cells take up and present endogenous heart-specific peptides (Eriksson et al. 2003). Activated and self-antigen loaded dendritic cells induce myocarditis and heart failure in genetically susceptible mice. The mechanism by which dendritic cells instigate damage to the myocardium is likely a combined effect from tissue damage and innate immunity activation that causes dendritic cells to activate autoreactive T cells and target the myocardium (Marty et al. 2006). This proposed mechanism was supported by Eriksson et al who have shown that injection of dendritic cells loaded with cardiac myosin peptide induces CD4+ T-cell-mediated autoimmune myocarditis (Eriksson et al. 2003). Interestingly, the dendritic cell-induced autoimmunity observed by Eriksson et al resulted only with TLR and CD40 stimulation. They demonstrated how TLR signalling following the onset and resolution of acute myocarditis instigates the reoccurrence of inflammatory infiltrates in the heart and the onset of autoimmunity. TLR signalling activation was also important for myocarditis induction in mice injected with damaged, immune stimulating cardiomyocytes (Eriksson et al. 2003). These few studies provide an insight into the possible role of dendritic cells in the induction of myocarditis and they offer an alternative, complete Freund's adjuvant-free method of inducing experimental induced autoimmune myocarditis (Afanasyeva et al. 2004).

The work done by Eriksson et al suggests targetting TLR signalling pathways may be needed as a therapeutic avenue to protect from heart-specific autoimmunity. In a scenario where microbial infections are acting concurrently with myocardial damage, such as with coxsackievirus B3 infection and myocarditis onset, self peptide–loaded dendritic cells might respond to the various pathogen associated molecular patterns in the environment that stimulate different TLRs and induce tolerance rather than act in antigenic mimicry. The end result may not be antigenic mimicry to instigate autoimmunity, but downregulation of autoreactive T cells and induction of tolerance (Eriksson et al. 2003; Blyszczuk et al. 2008). With this in mind, innate activation pathways such as TLR signaling, may be attractive targets for autoimmune myocarditis therapy.

Macrophages, another type of antigen presenting cell, play a critical role in the immune response to coxsackievirus B3 infection and have been implicated in the pathogenesis of coxsackievirus B3-induced autoimmune myocarditis. There are two groups of macrophages, type I or type II that are defined by their activation markers and cytokine production. Type II macrophages have been linked to the cardiac repair stage following acute myocarditis (Nahrendorf et al. 2007). The significance of macrophages in coxsackievirus B3 myocarditis was demonstrated in previous work by Richer et al and Horwitz et al, where a transgenic TGF-β mouse model demonstrated a protective role for TGF-β against autoimmune disease. This protection coincided with a reduction in macrophage maturation suggesting the important involvement of macrophage inflammatory properties (Horwitz et al. 2006; Richer et al. 2006). It has been suggested therefore, that a balance between the inflammatory macrophages that are necessary for defense against viruses and the macrophages necessary for the resolution of an immune response and tissue healing is critical for an appropriate antiviral immune response that avoids autoimmunity (Heath et al. 2004). Interestingly, macrophage phenotype can differ between male and female mice with coxsackievirus B3-induced myocarditis. Since coxsackievirus B3 infection induces severe myocarditis only in male mice it is possible that myocardial infiltrating macrophages detected in female mice will have a distinct functional phenotype that contributes to their protection from coxsackievirus B3-induced myocarditis. Li et al observed myocardial infiltrating

macrophages from coxsackievirus B3-infected male mice expressing high levels of classically activated macrophages (type I) markers, such as inducible nitric oxide synthase, IL-12, TNF-α, and CD16/32, whereas macrophages from females had increased expression of arginase 1, IL-10, macrophage mannose receptor and macrophage galactose type C-type lectin that are typically associated with alternatively activated macrophages (type II) (Li et al. 2009). Li et al also demonstrated a distinct myocardial-derived cytokine signature that is sex-biased and contributes to differential macrophage polarization after coxsackievirus B3 infection. With adoptive transfer experiments using *ex vivo* programmed M1 macrophages, Li et al observed significantly increased myocarditis in both male and female mice. However, the transfer of M2 macrophages into susceptible male mice protected mice from myocardial inflammation (Li et al. 2009). This protection was postulated to be the result of a modulated local cytokine profile that contributed to the promotion of peripheral regulatory T cells differentiation. This work has helped our understanding of a possible mechanism that underlies the gender bias in coxsackievirus B3 myocarditis susceptibility. Developing therapeutic strategies that manipulate macrophage polarization may be a promising avenue for the treatment of inflammatory heart diseases.

4.3 Cytokines and chemokines

Cytokines and chemokines also play critical roles in the detection of pathogens and the response by the innate immune system. Unfortunately, they are also actively involved in the pathogenesis and progression of viral myocarditis. Transgenic mouse models expressing cytokines have facilitated our understanding of interplay between cytokines at the sites of infection and the development of autoimmune disease.

During experimental induced autoimmune myocarditis, it has been thought that CD4+ Th cells differentiate into IL-2- and IFNγ-producing Th1 and IL-4-, IL-10- and IL-13-producing Th2 cell subsets and that the balance in T-helper cytokines can influence susceptibility and outcome of myocarditis (Horwitz et al. 2000). In recent research, it has been revealed that IL-1, IL-6, and IL-23 promote the differentiation of a distinct CD4+ T cell population that produces IL-17 and develops independently of Th1 and Th2 lineages (Blyszczuk et al. 2008). This new population denoted Th17, plays an important role for various models of immune-mediated tissue injury, including organ-specific autoimmunity diseases like myocarditis (Horwitz et al. 2000).

Work from our laboratory has established a critical link between IL-6 and disease severity. Work done by Poffenberger and colleagues has shown a significant increase in disease severity with the absence of IL-6 after coxsackievirus B3 infection in mice. An increase in inflammatory mediators associated with the progression of myocarditis such as TNF-α and MCP1 was observed in concordance with the increase in disease severity (Poffenberger et al. 2009). Without IL-6 to regulate the early immune response after infection, the early inflammatory response leads to increased chronic myocarditis severity as the disease progresses (Poffenberger et al. 2009).

An important factor affecting the immune response to the virus and viral clearance is the pro-inflammatory cytokine interferon-γ (IFNγ). Coxsackievirus B3 infection in mice deficient in IFNγ results in increased disease severity and increased viral replication in the heart (Eriksson et al. 2001; Fairweather et al. 2005). Expression of IFNγ in the pancreas can control viral replication as well as the virus-mediated damage in the heart and ensuing autoimmune disease (Horwitz et al. 2000). This cytokine also controls disease severity in an adjuvant induction disease model. In essence, IFNγ likely limits myocarditis pathology by

decreasing viral replication and virus-mediated damage. Resolution of inflammation and progressive remodeling are associated with high levels of another cytokine transforming growth factor-β (TGF-β) in the myocardium.

TGF-β is a pleiotropic, immunomodulating cytokine that greatly contributes to myocardial repair and remodelling (Khan et al. 2006; Rubtsov et al. 2007). Cardiac fibroblasts are the predominant source of secreted TGF-β within the heart. Secretion of TGF-β drives differentiation of cardiac fibroblasts into their more active myofibroblast form (Lijnen et al. 2002). It is with this active connective tissue cell form and stimulation by TGF-β that copious amounts of collagen can be secreted (Petrov et al. 2002). Fibrillar collagen is a leading contributor to extensive fibrosis. With disproportionate amounts of secreted collagen, ventricles tighten, restricting proper diastolic function (Kania et al. 2009). TGF-β contributes to the secretion of collagen via the TGF-β-Smad pathway that promotes collagen gene activation and translation (Khan et al. 2006). TGF-β also enhances the production of adhesion molecules that in turn, promote the longevity of myofibroblasts (Vaughan et al. 2000). Macrophages also secrete TGF-β in the heart (Riemann et al. 1994). They colocalize with myofibroblasts in fibrotic heart tissue and act as either initiating or supplemental sources of TGF-β1 (Hinglais et al. 1994; Kuwahara et al. 2004).

Coxsackievirus B3 first targets and replicates in the pancreas before reaching the heart. To inhibit coxsackievirus B3 spread to the heart and initiation of chronic disease, Horwitz et al developed a transgenic TGF-β mouse model where TGF-β is overexpressed in the pancreas. The expression of TGF-β in the pancreatic beta cells recruited macrophages into the pancreas, reduced viral replication, and inhibited the onset of coxsackievirus B3-induced autoimmune myocarditis. This study also demonstrated that the protective effect was strictly attributed to TGF-β and not IL-4, which has been linked to both autoimmunity suppression and antigen-presenting cell activation (Horwitz et al. 2006). Later on, Richer et al demonstrated that LPS from *Salmonella minnesota* and signalling through TLR-4 was capable of bypassing the protective effect provided by TGF-β in coxsackievirus B3-mediated autoimmune myocarditis (Richer et al. 2006). The authors also showed that neither antibody isotype switching, the extent of viral replication, nor the expression of CD40 was modulated with LPS induced TLR-4 signalling, rather the circumventing effect was due to failed APC expression of CD40 and inherent TLR-4 signalling effects such as the production of pro-inflammatory cytokines (Richer et al. 2006).

Though over-expression of TGF-β in the pancreas can protect from coxsackievirus B3-induced autoimmune myocarditis, there is still evidence that increased levels of TGF-β1 in the heart enhance, rather than protect from chronic disease. Elevated levels of TGF-β have also been tied to dilated, ischemic and hypertrophic cardiomyopathies (Khan et al. 2006). As mentioned previously, TGF-β can enhance collagen secretion and thus promote extensive tissue fibrosis. A recent study examined the effect of astragaloside IV, a Chinese medical herb that has anti-myocardial injury and immunoregulatory properties, to inhibit myocardial fibrosis in Balb/c mice inoculated with coxsackievirus B3 (Chen et al. 2011). Interestingly, astragaloside IV exhibited a protective effect alike TGF-β against myocardial fibrosis and significantly ameliorated survival in coxsackievirus B3-infected mice that developed dilated cardiomyopathy. The authors also suggest that the protective role exerted by astragaloside IV is likely due to its ability to interfere with TGF-β-Smad signalling through the direct downregulation of Smad2/3 and Smad 4 (Chen et al. 2011).

Lipopolysaccharide injection at the time of coxsackievirus B3 infection helps overcome genetic resistance in susceptible mice. This investigation also identified interleukin (IL)-1 as

the mediator responsible for causing the change in disease course. Injection of IL-1 alone overcomes the genetic resistance to induced myocarditis similarly to lipopolysaccharide treatment. The change in disease susceptibility is likely due to increased IL-1 production in the heart. Production of IL-1 begins during the acute stage of disease but persists into the chronic phase of disease. Interestingly, the levels of IL-1 in the heart correlate with the degree of fibrotic lesions during disease. Eriksson et al demonstrated that injection of an IL-1 receptor agonist prior to infection sufficiently decreases viral titres in the heart and reduced chances of mortality. They also showed that IL-1 receptor stimulation is required for efficient dendritic cell activation, the subsequent induction of autoreactive CD4+ T cells, and resulting autoimmune disease (Eriksson et al. 2003).

Another important immunomodulating cytokine that contributes to viral-induced myocarditis and controls macrophage activation is IL-10. IL-10 is produced in the myocardium during both the acute and chronic stages of virus-induced myocarditis. Chronic coxsackievirus B3-induced myocarditis features viral RNA persistence and chronic inflammation that is primarily mediated by macrophages and T cells therefore, cytokines like IL-10 that control these critical immune cells are important to investigate. IL-10 gene-deficient mice have been used to confirm the regulatory role of IL-10 in the outcome of coxsackievirus B3 myocarditis. Mice deficient in IL-10 have uncontrolled nitric oxide synthase production, which likely contributes to their ongoing myocardial injury (Szalay et al. 2006). IL-10 in experimental induced autoimmune myocarditis mice hearts is mainly detected in non-cardiomyocytic non-inflammatory cells (ie. fibroblasts, smooth muscle cells, and endothelial cells) and IL-10-targeting cells. The IL-10-targetting cells, which express both IL-10 receptors 1 and 2, are mainly T cells expressing αβT cell antigen receptors (αβT cells) and CD11b+ cells such as macrophages, dendritic cells, and granulocytes. Several studies have demonstrated a therapeutic effect for IL-10 in autoimmune and inflammatory diseases. In myocarditis models, IL-10 has been shown to inhibit the secretion of proinflammatory cytokines such as TNF-α, IFN-γ, iNOS, IL-2 and IL-12 and has displayed major effects on immune cells (Horwitz et al. 2000). These few studies suggest that the role of IL-10 in disease development could be predominantly protective.

IL-12, another key cytokine, is comprised of p40 and p35 subunits. IL-12 signals through a heterodimeric receptor composed of two units, IL-12Rβ1 and IL-12Rβ2. p40 and IL-12Rβ1 deficient mice show less or no myocardial disease with adjuvant-induction and treatment of wild type mice with IL-12, exacerbates disease suggesting IL-12 as a driving force in disease development similar to the actions of IL-1β and TNFα (Eriksson et al. 2001; Afanasyeva et al. 2004). However, Fairweather et al have found that IL-12 deficiency does not prevent myocarditis, rather viral replication significantly increases, causing more myocardial tissue damage. A decrease in inflammatory infiltrates was also observed and corresponded to with reduced TNF-α and IFN-γ levels in the heart. IL-12 and IFNg positively regulate each other and type I inflammatory responses. Type I inflammatory responses are believed to be responsible for tissue damage in autoimmune diseases. Eriksson et al further investigated the role of the IL-12/IFN-γ (Th1) axis in the development of autoimmune myocarditis. They observed resistance to disease in IL-12p40-deficient mice that were bred on a susceptible background. In the absence of IL-12, they suggested that autospecific CD4+ T cells proliferated poorly and exerted Th2 cytokine responses. IFN-γ-deficient mice developed fatal autoimmune disease. Interestingly, blocking IL-4R signalling did not confer susceptibility to myocarditis in IL-12p40-deficient mice. This suggests that IL-12 triggers autoimmunity in a manner independent of the cytokines IFN-γ and IL-4 (Eriksson et al. 2001).

IL-13 is another cytokine that can protect mice from both viral and adjuvant induced myocarditis. Cihakova et al demonstrated that IL-13 knock out BALB/c mice develop severe autoimmune myocarditis and their pathology is characterized by increased cardiac inflammation, increased total intracardiac CD45+ leukocytes, elevated anti-cardiac myosin autoantibodies, and increased cardiac fibrosis, with impaired cardiac function and heart failure. Hearts of IL-13 knock out mice showed elevated levels of the proinflammatory and profibrotic cytokines including IL-1β, IL-18, IFN-γ, TGF-β, and IL-4. CD4+ T cells were also highly increased in IL-13 knock out hearts. Splenic T cells from the knock out mice were greatly activated and with myosin stimulation, immensely proliferated. Regulatory T-cells harvested from spleens were also affected in knock out mice, where they showed a decrease in numbers compared to wild type mice. IL-13 knock out also reduced alternatively activated CD206(+) and CD204(+) macrophages and hightened levels of classically activated macrophages. Caspase-1 activation was increased, which then likely increased production of both IL-1β and IL-18. This study exemplified IL-13 as another important immunomodulating cytokine that protects against myocarditis by manipulating T cell and macrophage populations (Cihakova et al. 2008).

Examining T cells subsets is another avenue investigated to determine pathogenic or protective factors in myocarditis development. Mice deficient in T-bet, a T-box transcription factor required for Th1 cell differentiation and IFN-γ production, develop severe autoimmune heart disease. T-bet can also regulate autoimmunity by controlling nonspecific CD8+ T cell bystander functions in the inflamed target organ such as the heart. CD4+ Th1 cells producing INFγ are protective in experimental induced autoimmune myocarditis. This protection is likely attributed to regulation of IL-17 production by Th17 cells. Th17 cells produce IL-17, a proinflammatory cytokine that activates T cells and other immune cells to produce a variety of cytokines, chemokines and cell adhesion molecules. Rangachari et al have shown that Th17 cells are involved in acute viral myocarditis and enhance humoral responses (Rangachari et al. 2006). The relationship between Th17 cells and coxsackievirus B3 replication still remained unclear so they infected BALB/c mice with the virus and observed increased viral replication, expression of splenic Th17 cells, serum IL-17, and cardiac IL-17 mRNA that were all accompanied by progressive cardiac injury. Interestingly, Th1 and CD8+ T cell expression was elevated and the neutralization of IL-17 further upregulated splenic Th1 and CD8+ T cell numbers and levels of cardiac IFN-γ mRNA. Cardiac pathology was improved after IL-17 neutralization and correlated with reduced viral replication and decreases in cardiac inflammatory cytokines IL-17, TNF-α, and IL-1β. This study implicates Th17 cells in contributing coxsackeivirus B3 replication in viral myocarditis, and implicates IL-17 as a target for regulating antiviral immune responses (Yuan et al. 2010). Th17 cells are activated by IL-23, a cytokine likely produced by activated macrophages and dendritic cells, through receptor interactions made of IL-12Rβ1 and IL-23 receptor (Langrish et al. 2005). IL-23 is a heterodimeric cytokine like IL-12 that is composed of a p19 and p40 subunit similar to IL-12. IL-23–mediated immune responses have a different gene expression pattern than IL-12–driven T cell responses and IL-23 does not promote the development of IFN-γ-producing Th1 cells as does IL-12. IL-23 is however, one of the many contributors to pathogenic CD4+ T cell expansion. With its anti-IFN-γ and Th1 cell activity, IL-23 helps establish and maintain organ-specific inflammatory autoimmune diseases such as myocarditis (Langrish et al. 2005). Understanding the molecular basis for the differential gene expression pattern observed with IL-23–dependent T cell populations and investigating IL-23's cellular mechanism of action in autoimmunity could provide additional therapeutic targets for the treatment of inflammatory autoimmune diseases.

Experimental and preliminary human studies have demonstrated that TNF-α plays a crucial role in viral-induced myocarditis. Calabrese et al investigated the expression of TNF-α and both its receptors (TNFRI and TNFRII) in both viral and non viral myocarditis. Expression of TNF-α was significantly enhanced in viral myocarditis compared to non viral myocarditis. Importantly, cardiac myocytes express TNFα receptors TNFR1 (TNFRp55) and TNFR2 (TNFRp75), implicating the possible importance of enhance TNF-α in the myocardium. Histological analysis revealed that myocardial necrosis and cellular infiltration are more prominent in TNF-α-positive cases further supporting the notion that the expression of TNF-α significantly contributes to the pathogenesis of viral myocarditis and including the severity of cardiac dysfunction (Calabrese et al. 2004).

Chemokines act as chemotactic mediators in leukocyte trafficking to sites of infection (Groom et al. 2011). In addition to their chemo-attractant activity, chemokines can influence disease severity by modifying immune response strength and polarity. This immune modulating capability thus makes them attractive targets for viral myocarditis therapeutics. Chemokines apart of the CC chemokine family such as CCL2, CCL4, and CCL19 have been shown to mediate mononuclear cell migration to the heart in coxsackievirus B3-induced myocarditis (Chen et al. 2009). In fact, a recent gene therapy approach using a CCL2 mutant that lacked chemo-attractant activity in a Balb/c coxsackievirus B3-infection model impaired appropriate Th1 immune responses and significantly controlled myocardial disease (Yue et al. 2011). Blocking CCL2 with expression of the mutant did not assist in viral clearance. The potential therapeutic effect of blocking CCL2 lies in the CCL2 mutant's ability to weaken the pro-inflammatory Th1 immune response (Yue et al. 2011).

The CDC family of chemokines also contribute to myocarditis pathogenesis. CDC chemokines act on monoculear CXCR3 expressing cells and include such members as IFN-inducible protein 10 (IP10/CXCL10), monokine induced by IFN-γ (Mig/CXCL9), and IFN-inducible T-cell a chemoattractant (I-TAC/CXCL11) (Groom et al. 2011). CXCL10 and its interaction with its receptor CXCR3 have been implicated in many virus disease models. CXCL10 is a key contributor in the innate immune response to viral infection. By interacting with its receptor CXCR3, CXCL10 manipulates natural killer cell trafficking and their production of IFN-γ. Yuan et al recently found CXCL10 levels in the heart to be inversely related to viral titers after coxsackievirus B3 infection and a massive infiltration of $CXCR3^+$, $CD4^+$, and $CD8^+$ cells (Yuan et al. 2009). The production of associated inflammatory cytokines followed with the infiltration of leukocytes though the anti-viral response was not effective in clearing the virus or ensuring survival in coxsackievirus B3-infected CXCL10 transgenic mice (Yuan et al. 2009). CXCL10 did assist viral clearance and protect myocytes from damage in the early stages of infection by robustly attracting NK cells and enhancing IFN-γ production to infection sites (Yuan et al. 2009). Though CXCL10 appears to improve cardiac pathology and reduce viral persistence during initial infection stages, the caveat with using this chemokine as a potential coxsackievirus B3-induced myocarditis therapy lies with its inherent actions as a Th1-type chemeokine. Th1 immune responses escalate detrimental cardiac fibrosis during the reclamation stage of disease so enhancing important contributors to this repair process may acceleration rather than prevent the development of chronic disease.

Determining the effect of CXCL10 on viral replication and recruitment of innate immune cells in other organs than the heart such as the liver and pancreas must still be pursued. As seen with another immunomodulatory factor, TGF-β, the immune responses in other organs than the heart can affect susceptibility to severe myocardial injury and the development of chronic disease.

Recent research has demonstrated that coxsackievirus B3-infected Balb/c mice have increased levels of cardiac CXCL10 and this level fluctuates in a time and dose-dependent manner (Yue et al. 2011). The same research group treated coxsackievirus B3-infected mice with a CXCL10 mutant that lacks the critical chemo-attractant part. This mutant effectively blocked endogenous CXCL10 activity and protected coxsackievirus B3-infected mice from developing myocarditis (Yue et al. 2011). The CXCL10 mutant expressing mice had greater survival, less changes in body weight, less inflammation and necrosis in the heart. Cardiac Th1 cytokines IFN-γ, IL-12, TNF-α, were also found to be significantly reduced with CXCL10/CXCR3 signalling inhibition. This suggests that dampening the Th1 response to coxsackievirus B3 infection through blocking CXCL10 activity may present as an effective strategy to suppress immune inflammation and myocardial damage in coxsackievirus B3-mediated myocarditis (Yue et al. 2011).

The production of immunomodulating mediators such as cytokines and chemokines affects not only antigen presenting cell and T cell populations acting at the site of injury, but greatly influences the outcome of disease. The expression of particular cytokines and chemokines after a pathogenic insult, such as coxsackievirus B3 infection, influences the balance of effector versus regulatory responses that ensue. Once the balance between effector versus regulatory responses tips in a particular direction, chronic autoimmune-type disease or the prevention of disease will materialize (Rouse et al. 2010; Wing et al. 2010) (Figure 3). It is therefore critical to bear in mind the possibility of tipping the immunity balance when manipulating key immune players in coxsackievirus-induced myocarditis.

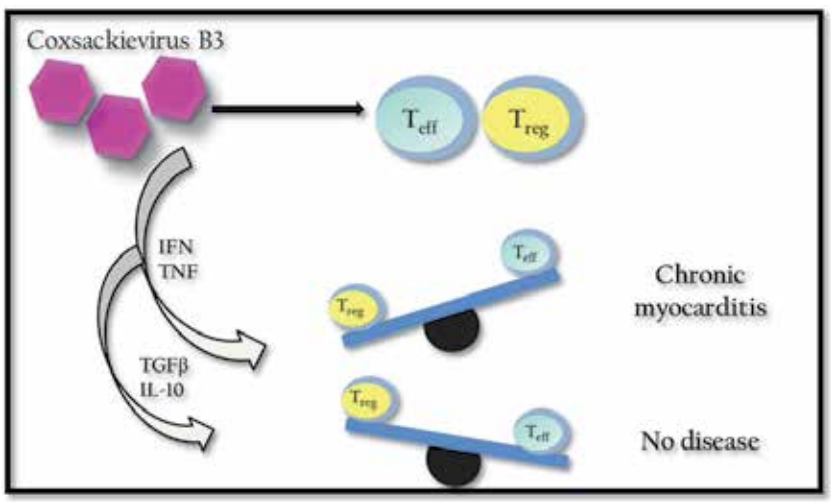

Fig. 3. Immunomodulation of the immune balance and the effects on disease outcome. With coxsackievirus B3 infection, populations of regulatory and effector cells are produced. It is with particular immunomodulating mediators such as cytokines interferon (IFN) and tumor necrosis factor (TNF) that enhanced production and proliferation of effector T cells (T_{eff}) is promoted and leads to the persistent destruction of the myocardium and chronic myocarditis disease. Pro-regulatory cytokines transforming growth factor-β (TGF-β) and interleukin-10 (IL-10) are examples of immune modulators that promote the enhanced production of regulatory T cells (regulatory T cells) and the prevention of autoimmune myocarditis disease.

5. Therapeutic challenges and future directions

Treatment for myocarditis mainly involves targeting the symptoms of heart dysfunction. If the disease progresses to dilated cardiomyopathy, heart transplantation is necessitated. Regrettably, for 50% of patients progressing to dilated cardiomyopathy, survival is limited to 5 years. Broad-spectrum antivirals tested in clinical trials with children and adults have been few and mildly successful. Specific therapies designed against viruses (enteroviruses) for prevention and/or management of viral myocarditis are still desperately needed. One challenge in designing specific antivirals or vaccines for viral myocarditis is the range of possible etiologies and immune mechanisms that may be responsible. Although enteroviral infection inducing or exacerbating an autoimmune response is a likely cause for viral myocarditis, supporting data defining a link between viral infection and the onset of acute and/or chronic myocarditis in humans must still be determined. The existence of many viral serotypes for promising etiological agents like coxsackievirus B also poses a problem in designing specific antiviral therapies.

Dampening the Th1 immune response in viral myocarditis is a therapeutic avenue consistently featured in past and recent studies. Many key innate immune players, such as cytokines and chemokines, are targets of potential Th1 skewing therapeutics. Cytokines presenting at the site of viral infection have a significant influence on whether a tolerant or autoimmune response is chosen. Targeting cytokines, chemokines and other immunomodulating factors for myocarditis therapy may depend on the timing and duration of the particular factor and the maintenance of immune balance to prevent the development of autoimmunity.

6. Conclusion

For quite some time scientists have investigated virus and host cellular and molecular events that underlie the pathogenesis of enteroviral-induced myocarditis. Our lab has demonstrated the significance of many key innate players that contribute to coxsackievirus-B3 induced myocarditis in mouse models. Horwitz et al first revealed the importance of IFN-γ expression in the pancreas for the protection from coxsackievirus B3 infection and the induction of myocarditis (Horwitz et al. 2000). Later on, Horwitz et al again demonstrated a protective role against coxsackievirus B3-induced myocarditis with cytokine expression in the pancreas, this time, with transgenic expression of TGF-β in the beta cells (Horwitz et al. 2006). This work was followed by Richer et al, who provided a role for TLR4 and stimulation by *Salmonella minnesota* lipopolysaccharide in bypassing the protective effect exerted by TGF-β in coxsackievirus B3-induced myocarditis (Richer et al. 2006). Our lab has also investigated the role of IL-6 in disease severity. Poffenberger et al demonstrated that in the absence of IL-6, a greater early immune response occurred, instigating a severe chronic disease pathology (Poffenberger et al. 2009). Recently, Poffenberger et al described susceptibility loci on chromosome 17 that implicate the highly suggested genetic component as a factor dictating viral myocarditis pathogenesis (Poffenberger et al. 2010). Though work from our lab has contributed a great deal to the understanding of coxsackievirus B3-induced myocarditis, there remains many unanswered questions with regards to the true key immune players that dictate disease pathogenesis. It is likely that an interplay of genetic and environmental factors influence the susceptibility and severity of virus-induced myocarditis however, we must not discount the significance of the innate immune response in shaping the outcome of virus-induced disease. It is possible that this early immune response may

ultimately dictate whether an acute or chronic immune response ensues with enteroviral infection.

7. References

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13

Cellular and Immunological Regulation of Viral Myocarditis

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1. Introduction

Since the discovery of coxsackievirus type B3 (CVB3) more than 50 years ago there has been considerable progress in the understanding of viral heart disease. Coxsackievirus was first discovered as a filterable agent associated with a paralytic syndrome, so named for its identification in Coxsackie, New York (coxsackievirus type A) (Dalldorf and Sickles, 1948). Coxsackievirus type B (CVB) was isolated the following year from patients with aseptic meningitis and by the mid-1950s an association with acute myocarditis in humans was apparent (Melnick et al., 1949). Many other viruses have since been shown to cause myocarditis and its long term sequelae, arrhythmias, dilated cardiomyopathy (DCM) and heart failure. By example, adenovirus, herpes viruses, and influenza can cause myocarditis in humans and models of heart failure. The viral agent most often reported as being the cause of viral myocarditis is CVB3. For example, CVB3 RNA can be detected in the heart muscle of 10 - 35 % of DCM patients (Feldman and McNamara, 2000; Hosenpud et al., 2001), depending on the study cohort.

1.1 Myocarditis etiologies

Inflammation of the heart muscle can have many causes, not least of which is viral infection. Cocaine [(Jentzen, 1989) reviewed in (Maraj et al., 2010)], virus induced, autoimmune (possibly due to resolved microbial infection), and even vaccine induced myocarditis (Cassimatis et al., 2004) have all been reported with a wide degree of occurrence. Though very rare, the vaccine induced aetiology is classified as idiopathic due to the poorly understood mechanism of inflammation. This manifestation of myocarditis is so rare it is probably very complex in cause and mechanism, requiring both environmental and genetic susceptibility factors of the host to trigger myocardial immune invasion. The subject of this chapter is one of the most common forms of myocarditis, and perhaps the most studied: myocarditis due to viral infection. In such cases, myocyte dropout and provisional matrix fibrosis, focal lesions of immune cell invasion are marked and key observations in the diagnosis of these cases.

1.2 Clinical presentation

With an experimental understanding of CVB3 induced myocarditis comes a better understanding of the breadth of symptoms presented by the admitted patient, before,

during and after a hospital visit. The patient is most often admitted to hospital with general chest pain or discomfort, combined with other symptoms of an infection, which are often suspected as other ailments (Brady et al., 2004). These symptoms of infection can be so apparent that they divert the attention of the clinician away from cardiac dysfunction. Further work-up of the case reveals elevated creatinine phosphokinase and troponin with rhythmic disturbances and reduced cardiac output, often presented by shortness of breath with minimal exertion (Brady et al., 2004). Most cases resolve with little or no supportive therapy, but a minor percentage of cases progress to dilated cardiomyopathy and congestive heart failure, for which there is no cure other than heart transplantation.

1.3 Diagnosis: the Dallas criteria

The Dallas criteria for myocarditis are a set of histology based criteria proposed by a publication in 1987 by Aretz et al. (Aretz et al., 1987). These criteria rely upon histological observations made of left ventricular biopsies, obtained by either the Cordis or Scholten bioptomes. Several biopsies of the ventricle are required for a definitive diagnosis and there is a high possibility of false negatives using this method. Though not necessarily a problem inherent in the method itself but the need for a large number of samples due to the often localised and focal nature of the disease. The chances of false negatives combined with procedural discomfort are driving forward a need to develop new and less invasive procedures like blood-based biomarker-detection.

The Dallas criteria for diagnosis of myocarditis require an inflammatory infiltrate with associated myocyte necrosis, or otherwise damage that is not characteristic of ischemia. Less intense immune infiltration with little or no evidence of myocyte destruction is classified as borderline myocarditis (Aretz et al., 1987).

Understanding the etiology of myocarditis will improve the success rate of diagnosis, which in turn, improves management of the disease and prognosis (Baughman, 2006; Felker et al., 2000). The Dallas criteria were a good stepping stone toward a standardised diagnostic method, however there remain significant drawbacks to this biopsy based histological criteria. It has been demonstrated that myocarditis could only be accurately diagnosed in 25 % of single biopsies obtained from patients who had died of myocarditis (Chow et al., 1989; Hauck et al., 1989), and more than 5 biopsies are required to diagnose myocarditis in two thirds of patients (Hauck et al., 1989), and 17 biopsies are required for an 80 % level of confidence in diagnosis (Hauck et al., 1989; Schultz et al., 2009). Therefore only positive samples obtained by endomyocardial biopsy should be considered diagnostic. These poor rates of definitive diagnosis are a product of the small size of the bioptome sample from a relatively large area afflicted with a disease that is often focal and of relatively narrow localisation.

The Dallas criteria were a good start for a disease that had no diagnostic criteria at the time of publication (1987). To this day, the Dallas criteria are used to diagnose myocarditis from endomyocardial biopsies, though the existence of sensitive methods for disease detection, particularly in the blood, biomarker discovery should make this methodology supplementary or even obsolete. Therefore, there is a need for the development of sensitive methods for the differential and definitive diagnosis of non-ischemic cardiomyopathies like myocarditis. This chapter will not cover diagnosis or new detection strategies any further as these topics are covered in greater detail, elsewhere in this book.

2. Viral replication as the central regulator of viral myocarditis

The advent of virus replication in the heart of an individual is thought to occur secondarily to a more general and systemic virus infection (Carthy et al., 1997; Mena et al., 2000; Vuorinen et al., 1994), the spleen, gut, pancreas and lymph nodes are often infected; patients often report malaise, fatigue and more general 'flu-like' symptoms about 1- 2 weeks prior to chest pains and cardiac rhythm disturbances. Immune infiltration in the heart has taken hold by the time the patient has been seen by a physician and any evidence of direct virus-induced damage has been superseded by T cell invasion at the foci of infection. It is not surprising that there have been many reports that claim the predominant source of damage is immune infiltration of the myocardium, as inflammation is what defines this disease. Autoimmune etiologies due to an antiviral response gone awry have been proposed as one of the major causes of pathology (Lv et al., 2011). Regardless, there are numerous publications that suggest a large amount of damage is inflicted by the virus itself, in the murine model, early post-infection, that is correlated with the number of myocarditic lesions spatially coincident with positivity by *in situ* hybridization for CVB3 genome (Cheung et al., 2008; Chow et al., 1992; Marchant et al., 2009a; McManus et al., 1993). These observations are supported by findings in autopsy cases, particularly in infants (Iwasaki et al., 1985a; Iwasaki et al., 1985b).

2.1 The cytotoxic nature of enterovirus replication

Coxsackievirus B3 is a member of the *Enteroviridae* of the *Picornaviridae* family, which are fast replicating and exquisitely cytotoxic viruses. Another member of this family is poliovirus, which is also highly cytotoxic, producing a paralytic syndrome reminiscent of the aseptic meningitis and encephalitis sometimes caused by CVB3. The very nature of enterovirus replication makes these viruses very cytotoxic because the infected cell must burst to permit the release of progeny virus virions for infection of bystander cells and further replication cycles. This aspect of CVB3 replication means that every virus infected cell will die within 8 – 24 hrs of infection, releasing numerous rounds of progeny virus into the heart before the innate immune system has had a chance to control infection. The degree of immune infiltration and damage inflicted on the heart muscle is therefore a function of the amount of virus inoculum that takes hold in the heart, dictating the degree of damage done to the myocardium.

The virus polarises the protein expression machinery entirely to the benefit of the virus by a number of mechanisms and so in combination with the inability of the cell to perform its natural housekeeping functions inevitably leads to cell destruction. The viral proteases act to cleave capped cellular mRNAs thereby skewing protein translation in the cell from cap-dependent translation initiation to internal ribosome entry site (IRES) translation. Coxsackievirus B3 translates it's RNA genome via IRES translation initiation so destruction of the cell's own capped mRNAs removes any competition of the virus' own IRES RNAs for the cell's ribosomes. This usurping of control of the cell's protein expression machinery effectively brings the normal housekeeping function of the cell to a halt.

The massive amount of protein produced by the virus, devolution of the endoplasmic reticulum (ER) and other membranous structures combined with the oxidative stress placed upon the cell by virus replication, all destabilise the homeostasis of the infected cell. The large protein-nucleic acid aggregates that make up progeny virion are not normal to the cell and induce numerous UPR responses; due to their toxic nature, protein aggregates are

normally sequestered to vimentin-cytoskeleton encased aggrosomes (Garcia-Mata et al., 1999; Johnston et al., 1998). For example, the irregular folding of viral proteins can lead to sequestering of virus protein products into cellular aggresomes (Spiropoulou et al., 2003). These combined facets of CVB3 replication contribute to the inherently toxic nature of virus replication, even in the absence of the onset of programmed cell death.

2.2 Virus entry into the host cell

The replication cycle of CVB3 in the cell begins in a very similar manner to poliovirus infection via receptor mediated endocytosis into the target host cell. Coxsackievirus B3 binds its cellular receptor(s), the coxsackie-adenovirus receptor (CAR) and ,sometimes, decay acceleration factor (DAF), to achieve entry via endocytosis into the host cell. Once the virus has achieved delivery inside the cell there is massive rearrangement of internal membrane structures and then complete usurping of the host cell protein translation machinery for the purposes of virus replication and virion assembly. The RNA polarity (positive-polarity RNA) of the CVB3 genome means that viral polypeptide can be translated directly after delivery into the host cell. All of the proteins encoded by the CVB3 genome are expressed as 1 poly-protein that is then cleaved by virus proteases, 2A and 3C, into the individual viral proteins VP4, VP2, VP3, VP1, 2A, 2B, 2C, 3A, 3B, 3C, and 3D.

2.3 Death and lysis of virus infected cells

Direct virus-induced damage inflicted by CVB3 upon the infected cell is through simultaneous activation of multiple cell death pathways, by direct and indirect mechanisms. For example, all of the known caspase cell death activation pathways are activated during CVB3 infection: the FAS/FADD receptor-mediated caspase- 8 pathway and the mitochondrial cytochrome c -caspase- 9 pathway are activated to ultimately trigger the death effector, caspase-3 (Carthy et al., 1998; Carthy et al., 2003). Normally, the activation of caspases during apoptosis leads to programmed cell death: cleavage of cellular DNA by endonucleases and shrinking of the cell's contents into a cytoskeleton-vimentin cage, for engulfment by phagocytes at some later time. This process is programmed, and most importantly, 'neat,' preventing the dissemination of the dead cell's contents throughout the tissue. The debris released from cells are hydrolytic and oxidative, some are apoptotic, which would lead to bystander necrosis of surrounding cells and tissue if not disposed of 'neatly' through programmed cell death. However, CVB3 infected cells do not die in a programmed and neat manner. The act of virus replication; viral proteases and activation of numerous cell death pathways all at once cause infected cells to rapidly lyse and release cellular contents, along with virus progeny virions, for further rounds of replication in surrounding bystander cells.

Necrotic cell death during CVB3 infection is most likely caused by a myriad of pathways that act in concert to result in the uncontrolled lysis of the cell for the purpose of virus progeny release. Evolution of the virus has favoured a more chaotic lysis/necrosis type death as opposed to a programmed and controlled method of killing the host cell. We will summarise the pathways of CVB3 induced cell death below:

2.3.1 Caspase-induced cell death during CVB3 infection

The caspase pathways of apoptosis were the first mechanisms of programmed cell death that were discovered [(Lazebnik et al., 1994; Miura et al., 1993; Yuan et al., 1993; Yuan and

Horvitz, 1990) reviewed in (Yuan and Horvitz, 2004)]. The first cellular pathways implicated in cell death due to CVB3 infection were also caspase mediated (Carthy et al., 1998; Colston et al., 1998), since these were the predominant mechanisms of cell death being studied at the time (Andrade et al., 1998; Atkinson et al., 1998; Barry et al., 2000). As new mechanisms of cell death were discovered, such as GSK-3β, some were attributed to virus induced cell death.

Comprehensive studies of caspase cleavage and activation during infection have demonstrated a pan-caspase activation profile during viral infection (Carthy et al., 1998; Carthy et al., 2003), from activation of the tumour necrosis factor (TNF) receptor activated caspase-8 to the mitochondrial cytochrome *c* release that results in the activation of caspase-9. All of the above mentioned pathways converge upon the activation of the effector caspase, caspase-3. The most notable caspase-8 activated pathway is triggered by Fas ligand binding and activation of Fas receptor, a member of the TNF superfamily of receptors. Various oxidative states, toxins and aberrant protein expression can lead to a permeable mitochondrial membrane that releases cytochrome *c* into the cytoplasm which leads to caspase-9 activation. Though the caspase-8 and -9 pathways are quite different in that they are extrinsic and intrinsic pathways of cell death, respectively, they both lead to cleavage and activation of caspase-3, a death effector caspase. In cells destined to die by either of these routes of apoptosis there is cleavage of poly ADP-ribose polymerase (PARP) and DNA fragmentation factor (DFF) by caspase-3. Cleavage of DFF by this caspase reveals an endonuclease that enters the nucleus and cleaves DNA, resulting in DNA fragmentation (Figure 1A).

Two of the experimental hallmarks of apoptotic cell death are PARP fragmentation, as observed on a Western blot, and fragmented genomic DNA as viewed by agarose gel electrophoresis. It was demonstrated that inhibition of caspase-3 PARP and DFF cleavage did not inhibit virus induced cell death completely (Carthy et al., 1998), however some alleviation of cytopathic effect was notable. The broad spectrum zVAD.fmk caspase inhibitor is not 100 % effective at inhibiting all caspases, and there is the possibility that in the absence of caspase activation that a redundant mechanism of cell death becomes dominant in mediating cytotoxicity. In this scenario we would expect zVAD.fmk to appear ineffectual at inhibiting cell death. It was apparent that the caspases were activated though this was clearly not the mechanism that was entirely responsible for CVB3 induced cytopathic effect. Below we will outline more mechanisms responsible for cell death and that may run parallel to caspase mediated cytotoxicity. Later in this chapter we will also cover new systems biology approaches that could be applied toward the understanding of the dominant/ pertinent pathways of CVB3 induced cell lysis and the networks employed by the virus to drive cell death.

2.3.2 Glycogen synthase kinase-3β

In addition to the roles of caspases during cell death, the actions of glycogen synthase kinase-3 β (GSK-3β) have also been implicated in the induction of cytopathic effect by CVB3 replication (Yuan et al., 2005; Zhang et al., 2003). Phosphorylation of transcription factors by GSK-3β can lead to either the activation or down-regulation of transcription, and activation of GSK-3β has been observed relatively early in the CVB3 replication cycle (Yuan et al. 2005). The catenins are poly-phosphorylated by activated GSK-3β, leading to degradation in the proteasome (Doble and Woodgett, 2003; Harwood, 2001). During CVB3 infection, the activation of GSK-3β has been proposed to lead to instability of cell viability due to

degradation of a protein called β-catenin (Figure 1C). The catenins interact with the actin cytoskeleton and act to maintain morphological integrity by connecting the actin cytoskeleton with the cell membrane. The loss of these connections through enhanced GSK-3β activation and β-catenin degradation is consistent with the profound cell rounding that occurs during intermediate to late phases of CVB3 replication. The accumulation of β-catenin in the cytoplasm leads to translocation into the nucleus where it transactivates survival signalling via T-cell factor (TCF)/lymphocyte enhancer factor (LEF) family members (Doble and Woodgett, 2003; Hardt and Sadoshima, 2002; Harwood, 2001). Consistent with these findings, the inhibition of GSK-3β or the over-expression of β-catenin resulted in the suppression of viral progeny release, but not viral protein production (Yuan et al., 2005). Therefore GSK-3β is pivotal in regulating the rupture of infected cells for progeny release.

2.3.3 The endoplasmic reticulum unfolded protein response

CVB3 induces the unfolded protein response (UPR), which is an acute stress response that leads to ER stress (Zhang et al., 2010). Poliovirus, CVB3 and other viruses have been shown to replicate their genomes and translate protein on the surfaces of double membrane vesicles derived from the ER and autophagosomes (Suhy et al., 2000; Wong et al., 2008; Zhang et al., 2011). This activity in combination with interruption of regular protein translation and a massive upregulation of protein production by the virus probably leads to activation of the UPR. This process involves 3 different pathways of independent activation, initiated by 3 respective stress sensors in the proximal ER: ATF6a, IRE1-XBP-1, (X box binding protein 1), and PERK (PKR-like ER protein kinase). It has been shown that infection with CVB3 invokes activation of ATF6a and XBP-1, possibly through replication of the virus on the surface of double membrane vesicles derived from the ER and autophagosome (Wong et al., 2008). Other viruses that replicate in the cytosol, such as West Nile Virus, have been shown to activate this pathway to promote virus replication and cell death ((Medigeshi et al., 2007) review). The UPR response to massive viral protein synthesis and usurped cellular pathways most likely acts to initiate or augment the onset of death in the infected host cell. However if these pathways are activated too early in the virus replication cycle the cell will die before virus protein production and virus progeny can be completed and released, respectively. This would surely result in the termination of virus replication. Zhang et al. showed that expression of the UPR response protein, XBP-1 acts to delay the onset of cell lysis and release of progeny virion (Figure 1B). Therefore, pathways like XBP-1 are used by the virus to counter early destruction of the cell; so CVB3 has evolved mechanisms to prevent premature death of the infected cell. Major cellular survival pathways, like Akt, are also invoked to counter the death reflex, which we will discuss later in the chapter.

2.3.4 Cell death and lysis through activation of many simultaneous pathways

The overwhelming and rapid activation of many different pathways simultaneously leads to cell death and lysis (Figure 1), liberating progeny virion. It is possible, that cell death pathways may even be synergistic, additively driving the cell toward more rapid and thus uncontrolled death, leading to lysis. The cytochrome c- caspase-9 pathway requires a minimum of 15 hours to kill a cell that has been chemically induced, almost twice as long as it takes CVB3 to lyse an infected cell (Qu and Qing, 2004). The plant toxin abrin requires upwards of 15 – 36 hours to kill a culture of HeLa cells (Qu and Qing, 2004), the E2 protein of human papillomavirus requires 20 – 24 hrs to induce apoptosis (Desaintes et al., 1999),

whereas CVB3 will have induced apoptosis and destroyed a similar culture of HeLa cells in just 8 – 10 hours. The rapid nature of CVB3 induced cell lysis suggests the synergy between numerous death pathways 'criss-cross' and interact to enhance their collective cell-death effect.

The loss of checked and contained cell death results in necrosis and lysis of the CVB3 infected host cell. Even though the pathways of cell death are clearly defined in this arena, the precise mechanisms of initiation are still unclear, and potential pathway interactions are unknown. Many reports have indicated that a particular pathway may be involved in CVB3 induced cell death although there hasn't been 100 % recovery of cell viability demonstrated from experiments that inhibit just one pathway. However, zVAD, a pan caspase inhibitor does not completely inhibit virus induced death and lysis just as GSK3β inhibition doesn't completely inhibit cell-rounding and death. Therefore there is still much to be learned about the initiation and interaction of cell death pathways during CVB3 infection, but we may never be able to completely inhibit CVB3 induced cell death because once virus replication is initiated the cell may already be past the threshold of viability.

2.3.5 Necroptosis?

Necroptosis (necrotic apoptosis) is a tumour necrosis factor receptor activated form of programmed cell death that is activated by the same class of receptors that activate caspase-8 mediated apoptosis (Declercq et al., 2009; Hitomi et al., 2008; Yuan and Kroemer, 2010). This is a more recently discovered pathway of cell death that has not been reported in the context of CVB3 infection. However, instead of caspase-8 activation that normally results from TNF receptor ligation there is activation of a receptor interacting protein (RIP)-1/RIP-3 pathway that results in rupture of the cell through loss of membrane integrity; a mode of cell death reminiscent of CVB3 induced cell lysis. Transgenic caspase-8 null animals undergo RIP-3 necroptosis instead of apoptosis, leading to embryonic lethality and non-viability of the line. However caspase-8 -/-, RIP-3 -/- double transgenic mice are viable, suggesting that caspase- 8 holds RIP-3 'in check', preventing initiation of programmed necrosis (Kaiser et al., 2011). The necroptosis pathway has not yet been described with respect to CVB3 infection but given the similarities between CVB3 induced cell lysis and necroptosis this pathway of cell death and the RIP-1/RIP-3 proteins may provide some insight.

2.4 Virus induced cell death and lysis – 'Timing is everything'

There have been reports showing that cell death activated early in the virus life cycle significantly inhibits viral replication (Zhang et al., 2010). Release of the cell contents prior to progeny virion production and more programmed processes of cell death will halt virus replication by killing the cell prior to assembly of progeny virus virion or confinement of progeny virion into vimentin cages, respectively.

Virus replication is cytotoxic and can cause cell death before production of progeny virion, terminating the life-cycle of the virus. For example, reactive oxygen species are a byproduct of CVB3 replication very early in the life-cycle of the virus, placing stress on the cell such that the mitochondrial cytochrome *c* pathway of caspase-9 mediated cell death might be activated prior to virion assembly. To prevent premature death of the host cell coxsackievirus triggers the activation of pro-survival proteins and their associated cascades to suppress cell lysis until progeny virion can be assembled. Perhaps the most studied pro-

survival protein, Akt, is activated during the CVB3 replication cycle (Esfandiarei et al., 2007; Esfandiarei et al., 2004; Liu et al., 2008). It may appear counter-intuitive for a cytolytic virus to evolve to activate pro-survival pathways, particularly if the virus requires the cell to lyse at the end of the cycle. However, as we discussed in section 2.1 CVB3 replication is cytotoxic from the onset so pro-survival pathways may be selected evolutionarily to support the survival of the cell while viral protein synthesis and viral progeny assembly are taking place. These mechanisms of cell survival during virus infection act in favour of viral replication because they allow maximum amounts of virus progeny to be assembled prior to cell lysis and release of viral progeny. Subsequently, these same survival pathways must be inhibited to then permit cell death and lysis for release of assembled viral progeny virions.

2.5 Coxsackievirus induced cell death: many dimensions of complexity

It is now apparent that several cell death pathways and perhaps dozens of proteins are activated in a web of molecular interaction in order to short circuit normal death mechanisms, to cause rapid and aberrant rupture of the infected cell (Figure 1). New pathways of cell death, such as necroptosis, may as yet be shown to mediate death of the CVB3 infected cell. Nevertheless, there already exists a large web of seemingly disparate pathways of CVB3 induced cellular lysis. As we discussed earlier, it is difficult to determine the foundation pathway that is responsible for virally induced cell lysis of infected cardiomyocytes, and which ones are triggered, merely as a matter of coincident activation. New methods of data organisation and analysis are clearly needed as more pathways and mechanisms of CVB3 induced cell death are revealed. Knowledge of the truly pertinent and central pathways of cell death could lead to the most robust antivirals. Toward the end of this chapter we will discuss the use of systems biology to organise the myriad of pathways activated during CVB3 infection (proteolytic and kinase) and how it could be applied to elucidate the dominant pathways of virus induced cell death.

3. Inflammatory regulation of myocarditis by the host: regulatory T cells and healthy vs pathological signalling

3.1 Mouse genetic determinants of immune infiltration

Once virus infection has taken hold in the heart there is infiltration and onset of immune responses to clear infection and lay down provisional matrix in place of damaged tissue. As we discussed earlier in the chapter, the severity of the immune response varies broadly from patient to patient, from mild to massive immune infiltration that can lead to DCM and congestive heart failure. We know from the mouse models of CVB3 induced myocarditis that immune infiltration is correlated with the amount of virus inoculum, and therefore with the degree of damage that has been mediated by virus. However experiments comparing the susceptibility of various inbred strains of mice to CVB3 induced myocarditis showed that there could be a genetic predisposition to myocarditis severity (McManus et al., 1993). These data suggest that a genetic predisposition of the individual patient to mild or severe myocarditis, combined with viremia and cardiac viral load could act in concert to determine severity and number of myocarditic foci. The studies conducted with inbred mouse strains have implicated the MHC class II (H2) locus (Wolfgram et al., 1986), in addition to other genetic loci (Traystman et al., 1991) associated with the severity and susceptibility to CVB3 myocarditis, both acute, chronic and autoimmune. Not surprisingly, the H2 locus of the MHC II complex has been implicated in clearance of acute coxsackievirus infection

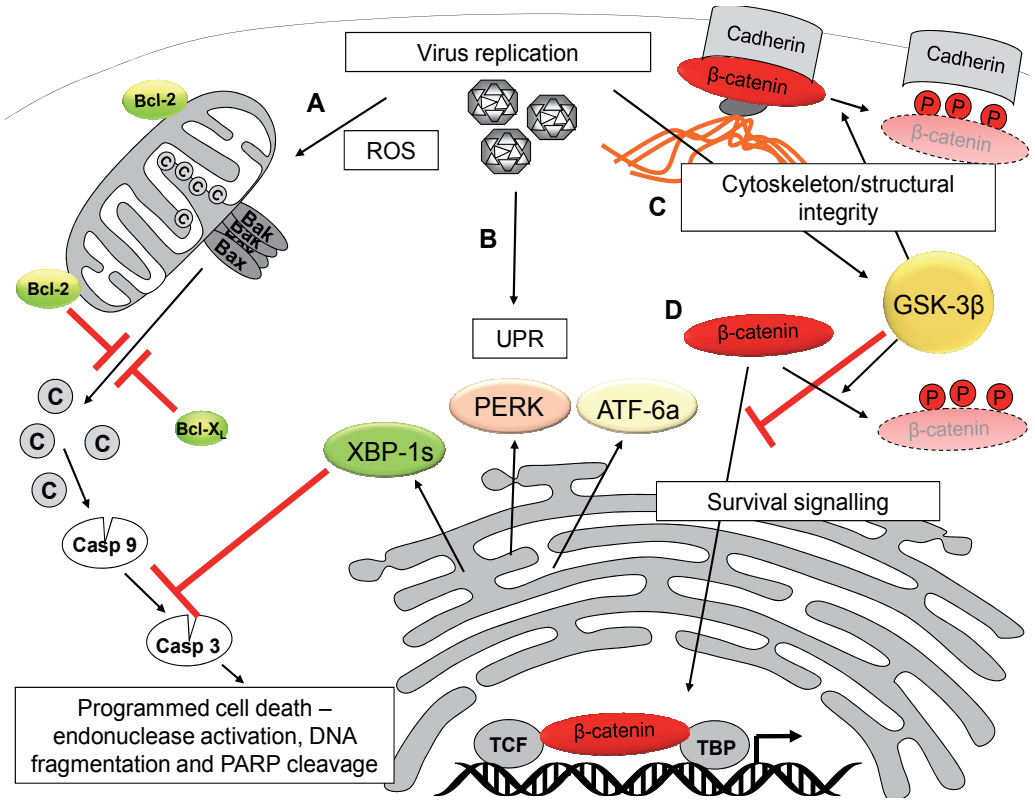

Fig. 1. Pathways of cell death in CVB3 infected cardiomyocytes. A. Coxsackievirus replication results in production of reactive oxygen species which promote the release of cytochrome c from mitochondria. The formation of Bak and Bax at the membrane surface mediate passage of cytochrome c into the cytosol. Overexpression of the anti-apoptotic proteins BCl 2 and BCl-XL during CVB3 infection significantly reduce cytochrome *c* release and activation of the caspase-9 pathway of apoptosis, which leads to caspase-3 death effector caspase activation. ROS = reactive oxygen species. B. The unfolded protein response (UPR) of the endoplasmic reticulum (ER) is activated during CVB3 infection of cardiomyocytes leading to activation of all 3 known pathways of the UPR: spliced XBP-1s, PERK and ATF6a ER stress sensors are all activated during CVB3 infection. Virus replication occurs on the surface of double membrane vesicles and membrane derived from the ER, activating the ER stress sensors. The result of XBP-1s activation is to slow the onset of cell death to prevent the cell from rupturing prior to the assembly of progeny virus virions. UPR = unfolded protein response. C and D. Glycogen synthase kinase -3β (GSK-3β) is activated during CVB3 infection resulting in phosphorylation and subsequent proteosomal degradation of its target, β –catenin, in the cytosol. C. Loss of β –catenin in the cytosol results in loss of connections between the actin cytoskeleton and cadherin receptors, via connections to α and β –catenins. D. Less β –catenin in the cytosol means that there will be less available to translocate across the nucleus to activate survival gene expression via TCF and TBP transcription factors.

(Wolfgram et al., 1986). The presence of a strong adaptive response that includes robust neutralising antibodies is required to control CVB3 infection in mice (Wolfgram et al., 1986) and patients (McKinney et al., 1987; Misbah et al., 1992), as well as other picornaviruses, like polio (MacLennan et al., 2004). Therefore, the host's own genetic makeup will determine how quickly and how completely virus infection is cleared from the body. An inability to mount a broad immune response will lead to more virus replication, more immune infiltraton of the myocardium, more matrix deposition and longer recovery times. In addition to these host dependent factors there may also be environmental factors that play roles during infection and inflammation: extrinsic source stress, pollution/allergens and population density could also contribute to susceptibility and severity of viral myocarditis.

3.2 Chronic infection and the cycle of virus-immune mediated damage

Over the last 3 decades it has become apparent that viral load is a primary determinant of viral myocarditis severity (Cheung et al., 2008; Chow et al., 1992; Lim et al., 2005; Marchant et al., 2009a; McManus et al., 1993), the virus causes extensive, early direct injury to the myocardium through its replication. The studies cited above all indicate significant injury to the myocardium prior to cardiac immune infiltration, supporting the view that considerable damage to the heart is directly mediated by the virus alone. An argument has been made previously for the importance of chronic virus replication in myocarditis and as a basis of dilated cardiomyopathy (Szalay et al., 2006) and chronic heart failure. Enhanced immunoproteasome expression in chronically CVB3 infected cardiomyocytes (Szalay et al., 2006) may increase the expression and presentation of auto-antigens, leading to more severe myocarditis through autoimmune avenues. Chronic virus replication will lead to consistent damage which will, in turn, result in provisional matrix turnover. This type of damage-repair loop will propagate the fibrosis that is evident during chronic myocarditis and DCM. As we covered in the apoptosis/death pathway sections of this chapter the release of cell constituents and debris, and progeny virus and proinflammatory cytokines, from an infected cell will lead to inflammation at the site of virus insult. Therefore, the damage inflicted by the virus itself will be exacerbated by the onset of cytolytic immune infiltration and fibrosis.

3.3 Regulatory T cells

Not all T cell infiltration and immunity are harmful as there are moderating arms of the immune response that solely act to moderate the strength of a response, preventing autoimmunity. Li et al recently reported that allografted M2 (anti-inflammatory) macrophages led to improvement of virus-induced myocarditis (Li et al., 2009) which was associated with enhanced levels of regulatory T cells (Treg). Huber et al have also reported decreased viral load and immune infiltration after adoptive transfer of a CD4+ CD25+ regulatory-like T cell population into a mouse model of CVB3 infection (Huber et al., 2006). Regulatory T cells are CD4 + cells that express the α-subunit of the IL2 receptor CD25, and are negative for CD127. The hallmark protein expressed in these cells is the transcription factor FoxP3 and they circulate as a functionally distinct T cell subpopulation, preventing autoimmune and other aberrant responses to innocuous environmental antigens (Wing and Sakaguchi). The T-reg population prevents the proliferation of self-reactive effector T cell populations and were aptly named suppressor T cells when first discovered for their ability to secrete IL10 and moderate Th1 immunity (Wing and Sakaguchi). Thus, it would be reasonable to postulate that adoptive transfer of T-regs into a mouse model of viral

myocarditis would suppress the immune response and allow virus replication to proceed unchecked.

In a study by Shi et al (Shi et al., 2010) T-reg cells were adoptively transferred into CVB3 infected mice and the effect of the transferred cells upon viral load and signalling within the heart were investigated. Included in the study were PBS injection controls, but also naïve T cells. Decreased immune infiltration and enhanced Akt activation, as compared to the naïve CD4 T cell and PBS grafted controls, were notable in the T-reg adopted mice (Figure 2). Perhaps the most surprising of the results was the decreased viral load, not only in the heart but also in the pancreas and spleen, associated with lower expression of the Coxsackie-Adenovirus Receptor (CAR). The authors adoptively transferred, not only T-reg cells, but CD25 (-) CD4 T cells (naïve) as well (Figure 2A), into a separate group of mice. In fact, the naïve CD4 T cell treated control group fared worse than either the PBS or T-reg treated groups, related to less Akt activation and higher CAR receptor expression. As a result, the naive T cell population brought on a more severe myocarditis phenotype due to higher viral loads. Taken altogether, Shi *et al.* describe the protective role that T-reg cells play in immune infiltration of the heart during viral myocarditis, suggesting that there is a balance to be struck between clearance of infection and immune-associated damage to the myocardium. In fact, in this study there was observed decreased viral loads consonant with a decrease in immune infiltration due to reduced TNF-α release and lower CAR expression (Figure 2B). The authors found that though TNF-α could enhance CAR expression in mouse cardiomyocytes the co-addition of TGF-β eliminated TNF-α-induced CAR expression. To conclude, these findings suggest a more intimate link between inflammation and virus replication than previously posited. Thus, moderating the immune response may be critical for prevention of chronic virus replication.

Although the dominant factor in virus suppression during T-reg treatment was most likely down-regulation of CAR expression, we propose that the alteration in signaling evoked by T-regs is also playing a significant role in modulating virus replication. The basis of our argument is that CAR expression was suppressed 3-fold *in vivo* and 5-fold in a dividing cardiomyocyte cell line *in vitro*, but the suppression of viral load in the Treg group was almost 2-logarithms reduced as compared to the control group. Although the correlation between cell receptor expression and virus infection may not be linear, the decreased levels of CAR receptor may not explain the entire antiviral effect of T-regs in the myocardium during virus infection. It is entirely possible that the signalling environment altered by the adoptive transfer of T-regs may have also contributed to a less favorable environment for virus replication. The authors reported activation of Akt in the myocardium of the T-reg treated groups, which suggests that the activation of other signalling proteins may have been altered by T-reg transfer. We have reported that Akt activation is required for successful CVB3 replication (Esfandiarei et al., 2007; Esfandiarei et al., 2004; Esfandiarei et al., 2006), a pro-survival protein required by the virus to optimize the longevity of the infected cell to promote optimal progeny virus production. On the other hand, the Akt activation reported by Shi *et al.* may merely be a reflection of a healthier environment created by allografted T-reg cells in the myocardium. It is quite clear that an unbraked immunologically active environment supports enhanced cellular signalling driven by viruses for successful replication (Esfandiarei et al., 2006; Marchant et al., 2009a; Marchant et al., 2008). For example, we have previously reported that the powerful immune-stimulating protein p38 is required for effective virus replication in a CVB3 myocarditis mouse model (Marchant et al., 2009a). The activation of p38 MAP kinase was not investigated by Shi *et al.*,

but we would predict less net activation of p38 in the presence of T-regs, and thus an environment that is less conducive to virus replication. Although p38 MAP kinase activation is required for suppressor (T-reg) T cell activity and function (Adler et al., 2007), we propose that adoptive transfer of T-regs may have decreased the immune infiltrate in the myocardium with less activation of p38 MAP kinase.

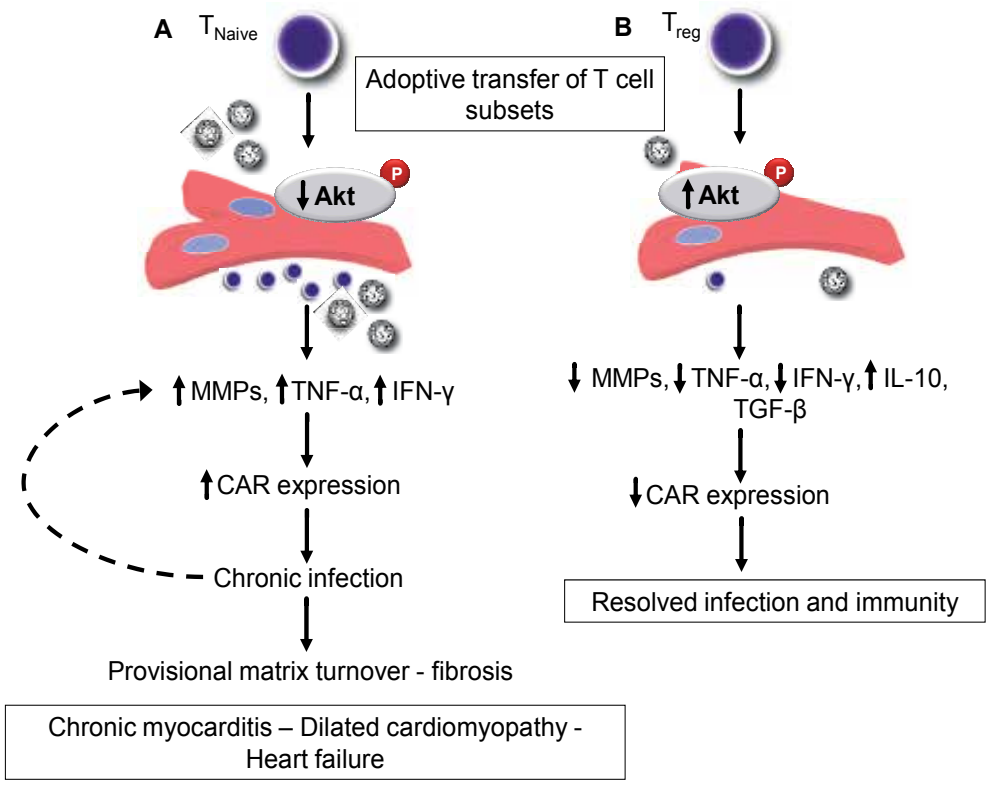

Fig. 2. Adoptive transfer of T cells into CVB3 infected mice has assisted in the elucidation of the beneficial arms of the immune response that aid in clearance of virus from the heart. A. Transfer of naïve T cells or control treatment with PBS, followed by infection with CVB3 results in high viral load in the heart followed by a large influx of T cells. An indication of myocarditis in the mouse model is the detection of decreased Akt activation, and MMP, TNF-α, and IFN-γ expression. Shi et al demonstrated that TNF- α induces coxsackie-adenovirus receptor (CAR) expression in myocardium during CVB3 replication which may promote chronic virus replication which could lead to dilated cardiomyopathy and heart failure through successive rounds of provisional matrix deposition and degradation. B. Adoptive transfer of syngeneic regulatory T cells reduces the immunologically reactive environment which results in less MMP, TNF-α, and IFN-γ expression. The result is lower CAR expression, lower viral loads and enhanced Akt activation, leading to a healthier, less immunologically reactive environment.

3.4 ERK and p38 MAP kinases

Activation of p38 MAP kinase is required for virus replication to take place, such that inhibition of p38 MAP kinase is an effective antiviral strategy *in vitro* (Si et al., 2005) and *in vivo* (Marchant et al., 2009a). The activation of ERK MAP kinase was first reported in the context of CVB3 infection both *in vitro* (Luo et al., 2002) and *in vivo* (Opavsky et al., 2002). The activation of this molecule by virus binding the CAR receptor may be involved in triggering endocytosis uptake of CVB3 during entry (Marchant et al., 2009b). The activation of p38 MAP kinase was later shown to be required for cell lysis and release of progeny virion. Si et al reported that inhibition of p38 MAP kinase could block progeny virus production but not virus protein expression (Si et al., 2005). Underlining the importance of p38 during virus replication was a relatively recent study that investigated the role of p38 during the life cycle of several different viruses. The activation of p38 MAP kinase was demonstrated as a common and dominant signalling molecule during the replication of many different types of viruses (Marchant et al., 2010). This molecule's intimate link to the immune response is perhaps not surprising; one might expect that Akt activation, p38 inhibition and T-reg immune control lead to improved outcome and result in lower viral load and immune infiltration.

With regard to new treatment strategies, this does not necessarily mean that we should be injecting T-reg cells into afflicted patients, but methods should be pursued that mediate the immune response to swing the pendulum in favor of viral clearance and reparation, and away from immune infiltration of the myocardium that favors higher viral loads. The work of several recent studies [(Li et al., 2009; Shi et al., 2010) reviewed in (Yajima and Knowlton, 2009)] have demonstrated the virus' evolved ability to bias immune activation and associated signaling queues for the benefit of virus replication. Given the results presented by Shi *et al.*, one might initially conclude that immune suppression would be a sound therapeutic strategy. However the Myocarditis Treatment Trial, wherein patients were treated with immunosuppressive agents (Mason et al., 1995), showed that such administration had no significant benefit for outcome of human myocarditis. As such, pan-immune suppression is not the way forward and better targeted methods of immune modulation are needed. Shi *et al.* demonstrated that TGF-β secreted by T-reg cells may have been responsible for the decreased CAR expression and enhanced Akt activation (Figure 1). Such suggests that we need not inhibit the immune response entirely, but rather target, modulate and encourage the arm of the immune system that promotes virus clearance. The way forward may not be adoptive transfer of T-regs, but administration of a drug that can promote T-reg differentiation and function.

3.5 The matrix metalloproteinases

The Matrix MetalloProteinases (MMPs) have been implicated in the immune response to CVB3 infection in addition to delaying matrix remodelling that takes place after acute infection.

The MMPs are collectively termed 'matrixins' and were first discovered for their ability to degrade the tails of tadpoles during frog morphogenesis (Visse and Nagase, 2003). These enzymes are zinc endoproteinases and there have since been 24 vertebrate MMP genes discovered that play a large number of roles during immune and developmental processes. There are several gene knock-out strains of MMPs that have been used to elucidate the roles of these enzymes during development and immunity. For example, MMP-8$^{-/-}$ mice are more prone to skin cancers due to delayed neutrophil infiltration and an aberrant immune response (Balbin et al., 2003). Matrix MetalloProteinase-12$^{-/-}$ mice are more susceptible to

bacterial infection due to an innate antimicrobial property of MMP-12 in intracellular compartments (Houghton et al., 2009). This MMP was shown to bind to bacterial membranes within the endosomes of macrophages and destabilise bacteria, thus acting as an antimicrobial.

3.5.1 MMPs as modulators of the antiviral immune response

MMP-9 was one of the first MMPs to be implicated in the mechanism of myocarditis and cardiac dilatation. Heymans et al first showed that inhibition of MMP action through suppression of urokinase-type plasminogen activator, a potent activator of MMPs, and exogenous expression of tissue inhibitor of metalloproteinase-1 (TIMP-1) decreased cardiac inflammation and reduced myocardial necrosis at 7 days, decreasing cardiac fibrosis at 35 days after CVB3 infection (Heymans et al., 2006). When the activity of MMP-2 and -9 were decreased there was a concomitant recovery of cardiac function with decreased immune infiltration. However, shortly after the publication of this paper Cheung et al demonstrated that MMP-9 was in fact a necessary constituent of the antiviral immune response (Cheung et al., 2008). They demonstrated no difference in viral load between MMP-8$^{-/-}$ mice and their WT counterparts. However, MMP-9$^{-/-}$ mice had higher viral loads and virus mediated myocardial damage, during CVB3 infection. Though the antiviral mechanism of MMP-9 action was not explicitly reported, there were significantly elevated levels of interferon-β (IFN-β) in the MMP-9$^{-/-}$ mice. This MMP has been shown previously to cleave and inactivate IFN-β (Nelissen et al., 2003), and IFN-β is known to have a negative feedback effect upon IFN-γ expression (Hanada and Yoshimura, 2002; Yoshimura et al., 2003). The viral loads were similar at early time points (3 – 5 days) post-infection between the genetic MMP-9 knock-outs and WT mice, suggesting that the innate-early immune response was intact, but there were higher viral loads seen in the MMP-9$^{-/-}$ mice 9 days post-infection. These results suggest that there was a deficiency in adaptive immunity in the MMP-9$^{-/-}$ mice, consistent with IFN-β negative feedback inhibition on IFN-γ regulated adaptive immunity in the MMP-9$^{-/-}$ mice.

4. Virus induced cell signalling and global aspects of cellular signalling responses

Thus far we have discussed a large number of parallel signalling molecules and pathways that are simultaneously activated during CVB3 infection, from proteolytic death pathways to phospho-signalling kinase cascades that activate a myriad of functions. Systems biology approaches now exist whereby one may survey a large number of pathways and molecules at the same time, query these results using complex statistical methods and elucidate the pathways that are truly required for a particular pathogenic function, and determine which of those that are merely coincident to viral infection.

4.1 Defining cell signalling network models in virus-host interactions

Host cells have evolved complex systems to detect and eradicate viruses; on the other hand, viruses have evolved mechanisms to compromise essential cellular processes and suppress the host cell defence. In these complex systems, protein phosphorylation events control most aspects of cellular function and homeostasis, and the failure of control mechanisms causes disease (Cohen and Tcherpakov, 2010). Most notably, protein phosphorylation events

mediated by kinases and phosphatases are used by viruses as they are crucial for pathogen replication, propagation, and evasion from host immune responses (Ribet and Cossart, 2010). Several studies have showed that multiple phosphorylated-proteins individually regulate the events that constitute virus replication [reviewed in (Esfandiarei and McManus, 2008)], yet the phospho-proteins are part of an intracellular signal-transduction network, composed of several pathways. Thus, systems perspective studies have enabled us to address network mechanisms active during host-virus interactions, with specific emphasis on elucidating key determinants of disease severity and the effective translation of these concepts into new systems-oriented therapeutic targets.

Virus infection is equivalent to a network perturbation, in that viruses have evolved effective strategies to manipulate multiple signalling pathways and induce crosstalk and feedback loops among pathways to form a network (Garmaroudi et al., 2010). In fact, viruses activate signal-transduction networks through multiple independent events, which include viral docking to receptors, viral protein synthesis, viral progeny release and virus-induced inflammatory responses (Tam, 2006). Thus, to properly understand how signal-transduction networks are disrupted by viruses, a global multivariate approach is required (Ideker et al., 2001; Kleppe et al., 2006).

One of the major challenges of defining a signal-transduction network model in the context of a disease is to study a holistic picture of molecular structures, coming together to make complex and dynamic networks. New tools are required to systematically perturb and monitor signalling processes and functions within cells. Indeed, the emergence of high-throughput, extremely parallel technologies enable biologists to monitor multiple cellular components all together (Albeck et al., 2008). These tools have provided researchers the opportunity to collect comprehensive and large datasets. Nowadays, in the era of "new biology" researchers are 'drowning' in data, in that one emerging challenge is how to organize, interpret and extract pertinent information. The complex web of death pathways discussed in section 2.3 or the complex nature of the immune response discussed in section 3.0, all argue a need for higher level analysis through machine learning. Though this analysis requires skilled computational biologists, using mathematical, statistical and computational techniques to put together the biological components into functional molecular and cellular network models in a systematic fashion (Janes and Yaffe, 2006).

One work that has emerged from recent studies of networked systems in the virus-infected host cell revealed ~260 host cellular factors, affecting virus infection (Brass et al., 2008), however only a small subset of these proteins played a role in the early stage of virus infection. Interestingly, a complementary study showed how a multi-parameter approach could unravel host-virus protein interactions that likely act as a network to facilitate the early steps of HIV-1 infection (Konig et al., 2008). Recently, small-molecule inhibitor pairs were used to perturb pairs of phospho-proteins to reveal causal mechanisms within the signal-transduction network response of cardiomyocytes to coxsackievirus B3 (CVB3) infection. Hierarchical cluster analysis of the resulting dataset showed that paired-inhibitor data was required for accurate predictions of the network. In this study we also depicted a high-confidence network based on partial correlations, which identified phospho-IκBα as a central "hub" in a measured phosphorylation signature (Garmaroudi et al., 2010). Now, biologists are poised to understand the network mechanisms in virus infection, in that these networks might be used to improve patient diagnosis, monitoring, and treatment.

4.2 Proposing novel therapeutic targets for viral myocarditis through systems biology analysis of host cell signal-transduction networks

As we have already discussed, cardio-tropic viruses like CVB3 can directly and indirectly further the progression of viral myocarditis to heart failure [(McManus et al., 1993) reviewed in (Marchant et al., 2008)]. To date, there is no curative treatment beyond heart transplantation for viral myocarditis-associated heart failure. Therapies that have been used in patients with myocarditis are immune serum globulin and pleconaril (Pevear et al., 1986; Rotbart, 1999). The anti-picornaviral agent, pleconaril perturbs viral uncoating and in turn blocks viral attachment to host cell receptors. Although, directly targeting viruses has been successful in controlling viral diseases, it suffers from some serious weaknesses, including failure to eliminate chronic viral myocarditis, narrow spectrum of action, and the inherent capacity to force outgrowth of drug resistance mutations (Tan et al., 2007). Thus, the discovery of novel antiviral targets, host cell-based antiviral agents is a more promising approach and deserves more attention (Saladino et al., 2010).

At the cellular level, studies have shown that host cell phospho-proteins essential for viral replication, are potentially targetable [(Marchant 2009) reviewed in (Marchant et al., 2008)]. It is known, however, that phospho-protein networks embrace a system that contains redundant, convergent and even distinct signaling pathways (Borisy et al., 2003). Such combinative properties of signaling networks may counteract the therapeutic efficacy of highly selective drugs due to signalling pathway redundancy. Thus, combination therapy may be necessary to achieve efficacy due to less treatment resistance (Fitzgerald et al., 2006). However, motivation for this initiative, therapeutic synergy of a combination is tempered by concerns about introducing synergistic side effects. Nevertheless, an *in vivo* study that has emerged from recent studies of the healing process in a rat asthma model showed that combining drugs performed better than single ones (Lehar et al., 2009).

Together, to propose novel and promising drugs and therapies requires us to understand network mechanisms at the cellular, tissue and organism level. Using high-throughput technologies in genomics, proteomics, phosphoproteomics, metabolomics and lipidomics, we can develop interactome network models, in that these models ultimately translate to network analysis and data-driven questions in a specific disease context, that can be used to analyse a broad range of different states and disease contexts. There have been recent reports that used systems biology analysis tools in an attempt to untangle the web of kinase signalling pathways that are activated during CVB3 infection of cardiomyocytes (Garmaroudi et al., 2010). There is a current unclaimed niche of research that exists to use similar systems biology tools to analyse the pathways of cell death in order to better understand not only the dominant pathways of virus induced cell death but to also characterise the type of cell death that causes necrotic rupture of infected cells. To perturb specific molecules or biological processes in a combination as directed by network models can be a capable strategy to treat complex diseases.

5. Conclusion

In every facet of CVB3 replication there are a large number of pathways and proteins involved, forming a large network of pathways and complexity too large to analyse by conventional means. The large number of death pathways activated by CVB3 may not all be required to mediate lysis of the infected host cell. The question still remains as to whether there are pathways activated as a matter of coincidence or whether death pathways are all

consequential. There are also a large number of immune constituents involved in the clearance but also the pathogenesis of myocarditis, from naive T cells to regulatory T cell subsets and a large number of MMPs. Systems biology should prove useful in the near future to elucidate the interactions of proteins and pathways and show the truly pertinent proteins and pathways responsible for cell death, virus replication or MMP mediated tissue remodelling. New questions regarding pathway coincidence and consequence can be investigated. New methods of analysis should assist in the understanding of beneficial vs pathological arms of the immune system. With this knowledge we should be able to design highly useful and robust biomarkers, antivirals and drugs to help direct appropriate repair and remodelling.

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14

Impaired Cardiac Function in Viral Myocarditis

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1. Introduction

Viral myocarditis, the inflammation of the myocardium caused by viral infection, is an important cause of dilated cardiomyopathy (DCM) – a major cause of morbidity and mortality worldwide (Mason, 2003; Esfandiarei & McManus, 2008; Cooper, 2009). In North America, viral myocarditis and DCM together account for 20% of the sudden deaths and heart failure in children and adolescents (Okuni *et al.*, 1975; Drory *et al.*, 1991). To date, there is no effective therapeutic against these diseases. Patients diagnosed with late stage DCM are limited to supportive treatments such as ventricular assist device implantation and heart transplantation.

The clinical presentation of viral myocarditis comes in various severities. Most people have contracted and subsequently recovered from multiple viral infections of the heart without overt symptoms. Yet, retrospective studies revealed that ~20% of subclinical cases later develop congestive heart failure. In addition, some may experience acute fulminant viral myocarditis or persistent chronic myocarditis symptoms. About one-third of these patients with viral myocarditis subsequently develop DCM (Esfandiarei & McManus, 2008). A combination of new diagnostic technologies for viral myocarditis such as cardiovascular magnetic resonance techniques with conventional diagnostics including clinical presentation, histopathological examination, cardiac antibody assessment, and viral polymerase chain reaction (PCR), now helps better define disease stage and its respective management protocol (Baughman, 2006).

The presence of viral genome in the myocardium is associated with significantly worse outcome over two years (Why *et al.*, 1994). Analysis of human failing hearts by PCR unveiled trails of previous viral infection. The identified viruses include enterovirus, adenovirus, parvovirus B19, herpes simplex virus 6, cytomegalovirus, hepatitis C virus, and human immunodeficiency virus, which are clinically associated with viral myocarditis (Grist & Reid, 1997; Calabrese *et al.*, 2010). Among them, coxsackievirus B3 (CVB3), an enterovirus in the picornavirus family, is highly implicated in clinical cases of viral myocarditis, particularly in neonates and young children, and is the most thoroughly studied causative agent in experimental viral myocarditis models (Froeschle *et al.*, 1966; Abelmann, 1971; Reyes & Lerner, 1985; McManus *et al.*, 1988). CVB3 replicates rapidly in short infection cycles that begin with viral receptor engagement and subsequent internalization, followed by translation of viral RNA, amplification of viral genome, viral assembly, and complete with viral progeny release.

CVB3 infection of myocarditis susceptible mice results in severe heart failure. The disease progression of viral myocarditis in the experimental infection model can be classified into three phases: acute (viremia), subacute (inflammatory), and chronic (remodeling) phases. The acute (viremia) phase is signified by active viral replication and direct virus-induced cardiomyocyte damage. The subacute (inflammatory) phase is characterized by the infiltration of immune cells that helps viral clearance but nonetheless adds to myocardial damage. The chronic (remodeling) phase is featured by the continual efforts of the impaired heart to meet the hemodynamic demand by remodeling the myocardium. Cardiac hypertrophy is triggered during remodeling to compensate for reduced contractile function due to myocyte loss and interstitial fibrosis in the earlier phases. However, such an adaption is unsustainable in the longer term in face of increasingly hostile environments, i.e. reduced blood supply and increased reactive oxidative stress, thus leading to cardiomyocyte death and triggering further inflammation and fibrosis. The pathological remodeling process eventually leads to DCM and heart failure.

This book chapter focuses on the virus-host protein interactions in cardiomyocytes during viral myocarditis. We discuss the role of virus-induced protein cleavage and dysregulation of the host protein degradation systems in the pathogenesis of viral myocarditis and its subsequent progression to DCM.

2. Host protein cleavages by coxsackieviral proteases contributing to cardiac dysfunction

2.1 Viral proteases and dilated cardiomyopathy

CVB3 encodes two viral proteases, 2A and 3C, both of which are cysteine proteases that have chymotrypsin-like activity and play critical roles in successful viral replication (Chau *et al.*, 2007). First, the viral proteases are required to process the large viral polyprotein, a product of mono-cistronic translation of RNA genome, into the individual functional, structural and non-structural proteins. Second, the viral proteases facilitate viral replication by cleaving a number of host proteins that are involved in various cell functions such as transcription, translation, cell signaling, and cellular structure.

Viral myocarditis is originally thought to be an immune response driven disorder. The initial observation that established the importance of direct virus-mediated myocardial damage in the pathogenesis of viral myocarditis was made in severe combined immunodeficient (SCID) mice, which lack functional T and B lymphocytes and yet developed early and severe myocyte damage upon enterovirus challenge (McManus *et al.*, 1993). The significance of viral proteins in the development of viral myocarditis and DCM was further explored by Dr. Knowlton's research group. First, they showed that the cardiac-specific expression of a replication-restricted CVB3 mutant genome in transgenic mice, which only allows the expression of viral proteins without generating viral progenies and subsequent immune response, results in DCM phenotype (Wessely *et al.*, 1998). Then, they demonstrated that mice with cardiac-restricted expression of viral protease 2A display a severe DCM phenotype (Xiong *et al.*, 2007). These findings suggest that viral proteases play an important role in the development of viral-induced dilated cardiomyopathy.

2.2 Cleavage of dystrophin during CVB3 infection may contribute to dilated cardiomyopathy

The observation that mouse cardiac expression of viral protease 2A induces DCM has been explained based on the landmark finding that dystrophin, which links the cytoskeleton to

the extracellular matrix (ECM) by forming the dystrophin glycoprotein complex (DGC), is cleaved during CVB3 infection by protease 2A (Badorff *et al.*, 1999; Badorff & Knowlton, 2004) (Fig. 1A). Dystrophin has three domains that serve different purposes. Its N-terminal domain anchors to the actin cytoskeleton and its rod domain provides the linkage to the C-terminal domain, which binds to β-dystroglycan that in turn connects to the sarcolemma and the extracellular matrix (Fig. 1A). Furthermore, dystrophin-deficient mice have been shown to have an increased susceptibility to viral myocarditis and develop severe cardiomyopathy (Xiong *et al.*, 2002). Human genetic mutations of the dystrophin gene cause Duchenne Muscular Dystrophy (DMD) (Nigro *et al.*, 1990). Approximately 20% of DMD patients suffer and die from a resultant cardiomyopathy. Other mutations of the dystrophin gene also cause X-linked DCM (Ferlini *et al.*, 1999). CVB3-induced dystrophin cleavage occurs at its 3′ hinge and therefore breaks its connection to the ECM. As a result, the sarcolemmal integrity is compromised and force transmission is reduced. This can further lead to cardiomyocyte necrosis due to the increased sarcolemmal permeability (Fig. 1A). Thereafter, dystrophin cleavage has been viewed as a major mechanism in enteroviral cardiomyopathy. However, dystrophin knockout mice (Mdx) display only mild cardiomyopathy phenotype, due to the compensatory upregulation of utrophin, a dystrophin homologue (Deconinck *et al.*, 1997; Grady *et al.*, 1997). Thus, other mechanisms may also contribute to the severe cardiomyopathy phenotype in viral protease 2A expressing mice.

2.3 Cleavage of serum response factor by viral protease 2A is associated with impaired cardiac function

Recent efforts have demonstrated for the first time that serum response factor (SRF) is cleaved in CVB3-infected mouse hearts and cultured murine cardiomyocytes (unpublished). SRF, which belongs to the MADS-box (MCM1, Agamous, Deficiens, and SRF) protein superfamily, is a muscle-enriched transcription factor that regulates the expression of contractile and regulatory genes, as well as microRNAs (miRNAs) (Miano, 2003; Niu *et al.*, 2007; Oka *et al.*, 2007) (Fig. 1B). SRF interacts with tissue specific cofactors such as myocardin, Nkx2.5, c-Fos, and binds to the serum response element (SRE) of its target genes. It contains two major domains: the N-terminal DNA binding and dimerization domain and the C-terminal transactivation domain (Fig. 1B). Genomic studies have identified over 1200 SRE containing genes and more than 250 of these have been verified (Sun *et al.*, 2006). Cardiac contractile genes under SRF regulation include cardiac α-actin, β-myosin heavy chain, myosin light chain, cardiac troponin I, etc. SRF is indispensable for mesoderm formation and plays a central role in cardiac development and function (Niu *et al.*, 2007). Therefore, SRF knockout results in embryonic lethality (Parlakian *et al.*, 2004). The construction of cardiac-specific inducible SRF knockout transgenic mice overcomes this problem and helps illustrate the importance of SRF in cardiac function (Parlakian *et al.*, 2005). It was shown that SRF knockout in the adult mouse heart results in damaged cardiac function, and subsequent progression to DCM. Genetic mutations of SRF in humans have not been described likely due to the associated lethality. However, the expression of alternatively spliced SRF isoforms, which was shown to have inhibitory effects on wild-type SRF, is increased in failing human and animal hearts (Davis *et al.*, 2002). Furthermore, a cleaved form of SRF lacking the transactivation domain was also found in human failing hearts as a result of caspase-3 activation during cardiomyocyte apoptosis (Chang *et al.*,

2003). This caspase-cleaved SRF fragment functions as a dominant-negative mutant that inhibits SRF-dependent activation of cardiac genes. On the other hand, SRF overexpression in transgenic mouse hearts leads to the development of cardiac hypertrophy and subsequent cardiomyopathy (Zhang *et al.*, 2001).

During CVB3 infection, SRF is cleaved into a ~47kD N-terminal fragment (SRF-N) and a ~20kD C-terminal fragment (SRF-C) by viral protease 2A (unpublished). As a result, the DNA-binding domain is detached from the transactivation domain, thus abolishing SRF function. In addition, the SRF-N fragment generated from the cleavage can compete with wild-type SRF for target gene binding, and thereby exhibits dominant-negative suppression of SRF target gene expression (Fig. 1B). This study suggests another important mechanism by which CVB3 damages cardiac function and leads to subsequent DCM. Further research using knock-in transgenic mice expressing non-cleavable SRF will help clarify the relative contribution of SRF cleavage in the pathogenesis of viral myocarditis.

2.4 Caspase activation by viral proteases

Both viral proteases 2A and 3C lead to late onset of host cell apoptosis through the direct cleavage of caspase-8 and the activation of caspase-3, as well as the cleavage of anti-apoptotic protein Bid that leads to mitochondrial cytochrome c release and subsequent initiation of the caspase cascade (Chau *et al.*, 2007). Cardiomyocyte apoptosis is a common phenomenon in various cardiac diseases. Apoptosis, also known as programmed cell death, is the self-destruction pathway activated when host cells decide to commit suicide. Apoptosis during viral myocarditis caused by viral protease-induced caspase activation, however, is "switched on" intentionally by viral mechanisms for the release of mature viral progenies. In cell culture, caspase activation results in cytoplasmic proteolysis and DNA fragmentation. However, despite ultrastructural evidence of cytochrome c release detected in many cardiomyocytes of heart failure patients, intact nuclei are seen in all of these myocytes (Narula *et al.*, 1998). This suggests that the terminally differentiated cardiomyocytes have evolved strategies to resist nuclear fragmentation despite ongoing cytoplasmic apoptosis. In fact, the number of apoptotic myocytes in a cardiomyopathic heart ranges only from 0.07% to 0.7% as compared to 0.003% in the normal myocardium (Narula *et al.*, 1998). Nevertheless, cytoplasmic apoptosis initiated during viral myocarditis may compromise mitochondrial ATP generation, as well as cause destruction to contractile proteins which add to systolic dysfunction in the disease pathogenesis.

2.5 Other mechanisms

Other mechanisms that viral proteases use to impair cardiomyocyte function include the interference of host gene transcription by the cleavage of cyclic AMP response element binding protein (CREB) (Yalamanchili *et al.*, 1997), the disruption of host protein translation through the cleavage of eukaryotic translation initiation factor 4γ (eIF4γ) (Chau *et al.*, 2007) and eIF5B (de Breyne *et al.*, 2008), the interception of cell signaling pathways via the cleavage of RasGAP (Huber *et al.*, 1999), and the weakening of the cytoskeletal network by the cleavage of cytokeratin-8 (Seipelt *et al.*, 2000). Although there are no known cardiac diseases associated with the aforementioned proteins, their cleavages exert additive effects to the final detriment of the infected cardiomyocytes.

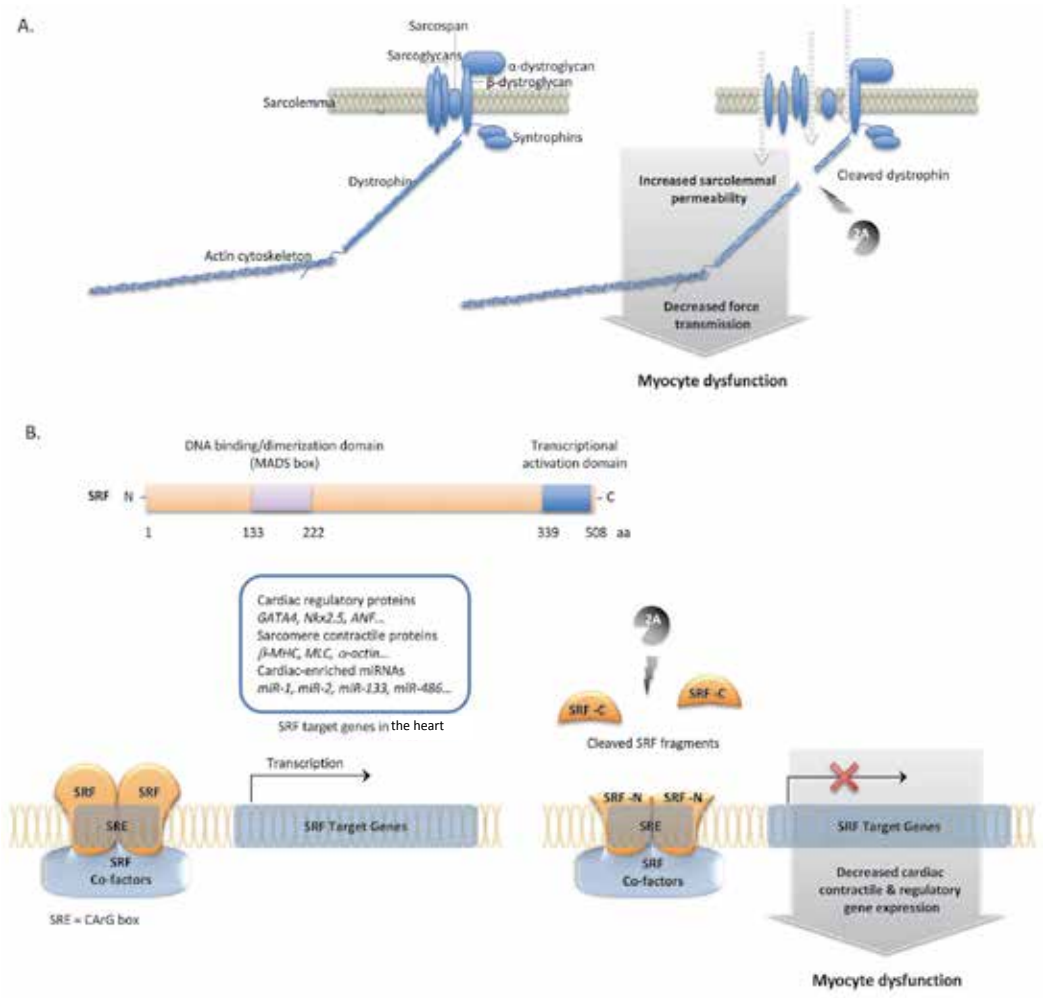

Fig. 1. Host protein cleavages by coxsackieviral proteases in viral myocarditis.
A. Coxsackieviral protease 2A cleaves dystrophin at its 3' hinge. Dystrophin is a component of the dystrophin-glycoprotein complex that links the cytoskeleton to the extracellular matrix. Dystrophin cleavage contributes to myocyte dysfunction by reducing contractile force transmission and increasing sarcolemmal permeability.
B. Viral protease 2A also cleaves serum response factor (SRF). SRF is a muscle-enriched transcription factor that regulates the expression of cardiac regulatory proteins, sarcomere contractile proteins, as well as cardiac-specific microRNAs (miRNAs). SRF associates with co-factors such as GATA4, Nkx2.5, MEF2, and myocardin and binds to serum response element (SRE) (also known as CArG box) to activate gene transcription. SRF cleavage results in myocyte dysfunction by the dissociation of the N-terminal DNA binding/dimerization domain from the C-terminal transcriptional activation domain, thus abolishing SRF-mediated gene expression. Furthermore, the N-terminal fragment (SRF-N) exhibits a dominant-negative effect on endogenous SRF function by competing for DNA binding.

3. Dysregulation of host protein degradation systems in viral cardiomyopathy

3.1 Protein degradation systems

Protein degradation is an integral part in the maintenance of cellular homeostasis. It allows for the selective removal of host proteins that are misfolded, damaged, and unnecessary, and balances the ongoing protein synthesis that is driven to provide the desirable cellular function in response to environmental changes. The dynamic interaction between protein synthesis and degradation is of particular importance to cardiomyocytes, the basic contractile units of the heart, due to the plasticity of the heart. It drives the hypertrophy or atrophy of individual cardiomyocytes, which contributes to sufficient contractile force generation that meets the hemodynamic demands. Consequently, the dysregulation of protein degradation jeopardizes cardiomyocyte vitality and function and plays an important role in the development of various cardiac diseases (Zheng & Wang, 2010).

Enteroviral infection leads to the dysregulation of host protein degradation pathways, which include the ubiquitin/proteasome system (UPS) and autophagy. Such viral-induced manipulation plays key roles not only in viral propagation, but also in the pathogenesis of viral myocarditis and the subsequent development of DCM. The following sections discuss the contributions of UPS and autophagy in viral propagation and the development of cardiomyopathy.

3.2 The ubiquitin/proteasome system

The ubiquitin/proteasome system is the major protein degradation pathway in eukaryotic cells that accounts for ~80% of host protein recycling (Zheng & Wang, 2010). By controlling the longevity/half-life of most proteins (predominantly short-lived but also some long-lived proteins), the UPS extends its role beyond protein recycling and regulates most aspects of cellular functions. UPS substrate selectivity is achieved by protein polyubiquitination, i.e. the attachment of ubiquitin (Ub) molecules onto the target protein. Ubiquitin is a small 76 amino acid protein modifier molecule that conjugates to target protein or to another Ub through one of its seven lysine residues. Ubiquitin linkage at different lysine residues serves different functions. For example, polyubiquitination via lysine 48 targets protein for UPS degradation, polyubiquitination via lysine 63 promotes signal transduction or targets degradation through the autophagy pathway, whereas monoubiquitination modulates protein intracellular localization and protein function.

Protein ubiquitination is regulated in a multi-step manner (Hershko & Ciechanover, 1998) (Fig. 2A). First, Ub is activated by the ubiquitin-activating enzyme (E1) using ATP. Then, it is transferred to ubiquitin-conjugating enzyme (E2). Finally, it is conjugated onto the target protein selectively brought in by the ubiquitin ligase (E3). A polyUb chain is formed by repeating the ubiquitination process. The expression of these important enzymes that regulate protein ubiquitination may change according to physiological stimuli. For instance, in cardiac atrophy mouse model, the E2 enzyme UbcH2 expression is upregulated in atrophic hearts to increase the capacity of protein degradation (Razeghi *et al.*, 2006).

The 26S proteasome is composed of the 20S proteolytic core and the 19S proteasome activator lid(s) (Luo *et al.*, 2010). The 20S proteolytic core is made up of two outer (α-subunits) and two inner (β-subunits) rings. It contains caspase-like, trypsin-like, and chymotrypsin-like protease activities conveyed by subunits β1, β2, and β5, respectively. The 19S lid(s), also known as proteasome activator 700 (PA700), helps the recognition and

docking of polyubiquitinated target protein. 19S also serves to detach and hence recycle the Ub by its deubiquitinating enzyme (DUB) activity. Furthermore, 19S unfolds the target protein and feeds it to the 20S core for degradation.

The immunoproteasome is an alternative version of the proteasome expressed to accommodate inflammatory responses upon stimulation with interferon-γ (Rivett & Hearn, 2004). The immunoproteasome has a 20S core that substitutes the constitutive catalytic β-subunits with inducible β-counterparts (β1i, β2i, and β5i), which offer different proteolytic function and activity to generate small peptides suitable for antigen presentation by major histocompatibility complex (MHC) class I (Griffin *et al.*, 1998) (Fig. 2A). In addition to the 19S proteasome, the immunoproteasome can also have a different lid(s) – the 11S proteasome, also known as PA28 (proteasome activator 28). Different compositions of 11S exist: heteroheptamer of PA28α and PA28β that are induced by interferon-γ under intensified immune response (Murray *et al.*, 2000) and homoheptamer of PA28γ that resides in the nucleus and assists ATP- and ubiquitin-independent proteasomal activity (Mao *et al.*, 2008). Sometimes, hybrid proteasomes with both 11S and 19S lids are also observed. However, their functions remain to be explored.

3.2.1 The UPS and heart diseases

UPS dysregulation is a common phenomenon in heart diseases. It is accentuated with the accumulation of Ub-protein conjugates and is associated with markedly reduced proteasome proteolytic activity in failing human hearts as compared to non-failing hearts (Predmore *et al.*, 2010). This suggests that ubiquitinated proteins in hearts are not degraded due to impaired proteasomal function. While no changes were noted in protein expression of proteasome subunits (i.e. 20S, 19S, 11S), elevated levels of protein carbonyls and 4-hydroxynonenylated proteins were observed in failing hearts. Also, oxidative modification to the 19S ATPase subunit Rpt5 was found in these failing hearts. Together, these oxidative modifications to proteasome subunits and substrate proteins may lead to impaired proteasomal function. On the other hand, microarray studies demonstrate reduced transcript levels of some 20s α- and β-subunits in the failing hearts as compared to controls (Hwang *et al.*, 2002; Kaab *et al.*, 2004). The incongruence between protein and mRNA expression of proteasome subunits may be attributed to myocyte loss and fibrosis in the failing hearts.

Animal models of cardiac diseases also have an increased ubiquitinated protein expression, but are acommpanied with changes in their proteasome expression profile. Upregulation in protein expression of proteasome subunits was observed in a left ventricular hypertrophy mouse model (Depre *et al.*, 2006). Post-translational modifications of the proteasome subunits were also reported in these hypertrophic hearts (Depre *et al.*, 2006). Treatment with proteasome inhibitor effectively prevents cardiac hypertrophy development, suggesting that the upregulation of proteasome expression is central to this physiological adaptation. Similar beneficial effects of proteasome inhibition in the regression of cardiac hypertrophy were observed in other studies (Meiners *et al.*, 2008; Stansfield *et al.*, 2008). Besides hypertrophic cardiomyopathy, the accumulation of Ub-conjugated proteins was observed in hyperglycemia-induced cardiomyopathy mouse model (Powell *et al.*, 2008). A parallel drop in the basal ATP-dependent proteasomal activity was observed in these mice. However, an increased ATP-independent chymotryptic proteasomal activity was observed, which is accompanied by an increased expression of 11S lid subunits PA28α and PA28β, as well as

the 20S subunits α3 and β5. Together, these data suggest a shift to immunoproteasomal activity is induced under hyperglycemic stress conditions to help the degradation of accumulated proteins.
The difference in cardiac proteasome expression profiles between human heart failure and various cardiomyopathy mouse models can be attributed to the limited time frame of animal studies. It is likely that stressed human hearts also induce the compensatory upregulation of proteasomal expression and activity at the earlier disease stages, but fail to maintain these changes over time.

3.2.2 The UPS and viral myocarditis

The UPS is also dysregulated in viral myocarditis (Fig. 2A). Accumulation of Ub-protein conjugates, as in other cardiomyopathies, was observed in CVB3-infected mouse hearts and cultured cells (Luo *et al.*, 2003; Gao *et al.*, 2008; Si *et al.*, 2008). The expression of several enzymes in the UPS pathway such as E1 enzyme E1A/E1B, E2 enzyme UbcH7, and DUB UCHL1 (ubiquitin carboxyl-terminal hydrolase L1) is upregulated in CVB3-infected mouse hearts, while ATP-dependent proteasomal activity is unaltered (Gao *et al.*, 2008). *In vitro* application of proteasome inhibitors such as MG132 (Luo *et al.*, 2003), lactacystin (Luo *et al.*, 2003), pyrrolidine dithiocarbamate (PDTC) (Si *et al.*, 2005), and curcumin (Si *et al.*, 2007) effectively attenuates viral RNA replication and protein synthesis. In addition, depletion of Ub by RNA interference also inhibits viral replication (Si *et al.*, 2008). Furthermore, it was shown that viral RNA-dependent polymerase 3D is ubiquitinated during viral replication, which may help its anchorage to intracellular membrane platforms required for the assembly of viral RNA replication machinery (Si *et al.*, 2008). *In vivo* administration of proteasome inhibitor to CVB3-infected mice also improves the outcome of viral myocarditis with reduced myocardial damage and inflammatory infiltration (Gao *et al.*, 2008). The viral titer, however, is not significantly reduced in the treated mice. This suggests that proteasome inhibitor treatment ameliorates viral myocarditis via multiple mechanisms: direct viral inhibition and immunomodulation. It was further demonstrated that the expression of 11S subunit PA28γ plays a role in CVB3 replication (Gao *et al.*, 2010). CVB3 infection leads to the redistribution of PA28γ from the nucleus to the cytosol, where it interacts with host proteins, such as tumor suppressor protein p53, and promotes their degradation via UPS. Overexpression of PA28γ enhances viral replication while its knockdown does the opposite.
Szalay et al. explored the involvement of the immunoproteasome in viral myocarditis. They found that the catalytic subunits of the immunoproteasome, LMP2 (β1i), LMP7 (β5i), and MECL-1 (β2i), are upregulated in CVB3-infected myocarditis-susceptible mouse hearts as compared to infected hearts from resistant mouse strains (Szalay *et al.*, 2006). Increased activity of the immunoproteasome in the susceptible myocardium helps generate the MHC class I-restricted peptide, boost antigen presentation and mount the subsequent adaptive immune response. A recent study demonstrates a differential immunoproteasome expression pattern between myocarditis-susceptible and -resistant mouse strains (Jakel *et al.*, 2009). In this study, immunoproteasome formation peaks early after CVB3 challenge in resistant mice, while it is postponed and expressed in greater extent in susceptible mice. The timing and magnitude of immunoproteasome activation determine in part the effectiveness of early viral clearance and the extent of direct viral-mediated damages, as well as the injury incurred during adaptive immune responses.

3.3 Autophagy

The other host protein degradation system is autophagy, i.e. "self-eating". It proceeds by the engulfing of a portion of the cytoplasm including long-lived and misfolded proteins and organelles by the autophagic membranes to form double-membraned vesicles called autophagosomes, followed by their delivery to lysosomes for degradation (Levine & Kroemer, 2008) (Fig. 2B). Autophagy is activated in two parallel cascades of enzymatic actions that are similar to the process of protein ubiquitination (Ravikumar *et al.*, 2010). First, Atg12 (autophagy-related gene 12) and Atg8 (also known as LC3, microtubule-associated protein light chain 3, in mammalian cells) are activated by the E1-like activating enzyme Atg7 using ATP. Then, Atg12 is conjugated to its E2 Atg10, while Atg8 is attached to another E2 Atg3. Atg12 is then transferred to its designated partner Atg5 forming the Atg12-Atg5 complex and further matures by the conjugation to Atg16. Finally, the Atg12-Ag5-Atg16 complex acts as an E3 to help Atg8 lipidation, forming the Atg8-PE (phosphatidylethanolamine) complex. Lipidation of Atg8 helps its incorporation onto the autophagic membrane. Atg8-PE then takes part in the elongation of the autophagic membrane and its enclosure to form the autophagosome.

3.3.1 Autophagy and cardiac diseases

Under baseline conditions, autophagy represents an important homeostatic mechanism. However, excessive activation of the autophagy machinery has been suggested to be involved in the pathogenesis of various disease conditions, including cardiac diseases. LC3 activation was observed early and was well-sustained in pressure-overload cardiomyopathy mouse model (Zhu *et al.*, 2007). Autophagy activation in this model promotes cardiac remodeling. Overexpression of Beclin-1, also known as Atg6, accentuates pathological remodeling and interstitial fibrosis, whereas heterozygous knockout of Beclin-1 improves systolic function and delays cardiac remodeling. In desmin-mediated cardiomyopathy mouse model, early activation of autophagy was observed well before any measurable decline in cardiac function (Tannous *et al.*, 2008). Autophagy pathway impairment by heterozygous inactivation of Beclin-1 leads to accumulation of polyubiquitinated protein aggregates, as well as acceleration to heart failure and early mortality (Tannous *et al.*, 2008). Similarly, autophagy is activated in both ischemia and subsequent reperfusion, but via two different initiation pathways (Takagi *et al.*, 2007). The AMPK pathway drives autophagy during ischemia, while Beclin-1 initiates autophagy upon reperfusion. Autophagy during ischemia is considered a cell survival response as it helps to sustain the starved cardiomyocytes during ischemia; however, autophagy during reperfusion is viewed as a pathological response as it promotes autophagic cell death.

3.3.2 Autophagy and viral myocarditis

Autophagy also plays an important part in the host innate defense system by direct sequestration of invading pathogens (bacteria, fungi, and virus) for clearance through lysosomal degradation (Jackson *et al.*, 2005). In addition, autophagy helps antigen presentation to class II MHC in order to mount an adaptive immune response (Dengjel *et al.*, 2005). However, this innate defense machinery is subverted by certain viruses to facilitate their replication (Jackson *et al.*, 2005; Wong *et al.*, 2008). It was shown that LC3-PE expression, a hallmark of autophagosome formation, is increased after CVB3 infection with dramatic reorganization of intracellular membranes (Wong *et al.*, 2008) (Fig. 2B). Inhibition

of autophagy by 3-methyladenine which targets the upstream signaling class III PI3-kinase, and by siRNA knockdown of Atg7 expression effectively block viral replication (Wong *et al.*, 2008). Recent work in mouse models also suggests that autophagy is activated *in vivo* after CVB3 infection (unpublished data). LC3-PE expression is elevated in CVB3-infected organs such as the heart, liver, and pancreas. Kemball *et al.* also reported the induction of autophagosome formation in pancreatic acinar cells in CVB3-infected mice (Kemball *et al.*, 2010). This theme of virus-induced autophagy activation is further extended to coxsackievirus B4-infected rat primary neurons (Yoon *et al.*, 2008). Nonetheless, virus-induced autophagy only serves to help viral replication without increasing protein degradation as suggested by the unchanged expression level of p62, a selective autophagy substrate, after virus infection (Wong *et al.*, 2008).

Subversion of the autophagy machinery by enteroviruses may contribute to the pathogenesis of viral myocarditis beyond impacting cardiomyocyte viability. Recent research demonstrates that cellular autophagy plays a role in nucleic acid-sensing toll-like receptor 3 (TLR3) signaling, which is necessary for the antiviral interferon pathway (Gorbea *et al.*, 2010). TLR3-deficient mice show vulnerability to CVB3 infection and develop acute myocarditis (Negishi *et al.*, 2008). Dysregulation of the autophagy pathway in CVB3-infected cardiomyocytes may interfere with TLR3-mediated antiviral response, resulting in compromised viral clearance and increased myocardial damage during viremia. In addition, autophagy is known to be a pro-survival response against apoptosis. The dysregulation of autophagy may decrease the viability of virus-infected cardiomyocytes because it cannot protect the host from virus-induced apoptosis. Furthermore, angiotensin II receptors type I & II (AT1 & AT2) regulate cardiomyocyte autophagy activity (Porrello *et al.*, 2009). AT1 expression triggers autophagy in neonatal cardiomyocytes as well as subsequent autophagic cell death, while AT2 expression counteracts AT1-induced autophagic activity. Further modulation by angiotensins may have an adverse effect on virus-infected cardiomyocytes as it may further activate autophagy, thus triggering autophagic cell death.

4. Potential therapeutics targeting viral proteases, UPS, and autophagy

The current knowledge of the roles of viral proteases and the host protein degradation systems in viral myocarditis may lead to new diagnostic and therapeutic approaches for the disease. Virus-induced SRF cleavage fragments may be utilized as a biomarker to detect acute phase myocarditis. Early diagnosis of viral myocarditis opens up the optimal timeframe for treatment. Successful medical interventions during acute infection can limit viral replication and its associated damage, limit viral spreading, as well as minimize the damage caused by immune activation. Since the viral proteases and the protein degradation systems all play important roles in viral propagation, a combinatorial therapy of highly specific viral protease inhibitors, proteasome inhibitors, and autophagy inhibitors during viremia would limit viral infection effectively. Furthermore, application of proteasome inhibitors and autophagy inhibitors provides additional benefits in immunomodulation to control the inflammatory response. On the other hand, viral myocarditis patients in the chronic phases may be managed differently. Since DCM patients have depressed proteasomal function, proteasome inhibitor treatment may further exacerbate myocardial damage. Moreover, long-term application of inhibitors against UPS will have adverse effects as demonstrated in the increased incidence of heart failure in cancer patients undergoing proteasome inhibitor treatment (Enrico *et al.*, 2007; Hacihanefioglu *et al.*, 2008).

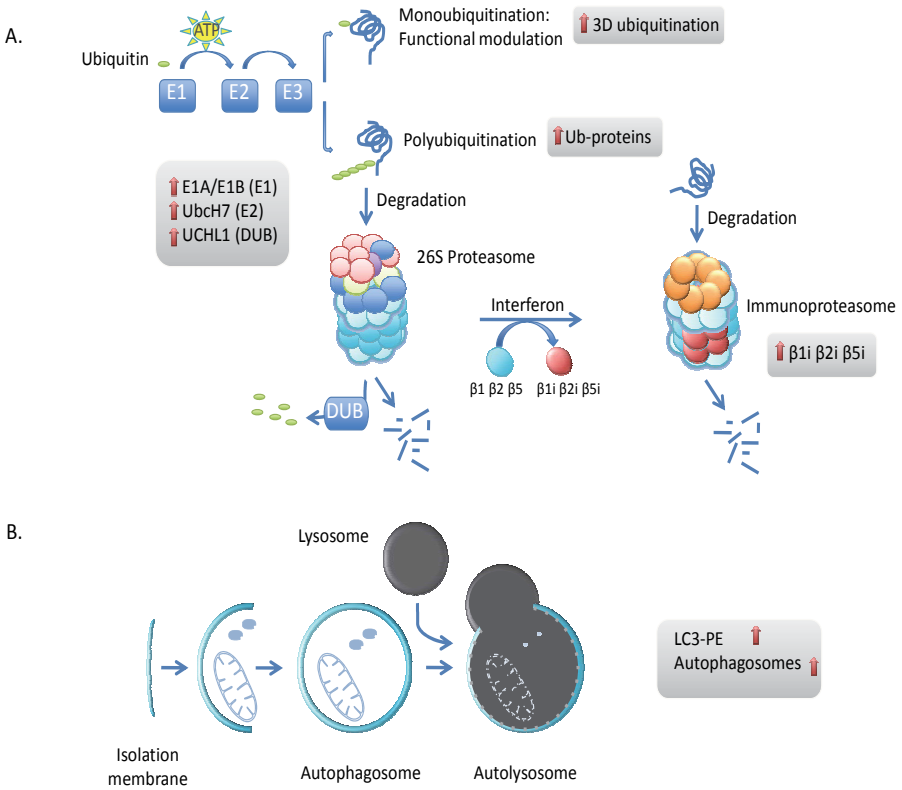

Fig. 2. Dysregulation of the host protein degradation systems in viral cardiomyopathy. The ubiquitin/proteasome system (UPS) and autophagy are the two major protein degradation mechanisms in eukaryotic cells. A. The UPS function by a cascade of enzymatic reactions (El - ubiquitin activating enzyme, E2 - ubiquitin conjugating enzyme, E3 - ubiquitin ligase) that conjugate ubiquitin, a small protein modifier, onto the target proteins. The type of ubiquitin conjugation linkage determines the target protein's fate: functional modulation or degradation. Polyubiquitinated target proteins are recognized by the 26S proteasome for degradation, whereas monoubiquitination serves to help endocytosis, endosomal sorting, DNA repair, histone regulation, and nuclear export. Ubiquitins are recycled by the deubiquitinating enzymes (DUBs). CVB3 infection causes the dysregulation of the UPS. An increased expression of ubiquitin-protein conjugates, E1A/E1B (El), UbcH7 (E2), UCH-L1 (DUB) was observed in CVB3-infected mouse hearts. Proteasome inhibitor application attenuates viral replication *in vitro* and reduces myocardial lesion and fibrosis *in vivo*. B. Autophagy begins with the enwrapping of organelles and cytoplasmic proteins by the isolation membrane which elongates and encloses to form a double-membraned vesicle called the autophagosome. The autophagosome fuses with lysosomes to degrade the sequestrated materials. Autophagy plays an important role in host defense by trapping and degrading invading pathogens. However, certain viruses including CVB3 evolve strategies to subvert autophagic mechanism to facilitate their own replication. Autophagosome formation is upregulated during CVB3 infection. Inhibition of the autophagy pathway has been shown to block viral replication *in vitro*.

5. Conclusion

Viral-induced protein cleavage and host protein degradation dysregulation play important roles in the pathogenesis of viral myocarditis and its subsequent progression to DCM. Identification of viral-induced protein cleavage fragments may allow early diagnosis of viral myocarditis, which opens up the optimal treatment window. A combination of antiviral therapies including specific viral protease inhibitors, proteasome inhibitors, and autophagy inhibitors presents new strategies for effective early viral clearance and minimization of viral-induced, inflammation-associated damage. Further studies employing system-like approaches, such as ubiquitomics, degradomics, and RNAi screens, are required to decipher the complex interactions between host and virus during different stages of viral myocarditis. Efforts in clarifying the precise functions and regulatory mechanisms of the host protein degradation systems in the disease progression of viral myocarditis will lead to novel therapeutic targets to improve treatment in different disease stages.

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15

Experimental Autoimmune Myocarditis: Role of Renin Angiotensin System

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1. Introduction

Myocarditis is defined as myocardial inflammation allied with edema, cellular infiltration, apoptosis and necrosis of cardiomyocytes (Rosenstein et al., 2000). It is likely to be a complex disease and its etiology has been associated with various infections, systemic diseases, drugs, and toxins. Among them, a wide array of organisms, including viral, bacterial, rickettsial, fungal, and parasitic organisms have been implicated as causative agents (Feldman & McNamara, 2000). There are two types of myocarditis viz, lymphocytic and giant cell myocarditis.

Acute myocarditis must be considered in patients who present with recent onset of cardiac failure or arrhythmia, though the onset of clinical cardiac symptoms may be vague in many patients. Fulminant myocarditis is a distinct entity characterized by the sudden onset of severe congestive heart failure or cardiogenic shock, usually following a flu-like illness. Giant cell myocarditis is a rare, frequently fatal disorder of unknown origin characterized by the presence of giant cell inflammatory infiltrate in the myocardium with widespread necrosis and degeneration of myocardial fibers (Batra & Lewis, 2001). It may be associated with various systemic autoimmune diseases (Kuhl & Schultheiss, 2010).

Heart reactive autoantibodies are found in a high percentage of patients with myocarditis. Identified autoantigens include the β1 adrenoreceptor adenine dinucleotide translocator, branched-chain keto acid dehydrogenase, cardiac myosin, sarcolemmal and myolemmal proteins, connective tissue, and extracellular matrix proteins including laminin. Antigenic mimicry between the dominant self molecules and the infectious agents also contributes to the disease process (Caforio et al., 1997; Neumann et al., 1990; Maisch, 1989).

2. Experimental autoimmune myocarditis (EAM)

The EAM model has been extensively used as a disease model of human myocarditis (Kodama et al., 1990). Experimental data revealed several similarities between this model

and the original disease in human. The current EAM model will provide the opportunity for further fundamental research into myocarditis.
EAM is similar to the giant cell myocardits and is more likely to progress into dilated cardiomayopathy (DCM) (Kodama et al., 1990). EAM can be induced by injection of cardiac myosin with Freund's adjuvant in to the footpads of Lewis rats. The immunized rats become ill and immobile at day 14 and then their activity gradually recovers beginning in the fourth week. The diseased rats show severe myocardial damage with inflammatory cell infiltration. Rats with EAM that survive the acute phase develop postmyocarditis DCM after 4 months or more (Watanabe et al., 2001).
Rat EAM is characterized by its high morbidity and mortality (Kamal et al., 2010). Pericardial effusions, cardiac enlargement and discoloration of the cardiac surface are the macroscopic findings of EAM (Kodama et al., 1992). These findings are common in the autopsy of the patients with myocarditis and EAM Lewis rats but rarely reported in other experimental animal models of myocarditis.

2.1 Pathogenesis of EAM

In the rat model of EAM, cardiac myosin is one of the major inflammation inducing agents used commonly. It is composed of two heavy chains of about 2000 amino acids and four light chains. This protein acts as antigen and stimulates the inflammatory reactions in the rat especially targeting the myocardium. In the rat immune system, T cells recognize 10-20 amino acid residues, and B cells recognize 5-10 amino acid peptides as antigens. Amino acid residues 1539-1555 of the rat cardiac myosin α-chain would be the myocarditogenic epitope of EAM (Pummerer et al., 1996). Direct sub fragment analysis revealed that actually several myocarditogenic epitopes existed on cardiac myosin. The most effective epitope is present on the residues 1070-1165 of the porcine cardiac myosin β-chain (Inomata et al., 1995). Antigen-specific breakdown of self-tolerance due to the molecular mimicry of myocarditogenic epitopes initiates the autoimmune reaction (Jones et al., 1997). This is followed by the stimulation and proliferation of myocarditogenic T cells. Activated T cells secrete many cytokines, chemokines and other mediators, which recruit and activate other inflammatory cells. The inflammatory mediators damage the myocardium and interfere with the cardiac function (Smith & Allen, 1992; Goren et al., 1998; Ishiyama et al., 1998).
Administration of porcine cardiac myosin with complete Freund's adjuvant, which comprises of inactivated and dried *Mycobacterium tuberculosis* leads to antigen presentation of cardiac myosin-specific T cells in the peripheral lymphatic organs. Freund's adjuvant, an immunopotentiator, plays an important role in the cell-mediated immunity leads to the breakdown of self-tolerance by activation of antigen presenting cells, enhancement of the expression of major histocompatibility molecules as well as increases in vascular permeability. T cells produced de novo in the bone marrow play a major role in the pathogenesis of the autoimmune myocarditis (Bergelson et al., 1997). Release of cardiac myosin from the damaged heart leads to further activation of specific T cells. Autoantibodies developed against the myosin bind to the injured heart and destroy them (Fedoseyeva et al., 2002).

2.2 Role of inflammation in myocarditis

Cytokines play important roles in the pathogenesis of myocarditis (Ding et al., 2010; Yuan et al., 2010; Huang et al., 2009; Ingkanisorn et al., 2006). Interleukin (IL) -2 mRNA appears in

the EAM hearts at the onset of the disease (Okura et al., 1998). Subsequently, mRNA of IL-1β, interferon γ, and tumor necrosis factor (TNF) α increases (Maeda et al., 2005). In the recovery phase IL-10 appears in the heart. IL1, IL2, IL6 and TNFα are involved in the impairment of cardiac contractility, IL1 and IL6 may induce hypertrophy of myocytes whereas IL1 and TNFα may play a role in the development of myocardial fibrosis. Th2 cytokine IL-10 plays a protective role in the development of EAM (Watanabe et al., 2001). IL10 possesses immunomodulatory properties involving the inhibition of macrophage function and the production of proinflammatory cytokines (Nishio et al., 1999).
In rat EAM, infiltration of the inflammatory cells into the myocardium may be mediated by monocyte chemoattractant protein-1 (MCP-1) or other chemokines as MCP-1 mRNA is strongly expressed in the heart coincident with the onset of the disease and persists until the recovery phase (Goser et al., 2005). Nitric oxide may also play a role in the development of autoimmune myocarditis, by exacerbating inflammatory responses to cardiac infections (Kittleson et al., 2005).
Angiotensin (Ang) II, the principal effector peptide of the renin–angiotensin system (RAS), has been reported to induce immune and inflammatory response in various cardiac disease conditions, including atherosclerosis, hypertension, left ventricular hypertrophy, myocardial infarction, heart failure and myocarditis (Ferrario & Strawn, 2006; Schmieder et al., 2007).

3. Renin angiotensin system (RAS)

The RAS is a central element of the physiological and pathological responses of the cardiovascular system. Its primary effector hormone, Ang II, not only intercedes immediate physiological effects of vasoconstriction and blood pressure regulation, but is also implicated in inflammation, endothelial dysfunction, hypertension and heart failure (Opie & Sack, 2001). Many of the cellular effects of Ang II appear to be mediated by ROS generated by NAD(P)H oxidase (Koumallos et al., 2011). Two subtypes of Ang II receptors have been defined on the basis of their differential pharmacological and biochemical properties: Ang II type 1 receptors (AT1), which are involved in most of the well-known physiological effects of Ang II, and Ang II type 2 receptors (AT2), which have a less well-defined role but appear capable of counterbalancing some of the effects of AT1 stimulation. AT1 transactivates growth pathways and mediates major Ang II effects such as vasoconstriction, increased cardiac contractility, renal tubular sodium reabsorption, cell proliferation, vascular and cardiac hypertrophy, inflammatory responses, and oxidative stress. AT2 is believed to induce essentially opposite effects, including vasodilation, antigrowth and antihypertrophic effects, and to play a significant role in blood pressure (BP) regulation (Oudit & Penninger, 2011; Horiuchi et al., 1999; Matsubara, 1998; Siragy, 2000; Carey et al., 2001).
The discovery of Ang (1–7), an endogenous peptide which opposes the pressor, proliferative, profibrotic, and prothrombotic actions mediated by Ang II has contributed to the realization that the RAS is composed of two opposing arms: the pressor arm constituted by the enzyme angiotensin-converting enzyme (ACE), Ang II as the product, and the AT1 receptor as the main protein mediating the biological actions of Ang II; the second arm is composed of the monocarboxypeptidase ACE2, Ang (1–7) produced through hydrolysis of Ang II, and the Mas receptor as the protein conveying the vasodilator, antiproliferative, antifibrotic, and antithrombotic effects of Ang (1–7) (Petty et al., 2009; Ferrario, 2011).

Hypertrophy of cardiac myocytes is an adaptive response in the damaged heart. Initially, hypertrophy acts as a compensatory mechanism to preserve cardiac function, but when sustained, it becomes a major risk factor for congestive heart failure and sudden cardiac death. Until recently, most in vitro and in vivo studies of the roles of AT1 and AT2 indicated that AT1 mediates the growth promoting, fibrotic, and hypertrophic effects of Ang II on cardiovascular tissues and that AT2 exerts counterbalancing suppressant effects (Gao & Zucker, 2011). Evidence has been provided that the circulating and local RAS promote the development of myocardial fibrosis in hypertensive heart disease and chronic heart failure where both Ang II and aldosterone stimulate collagen synthesis in a dose-dependent manner while Ang II additionally suppresses the activity of matrix metalloproteinase 1, the key enzyme of interstitial collagen degradation, that synergistically leads to progressive collagen accumulation within the myocardial interstitium (Lijnen & Petrov, 2003). Therefore, the physiological role of RAS on the development of myocardial fibrosis could be established.

In addition to its role in the regulation of arterial pressure, Ang II is known to mediate effects on cell growth and apoptosis and to have pro-oxidative and proinflammatory effects. Apoptosis can be induced in cardiomyocytes by a variety of factors and pathways, a number of findings suggest that the effectors of the RAS can be critically involved in cardiomyocyte apoptosis (Fabris et al., 2011; Guleria et al., 2011; Yamada et al., 1996).

Peroxisome proliferator activated receptors (PPARs), members of the superfamily of ligand regulated transcription factors, are expressed in the cardiovascular system and control diverse vascular functions by mediating appropriate changes to gene expression. PPARα and PPARγ modulate the RAS by transcriptional control of renin, angiotensinogen, ACE and AT1 (Takeyama et al., 2000; Lansang et al., 2006).

4. Angiotensin receptor blockers (ARBs)

ARBs preferentially block AT1 and leave AT2 unopposed. Long-term administration of ARBs results in a several-fold increase in plasma Ang and thus a possible overstimulation of AT2. It is generally accepted that the effects of stimulation of AT2 on the cardiovascular system are beneficial and that no harm would result from increased activation of these receptors; indeed, activation of AT2 is believed to contribute to the benefits of blocking AT1 (Levy, 2004).

Various ARBs were screened for their role in the treatment of acute myocarditis and are found to have significant activity against acute myocarditis. ARBs prevent progression of systolic heart failure, thereby reducing cardiac morbidity and mortality (Lindholm et al., 2002; Cohn & Tognoni, 2001). They also reduce myocardial damage during myocarditis. The major cardiovascular actions of Ang II have been reported to be mediated by the AT1, and AT1 antagonists are therapeutically effective for the treatment of patients with heart failure by reducing cytokines and oxidative stress through their anti-inflammatory effects. Thus, the blockade of AT1 is an important way to interrupt the RAS (Sukumaran et al., 2010). Recently, an AT1 antagonist has been shown to ameliorate EAM by the suppression of myocardial damage and inflammatory events in the myocardium in addition to hemodynamic modifications, and it has been reported to inhibit nitric oxide (NO) production in macrophages and IL 1β production. ARB treatment decreased myocardial fibrosis and its marker molecules (i.e. RNA expression of TGF-β1 and collagen-III), and improved the survival rate and cardiac function in rats with DCM after myocarditis in a

dose dependent manner. Treatment with oral ARB improved both systolic and diastolic functions, increased neurohormonal parameter, such as plasma Ang II, and ameliorated myocardial remodeling and its marker molecules (Sukumaran et al., 2010, 2011a, 2011b; Shirai et al., 2005).

4.1 ARB and oxidative stress

EAM rats also suffer from various stresses including reactive oxygen species (ROS) mediated oxidative stress. There are several evidences for the adverse cardiac effects triggered by redox cycling of ROS, generated in part by an NADPH oxidase dependent pathway. Reports also add the role of Ang II in triggering the oxidative stress in which increase in the levels of NADPH oxidase subunits like gp91phox, NOX4, p22phox, p40phox, p47phox, p67phox, rac1 and 3-Nitrotyrosine in rat EAM. ARBs can block the myocardial oxidative stress in EAM evidenced by the decreased levels of these markers (Sukumaran et al., 2011b; Seko, 2006; Singh et al., 2008).

4.2 ARB and hemodynamics

Central venous pressure (CVP) and left ventricular end diastolic pressure (LVEDP) were significantly higher and mean blood pressure (MBP), LVP and +dP/dt were significantly lower in EAM rats indicating systolic and diastolic dysfunction. CVP and LVEDP were significantly decreased in the ARB treated EAM rats. Myocardial contractility parameters including intraventricular pressure change were improved in EAM rats treated with ARB. Echocardiographic analysis also showed the improvement of cardiac remodeling with ARB treatment evidenced by decreased LVDd and LVDs and increased fractional shortening and ejection fraction (Sukumaran et al., 2010; 2011a; 2011b; Shirai et al., 2005; Tsutsui et al., 2007).

4.3 ARB and cardiomyocyte apoptosis

Inappropriate apoptosis contributes to the pathogenesis of a number of cardiac diseases and is recognized as an important factor in cardiovascular remodeling. AT1 mediated cardiomyocyte apoptosis is due to the pathologic involvement of RAS where ARB can block the actions of Ang II on AT1 thereby preventing the cellular apoptosis in the myocardium. There were several reports indicating the myocardial apoptosis in the EAM rats and ARBs can effectively prevent it due to their action on RAS. For instance, ARB can block the endoplamic reticulum stress and caspase12 activation in the EAM rats thus prevents cardiomyocyte apoptosis (Singh et al., 2008; Tsutsui et al., 2007; Ye et al., 2010; Matsusaka et al., 2006) (Figure 1).

4.4 ARB and inflammation in EAM

AT1 antagonists are reported to suppress cytokine production and the transcription of cytokine genes in vitro and in vivo (Matsubara, 1998; Siragy, 2000; Carey et al., 2001). ARBs can decrease the expression of IFN-gamma (interferon-gamma), FasL (Fas ligand), iNOS (inducible nitric oxide synthase) and PFP (pore-forming protein) in myocardial tissue, indicating suppression of the activation of infiltrating killer lymphocytes (Seko, 2006). ARB administration downregulates Th1 cytokines (IFN-gamma and IL-2) while upregulating Th2 cytokines (IL-4 and IL-10). Thus, studies of RAS antagonists in inflammatory diseases suggested that Ang II was involved in immune and inflammatory responses and ARBs are useful candidates in preventing the inflammation associated with those disorders. In our lab

we have studied the action of ARB against EAM in rats. Out of 10 EAM rats used for treatment with ARB only 20% mortality was observed whereas control group showed 60% mortality. The disease severity was also decreased in the ARB treated group as shown by the less number of apoptotic cells, lesser fibrotic tissue replacement and also low level of inflammatory cellular infiltration when compared with the control group rats (Fig. 1).

5. Conclusion

ARBs, when added to conventional treatment for patients with heart failure, are associated with a reduction in morbidity and mortality as well as an improvement in quality of life. Clinical trials of ARB therapy indicate that these agents are generally well tolerated, both alone and in combination with other neurohormonal inhibitors (Patterson, 2003). Studies with ARB against EAM in rats provided several evidences for their protective role against the pathological alterations induced by Ang II (Figure 2). The treatment option with ARB against the cardiac complications involving Ang II has widened the area of cardiovascular research which will benefit the number of suffering population.

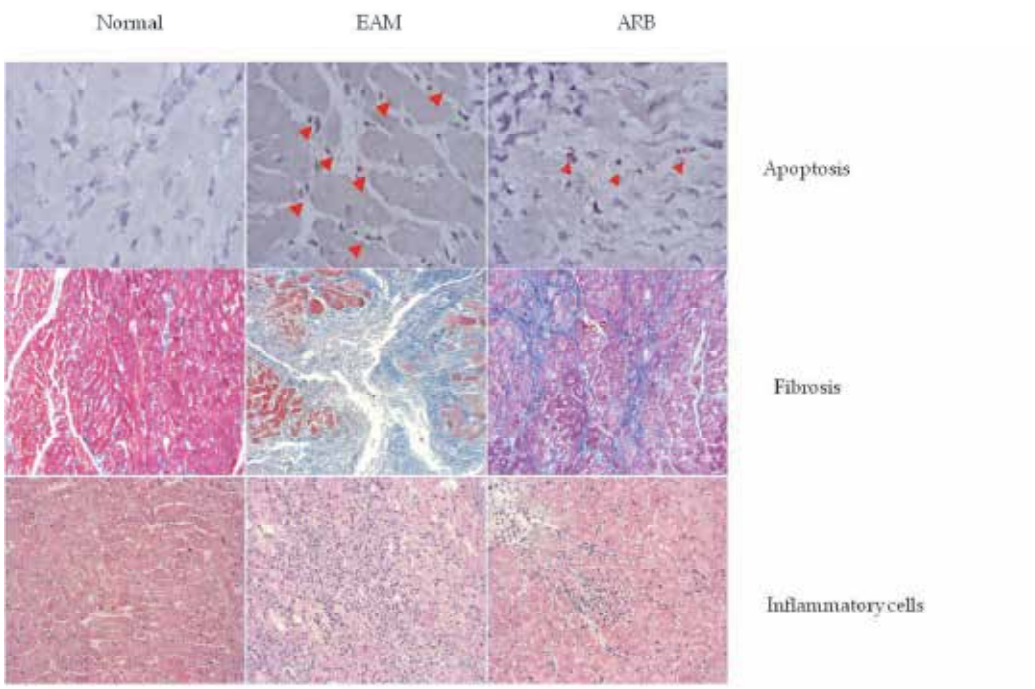

Fig. 1. Cross-sectional cardiac tissue slices with TUNEL staining depicting myocardial apoptosis, Azan-Mallory staining for fibrosis (blue area) and Hematoxylin and eosin staining depicting interstitial edema, vacuolization and degeneration of cardiac fibers respectively (X200). Normal, age-matched normal rats; EAM, Immunized rats without treatment; ARB, Immunized rats administered with ARB.

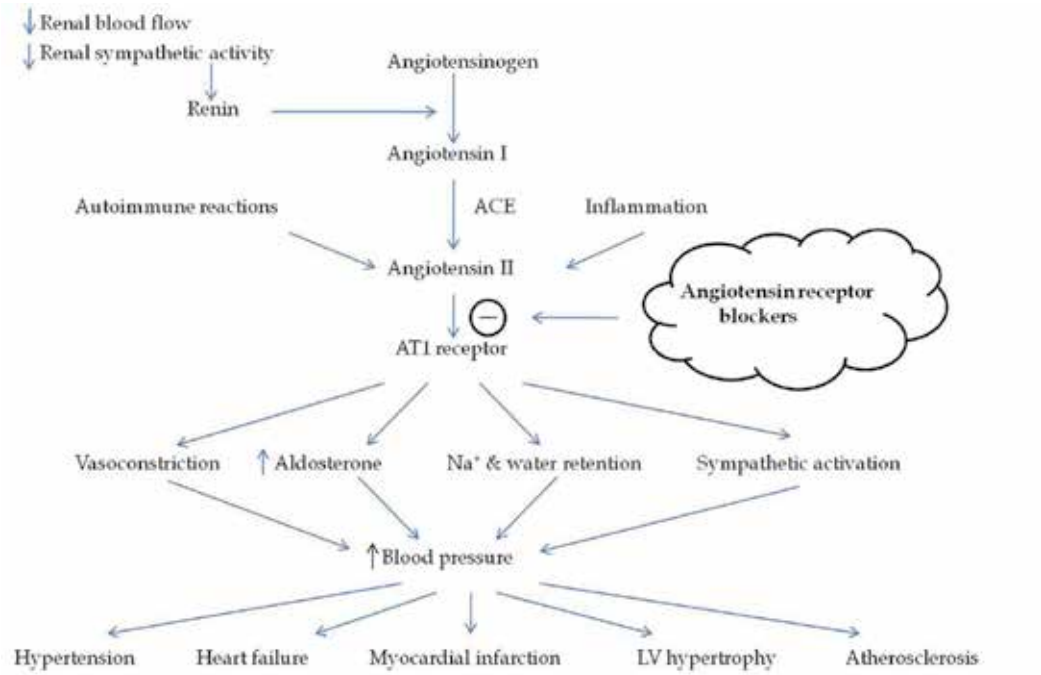

Fig. 2. Schematic representation of angiotensin pathway following angiotensin production. Angiotensin II binding to AT1 receptors leads to maintenance of homeostasis in normal physiology whereas pathological stimulation involves major cardiovascular complications. Treatment with ARB can potentially benefit the patients with these complications acting by blocking the actions of angiotensin II on AT1 receptors.

6. References

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Part 3

Recent Advances in Myocarditis

16

Biomarkers of Heart Failure in Myocarditis and Dilated Cardiomyopathy

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1. Introduction

There are many reviews that discuss the role of biomarkers in cardiovascular disease (CVD) and heart failure (Braunwald, 2008; Chen et al., 2010; Hochholzer et al., 2010), but little information exists regarding the presence and usefulness of biomarkers for heart failure in myocarditis and dilated cardiomyopathy (DCM) patients. Heart failure (HF) is the end consequence of many CVDs including atherosclerosis, myocardial infarction, myocarditis and DCM. CVD is the leading cause of morbidity and mortality in Western nations (Roger et al., 2011), and a growing concern worldwide (Gaddam et al., 2011). As treatments for CVD prolong survival, the prevalence of chronic HF has increased. It is now estimated that 5.8 million people in the United States live with HF and over 23 million worldwide (Bui et al., 2011). Approximately 550,000 new cases of HF are diagnosed each year, with a lifetime risk for developing disease of one in five (Chen et al., 2010; Krumholz et al., 1997). Hospitalizations for HF have also increased dramatically in the United States from 402,000 in 1979 to 2.4 million in 2007 with the cost of treating HF patients estimated at $39 billion annually (Bui et al., 2011; Chen et al., 2010; Roger et al., 2011). Biomarkers have become an increasingly important clinical tool for assessing CVD and progression to HF. Biomarkers are used in early detection of sub-clinical disease, diagnosis, risk stratification, monitoring disease state, and to determine therapies (Hochholzer et al., 2010). Many biomarkers are also risk factors directly involved in the pathogenesis of disease.

2. Heart failure

In spite of advances in diagnosis and treatment, HF remains a growing medical problem associated with major hospitalization, mortality and poor prognosis. Heart failure is characterized by significantly reduced cardiac output resulting in an inability to meet the metabolic needs of the body. Most cases of HF are caused by systolic dysfunction, or reduced myocardial contractile function, as occurs during ischemic injury, pressure or volume overload and DCM. However, HF can also occur because of an inability to relax, expand or fill the ventricle resulting in diastolic dysfunction as observed during myocardial fibrosis and constrictive pericarditis (Afanasyeva et al., 2004; Kumar et al., 2005). The prevalence of HF is higher in men than women and sex is a major risk factor along with age, hypertension, left ventricular (LV) hypertrophy, valvular heart disease, obesity and diabetes (Bui et al., 2011; Roger et al., 2011) (Table 1). The New York Heart Association (NYHA) has

defined four stages of HF in patients called the NYHA classification: class I) patients who have no symptoms or limitations in ordinary activities; class II) patients who have no symptoms in rest or in mild exercise, but symptoms appear with intense activity resulting in slight or mild limitations; class III) patients who have a marked limitation of activity, but no symptoms at rest; and finally class IV) patients who have symptoms when resting and who are restricted to bed or chair. This classification is still widely used in clinical practice but is not always a reliable guide to evaluate prognosis and therapy of patients with systolic HF (Hebert et al., 2011). Echocardiography is the imaging method most commonly used for the initial clinical assessment of patients with suspected HF because it is widely available, versatile, non-invasive, and has a low cost (Blauwet & Cooper, 2010). However, causative diagnosis cannot be established in a significant number of patients with HF despite significant advancement in echocardiographic techniques. Cardiac magnetic resonance imaging (MRI) is being used with increasing frequently and often provides additional information to echocardiography in patients with suspected or known HF (Blauwet & Cooper, 2010; Karamitsos & Neubauer, 2011; Olimulder et al., 2009). Although echocardiography and MRI are good at defining changes in volume, they do not assess pressure.

Nonmodifiable risk factors	Potentially controllable risk factors
Increasing age	Infections
Male gender	Inflammation
Family history	Obesity
Genetic abnormalities	Alcohol
Left ventricular hypertrophy	Stress
Myocardial infarction	Air pollution
Valvular heart disease	Diabetes mellitus
Hypersensitivity	Hypertension
	Tobacco smoking
	Hyperlipidemia

Table 1. Risk factors for developing heart failure. (Adapted from Bui et al., 2011 and Kumar et al., 2005)

The cardiac myocyte is generally considered to be a terminally differentiated cell that has lost its ability to divide or regenerate. Increased mechanical load causes an increase in the cellular content resulting in an increase in cell size termed hypertrophy (Kumar et al., 2005). The extent of hypertrophy varies depending on the underlying causes (Figure 1). Volume overload hypertrophy is characterized by dilation and an increase in muscle mass and wall thickness. Cardiac hypertrophy is also accompanied by many transcriptional and morphologic changes including generation of myofibroblasts, collagen deposition and fibrosis (Figure 1). Sustained hypertrophy and/or dilation may evolve to HF.

2.1 Myocarditis, dilated cardiomyopathy and heart failure

Myocarditis, or inflammation of the myocardium, leads to around half of all DCM cases in the United States (Roger et al., 2011). DCM is the most common form of cardiomyopathy requiring a heart transplant (Cooper, 2009, Wexler et al., 2009). Additionally, DCM contributes to approximately one third of all congestive HF cases (Jameson et al., 2005). The

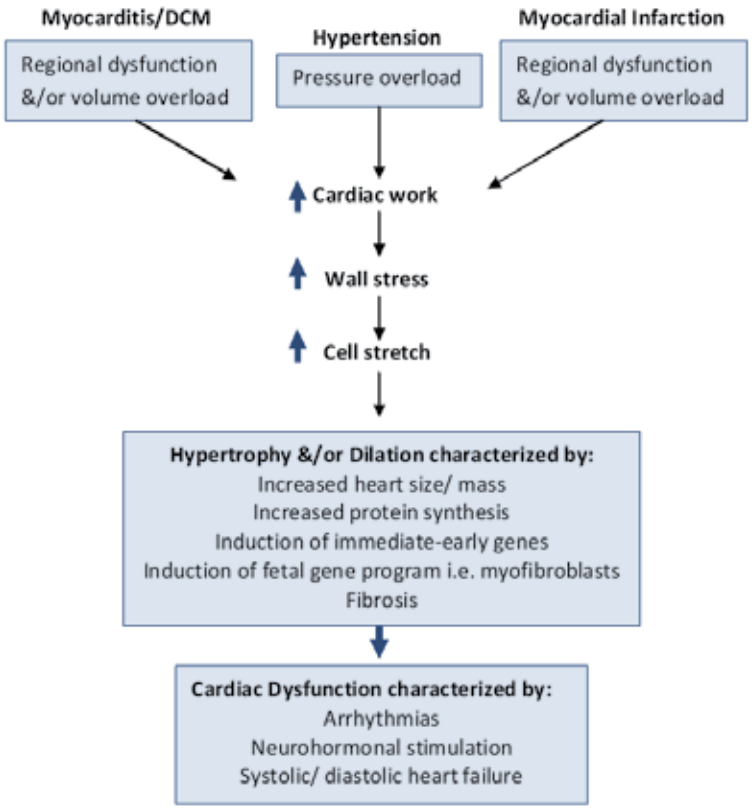

Fig. 1. Representation of the sequence of events leading to the development of heart failure. Viral infection or other causes of myocarditis lead to cardiac dysfunction resulting in increased cardiac work. Adapted from Kumar et al., 2005.

prognosis for patients with acute myocarditis varies but depends on ejection fraction (EF), clinical presentation and pulmonary artery pressure (Schultz et al., 2009). The life expectancy after diagnosis of myocarditis is 50% at 10 years and only 50% at 4 years after diagnosis of DCM (Grzybowski et al., 1996; Gupta et al., 2008). Over 20% of sudden deaths among young adults, the military, and athletes are due to myocarditis (Gupta et al., 2008). Similar to coronary artery disease and HF, myocarditis and DCM occur more frequently in men than women (Cooper, 2009; Roger et al., 2011). The clinical presentation of acute myocarditis in adults is highly variable often presenting as myocardial infarction or angina with nonspecific symptoms. Symptoms suggesting a viral infection including fever, rash, myalgias, arthralgias, fatigue, and respiratory or gastrointestinal symptoms frequently occur several days to weeks before the onset of myocarditis (Blauwet & Cooper, 2010). Electrocardiogram is widely used as a screening tool for myocarditis, but only detects about 47% of cases (Morgera et al., 1992). Echocardiography is useful for evaluating cardiac chamber size, wall thickness, systolic and diastolic function, and the presence of intramural thrombi in suspected myocarditis patients (Blauwet & Cooper, 2010). However, there is no specific echocardiographic feature for myocarditis, and patterns consistent with hypertrophy, DCM and/or ischemic heart disease can be observed in myocarditis patients. Cardiac MRI is an important noninvasive method to assess patients suspected to have acute

myocarditis. Unique features of myocarditis including myocardial edema, hyperemia, increased capillary permeability due to inflammation, and fibrosis can be identified using a combination of T1 and T2-weighted images (Blauwet & Cooper, 2010; Karamitsos & Neubauer, 2011). Endomyocardial biopsy is used to verify inflammation in the heart and to assess whether certain cell populations such as eosinophils or giant cells are present in the myocardium for diagnostic purposes (i.e. eosinophilic or giant cell myocarditis). Due to the focal nature of myocarditis and the fact that foci are frequently located in the peri/myocardium, endomyocardial biopsies often miss inflammation and so often do not aid in diagnosis (Maisch, 1994; Olimulder et al., 2009). Although the complication rate is low, patients are at a risk of death from the procedure.

3. Biomarkers in heart failure

Biomarkers are frequently used in cardiovascular medicine where they provide valuable information regarding diagnosis, treatment, identification of individuals at risk for HF, and potentially the pathogenesis of disease. For a biomarker to be clinically useful it should fulfill several criteria: 1) biomarker levels should be able to be accurately assessed using widely available and cost-efficient methods, 2) biomarkers should provide additional information from the tests already conducted such as MRI, and 3) biomarker information should aid in medical decision making (Braunwald, 2008; Morrow & de Lemos, 2007). A growing list of enzymes, hormones, markers of cardiac stress or necrosis, cytokines and other biological agents have been examined as possible biomarkers for HF (Table 2). Although biomarkers are discussed in this review by category (e.g. those associated with cardiac damage or inflammation), in reality many of these biomarkers interact or are associated with one another suggesting that combinations of biomarkers are likely to provide the best assessment of HF risk. This review will focus on HF biomarkers from Table 2 that have been studied in myocarditis/ DCM patients or experimental models.

3.1 Biomarkers of cardiovascular injury or stress

Myocyte injury can occur from many causes including infections, oxidative stress, inflammation or severe ischemia (Brauanwald, 2008). Cardiac myosin is typically not used as a biomarker for HF because it is rapidly cleared from the circulation. Cardiac troponin I and T are current standard biomarkers used to diagnose acute myocardial infarction and to stratify patient risk in acute coronary syndromes (ACS) because of their long half-life in the circulation (Hochholzer et al., 2010). B-type natriuretic peptide (BNP) and N-terminal pro-brain natriuretic peptide (NT-pro-BNP) are important indicators of cardiovascular stress having the advantage of being able to distinguish acute from chronic HF. Recently ST2 has emerged as another biomarker of cardiac damage that has prognostic ability in patients suspected of HF.

3.1.1 Troponins I and T

The cardiac troponins are proteins located in myocytes that are responsible for regulating cardiac muscle contraction. Cardiac troponin is composed of three subunits that are products of different genes- troponin C, troponin I and troponin T. Compared to myosin and actin, troponins are present at low levels in the heart. However, both troponin I and T are ideally suited to detect myocardial damage because they are expressed as cardiac-specific isoforms (Agewall et al., 2011). Elevated sera troponins are associated with

Biomarker
Myocyte inury
Cardiac-specific troponins I and T
Myosin light-chain kinase I
Creatine kinase MB fraction
Myocyte stress
Brain natiuretic peptide
N-terminal pro-brain natriuretic peptide
ST2/ interleukin-33
Inflammation
C-reactive protein
Tumor necrosis factor
Fas (APO-1)
Interleukins 1, 6, 18
Galectin-3
Oxidative stress
Oxidized low-density lipoproteins
Myeloperoxidase
Urinary and plasma isoprostanes
Neurohormones
Norepinephrine
Renin
Angiotensin II
Aldosterone
Endothelin
Extracellular-matrix remodeling
Matrix metalloproteinases
Tissue inhibitors of metalloproteinases
Collagen propeptides

Table 2. Biomarkers in heart failure. (Adapted from Braunwald, 2008)

myocardial ischemia and necrosis and have been found to be excellent diagnostic and prognostic biomarkers for thrombotic ACS. But many cardiac conditions can lead to elevated troponins in addition to ACS including myocarditis, DCM and HF (Table 3). With new high-sensitivity detection methods for troponins, very minor changes in cardiac damage can be detected. Although troponins released to the circulation do not identify the type of heart damage, their levels may indicate the severity of damage (Figure 2) (Miller et al., 2007). A number of studies have been conducted examining troponin release during myocarditis, DCM or heart failure in patients (Smith et al., 1997; Brandt et al., 2001; Imazio et al., 2008; Miller et al., 2007; Peacock et al., 2008). Troponin I and T have also been found to predict the severity of myocarditis and the short-term prognosis in children with acute and fulminant myocarditis and DCM (Soongswang et al., 2002; Al-Biltagi et al., 2010). Overall, troponin levels were found to increase in relation to the severity of myocardial inflammation or ventricular wall stress caused by remodeling (Agewall et al., 2011; Miller et al., 2007). DCM patients with elevated serum troponin I levels were more dilated and had a worse outcome that troponin I-negative patients (Miettinen et al., 2008). Additionally, acute decompensated heart failure patients who were positive for troponin had a lower EF and

higher in-hospital mortality, independent from other predictive values, than those who were negative for troponin (Peacock et al., 2008). Troponin T was found to be an important independent variable that predicted increased risk of death in patients with chronic HF (Latini et al., 2007). These findings demonstrate that troponin measurement is an important tool in early risk assessment of myocarditis/ DCM patients.

Condition
Acute coronary syndrome
Myocarditis
Pericarditis
Endocarditis
Dilated cardiomyopathy
Acute heart failure
Pulmonary embolism
Stroke
Sepsis
Acute aortic dissection
Tachyarrhythmias
Cardiac contusion
Tako-tsubo cardiomyopathy
Strenuous exercise e.g. marathon runners
Sympathomimetic drugs
Chemotherapy

Table 3. Cardiac conditions that can result in acutely elevated troponins. (Adapted from Agewall et al., 2011)

Interestingly, autoantibodies against circulating troponin I have been found in patients with ACS and acute myocardial infarction (Eriksson et al., 2005; Leuschner et al., 2008; Shmilovich et al., 2007). These autoantibodies were discovered because they interfered with troponin detection assays (Eriksson et al., 2005). This discovery led to the realization that autoantibodies against troponin I were also present in the sera of patients with DCM and heart failure (Miettinen et al., 2008; Shmilovich et al., 2007). One study of DCM patients found that troponin I, but not troponin I autoantibodies, were associated with dilation and poor outcome (Miettinen et al., 2008). In another study a significant number of DCM patients had autoantibodies against troponin I, but the autoantibodies were not found to bind to cardiac myocytes or activate Ca^{2+} currents (Shmilovich et al., 2007). Myocardial infarction patients with elevated troponin I autoantibodies had poor recovery of LV EF suggesting that troponin autoantibodies affect heart function (Leuschner et al., 2008). Further evidence that troponin autoantibodies may directly affect heart function comes from animal studies. PD-1 receptor deficient mice were found to develop severe DCM with high levels of autoantibodies against tropoinin I (Kaya et al., 2010; Nishimura et al., 2001). These troponin I autoantibodies were found to bind to heart tissue and to induce Ca^{2+} influx in cardiac myocytes. Inoculation of mice with recombinant troponin I with complete Freund's adjuvant was found to induce severe myocarditis and increased proinflammatory cytokines that progressed to fibrosis, DCM and heart failure (Goser et al., 2006; Kaya et al., 2008; 2010). However, this inflammatory response was only observed for troponin I but not troponin T inoculation. More research is needed to better understand the relationship of circulating

troponin I and its autoantibodies in the progression of myocarditis to DCM and heart failure.

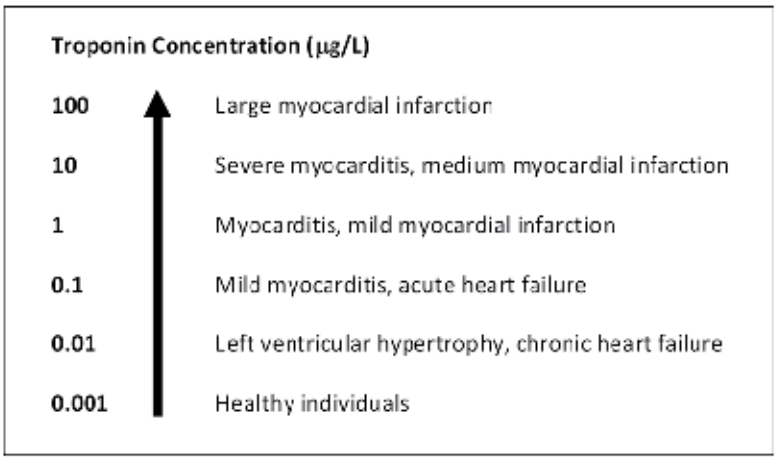

Fig. 2. Relationship between serum troponin levels and the severity of cardiac damage caused by myocarditis, heart failure or myocardial infarction. Adapted from Agewall et al., 2011.

3.1.2 BNP and NT-pro-BNP

In contrast to cardiac myosin or troponins that are released due to cell wall compromise, BNP is synthesized in healthy cardiac myocytes from its precursor NT-pro-BNP (Braunwald, 2008; Chen et al., 2010). The prohormone BNP is only released to the circulation when the ventricles become dilated, hypertrophic or during other conditions that induce wall distension and stretching, and by neurohormonal activation (Table 4). Prohormone BNP is cleaved by an endoprotease, corin, in the circulation into two polypeptides: the inactive NT-pro-BNP and the bioactive BNP. BNP causes arterial vasodilation, natriuresis and diureses while reducing the renin angiotensin system and adrenergic response (Braunwald, 2008; Palazzuoli et al., 2011). Elevated plasma BNP levels occur during hypertrophic cardiomyopathy, diastolic dysfunction and LV hypertrophy, and have been shown to be directly proportional to NYHA class and inversely related to cardiac output (Silver et al., 2004). Although few studies specifically address the topic, BNP has been found to be elevated in the serum of patients with myocarditis or DCM and in animal models of myocarditis (Grabowski et al., 2004; Miller et al., 2007; Ogawa et al., 2008; Talvani et al., 2004; Tanaka et al., 2011). Plasma BNP levels are also elevated in patients with acute myocardial infarction and this relationship persists into the late phases of cardiac remodeling (Hirayama et al., 2005). Many studies have linked higher levels of circulating BNP with heart failure diagnosis and worse outcome (Miller et al., 2007; Palazzuoli et al., 2011). BNP levels are a better predictor of death than norepinephrine or endothelin-1 (Braunwald, 2008). Several studies have found that NT-pro-BNP was better than BNP for predicting death or re-hospitalization for heart failure, probably due to the longer half-life of NT-pro-BNP in sera (Masson et al., 2006; Omland et al., 2007). However, BNP is a better

predictor than NT-pro-BNP of worse outcome in ACS (Palazzuoli et al., 2011). Natriuretic peptides were also found to be useful in screening asymptomatic subjects at risk of developing HF such as those with hypertension, diabetes and coronary artery disease (Braunwald, 2008). Noncardiac conditions that increase plasma BNP levels such as age, race, obesity and renal dysfunction should be taken into consideration when using this biomarker (Table 4). Overall, BNP and NT-pro-BNP are biomarkers with a high sensitivity and specificity in predicting HF in a number of conditions including myocarditis and DCM.

Cardiac condition	Noncardiac condition
Systolic dysfunction	Acute pulmonary embolism
Diastolic dysfunction	Pulmonary hypertension
Myocarditis	Anemia
Pericarditis	Septic shock
Myocardial fibrosis	Hyperthyroidism
Dilated cardiomyopathy	*Cor pulmonale*
Heart failure	Renal failure
Valvular heart disease	
Coronary artery disease	
Hypertension	
Atrial fibrillation	

Table 4. Conditions that increase plasma natriuretic peptide levels. (Adapted from Braunwald, 2008 and Palazzuoli et al., 2011)

3.1.3 Soluble ST2

Soluble ST2 (sST2), an interleukin (IL)-1 receptor (R) (IL-1R) family member, is basally expressed by cardiomyocytes and upregulated in the heart by mechanical strain and IL-1β (Weinberg et al., 2002). The ST2 gene is known to encode at least 3 isoforms of ST2 by alternative splicing: ST2L, a transmembrane receptor; sST2, a secreted soluble form of ST2 that can serve as a decoy receptor for the ST2 ligand, IL-33; and ST2V, a variant of ST2 found in the gut of humans (Miller & Liew, 2011). ST2L is a member of the Toll-like receptor (TLR)/IL-1R superfamily that share a common structure with an extracellular domain of three linked immunglobulin-like motifs, a transmembrane segment and a cytoplasmic Toll-interleukin-1 receptor (TIR) domain. ST2L forms a complex with IL-1R accessory protein (IL-1RAcP) that is required for IL-33 signaling (Ali et al., 2007). IL-33 signaling recruits the adaptor protein MyD88 to the TIR domain leading to activation of the transcription factors NF-κB and AP-1 and production of inflammatory mediators including proinflammatory tumor necrosis factor (TNF), IL-1β, IL-6, and the T helper (Th)2-associated cytokines IL-5, IL-13 and IL-10 (Liew et al., 2010; Miller & Liew, 2011). Both ST2L and sST2 are induced in cardiomyocytes by biomechanical strain (Weinberg et al., 2002). Elevated levels of sST2 in the sera are associated with poor prognosis in patients with acute myocardial infarction or chronic heart failure where sST2 levels correlate positively with creatine kinase and negatively with EF (Weinberg et al., 2002; 2003). In patients with severe chronic NYHA class III or IV heart failure, the change in sST2 levels was an independent predictor of subsequent mortality or transplantation (Weinberg et al., 2003). IL-33 is expressed largely within fibroblasts in the heart and is thought to be released from necrotic cells due to tissue damage caused by direct damage or infection (Liew et al., 2010). IL-33 has been shown to be

cardioprotective using animal models where recombinant (r)IL-33 treatment reduced hypertrophy and fibrosis following pressure overload induced by transverse aortic constriction (TAC) (Sanada et al., 2007). This effect was reversed by treating mice with sST2 prior to TAC, providing evidence that sST2 functions as a decoy receptor for IL-33. Additionally, rIL-33 treatment was found to decrease atherosclerosis in ApoE deficient mice fed a high fat diet by skewing the immune response from a Th1 to a Th2 response (Miller et al., 2008).

Currently there are no reports on the role of sST2 or IL-33 in the development of myocarditis or DCM even though sST2 is known to be a good biomarker predicting heart failure. Our laboratory is investigating the role of sST2/IL-33 signaling in an autoimmune model of coxsackievirus B3 (CVB3) myocarditis and DCM in mice. We found that IL-33 mRNA was upregulated in the heart during acute CVB3 myocarditis and chronic DCM (Figure 3A,B). Additionally, sST2 levels were elevated in the sera during acute CVB3 myocarditis in mice (Figure 3C) and correlated with poor heart function as assessed by echocardiography (not shown) or pressure-volume relationships (Figure 4). Serum sST2 is a good marker of disease because it could not be detected in the sera of undiseased mice (Figure 3C). Our findings of elevated sST2 levels in the sera of mice with CVB3 myocarditis and its relation to poor heart function suggest that sST2 may serve as a useful biomarker to predict progression to HF in myocarditis and DCM patients.

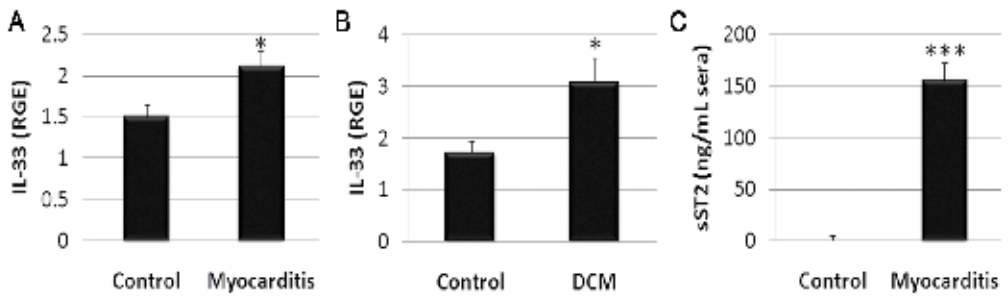

Fig. 3. IL-33 and sST2 are increased during autoimmune CVB3 myocarditis in mice. Male BALB/c mice were infected intraperitoneally with heart-passaged coxsackievirus B3 (CVB3) containing infectious CVB3 (10^3 plaque forming units) and heart proteins on day 0 and myocarditis examined at day 10 post infection (pi) and dilated cardiomyopathy (DCM) at day 90 pi. Saline inoculated age-matched mice were used as controls. Interleukin (IL)-33 mRNA was assessed by quantitative RT-PCR in the heart at day 10 (**A**) and 90 (**B**) pi and normalized to hypoxanthine phosphoribosyltransferase (HPRT) levels. sST2 levels were assessed in the sera of mice during acute myocarditis at day 10 pi by ELISA (**C**). Data are expressed as mean relative gene expression (RGE) ±standard error of the mean (SEM) in 7 to 10 mice per group. $*P < 0.05$; $***P < 0.001$.

3.2 Biomarkers of inflammation

Inflammation is important in the pathogenesis of many of the conditions that lead to HF. Traditionally, inflammatory biomarkers have been considered to be risk markers rather than risk factors because their role in disease pathogenesis is not always clear (Rao et al., 2006). Many inflammatory biomarkers found in the circulation, such as C-reactive protein (CRP),

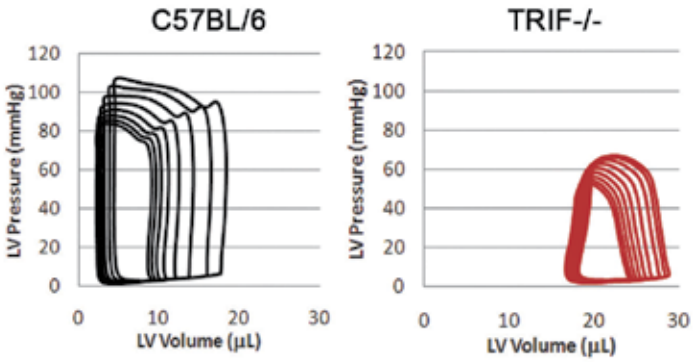

Fig. 4. Poor heart function in TRIF deficient mice is associated with elevated sST2 during acute CVB3 myocarditis. Male C57BL/6 (BL/6) or TRIF deficient (TRIF-/-) mice were infected intraperitoneally with heart-passaged coxsackievirus B3 (CVB3) containing infectious CVB3 (10^3 plaque forming units) and heart proteins on day 0 and myocarditis examined at day 10 post infection (pi) using end systolic pressure-volume relationships (ESPVR). Ees, a measure of LV end systolic stiffness/elastance, was 7.1 in BL/6 and 5.1 in TRIF-/- mice ($P = 0.04$) while V_0, the X-intercept of the ESPVR, was -5.4 in BL/6 and 26.8 in TRIF-/- mice ($P = 0.0001$). End diastolic volume (EDV) was 24±1.2 in BL/6 and 33±3.3 in TRIF-/- mice ($P < 0.01$). Thus, elevated sST2 in the sera of TRIF-/- mice (not shown) was associated with dilation and heart failure in TRIF deficient mice in an autoimmune model of CVB3 myocarditis.

IL-6 and serum amyloid A protein (SAA), are part of the acute phase response arising in the liver and although they are strongly associated with disease they may simply infer the presence of an inflammatory state. In clinical studies inflammatory mediators have been found to predict progression to HF similar to injury biomarkers and/or neurohormones (Table 2) (Mann, 2005). Inflammatory biomarkers have been shown in animal studies and the clinical setting to increase LV dysfunction, increase edema, and induce endothelial dysfunction and cardiomyocyte apoptosis, as well as other deleterious effects (Table 5).
A recent long-term study of myocarditis patients revealed that inflammation was the best predictor for the progression to HF following acute myocarditis (Kindermann et al., 2008). Viruses like CVB3, adenovirus, parvovirus B19 and hepatitis C virus are often detected in patient myocardial biopsies (Cooper, 2009; Gupta et al., 2008). Antiviral treatments such as interferon-β reduce inflammation and HF in animal models and patients, implying that viral infections are an important cause of myocarditis cases that lead to HF (Kuhl et al., 2003; Wang et al., 2007). Inflammation appears to be etiologically linked with the development of HF, not only because heart failure is a consequence of inflammatory CVDs but because patients with chronic HF that have elevated levels of inflammatory mediators have a worse prognosis (Robinson et al., 2011). Evidence exists that both cellular and auto/antibody-mediated damage contribute to the progression to DCM and HF following myocarditis (Cooper, 2009; Fairweather et al., 2008; Kallwellis-Opara et al., 2007). Similar to atherosclerosis, acute myocardial inflammation is associated with an elevated Th1 response in males (Daniels et al., 2008; Frisancho-Kiss et al., 2007; Huber and Pfaeffle, 1994; Nishikubo et al., 2007). A Th17 response has been shown to increase fibrosis leading to DCM in the experimental autoimmune myocarditis (EAM) model in mice (Baldeviano et al., 2010).

Interestingly, Th2 responses have also been implicated in the pathogenesis of myocarditis leading to HF (Afanasyeva et al., 2001; Fairweather et al., 2004a; 2004b). IFN-γ deficient mice, which have elevated IL-4 levels and a Th2 response, progress to severe DCM and HF following CVB3 myocarditis (Fairweather et al., 2004a).

Deleterious effects
Known
Left ventricular dysfunction
Pulmonary edema
Cardiomyopathy
Inflammation
Left ventricular remodeling
Endothelial dysfunction
Inducible nitric oxide synthase activation
Decreased skeletal-muscle blood flow
Anorexia and cachexia
Potential
Receptor uncoupling from adenylate cyclase
Activation of the fetal-gene program
Apoptosis of cardiac myocytes

Table 5. Deleterious effects of inflammatory biomarkers on acute and chronic heart failure. (Adapted from Anker & von Haehling, 2004; Braunwald, 2008; Mann, 2005)

3.2.1 C-reactive protein

Interest in the study of inflammatory mediators in patients with HF began in 1954 when an assay for CRP was first developed (Braunwald, 2008). CRP is an acute phase protein synthesized in the liver in response to IL-6 and released to the circulation during inflammation (Pepys & Hirschfield, 2003). Its levels are synergistically increased by IL-1β. In phagocytes CRP has been shown to bind Fcγ receptor I and II and to function in the clearance of apoptotic and necrotic cells (Devaraj et al., 2009; Rhodes et al., 2011). In 1956 a study was published showing that CRP was detectible in the sera of 30 out of 40 patients with chronic HF, and that elevated CRP levels were associated with more severe disease (Braunwald, 2008; Elster et al., 1956). Since then many studies have shown that CRP independently predicts adverse outcomes in patients with acute or chronic HF (Braunwald, 2008; Osman et al., 2006). Higher levels of CRP are associated with more severe HF and independently associated with morbidity and mortality (Anand et al., 2005). Additionally, elevated CRP levels identified asymptomatic elderly individuals who were at a high risk of developing HF in the future (Vasan et al., 2003). The main problem with CRP as a biomarker is that it lacks specificity for CVD. That is, CRP levels are elevated in the sera during most conditions that increase inflammation such as acute or chronic infection, cigarette smoking, ACS and some autoimmune diseases (Pepys & Hirschfield, 2003; Perez-De-Lis et al., 2010; Rhodes et al., 2011). There is increasing evidence that CRP may be able to exert direct proinflammatory effects on the heart by increasing matrix metalloproteinase-1 (MMP)-1 and IL-8 in endothelial cells and by increasing CD11b and CC-chemokine receptor 2 (CRR2) in monocytes, for example (Table 6) (Devaraj et al., 2009; Osman et al., 2006; Venugopal et al., 2005).

Endothelial cells	Monocyte-macrophages	Smooth muscle cells
Increased VCAM, ICAM-1, E-selectin, MCP-1 and monocyte adhesion	Increased tissue factor	Increased AT-1 and VSMC migration and proliferation
Increased PAI-1, IL-8, CD40/CD40L, MMP-1, ET-1 and M-CSF	Increased superoxide and myeloperoxidase	Increased neointimal formation *in vivo*
Decreased tPA	Increased proinflammatory cytokines (e.g. TNF) and decreased IL-10	Increased iNOS
Decreased prostacyclin	Increased CD11b and CCR2	Increased ROS
Increased superoxide and iNOS	Promoted oxLDL uptake and decreases cholesterol efflux	Increased tissue factor
Promoted endothelial dysfunction *in vivo*	Increased MMPs and HMGB1	
Impaired EPC number and function *in vitro*	Increased M-CSF and proliferation	

Table 6. Inflammatory effects of C-reactive protein. (Adapted from Devaraj et al., 2009) Abbreviations: AT, angiotensin receptor; CCR2, CC-chemokine receptor-2; CD40L, CD40 ligand; EPC, endothelial progenitor cell; ET, endothelin; HMGB1, high-mobility group protein B1; ICAM, intercellular adhesion molecule; IL, interleukin; iNOS, inducible nitric oxide synthase; MCP, monocyte chemotactic protein; M-CSF, macrophage colony-stimulating factor; MMP, matrix metalloproteinase; oxLDL, oxidative low-density lipoprotein; PAI, plasminogen activator inhibitor; ROS, reactive oxygen species; TNF, tumor necrosis factor; tPA, tissue plasminogen activator; VCAM, vascular cell adhesion molecule; VSMC, vascular smooth muscle cell.

Although many studies have examined the relationship between serum CRP levels and HF, few studies have examined CRP levels in myocarditis patients. In one study, 31 patients with clinical and histological evidence of lymphocytic myocarditis were found to have elevated plasma CRP levels that correlated positively with the NYHA functional class (Kaneko et al., 2000; Osman et al., 2006). Five of these patients who died of HF during the study had significantly higher levels of CRP, suggesting that CRP measurement may be a useful tool for determining prognosis in myocarditis patients. A separate study found that 80% of patients with clozapine-induced myocarditis (an antipsychotic drug used to treat schizophrenic symptoms) had elevated levels of CRP compared to a control group (Ronaldson et al., 2010). Several studies have examined CRP levels in idiopathic/ non-ischemic DCM patients where CRP levels have been found to independently predict disease outcome (Ishikawa et al., 2006; Kaneko et al., 1999; Senes et al., 2008). CRP levels increased with the severity of symptoms and the level of systolic impairment in DCM patients, while ongoing statin treatment was found to decrease CRP levels (De Gennaro et al., 2008). Interestingly, CRP has been found to co-express with TNF, macrophages and complement in the myocardium of DCM patients suggesting that CRP may play a role in the pathogenesis of disease (Satoh et al., 2005; Zimmermann et al., 2009). One of the obstacles to understanding the role of CRP in myocarditis and DCM is that mouse CRP appears only in trace amounts during the acute phase response (Pepys & Hirschfield, 2003). Instead of CRP mice upregulate serum amyloid P component (SAP), which is a non-acute phase protein in

humans. Thus, CRP deficient or transgenic mice may provide only limited information on the role of CRP in HF promotion.

3.2.2 Cytokines: TNF, IL-1β and IL-18

The inability of hemodynamic factors to fully explain HF cases led to the hypothesis that cytokines released from cardiac tissue and/or inflammatory cells also contribute to disease progression. According to the "cytokine hypothesis", HF develops because cytokine cascades that are activated following myocardial injury or stress exert deleterious effects on heart function (Table 5) (Anker & von Haehling, 2004; Braunwald, 2008; Seta et al., 1996). Cytokines can induce hemodynamic abnormalities and/or direct toxic effects on the heart. Since the original report in 1990 by Levine et al. there have been many studies showing that an increase in circulating TNF levels directly relates to a patient's NYHA classification and predicts patient mortality (Anker & von Haehling, 2004; Mann, 2005; Seta et al., 1996; Vasan et al., 2003). Similar relationships between IL-1β, IL-6 or IL-18 and HF have been found (Anker & von Haehling, 2004; Hedayat et al., 2010; Jefferis et al., 2011; Vasan et al., 2003). Cardiac myocyte hypertrophy, contractile dysfunction, cardiac myocyte apoptosis and extracellular matrix (ECM) remodeling contribute critically to the progression from cardiac injury to HF (Hedayat et al., 2010).

More than any other category of biomarker (Table 2), the role of cytokines in the pathogenesis of myocarditis and DCM has been studied by researchers (Fairweather & Rose, 2005; Hedayat et al., 2010). TNF, IL-1β and IL-18 have all been shown to play a role in myocarditis by inducing myocyte hypertrophy, contractile dysfunction, myocyte apoptosis and contributing to ECM remodeling, a step critical in the progression from myocarditis to DCM (Cain et al., 1999; Fairweather et al., 2004a; 2004b; Hedayat et al., 2010). In one study, TNF mRNA expression was found to be elevated more often in myocarditis patients when viral genomes were also detected, and greater mRNA levels of TNF and its receptor TNFRI correlated with impaired cardiac function (Calabrese et al., 2004). In a mouse model of CVB3 myocarditis, TNF was found to increase CD1d expression on lymphocytes resulting in increased inflammation in males (Huber, 2010). However, viral replication and acute CVB3 myocarditis was not altered in TNFRI deficient mice (Fairweather et al., 2005). Out of the 13 or so TLRs that have been described so far in humans and mice, TLR4 is unique in its ability to work with the inflammasome to produce bioactive IL-1β and IL-18 in the heart (Fairweather et al., 2003; Vallejo, 2011). TLR2 and TLR4 signaling increase TNF levels, and TLR2 can act with TLR4 to increase IL-1β levels (Vallejo, 2011). TLR4 mRNA expression has been found to be higher in patients with myocarditis than controls, and to correlate with viral RNA levels in the heart (Satoh et al., 2003). Myocarditis patients with active viral replication had higher levels of TLR4 that was associated with lower systolic function. In a mouse model of CVB3 myocarditis, our laboratory found that TLR4 deficient mice develop reduced acute inflammation and lower IL-1β and IL-18 levels in the heart (Fairweather et al., 2003). The importance of TLR4 signaling in a strictly autoimmune model of myocarditis was demonstrated by Nishikubo et al. where TLR4 signaling was found to be necessary to mount a Th1-type immune response (Nishikubo et al., 2007). We have shown that TLR4 is upregulated on macrophages and mast cells during the innate immune response to CVB3 and during acute CVB3 myocarditis and this response results in increased inflammation and progression to DCM and HF in males compared to females (Frisancho-Kiss et al., 2007; 2009; Onyimba et al., 2011). A Th1 response in male mice was found to be due to TLR4-derived IL-

18, which was originally named IFN-γ-inducing factor, rather than to a classical IL-12/STAT4-induced Th1 response (Frisancho-Kiss et al., 2006). We were surprised to find that TLR4 was expressed on alternatively activated M2 macrophages (induced by Th2 cytokines) rather than classically activated M1 macrophages (induced by Th1 cytokines) within the heart during acute CVB3 myocarditis (Fairweather & Cihakova 2009; Frisancho-Kiss et al., 2009). These CD11b+GR1+F4/80+ M2 macrophages expressed TLR4 and IL-1β (Frisancho-Kiss et al., 2009). We, and others, have shown that IL-1β is particularly important in the cardiac remodeling that leads to fibrosis, DCM and HF following acute myocarditis (Blyszczuk et al., 2009; Cihakova et al., 2008 Fairweather et al., 2004a; 2006). Further work is needed to better understand the role of innate TLRs and cytokine production/regulation in the heart in order to determine whether anti-cytokine therapies will be effective once disease has progressed to the point of being clinically apparent (Mann, 2005). Another area that needs to be addressed in animal models is the relationship between sera levels of proinflammatory cytokines and the stage of disease (i.e. acute myocarditis vs. DCM) and whether increases in sera levels of cytokines predict HF.

3.3 Biomarkers of extracellular matrix remodeling

Remodeling of the ventricles plays an important role in the progression to HF (Braunwald, 2008). The extracellular matrix provides a framework for cardiac myocytes, mediates cell adhesion and cell-to-cell communication, mediates diastolic stiffness, promotes cell survival or apoptosis, and is a reservoir for growth factors and cytokines (Liu et al., 2006). Release of cytokines and growth factors at the site of tissue injury and by inflammatory cells induces fibroblast proliferation and deposition of collagen, which is the primary component of the ECM resulting in scar tissue (Figure 5). Profibrotic cytokines, such as TNF and IL-1β, and growth factors, like transforming growth factor (TGF)β1 and fibroblast growth factor (FGF), induce collagen production from fibroblasts. Normally a balance exists between matrix metalloproteinases (MMPs) that proteolytically degrade fibrillar collagen and tissue inhibitors of MMPs (TIMPs). However, during inflammatory CVDs an imbalance in MMPs and TIMPs contributes to collagen deposition, ventricular dilatation and remodeling resulting in DCM and HF. The activity of MMPs has been shown to be increased in the progression to HF (Bradham et al., 2002; Tyagi et al., 1996). Serum MMP9, for example, has been found to predict CVD mortality better than other traditional prognostic markers such as cholesterol, CRP or IL-6 (Blankenberg et al., 2003; Liu et al., 2006).

The progression from myocarditis to fibrosis, DCM and HF has been well documented in clinical studies and animal models (Fairweather et al., 2004a; Kania et al., 2009; Looi et al., 2010). Studies in our laboratory have revealed a two-stage process where increases in profibrotic mediators during acute CVB3 myocarditis, which occurs from day 8 to 12 post infection, result in a gradual remodeling that progresses to fibrosis and DCM by day 35 post infection (Figure 5) (Fairweather et al., 2004a; 2006; Fairweather & Rose, 2007). In both animal and human studies of DCM, genes associated with extracellular matrix remodeling and fibrosis are upregulated with disease (Piro et al., 2010; Yung et al., 2004). TNF, IL-1β, IL-4, IL-6, IL-17 and TGF-β have all been found to initiate remodeling (Baldeviano et al., 2010; Blyszczuk et al., 2009; Fairweather et al., 2004a; Heymans, 2006; Kania et al., 2009). There are many MMPs, but only 4 known TIMPS. MMPs are upregulated in the heart during EAM and during viral myocarditis (Marchant & McManus, 2009; Tang et al., 2007; Westermann et al., 2010). Individual MMPs and TIMPs have been found to differ in their effects on

myocarditis in rodents (Liu et al., 2006; Marchant & McManus, 2009; Westermann et al., 2010). For example, MMP9 deficient mice had increased CVB3 replication and inflammation, and worse heart function than wild type controls, indicating that MMP9 protects the heart from CVB3 myocarditis (Cheung et al., 2008). In contrast, TIMP-1 deficient mice were protected from CVB3 myocarditis indicating that TIMP-1 increases disease (Crocker et al., 2007). Although a number of studies have examined the role of MMPs and TIMPs in myocarditis, the relationship between serum levels of these factors and the progression to DCM and HF is not yet clear from animal models.

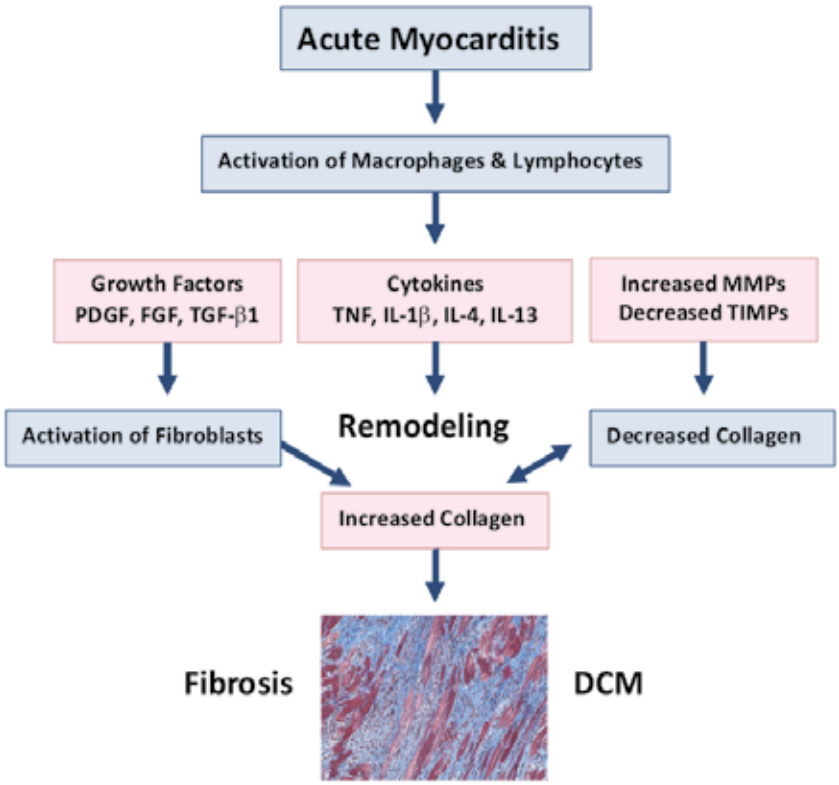

Fig. 5. Development of fibrosis and DCM following acute myocarditis. Macrophages and lymphocytes present within the myocardium during acute myocarditis release profibrotic cytokines such as tumor necrosis factor (TNF), interleukin (IL)-1β, IL-4 and IL-13 that activate fibroblasts to release collagen. Inflammation additionally stimulates the release of growth factors like platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) and transforming growth factor (TGF)-β1, which act with cytokines to increase collagen production. Cytokines and growth factors released from inflammatory cells and cardiac tissues contribute to pathology. Normally a balance exists between matrix metalloproteinases (MMPs) that proteolytically degrade fibrillar collagen and tissue inhibitors of MMPs (TIMPs). However, during acute myocarditis an imbalance in MMPs and TIMPs contributes to collagen deposition, ventricular dilatation and remodeling resulting in dilated cardiomyopathy (DCM) and heart failure.

3.4 Thrombosis biomarkers

Atherosclerosis is a major initiator of thrombi formation that can restrict blood flow and lead to a heart attack (Carter, 2005). Cardiac mural thrombi can arise from a myocardial infarction, infection, inflammation (e.g. myocarditis) or rheumatic heart disease, for example. Although research has led to a clear understanding of conditions that induce thrombosis, the precise pathology leading to disease remains unclear. Thrombi can develop anywhere in the cardiovascular system like in the ventricular or atrial chambers, arteries, veins or capillaries. The size and shape of individual thrombi vary depending on the circumstances leading to their development. They often are found at sites of endothelial injury. Once thrombi have formed (acute) they may accumulate more platelets and fibrin and grow larger, or dislodge and travel to other sites, or be removed by fibrinolytic activity, and finally they can attract inflammation, undergo remodeling with deposition of collagen and be reincorporated into the vessel or myocardium (Kumar et al., 2005) (Figure 6).

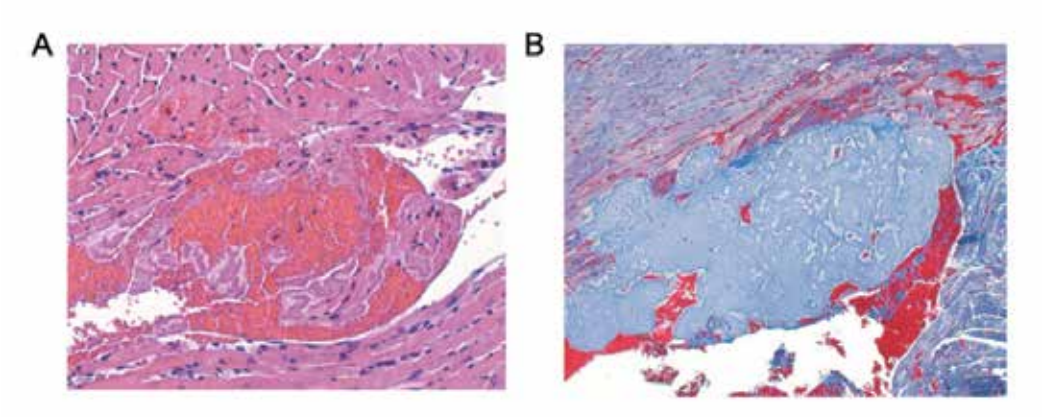

Fig. 6. Mural thrombi develop during acute CVB3 autoimmune myocarditis in mice and reincorporate into the myocardium. Male BALB/c mice were infected intraperitoneally with heart-passaged coxsackievirus B3 (CVB3) containing infectious CVB3 (10^3 plaque forming units) and heart proteins on day 0 and thrombus formation examined at day 10 post infection (pi) during acute myocarditis (**A**) or during chronic myocarditis/DCM at day 35 pi (**B**). H&E staining shows ventricular mural thrombus at day 10 pi (magnification, x100) (**A**). Masson's trichrome stains bright blue revealing collagen composition of mural integrated ventricular thrombus at day 35 pi (magnification, x100) (**B**).

Damage of cardiac tissue by viruses and inflammation is known to release tissue factor (TF), the main initiator of the coagulation cascade that results in the formation of fibrin and thrombotic clots (Mackman, 2009). Mural thrombi are known to occur in viral models of myocarditis in mice as well as in myocarditis patients (Antoniak et al., 2008; Kojima et al., 1988; Kuh & Seo, 2005). Furthermore, DCM patients demonstrate a high frequency of LV thrombi and prothrombotic characteristics like high levels of circulating fibrinogen and antithrombin III (Abdo et al., 2010). Many studies have shown that TF can increase inflammation by stimulating release of IL-6, a cytokine that along with TNF and IL-1β has been strongly associated with poor CVD outcome (Braunwald, 2008; Carter, 2005; Mackman, 2009; Rao et al., 2006). Complement components also contribute to thrombosis by depositing at sites of tissue damage and by activating platelets (Fairweather et al., 2006; Peerschke et al.,

2010). Eosinophils are potent inducers of ECM remodeling, releasing many profibrotic factors including IL-1β, IL-6, TGF-β, MMPs, and TIMPs (Shamri et al., 2011). Additionally, eosinophils release potent prothrombotic agents such as major basic protein, eosinophilic cationic protein, and eosinophil peroxidase, as well as directly and/or indirectly activating TF (Ames et al., 2010). Eosinophilia, fibrosis and thrombosis are characteristics of eosinophilic cardiovascular diseases like Churg Strauss syndrome, a form of vasculitis, hypereosinophilic syndrome, eosinophilic myocarditis and giant cell myocarditis (Ames et al., 2010; Cooper, 2000; 2009; Kleinfeldt et al., 2010; Rezaizadeh et al., 2010). Circulating biomarkers that may indicate a hypercoagulable state include complement C3, C4, IL-6, fibrinogen or antithrombin III (Abdo et al., 2010). Complement components were recently found to be elevated in the sera of mycarditis and DCM patients (Cooper et al., 2010). We have found that complement receptor 1 deficient mice, a receptor that regulates C3 levels, develop severe CVB3 myocarditis, dilation and HF with elevated levels of IL-1β in the heart and fibrosis (Fairweather et al., 2006). Overactivation of the terminal complement complex (C5b-9) has been shown to contribute to the progression of myocarditis to DCM in mice, indicating the importance of complement in regulating disease (Zwaka et al., 2002). Overall, these findings suggest that biomarkers of coagulation and/or thrombosis are likely to be important indicators of progression to DCM and HF following myocarditis.

4. Conclusions

Biomarkers are an important clinical tool for assessing progression to heart failure. DCM often leads to HF, and myocarditis is an important cause of acute (sudden death) and chronic (arising from DCM) forms of HF. Even though myocarditis is known to be an important cause of HF, few clinical studies have been conducted to determine the presence or usefulness of HF biomarkers in predicting adverse outcomes in myocarditis patients. Studies that have been conducted in myocarditis/DCM patients or animal models suggest that many of the biomarkers used to assess the likelihood of progression to heart failure in other CVDs will also provide useful information in myocarditis/DCM patients. More studies examining the ability of circulating HF biomarkers to predict poor outcome and HF in animal models of myocarditis/DCM are needed. Animal models will also provide valuable information on the potential role of HF biomarkers in the pathogenesis of disease. This knowledge is critical in determining the ability of therapies targeted to these biomarkers to prevent disease progression.

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17

A Proteomic Approach to Investigate Myocarditis

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1. Introduction

Myocarditis is an inflammatory disease of the cardiac muscle which might be related to viral (mainly parvovirus B19 and many others), protozoan (*Borrellia burgdoferi, Trypanosoma cruzi, Toxoplasma gondi*), bacterial (*Brucella, Corynebacterium diphtheriae, Gonococcus, Haemophilus influenzae, Actinomyces, Tropheryma whipplei, Vibrio cholerae, Borrelia burgdorferi, Leptospira, Rickettsia*), fungal (*Aspergillus*) and other non viral pathogens (Rezkalla SH et al., 2010, Blauwet LA et al., 2010, Cihakova D et al., 2010) infections; It has been reported, however, that this kind of inflammation might be caused by an hypersensitivity response to drugs (Kühl U et al., 2009). The final effect in each case is represented by myocardial infiltration of immunocompetent cells following any kind of cardiac injury.
Myocarditis presents with many symptoms, from chest pain that spontaneously resolves without treatment to cardiogenic shock and sudden death (Kühl U et al., 2010, Taylor CL et al 2010). The major long-term consequence is dilated cardiomyopathy with chronic heart failure(Lv H et al. 2011, Stensaeth KH et al 2011).
Nowadays, diagnostic tools available for this disease are mainly related to general investigations (such as electrocardiography) and analysis of the most abundant serum proteins, whose alteration is related to cardiac pathology, even if it's not specifically connected to myocarditis itself. Here we propose preliminary speculations on the serum proteins profiling (both in the expression level and in the characterization of post translational modifications) and on free peptides identification in myocardytis affected patients (compared to healthy individual), that could be helpful in finding specific markers for this pathology.
As many other inflammatory events this disease involves in fact different kinds of biological macromolecules, here we focus in particular on proteins. More than 50% of total protein content in a cell is post translationally modified. The pattern of Post Translational Modifications (PTMs) on proteins constitute a molecular code that dictates protein conformation, cellular location, macromolecular interactions and activities, depending on cell type, tissue and environmental conditions (Diernfellner AC et al., 2011, Savidge TC,

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2011, Hao P et al.,2011). It is very well known that biological function of many proteins is strictly related to the presence of the appropriate set of PTMs. Most importantly, PTMs deregulation might be involved in the development of diseases , in fact, as a consequence of many pathologies, PTMs set might be altered (Dell A et al., 2001, Kim YJ. Et al 1997, Dube DH. Et al., 2005, Granovsky M. Et al 2000).

Proteomics investigations offer all useful tools to deeply investigate this kind of alterations. Two dimensional electrophoresis coupled with software mediated image analysis, affinity chromatographies and especially Mass Spectrometry (MS) present high levels of sensitivity, accuracy and reproducibility and these are all fundamental requirements that this kind of study needs.

As previously described by our group (Carpentieri A, Giangrande C, et al., 2010), serum glycoproteome characterization might be crucial in clinical investigation. In fact we showed that the *N*-glycan profiling of serum glycoproteins extracted from myocarditis affected donors compared to the ones of healthy people shows many peculiarities. We demonstrated that many of the extracted oligosaccharides are in fact incomplete or truncated structures whilst others show a high level of fucosylation, all these results fully matched previously published data about the glycosylation in proteins during chronic inflammation events (Dell A et al., 2001, Kim YJ. Et al 1997, Dube DH. Et al., 2005, Granovsky M. Et al 2000, Carpentieri A, Giangrande C, et al., 2010).

Thanks to the high sensitivity of the most modern analytical techniques, several research groups focus the research at level of peptides rather than at protein level (Taylor-Papadimitriou J. et al., 1994 Amado F et al., 2010, Menschaert G et al. 2010). In fact, these endogenous peptides are referred to as the peptidome. Initially, peptidomic analyses were conducted as a method to study neuropeptides and peptide hormones; these are signaling molecules that function in a variety of physiological processes (Ludwig M. 2011, Colgrave ML et al., 2011). Recent studies have found large numbers of cellular peptides with half-lives of several seconds, raising the possibility that they may be involved in biological functions (Gorman PM et al 2003).

Here we propose preliminary data to show molecular basis of myocarditis using a proteomic approach. The analyses were focused on the serum proteins profiling, namely the study of the expression level and the characterization of glycoproteins involved in the pathology. A classical two-dimensional gel electrophoresis procedure to obtain protein maps and a "gel-free" comparison of the glycoproteomes in healthy and myocarditis human sera by using advanced mass spectrometry were reported. Free peptides identification in myocardytis affected patients (compared to healthy individual), was achieved in order to provide possible specific markers for this pathology.

2. Materials and methods

2.1 Materials

Human serum samples from 8 healthy donors (all Caucasian 4 males and 4 females aged between 60 and 80 years, all other clinical informations were covered by laws on privacy) and from 5 myocarditis affected patients (all Caucasian 3 males and 2 females aged between 60 and 85 years, all other clinical informations were covered by laws on privacy), respectively, have been obtained from the "Servizio Analisi" Policlinico, Napoli. Aliquots of serum samples from different donors were pooled in order to obtain an average overview of glycoforms distribution.

Guanidine, dithiothreitol (DTT), trypsin, α-cyano-4-hydroxycinnamic acid were purchased from Sigma. Iodoacetamide (IAM), tris(hydroxymethyl)aminomethane, calcium chloride and ammonium bicarbonate (AMBIC) were purchased from Fluka as well as the MALDI matrix 2,5-, α-cyano-4-hydroxycinnamic. Methanol, trifluoroacetic acid (TFA) and acetonitrile (ACN) are HPLC grade type from Carlo Erba, whereas the other solvents are from Baker. Gel filtration columns PD-10 are from Pharmacia, the HPLC ones from Phenomenex, whereas the pre-packed columns Sep-pak C-18 are from Waters.
Comassie Brilliant Blue was from Bio-Rad. PNGase F were purchased from Boheringer. Ion exchange resins Dowex H+ (50W-X8 50-100 mesh) was provided by BDH. Concanavalin A sepharose resin was purchased from Amersham Biosciences.

2.2 Protein concentration determination

Sera protein concentration was determined by Bradford assay method, using bovine serum albumin (BSA) as standard. Known amounts of BSA were diluted in 800 µL of H2O and then mixed to 200 µL of Comassie Brilliant Blue. 5 different BSA concentrations were determined by measuring absorbance at 595 nm and used to obtain a linear calibration curve. Three different sera dilutions were measured at 595 nm. Absorbance data were interpoled on the calibration curve, allowing the determination of protein concentration in the different samples.

2.3 Serum depletion

The depletion of the most abundant proteins from each serum sample was performed using Multiple Affinity Removal Spin Cartridges from Agilent. The procedure was performed at room temperature according to manufacturer's instructions and then immediately frozen to -20°C.

2.4 Free peptides analysis

300 µL acetonitrile was added to 100 µL of serum, incubated at room temperature for 30 minutes and then centrifuged at 13000 rpm. Supernatants were collected and concentrated by vacuum centrifugation (SAVANT) and resuspended in 20 µL formic acid 0.1%. Samples were desalted by C18 Zip Tip (Millipore) and diluted 100 times prior mass spectrometry analyses.

LC/MS-MS HPLC-Chip/Q-TOF 6520

Peptides were analyzed by a HPLC-Chip/Q-TOF 6520 (Agilent Technologies). The capillary column works at a flow of 4 µL/min, concentrating and washing the sample in a 40 nL enrichment column. The sample was then fractionated on a C18 reverse-phase capillary column (75 µm~43 mm in the Agilent Technologies chip) at flow rate of 400 nl/min, with a linear gradient of eluent B (0.2% formic acid in 95% acetonitrile) in A (0.2% formic acid in 2% acetonitrile) from 7% to 60% in 50 min.
Data were acquired through MassHunter software (Agilent Technologies). Proteins identification was achieved by using Mascot software (Matrix science), with a tolerance of 10 ppm on peptide mass, 0.6 Da on MS/MS, and choosing methionine oxidation and glutamine conversion in pyro-glutamic acid as variable modifications.

MALDI-TOF/TOF

Peptides were also analyzed by MALDI-TOF/TOF using a 4800 Plus MALDI-TOF/TOF (Applied Biosystems). Samples were mixed on MALDI plate to the matrix consisting of a

solution of 10 mg/mL α-cyano-4-hydroxycinnamic, whose preparation consisted in the resuspension of α-cyano-4-hydroxycinnamic in water and acetonitrile 10:1 (v/v). The instrument was calibrated using a mixture of standard peptides (Applied Biosystems). Spectra were register even in reflector positive. MS/MS spectra were performed with CID using air as collision gas. Spectra were manually interpreted.

2.5 2D-gel electrophoresis

IEF (first dimension) was carried out on non-linear wide-range immobilized pH gradients (pH 4-7; 7 cm long IPG strips; GE Healthcare, Uppsala, Sweden) and achieved using the EttanTM IPGphorTM system (GE Healthcare, Uppsala, Sweden). Analytical-run IPG-strips were rehydrated with 125µg of total proteins in 125µl of rehydratation buffer (urea 8 M, CHAPS 2%, 0,5% (v/v) IPG Buffer, bromophenol blue 0,002%) for 12 h at 20°C. The strips were then focused according to the following electrical conditions at 20°C: 500 V for 30 min, from 1000 V for 30 min, 5000 V until a total of 15000 Vt was reached. After focusing IPG strips were equilibrated for 15 min in 6 M urea, 30% (vol/vol) glycerol, 2% (wt/vol) SDS, 0.05 M Tris-HCl, pH 6.8, 2% (wt/vol) DTT, and subsequently for 15 min in the same urea/SDS/Tris buffer solution but substituting the 2% (wt/vol) DTT with 2.5% (wt/vol) iodoacetamide. The second dimension was carried out on 12% polyacrylamide gels at 25 mA/gel constant current until the dye front reached the bottom of the gel. MS gel was stained with colloidal comassie.

2.6 Image analysis

Gels images were acquired with an Epson expression 1680 PRO scanner. Computer-aided 2-D image analysis was carried out using the ImageMasterTM 2D Platinum software (GE Healthcare, Uppsala, Sweden). Differentially expressed spots were selected for MS analysis.

In order to find differentially expressed proteins, comassie stained gel image of serum proteins from healthy individual was matched with the one of myocarditis affected patient. The apparent isoelectric points and molecular masses of the proteins were calculated with ImageMaster 2D Platinum 6.0 using identified proteins with known parameters as references.

Relative spot volumes (%V) (V=integration of OD over the spot area; %V = V single spot/V total spot) were used for quantitative analysis in order to decrease experimental errors. The normalized intensity of spots on three replicate 2-D gels was averaged and standard deviation was calculated for each condition.

2.7 Protein identification by mass spectrometry

In situ digestion

Protein spots were excised from the gel and destained by repetitive washes with 0.1 M NH4HCO3 pH 7.5 and acetonitrile. Enzymatic digestion was carried out with trypsin (12.5 ng/µl) in 10 mM ammonium bicarbonate buffer pH 7.8. Gel pieces were incubated at 4 °C for 2 h. Trypsin solution was then removed and a new aliquot of the same solution was added; samples were incubated for 18 h at 37 °C. A minimum reaction volume was used as to obtain the complete rehydratation of the gel. Peptides were then extracted by washing the

gel particles with 10mM ammonium bicarbonate and 1% formic acid in 50% acetonitrile at room temperature.

Mass spectrometry and protein identification

LC-MS/MS analyses were performed as previously described for free peptides.
Mass spectrometric obtained data were used for protein identification using the software MASCOT that compare peptide masses obtained by MS and MS/MS data of each tryptic digestion with the theoretical peptide masses from all the proteins accessible in the databases (Peptide Mass Fingerprinting, PMF). Database searches were performed in NBCI databank (National Center for Biotechnology Information), restricting the analysis to the pertinent taxonomies. The parameters used for the identification were: tolerance of 10 ppm on peptide mass, 0.6 Da on MS/MS, and cysteine carbamidomethylation as fixed modification. Variable modifications were methionine oxidation, glutamine conversion in pyro-glutamic acid, and asparagine deamidation.

2.8 Boronate affinity chromatography

Glycoproteins were purified using PBA-bound agarose (Sigma-Aldrich, Munich, Germany). 500 µl of sample previously diluted (1 : 1) with equilibration buffer (50 mM taurine/NaOH, pH 8.7, containing 3–10 mM MgCl 2) was incubated with 200 µl of pre-washed immobilized ligand resin for 1 h on ice and with gentle shaking. After transfer of the resin into 1,5 ml eppendhorf tubes, the non-binding fraction was collected by low speed centrifugation (10 s, 500 × g). The resin was then thoroughly rinsed with equilibration buffer (six washes of 150 µl each) and 1 N NaCl (three washes of 150 µl each). For final elution of the bound fraction, a total of six washes (150 µl each) with taurine buffer containing 50 mM sorbitol were used. Three successive fractions (150 µl each) were pooled before further analysis.

2.9 Deglycosylation

Glycopeptides were lyophilized, resuspended in 10 mM AMBIC and incubated with PNGase F (5 U), for 12-16 h at 37°C. Deglycosylation was carried out also on unbound peptides, in order to release the glycans not recognized by Boronate affinity chromatography.

3. Results and discussion

3.1 Free peptides analysis

The analyses to investigate the "peptidomic" both in healthy and in pathological samples were accomplished by using a gel-free approach. The peptide component of the eluted fraction was analyzed by tandem mass spectrometry. The stringency of scoring parameters of the MASCOT algorithm minimized the number of false positive identifications. Most MS/MS spectra giving positive hits were derived from doubly and triply charged precursor ions that resulted predominantly in y-ion series.
Triplicate LC-MS/MS analysis of supernatants after ACN precipitation of serum proteins, showed the occurrence of many free peptides in both analyzed sera. As reported in Table 1, the total number of detected and identified peptides was 41. Among these, 9 peptides were unique in the pathologic sample. It should be noted that some peptides were identified in both samples.

m/z	RT	Sequence	Peptide	Protein	H/M ratio
567.95	17.09	QAGAAGSRMNFRPGVLS	(650-666)	**Q14624** Inter-alpha-trypsin inhibitor heavy chain H4	H:not detected
513.78	17.70	YYLQGAKIPKPEASFSPR	(627-644)	**Q14624** Inter-alpha-trypsin inhibitor heavy chain H4	H:not detected
823.17	22.11	MNFRPGVLSSRQLGLPGPPDVPDHAAYHPF	(658-687)	**Q14624** Inter-alpha-trypsin inhibitor heavy chain H4	H:not detected
1005.98	22.78	QLGLPGPPDVPDHAAYHPF	(669-687)	**Q14624** Inter-alpha-trypsin inhibitor heavy chain H4	0.68±0.18
757.71	19.96	SRQLGLPGPPDVPDHAAYHPF	(667-687)	**Q14624** Inter-alpha-trypsin inhibitor heavy chain H4	0.27±0.09
681.85	22.07	PGVLSSRQLGLPGPPDVPDHAAYHPF	(662-687)	**Q14624** Inter-alpha-trypsin inhibitor heavy chain H4	0.18±0.03
576.90	20.69	PGVLSSRQLGLPGPPDVPDHAAYHPFR	(662-688)	**Q14624** Inter-alpha-trypsin inhibitor heavy chain H4	H:not detected
379.71	0.55	LAEGGGVR	(28-35)	**P02671** Fibrinogen alpha chain	25±3.11
453.24	3.65	FLAEGGGVR	(27-35)	**P02671** Fibrinogen alpha chain	2.48±0.57
510.76	11.29	DFLAEGGGVR	(26-35)	**P02671** Fibrinogen alpha chain	1.33±0.76
539.27	11.70	GDFLAEGGGVR	(25-35)	**P02671** Fibrinogen alpha chain	3.15±1.02
597.77	15.59	SGEGDFLAEGGGV	(22-34)	**P02671** Fibrinogen alpha chain	0.85±0.23
603.79	12.57	EGDFLAEGGGVR	(24-35)	**P02671** Fibrinogen alpha chain	2.42±0.63
632.30	13.50	GEGDFLAEGGGVR	(23-35)	**P02671** Fibrinogen alpha chain	2.72±0.71
655.28	16.03	DSGEGDFLAEGGGV	(21-34)	**P02671** Fibrinogen alpha chain	1.23±0.36
675.81	13.67	SGEGDFLAEGGGVR	(22-35)	**P02671** Fibrinogen alpha chain	2.32±0.58
690.80	16.13	ADSGEGDFLAEGGGV	(20-34)	**P02671** Fibrinogen alpha chain	3.25±0.82
733.33	14.04	DSGEGDFLAEGGGVR	(21-35)	**P02671** Fibrinogen alpha chain	1.59±0.12
768.85	14.08	ADSGEGDFLAEGGGVR	(20-35)	**P02671** Fibrinogen alpha chain	6.16±1.01
851.71	13.58	SSSYSKQFTSSTSYNRGDSTFES	(576-598)	**P02671** Fibrinogen alpha chain	0.65±0.17

m/z	RT	Sequence	Peptide	Protein	H/M ratio
693.06	13.06	SSSYSKQFTSSTSYNRGDSTFESKS	(576-600)	**P02671** Fibrinogen alpha chain	M: not detected
733.83	14.48	SSSYSKQFTSSTSYNRGDSTFESKSY	(576-601)	**P02671** Fibrinogen alpha chain	M: not detected
619.75	17.89	QGVNDNEEGFF	(31-41)	**P02675** Fibrinogen beta chain	3.25±0.89
663.26	16.54	QGVNDNEEGFFS	(31-42)	**P02675** Fibrinogen beta chain	1.97±0.41
696.28	17.64	QGVNDNEEGFFSA	(31-43)	**P02675** Fibrinogen beta chain	2.31±0.53
404.55	18.90	RIHWESASLL	(1310-1319)	**P01024** Complement C3	H: not detected
402.22	14.29	THRIHWESASLLR	(1308-1320)	**P01024** Complement C3	H: not detected
445.25	16.98	SKITHRIHWESASLL	(1305-1319)	**P01024** Complement C3	H: not detected
415.20	7.60	HWESASL	(1312-1318)	**P01024** Complement C3	H: not detected
471.74	16.21	HWESASLL	(1312-1319)	**P01024** Complement C3	H: not detected
851.07	22.22	TLEIPGNSDPNMIPDGDFNSYVR	(957-979)	**P0C0L4** Complement C4-A	H: not detected
1054.53	25.86	DDPDAPLQPVTPLQLFEGR	(1429-1447)	**P0C0L4** Complement C4-A	H: not detected
489.96	20.26	RHPDYSVVLLLR	(169-180)	**P02768** Serum Albumin	0.43±0.17
417.91	16.67	KFQNALLVRY	(426-435)	**P02768** Serum Albumin	H: not detected
547.31	16.25	KVPQVSTPTLVEVSR	(438-452)	**P02768** Serum Albumin	H: not detected
868.11	21.57	AVPPNNSNAAEDDLPTVELQGVVPR	(14-38)	**P00488** Coagulation factor XIII A	2.40±0.84
920.14	21.09	RAVPPNNSNAAEDDLPTVELQGVVPR	(13-38)	**P00488** Coagulation factor XIII A	23.60±3.61
837.93	21.39	TAFGGRRAVPPNNSNAAEDDLPTVELQGVVPR	(7-38)	**P00488** Coagulation factor XIII A	91.32±7.39
781.37	17.84	TATSEYQTFFNPR	(315-327)	**P00734** Prothrombin	H: not detected
868.46	25.47	TGIFTDQVLSVLKGEE	(86-101)	**P02655**Apolipoprotein C-II	H: not detected
572.95	12.91	DALSSVQESQVAQQAR	(45-60)	**P02656**Apolipoprotein C-III	H: not detected
803.76	21.14	AATVGSLAGQPLQERAQAWGERL	(210-232)	**P02649**Apolipoprotein E	0.02

Table 1. List of free peptides identified by LC-MS/MS. Averaged area of chromatographic peaks from healthy(H) and myocarditis (M) ratio, indicates that some of them were differently represented. Peptide sequences were validated by MALDI-TOF/TOF analyses too.

As a whole, the LC-MS/MS proved to be a very sensitive and reproducible analysis which led to the identification of very weakly present peptides, these data were confirmed by another fragmentation technique, MALDI-TOF/TOF. As Fig. 1 shows, the fragmentation pattern of the peptide –ADSGEGDFLAEGGGVR- (from alpha fibrinogen protein) confirm what we found using LC-MS/MS analysis. Some of these peptides are related to different proteolytic activities on proteins involved in acute phase or inflammatory events, such as myocarditis itself. Among identified peptides, the molecular species , namely, peptide 662-668 from inter-alpha-trypsin inhibitor heavy chain H4 and peptide 1305-1319 from complement C3 correspond to free bioactive peptides, whose activity might be related again to inflammatory events (van den Broek I et al, 2010, ter Weeme M, et al, 2009). Preliminary qualitative and quantitative differences were detected in the analysis of the peptidomas from healthy and pathological samples. In fact, as reported in the Table 1 , averaged area of each peak of all detected peptides resulted to be different, showing that some of them were differently represented in each sample. The above mentioned peptides from complement C3 and inter-alpha trypsin inhibitor were poorly represented in the healthy serum samples, whereas fibrinogen alpha chain peptides were strongly represented in these sera, as well as coagulation factor XIII A peptides.

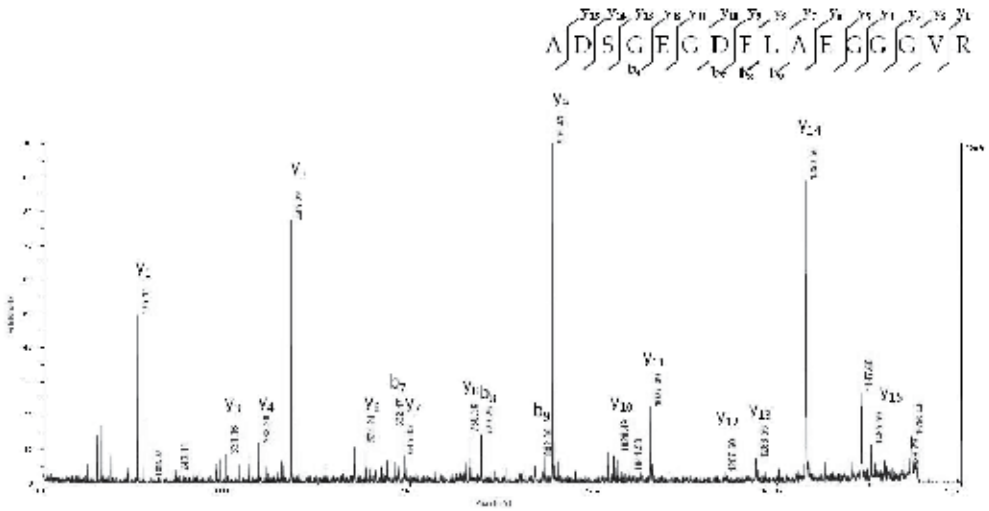
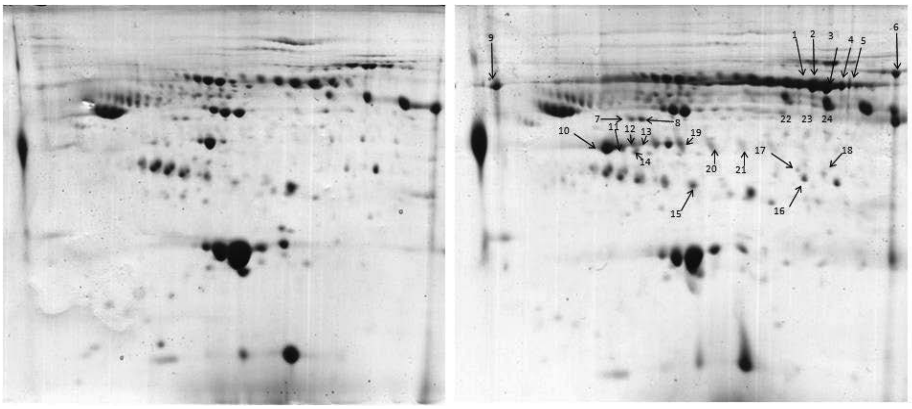


Fig. 1. Positive ion mode MALDI-TOF/TOF fragmentation spectrum of m/z 1536.44 corresponding to peptide 20-35 from fibrinogen alpha chain protein from healthy serum.

3.2 The proteins profile from 2D-electrophoresis is different

After depletion of the most abundant proteins, a two dimensional electrophoretic separation of the two sera samples was performed, thus leading to the construction of 2D protein maps of healthy and myocarditis samples. Each gel was blue comassie stained. Image analysis was performed on the two sets of 2D maps (from healthy control and pathologic sera) clearly showing that the protein profile is quite different, as reported in figure 2. The protein spots which resulted to be differentially expressed in the two samples were then submitted to mass spectral identification. Fig 2 shows all the spots we chose.



(a) (b)

Fig. 2. Two-dimensional electrophoresis gels of healthy (A) and myocarditis affected (B) sera proteins. Arrows and numbers in (B) correspond to numbers of identified spots in Table 2.

It should be underlined that all the spots we further analysed were taken from the pathologic serum and results are summarized in Table 2.

Spot	Accession number	Protein
1	P02790	Hemopexin
2	P02790	Hemopexin
3	P02790	Hemopexin
4	P02790	Hemopexin
5	P02790	Hemopexin
6	P02787	Serotransferrin
7	P01008	Antithrombin III
8	P01008	Antithrombin III
9	P01011	Alpha-1 antichymotrypsin
10	P01024	Complement C3
11	P04196	Histidine rich glycoprotein
12	P01024	Complement C3
13	P00738	Haptoglobin
14	P04196	Histidine rich glycoprotein
15	Q14624	Inter-alpha-trypsin inhibitor heavy chain H4
16	P08603	Complement factor H
17	P08603	Complement factor H
18	P03952	Plasma kallikrein
19	P06727	Apolipoprotein A-IV
20	P00738	Haptoglobin
21	P00738	Haptoglobin
22	P36955	Pigment epithelium-derived factor
23	P36955	Pigment epithelium-derived factor
24	P36955	Pigment epithelium-derived factor

Table 2. List of proteins identified by LC-MS/MS of spots excised from two-dimensional gels of sera taken from myocarditis affected patients.

Proteins excised from the gel were reduced alkylated and, in situ, digested with trypsin. The resulting peptide mixtures were directly analysed by LC-MS/MS according to the peptide mass fingerprinting procedure. MS and MS-MS obtained data were used to search for a non-redundant sequence using the in-house MASCOT software, taking advantage of the specificity of trypsin and of the taxonomic category of the samples. The number of measured masses that matched within the given mass accuracy of 20 ppm was recorded and the proteins that had the highest number of peptide matches were examined.
Thanks to this approach, we could identify many proteins differently expressed in the two sera. Among identified proteins, hemopexin (Dooley H et al., 2010) , complement C3 (Adamsson Eryd S et al., 2011, Onat A et al., 2011), plasma kallikrein (Kolte D et al.,2011) are undoubtedly related to inflammatory events and most interestingly they resulted clearly over expressed in the pathologic sample.
A preliminary speculation on identified proteins might involve the function of hempopexin. As shown by literature data (Dooley H et al., 2010, : Mauk MR et al., 2011, Larsen R et al.,2010), hemopexin is a serum protein with the very well known function of scavenging the heme released or lost by the turnover of heme proteins such as haemoglobin or by haemolysis caused by parasitic infection, and thus protects the body from the oxidative damage that free heme can cause (Larsen R et al.,2010). Myocarditis itself it's not related to haemolysis phenomena, but some viral infections may cause it, therefore, finding a very high level of hempoxin in a myocarditis affected patient might be a putative marker of the inflammation itself (quite common are in fact viral myocarditis).
Moreover we found some connections between differently expressed proteins in pathologic serum and identified free peptides; in particular we could detect some free peptides some peptides from proteins Complement C3, Inter-alpha-trypsin inhibitor heavy chain H4, Antithrombin III which results over expressed in pathologic serum, (see Tab 1).

3.3 Boronate affinity chromatography

The third part of this work focus on the investigation of one of the most important post-translational modification, the glycosylation. The importance of investigation of post-translational modifications (PTM) is notably increased in the proteomic era, as they play a critical role in cellular functioning and they vary in response to environmental stimuli, signalling modulators or development of diseases (Laurell E et al., 2011). PTMs can affect biological functions thus playing a critical role in cellular functioning. Moreover, they can vary in response to environmental stimuli, thus finely tuning cellular mechanisms and their deregulation might be involved in the development of diseases. A huge number of different types of PTMs have been identified but only a few are reversible and important for regulation of biological processes (Wu C et al. 2011). The pattern of PTMs on proteins constitute a molecular code that dictates protein conformation, cellular location, macromolecular interactions and activities, depending on cell type, tissue and environmental conditions. Understanding this code is the major challenge of proteomics in post-genomic era. Existing methodologies for PTMs identification essentially rely on specific enrichment procedures able to selectively increase the amount of modified peptides. These procedures have to be integrated with sophisticated mass spectrometric experiments to address the identifications of PTMs. The development of a variety of new technologies for exploring the structures of the sugar chains has opened up a new frontier in the glycomics field. Moreover recent progress in mass spectrometry led to new challenges in glycomics, including the development of rapid glycan enrichment. Recently our group introduced an

easy to handle strategy to give preliminary insights for the comparison of glycoproteomes in healthy and pathological human sera, by using a single Con A affinity chromatography step coupled with mass spectrometry techniques. The strategy led both to the identification of 69 different glycosylation sites within 49 different proteins and to the definition of the glycosylation patterns. Moreover, glycoform distribution in myocarditis and hepatic carcinoma has been reported. The analysis of glycan profiling, once extracted from serum glycopeptides, is essential for comparative studies on different sera samples thus providing a useful tool for the development of screening procedures (Carpentieri A, Giangrande C, et al., 2010).

In this paper, a different simple and rapid procedure to obtain an overview of the glycosylation sites profiling in the two samples was accomplished. This was achieved by enriching for the N-linked glycopeptides resulting from trypsin digestion of sera samples in order to enhance the identification of N-glycosylation sites using LC-MS/MS. The analyses have been carried out by using healthy sera as control.

To reduce the complexity of the whole sample, Boronate affinity purification was rapidly performed in batch after tryptic digestion. Thanks to the vicinal diols binding capacity no discrimination on the basis of the glycan type was performed thus, in a proof of principle, all glycopeptides could be selected. The recovered glycopeptides were then deglycosylated by PNGase F treatment and the peptide mixtures directly analysed by LC-MS/MS.

The analyses were performed on intact serum samples without any pre-purification step or removal of most abundant proteins. The peptide component of the eluted fraction was analysed by tandem mass spectrometry. Similar analyses were carried out on the unbound Boronate fractions, mainly containing non-glycosylated peptides.

The data were then pooled and summarised in Table 3. The results presented here demonstrated that Boronate affinity chromatography on serum tryptic digests is a useful tool to enhance the detection by LC-MS/MS of glycopeptide. Another advantage of the strategy relies on the fact that it was performed on glycopeptides instead of glycoproteins, therefore there were no SDS-PAGE step, no isolation of the individual glycoproteins and no *in situ* digestion. This allowed the detection of less abundant glycopeptides together with the most represented ones, such as those deriving from albumins or immunoglobulins.

As shown in Table 3, all the selected peptides still contained the conserved N-glycosylation motif (Asn-X-Ser/Thr), thus indicating that N-glycosylation peptides were isolated with high selectivity. This analysis led both to the localization of the modification sites and identification of glycoproteins.

The presence of a putative N-glycosylation site was confirmed by the fact that peptides mass was increase of 1 Da, due to the conversion of Asn into Asp after PNGase F incubation. However, some non-specific peptides, namely non-glycosylated peptides, were detected in the eluted Boronate fraction, and identified as belonging to most abundant proteins like albumin.

Spontaneous deamidation seems rather unlikely for generating the results presented, although it cannot be excluded completely. Most MS/MS spectra giving positive hits were derived from doubly charged precursor ions that resulted predominantly in y-ion series.

As a whole, using Boronate affinity approach we could confirm the previously identified glycosylation sites based on ConcanavalinA enrichment and 5 more glycosylation sites were identified thus refining previous data (Carpentieri A, Giangrande C, et al., 2010) on myocarditis glycoproteome.

	Protein	Sequence	Peptide
P01011	Alpha-1-antichymotrypsin	YTGN*ASALFILPDQDKH,M	268-283
P02763	Alpha-1-acid glycoprotein	SVQEIQATFFYFTP**N***KTEDTIFLR[H,M] QDQCIY**N***TTYLNVQR[H,M]	58-81 87-101
P01009	Alpha-1-antitrypsin	YLGN*ATAIFFLPDEGKH,M QLAHQSN*STNIFFSPVSIATAFAMLSL GTKH ADTHDEILEGLNFN*LTEIPEAQIHEGF QELLRH,M	268-283 64-93 94-125
P01023	Alpha-2-macroglobulin	SLGNVN*FTVSAEALESQELCGTEVPS VPEHGRKH	864-886
P43652	Afamin	DIENFN*STQKH,M YAEDKFN*ETTEKH	28-37 396-407
P01008	Antithrombin III	LGACN*DTLQQLMEVFKH,M SLTFN*ETYQDISELVYGAKH,M	124-139 183-201
P04114	Apolipoprotein B-100	FN*SSYLQGTNQITGRH,M FVEGSHN*STVSLTTKH AEEEMLEN*VSLVCPKM YDFN*SSMLYSTAKM	1522-1536 3405-3419 27-41 3462-3474
P05090	Apolipoprotein D	ADGTVNQIEGEATPVN*LTEPAKM	83-104
P02749	Apolipoprotein H	VYKPSAGN*NSLYRH LGN*WSAMPSCKM	155-167 251-261
O75882	Attractin	IDSTGN*VTNELRH,M GPVKMPSQAPTGNFYPQPLLN*SSMC LEDSRH	411-422 1023-1052
P00450	Ceruloplasmin	EHEGAIYPDN*TTDFQRH,M EN*LTAPGSDSAVFFEQGTTRH,M	129-144 396-414
P08603	Complement factor H	ISEEN*ETTCYMGKH MDGASN*VTCINSRH,M IPCSQPPQIEHGTIN*SSRM SPDVIN*GSPISQKH	907-919 1024-1036 868-885 212-224
P10909	Clusterin	LAN*LTQGEDQYYLRH,M	372-385
Q8IWV2	Contactin-4	LN*GTDVDTGMDFRM	64-76
P05156	Complement factor 1	FLNN*GTCTAEGKH,M	100-111
P01024	Complement C3	TVLTPATNHMGN*VTFTIPANRH	74-94
P0C0L4	Complement C4-A	GLN*VTLSSTGRH,M	1326-1336
P02748	Complement component C9	AVN*ITSENLIDDVVSLIRH,M	413-430
Q14517	Protocadherin-fat 1	QVYN*LTVRAKDKM FSMDYKTGALTVQN*TTQLRSRM	994-1005 1930-1950
P02765	Alpha-2-HS-glycoprotein	VCQDCPLLAPLN*DTRH,M AALAAFNAQNN*GSNFQLEEISRH	145-159 166-187
Q03591	Complement factor H related protein 1	LQNNENN*ISCVERH	120-132

	Protein	Sequence	Peptide
Q13439	Golgin subfamily A member 4	HN*STLKQLMREFNTQLAQKH	1990-2008
P02790	Hemopexin	SWPAVGN*CSSALRH,M ALPQPQN*VTSLLGCTHH,M	181-193 447-462
P00738	Haptoglobin	VVLHPN*YSQVDIGLIKH,M MVSHHN*LTTGATLINEQWLLTTAKH,M NLFLN*HSEN*ATAKH,M	236-251 179-202 203-215
P04196	Histidine rich glycoprotein	VEN*TTVYYLVLDVQESDCSVLSRH VIDFN*CTTSSVSSALANTKH,M	61-83 121-139
P05155	Plasma protease C1 inhibitor	VGQLQLSHN*LSLVILVPQNLKH,M	344-364
P01857	Ig alpha-1 chain C region	LSLHRPALEDLLLGSEAN*LTCTLTGLRH	127-153
P01859	Ig gamma-2 chain C region	TKPREEQFN*STFRH TPLTAN*ITKH	168-180 200-208
P01860	Ig gamma-1 chain C region	EEQYN*STYRH,M	136-144
P01591	Immunoglobulin J chain	EN*ISDPTSPLRH,M	48-58
P40189	Interleukin-6 receptor beta	QQYFKQN*CSQHESSPDISHFERM	812-833
P56199	Integrin alpha-1	SYFSSLN*LTIRM	1096-1106
P29622	Kallistatin	DFYVDEN*TTVRH	232-242
P01042	Kininogen-1	LNAENN*ATFYFKH,M	389-400
P11279	Lysosome-associated membrane glycoprotein 1	DPAFKAAN*GSLRM	314-325
P01871	Ig mu chain C region	YKN*NSDISSTRH GLTFQQN*ASSMCVPDQDTAIRM	44-54 204-223
Q9HC10	Otoferlin	NEMLEIQVFN*YSKVFSNKH,M	59-76
Q5VU65	Nuclear pore membrane glycoprotein 210-like	EVVVN*ASSRH	1551-1559
Q9Y5E7	Protocadherin beta 2	ETRSEYN*ITITVTDFGTPRM	414-432
P36955	Pigment epithelium derived factor	VTQN*LTLIEESLTSEFIHDIDRH	282-303
P27169	Serum paraoxanase/arylesterase1	HAN*WTLTPLKH	250-259
P49908	Selenoprotein P	EGYSN*ISYIVVNHQGISSRH	79-97
Q9Y275	Tumor necrosis factor ligand superfamily member 13B	CIQNMPETLPN*NSCYSAGIAKM	232-252

	Protein	Sequence	Peptide
P40225	Thrombopoietin	IHELLN*GTRGLFPGPSRRM	250-267
P02787	Serotransferrin	QQQHLFGSN*VTDCSGNFCLFRH,M CGLVPVLAENYN*KSDNCEDTPEAGY FAVAVVKM	622-642 421-452
P07996	Thrombospondin 1	VVN*STTGPGEHLRH,M	1065-1077
P04004	Vitronectin	NN*ATVHEQVGGPSLTSDLQAQSKM	85-107
P25311	Zinc-alpha-2-glycoprotein	DIVEYYN*DSN*GSHVLQGRH,M	100-117
P02766	Transthyretin	ALGISPFHEHAEVVFTA**N***DSGPR^{H}	101-123

Table 3. LC/MSMS analysis for the identification of glycosylation sites, H indicates peptides deriving from healthy serum and M indicates the ones from myocarditis serum.

4. Conclusions

In biomedical applications, a comparative approach is usually employed to identify proteins that are up and down regulated in a disease specific manner for use as diagnostic markers or therapeutic targets. This report represents an overview of the investigation at molecular level of myocarditis by using a proteomic approach .

Serum proteins (including the N-glycosylation sites profiling) and glycoproteins and free peptides occurring in human sera from healthy donors were compared to the ones from myocarditis patients. This procedure, allowed the identification of several N-glycosylation sites by a single-step proteomic approach, contemporarily probing an entire complex sample by LC-MS/MS. Thanks to the depletion of the serum most abundant proteins, we could detect some of the very weakly represented free peptides, whose presence is connected to the pathology itself. The high resolution, the sensitivity and the reproducibility of the used techniques led to the identification of some up regulated proteins in the serum from a myocarditis affected patient, all these proteins are connected to inflammatory events and one in particular (hemopexin) opens the way to new speculations in serum proteins as a specific marker for pathologic state.

Finally, this proteomic approach represents a new opportunity for therapeutics and early diagnostics, for the screening of proteic biomarkers in pathological status. Finding a biomarker molecule that precisely indicates certain kind of pathology, is something quite difficult to achieve since it requires a huge background in many different fields of clinical investigation. Here we contribute with putative diagnostic species that could really be helpful for an early diagnosis myocarditis event.

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Myocarditis Presenting with Ventricular Arrhythmias: Role of Electroanatomical Mapping-Guided Endomyocardial Biopsy in Differential Diagnosis

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1. Introduction

Myocarditis is defined as a disease characterized by myocardial inflammation associated with myocyte necrosis. It can be caused by infections, autoimmune response primarily affecting the myocardium or by systemic autoimmune or inflammatory disorders (Aretz et al., 1986). Viral infections are the most frequent cause and account for the vast majority of cases in North America and Europe (Cooper, 2009).

Cardiac symptoms that develop during myocarditis may follow after a delay of days to weeks from the beginning of the pathological process; they are quite unspecific and include fatigue, dyspnoea, palpitations, malaise and atypical chest discomfort. Even the clinical cardiac signs may be vague in many patients and generally include cardiac murmurs, gallop rhythms and other signs of heart failure and sometimes pericardial rubs when the pericardium is also involved in the inflammatory process. Myocarditis is often associated with various types of ECG abnormalities (including bundle branch blocks, Q waves resembling those related to myocardial infarction, repolarization abnormalities and QRS prolongation) and rhythm disturbance such as atrio-ventricular blocks, supraventricular tachycardias and ventricular ectopies and tachycardias. Echocardiography may reveal overt systolic dysfunction or a reduction of peak systolic velocities at TDI; moreover, regional wall motion abnormalities and diastolic dysfunction may be found (Cooper, 2009; Feldman et al. 2000). Therefore, this disease should always be considered in patients who present with rapidly progressive cardiomyopathy, chest pain with ECG anomalies that mimic an acute coronary syndrome but with normal coronary arteries or idiopathic ventricular arrhythmias. Furthermore, in young people myocarditis may be frequently responsible for sudden cardiac death, particularly after strenuous physical exertion (Doolan et al., 2004; Corrado D et al. 2001). With this regard it should be highlighted that the recognition of myocarditis in patients presenting with aborted sudden death or major ventricular arrhythmias is actually challenging in everyday clinical practice, as the diagnosis may be difficult and may require the use of invasive procedures. Nevertheless, the detection of myocarditis in patients presenting with ventricular arrhythmias may have a pivotal importance, because the

identification of myocarditis as the substrate of arrhythmias is actually important for targeting therapies.
In the last decades the development of new diagnostic techniques, in particular cardiac magnetic resonance, has led to an increased recognition of myocarditis as a cause of ventricular arrhythmias. However endomyocardial biopsy still represents the gold standard for the diagnosis of myocarditis. The main criticism against a wider use of endomyocardial biopsy in the diagnostic approach to patients with ventricular arrhythmias is represented by the possible sampling error in the presence of a focal myocarditis. We recently demonstrated that three dimensional electroanatomical mapping (3D-EAM) may guide endomyocardial biopsy identifying the segments of ventricular wall presenting an abnormal voltage, thus reducing sampling error and increasing sensitivity of biopsy. The systematic association of endomyocardial biopsy with electroanatomical mapping represents a significant improvement of the diagnostic tools available to identify the substrate of ventricular arrhythmias.
In this chapter we describe the role of electroanatomical mapping-guided endomyocardial biopsy in the differential diagnosis of myocardial pathological substrates in patients with ventricular arrhythmias. We report the results of our group and other groups adopting this technique in different categories of patients, including subjects with a clinical diagnosis of arrhythmogenic right ventricular cardiomyopathy (ARVC), competitive athletes and patients with electrocardiographic diagnosis of Brugada syndrome.

2. Myocarditis and ventricular arrhythmias

Endomyocardial biopsy and autopsy findings have clearly demonstrated that myocarditis represents a frequent cause of life-threatening ventricular arrhythmias and sudden death. Post-mortem studies suggest that myocarditis is a major cause of sudden, unexpected death in adults less than 40 years of age (accounting for approximately 20% of cases - Fabre & Sheppard, 2006; Doolan et al., 2004). Indeed myocarditis may frequently cause ventricular arrhythmias associated with systolic dysfunction of left or right ventricle or both. In both its acute and chronic phase myocarditis may be associated with severe arrhythmias that can significantly affect the natural course of the disease, as they can further contribute to the deterioration of cardiac systolic and diastolic function and can be the ultimate cause of death in these patients (Magnani et al. 2006; Zeppenfeld et al. 2007; Graner et al, 2007). Mechanisms of arrhythmogenesis in the context of myocarditis include myocyte necrosis, replacement fibrosis (favoring re-entry mechanism), proarrhythmic effects of cytokines and inflammatory mediators possibly through a modulation of ion channel function.
In patients with chronic active myocarditis, the perpetuation of inflammation is often related to viral persistence or to autoimmune self-maintaining mechanisms, and replacement fibrosis seems to represent a major arrhythmogenic substrate in this case, together with the permanence of an inflammatory milieu surrounding myocardiocytes. Moreover it has been demonstrated that enteroviral persistence perpetrates myocardial damage even in the absence of overt myocardial inflammation, through the release of proteases capable of cleaving dystrophin (Andreoletti et al., 2007). This in turns causes cytoskeleletal anomalies that can affect myocyte mechanical and even electric properties and finally lead to myocyte death, possibly contributing to arrhythmogenesis.
Myocarditis may be the cause of ventricular arrhythmias even in subjects with no previous symptoms or presenting with an apparently normal heart or minimal electrocardiographic

and cardiac structural abnormalities (Theleman et al., 2001; Friedman et al., 1994). Subtle abnormalities not detectable by first-line examinations such as echocardiography may be present in patients with myocarditis presenting with ventricular arrhythmias. Other imaging modalities such as cardiac magnetic resonance (CMR) and even ventricular angiography are generally needed to detect these abnormalities. In a study published in 2001 we found small aneurysms at ventricular angiography in patients with apparently idiopathic major ventricular arrhythmias; it should be underlined that all the patients enrolled in this study were also submitted to CMR that failed to detect microaneurysms in most patients (It is possible that currently used cine-sequences for functional analysis of both ventricles may enhance the diagnostic performance of CMR even in this setting). Histological examination of myocardial samples drawn from areas surrounding the aneurysms revealed the presence of active lymphocytic myocarditis with intense myocytolisis. Notably, no patient suffered from cardiac sudden death or malignant ventricular arrhythmias during a 1-year follow-up and sequential Holter recording showed progressive reduction of the arrhythmic burden. Furthermore neither heart failure episodes nor decrease of LV function were reported in the study population (Chimenti et al., 2001). Under this respect it should be noticed that inflammatory microaneurysms were also found in experimental animal models of myocarditis, namely in hamster and mice which survived to acute viral myocarditis, and they seem to cause electroanatomical abnormalities that are associated with development of severe ventricular arrhythmias; interestingly, in the inoculated animals the prognosis associated with the development of microaneurysms in the setting of experimental viral myocarditis was good with a survival similar to normal animals who had no aneurysms (Hoschino et al., 1984; Matsumori et al 1983).

Myocarditis may selectively affect the right ventricle causing structural abnormalities, including microaneurysms, and arrhythmic manifestations typical of arrhythmogenic right ventricular cardiomyopathy. In fact myocardial inflammatory infiltrates associated with myocyte necrosis and replacement fibrosis, may lead to functional and structural changes of right ventricular myocardium resembling those produced by fibrofatty replacement, and representing the substrate of abnormal voltage map and ventricular arrhythmias (Hoffmann et al, 1993; Pieroni et al., 2009). Namely, our group demonstrated that biopsy-proven myocarditis is present in up to 50% of patients fulfilling current diagnostic criteria of arrhythmogenic right ventricular cardiomyopathy at non-invasive evaluation (including cardiac magnetic resonance), moreover myocarditis was associated to the presence of low-voltage areas, as detected by 3D electro-anatomic mapping (Pieroni et al., 2009). Consistently it has been recently reported that also right ventricular sarcoidosis may mimic electroanatomic mapping features and arrhythmic presentation of arrhythmogenic right ventricular cardiomyopathy (Ott et al., 2003; Koplan et al. 2006; Vaisawala et al., 2009).

Myocarditis represents a frequent cause of ventricular arrhythmias also in competitive athletes and its recognition and differential diagnosis with other cardiomyopathies with different prognosis may have important implications for sport eligibility (Basso C et al., 2007). In fact, a recent study by our group demonstrated the presence of myocarditis, diagnosed by endomyocardial biopsy, in most elite athletes presenting with major arrhythmias and apparently normal heart. Moreover it should be emphasized that according to current diagnostic recommendations (the 2nd consensus document on Brugada Syndrome underlined the need to exclude other pathological conditions), the presence of a myocarditis should be always excluded in patients with electrocardiographic features leading to the diagnosis of Brugada syndrome. Among the pathologic conditions that can lead to a

Brugada-like phenotype, myocarditis of the right ventricle is one of the most frequently found when patients with Brugada syndrome are submitted to an extensive invasive and non-invasive evaluation (Frustaci et al., 2005; Okubo et al., 2010). In facts, a study on 18 patients with Brugada Syndrome (diagnosed according to the consensus criteria) presenting with sustained ventricular arrhythmias or syncope, revealed the presence of biopsy-proven myocarditis in most patients (Frustaci el al., 2005). The complex links between myocarditis and genetically determined arrhythmic disorders is further discussed below in this chapter.
Various studies on experimental animal models contributed to demonstrate that both autoimmune and viral myocarditis may cause major ventricular arrhythmia. Studies on murine models of myocarditis demonstrated that persistence of inflammation after the acute phase is associated with electrical abnormalities associated with ventricular arrhythmias; interestingly, a close correlation between the location of inflammatory burden (associated with myocyte necrosis), the reported electric abnormalities and the site of origin of arrhythmias was found (Kishimoto et al., 1983; Hoshino et al., 1982;). The major mechanisms involved in the genesis of arrhythmias seems to be the formation of micro-reentry circuits, favored by myocyte injury and replacement fibrosis, and triggered activity mainly due to the pro-arrhythmic effects of cytokines. Moreover it was reported that the environment surrounding myocytes could influence the electrophysiological properties of the myocardium, this phenomenon is known as electrical remodeling. As previously pointed out the inflammatory process of the myocardium may itself be arrhythmogenic, nonetheless the change in the electrophysiological properties of the myocardiocytes seems also play a role in causing the arrhythmias. Changes in expression of surface ion channels, as assessed by quantitative determination of mRNA expression, have been demonstrated in animal experimental models of both viral and autoimmune myocarditis; these changes, affecting mainly potassium and calcium channels, causes modification of the ventricular effective refractory period, which appear to be longer in myocarditis, an increase in monofasic action potential duration of the ventricular myocardium and an augment of ventricular vulnerability that ultimately favors the genesis of ventricular arrhythmias (Saito et al., 2006). Interestingly, some data suggests that these changes may be transient and can reverse after the healing of the inflammatory process. Altogether, these data further support the hypothesis that active inflammation represents a major substrate of ventricular arrhythmias and suggests that pro-arrhythmic changes of myocardial electrical properties caused by the inflammation might be, completely or partially, reversed after the healing of the inflammatory process.

2.1 Myocarditis and sudden arrhythmic death

In the last decades myocarditis has emerged as an important cause of sudden arrhythmic death also in patients with a structurally and functionally normal heart. The most striking evidence that myocarditis may be the substrate of sudden arrhythmic death is represented by a study on Air Force recruits victims of cardiac sudden death in which myocarditis was recognized as the cause of death in 40% of cases (Phillips et al., 1986)
Although no data on cardiac function before death were available in the study, the percentage of cases in which myocarditis was observed at necropsy studies indicates the high arrhythmic risk related to myocarditis. In more recent studies the percentage of myocarditis detected at necropsy in subjects with sudden arrhythmic death and an apparently normal heart ranged from 5 to 12% (Doolan et al., 2004; Thelemann et al., 2001).

In a study performed in our Institution in 1994 seventeen young patients (10 males and 7 females, aged 14 to 38 years, mean 26.4) without overt organic heart disease, who had been resuscitated from sudden cardiac arrest were submitted to noninvasive (electrocardiography, 2D-echocardiography, and magnetic resonance imaging) and invasive (coronary angiography with ergonovine testing, electrophysiologic study and biventricular angiography and endomyocardial biopsy) cardiac studies. Six to 8 biopsy fragments per patient were processed for histology and electron microscopy and read by a pathologist blinded to clinical data. Two groups of patients were distinguished by invasive and noninvasive examinations: Group 1 consisted of 9 patients with entirely normal parameters; Group 2 consisted of 8 patients with structural, nonspecific cardiac abnormalities. In this latter group, mild to moderate dilatation and hypokinesia of the left ventricle were documented in 4 patients, concentric left ventricular hypertrophy was seen in three patients, and RV dysfunction was noted in 1 patient. Histologic examination was abnormal in all patients and revealed specific lesions in 65% of them; in particular an active myocarditis was diagnosed in 6 out of 9 patients in the Group 1. In these 6 patients immunosuppressive therapy (steroids plus azathioprine) administered on top of conventional antiarrhthmic treatment led to dispappearance of ventricular arrhythmias and healing of myocardial inflammation at follow-up endomyocardial biopsies (Frustaci et al., 1994) Similarly in a study from the Padua group in a series of 273 sudden cardiac deaths victims, 76 cases (28%) presented a macroscopically normal heart: of these 60 (79%) had abnormal histology with a 36% of cases of active myocarditis (Corrado et al., 2001). These data suggest that myocarditis represents an important, frequently unrecognized and underestimated, cause of sudden arrhythmic death. Moreover these studies dramatically demonstrate that sudden arrhythmic death may represent the very first clinical manifestation of myocarditis. Accordingly the consensus document based on the two main registries on sudden arrhythmic death (the UCARE, Unexplained Cardiac Arrest Registry of Europe and the IVF-US, Idiopathic Ventricular Fibrillation Registry of United States) includes myocarditis among the subclinical disorders that may cause sudden death (UCARE, 1997).

It should be also emphasized that myocarditis is a cause of sudden arrhythmic death even in competitive athletes, more frequently exposed to viral infections of upper airways. With this regard it has been claimed but never conclusively demonstrated that exercise in the context of an active myocarditis may represent a further arrhythmic trigger.

2.1.1 Myocarditis and genetically determined arrhythmic syndromes

In the last decades several studies suggested a role for myocardial inflammation in the pathogenesis of different genetically determined arrhythmic syndromes and cardiomyopathies. In the presence of gene mutations leading to abnormal structure and/or function of structural and functional proteins, myocardial inflammation may act as a mechanism of myocardial damage, amplificating the dysfunction related to the genetic abnormalities, or rather as trigger of specific clinical manifestations, such as arrhythmias, occurring later in the natural history of the disease. Myocardial inflammation has been observed in necropsy and endomyocardial biopsy studies in genetically determined myocardial disorders, including ARVC, Brugada syndrome and hypertrophic cardiomyopathy.

In ARVC myocardial inflammation may be seen in up to 75% of hearts at autopsy, and probably it plays a role in triggering ventricular tachyarrhythmias (Thiene et al., 2001). Nobody knows whether inflammation is a reactive phenomenon to cell death, or whether it

is the consequence of an infection or immune mechanism. Viruses have been detected in the myocardium of some ARVC patients and have been claimed to support an infective etiology of the disease (Bowles et al., 2002). Others say that the viruses are innocent bystanders or that spontaneous cell degeneration may serve as a milieu favoring viral settlement in the myocardium (Calabrese et al., 2006). In addition we and others demonstrated that myocarditis may present with clinical and arrhythmic features and structural right ventricular abnormalities resemblig those observed in ARVC. (Chimenti et al., 2004; Pieroni et al., 2009).

With regard to Brugada syndrome we studied 18 consecutive patients with clinical phenotype of Brugada syndrome and normal cardiac structure and function on noninvasive examinations. Clinical presentation was ventricular fibrillation in 7 patients, sustained polymorphic ventricular tachycardia in 7, and syncope in 4. All patients underwent cardiac catheterization, coronary and ventricular angiography, biventricular endomyocardial biopsy, and DNA screening of the *SCN5A* gene. Biopsy samples were processed for histology, electron microscopy, and molecular screening for viral genomes. In 14 patients histology showed a prevalent or localized right ventricular myocarditis, arrhythmogenic right ventricular cardiomyopathy in 1 patient and cardiomyopathic changes in 3. In the latter 4 patients genetic studies identified *SCN5A* gene mutations causing in vitro abnormal function of mutant proteins. In these patients, myocyte cytoplasm degeneration and a significant increase of apoptotic myocytes in right and left ventricle versus normal controls were observed (Frustaci et al., 2005). This study highlighted the complexities between the clinical manifestations of Brugada syndrome, the presence of *SCN5A* mutations, and the presence of structural heart disease and provided striking new evidence implicating myocarditis in the transient development of Brugada-like ECG abnormalities and arrhythmias.

However it still remains unclear whether myocardial inflammation may be part of the structural changes following an abnormal ion channel function, or rather right ventricular myocarditis may mimic ECG and arrhythmic features of Brugada syndrome. Further studies combining genetic analysis and accurate pathology studies are needed to clarify the complex menage a trois between abnormal gene function (whether affecting desmosomal or ion channel proteins), myocardial inflammation and inherited arrhythmic syndromes.

Myocarditis has been also observed in patients with hypertrophic cardiomyopathy and severe arrhythimic manifestations, suggesting that inflammation may act as a trigger for life-threatening ventricular arrhythmias when affecting a myocardium already prone to electrical instability (Frustaci et al., 2007). Even in this context it is not clear whether myocardial inflammation was the result of a reactive process related to ischemic myocyte necrosis or rather a superimposed autoimmune or viral process.

3. The diagnosis of myocarditis in patients with ventricular arrhythmias

Myocardial inflammation represents a potent pro-arrhythmic substrate, myocarditis should always be taken into account when facing with ventricular arrhythmias. Furthermore the detection of myocarditis as the pathological substrate underlying ventricular arrhythmias may be crucial as the identification of myocarditis as the etiology of the arrhythmias is actually important for targeting therapies and may have implications even for patients' relatives as discussed below in the chapter. In the last decades the development of new imaging techniques, in particular CMR, led to an increased recognition of myocarditis as a cause of ventricular arrhythmias.

3.1 Cardiac magnetic resonance

Cardiac Magnetic resonance offers a combination of clarity of anatomical visualization, interobserver consistency and quantitative accuracy, even when evaluating the right ventricle, which enables to overcome the major pitfalls of echocardiography. Furthermore it is characterized by the unique feature of providing some degree of tissue characterization, thus allowing to obtain non-invasively information about the presence of necrosis, fibrosis and edema. Nonetheless CMR has some limitations due to the technical difficulty in obtaining good images in patients with arrhythmias that cause high variability of cardiac cycle length and also due to safety issues related to the presence of cardiac rhythm devices. Recently a panel of international experts on CMR has published a white paper in which imaging protocols and diagnostic criteria are defined that should be used in the setting of myocarditis. According to this consensus (Friedrich et al., 2009), a diagnosis of myocarditis can be set when at least two of the following features are present: one area of non-ischemic (i.e. sparing of sub-endocardial layers of the myocardium and/or distribution of areas not consistent with coronary perfusion territories) late gadolinium enhancement (consistent with myocyte injury and scarring), global or regional signal increase in T2-weighted images (indicative of tissue oedema) and global early gadolinium enhancement (consistent with hyperemia) (Figure 1).

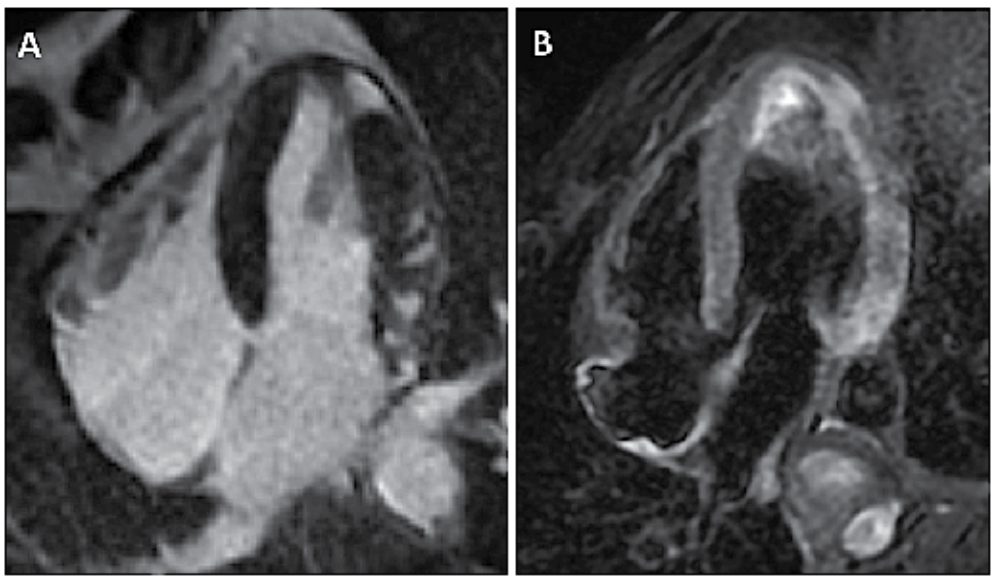

Fig. 1. Diagnosis of myocarditis through CMR in a patient with sustained ventricular arrhythmias. Evidence of spotty areas of sub-epicardial and midwall late enhancement (non-ischemic pattern) in the lateral wall (A), associated with areas of edema (high signal intensity on T2-weighted images) in the same location (B). The presence of both late enhancement and edema fulfils the criteria proposed by the consensus document.

In many studies CMR has demonstrated to be useful for the assessment of the presence of pathological substrates underlying ventricular arrhythmias. Its use allowed to detect the presence of structural cardiomyopathies including myocarditis (Ordovas et al. 2008) in up to 30% of patients. Clinical studies specifically addressing the diagnostic yield of CMR in the

setting of myocarditis presenting with either arrhythmia or heart failure reported a sensitivity and specificity of more then up to 90% (Mahrholdt et al., 2004). Moreover, CMR was able to differentiate active from healing inflammation in patients presenting with chronic myocarditis.

In particular, De Cobelli and colleagues studied patients presenting with chronic myocarditis assessed with EMB and CMR; they found areas of high signal intensity in T2-weighted images in 36% of patients with histological evidence of active myocarditis, but not in patients with borderline myocarditis. They also found areas of late gadolinium enhancement in 84% and 44% of patients with active and borderline myocarditis, respectively. A midwall pattern of late enhancement was a frequent finding in patients with both active myocarditis and borderline myocarditis, whereas a subepicardial pattern was only observed in patients with histological evidence of active myocarditis (De Cobelli et al., 2004). Taken together, these data support the need to include sequences for the detection of myocardial inflammation when CMR is performed in the non-invasive diagnostic work-up of patients presenting with ventricular arrhythmias (Figure 2). In addition CMR represents an important noninvasive tool in the follow-up of patients with a diagnosis of myocarditis.

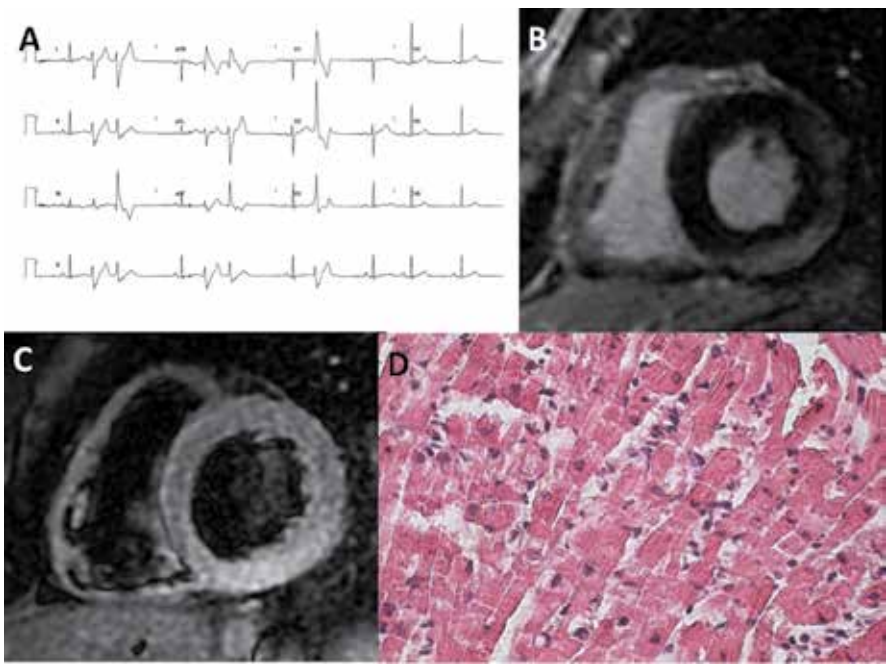

Fig. 2. CMR findings in a patient with chronic myocarditis presenting with ventricular arrhythmias. ECG shows repetitive polymorphic ventricular ectopic beats (A). CMR shows sub-epicardial late enhancement in the posterior and postero-lateral walls (B) and transmural edema in the same location (C). Histology shows the presence of active myocarditis (D).

3.2 Endomyocardial biopsy

Despite the improvement of imaging techniques, endomyocardial biopsy still represents the gold standard for the diagnosis of myocarditis as allow the recognition and the

characterization of the myocardial inflammatory process, the assessment of myocyte necrosis and fibrosis as well as the possible detection of viral genomes.

A recent statement from AHA/ACC/ESC (Cooper et al., 2007) aimed to define the role of endomyocardial biopsy in the management of cardiovascular disease, identified 14 clinical scenarios in which the incremental diagnostic, prognostic and therapeutic value of endomyocardial biopsy could be estimated and compared with the procedural risks. According to this statement, in the absence of randomized clinical trials or multicenter studies, endomyocardial biopsy may be considered in the setting of unexplained ventricular arrhythmias (Clinical scenario 13) only in exceptional cases in which the perceived likelihood of meaningful prognostic and therapeutic benefit outweighs the procedural risks (Class of recommendation IIb, Level of evidence C).

Nevertheless there is growing evidence that endomyocardial biopsy may be crucial to clarify the pathological substrate and therefore the cause of otherwise unexplained ventricular arrhythmias, with a possible impact on both treatment and prognosis as detailed further in this chapter.

Accordingly a recent document from the Italian federation of Cardiology (Leone et al., 2009) suggested indications to endomyocardial biopsy different from the above-mentioned statement, in particular with regard to patients with ventricular arrhythmias. The authors graded clinical indications according to the following scheme:

- Grade 1: there are no alternative tools to obtain a definite diagnosis and the clinical implications of diagnosis are certain.
- Grade 2A: there are no alternative tools to obtain a definite diagnosis and the clinical implications of diagnosis are uncertain.
- Grade 2B: there are no alternative tools to obtain a definite diagnosis but the diagnosis has scientific but no clinical implications.
- Grade 3: there are alternative tools to obtain a definite diagnosis.

According to this grading, taking into account the potential diagnostic usefulness of endomyocardial biopsy in different myocardial disorders, the authors identified several clinical scenarios. In the presence of sustained and/or life-threatening ventricular arrhythmias, when a myocarditis is suspected or is a possible diagnosis, the grade of recommendation to perform endomyocardial biopsy is 1. Similarly in the presence of AV blocks associated with a clinical context consistent with a diagnosis of myocarditis, recommendations are Grade 2A and 2B in the presence or in the absence of left ventricular dysfunction respectively. Therefore the Italian Federation of Cardiology document seems to more adequately address the diagnostic and therapeutic issues that are frequently faced by cardiologists in everyday clinical practice when dealing with patients with severe ventricular arrhythmias or atrioventricular conduction disturbances and no evidence of ischemic heart disease or overt valvular or myocardial disease.

Both documents do also indicate how many samples are needed in different clinical conditions, how to handle and process myocardial samples, and which studies are necessary to obtain the desired diagnostic information. In the case of clinically suspected myocarditis 5 to 10 samples must be obtained to perform histology and immunohistochemistry studies and molecular bioloy studies to detect the presence of viral genomes. Samples for molecular biology must be flash-frozen in liquid nitrogen and stored at -80°C. Endomyocardial biopsy should better not be performed in patients with clinically suspected myocarditis, if immunohistochemistry and virology studies are not available.

The main criticism against a wider use of endomyocardial biopsy in the diagnostic approach to patients with ventricular arrhythmias is represented by the possible sampling error in the presence of a focal myocarditis. In fact, as previously mentioned, the myocardial inflammation in patients presenting with arrhythmias may present a patchy distribution and therefore myocardial samples drawn from a same conventional site (the right side of interventriculrar septum or the left ventricular apex) may not include myocardial tissue involved by the inflammatory process. Increasing the number of samples obtained or arbitrarily changing the site of biopsy can in part minimize sampling error, but these tricks may increase the risks of the procedure. To overcome the sampling error and increase the diagnostic sensistivity of endomyocardial biopsy, we recently demonstrated that in patients presenting with ventricular arrhythmias, electroanatomical mapping may guide the execution endomyocardial biopsy identifying the segments of ventricular wall presenting an abnormal voltage, suggesting an abnormal histological substrate (Pieroni et al, 2009; Casella & Dello Russo, 2009). This technique represents an important innovation in the field of endomyocardial biopsy as reduces sampling error and increases the sensitivity of biopsy, thus possibly reducing the number of samples needed for a complete study of myocardial tissue and therefore reducing the risks of the procedure.

3.3 Electroanatomic mapping

Electroanatomic mapping allows operators to record intracardiac electrical activation in relation to anatomic location in a cardiac chamber of interest, even during arrhythmia mapping. Several 3D-EAM systems are currently available that accomplish these tasks. When applied properly, such technology allows one to accurately determine the location of arrhythmia origin, define cardiac chamber geometry in three dimensions, delineate areas of anatomic interest, and allow catheter manipulation and positioning without fluoroscopic guidance. These systems often simplify mapping efforts and can enhance procedural success, particularly in cases in which complex arrhythmias and unusual cardiac anatomy are encountered.

The CARTO mapping system (Biosense, Diamond Bar, CA, USA) is a three-dimensional nonfluoroscopic mapping system that utilizes a low-level magnetic field (5×10^{-6} to 5×10^{-5} Tesla) delivered from three separate coils in a locator pad beneath the patient. The magnetic field strength from each coil is detected by a location sensor embedded proximal to the tip of a specialized mapping catheter. The strength of each coil's magnetic field measured by the location sensor is inversely proportional to the distance between the sensor and coil. Hence, by integrating each coil's field strength and converting this measurement into a distance, the location sensor (and therefore, catheter tip location) can be triangulated in space. The mapping catheter has proximal and distal electrode pairs, and a tip electrode capable of radiofrequency energy delivery. This catheter can be moved along a chamber's surface to record local endocardial activation times for arrhythmia mapping, while simultaneously recording location points to generate 3D chamber geometry. Validation studies have shown CARTO to have substantial accuracy in navigating to single points, in returning to prior ablation sites, and in creating a desired length of ablation line. Electrograms recorded from the specialized mapping (NaviSTAR) catheter showed excellent correlation with recordings from standard EP catheters. Moreover human validation studies have shown a good level of spatial precision and accuracy, and realistic reconstruction of chamber geometry and electroanatomic activation during arrhythmia mapping. Nevertheless in our experience, the comparison between biplane right ventricular angiography and the 3D reconstruction

obtained by CARTO showed that small structures such as microaneurysms, can be missed by the mapping catheter and therefore are not properly visualized. These limitations would be partially overcome by importing CT and CMR images and merging them with 3D maps so that during mapping the catheter can be visualized inside the anatomical images.
Similar to CARTO the NavX system is a mapping and 3D nonfluoroscopic intracardiac browsing system that can reconstruct in real time geometry as well as the electric activation of the heart chamber. NavX technology makes use of six surface patch electrodes that generate, once correctly positioned, an electric filed. Within this electric field it is possible to locate in real time the position of every electrophysiology catheter employed during the procedure, including the cardiac bioptome when electically connected to the mapping system. We tested both systems in guiding endomyocardial biopsy execution. Although a comparison between the systems exceeds the scopes of this chapter, CARTO system probably offers a more detailed reconstruction of the ventricular chambers, while NavX system has the advantage of direct real time visulization of the bioptome in the 3D map. In this chapter we will refer to the CARTO system as it was more frequently used in our and other studies.
High-density mapping must be obtained in sinus rhythm (reference channel: QRS complex) by sampling at least 100 points in each chamber, uniformly distributed. The voltage maps are then edited setting the point density (fill threshold) at 15 mm and manually eliminating intracavitary points. According to current literature "electroanatomic scar" is defined as an area including at least 3 adjacent points with bipolar signal amplitude <0.5 mV; the reference value for normal endocardium is usually set at 1,5 mV. In the CARTO system the color display to identify normal and abnormal voltage myocardium ranges from red (electroanatomic scar tissue; amplitude <0.5 mV), to purple (electroanatomic normal tissue; amplitude ≥1.5 mV). Intermediate colors represent the electroanatomic border zone (amplitude >0.5 and <1.5 mV) (Figure 3).

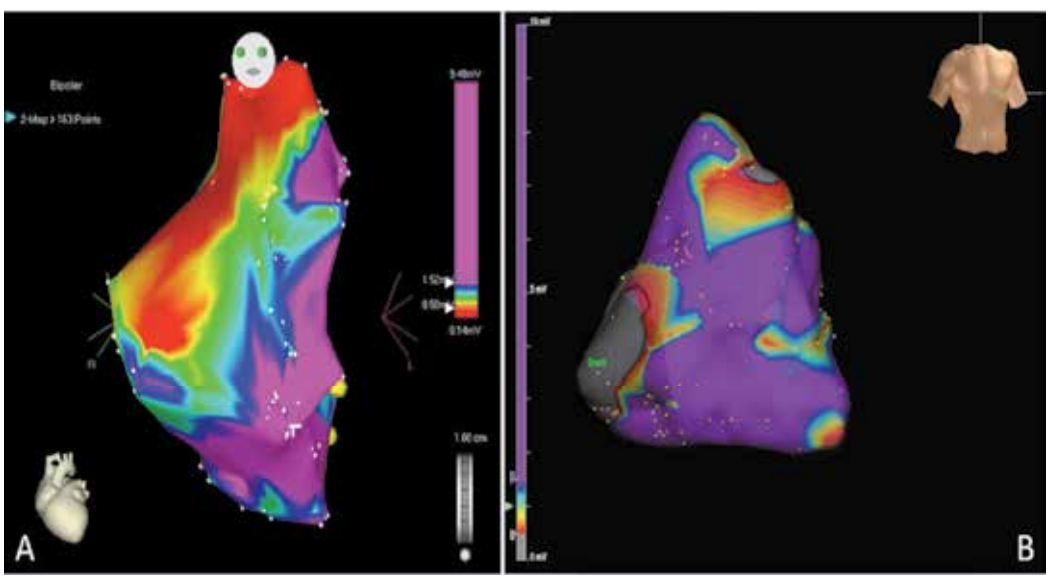

Fig. 3. Example of electroanatomic maps obtained with CARTO (A) and NavX (B) systems. For colours interpretation see text.

Adequate catheter contact should be confirmed by concordant catheter tip motion with the cardiac silhouettes on fluoroscopy and by adherence of voltage map to angiographic right ventricular shape. To avoid low voltage recordings due to poor contact, the following tools can be used: 1) the signal has to satisfy 3 stability criteria automatically detected by CARTO system in terms of cycle length, local activation time and beat-to-beat difference of the location of the catheter (<2%, <3 ms, and <4 mm, respectively); 2) both bipolar and unipolar signals are simultaneously acquired to confirm true catheter contact through the analysis of local electrogram (in particular the shape of the unipolar electrogram); 3) in the presence of a low voltage area, at least 3 additional points should be acquired in the same site to confirm the reproducibility of the voltage measurement. The anatomical distribution of the pathological areas is evaluated dividing the right ventricular voltage map into five segments: outflow tract, free (anterolateral) wall, inferior and posterior basal segments, apex, and interventricular septum.

3.4 Electroanatomic mapping-guided endomyocardial biopsy

The main limitation of endomyocardial biopsy to provide a specific diagnosis in patients with ventricular arrhythmias caused by focal myocardial diseases, (i.e. myocarditis or initial forms of arrhythmogenic right ventricular cardiomyopathy), is represented by the sampling error due to the lack of an effective guide in selecting ventricular areas where to perform biopsies. In the last years, after the development of 3D-EAM systems, we introduced a new technique for the execution of endomyocardial biopsies in patients with ventricular arrhythmias (Pieroni et al., 2009).

3.4.1 Technique

The new approach is aimed to perform endomyocardial biopsies in the ventricular segments presenting electrical abnormalities at electroanatomical mapping. When using the CARTO system, once the electroanatomical map is completed, the mapping catheter is placed in a region of interest of the ventricular wall and the preformed sheath is positioned closed to the catheter tip (Figure 4). Endomyocardial biopsy is then performed in the area with abnormal electrical properties as showed by the map. As previously mentioned, another approach we tested requires the electrical connection of the bioptome to the mapping system: the presence of the metallic jaws makes the bioptome similar to a mapping catheter and it is therefore visualized in the 3D electroanatomical map of the ventricle. In this case, the site of biopsy can be chosen "live", directly mapping the ventricular wall with the bioptome (Figure 4). The latter technique can be adopted when using the NavX mapping system. With both systems we usually performed ventricular angiography before the execution of ventricular mapping, in order to improve the anatomical accuracy of the map, as angiography still represents the gold standard to detect wall motion abnormalities and small aneurysms of the right venticle.

Although no data on specificity and sensitivity of 3D-EAM-guided vs. conventional technique are currently available in literature, it is reasonable that obtaining myocardial samples from areas of the ventricular wall presenting electrical abnormalities will probably reduce the sampling error and the need for multiple biopsies from the same patient (See below). Moreover the combination of 3D-EAM with other imaging tools such as cardiac MRI would further improve our ability to obtain samples from selected regions that present structural, functional and electrical abnormalities.

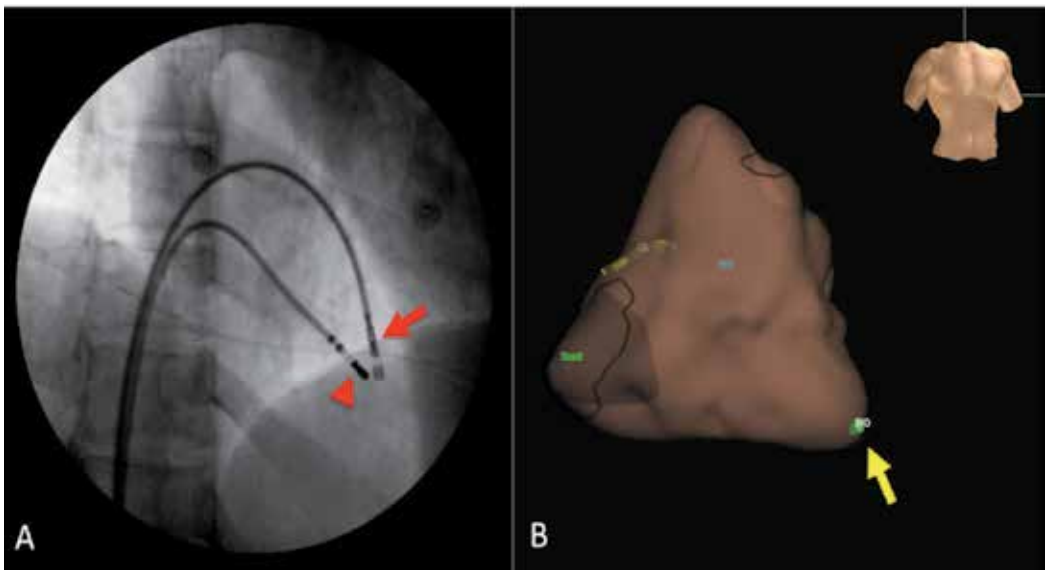

Fig. 4. 3D-EAM-guided EMB. With the CARTO system (A) the long sheath with the bioptome inside (arrow) is positioned near to the mapping catheter (arrowhead). With the NavX system (B) the bioptome tip (green point and arrow) can be directly visualized in the 3D ventricular map.

3.4.2 Safety

The execution of endomyocardial biopsies drawing myocardial samples from non conventional sites, including the right ventricular free-wall and outflow tract do not increase the risks of the procedure. Since 2006 we perfomed 3D-EAM-guided endomyocardial biopsy in more than sixty patients with about 400 samples obtained. In order to evaluate the safety of this new approach we prospectively analysed the rate of major and minor complications through continuous ECG monitoring during the procedure, ECG and 2D-echocardiography at the end the procedure and after 3 and 6 hours. Major complications included pericardial tamponade with need for pericardiocentesis, hemo- and pneumopericardium, permanent atrioventricular block requiring permanent pacemaker implantation, myocardial infarction, transient cerebral ischemic attack and stroke, severe valvular damage, and death, whereas minor complications included transient chest pain, transient ECG abnormalities, transient arrhythmias, transient hypotension, and small pericardial effusions. The major complication rate was 0% and the minor complication rate was 4.5%: minor complications were represented by small pericardial effusions in 2 patients and chest pain in 1. These rates are in line with those observed in a recent large two-centers study including 755 procedures (right, left and biventricular endomyocardial biopsy) and reporting a major and minor complication rate of 0.82% and 5.1% respectively for right ventricular endomyocardial biopsy (Yilmaz et al., 2010). In literature another study in which 3D-EAM-guided endomyocardial biopsy was performed in 22 patients, reported a higher rate of major complications (1.1%) and minor complications (5.7%), thus suggesting that the expertise of the operators besides the clinical condition and the underlying disorder may influence the safety of the procedure (Avella et al., 2008).

4. Electroanatomic mapping-guided endomyocardial biopsy in patients with ventricular arrhythmias

There is growing evidence that 3D-EAM-guided endomyocardial biopsy represent a new important tool to improve the diagnosis and the treatment of patients with arrhythmias, as well as the knowledge of the pathological substrates and mechanisms ubderlying arrhythmic syndromes. Since 2006 we performed 66 3D-EAM-guided endomyocardial biopsy procedures. In all cases the biopsies where drawn from the right ventricle, with biventricular mapping and 3D-EAM-guided biopsy being performed in 10 patients. Although data on a direct comparison between 3D-EAM-guide and conventional technique are not yet available, the 3D-EAM-guided approach seems to significantly improve the diagnostic sensitivity and specificity of endomyocardial biopsy. In fact, when applying immunohistochemistry and molecular biology techniques, a definite histologic diagnosis was obtained in all cases. The new technique was firstly adopted in a series of 30 patients with a noninvasive diagnosis of ARVC according to current diagnostic criteria (Pieroni et al., 2009). Twenty-nine (97%) of 30 patients presented an abnormal voltage map. Histology and immunohistochemistry confirmed the diagnosis of ARVC in 15 patients, while showed an active myocarditis in the remaining 15 patients (Figure 5). Patients with ARVC were not distinguishable on the basis of clinical features, presence and severity of structural and functional right ventricular abnormalities and three-dimensional 3D-EAM findings.

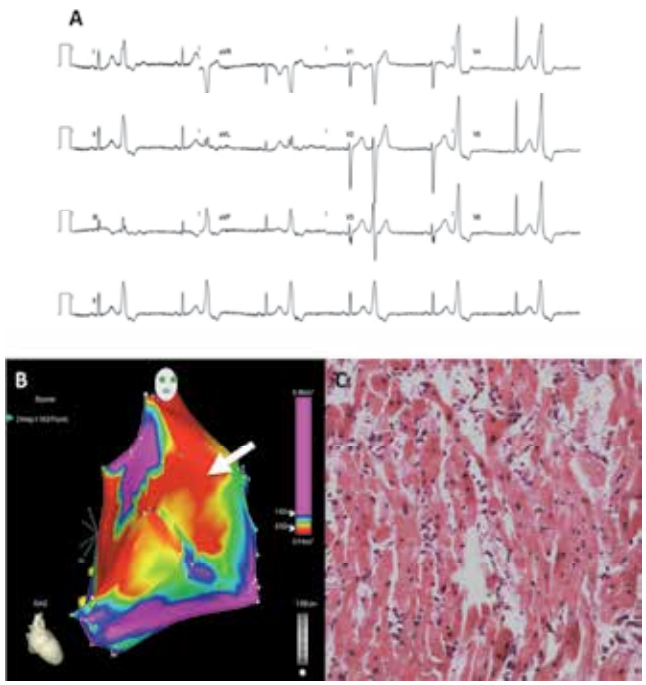

Fig. 5. 3D-EAM-guided EMB, in a patient with bigeminism due to ventricular ectopic beats with LBBB morphology and inferior axis (A). 3D-EAM shows low voltages in the outflow tract and free wall (B). Endomyocardial biopsies drawn from free wall (arrow) shows active myocarditis (C).

On the basis of clinical and histological features, a cardioverter defibrillator was implanted in 13 patients with biopsy-proven ARVC and in 1 patient only with myocarditis. At a mean follow-up of 21±8 months, 7 (47%) patients with ARVC experienced recurrence of symptomatic sustained ventricular arrhythmias with appropriate defibrillator intervention in all cases. All patients with myocarditis remained asymptomatic and free from arrhythmic events. Our study was the first to demonstrate that 3D-EAM-guided EMB may allow obtaining a differential diagnosis in patients with otherwise undistiguishable clinical, arrhythmic and imaging features. In addition our study clearly showed that patients with similar arrhythmic presentation may have a dramatically different prognosis in terms of arrhythmias' recurrence according to the underlying disorders, as only 53% of biopsy-proven ARVC patients remained free from arrhythmias at a mean follow-up of 21±8 months years, while no patients with biopsy-proven myocarditis experienced major arrhythmic events during the same time (Figure 6).

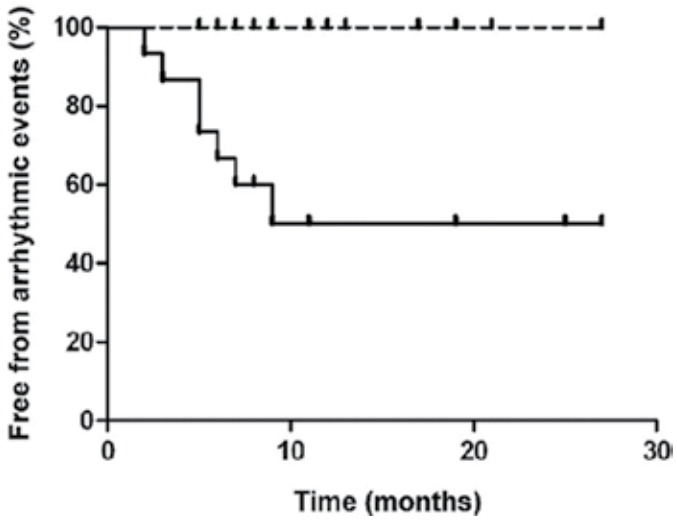

Fig. 6. Kaplan-Meier analysis of arrhythmic event-free survival depending on the histological findings (Modified from Pieroni et al. 2009).

In a more recent study (Pieroni et al unpublished data) we performed 3D-EAM-guided EMB in a series of elite competitive athletes presenting with sustained ventricular arrhythmias. We studied 13 consecutive competitive athletes with evidence of sustained ventricular arrhythmias within the previous six months on 12-lead ECG, 24-hour Holter ECG or ECG exercise testing and who were judged as having a structurally normal heart after a thorough non-invasive evaluation, including signal-averaged electrocardiogram, transthoracic echocardiogram and CMR. Depending on the presumed site of arrhythmias origin according to 12-lead ECG criteria, patients underwent right or left ventricular 3D-EAM and 3D-EAM-guided endomyocardial biopsy. Twelve (92%) patients presented at least 1 low-voltage region at 3D-EAM, while the histologic diagnosis was active myocarditis in 7 patients, and of arrhythmogenic RV cardiomyopathy in 5. In one patient the histological evidence of contraction-band necrosis allowed to unmask caffeine and ephedrine abuse.
The identification of the underlying histological substrate in patients with ventricular arrhythmias may be relevant also when radiofrequency catheter ablation (RFCA) is

considered as a possible therapeutic strategy. In fact there is growing evidence that in patients with ARVC, RFCA is effective in abolishing ventricular tachycardias, but the rate or recurrence of arrhthmias with the same or a different morphology may be very high even when an epicardial approach is adopted (Dalal et al., 2007). On the contrary several reports have demonstrated that RFCA may be effective in eliminating ventricular arrhythmias in myocardial inflammatory disorders, including Chagas cardiomyopathy and sarcoidosis (Sosa et al., 1999; Sarabanda et al., 2005; Jefic et al., 2009; Henz et al., 2009). Recent reports and also our experience suggest that endocardial ablation cannot be sufficient in some patients and a combined endo-epicardial approach is required for definitive results. (See below, Clinical implications of myocarditis diagnosis)

5. Clinical implications of myocarditis diagnosis in patients with ventricular arrhythmias

Despite the improvement of diagnostic techniques in defining the characteristics and the etiology of the inflammatory process and the more specific comprehension of the mechanisms leading to myocardial damage, a specific standardized treatment of myocarditis is not yet available. However in the last decades several studies and trials have clearly demonstrated that an etiologic definition of myocarditis based on the immunohistological evaluation of inflammation and proof of viral infection by PCR on biopsies is essential for targeting appropriate treatment strategies indicating that myocarditis patients may benefit from immunosuppression, provided that viral persistence is excluded, while patients with viral persistence fail to respond favourably or deteriorate by immunosuppression.

Several studies have demonstrated the negative prognostic role of viral persistence. In a recent study Kuhl and colleagues analyzed 172 patients with dilated cardiomyopathy and biopsy-proven viral infection in endomyocardial biopsies. The authors reported a high prevalence of enteroviral and parvovirus B19 infection (32.6% and 36.6%, respectively) and found that viral persistence was associated with a progressive impairment of left ventricular ejection fraction, whereas spontaneous viral elimination was associated with a significant improvement in left ventricular function (Kuhl et al., 2005).

Autoimmunity, on the other hand, may play an outstanding role in the progression of myocardial damage and persistence of symptoms, including ventricular arrhythmias. Notably, both acute viral infection and chronic viral persistence can induce autoimmunity by mechanisms not fully elucidated. Molecular mimicry has been suggested as one mechanism to explain chronic myocarditis (Caforio et al., 2002; Rose et al., 2006) and several cellular antigens have been identified to cross-react with viral antigens, thus providing possible targets in virus-induced myocarditis. Autoantibody production also may be caused by the initial myocyte damage by viral infection, releasing increased amounts of self-antigens into the circulation. Autoantibodies against adrenergic and acetylcholine receptors contractile structures, extracellular matrix proteins, proteins involved in energy metabolism, calcium receptors and homeostasis and antistress proteins have all been detected in patients with myocarditis (Neumann et al., 1994; Pankuweit et al., 1997). Furthermore, autoimmunity is considered the main pathogenetic mechanism underlying eosinophilic, giant-cell and granulomatous myocarditis (Cihakova et al., 2008), as well as myocarditis associated with connective tissue diseases, peripartum cardiomyopathy (Ansari et al., 2002) or heart transplant, in which immunosuppression has been proven effective.

Immunosuppression is clearly recommended essentially for the treatment of eosinophilic, granulomatous, giant-cell myocarditis and lymphocytic myocarditis associated with connective tissue diseases or with the rejection of a transplanted heart. With regard to idiopathic lymphocytic myocarditis, beyond acute phase where a spontaneous resolution has been reported in up to 40% of the cases, many patients with idiopathic myocarditis and chronic heart failure are likely to benefit from immunosuppression. An up-regulation of HLA antigens in the myocardial tissue of patients with lymphocytic myocarditis has been proposed as a marker of susceptibility to beneficial effects of immunosuppression (Wojnicz et al., 2001). Recently the data from a randomized double-blind, placebo-controlled study designed to evaluate the efficacy of immunosuppression in virus-negative inflammatory cardiomyopathy have been reported (Frustaci et al., 2009). Eighty-five patients with myocarditis and chronic (>6 months) heart failure unresponsive to conventional therapy and no evidence of myocardial viral genomes were randomized to receive either prednisone and azathioprine (43 patients, Group 1) or placebo (42 patients, Group 2) in addition to conventional therapy for heart failure. Primary outcome was the 6 months improvement in left-ventricular function. Group 1 showed a significant improvement of left-ventricular ejection fraction and a significant decrease in left-ventricular dimensions and volumes compared with baseline. None of Group 2 patients showed improvement of ejection fraction, that significantly worsened compared with baseline. No major adverse reaction was registered as a result of immunosuppression. These data confirmed the efficacy of immunosuppression in virus-negative inflammatory cardiomyopathy. Lack of response in 12% of cases suggested the presence of not screened viruses or mechanisms of damage and inflammation not susceptible to immunosuppression. Antiviral therapy is a logical strategy advocated for the treatment of myocarditis in patients with evidence of viral presence in myocardial tissue. In a small phase II study 22 patients with myocarditis and biopsy-proven viral persistence (15 enteroviral and 7 adenoviral) were treated with interferon-beta $18x10^6$ IU/week for 24 weeks. Interferon-b treatment resulted in complete elimination of both enteroviral and adenoviral genomes and hemodynamic improvement, as shown by improvement of EF and amelioration of heart failure symptoms. Importantly, treatment with interferon-beta was safe and well-tolerated, with no adverse cardiac effects, and flu-like side effects could be efficiently eliminated by non-steroidal anti-inflammatory drugs (Kuhl et al., 2003). After these encouraging results, a prospective placebo-controlled randomized multicenter study, the Betaferon® in Chronic Viral Cardiomyopathy (BICC) trial, was initiated in 2002 and presented at the 2008 American Heart Association scientific sessions. The primary endpoint was virus elimination or reduction of virus load. At 24-week follow-up, compared to placebo, virus elimination and/or viral load reduction were significantly higher in the interferon group. Moreover, interferon-beta showed a good safety profile and was associated with beneficial effects on clinical secondary endpoints, such as NYHA functional class and quality of life, as assessed by Minnesota questionnaire, although echocardiographic and hemodynamic parameters were not statistically significantly different. This interesting phase II study showed proof of concept for targeted therapy based on molecular diagnosis in inflammatory cardiomyopathy. Future directions in the field of antiviral therapy include preventing direct viral damage and inhibition of viral proliferation by preventing the interaction of viruses with their cellular receptor and their consequent signalling amplification systems, such as the tyrosine kinase p56lck, phosphatase CD45 and downstream ERK1/2 (Liu et al., 2000). Therefore, additional research is required to further understand the mechanisms of viral heart disease and consequently identify new potential targets for therapy.

No specific trial or data from single-center studies on the treatment of myocarditis presenting with ventricular arrhythmias are currently available in literature. In patients with acute myocarditis, therapy for arrhythmias is essentially supportive, since arrhythmias usually resolve after the acute phase of the disease, which can last several weeks, together with the resoluton of the inflammatory process. Even in chronic myocarditis, management of ventricular arrhythmias is currently confined to antiarrhythmic drug therapy, with limited efficacy, and to implantable cardioverter-defibrillator for higher-risk patients, including those with hemodynamically unstable ventricular tachycardias (Zipes et al., 2006) and those with aborted sudden death. Radiofrequency catheter ablation has been demonstrated to be effective in reducing ventricular tachycardia occurrence in patients with Chagas cardiomyopathy and sarcoidosis. Regarding ventricular arrhythmias in patients with lymphocytic myocarditis, isolated reports suggest that RFCA may be effective, (Chauhan et al., 2008; Hama et al. 2009; Kettering et al., 2009; Zeppenfeld et al., 2007) but its safety and long-term efficacy in this setting is unclear. In our Institution we documented the safety and efficacy of RFCA in a series of consecutive patients with chronic active myocarditis presenting with drug-refractory ventricular tachycardias. We enrolled 20 pts with biopsy-proven myocarditis and drug-refractory ventricular tachycardia including 5 pts presenting with electrical storm. All patients underwent endocardial RFCA with an irrigation catheter, using contact electroanatomical mapping. Recurrence of sustained ventricular tachycardia after endocardial RFCA was treated with additional epicardial RFCA. Endocardial RFCA was acutely successful in 14 pts (70%), while in the remaining 6 pts (30%) clinical ventricular tachycardia was successfully ablated by epicardial RFCA thus demonstrating that in patients with myocarditis, RFCA of drug-refractory ventricular tachycardia is feasible, safe and effective and that epicardial RFCA should be considered as an important therapeutic option to increase success rate (Pieroni and colleagues in press).

6. Conclusion

Myocarditis represents a frequent but often underestimated cause of life-threatening ventricular arrhyhmias and sudden arrhythmic death and therefore should be always suspected in these clinical settings. The recognition of myocarditis as the cuase of ventricular arrhythmias may have dramatic implications on treatment and prognosis. Imaging techniques such as CMR have improved our ability to recognize myocardial inflammatory damage, and increased the awareness of the disease among physicians. Nevertheless EMB still represents the gold standard for the diagnosis of myocarditis as offers a immunohistological and virological characterization of thr inflammatoty process that may influence the treatment. Electroanatomic mapping-guided EMB represents a new important technique that allows increasing diagnostic sensitivity and minimizing the sampling error in patients with myocarditis presenting with arrhythmias. In the next future 3D-EAM-guide EMB will also represent an important research tool in the study of channellopathies and other genetically determined arrhythmic syndromes.

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Acute Myocarditis – A Trigger of Cardiac Autoimmunity? Expected Insights from the Etiology, Titre-Course, and Effect on Survival of Cardiac Autoantibodies (ETiCS) Study

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1. Introduction

Progressive cardiac dilatation and pump failure of unknown aetiology - termed "idiopathic" dilated cardiomyopathy (DCM) (Richardson et al., 1996; Maron et al., 2006) - represents one of the main causes of severe heart failure in Western populations with an annual incidence of about 100 and a prevalence of 300-400 patients per year (American Heart Association, 2009). The large majority of cases are thought to arise from an initial (mostly viral) infection leading to acute myocardial inflammation. Acute myocarditis may either heal (about one third of the cases) or progress to a chronic inflammatory process with continued fibrotic repair, subsequent dilatation of the left and/or right ventricle and –finally– severe congestive heart failure (about another third of the patients). Progression to DCM appears to occur particularly, when associated (a) with chronic inflammation of the myocardium due to viral persistence (Kühl et al., 2005) and/or (b) with the development of autoantibodies directed against distinct sarcoplasmatic or myocyte membrane proteins that are essential for cardiac function (Freedman & Lefkowitz, 2004; Jahns et al., 2006). The latter findings are further strengthened by the fact that patients with DCM often have alterations in both, their innate and their adaptive immune system (Limas, 1997; Luppi et al., 1998; Jahns et al., 2006; Mahrholdt et al., 2006). Thus, under certain conditions an initial acute inflammatory reaction may proceed into a kind of low-grade inflammation (MacLellan & Lusis, 2003) facilitating the development of abnormal or misled immune responses to the primary (infectious)

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trigger (MacLellan & Lusis, 2003; Freedman & Lefkowitz, 2004; Kühl et al., 2005; Smulski et al., 2006; Maekawa et al., 2007). More recently, a detailed molecular analysis of T cell infiltrates in human endomyocardial biopsies (EMBs) from both, patients with acute myocarditis and patients with (post-)inflammatory DCM revealed an increased expression of CD3d, CD3z, and T cell receptor beta constant region (TRBC) in both disease entities. However, differential expression of functional T cell markers was found in DCM EMBs only (dominance of Th1 markers, regulatory [FoxP3] T cells, and cytotoxic T cells (CTLs)) and not in acute myocarditis. Additionally, in DCM EMBs some Th2 marker genes were increased, indicating that a Th2 response (required for T/B cell interactions) also participates in the T cell infiltrates in DCM (Noutsias et al., 2011). This might explain, why a substantial number of DCM patients have been found to develop cross-reacting antibodies and/or autoantibodies to different cardiac self-antigens, including mitochondrial proteins (e.g., adenine nucleotide translocator, lipoamide and pyruvate dehydrogenase (Schultheiss & Bolte, 1985; Schultheiss et al., 1988; Pohlner et al., 1997; Schulze et al., 1999)), sarcoplasmatic proteins (e.g., actin, laminin, myosin, troponin (Neumann et al., 1990; Caforio et al., 2002; Okazaki et al., 2003; Göser et al., 2006; Li et al., 2006)), and membrane proteins (e.g., cell surface adrenergic or muscarinergic receptors (Fu, L.X.M. et al., 1993; Magnusson et al., 1994; Jahns et al., 1999; Christ et al., 2006)).

Irrespective of whether development of DCM is primarily due to chronic myocardial infection (Kühl et al., 1996) or to abnormalities in the adaptive or innate immune system (Luppi et al., 1998; Eriksson et al., 2003), in both cases cardiac tissue injury is believed to be mediated mainly by cytokines and/or heart-specific autoantibodies (Caforio et al., 1995; Limas, 1997; Eriksson et al., 2003). However, the pathophysiological relevance of each of the aforementioned cardiac autoantibodies (aabs) is far from clear. Low titers of autoantibodies to various house-keeping antigens can also be detected in healthy subjects as a part of the natural immunological repertoire (Rose, 2001).

In addition, under physiological conditions at least the intracellularly localized cardiac antigens are not easily accessible for the immune system. Thus, the mechanisms by which autoimmune-mediated myocardial injury is initiated are mostly based on indirect or circumstantial evidence (Limas, 1997). The potential pathophysiological - and thus clinical – relevance of a heart-specific autoantibody depends on its disease-inducing or -aggravating potential, which in turn is supposed to be associated with both the *accessibility* and the *functional relevance* of its target. Therefore, autoantibodies directed against cell surface key constituents, and in particular aabs that have the potential to affect myocardial contraction and relaxation (e.g. , by interaction with the cardiac beta1-adrenoceptor (beta1-AR)) and/or the M2-muscarinic acetylcholine receptor (M2-AchR)) represent key candidates involved in the initiation and/or progression of DCM (Fu et al. 1993, 2008; Magnusson et al., 1996; Limas, 1997; Engelhardt et al., 2004; Jahns et al., 2004; Freedman & Lefkowitz, 2004). Whereas anti-muscarinic antibodies (exhibiting an agonist-like effect on cardiac M2-AchR) have been associated with negative chronotropic effects at the sinuatrial level (e.g., sinus node dysfunction, atrial fibrillation (Wang et al., 1996; Baba et al., 2004)), functionally activating anti-beta1-AR antibodies have been associated with both the occurrence of severe arrhythmias at the ventricular level, and the development of (maladaptive) left ventricular hypertrophy, finally switching to left ventricular enlargement and progressive heart failure (Jahns et al., 1999, 2006; Iwata et al., 2001; Engelhardt et al., 2004; Störk et al., 2006). In addition, both autoantibodies seem to target the (easily accessible) second extracellular loop of the respective receptors.

To generate an autoimmune response, myocyte membrane proteins (including receptors) must be degraded to small oligopeptides able to form a complex with a major histocompatibility (MHC) class II or human leukocyte antigen (HLA) molecule of the host (Hoebeke et al., 1996). In previous clinical studies autoimmunity has been found to be associated with certain HLA- and MHC class II-phenotypes (Limas, 1996), and also with the expression and/or activity of the T-lymphocyte antigen 4 (CTLA-4) - known as a potent (indirect) suppressor of the immune system (Golden et al., 2005). Therefore, another important point to consider in the development of (human) post-inflammatory and/or post-ischemic cardiomyopathy is the patients' genetic pre-disposition, which will determine both, the susceptibility to self-directed immune reactions and the phenotypic expression of the myocardial disease (MacLellan & Lusis, 2003; Limas et al., 2004).
On this background the following book-chapter will review current knowledge and recent experimental and clinical evidence for the potential role of cardiac autoantibodies in the pathogenesis of DCM focussing on the rationale and expected insights from the prospective diagnostic multicentre **E**tiology, **Ti**tre-**C**ourse, and (effect on) **S**urvival of cardiac autoantibodies (**ETiCS**) study.

2. Rationale and scope of the ETiCS study

Evidence for a pathophysiologic role of autoimmunity in human heart disease has substantially increased during the past two decades, but the true prevalence and clinical impact of cardiac autoantibodies (aabs) in human heart disease are still unclear, as are the events leading to their formation, their frequency of appearance, and their kinetics (that is, their patterns of clearance and/or persistence).
In this regard, the investigator-initiated diagnostic multicentre ETiCS study will prospectively address the hypothesis that a first inflammatory (i.e., acute myocarditis (AMitis)) or ischemic injury of the myocardium (i.e., first acute myocardial infarction (FAMI)) may trigger the development of heart-directed autoimmune reactions (**Fig. 1**).

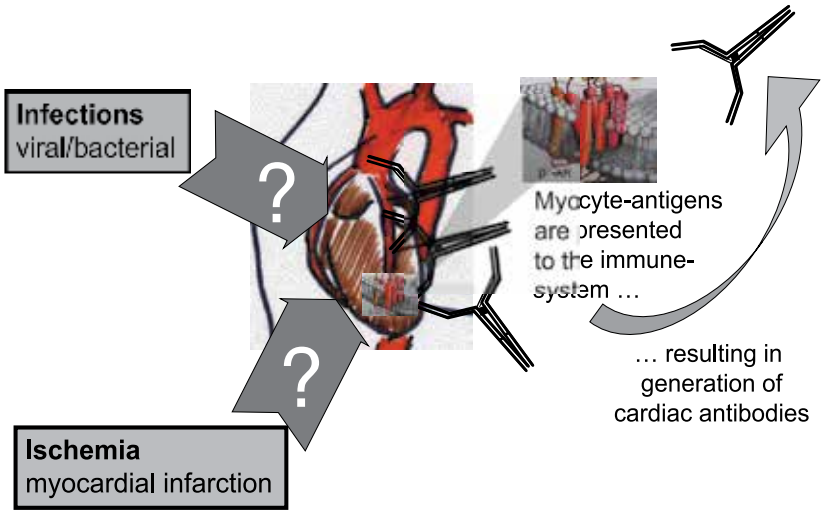

Fig. 1. Formation of autoantibodies against myocardial self-antigens.

Immunogenicity is defined as the property of a molecule to induce an immune response (Hoebeke, 1996). To serve as a potential antigen, myocyte constituents (e.g., cardiac membrane receptors) must be degraded by proteolysis into small fragments (oligopeptides), and one or several of the generated fragments must be able to form a complex with one of the major histocompatibility complexes (class II-MHCs) or human leukocyte antigen (HLA) molecules of the host. When presented to T cells (Harding et al., 1990; Mobini et al., 1999) antigenic parts of such myocyte-derived (self) peptides may engender an immunological response. Acute inflammatory processes are supposed to enhance the occurence of self-directed immune responses (e.g., acute viral or bacterial myocarditis and/or acute ischemic events (Borda et al., 1984; Latif et al., 1993; Kühl et al., 1996; Limas, 1997; Noutsias et al., 1999; Liu & Mason, 2001; Rose, 2001; Caforio et al., 2002)).
Therefore, we assume that a first substantial inflammatory or ischemic myocyte damage – through liberation of a "critical amount" of cardiac self-antigens previously hidden to the immune system – might induce and perpetuate a disease-causing and/or -modulating autoimmune reaction that deteriorates cardiac function and ultimately results in progressive heart failure.
As a consequence, the ETiCS study has been designed to provide a maximum of sequential clinical and serological data from patients after a first inflammatory or ischemic cardiac event. The development/prevalence and titre-course (clearance/persistence) of distinct cardiac aabs after 0, 3, 6, and 12 months of either event will be prospectively assessed and correlated with the corresponding cardiac functional parameters, cardiac imaging (echocardiography, cardiac magnetic resonance imaging), and clinical outcome. A limited number of ETiCS sub-studies will focus on components of the patients' immune system potentially involved in the generation of cardiac receptor-aabs – including a search for predisposing genotypes (Limas et al., 2004; Caforio & Iliceto, 2008) – and on the possible impact of conformational adrenoceptor-aabs on renal function (Boivin et al., 2001).
Expanding the scope of ETiCS beyond adrenoceptor-directed autoimmunity other known cardiac aabs will be investigated by the respective expert core centres, including aabs against the muscarinic acetylcholine receptor 2 (Fu, L.X.M. et al., 1993), against troponin I (Göser et al., 2006), organ-specific and skeletal muscle cross-reactive anti-heart-aabs (Caforio et al., 2007), and cardio-depressant aabs (Felix et al., 2002). This joint venture will enable a comprehensive characterisation of heart-directed autoimmunity after inflammatory or ischemic disruption of myocardial integrity, and allow for cross-correlations of the titre-course and prognostic impact of all cardiac aabs prospectively analyzed.

3. Design of the ETiCS study

The prospective ETiCS study complies with the standards of Good Clinical Practice (GCP) and has been approved by the Ethics committees of all participating institutions. Within a two years-period ETiCS will include 400 patients with a first cardiac event, 200 of them with acute myocarditis (AMitis), and 200 patients with a first myocardial infarction (FAMI; acute ST-elevation MI only, without any history or signs of previous myocardial infarction). After inclusion and baseline assessment, the patients will undergo three follow-up visits after 3, 6, and 12 months (Fig. 2). Diagnosis of acute myocarditis is based on at least one major and two minor clinical criteria and/or symptoms (see table 1) and must be confirmed by endomyocardial biopsy (EMB) using either the WHO/ISFC (Richardson et al., 1996; Elliott et al., 2008) or the Dallas criteria (Aretz et al., 1987). This proceeding is in full accordance

with the actual AHA/ACC/ESC scientific statement on the role of EMB in the management of cardiovascular disease (Cooper et al., 2007), which strongly recommends EMB in acute myocarditis because of its potential relevance for outcome and therapeutic decisions (Kindermann et al., 2008; Frustaci et al., 2009). In addition, immunohistology and molecular analysis of the EMBs may also significantly contribute to further elucidate the clinical impact of heart-directed autoimmune reactions.

Suspicion of acute myocarditis (One major and two minor criteria fulfilled)	
Major criteria	**ST/T wave changes in the electrocardiograms (ECG)** **Ventricular arrhythmia (ECG)** **Pericardial effusion on echocardiography** **Impairment of LVEF on echocardiography with CAD excluded by coronary angiography (no stenosis >50%)**
Minor criteria	**Dyspnea, new onset within the past 30 days** **Chest pain, new onset within the past 30 days** **Palpitations, new onset within the past 30 days** **History of infection within the past 30 days** **Fever >38.0° C within the past 30 days**

Table 1. Diagnostic criteria for suspicion of acute myocarditis.

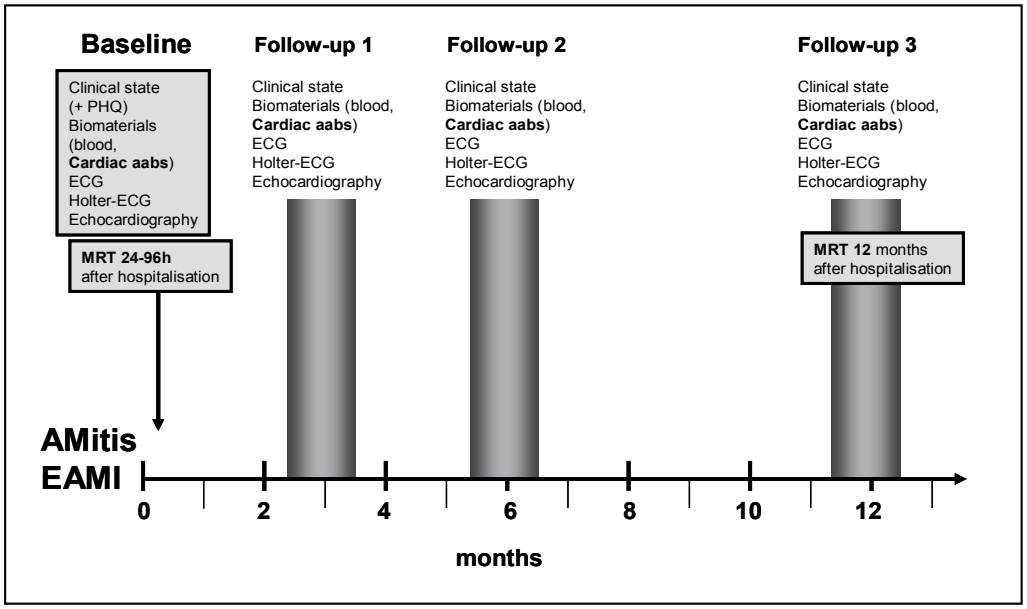

Fig. 2. Time schedule of the follow-up examinations in the ETiCS study.

Echocardiography and cardiac magnetic resonance imaging (cMRI) will be carried out in all patients within 24 to 96 hours of hospitalisation and one year after the respective cardiac

event. Follow-up visits at 3, 6, and 12 months comprise a detailed patient history including medication, physical examination, a standardized health questionnaire, echocardiography, ECG, Holter-ECG, and blood sampling (**Fig. 2**). The sequentially obtained blood samples will serve to determine the time course of formation and the titre-course (clearance/persistence) of distinct cardiac aabs at 0, 3, 6, and 12 months after inflammatory or ischemic cardiac injury (AMitis, FAMI). Three-hundred healthy subjects with normal blood pressure, ECG, and exercise-stress test will serve as a control collective (male to female ratio 1:1, n=50 per five-years age range, no history of myocardial infarction, diabetes, or peripheral vascular disease). The primary endpoint of the planned cross-sectional analyses is the association of a specific cardiac aab status at diagnosis (aab-positive/aab-negative) with (**a**) the change in cardiac function as derived from sequential echocardiograms and cMRI (baseline *versus* 12 months), and (b) the severity and clinical course of the index disease. Longitudinal primary endpoints are titre-changes of a given cardiac aab over time, conversion rates (persistence/clearance), and the "time to first cardiovascular event" (see table 2 for definition of composite endpoints). Pre-specified secondary endpoints for each of the different cardiac aabs at diagnosis are time to cardiovascular death and time to all-cause death (table 2).

Primary endpoint: prospective ETiCS study
Titre course of conformational cardiac aabs
Secondary endpoints: prospective ETiCS study
Change in left ventricular enddiastolic diameter
Change in left ventricular ejection fraction
Occurrence of ventricular arrhythmias
Change of clinical NYHA classification
Severity of inflammation as assessed by EMB/cMRI
Time to cardiovascular death/all-cause death
Composite endpoints
Cardiovascular Death
Myocardial infarction
Resuscitation (successful or not successful)
ICD discharge, if appropriate
Cardiac transplantation
Sudden cardiac death
Death due to progressive heart failure
Death due to myocardial infarction

Table 2. Pre-defined endpoints of the ETiCS study.

4. ETiCS substudies

4.1 Immunobiology of heart-directed autoreactivity

This substudy attempts to unravel the step-by-step changes occurring in a patients' immune system whilst autoantibody-mediated immune cardiomyopathy develops after inflammatory or ischemic myocardial injury, including the time course and cell types (T and B cell subpopulations) involved in the generation of cardiac aabs. The reactivity and prevalence of receptor (auto)antigen-specific T and B cell populations will be assessed by

antigenic recall assays as well as FACS and Elispot analyses. In addition, the respectively activated immunologic paths will be derived from the Th1/Th2/Th17 cytokine profiles determined in the sera from cardiac autoantibody-positive patients with acute myocarditis (including an analysis of the corresponding EMBs (Noutsias et al, 2011)) or acute transmural myocardial infarction. Since the cytotoxic T-lymphocyte antigen 4 (CTLA-4) is a potent (indirect) suppressor of the immune system, its mutation or hampered expression might promote hyperreactivity to autoantigens (Golden et al., 2005). Thus, in all ETiCS patients CTLA-4 expression and CTLA-4 alleles will be determined by FACS and by PCR, respectively. In addition, in all patients the HLA-DR/DQ and MHC class II haplotypes (Limas et al., 2004; Caforio & Iliceto, 2008) will be determined and correlated with the formation and titre-course of distinct cardiac aabs in order to unravel the individual genetic susceptibility for heart-directed autoimmune reactions.

4.2 Adrenoceptor-autoantibodies and their impact on kidney function

The possible clinical implications of adrenoceptor-directed autoimmunity have gained increasing interest in human heart disease. By contrast, the renal effects of functionally active adrenoceptor-aabs have been almost neglected. Previous immuno-histologic studies on the rat kidney strongly suggest that functionally stimulating adrenoceptor aabs are capable of modulating the secretion of renin from the juxtaglomerular cells *via* renal beta1-AR and, also, to increase the sodium-reabsorption in the distal tubulus (Boivin et al., 2001). Thus, through renal beta1-AR-mediated activation of the renin-angiotensin-aldosterone-system (RAAS) and/or augmentation of the circulating blood volume such aabs might contribute to further worsening of the prognosis of antibody-positive heart failure patients.
To assess the impact of adrenoceptor-aabs on kidney function (and clinical outcome), a number of pre-specified renal functional parameters will be sequentially determined in all ETiCS patients (e.g. serum *versus* urine creatinine/sodium/pH; glomerular filtration rate (GFR), urinary protein excretion). Primary endpoints are the relationship between antibody-titres and renal function at baseline and the "time to first cardiovascular event", adjusted for renal function. Secondary endpoints include the change in renal function dependent on the formation and conversion rates (clearance/persistence) of adrenoceptor-aabs.
These data might furnish a rationale for the development of novel therapeutic strategies to protect the kidney(s) from functional adrenoceptor-aabs in aab-positive heart failure patients.

5. Conclusion and expected insights from the ETiCS study

In the last two decades, much knowledge has accumulated with respect to the possible pathophysiologic and clinical implications of heart-directed aabs (Jahns et al., 2006; Fu, M., 2008).
Homologies between cardiomyocyte surface molecules (in particular membrane receptors) and viral or bacterial proteins have been proposed as a mechanism for the elaboration of endogenous cardiac autoantibodies by antigen mimicry (Elies et al., 1996; Hoebeke et al., 1996). Chagas' heart disease, a slowly evolving inflammatory cardiomyopathy, is one of the best investigated examples to highlight this mechanism. The disease is induced by the protozoon *Trypanosoma cruzi*. About 30% of the Chagas' patients develop antibodies that cross-react between the ribosomal P2beta-protein of *T. cruzi* and some specific amino acids present in the second extracellular loop of the human beta1-adrenoceptor (Elies et al., 1996).

Because the large majority of functionally active beta1-aabs detected in DCM patients seem to be directed against the same receptor loop, it was speculated that these antibodies might also originate from molecular mimicry between the beta1-adrenoceptor and hitherto non-identified viral pathogen (Hoebeke, 1996). Another example is Chlamydia–associated heart disease, which in BALBc mice appears to be induced by antigen mimicry between Chlamydia antigens and the alpha-myosin heavy chain molecule, resulting in activation of autoreactive T- and B-cells (Bachmaier et al., 1999).

However, irrespective of (potentially occurring) immunologic cross-reactions, to date no prospective clinical study has ever addressed the key question, whether structural damage to the heart muscle (e.g., necrosis or apoptosis) is a mandatory pre-requisite for the formation of heart-directed aabs.

In general, the immune system will not attack cardiac self-proteins. On a susceptible genetic background, however, this self-inhibition of immune effector cells after cardiac injury may be hampered. It is presently unclear, whether this autoreactivity depends on the kind and extent of injury, the kind and amount (or "dose") of self-antigens presented, or the kind and quality of the subsequently engaged immunologic paths. Regarding the latter aspect, a recent analysis of the expression of functional T cell markers in EMBs from patients with acute myocarditis and patients with chronic (post-inflammatory) DCM found an increased expression of CD3d, CD3z, and TRBC in both disease entities, whereas Th1 (more than) Th2 marker-genes as well as regulatory and cytotoxic T cells were differentially up-regulated in DCM EMBs only (Noutsias et al., 2011). Interestingly, in human DCM biopsies any clues for a Th17 response were lacking, which is in sharp contrast to findings in murine models of (experimental) autoimmune myocarditis (Valaperti et al., 2008). Nevertheless, from these recent molecular data it seems clear now that in human DCM a Th2 response also participates in the myocardial T cell infiltrates and serves as a pre-requisite for the stimulation (that is, maturation) of autoreactive B cells that, e.g., produce cardiac autoantibodies.

In acute myocarditis diffuse and/or focal inflammation causes structural damage to the heart. If myocardial inflammation persists, in a majority of cases cardiac function does not recover and finally may result in severe DCM. Acute ischemia also causes structural damage to the heart and to date represents the most common aetiology of heart failure. Thus, a structured follow up of patients with either disease is both pathophysiologically and clinically relevant.

The ETiCS study will follow such patients 3, 6, and 12 months after their index events, because the formation of autoreactive immunoglobulin G (IgG) is supposed to take place within the first 6 weeks (up to three months) after the index event.

A variety of autoreactive IgG have been identified, but only few of them have been investigated more in detail. Because myocyte surface receptors are easily accessible to circulating autoantibodies, the cardiac beta1-adrenergic receptor (which is the predominant adrenocepter subtype in the heart) and the M2-muscarinic acetylcholine receptor represent key targets for autoreactive antibodies that might affect heart function to some extent.

To generate an autoimmune response, membrane receptors must be degraded to small oligopeptides able to form a complex with a MHC class II or HLA molecule of the host (Hoebeke, 1996). In case of the human beta1-adrenoceptor, a computer-based search for potential immunogenic amino-acid streches within this (seven) transmembrane spanning protein revealed, that the only portion of the molecule containing B- and T-cell epitopes (and accessible to antibodies) was in fact the predicted second extracellular receptor loop

(beta1-ECII). This might explain the successful use of ECII-mimicking peptides for the induction of specific anti-beta1-ECII antibodies in different animal-models (Magnusson et al., 1996; Jahns et al., 2004, 2006). Finally, by isogenic transfer of anti-beta1-ECII antibodies in a human-analogous rat model the (autoimmune-)attack against the hearts of healthy recipients by activating anti-beta1-antibodies has been identified as a possible cause of heart failure (Jahns et al., 2004).

In the last decade several research groups have independently shown that anti-beta1-ECII-antibodies preferentially recognize native membrane beta1-receptors in various immunological assays (cell-ELISA, immunoprecipitation, immunofluorescence), indicating that such antibodies or autoantibodies are conformational (Jahns et al., 1999, 2006; Fu 2008). In addition, functional tests revealed that such anti-beta1-ECII may also act as allosteric regulators of beta1-adrenoceptor activity through modulation of cellular cAMP-production and/or cAMP-dependent protein kinase (PKA) activity (Wallukat et al., 1991; Limas et al., 1992; Jahns et al., 1999; Nikolaev et al., 2007). The use of different screening-techniques renders direct comparisons of the available data difficult, however. Hence, so far reported prevalences should be always interpreted in the context of the detection method utilised. Nevertheless, taken all these reports together, there is wide consent that a substantial fraction of patients with DCM and ICM, but only very few healthy subjects have ciculating functionally active adrenoceptor-aabs (Wallukat et al., 1991; Magnusson et al., 1994; Jahns et al., 1999). An association of such aabs with impaired cardiac function (Jahns et al., 1999), a higher incidence of ventricular arrhythmias (Iwata et al., 2001; Störk et al., 2006), and a higher incidence of sudden cardiac death (Iwata et al., 2001) have been demonstrated. In addition, a previous clinical follow-up study also implies an increased risk for (cardiovascular) mortality in adrenoceptor aab-positive DCM patients (Störk et al., 2006).

To further analyse the time-course and sequentially engaged immunologic processes in autoimmune-mediated heart disease, the ETiCS study will prospectively follow the evolution of cardiac morphology and function after a first inflammatory or ischemic myocyte damage. In total 400 patients will benefit from a structured follow-up and best standard available medical care. The central hypothesis of ETiCS is that both inflammatory and ischemic myocardial injury may trigger a sequence of immunologic reactions which result in the formation of functionally active cardiac receptor-aabs (Jahns et al., 2006). Thus, the pre-specified primary endpoint is the titre of receptor-aabs at diagnosis compared to 3, 6, and 12 months after the index event. Since these aabs are thought to confer additional risk, pre-specified secondary endpoints of the ETiCS study comprise occurrence of life-threatening arrhythmias, changes in cardiac diameters and function, changes in clinical status, time to cardiovascular death, and time to all-cause death.

Endomyocardial biopsies will allow for a correlation of cardiac aab-titres with the severity of myocardial inflammation (Aretz et al., 1987; Elliott et al., 2008; Kindermann et al., 2008) , with the kind of T cells engaged (Th1 vs. Th2-response, Treg, CTLs (Noutias et al. 2011)), and with the presence and/or activity of infective agents detected (that is, the type and load of pre-specified viral/microbial pathogens, as determined by PCR (Kühl et al., 2005)).

Once available, the results of the ETiCS study will significantly contribute to a number of important diagnostic, pathophysiologic and prognostic issues in autoantibody-mediated heart disease (Fu, M., 2008). We expect insights on the role of inflammatory (and ischemic) cardiac damage in triggering autoimmune processes (including the involved immunologic paths), and on the relevance of heart-directed autoimmune reactions for the initiation or progression of heart failure (Jahns et al., 2006). In particular, we might learn and better

understand whether –and if so, then to which extent– the specific target of a heart-directed autoantibody, but also its respective titre and biological activity and –last not least– its respective kinetics (that is, antibody-persistence or -clearance over time) relate to the complex process of cardiac wounding and healing. Different cardiac aabs might have distinct propensities to induce a certain cardiac phenotype, and the ETiCS study will allow for a differentiation of such features in a prospective manner. Thereby, additional prognostic markers for patients with an unfavourable course of autoimmune heart failure might be recognised and, as a consequence, conventional treatment modalities could be optimized earlier and/or novel -more specific- treatment strategies could be developed.

6. Future perspectives and therapeutic implications

Despite available treatment guidelines, the recent progress in conventional pharmacotherapy, and promising novel device-based therapeutic approaches, the outcome of patients suffering from heart failure remains unsatisfactory. This has stimulated the search for causal treatment strategies aiming to block or neutralize factors thought to play a role in heart failure progression.

In a large number of neurologic, rheumatologic, and endocrine disorders autoimmune phenomena have been recognised as main disease-causing factors. Their relevance in human heart disease and failure, however, still need to be substantiated (Jahns et al., 2006; Fu, M., 2008) although preliminary clinical data suggest that the presence of certain cardiac aabs clearly worsens the prognosis of patients with idiopathic DCM (Störk et al., 2006). Therapeutic strategies known from other autoimmune disorders, such as application of peptide-ligands (for multiple sclerosis (Warren et al., 2006)) or immunoadsorption of disease-causing aabs (for myasthenia gravis (Tzartos et al., 2008)) might thus also offer treatment options for a variety of human cardiac disorders.

In this regard, recent *in vitro* experiments with functionally active receptor-aabs isolated from a smaller number of DCM-patients indicate, that aab-induced adrenoceptor activation might be abrogated by incubation with epitope-mimicking peptides (Nikolaev et al., 2007; Jahns et al. 2010). Although clinical *in vivo* data with epitope-derived antibody-scavengers are still lacking, the latter *in vitro* findings together with the results from the ETiCS study should stimulate further research in the field of specifically antibody-directed therapeutic strategies.

In addition to established anti-adrenergic drugs like cardioselective beta-blockers such strategies might comprise (a) the aforementioned epitope-derived peptides as antibody-scavengers (Jahns et al., 2010), (b) an elimination of functionally active cardiac aabs by selective or non-selective immunoadsorption (studies currently under way (Felix & Staudt, 2008; Müller et al., 2008)), or (c) the direct targeting/suppression of autoantibody-producing B cells and/or plasma-cells themselves (Neubert et al., 2008).

At least in animal models of antibody-induced immune-cardiomyopathy and –nephropathy some of these novel therapeutic approaches have already been successfully applied (Jahns et al., 2005; Matsui et al., 2006; Neubert et al., 2008; Jahns et al., 2010). Hence, the results from the clinical (diagnostic) ETiCS study might also furnish a basis for and accelerate further pre-clinical development of such novel therapeutic approaches and agents targeting at cardio-noxious aabs, and –hopefully– for a faster transfer into clinical practice. Moreover, by initiating a joint venture of the leading European research institutes in the field of cardiac autoimmunity, ETiCS could equally serve as a starting point for future common efforts to

further understand and therapeutically modulate the immune system with respect to immune-mediated (post-inflammatory) human cardiac disorders.

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20

Nucleic Acid-Based Strategies for the Treatment of Coxsackievirus-Induced Myocarditis

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1. Introduction

Viral myocarditis is the most common heart disease in infants, children, young adults and pregnant women. Although a number of viruses from different genera, such as adenovirus, hepatitis C virus (HCV), parvoviruses and cytomegalovirus have been reported to cause myocarditis (Bowles et al., 2003; Kindermann et al., 2008; Kuhl et al., 2005a; Kuhl et al., 2005b; Kyto et al., 2005; Mahrholdt et al., 2006; Matsumori, 2005; Matsumori et al., 2006), coxsackievirus, particularly coxsackievirus B3 (CVB3), is generally considered the primary etiological agent of myocarditis (Blauwet, 2010; Kuhl et al., 2005a; Mahrholdt et al., 2006). CVB3 infection of the heart is often persistent and enters the chronic phase, leading to dilated cardiomyopathy (DCM)(Andreoletti et al., 2009; L. T. Cooper, Jr., 2009; Kuhl et al., 2005b; Yajima& Knowlton, 2009), a squelae of viral myocarditis characterized by ventricular chamber dilation, increased wall thickness, weaker beating and abnormal heart function. Patients with DCM eventually develop into congestive heart failure.

To date, there is no clinically proven specific treatment available for viral myocarditis and DCM. Patients with DCM eventually need heart transplantation as the final treatment (Schultz et al., 2009). The managements for viral myocarditis are usually supportive therapies, such as improvements in cardiophysiology with medicine used to treat other kinds of heart diseases, and application of non-specific antiviral agents to decrease the viral load. The former measurements include administration of angiotensin-converting enzyme inhibitor or angiotensin receptor blockade, beta-adrenergic blockade, diuretics, etc (Dennert et al., 2008; Rose, 2009; Schultz et al., 2009); the later measurements include application of type I interferons or nucleotide analogs such as ribavirin, which is reviewed elsewhere (Blauwet, 2010; Dennert& Crijns& Heymans, 2008; Schultz et al., 2009). If myocarditis was caused by an autoimmune disorder, it would be appropriately treated by immunosuppression (Rose, 2009; Schultz et al., 2009). However, the effectiveness of treatment with immunosuppressive therapies has not reached a consensus amongst different studies. This can probably be attributed to the difficulty of confirmation and diagnosis of the etiology and pathogenesis of myocarditis. Thus it is very important to distinguish infectious and autoimmune disease since the same methods of treatment will not be optimal for both forms of heart muscle diseases. The diagnostic gold standard is endomyocardial biopsies with the histological Dallas criteria, in association with new

immunohistochemical and viral PCR analyses of cardiac tissues (L. T. Cooper et al., 2007). In case of confirmed autoimmune-related disease and lack of detectable viral infection, an immunosuppressive treatment combining corticoids and azathioprine may be beneficial to the patients (Frustaci et al., 2003). However, if the disease is primarily caused by viral infections, more specific antiviral agents would be the ideal drugs of choice. In recent years, the search for such antiviral drugs has become a new trend in drug development for treatment of viral myocarditis. The strategies for developing such antivirals include i) screening chemical compounds, such as Pleconaril, capable of interacting with picornavirus (particularly human rhinovirus) antireceptor to block viral entry of the host cells (Groarke& Pevear, 1999; Kaiser et al., 2000; Reisdorph et al., 2003), ii) application of herb medicine to reduce viral load or boost immune responses to limit viral replication (Si et al., 2007; Y. F. Wang et al., 2009), iii) development of small peptide inhibitors of viral proteases to block CVB3 replication cycle (Maghsoudi et al., 2010) and iv) production of recombinant soluble protein of coxsackievirus-adenovirus receptor (CAR) fused to a human immunoglobulin (sCAR-Fc) to block coxsackievirus B3 entry (Pinkert et al., 2009; Werk et al., 2009; Yanagawa et al., 2003; Yanagawa et al., 2004). Another very attractive and promising trend in drug development is the nucleic acid (NA)-based approach to target viral genome or cellular genes to block viral translation and transcription. These strategies include design and synthesis of antisense oligonucleotide (ASON), ribozyme, short interfering RNA (siRNA) and artificial microRNA (miRNA). In this chapter we will focus our discussion on the recent state of this group of antiviral agents for the treatment of myocarditis caused by CVB3 and other viruses that have been recently reported as causal agents of myocarditis.

2. CVB3 genome organization and its receptor

CVB3, a member of enterovirus in the *Picornaviridae* family, is a positive single-stranded, non-enveloped RNA virus. Its genome is ~7.4 kb long containing a single long open reading frame (encoding 11 proteins) flanking by the 5′ and 3′untranslated regions (UTRs) (Klump et al., 1990). The 5′UTR is unusually long (741 nucleotides (nt)) and harbors a number of *cis*-acting translational elements, such as internal ribosomal entry site (IRES) and cloverleaf sequence (Cheung et al., 2007; Z. Liu et al., 1999; Verma et al., 2010; Yang et al., 1997), which are crucial structures for viral translation and transcription. The 3′UTR is a 99-nt long segment attached with a poly-A tail. The 3′UTR folds to form kissing-loop tertiary structures, which are believed to play a role in facilitating viral transcription of the negative strand of CVB3 replication intermediate (Melchers et al., 1997; J. Wang et al., 1999). The viral genomic RNA can directly serve as a mRNA template for translation of a single long polyprotein, which is processed by viral proteases to produce eleven individual proteins, among which four are structural proteins, VP1-VP4, and seven are non-structural proteins including proteases, 2A and 3C, as well as a RNA-dependent RNA polymerase 3D. These three enzymatic proteins play important roles in viral life cycle and pathogenesis (Knowlton, 2008).

CVB3 is a cardiotropic virus. It infects cardiomyocytes by endocytosis through viral receptor CAR (coxsackie and adenovirus receptor) colocalized with tight junction protein (e.g., occludin) (Raschperger et al., 2006). Structural analysis of CAR D1 domain supports the proposed function of CAR as a mediator of cell adhesion (Honda et al., 2000) in the junction complexes of epithelial cells in many tissues (Cohen et al., 2001). It is also known that CAR binding site (anti-receptor) on CVB3 particle lies in the canyon on the capsid surface. Upon

attachment of CVB3 particles to CAR, the receptor changes conformation to form the viral A-particle, a product of the interactions between CVB3 and CAR, which then allows for the release of viral RNA into host cells and begins viral translation and transcription. The observation that soluble CAR can function as a virus trap leading to inactive A-particles has been suggested as a strategy for CVB3 therapy (Pinkert et al., 2009; Werk et al., 2009; Yanagawa et al., 2004). Depending on the different combination of viral strains and mouse models in study of CVB3 infection, a CVB3 co-receptor called decay accelerating factor (DAF, CD55) is sometimes also necessary for CVB3 entry of the host cells (Freimuth et al., 2008; Shafren et al., 1997). Thus, genes encoding CAR and DAF are important candidates for study of viral tropism and rationale targets for antiviral drug design.

3. NA-based antiviral strategies

3.1 Antisense oligonucleotide (ASON)

ASON is probably the earliest NA-based antiviral agent developed. They are designed to bind a complementary sequence in the target mRNA to form RNA-DNA heteroduplexes. These double-stranded hybrid sequences are recognized by RNase H, which digests the RNA strand in the duplex, releasing the ASON to bind another target and so on, effectively silencing the encoded gene (Walder & Walder, 1988). Certain ASONs are not capable of activation of RNase H; instead they inhibit gene translation by steric competition with the translational machinery. In addition, ASONs, if bound to pre-mRNA at intron-exon junctions, can disrupt mRNA splicing (Munroe, 1988). Furthermore, ASONs can also disrupt RNA trafficking by occupying protein-RNA interaction sequences necessary for correct intracellular localization. For example, hnRNP A2 response element (A2RE) is identified as a key sequence required for the trafficking of myelin basic protein (Shan et al., 2000).

Due to major problems including instability, non-specific delivery and unwanted side effects of the ASONs, the structure of this molecule has been modified extensively at different components (i.e., the bases, sugar or phosphate backbone) and has entered its third generation (Fig. 1). The first generation of chemical modification was designed to enhance nuclease resistance of ASON in serum (Stein et al., 1997). The representative of such is the phosphorothioate (PS) oligonucleotide (ON), in which one of the non-bridging oxygen atoms in the phosphodiester bond is replaced by sulfur, intended to prevent cleavage by nucleases. Early antiviral PS-modified ASONs exhibited the antisense properties of phosphodiester ASONs, such as the ability to induce RNase H activation, while showing enhanced stability *in vitro* for up to 48 hours (Hoke et al., 1991); reviewed in (Kurreck, 2003). One notable property of PS-ASON is their tendency to form aptamers, i.e., nonspecific interactions with proteins due to its negative charge. This is disadvantageous intracellularly because aptamer interactions can impede ASON interaction with its intended target, and hence its function. Conversely, the tendency for PS-ASONs to bind serum proteins albumin and alpha-2 macroglobulin in circulation actually improves their bio-distribution throughout the body *in vivo* and prevents them from being cleared for excretion (Crooke et al., 1996).

Another strategy to increase the stability of ASONs is the addition of alkyl groups at the 2′ position of the ribose. 2′-*O*-methyl (OMe) and 2′-*O*-methoxy-ethyl (MOE) substitutions sterically shield the backbone from nuclease access, and also increase affinity to the target, shown by increased T_m, thus stabilizing the duplex (Cotten et al., 1991). 2′-*O*-alkyl ASONs

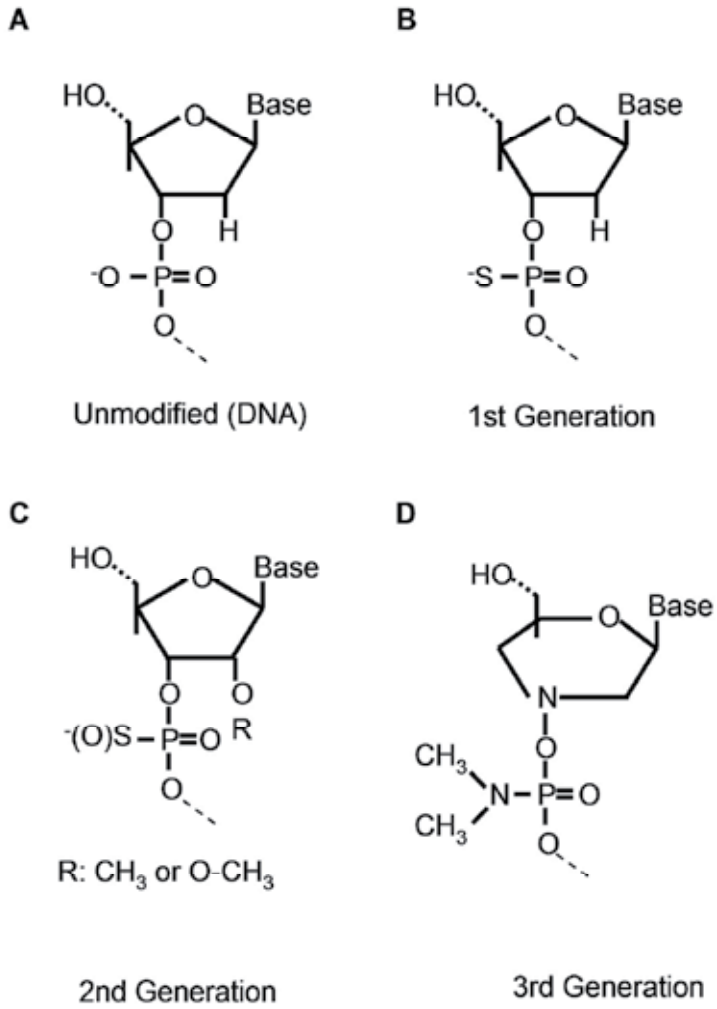

Fig. 1. Structures of certain nucleic acid analogs used to synthesize different generation ASONs. (A) unmodified deoxyribonucleotide. (B) Phosphorothioate modification of the phosphodiester backbone replaces the non-bridging oxygen with a sulfur atom. (C) Second generation ASONs with a 2′-akyl or 2′-methoxy ethyl groups further stabilize the molecule. (D) Phosphorodiamidate morpholino oligos have a modified backbone and modified sugar ring and are electrically neutral.

are also less toxic than PS-ASONs (Cotten et al., 1991); however, the 2′-*O*-alkyl group simultaneously shields heteroduplexed ASON-RNA from RNase H and therefore cannot induce direct cleavage of the target RNA. These modified ASONs function mainly by blocking translation via steric hindrance of elongating ribosome. In order to retain the advantage of the RNAse H mechanism while still conferring some benefits of 2′-O-alkyl protection, chimeric oligos containing both 2′ unmodified and 2′-modified DNAs, called gapmers, were conceived. Gapmers are typically end-modified, allowing a normal DNA-RNA heteroduplex to form mid-strand, although they may also consist of centre-modified

ASON flanked by normal DNA, or more commonly, phosphorothioate-linked DNA so as to capitalize on the advantages of both types of modifications (Turner et al., 2006). Both designs also reduce the polyanionic side effects of the phosphorothioate modification (Monia et al., 1993). 2′-O-alkyl modified ASONs and mixed backbone gapmer ASONs represent a second generation of ASON.

Third generation ASONs are phosphorodiamidate morpholino oligonucleotides (PMOs). PMOs are nonionic DNA analogues originally proven in loss of function knockdown studies in developmental systems such as zebrafish. The success and limitations of their usage have been recently reviewed comprehensively (Amantana& Iversen, 2005; Heasman, 2002). PMOs have an altered structure in which the ribose is replaced by a morpholine moiety and phosphorodiamidate (O-PONH$_2$-O) linkers are used instead of phosphodiester bonds. Thus PMOs are resistant to digestion by nucleases and are electrically neutral, a property that reduces nonspecific interactions with intracellular proteins. Morpholinos form base pairs with target sequences, but the binding ability is no greater than binding of analogous DNA and RNA oligomers, necessitating the use of relatively long 25-base oligomers for antisense inhibition. In addition, PMO-RNA hybrids do not activate RNase H. Therefore, the mechanism by which the PMOs inhibit protein synthesis is via binding the critical mRNA elements, such as the mRNA 5′UTR or the start codon region, to prevent ribosomes from binding or scanning. Alternatively, PMOs may occupy the mRNA splice recognition site to block the normal posttranscriptional processing required for synthesis of the functional protein. A good example is the report on therapeutic application of PMOs to correct aberrant splicing of mutated β-globin precursor mRNA (Lacerra et al., 2000).

The limitations of PMOs are their low cellular uptake levels as compared to unmodified ASONs. To address this shortcoming, PMOs can be conjugated to certain positive peptide carriers such as arginine-rich HIV-TAT and *drosophila* antennapedia sequences (Cardarelli et al., 2007). Because PMOs have a standard phosphodiester linkage and are uncharged, the addition of a positive peptide conjugate does not cause the same aptamer interaction as that caused by PS-ASON, which contains negative oxygen atoms.

3.2 Ribozymes

Ribozymes are catalytically active small RNA (~30-100 nts) molecules that act as enzymes to specifically cleave single strand RNA without the need of proteins. A major therapeutic advantage of ribozymes is the ability to make them *trans-acting* and to confer specificity to virtually cleave any target sequence (Peracchi, 2004). This can be achieved by fusing the ribozyme core sequence at the 5′ and 3′ ends with the sequences that are complementary to the target sequence. Of the nine groups of ribozymes, the hammerhead and hairpin ribozymes have received a great deal of attention (Scherer& Rossi, 2003). Hammerhead ribozymes, originally identified from plant viroid and viroid RNA, are composed of about 30 nts and have minimal requirements for the cleavage site, in which virtually any motif with the dinucleotide sequence UU, UC or UA can be targeted (Haseloff& Gerlach, 1992). For this reason, hammerhead ribozyme is very popular for the design of therapeutic ribozymes. On the other hand, the hairpin ribozyme has a more complex structure and requirements for target sequences, with a preference for GUC and cleavage occurring directly upstream of the G residue (Kore et al., 1998).

An advantage of ribozyme over ASON is its catalytic mode of action, which should in principle require a much lower concentration of ribozymes as compared to non-catalytic

ASONs. In addition, chemical modifications of ribozyme can increase its stability and improve therapeutic potential (Gonzalez-Carmona et al., 2006; Jakobsen et al., 2007). Antiviral ribozymes have been extensively tested in different gene therapy settings (Haasnoot et al., 2007). On the other hand, ribozyme also has its limitation, which is that target site selections are limited due to sequence requirements at the cleavage site and to structural constrains that interfere with ribozyme function to a higher extent (Frese, 2006). Therefore, the selection of appropriate target sites is of utmost importance which can not be predicted but must rather be determined empirically and which depends on the particular ribozyme used.

3.3 RNAi-based strategies

The term of RNA interference (RNAi) refers to a cellular process by which a double-strand RNA (dsRNA) sequence specifically inhibits the expression of a gene. This very efficient process of posttranscriptional gene silencing (PTGS) was discovered first in plant (Napoli et al., 1990) and served as a protection against viruses and genetic instability arising from transposons (Bartel, 2004). Accumulated evidence suggests that RNAi also plays a role in the antiviral defense mechanism in mammalian cells (Bennasser et al., 2005; Berkhout& Jeang, 2007; Cullen, 2006; Lecellier et al., 2005). These findings fuel the interests of the researchers to use RNAi not only for study of gene regulation but also for antiviral drug development (Lecellier et al., 2005; Otsuka et al., 2007).

The specificity of RNA silencing is mediated by small RNAs called short interfering RNAs (siRNA) and microRNA (miRNA). Both types of RNAs are generated by members of the Dicer family. This group of class III endoribonucleases cleaves double stranded non-coding RNA into fragments with a length of 21-25 nts. For siRNA, the long dsRNA or transgene-expressed short hairpin RNA (shRNA) is cleaved by Dicer. These RNAs are assembled into a multi-component complex, known as the RNA-induced silencing complex (RISC), which incorporates a single strand (antisense strand) of the siRNA serving as a guide sequence to silence the target gene (Hannon, 2002; Tomari& Zamore, 2005) (Fig. 2). For miRNA, this endogenous gene regulator is processed from primary RNA (priRNA) transcripts of non-coding regions or introns of protein-coding polymerase II transcripts. They are processed by RNase III Drosha to approximately 70-nt long pre-miRNAs, which are transported into cytoplasm by exportin-5 and are cleaved by Dicer to become the functional miRNA. Similar to siRNA, they also form a RISC with Argonaut proteins (having RNse H activity) and bind to their target mRNAs. The modes of actions of siRNA and miRNA depend on the degree of complementation between the siRNA or miRNA and their target sequences. siRNAs usually target coding regions by complementary base-paring and induce sequence-specific cleavage of mRNA substrate (Caudy et al., 2003); however, miRNA preferentially recognize target sequences in the 3′UTR of mRNAs and this target site is often in multi-copy (Brennecke et al., 2005; Grimson et al., 2007; Krek et al., 2005; Lewis et al., 2003). The binding of the miRNA often takes place with an incomplete homology, although a perfect base-pairing in the seed region (positions nt 2-8 from 5′ end of the antisense strand) of miRNA forms the core of interaction. Depending on the complete or partial homology between the miRNA and mRNA, the result can be cleavage of the target mRNA or repression of translation (Fig. 2) (Doench et al., 2003; Parker et al., 2005).

The precise mechanisms of RNAi-mediated suppression of gene expression have been studied extensively and made significant progress. The proposed mechanisms include the translation suppression by blocking the binding and scanning of ribosome and other

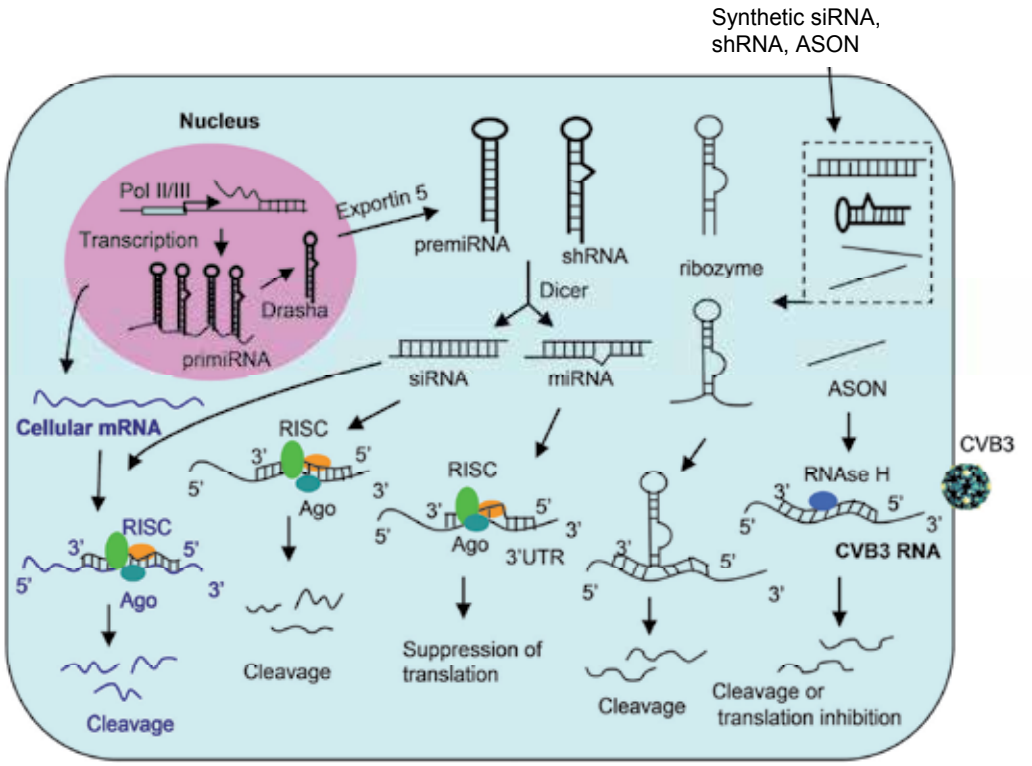

Fig. 2. NA-based antiviral strategies for pathogens of viral myocarditis. Antiviral nucleic acids can either be transfected into cells or expressed intracellularly. ASONs hybridize to viral mRNA to induce RNase H-mediated cleavage of RNA strand of the DNA-RNA duplexes. Some modified ASONs cannot induce RNase H but they have a high affinity for the target and inhibit translation by steric hindrance of ribosome or splicing. Binding of ribozymes to the target sequence can trigger cleavage of the viral RNA. siRNAs incorporated in the RISC target the viral RNA by perfect sequence complementation and induce cleavage of the target sequence by RNAse H activity of Ago protein. miRNAs (or AmiRNAs) target viral RNA by imperfect sequence complementation and induce gene silencing by destabilizing mRNAs (e.g., 3′ deadenylation or 5′ de-capping) and suppression of translation initiation and elongation. In addition, siRNAs can also target cellular genes (e.g., CAR and signal molecules) involved in viral entry and replication.

initiation factors on the mRNA or by disassociation of the premature translation initiation complexes. According to an alternative model, the mRNA destabilization is via miRNA-induced 3′ end de-adenylation or 5′ end de-capping, which results in degradation of mRNA after cleavage. Recently, some other mechanisms have been suggested. As review of the detailed mechanisms of RNAi action is beyond the scope of this article, the readers can refer to the recent reviews (Bartel, 2009; Carthew& Sontheimer, 2009; Q. Liu, Paroo, Z., 2010; Moazed, 2009). It should be pointed out that RNAi strategies for antiviral design have some advantages over the ASONs. Although they all cleave target mRNAs by RNase H, the modified ASON DNA induces activation of RNase H and cleavage of target sequence in nucleus, while the dsRNA functions primarily in cytoplasm. Ago2, the most important

component of the RISC, is located in the p-bodies (Sen & Blau, 2005). Its cytoplasm localization is critically important for anti-coxsackievirus action as this virus is replicated only in cytoplasm. In addition, in the case of RNAi, an endogenous cellular pathway is followed, which could explain the high efficiency with which siRNAs are able to reach 1000 times higher than the ASON in cleavage of the same target molecule (Bertrand et al., 2002; Grunweller et al., 2003). However, the limitations for RNAi are present (Hemida et al., 2010); similar to the ribozymes, the selection of the suitable target for binding is restricted, particularly for miRNA, as the search for effective targeting sites are often limited in the 3'UTR of mRNA.

4. NA-based antivirals against CVB3 infection

4.1 Anti-CVB3 ASONs

CVB3, one of the most frequently used model systems for study of viral replication and pathogenesis, is also widely employed for evaluation of NA-based antiviral agents. The early investigations mainly focused on the application of the second and third generations of the ASONs. McManus and coworkers are one of the pioneer groups to study the potential possibility to inhibit CVB3 replication using ASONs. Their earliest work using regular ASONs to target the different sites of 5'UTR of CVB3 genome successfully mapped the IRES by *in vitro* translation inhibition assay (Yang et al., 1997). This study provided useful information for the design of ASON for inhibiting CVB3 replication *in vitro* and in mouse models. Later, they used PS-ASONs targeting the 5' and 3'UTRs as well as the start codon region and found that the oligomers targeting the 5' and 3' proximate ends of the CVB3 genome are the most effective candidates to inhibit viral replication in HeLa cells. Each of these two ASONs resulted in ~80% reduction of viral particle production, which is followed by the candidates targeting the IRES and the initiation codon region (A. Wang et al., 2001). The importance of these sites for ASON binding was further confirmed by *in vivo* evaluation using a murine myocarditis model, although the antiviral efficiency is not as high as that obtained from *in vitro* evaluation (Yuan et al., 2004).

To improve the stability of the oligomers, our group designed eight phosphorodiamidate morpholino oligomers (PMO) targeting both the sense and antisense strands of the CVB3 replication intermediate. To increase the efficiency of drug internalization, the PMO were conjugated to a cell-penetrating arginine-rich peptide. These modified ASONs were evaluated in HeLa cells and HL-1 cardiomyocytes in culture and in a murine myocarditis model (Yuan et al., 2006). One of the oligomers, designed to target a sequence in the 3' portion of the CVB3 IRES, was found to be especially potent against CVB3. Treatment of cells with this oligomer prior to CVB3 infection produced an approximately 3-$\log_{10}$ decrease in viral titer and largely protected cells from a virus-induced cytopathic effect. A similar antiviral effect was observed when this oligomer treatment began shortly after the virus infection period. A/J mice receiving intravenous administration of this oligomer once prior to and once after CVB3 infection showed an ~2-$\log_{10}$-decreased viral titer in the myocardium at 7 days post infection and a significantly decreased level of cardiac tissue damage, compared to the controls (Yuan et al., 2006).

In addition to the many ASON reports, another strategy using CpG containing oligodeoxynucleotide to activate antiviral immunity has been reported (Cong et al., 2007). The mechanism is that the C-type of CpG oligomer can induce anti-CVB3 activity in human peripheral blood mononuclear cells (PBMCs) through the induction of synthesis of natural mixed interferons.

4.2 Antiviral ribozymes

Ribozyme as an antiviral agent has been tested for many virus infections; however, report on anti-CVB3 has not been documented. Here, we will take HCV as an example to briefly discuss the potential application of ribozyme for the treatment of HCV infection, as many recent reports found that HCV is a new causal agent of myocarditis (Matsumori, 2005; Matsumori et al., 2006). To investigate the potential application of synthetic, stabilized ribozymes for the treatment of chronic HCV infection, Macejak *et al*. designed and synthesized hammerhead ribozymes targeting 15 conserved sites in the 5'UTR of HCV RNA including the IRES (Macejak et al., 2000). It was shown that the inhibitory activity of ribozyme targeting site at nucleotide 195 of HCV RNA exhibited a sequence-specific dose response, required an active catalytic ribozyme core, and was dependent on the presence of the HCV 5'UTR. In an investigation of new genetic approaches on the management of this infection, six hammerhead ribozymes directed against a conserved region of the plus strand and minus strand of the HCV genome were isolated from a ribozyme library that was expressed using recombinant adenovirus vectors (Macejak et al., 2001). Treatment with synthetic stabilized anti-HCV ribozymes and vector-expressed HCV ribozymes has the potential to aid in treatment of patients who are infected with HCV by reducing the viral burden through specific targeting and cleavage of the viral genome. Gonzalez-Carmona and colleagues used RNA transcripts from a construct encoding a HCV-5'-NCR-luciferase fusion protein to test four chemically modified HCV specific ribozymes in a cell-free system and in HepG2 or CCL13 cell lines. They found that ribozyme (Rz1293) showed an inhibitory activity of translation of more than 70% thus verifying that the GCA 348 cleavage site in the HCV loop IV is an accessible target site in cell culture and may be suitable for the development of novel optimized hammerhead structures (Gonzalez-Carmona et al., 2006).

4.3 Anti-CVB3 siRNAs

RNAi-mediated antiviral strategies can achieve much higher efficiency than ASONs. Thus, recent studies have focused on the design and evaluation of anti-CVB3 siRNAs. This group of small double-stranded RNAs, as a silencer of target gene expression, can virtually inhibit any genes of virus and cell if the site of targeting within the gene is unique. Thus, the target search for anti-CVB3 siRNAs is not only concentrating on CVB3 genome but also extending to the host cellular genes required for viral infection or replication.

4.3.1 Targeting the CVB3 genome

CVB3 genome harbors many *cis*-acting sequence elements for viral transcription and translation, such as the 5′ and 3′ UTRs, the IRES and other segments for binding of transcription and translation initiation factors. In addition, the viral genome also encodes many essential enzymes for CVB3 multiplication, such as proteases 2A and 3C as well as the RNA-dependent RNA polymerase 3D. These structures are rationale targets for design of anti-CVB3 siRNAs. This hypothesis has been tested by a number of groups. The earlier selection of the siRNA targets was focused on CVB3 protease 2A. Almost at the same time, two groups independently found that inhibition of 2A protease by specific siRNAs significantly reduced CVB3 replication. The first group by Yuan et al., evaluated five siRNAs targeting the 5'UTR, AUG start codon, VP1, 2A and 3D, respectively and found that siRNA targeting 2A (nts 3543-3561) showed strongest anti-CVB3 activity in HeLa cells, resulting in 92% reduction of viral replication and siRNAs targeting VP1, 3D and the 5'UTR

showed modest antiviral effects, respectively. By mutational analysis of the mechanism of siRNA action, they found that siRNA functions by targeting the positive strand of the virus and requires a perfect sequence match in the central region of the target, but mismatches were more tolerated near the 3′ end than the 5′end of the antisense strand (Yuan et al., 2005). This finding on the targeting of siRNA to positive strand of CVB3 was further supported by a later study using siRNA targeting the CVB3 3D gene (Schubert et al., 2007). The second group that studied siRNA targeting CVB3 2A by Merl and co-workers evaluated antiviral activity of siRNA-2A (nts 3637-3657) *in vitro* and in highly susceptible type I interferon receptor-knockout mice. They found that siRNA-2A led to significant reduction of viral tissue titers, attenuated tissue damage and prolonged survival of mice (Merl et al., 2005). It is very interesting to point out that although the two groups used different targeting sequences within the 2A RNA, they all achieved high efficiency of antiviral effects. However, the later work by Racchi et al., which used these two siRNAs together to transfect HeLa cells and then infect with CVB3 did not potentiate the anti-CVB3 effect compared with an equimolar concentration of either siRNA (Racchi et al., 2009).

CVB3 RNA polymerase 3D is probably the most frequently used target for design of anti-CVB3 siRNAs as it is the only viral enzyme involved in CVB3 RNA replication. To date, at least a half dozen of studies on 3D have been reported. The earlier *in vitro* investigations using either un-modified or LNA-modified siRNAs or plasmid vector-expressed shRNAs all achieved significant reduction of viral replication in CVB3-infected HeLa or Cos-7 cells (Ahn et al., 2005; Schubert et al., 2005; Schubert et al., 2007; Werk et al., 2005; Yuan et al., 2005). The *in vivo* evaluation using mouse models also showed very promising results. One study employing transient transfection for *in vivo* mouse models demonstrated that two of the six candidate siRNAs targeting 3D and VP1, respectively, exerted strong anti-CVB3 effects in viral replication, accompanied by attenuated pancreatic tissue damage (J. Y. Kim et al., 2007). Another *in vivo* study is the intravenous treatment of mice with an adeno-associated virus vector (AAV2.9) expressing a shRNA targeting 3D (Fechner et al., 2008). Intravenous injection of recombinant AAV2.9 significantly attenuated the cardiac dysfunction compared to vector-treated control mice on day 10 after CVB3 infection. Recently, a study by combination of soluble CAR receptor (sCAR-Fc) and siRNA targeting 3D achieved a synergistic effect in antiviral effect in human myocardial fibroblast cell culture (Werk et al., 2009).

Other less frequently used CVB3 target genes are protease 3C, structural protein VP1 and non-structural protein 2C. Like protease 2A, protease 3C also plays an important role in the viral life cycle by processing CVB3 polyproteins to generate mature individual structural and non-structural proteins after initial cleavage by 2A (Chau et al., 2007; L. E. C. Leong, Cornell, C. T., Semler, B. L., , 2002). One study designed three siRNAs targeting genes encoding 3C, 2A and 3D of CVB4. Evaluation by transfection of rhabdomyosarcoma (RD) cells demonstrated that siRNA-3C was the most potent siRNA among these three in inhibition of CVB4 replication. This antiviral activity was followed by siRNAs targeting 3D and 2A (Tan, 2010). The difference in efficiency of these siRNAs was discussed by these authors and they proposed that this may be due to the differences in function of these viral enzymes, which are encoded by these regions: the 3C region encodes a protease 3C which is responsible for majority of the cleavage of the viral polyprotein (L. E. C. Leong, Cornell, C. T., Semler, B. L., , 2002) and 3C as well as its precursor 3CD also plays an important role at the level of viral transcription (Parsley et al., 1999). Protease 3C has been shown to be critical for interaction with the cloverleaf structures found at the 5′UTR of the viral genome to

deliver the 3D to the replication complex (L. E. Leong et al., 1993). They also indicated that since the function of 3C is required prior to 3D, a down-regulation in 3C would have a detrimental effect on viral transcription as available 3D would not be able to carry out replication of CVB4 replication without the assistance of 3C. The authors' discussion seems to be reasonable; however, according to the order (timing) of action for these enzymes, 2A cleaves the polyprotein prior to 3C's cleavage. For this situation, it may be difficult to explain why the siRNAs targeting 2A did not achieve a more efficacious anti-CVB3 activity than siRNA targeting 3C. Obviously, many issues relating to the mechanisms of action need to be further studied. However, according to the present reports, one point is clear that 2A, 3C and 3D are three important targets for design anti-CVB3 siRNAs.

Viral structural protein VP1 was also a selected target for testing anti-CVB3 siRNAs; however, the data from literature often showed a lower effectiveness of the siRNA targeting this structural gene as compared to that targeting other genes (Ahn et al., 2005; J. Y. Kim et al., 2007; Yuan et al., 2005). Due to the absence of a proof-reading activity in 3D, the mutation rate for RNA viruses is as high as 10^{-3} -10^{-4} (Cann, 2005). Thus, in recent years, the discovery of the occurrence of escape mutants due to siRNA treatment of HCV, poliovirus and HIV infections (Boden et al., 2003; Gitlin et al., 2005; Wilson& Richardson, 2005) greatly encouraged researchers to search for new approaches to counteract the drug resistance. One direction is the application of multiple distinct siRNAs or a siRNA pool to target more than one target genes of the virus (Merl& Wessely, 2007; Nygardas et al., 2009). The other direction is the identification of conserved *cis*-acting replication elements (CRE) (van Ooij et al., 2006). Theoretically, the 5'- and 3'-UTRs are the ideal target regions for siRNAs as they harbor a number of conserved *cis*-acting elements. However, studies with poliovirus and CVB3 found that siRNA residing in these regions are less efficient than siRNAs targeting on other regions (e.g., the coding region and particularly the non-structural coding region) in inducing antiviral activity (Gitlin& Stone& Andino, 2005; Merl& Wessely, 2007; Saleh et al., 2004; Yuan et al., 2005). This low antiviral potency seems to be due to the highly ordered structure of the UTRs itself, as well as the formation of the protein-RNA complexes in the region, which may block the access of the RISC complexes to its target sequences. To address this issue, Lee and coworkers selected a CRE within the coding region of 2C. Evaluation in HeLa cells demonstrated the downregulation of virus replication and attenuation of cytotoxicity in various strains and clinic isolates. Cells treated with this siRNA were resistant to the emergence of viable escape mutants and showed sustained antiviral ability (Lee et al., 2007). Based on this study, a similar experiment using siRNA targeting CRE of CVA24 2C was conducted and the authors reported the similar observations (Jun et al., 2008). These findings from *in vitro* studies were further solidified by *in vivo* evaluation, in which recombinant lentivirus was employed to express shRNAs targeting the CRE of CVB3 2C. Mice injected intraperitoneally with recombinant lentiviruses had significant reductions in viral titers, viral myocarditis and proinflammatory cytokines as well as improved survival rate, after being challenged with CVB3 (Y. J. Kim et al., 2008). Recently, this CRE was further confirmed for a number of enteroviruses, by using a novel program and *in vitro* evaluation (Lee et al., 2009).

4.3.2 Targeting host cellular genes

Another approach to fight the drug resistance caused by escape mutants is the selection of therapeutic targets within the host cellular genes that are necessary for virus entry or viral

replication. In this regard, the CAR receptor which is shared by CVB3 and adenovirus is an attractive candidate since both CVB3 and adenovirus are considered as the common causal agents of myocarditis. To date, two studies have been reported to silence CAR expression with specific siRNAs. One study reported that transfection of HeLa cells with siRNAs, siCAR2 or siCAR9, almost completely silenced the expression of CAR and that further analysis by viral plaque assay revealed ~60% reduction of CVB3 particle formation (Werk et al., 2005). Another study using cardiac-derived HL-1 cell line and primary neonatal cardiomyocytes (PNCMs) demonstrated that treatment with recombinant adenoviruses expressing shRNAs against CAR resulted in almost completely silencing of CAR expression in both HL-1 cells and PNCMs. CAR knockout resulted in inhibition of CVB3 infections by up to 97% in HL-1 and up to 90% in PNCMs. Adenoviruses were inhibited by only 75% in HL-1, but up to 92% in PNCMs (Fechner et al., 2007).

Another host gene, the tissue inhibitor of matrix metalloproteinase-1 (TIMP-1), has been suggested to be a potential target for siRNA to ameliorate CVB3-induced myocarditis. This suggestion is based on the investigation of Crocker and colleagues on a new role of TIMP-1 in exacerbating CVB-induced myocarditis (Crocker et al., 2007). They found that TIMP-1 expression was induced in the myocardium by CVB3 infection. Surprisingly, TIMP-1 knockout mice exhibited a profound attenuation of myocarditis, with increased survival. The amelioration of disease in TIMP-1 knockout mice was not attributable to either an altered T-cell response to the virus or to reduced viral replication. These data allowed the authors to propose and prove a novel function for TIMP-1: its highly localized up-regulation might arrest the matrix metalloproteinase (MMP)-dependent migration of inflammatory cells at the sites of infection thereby anatomically focusing the adaptive immune response. Finally, the benefits of TIMP-1 blockage in treating CVB myocarditis were confirmed by administration of siRNAs targeting TIMP-1, which diminished CVB3-induced myocarditis. However, this improvement of the disease is not due to the changes of viral titers, as demonstrated by viral plaque assay (Crocker et al., 2007).

Recently, the active investigations on CVB3-induced signal transduction pathways have provided new avenues for the search of therapeutic targets for the treatment of myocarditis. Since CVB3, like other picornaviruses, requires the activation of certain signal pathways for initiating their life cycle, inactivation of some signal molecules in the signal cascade with specific siRNAs would block CVB3 replication. Such kind of studies that have been documented thus far include i) the knockdown of ubiquitin expression by siRNAs to down-regulate the ubiquitination and subsequent alteration of protein function and/or degradation (Si et al., 2008); ii) silencing of proteosome activator REGγ to inhibit the REGγ-mediated degradation of several important intracellular proteins (Gao, 2010), such as cyclin-dependent kinase inhibitors p21, p16 and p19 and tumor suppressor p53; and iii) knockdown of genes critical important for autophagy formation, these genes include ATG7, Beeclin-1 and VPS34 (J. Wong et al., 2008). Although these target genes mentioned above have been tested *in vitro* using specific siRNAs in signal transduction studies and showed promising outcomes, their potential serving as a therapeutic target for treatment of CVB3 infection needs a further evaluation by pharmacological study in animal models.

4.4 Anti-CVB3 artificial miRNAs

miRNAs are a group of recently discovered new regulators of gene expression. These endogenous regulators control one third of human gene expression (Bartel, 2009; Q. Liu,

Paroo, Z., 2010). Thus, endogenous miRNAs are important targets for gene therapy and artificial miRNAs (AmiRNA) are useful tools for inhibiting disease-causing gene expression (Z. Liu et al., 2008; Sall et al., 2008). In this regard, although numerous studies have been documented for treatment of cancers, cardiovascular diseases, genetic diseases and other viral infections, only one study on anti-CVB3 from our group has been published so far. Here, I briefly summarize our work on design and evaluation of anti-CVB3 AmiRNAs (Ye et al., 2011). We constructed three short hairpin AmiRNAs (AmiR-1, -2 and -3) targeting the stem-loop of the 3'UTR of CVB3 with mismatches at the middle region of the target. Transfection of HeLa cells showed over-expression of these mature AmiRNAs as determined by real time quantitative RT-PCR. After these AmiRNA-expressing cells were infected with CVB3, the viral titers were reduced ~100 folds in cell cultures treated with AmiR-1 or AmiR-2 but not that treated with AmiR-3, at 24 h post infection. Mutational analysis of the targeting sites of AmiRNAs demonstrated that the central region but not the seed region of AmiRNAs is more tolerant to target mutation. In this study we also performed targeted delivery of the AmiRNAs to host cells through ligand-receptor interactions, which will be discussed in next section.

5. Drug delivery

NA-based agents are inefficiently taken up by mammalian cells and would therefore benefits from additional vehicles or modifications that facilitate drug delivery. Depending on transient delivery or long-term treatment, the delivery approaches can be divided into non-viral delivery of chemically synthesized agents and viral delivery of drug-expressing cassettes (Table 1). For the non-viral delivery measures, they can be further divided into un-specific and cell-type specific delivery. The former method has been widely used for delivery of many chemically synthesized ASONs, ribozymes and siRNAs as well as plasmids encoding shRNAs through transfection of tissue culture cells, hydrodynamic transduction/transfection of mice or intravenous (IV) injection of mice via tails using cationic lipoplexes or liposomes. The successful examples for inhibition of viral pathogens of myocarditis includes deliveries of i) ASONs targeting CVB3 IRES (Yuan et al., 2006) and both ends of the CVB3 genome (A. Wang et al., 2001), ii) siRNAs targeting CVB3 2A (Merl et al., 2005; Yuan et al., 2005) and 3D (Ahn et al., 2005; Schubert et al., 2005; Schubert et al., 2007), iii) plasmids expressing shRNAs targeting 3D and VP1 (J. Y. Kim et al., 2007) and vi) ribozymes targeting HCV RNA (Gonzalez-Carmona et al., 2006). For the cell-type specific method, proper modification and conjugation of 'naked' raw therapeutic molecules are required to achieve targeted delivery. The different chemical modifications described earlier for ASONs are applicable to all NA-based agents. The conjugation of these antivirals can be achieved by covalent linkage of a ligand to the molecules, which enables the drug internalization via specific interactions between the ligand and its receptor. The ligands can be an antibody, vitamin, short peptide, RNA aptamer, folic acid, etc. The details were reviewed elsewhere (X. Ye & Yang, 2009). By this strategy, we have specifically delivered siRNAs targeting CVB3 2A to HeLa (cancer) cells, a cell line susceptible to CVB3, through interactions between folate and its receptor highly expressed on the surface of all cancer cells but not the normal cells (Zhang et al., 2009). This study was carried out by covalent linkage of the siRNA targeting CVB3 2A to a bacterial phage-29 packaging RNA (pRNA). This small pRNA (~170 nts) can form dimer, trimer and hexamer by base pairing through its

Category	Target	Model system	Delivery route	Reference
PS-ASON	5′ & 3′UTRs, IRES, start codon,	HeLa cell, mice	Transfection	Wang 2003
PS-ASON	3′end of CVB3	HL-1 cells, mice	Transfection, IV injection	Yuan 2004
MOP-ASON	IRES, 5′ & 3′UTR, start codon, minus strand	HeLa, HL-1 cell, mice	Transfection and IV injection	Yuan 2006
CpG oligoer	no	PBMCs	Treatment	Cong 2007
siRNA	2A, VP1, 3D	HeLa cells	Transfection	Yuan 2005
siRNA	2A	HeLa cells, mice	Hydrodynamic,	Merl 2005
	2A	HeLa cells	Transfection	Racchi 2009
	2A	HeLa cells	pRNA vector	Zhang 2009
shRNA	3D	HeLa cells	Transfection of double expression plasmid	Schubert 2005
siRNA	3D, VP1	HeLa cells	Transfection	Ahn 2005
LNA-siRNA	3D	Cos-7 cells	Transfection	Schubert 2007
siRNA	siRNA pool	LLC-MK2 cells	Transfection	Nygardas 2009
shRNA	VP1, 3D, 5′ & 3′UTR	Cos-7 cells, mice	hydrodynamic, transfectin of plasmid	Kim J-Y 2007
siRNA & sCAR-Fc	3D	HMF	Transfection	Werk D 2009
shRNA	3D	HeLa, PNCMs, Mice	Transduction, IV, AAV vector	Fechner 2008
siRNA (CVB4)	3D, 3C, 2A	RD cells	Transfection	Tan , 2010
shRNA	2C	Mice	IP injection, lentivirus vecotr	Lee 2007
shRNA (CVA24)	2C	HeLa, HCC	Transfection, plasmid	Jun 2008
siRNA (enteroviruses)	2C	HeLa, Vero cells	Transfection	Lee 2009
shRNA	CAR	HL-1, PNCMs	Adenovirus vector	Fecherner 2007
siRNA	TIMP-1	Mice	IV injection	Crocker 2007
siRNA	CAR, 3D	HeLa,Cos-7 cells	Transfection	Werk 2005
siRNA	Ubiquitin	HeLa cells	Transfection	Si 2008
siRNA	ATG7, Beclin-,VPS34	HeLa cells	Transfection	Wong 2008
siRNA	Proteasome activator REGγ	HeLa cells	Transfection	Gao G 2010

Abbreviations: LNA: locked nucleic acid; RD: rhabdomyosarcoma; PBMC: peripheral blood mononuclear cell; HMF: human myocardial fibroblast; HCC: human conjunctive cell.

Table 1. NA-based agents for the treatment of CVB3 infection.

left- and right-hand loops (P. Guo, 2002) (Fig. 3). Thus, this pRNA multimer can carry multiple siRNAs and has the potential to overcome issues associated with drug resistance of viruses (P. Guo, 2005). In addition, this small pRNA vector has lower immunogenicity than big DNA vectors. Thus it is a safe vehicle for transportation of antiviral drugs (S. Guo et al., 2006). We labeled, by *in vitro* transcription, the 5′end of the pRNA with AMP-folic acid,

which guided the targeted delivery of siRNAs via ligand-receptor interactions and achieved strong inhibition of CVB3 replication (Zhang et al., 2009). The effectiveness of this strategy on targeted delivery of NA-based drugs was further solidified in targeted delivery of AmiRNAs to inhibit CVB3 replication (X. Ye, Liu, Z., Hemida, G. M., and Yang, D. C., 2011).

Viral vector-mediated delivery of NA-based agents is another promising approach for treatment of persistent infection such as CVB3, HCV and many other viruses. This is because that the vector-encoded shRNA can produce relative long-term and continuous silencing. Most viral vectors are modified viruses, which can be applied to deliver a cargo sequence to cells. Currently the most commonly used viral vectors for the delivery of NA-based drugs are derived from the adenovirus, adeno-associated viruses (AAV) and lentiviruses. These vectors have been widely used and have achieved exciting promise (Fechner et al., 2008; Henry et al., 2006; Kuhlmann et al., 2008). Recent trends in further improvement of these vectors focus on modifications of their structure to increase the capability for targeted delivery. The efforts of this goal can be summarized into three categories (X. Ye & Yang, 2009): i) the genetic and chemical modifications of the vector to express a unique chimeric surface protein, such as adenovirus capsid proteins: fiber knob, penton and hexon. The common strategy is the insertion of a foreign peptide into fiber knob, which enables the vector to be capable of binding the specific cellular receptor (Koizumi et al., 2003; Mizuguchi& Hayakawa, 2002); ii) incorporation of heterologous protein from another virus with a restricted range of tissue tropism to the viral envelope; this approach is also called pseudotyping. An example of this strategy is the pseudotyping of lentivirus vector with the neurotropic rabies virus glycoprotein allows retro-axonal and trans-synoptic spread, thereby enhancing the transgene expression within the brain (L. F. Wong et al., 2004); and iii) the application of a tissue-specific promoter to express the vector-carried gene in a specific organ or cell type. An impressive finding has been reported regarding the utilization of cardiac myosin light chain 2v promoter and the hypoxia-response element by AAV vector to express vascular endothelial growth factor, an angiogenic factor, specifically in myocardium, leading to cardiac functional improvement (Su et al., 2004). Here we will briefly discuss the delivery of NA-based antivirals using these viral vectors for CVB3 infection.

Adenovirus is known to share the CAR receptor with CVB3. This receptor is highly expressed on the surface of cardiomyocytes. Thus, adenovirus-derived vector is an ideal carrier for delivery of NA-based antivirals to the heart. This vector has successfully delivered shRNAs targeting the CAR gene in a cardiac-derived HL-1 cell line and isolated PNCMs, resulting in the strong reduction of replication of both CVB3 and adenovirus (Fechner et al., 2007). Lentivirus vectors are derived from HIV. They have the ability to transduce quiescent as well as proliferating cells, thus increasing their therapeutic ranges. Particularly, after pseudotyping with G glycoprotein of vesicular stomatitis virus, they can transduce almost any cell type (Kurreck, 2009). Kim Y-J et al. constructed recombinant lentiviruses that express shRNAs targeting the CRE within CVB3 2C. Intraperitoneal injection of mice with these viruses clearly showed a protective effect against viral myocarditis by elimination of CVB3 infection and reducing pro-inflammatory cytokines, such as IL6 and INF-α. (Y. J. Kim et al., 2008). AAVs are attractive vectors for gene transfer since they efficiently transduce target cells and are nonpathogenic for humans. Fechner H et al. have employed a pseudotyped AAV2.9 vector, carrying the most cardiotropic AAV capsid currently known to successfully transduce PNCMs. This vector expressed siRNAs targeting CVB3 3D and reduced CVB3 replication by >3 $\log_{10}$ steps. Further evaluation by

intravenous injection of mice demonstrated significant reduction of virus titers and improvement of heart function compared to the control. This study showed for the first time that intravenously injected AAV2.9 has the potential to target RNAi to the heart and suggests AVV2.9-shRNA vectors as a novel therapeutic approach for cardiac disorders (Fechner et al., 2008).

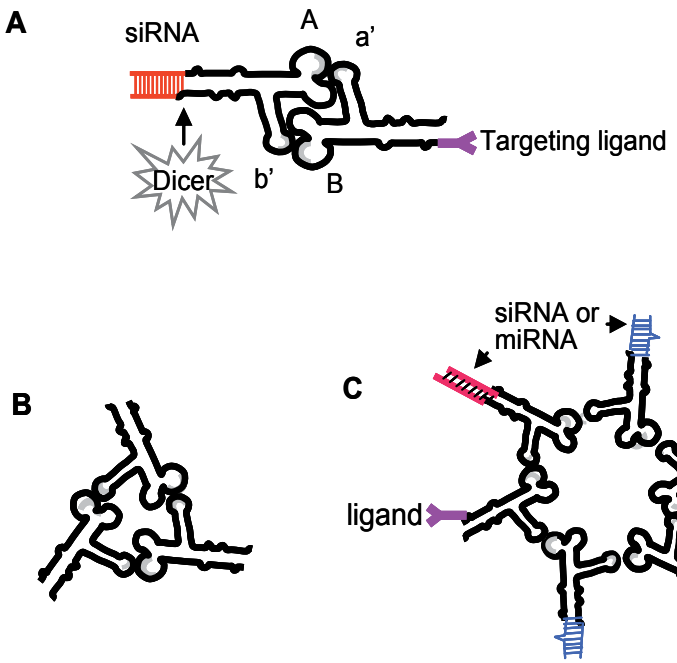

Fig. 3. Structural schematic of packaging RNA (pRNA) multimers as a drug targeted delivery vehicle. (A) pRNA dimer forms through the base-paring between the loops of an A'b-pRNA-siRNA and a B'a-pRNA-ligand. The shaded areas on the loops indicate the base-pair interactions between the monomers. siRNA is released when intracellular Dicer cleaves the dsRNA, which is indicated by an arrow. (B) pRNA trimers can be stoichiometrically formed by hand-in-hand loop interactions, which contains 1:1:1 of their linked conjugates. (C) hexamers allow for a customizable, combined therapy where multiple drugs may be added to the same complex.

6. Concluding remarks

NA-based gene silencing techniques have been successfully used in drug development. The major progress on ASON research is the chemical modifications and ligand conjugation to enhance drug stability and efficacy of delivery. The emergence of RNAi-mediated gene silencing techniques further provided new hope for this regard. Basically, siRNA silencing techniques can be used against any viral infection. Two major obstacles must, however, be overcome before it can become a broadly applicable standard therapy: the question of their specificity and efficient delivery to target cells. As siRNA can potentially cause off-site targeting and activate the immune system, minimizing the undesired effects must be considered in the drug design. Immense efforts have been undertaken to develop carrier

system with which siRNAs can be delivered to their target cells. Despite great advances in the last years, further developments are still required to get systematically applied siRNAs to their required sites of action. Here, viral vectors systems for shRNA expression cassettes offer additional options for efficient and organ-specific delivery. However, this approach must be first overcome the reservations based on the negative experience with gene therapy. As discussed earlier, pRNA is a promising vehicle for targeted delivery of NA-based therapeutic molecules. For treatment of myocarditis, a myocardium-specific ligand such as peptides from the CVB3 antireceptor protein or RNA apatamers of cardiomyocytes should be identified, which will be used to replace folic acid on the pRNA vector.

Very recent advances in the understanding of miRNA biology and particularly their association with the molecular pathogenesis of a variety of diseases have served as a theoretical basis for drug development. On the one hand, miRNA, as one of the key factors for regulation of viral replication, tissue tropism and latency, are the ideal targets for inhibition. In this regard, construction of mRNAs that contain multiple tandem binding sites of a given miRNA may be useful to produce decoys or "miRNA sponges" to inhibit the function of a specific miRNA. In addition, chemically synthesized antisense RNA oligomers ('antagomirs') targeting a miRNA of interest could be also a promising approach to inhibit miRNA activity (Ebert et al., 2007; Krutzfeldt et al., 2005). Other strategies include i) overexpression of specific miRNAs using an expression vector to achieve a long-term effect of reversing the imbalance of miRNA expression caused by infections, and ii) introduction of pre-miRNA mimetics for transient replacement of a down-regulated miRNA. On the other hand, miRNA can serve as a useful tool for therapy. Since miRNA is tolerable to target mutation at its center region, application of multiple artificial miRNAs to target the 3'UTR and/or other regions of CVB3 RNA may improve the drug resistance. Given the immense interest in NA-based drug research and the rapid progress made in this field and other areas such as nano-biotechnology for drug delivery, the coming years are likely to see an increasing range of clinical applications, particularly for the RNAi-based drug candidates. The realization of the potential of NA-based therapies to address human viral pathogens suggests that this field has a very promising future.

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Myocarditis, the inflammation of the heart muscle, could be in some cases serious and potentially fatal disease. This book is a comprehensive compilation of studies from leading international experts on various aspects of myocarditis. The first section of the book provides a clinical perspective on the disease. It contains comprehensive reviews of the causes of myocarditis, its classification, diagnosis, and treatment. It also includes reviews of Perimyocarditis; Chagas' chronic myocarditis, and myocarditis in HIV-positive patients. The second section of the book focuses on the pathogenesis of myocarditis, discussing pathways and mechanisms activated during viral infection and host immune response during myocarditis. The third, and final, section discusses new findings in the pathogenesis that may lead to new directions for clinical diagnosis, including use of new biomarkers, and new treatments of myocarditis.

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