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Physiology and Pathology of Immunology

Edited by Nima Rezaei

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Notice

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Meet the editor

Nima Rezaei obtained his MSc degree and then PhD degree in Clinical Immunology and Human Genetics from the University of Sheffield after gaining his medical degree (MD). Since 2010, Dr. Rezaei has been a faculty member of the Department of Immunology, Tehran University of Medical Sciences (TUMS). He is now the vice dean of International Affairs, School of Medicine, TUMS; cofounder and deputy president of the Research Center for Immunodeficiencies; and founding president of the Universal Scientific Education and Research Network (USERN). Dr. Rezaei is an editorial assistant or a board member for more than 30 international journals. He has edited more than 10 books, has presented more than 400 lectures/posters in congresses/meetings, and has published more than 600 articles in international scientific journals so far.

Contents

Preface

The immune system, like every other organ system, is tuned to maintain a state of balance between activation (when facing foreign molecules) and rest (when encountering various "familiar" molecules of the human body). Modern immunology goes even further to unveil darker shades of the complex immune network to identify more elusive signs of change and subtle variations in the body's anatomy. This way, immunity is equivalent to a "whole body portrait" of all that is good and all that should be reserved.

I always believed science to have a multidisciplinary nature, and the current inventory is one of the many examples, a proof to the notion of immunology science as spectrum of entangled knowledge from molecular and cellular biology, to genetics, microbiology, and to almost every specialty in medicine. Within the current book, you'll find harmony in the content from the basics of the immune system function to new technologies in immunology fundamentals, offering promising solutions for multidrug resistance in pathogens. Chapters 1 and 2 give a head start in understanding the building blocks of the immune system and the everlasting challenge of the immune system, anergy versus response. Three of the eleven chapters focus on molecular infrastructure of normal immune system (Chapters 3, 4, and 5). Chapters 6 and 7 redefine the role of immune system in infections, bringing about the human response to *Trypanosoma* parasite as a model of complexity of host-pathogen interactions. Autoimmune disorders were once believed to be the sole disorders of the immune system. Chapter 8 looks back onto the pathology of rheumatoid arthritis, to go ahead with describing the role of $CD4^+$ T cells in the most common inflammatory disorders of the musculoskeletal system. With the growing body of evidence on the footsteps of immunity in neurodegeneration, authors of Chapter 9 focus on the role of inflammation in Parkinson's disease. In Chapters 10 and 11, you can read about how multidrug-resistant bacteria can be combatted by highly selective, specially tailored vaccines that target the pathogen and use of phages and virus particles that have served faithfully in molecular engineering, for selective eradication of drug-resistant organisms. This highly readable book explores novels around the basic and presents the unseen to be a valuable resource for every student and senior in immunological sciences. Special attention is given to figures where you'll find aesthetically pleasing illustrations from authors of each chapter.

I highly recommend this book and encourage young scientists to have the courage to further explore this arena. I also want to give special thanks to InTechOpen for giving me the chance to publish and to all authors whose contribution is greatly appreciated.

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Chapter 1

Introductory Chapter: Introduction on Physiology and Pathology of Immunology

Farzaneh Rahmani, Mohammad Reza Rahmani and Nima Rezaei

Additional information is available at the end of the chapter

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1. Introduction

Dynamic, variable, and diverse overtime and in action, the entire effort of the human body is to survive. Whether preplanned, i.e. innate immune reactions, or partly planned, i.e. adaptive immunity, immunity has evolved to counteract changes. Human immune response follows the inexorable schemes based on four main principals of action:

First – Early detection of unwelcome factor of change and activation of response systems.

Second – Setting the stage for an effective, least interfering, response with normal body function and activation of systemic responses.

Third – Activation of long-term survival and adaptation signals, and natural repair systems.

Fourth – Timely termination of the response and learning from the experience if need be!

The advent of molecular genetics, molecular pathophysiology, utilization of new imaging techniques, and advances in bioinformatics and health data, have shed light over novel etiologic factors of disorders and opened eyes to more pieces of the puzzle of diseases of the human body. Researchers in clinical medicine and basic science are deciphering the complex trails by which our immune response is both regulated by and controls many functions of a living creature, from fundamentals of a local response at the site of injury to the neurodevelopmental function of immune pathways in the developing brain, and to the inclusive mechanisms by which immunity regulates one's longevity and survival. It is not at all a daring claim that our immune system is the executive element in almost every defense mechanism of the human body to counteract threats. Whether it be invading microorganisms into the guts, an abnormally proliferating cell in the lining of our lungs, a blood clot in one of the cerebral

arteries, aberrant aggregation of cholesterol in endothelium lining of the coronary artery, or an aging cell in the macula of the eye. Immune aspects of physiology and pathology of the human being are now the prevailing notion in research, therapeutic modalities, and day-to-day practice of clinicians.

This book is presented as the result of an effort to provide basics to this vast area of growing knowledge, on the basic actions of the immune system in health and disease. Uncertain nature of the "change", temporally and regionally, necessitates a 24-hour alert system to summon immune effectors and exert the proper scenario of action. The effector cells of the immune system are therefore distributed in a tightly regulated manner all throughout the body and over time. The mucosal-associated lymphoid tissue comprises the largest pool of immune effectors, followed by bone marrow and spleen, which are the primary training and regulation sites of immunity. Most interesting is that each and every organ system and each cell in the body is conferred with the intrinsic ability to respond to change, injury, or an adverse event, and set off a cascade of adaptation or maladaptation.

This makes us to the important crosstalk of immunity with three major regulatory bodies in human body. Immunity acts in consort with hemostatic response, neuroendocrine system, and circulatory/lymphatic system to exert potent internal regulatory signals. Later on, in this section, we look briefly at the fundamentals of the function of the human immune system to be able to move on to the exciting new dimensions of the role of immunity in cancer immunology, immunology of transplantation, autoimmunity, and immunodeficiencies.

Conventionally, there has been a trend to define the immune system, first by introducing the immune mediators, cells and organs involved in various responses, and then by dividing the responses into "innate" and "adaptive or acquired" response.

The innate immunity is as diverse and ancient as the structures of different parts of the human body. Each organ system has developed over time, barriers to minimize the scope of pathogen invasion, or better neutralize the attack at the site of entry. We believe that the evolution of human immune system is the product of and currently shaped by, an everlasting struggle with rapidly reproducing and frequently changing microbial pathogens. The various mechanisms of innate immunity are developed in order to quickly identify the stereotypes in pathogen structures [i.e., pathogen-associated molecular patterns (PAMPs)] and provide either mechanical barriers or respond by secretion or cell surface expression of antagonizing molecules, most importantly known as antimicrobial peptides. Pattern recognition in innate immunity is based on, but not confined to, identification of peptides, as well as carbohydrates and pathogen-associated nucleic acid segments [1]. Inflammation is a transitory and ongoing nonspecific mechanism of innate immunity. A full-armed activation of defense and repair mechanisms, and involvement of the pathogen-specific, acquired immune responses follow the initial inflammatory response. Inflammation could be defined as a harmonic array of activation of plasma proteins (complement system, antimicrobial peptides such as defensins and cathelicidins, cytokines, and vasoactive mediators), circulating and infiltrating leukocytes (polymorphonuclear leukocytes, lymphocytes, macrophages/monocytes etc.). Endothelial lining of the vessels and indolent cells of the parenchyma are known as non-specified, yet highly active players of inflammation.

The primary goal of the inflammatory response is to recruit immune cells to the site of invasion. Yet, failure to remove the pathogen from the site of entry, as happens following the invasion of *Mycobacterium tuberculosis* to a hilar lymph node of the lung, failure to terminate the response, such as when antigen:antibody complex deposits in glomerular basement membrane a few weeks after streptococcal pharyngitis, excessive response to a benign pathogen, when resident alveolar macrophages, eosinophils, and neutrophils are drawn to the site where Aspergillus spores penetrate the respiratory system, each underlie formation of different types of immune diseases.

Before we move to describe the common scenarios by which the immune system operates its actions and the distinct disciplines in emerging fundamental of cancer immunology, immunology of transplants, systemic and organ-specific autoimmune disorders, and immunodeficiencies, let us move to the building blocks of immunity, tissue/cellular components of the immunity.

2. Cells of the immune system

2.1. Granulocytes

The polymorphonuclear granulocytes (PMNs) or neutrophils are the soldiers in the front of acute infection and inflammation. Other two types of granulocytes, the eosinophils and basophils deliver target specific phagocytosis and cytotoxicity. Ingestion and degradation of microbes and cellular debris, from the site of infection or tissue damage, is the primary role of PMNs. They do so with the help of surface display of pathogen-associated molecular pattern receptors, such as toll-like receptors, NOD-like receptors, and also receptors for the constant part of various antibodies (e.g., fc IgG) and complement system receptors (e.g., C3a). The macrophages, in turn, appear late in the inflammatory response, continue phagocytosis and elaborate long-term tissue remodeling mechanisms. IL-8 and specific chemotactic agents from injured cells, and complement mediators (C3 and C5) in the plasma, attract neutrophils, eosinophils, and basophils to the site of infection. Together they produce soluble products, IL-5, eotaxin, histamine, or reactive nitrogen and oxygen species, exerting potent cytotoxic or chemotactic on the bacterial, viral, protozoal, or allergic pathogen. Inborn errors in adhesion, migration, or degranulation of granulocytes are the basis of leukocyte adhesion deficiency (LAD) and chronic granulomatous disease (CGD), the two types of "primary immunodeficiencies."

2.2. Lymphocytes

Lymphocytes are categorized into four types, based on function, the B lymphocytes, T lymphocytes, natural killer cells (NKCs), and NK T cells. They all share the same common lymphoid progenitor in the bone marrow, yet, the first two cell lines develop their clonally unique surface receptor immunoglobulin (Ig) or T-cell receptors (TCR), in the bone marrow, ensuring strict selection criteria, before they release into the periphery for further maturation. As for the B cells, they later undergo involution into mature antibody producing plasma cells. The safe/nonsafe and self/nonself discrimination, the paradigm of action of the immune system, is

perceived to be a primary consequence of T-cell discrimination of self-/nonself-antigens. No T cell can recognize a self-protein unless it is endocytosed, digested, and presented in the form of a complex with either type of major histocompatibility (MHC) molecules, on the surface of an antigen presenting cell (APC). In contrast, the antibody on the surface of B cells can recognize and reproduce soluble immunoglobulins, as they travel from lymph nodes to the blood and the sites of invasion, against a wide array of antigens from polysaccharides, lipids, nucleic acids, and larger proteins.

By virtue of their membrane expression of killer cell inhibitory receptors (KIR) and CD16, NK cells and NK T cells, are omnipresent circulating security check systems. Via a direct cell-to-cell contact, mediated by KIR, the NK cells "scan and inspect" almost every cell type in the body, for uneventful intracellular events, a neo-epitope or a mutant protein from neoplastic alteration of the cell or a viral antigen, etc. Antigenic particles formed in this process are presented by class I of MHC molecules and consist of peptides of various sources, mostly from denatured and worn out proteins after they undergo normal senescence and degradation by proteasome complex. The NK cells own a unique receptor for the constant part of the IgG, that upon recognition of the Ag:Ab complex, mediates a burst out of cytotoxic granules from the lymphocyte. A targeted, direct cell-to-cell cytotoxicity is the result. NK cells provide the first line of antitumoral/antiviral defense of the immunity, while the machinery of antigen presentation to cytotoxic and helper variants of T cells is setting off in action.

2.3. Antigen presenting cells (APCs)

Antigen presentation is the groundwork for various types of cells that are active in displaying protein antigens to the naïve T-cell lymphocytes. The monocytes/macrophages, constituents of the reticuloendothelial system, the B cells, and foremost, the dendritic cells, are labeled as APCs, based on their mutual ability to express both class I and class II MHC molecules. Some APCs (monocytes/macrophages), particularly express surface receptors for Fc gamma of IgG and C3b and are very potent phagocytosis. Some, like dendritic cells, are less potent phagocytes but have evolved into very efficient antigen detectors. Conventional dendritic cells are strategically located at the body entrance sites, to capture microbes and to migrate to T-cell zones of sentinel lymph nodes to instruct further deployment of adaptive immune response. Cross-talks between TCR, costimulatory molecules on APCs, and the MHC class II molecules are the core of a series of tightly regulated mechanisms that orchestrate a robust and accurate plan to counteract offenses.

2.4. Lymphoid tissues

It is an incomplete introduction on immune cells if one does not mention the diffuse, yet amazingly systematized organs with a lead role in human immunity. While every cell and organ in the body is endowed with a primary and nonspecific defense system (e.g., mucosal membrane of mouth, lung, etc., special circulation conduits of the GI system, the urine flushing the urethra, or the mucociliary escalator of the bronchi), there are accumulations of lymphoid tissue in human body in charge of training lymphocytes, fostering immune interactions, and providing long-term reservoirs for memory cells residence. Based on the main lymphocyte population, they are divided into primary (Thymus and bone marrow)

and secondary (lymph nodes, spleen, and mucosal-associated lymphoid tissues). Primary lymphoid organs, harbor lymphocytes proliferation, selection, clonal expansion, and maturation. While the secondary organs, serve as homing and expansion sites for mature lymphocytes and facilitate acquired immune response via exclusive structural delicacies.

3. Stories about the immune system

3.1. Inflammation

Inflammation, as described, is the effort of injured cells, to communicate the danger signal, on the spot, to the first-line innate mechanisms to minimize the invasion hazards. These include, but are not confined to, vascular response, regarding vasodilation, increase in permeability, and activation of endothelial cells, leading to cellular response, with an increase in leukocyte chemotaxis, adhesion, and transmigration, into extracellular tissue. Infection is the most common trigger for inflammation, yet, tissue necrosis, aseptic trauma with or without necrosis, foreign bodies, and in the case of inflammatory disorders, hypersensitivity to a sustained assault or autoantigens, could all be the souls behind the face of inflammation. Resident phagocytes, dendritic cells, and epithelial cells, and depending on whether endothelial damage has occurred, platelets are the first to confront the products of tissue assault. These cells recognize the danger via the pathogen/danger-associated molecular pattern (PAMP) receptors. They produce a wide array of danger signals including proinflammatory cytokines, and in turn respond to histamine, thrombin, TNF-α, IL-1, and IL-6 by secretion of chemotactic agents, further facilitating leukocyte transmigration.

The endothelial expression of adhesion and selectin molecules (e.g., E-selectin surfaced in response to TNF-α and IL-1), and their interaction with a multitude of surface integrin and cell adhesion molecules on leukocytes, facilitates leukocytes allocation to the infection site. Activated indigenous or infiltrated phagocytes, in turn, ingest microorganisms and dead cells, produce reactive oxygen and nitrogen species, extracellular digestive enzymes, and products of lipoxygenase cascade such as leukotrienes and prostaglandins. These, together with the complement system and antibody response, form the main body of chemical moderators of inflammation.

The face of an inflammatory response changes when antigen presenting cells set the stage for specific recognition of the antigens and presentation to T cells. Antigen-specific T cells, macrophages, circulating plasma cells, and memory B cells are leading characters of immunity, in a durative inflammation.

3.2. B-cell and T-cell development and maturation

Genetic recombination is the most noteworthy feature acquired by the immune system, endowing an adaptive ability to generate a limitless array of receptors, while maintaining genetic stability and frugality of genetic material of a vertebrate cell.

The B-cell receptor (BCR) light and heavy chains (IgL and IgH), and the TCR α, β, γ, and δ chains, each form as result of a matchless system of genetic recombination/rearrangement of V(D)J regions, their assembly with different types of immunoglobulin constant regions to form an clonally unique and specific receptor for two main types of lymphocytes.

Either as a soluble immunoglobulin or as B-cell surface receptor, the BCR is a heterodimer of two heavy chains (with five types of constant regions, to which they are designated for IgG, IgA, IgM, IgD, and IgE) and two light chains, either kappa,or lambda, based on the sequence of their constant region. The TCR is either an $\alpha\beta$ chains or a $\gamma\delta$ chains heterodimer, with the $\alpha\beta$ being the most common type. Alike Ig heterodimers, each of the α, β, γ, or δ chains also has a constant and variable region.

Within the precursors of B cell and T cell in the bone marrow, the immunoglobulin gene segment undergoes a sequential allelic exclusion to recombine one of each type of V, D, or J genes at each locus, at a time. If the rearrangement is productive, and full-length light and heavy chains are produced, the resultant, pro–B cells and pro–T cells, undergo selection either through bone marrow stromal cells for B cells or the cortex epithelial and medullary cells of the thymus. Induction of "central tolerance" in lymphocytes, follows one principle, that is high avidity and low avidity self-antigen recognition are both discouraged, and only the nonantigen-responsive lymphocytes are selected and given a chance to survive. These later migrated from primary lymphoid organs to the periphery to be exposed to a set of secondary immune surveillance mechanisms or "peripheral tolerance."

Receptor selection of TCR is different from the BCR, as TCR must acquire the potential to react to self-antigen-MHC complexes, rather than soluble antigens, an eligibility acquired via positive and negative selection. Only thymocytes that can bind to bare MHC class I and II in the cortex of the thymus (positive selection), and later unable to bind or bind with low affinity to the medullary expressed self-antigen-MHC complexes (negative selection), will receive life signals to release to the periphery. Pre–B cells with "high avidity self-antigen recognition" are either given a second chance to rearrange their variable V(D)J region of the light chain (with the remaining options of the same gene or their pair on the homologous chromosome), before either receiving a go-ahead signal or undergoing apoptosis.

A final leap is required for a B cell to get matured, and that is, the naïve B cell undergoes switching of the receptor subtype (class switching), changing the antibody subtype of the BCR from IgM to IgG, and later into other immunoglobulin subtypes to be able to mediate various types of humoral responses to different antigens. The peripheral encounter of a B cell or T cell, bearing a self-reactive receptor, evokes peripheral tolerance mechanism, leading to activation-induced death or anergy of the autoreactive lymphocytes.

3.3. Humoral and cellular response to an infection

In this paragraph, we look briefly into a typical humoral and cellular response to an extracellular pathogen; e.g., *Streptococcus pyogenes* cultivating the throat. Acute innate immune response to an infection is neutrophils infiltration, chemo-attraction of distinct populations of leukocytes, and secretion of TNF-α, IL-1, IL-6, nitrous oxide, and proteases. Next, macrophages, dendritic cells, along with other tissue-specific and nonspecific APCs, present parts of the degraded pathogen on their surface MHC receptors, which are in this case the class II of MHC molecules.

Regulation of lymphocyte circulation is essential, for appropriate interaction of APCs and lymphocytes to happen. An elaborate system of chemokines and surface receptors, makes sure that the meticulously selected mature B and T lymphocytes, not only travel in a nonstop

trip, trafficking in and out of secondary lymphoid organs, but also, receive appropriate "homing signals," to the site or sites where the interaction of immune cells predominantly occur.

Recognition of antigens via Ig or TCR provides the primary signal for lymphocyte activation, named as signal 1. As a consequence of the negative selection of lymphocyte in bone marrow and thymus, T lymphocytes that bind with moderate to low affinity to MHC/antigen complex, do not receive a "go" signal for maturation, unless triggered with costimulatory molecules, usually from activated APCs, that provide the additional "signal 2."The innate immune response activates APCs for a more efficient phagocytosis and antigen presentation to T cells, upregulating costimulatory molecules and expressing IL-2 that is essential for T-cell proliferation and differentiation.

A good sample of APCs and lymphocytes reciprocal signals and cross-activation during formation of adaptive immune response is when activated B cells, act as antigen presenting cells, give and receive signal 2 for maturation, to and from naïve T lymphocytes. Cross-bridging of two Ig on the surface of B cell activates B cells and mediates a "receptor-mediated internalization of antigen," which is then presented on the surface as a complex with MHC class II molecule and upregulation of CD80 (B7). The antigen/MHC complex on the B-cell membrane provides signal 1 for the naïve T cell. Later, CD80 binding to CD28 (a constitutive B7 receptor on T cells) provides signal 2, and the activated T cell upregulates CD40L (CD154), a costimulator for B-cell maturation, class switching, and immunoglobulin expression.

4. What does the immunity has to say about?

4.1. Immunology of neoplasia

Footprints from the immune system could be found in many aspects of cancer pathogenesis. Cancer is not only defined now, by the genetic mechanisms that culminate in transformed cells with a senseless tendency to proliferate but also as an everyday challenge of the body with cells that undergo subtle yet malignant intracellular changes and mutations. Two theories of "cancer immune surveillance" and "cancer immune editing" focused on the crucial role of immunity in cancer [2]. The identification of tumor's ability to selectively and efficiently suppress components of the immune system in favor of its longevity and invasion put a further spin on this notion [3]. Immune elimination of cancer releases tumor-specific antigens and danger signals and creates a tumor-edited immunity. Everyone working in the field of cancer immunology is familiar with new entries added to the dictionary of cancer, tumor antigens, tumor infiltrating lymphocytes, and myeloid-derived suppressor cells [4]. Finally, the advent of drugs targeting the cutting-edge knowledge of immune culprits of cancer and "tumor-specific antigens" has brought hope for effective cancer immunotherapy for tumor suppression or even tumor ablation [5], and cancer vaccination has become a trending topic in cancer research [6].

4.2. Immunology of infection

It was the microbes that drew our attention to our immune system and led us to know more about it. Studying immunology has diverged into many branches and disciplines, yet studying the immunity of infection still has galaxies to explore. Microbes are the primary reason for

the evolution of immunity, the phylogenetic of this phenomenon tells us that if it were not for smart eukaryotes, learning how to fight back prokaryotic within a complex, multipotent herd, the human system would have never maturated into its current shape and complexity. The danger theory and the self-/nonself-paradigm that reigned the knowledge of immunology for years was a direct revelation from years of studying host defense to infectious agents [7].

The emergence of HIV/AIDS pandemic has made researchers to turn a clever eye on "immunity in infection" and "infections of immunity." Rays of hope have shed over this situation with endeavors to design and test an ultimate HIV vaccine [8].

4.3. Immunology of organ transplant

Transplant of solid organs was restricted, first due to technical problems of heavy surgeries, infections, and hardships of ligating blood flow to major organs for long. Next, the daring first transplant surgeons became aware of the fact that the first reason of the transplant failure was not complications of the surgery, but rather it was the failure of the transplanted organ that killed the patient. The development of efficient and target specific immunosuppressive therapy and biological agents has turned organ transplant, into a routine therapeutic option for various types of end-stage organ failures. Nowadays, it is a problem of organ supply, as up to one-third of the patients waiting on the list die before a donor can be found. Cadaveric organ transplant, marginal donor transplants are still facing a dilemma to achieve a long-term reduction in mortality [9]. The overall success rate, however, is approximated between 80 and 90%, and it is now the duty of immunological research to open a landscape for alternatives of organ donation, such as xenografts, to overcome the problem of organ shortage [10].

4.4. Autoimmunity

Autoimmunity is a term used to describe the chronic response of the immunity to self-antigens, resulting in tissue damage. Autoimmunity happens in a sequence of distinct phases, from genetic susceptibility, which involves at least one of the factors; impaired tolerance, reduced production, or activation of the regulatory subset of T cells, impaired clearance of antigen: antibody complexes, etc. Next, there is a trigger, an initiation phase that the self-antigens are exposed to the faulty system of antigen recognition, or a full-blown immune response that activates the dormant nonresponsive alive autoreactive lymphocytes. The initiation of the reaction further releases autoantigens and results in progression of disease and establishment of chronic tissue damage. Credentials for these phases have been investigated regarding specific auto-antibodies, markers of initiation, and progression and clinical stages of the disease. The next challenge would be the implementation of this knowledge into a precise diagnosis, appropriate monitoring, and care of patients with autoimmune diseases.

4.5. Immunodeficiencies

Immunodeficiencies could be divided into two groups of inherited (primary) or acquired (secondary) immunodeficiencies, the latter group being a cause of acquired factors such as HIV infection, protein-losing enteropathy, malnutrition, cancers, or immunosuppressive

drugs that impair immune-related functions in a previously healthy immune system. Primary immunodeficiencies are a heterogeneous group of disorders, caused by a gene defect, leading to defect(s) of the immune system. More than 300 different types of primary immunodeficiencies have already been described [11]. Haematopoietic stem cell therapy, immunoglobulin replacement, and the use of antibiotics have extended lives of patients with immunodeficiencies to an almost normal span [12].

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Chapter 2

Physiology and Pathology of Immune Dysregulation: Regulatory T Cells and Anergy

Maria Isabel Torres, Miguel Angel López-Casado, Candelaria Ponce de León, Pedro Lorite and Teresa Palomeque

Additional information is available at the end of the chapter

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Abstract

The immune system is responsible for the defense of the organism. It controls what is introduced into it and identifies it as self from non-self. The defensive mechanisms activated by the immune system are directed against pathological microbes and toxic or allergenic proteins, and it must avoid responses that produce excessive damage of self-tissues, inducing tolerance to avoid autoimmunity and other immunopathologies. Regulatory T cells play an essential role in these active processes, using several distinct suppressive mechanisms. The immune dysregulatory diseases result from defects affecting regulatory T cell development and/or function, including the impact of essential genes mutations for T regulatory cell functions and the associated autoimmune syndromes.

Keywords: anergy, T cell exhaustion, regulatory T cells, IPEX syndrome, tolerance, autoimmunity

1. Introduction

The immune system requires strict control and self-regulation in order for its functioning to be the most efficient possible and adjusted to the defensive needs of each moment, thus inducing an appropriate immune response against pathogens and tumors. Immune tolerance is based on the fact that the immune system has to distinguish between itself and any non-self in order not to destroy its own components, which must be previously recognized as such in the thymus and bone marrow. When tolerance for some reason fails, multiple pathologies appear, as autoimmune diseases. In this chapter, we analyze general aspects of dysfunctional T cell responses such as anergy and T cell exhaustion, some of the phenotypic markers associated

with them, and the importance of these processes in the establishment of tolerance and autoimmunity. Also, we consider the main pathogenic event of regulatory T cell dysfunction leading to multi-organ autoimmunity in the immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome. Clonal anergy, a well-known regulatory mechanism, can be deemed a hyporeactive state arising when the T cell antigen receptor activates T cells despite the lack of suitable co-stimulatory signals. T cells have proved to be important in stimulating and/or maintaining anergy, so the anergic T cells may change their transcriptional and epigenetic programs and turn into regulatory T cells. Anergic T cells appear to represent the intermediate reprogramming stage before becoming regulatory T cells, which maintain self-tolerance. T cell exhaustion is a state phenotypically similar to anergy. When exhausted, T cells neither secrete cytokines nor lyse target cells, and furthermore fail to proliferate. Such chronic stimulation prompts the sustained high expression of co-inhibiting molecules, including TIM-3, 2B4, PD-1, and LAG-3, which act blocking the activation of T cells. Whereas anergy is a programmed transcriptional process induced by minimal signaling, exhaustion occurs at the pathological level by the presence of abundant inflammatory signals maintained over time. Certain conserved mechanisms promote both anergy and depletion of T cells in the immune system. The dysfunction of Treg cells is the main pathogenic event leading to the multi-organ autoimmunity that characterizes the IPEX syndrome, a paradigm of genetically determined primary immunodeficiency due to mutations of *FOXP3*, a key transcription factor for naturally occurring Treg cells, with autoimmunity.

2. Dysfunctional T cell responses

Mechanisms have been developed by the immune system to direct effective responses to a broad gamut of pathogens. Responses of the immune system protect against many lymphocyte antigen receptors that are generated by realignments of somatic genes. Although this process enables hosts to combat pathogens effectively, these organisms quickly evolve to present many challenges, prompting detrimental immune responses to, for example, self-tissue antigens and non-harmful components including food antigens or non-pathogenic agents of the intestinal tract [1]. Various states of T cell dysfunction have been described as a consequence of altered activation and differentiation processes. Terms such as exhaustion, tolerance, anergy, senescence, and even ignorance have been used to describe the dysfunctional state of T cells, depending on the clinical settings and the phenotypic and functional features of the T cells.

Autoimmunity, one of the most serious problems of the immune system, causes many diseases that are difficult to cure. One cause of autoimmunity is self-reactive T cells that start to attack the body of the host in the periphery [2, 3], although most self-reactive immature T cells are eliminated by negative selection in the thymus [4]. Multiple mechanisms are at work to prevent autoimmunity, including regulatory T cells [5], T cell ignorance [6], and T cell anergy [7]. The development the other pathologies such as chronic infections and cancer is facilitated by a variety of immune-subversion mechanisms, with the production of anti-inflammatory cytokines, induction of regulatory T (Treg) cells, and expression of immune checkpoint molecules.

3. Regulatory T cells

Regulatory cells (Tregs) play a critical role in the establishment and maintenance of immune homeostasis as well as in the limitation of chronic inflammatory responses directed against pathogens and environmental factors [8–10]. This cell-mediated suppression is considered a vital mechanism of negative regulation of immunomediated inflammation, and plays a prominent role in autoimmunity and auto-inflammatory disorders, allergies, acute and chronic infections, cancer, and metabolic inflammation; these are important candidates for the therapeutic treatment in these inflammatory and autoimmune diseases.

Treg cells represent 5–10% of peripheral CD4+ T cell compartment in humans. In this section, we present the characteristics that define regulatory T cells, the phenotypic and functional heterogeneity that they present, with particular reference to the consequences of T cell dysfunction in contributing to the development of autoimmunity and deregulation of the immune system [11].

3.1. Treg phenotypes

Treg cells represent highly differentiated populations in that they are distinguished phenotypes based on the expression of specific markers and mechanism of action. Different Treg subsets have been identified, but two major types expressing Foxp3+ transcription factor can be distinguished based on their origin: (i) natural or Treg cell thymus-derived (nTreg or tTreg) and (ii) induced Tregs that develop in the periphery from naïve conventional CD4+ T cells (iTregs or pTregs) [12, 13]. The nTregs are the major mediators of central immune tolerance, whereas iTregs are involved in the regulation of peripheral immune tolerance in sites of inflammation [14].

The phenotype as well as function of nTregs, as opposed to iTregs, have been difficult to study in humans, given the shortage of markers used for discriminating these cell types. It has recently been argued that the expression of Helios, which is a transcription factor of the Ikaros family, can discriminate nTregs from iTregs on the basis of most thymically derived FOXP3+ cells expressed by Helios [15]. Nevertheless, the Helios used as a marker for nTregs has been disputed because, depending on the cell-activation conditions, Helios is also expressed in conventional T cells (T conv) of humans [16]. Helios cannot be used as an nTreg/iTreg discrimination marker but may serve as a useful activation/differentiation marker for Tregs. In this sense, the subset of nTreg cells could be subdivided on the basis of Helios expression, representing a stable and suppressive Treg population that differs only in cytokine/chemokine production [17].

Other Treg cells are found in the periphery, such as Tr1 cells, which lack the expression of the transcription factor FOXP3 [18] with immunosuppressive functions as IL-10 and TGF-β secretion [19], and Th3 cells with a variable level of FOXP3 expression [20]. CD8+CD25+ Treg cells are also developed in the thymus, expressing several molecules characteristic of nTregs, namely CD25, FOXP3, CTLA-4, and TNF-receptor. CD8+CD28+ Tregs inhibit priming of CD8 + and CD4+ T cells, and antibody-mediated against oral antigens. CD8+CD28-Tregs can be induced from naïve CD8+ T cells upon activation by allogenic antigen presentation cells (APCs) in the presence of IL-2 and granulocyte-macrophage colony-stimulating factor (GM-CSF). The γδ T cells are commonly of the CD8 + FOXP3-phenotype and are found mainly in the

intestinal epithelium associated with mucosal tolerance. These cells can also regulate autoimmunity and tumor immunity by producing IL-10 and TGF-β, similarly to Tr1 cells [21].

3.2. Treg functions

Treg cells have been considered key players in dominant immune tolerance [22]. Treg cells have performed functions such as to suppress inflammatory responses in mucosal interfaces that are constantly exposed to allergens [23], commensal gut microbiota [24, 25], transplanted organs [26], pathogenic infections [24], and tumors [27]. Recent studies have suggested a role for Tregs in other situations, such as adipose tissue resident Tregs controlling metabolic disorders [28, 29] and Tregs limiting organ rejection [30]. In certain cases, the suppressive function of Tregs limits beneficial effector responses of the host against tumors and chronic infections [31, 32]. Hence, the activities of this suppressive population need to be controlled by allowing the balance between restricting deleterious inflammatory and autoimmune insults, while facilitating protective responses against infections and tumors.

While FOXP3 is an indispensable transcription factor to define the majority of the Treg transcriptional and functional subsets, FOXP3+ Treg cells express on the cell surface high levels of interleukin-2 receptor α (CD25) and a low level of IL-7 receptor α (CD127) [33]. Thus, the majority of Treg cells constitutively express high levels of the inhibitory molecule cytotoxic T lymphocyte-associated antigen 4 (CTLA4) and the glucocorticoid-induced TNFR family related (GITR), as well as the regulatory cytokines IL-10 and TGF-β [34–36].

According to a number of studies, not all FOXP3+ T cells are functional Tregs, and it is possible to induce a portion of the Treg signature without the presence of FOXP3 [37, 38] since activated human T cells express Foxp3 transiently without acquiring suppressor capacity [39, 40] The essential aspect of the Treg cell (FOXP3 expression and suppressive capability) can be maintained in differing Treg sub-populations identified in various anatomical locations as well as under pathological conditions [41–43]. Their characteristics allow phenotypic/functional adaptation to block full immune responses. Within the FOXP3+ Treg subsets, the diversity can be characterized by: (i) differential transcription-factor expression [44–47]; (ii) different expression of chemokine receptors [41, 42, 47], and (iii) differing expression of suppressor markers that control various types of target cell in diverse environmental and pathological conditions [48–50].

Treg cells, on losing FOXP3 expression as well as their suppressive capability, form an unstable population, taking on characteristics similar to those of the effector T cell reacting to environmental cues [51–53]. Though convincing evidence is available for Treg cell stability under healthy immune conditions [54, 55], numerous studies propose that inflammatory conditions may be related to downregulation/loss of FOXP3, secretions of effector cytokines, and also the proliferation of the so-called "ex-Treg" cells [13, 56]. This implies that Treg cells may be reprogrammable as inflammatory cells in reaction to microenvironmental signals. Treg cells show no terminal differentiation, though they do retain plasticity and can differentiate into specialized hybrids to control immune responses [57]. Thus, for Treg function, two models have been proposed: one in which Treg-specific expression of FOXP3 would encode the expression of Treg suppressor characteristics (greater CD25 and CTLA-4), whereas their ability to adapt to the shifting environmental cues would induce further suppressive modules (e.g. miRNAs, suppressive pathways, transcription factors, and chemokine receptors) for suitable immune regulation [58].

Another key question is the role of Treg cells in preventing autoimmunity and their therapeutic potential based on Treg cell transfer or activation leading to the definition of the signals responsible for generating and maintaining of Treg cells [59]. Several studies have focused on two sets of signals—interleukin-2 (IL-2) and antigen itself [60, 61]. Thus, IL-2 is required for the survival of Treg and for maintaining their functional activity by promoting expression of FOXP3 and mediators of suppression, particularly CTLA-4 [62]. Answers to environmental antigens may provide enough IL-2 to maintain a Treg cell repertoire in healthy individuals. The dependence of Treg cells based on IL-2 received from conventional T cells provides a negative feedback through which the ratio of Treg cells and conventional T cells is controlled [63].

3.3. Regulatory T cells and tolerance

Oral tolerance to foods is an active immunological process that involves allergen-specific Treg cells [64–66]. Genetic and immunological evidence supports an important role for Treg cells in enforcing oral tolerance to foods [67–69]. This tolerance depends on iTreg-cell development from naïve conventional CD4+ T cells (CD4+ Tconv), which are activated in presence of TGF-β1 and CD103+ dendritic cells (DCs) [70–72] regulating T helper 2 cell responses at the mucosal surfaces [73, 74]. In food allergy, a deficient formation and impaired function of allergen-specific Treg cells is present.

Treg cells in the intestine are important in bringing about a tolerogenic environment for maintaining immune homeostasis in commensal bacteria [75, 76]. The question of commensal bacteria-inducing Treg and effector cells is basic in explaining the way in which the immune system receives instructions from particular species of bacteria and in determining the dynamics of Treg versus effector-cell selection of bacterial antigens [77–79]. T cell differentiation may be guided by innate stimulators of commensal bacteria as TLRS selectively activate cytokine production from APC subsets, TLRS being major sensors capable of recognizing conserved molecular motifs in bacteria [80, 81]. However, the adaptive immune system may react to pathogenic rather than commensal bacteria, so that the pre-existing effector and Treg cell reactions to commensal bacteria may alter the course of the infection. In addition, infection may upset the balance between effector versus Treg cell reactions to commensal bacteria, disturbing immune homeostasis as well as potential immunopathology [75, 80].

A dynamically regulated Treg cell population would be in tune with the commensal microbiota and thus would be more responsive when confronted with a strong influx of commensal antigens after mucosal injury, limiting the activation of effector T cell, and controlling excessive inflammation [75–77]. By contrast, bacteria new to the digestive system would not trigger Treg or effector T cells already present, but rather would need a new selection of effector versus regulatory T cell reactions. This situation would enable quicker effector responses to microbes, limiting the generation of effector T cells meeting commensal bacteria, and this could prompt inflammatory bowel disease (IBD) development [82].

Commensal bacteria are major initiators of effector T cell reactions that lead to inflammation. The immune system responds to commensal antigens as non-self, not only because bacterial antigens are unlikely to be present during the selection of thymic T cells, but also because bacteria bear a number of ligands used in recognizing immune receptors [83]. It is widely accepted that commensal bacteria also induce T cells that decrease inflammation in order to

sustain intestinal tolerance. Thus, Treg cells play a vital part in maintaining homeostasis of the gut immune system and in deterring effector cells from triggering immunopathology as a response against commensal bacteria.

Several studies have identified microbial products from a specific bacterial species that affects Treg cell function. Polysaccharide A (PSA) from *Bacteroides fragilis* was found to activate TLR2 expressed on Treg cells, inducing the production of IL-10 [84], facilitating the persistence of *B. fragilis*. Many studies have reported a possible "universal" mechanism driving Treg cell expansion that is mediated by bacterially derived short-chain fatty acids (SCFAs) produced through the metabolism of dietary fiber [85–87]. The microbial products are perceived by the intestinal immune system to facilitate homeostasis and tolerance instead of inflammation, consistent with the notion of an evolutionary mutualistic relationship between commensal bacteria and the host [84, 88]. In this sense, colonic Treg cells utilize a unique set of T cell receptors (TCRs), suggesting that they recognize antigens found only in this tissue including colon-specific self-antigens and antigens derived from commensal bacteria [89].

Alterations in the composition of commensal bacterial populations are linked to multiple metabolic and inflammatory diseases including, but not limited to, inflammatory bowel disease (IBD), obesity, type 2 diabetes, atherosclerosis, allergy, and colon cancer [90–92]. Recent studies have identified a critical role for commensal bacteria and their products in regulating the development, homeostasis, and function of innate and adaptive immune cells [93–95]. However, an emerging and interesting area that has received relatively little attention is how metabolites and nutrients derived from commensal bacteria regulate the host immune system.

4. Immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome

As discussed above, Treg cells play a key role in immune homeostasis by maintaining a balanced adaptive immune response. The spectrum of manifestations due to Treg cell defect might range from mild allergy or autoimmunity to lethal immune dysregulation disorders (IPEX) [96]. Several human genetic disorders have recently been described and noted to have an extraordinary impact on Treg cell development and functional activity [97]. A loss of function mutation in FOXP3, the key transcriptional factor for Treg cell differentiation, leads to an IPEX phenotype. Subsequently, a number of other gene defects have been reported to cause IPEX-related phenotypes, including the loss of function mutations in theCD25, STAT5B, LRBA, and CTLA4 gene [98].

IPEX, a rare genetic disorder, results from a dearth of functional Treg cells caused by losses of function mutations in FOXP3. It affects only males because of its X-linked recessive inheritance. Also, it is frequently fatal in the early years of life if the patient receives no bone marrow transplant [99]. In clinical terms, IPEX presents three maladies: autoimmune enteropathy, autoimmune endocrinopathy, and eczematous dermatitis. The most frequent manifestation, enteropathy, gives way to endocrinopathy particularly insulin-dependent type 1 diabetes mellitus [99]. Other manifestations include lung disease, immune-mediated cytopenia,

autoimmune nephropathy, anemia and/or thrombocytopenia, and hepatitis. Furthermore, food allergies with high serum IgE and peripheral eosinophilia prove very common, indicating a clear failure of oral tolerance in this disorder. Usually, IPEX patients show a broad range of autoantibodies because of adaptive immune dysregulation. With over 60 FOXP3 mutations reported up to now, observations from the clinical phenotype reported for these mutations have led to postulations of genotype/phenotype relationships [100].

CD25 deficiency properties shared with IPEX include chronic eczema, enteropathy, lymphoproliferation, and autoimmunity disorders such as alopecia, diabetes mellitus, thyroiditis, and autoimmune hemolytic anemia [101–103]. CD25 deficiency is permissive to Treg cell differentiation, with normal count of FOXP3+ Treg cells found in circulation [104]. The loss of CD25 expression impairs Treg cell suppressive function by defective production of suppressive cytokine IL10. Their failure deprives Tconv cells of IL-2 production, leading to their apoptosis [62, 101]. Finally, the decreased sensitivity of CD25-deficient Treg cells to IL-2 impairs their metabolic competence in the context of an immune response [105, 106].

Evidence from studies on human and murine models show that Type-1 regulatory T (Tr1) cells can contribute to suppressing the development of autoimmunity in addition to nTreg cells [106, 107]. Tr1 cells can develop in IPEX patients regardless of FOXP3 expression [108]. This observation suggests that FOXP3-independent immune regulation can potentially help control the disease, although Tr1 cells alone do not seem adequate to suppress the initial acute phase of the disease.

5. T cell exhaustion

T cell exhaustion is distinguishable from other dysfunctions such senescence or anergy, based on molecular mechanisms [109, 110]. That is, exhausted T cells come from cells that initially developed an effector function but then gradually lose it because of continuous stimulation of the T cell receptor (TCR) from the persistent antigen helping to build peripheral tolerance as well as to modulate immune responses [111, 112]. As such, exhausted T cells present in patients having autoimmune disorders correlate with positive prognoses [113]. However, in cancers, exhausted T cells may block tumor clearance, thereby contributing to immune escape [114, 115]. This also leads to chronic infections, and viral immune evasion results from the persistence of activated T cells that have no effector function [116].

Regarding the origin of exhausted T cells, recent work has shown that exhausted CD4+ and CD8+ T cells bear a notably different transcriptional profile from that of effector and memory CD4+ or CD8+ T cells. These differences include shifts in the expression of co-stimulatory and inhibitory receptors (IRs), as well as signaling molecules, transcription factors, chemokines receptors, cytokines, and genes that are involved in metabolism. Also, genomic research supports the contention that exhausted T cells constitute a unique stage of T cell differentiation [110, 117].

With respect to the causes behind T cell exhaustion, CD8+ T cell exhaustion likely involves altered inflammatory and tissue microenvironments as well as other populations of

lymphocytes such as CD4+ T cells, regulatory T cells, B cells, and inhibitory cues from cytokines and inhibitory as well as co-stimulatory cell-surface receptors [110]. The major feature appears to be a chronic and presumably continual antigen exposure instead of acutely terminated or intermittent exposure. Also, the severity of the exhaustion and the deletion of antigen-specific T cells have been found to correlate with (i) the expression of stimulatory and inhibitory receptors; (ii) the levels of stimulatory and suppressive cytokines; and (iii) the degree of antigen stimulation [118, 119].

The gradual dysfunction of exhausted T cells is accompanied by the expression of multiple inhibitory receptors, by progressive loss of IL-2 production and TNF-α and IFN-γ depletion [112], as well as by altered cell metabolism with a markedly different transcriptional profile [120, 121]. T cells do not exhaust uniformly during chronic diseases or cancer, but instead specific subsets with different memory and proliferative potentials emerge after exposure to persistent antigen [122, 123].

While exhaustion was first viewed as a dysfunctional T cell state, this phenotype is now considered an appropriate response to chronic infection, because a persistent effector function could cause excessive damage to healthy cells. T cell exhaustion prevents optimal control of infection and tumors, modulating pathways overexpressed in exhaustion that can reverse this functional state and reinvigorate immune responses [124] by targeting programmed cell-death protein 1 (PD1) and cytotoxic T lymphocyte antigen 4 (CTLA4) [125, 126]. Exhausted T cells are not inert, given that they retain crucial functions at the suboptimal level that limits ongoing pathogen replication or tumor progression. These cells are not effective at eradicating pathogens or tumors, and have been considered of interest in avoiding or reversing exhaustion.

Inhibitory receptors (IRs) are negative regulatory pathways that control autoreactivity and immunopathology and are transiently expressed in functional effector T cells during activation. A higher and sustained expression of inhibitory receptors is a hallmark of exhausted T cells. The molecular mechanisms by which inhibitory receptors control T cell exhaustion are not entirely known. Although PD1 is the best characterized inhibitory receptor, exhausted T cells express a range of other cell-surface inhibitory molecules to impair T cell responses during chronic infections, such as CTLA4, LAG3, 2B4, TIM3, CD160, and many others [127]. The co-expression of multiple inhibitory receptors is a chief feature because the simultaneous blockage of IRs results in synergistic reversal of T cell exhaustion. Results of several clinical trials using immune checkpoint inhibitors are very encouraging. Blocking antibodies for CTLA-4, PD1, and PDL1 appear to have a strong therapeutic potential given alone or in combination with standard treatment in many tumors.

In addition, the soluble molecules regulate T cell exhaustion. These include immunosuppressive cytokines such as IL-10, TGF-β, and inflammatory cytokines such as IFNs type I and IL-6 [110]. Blockage of IL-10 restores T cell function and improves viral control during chronic viral infections, demonstrating that IL-10 promotes T cell exhaustion [128, 129]. Many cell types can be the source of IL-10 during chronic infection, including dendritic cells (DCs), monocytes, and CD4+ T cells [130, 131]. The blocking of IL-10 and the PD1 pathway in a simultaneous manner, synergistically reverses CD8+ T cell exhaustion and enhances viral control, indicating a role for IL-10 in controlling CD8+ T cell exhaustion [132]. Depletion of CD4+ T cells help during pathogen persistence and can contribute to defective CD8+ T cell responses. Therefore, in HIV

infection, the loss of the CD4+ T cell response can result in exhausted CD8+ T cells and disease progression [133].

6. T cell anergy

Immunological tolerance is the essential mechanism for maintaining immune homeostasis. T cell anergy, one of the major mechanisms involved in immunological tolerance [134–136], is a hyporesponsive state of T cells under antigen stimulation. The expression of several anergy-specific genes are known to change in anergic T cells, such as DGK-a, an intracellular signaling molecule (also known as an anergy-related gene) and EGR2, a transcription factor, and this reportedly increases in anergic T cells [137, 138]. However, the degree of contribution and relevance of each anergic gene and the mechanism of this gene regulation are not understood. It is known that the increased expression of anergic genes is maintained over the long term. However, it seems unlikely that every gene associated with anergy induction would be epigenetically regulated, because there are too many genes with an altered expression level in anergic T cells to be independently regulated [139, 140].

Effective mechanisms of peripheral tolerance are required to eliminate circulating autoreactive T cells and thereby prevent undesired immune responses against self-antigens. The key players in this process are DCs, which induce tolerance by different control mechanisms such as T cell deletion, the generation of Tregs, and/or the induction of anergy [141, 142]. Interaction between DCs and T cells occurs through three independent signals: (i) recognition of peptide-MHC complexes presented on DCs via specific TCR on T lymphocytes, (ii) binding of co-stimulatory molecules expressed on DCs to their respective receptors on T cells, and (iii) polarizing cytokines secreted by DCs [143]. When antigen peptides are presented by DCs in the absence of co-stimulation, T cells become anergic [144].

The induction of T cell anergy occurs when negative signals acquire more weight than the activatory signals from APCs. Anergy was originally defined as an unresponsive state provoked in T cells recognizing an antigen without co-stimulatory signals [145], normally when CD28 on T cells binds to its ligands, that is, B7 molecules, and expresses on DCs [146]. As a result, T cell proliferation and cytokine production are impaired when the same antigen is encountered again. Anergy also results from coinhibitory signals by PD-1 or CTLA-4 receptors [147, 148]. The latter interacts with B7 molecules, with preference toward CD80, whereas PD-1 binds to PD-L2 and/or PD-L1 ligands on DCs. Furthermore, adenosine from tissue, acting by the adenosine A2A receptor (A2AR), acts as another key negative regulator for the activation of T cells, having the ability to drive long-term anergy, even with co-stimulation [149]. Therapies known as the "checkpoint blockade" treat cancer patients using blocking antibodies against those receptors. This approach is clinically quite promising given that blocking antibodies can alleviate hyporesponsiveness and encourage the rejection of tumors. Unraveling this process is the focus in designing therapies to counteract autoreactive T cells involved in autoimmune diseases [150].

Treg cells, important for inducing and/or maintaining anergy and anergic T cells, can in turn alter their epigenetic and transcriptional programs to become Treg cells [151]. Anergic T cells may represent the intermediate reprogramming stage before they themselves become surveying Treg

cells that maintain self-tolerance. T cell anergy and Treg induction are crucial mechanisms for re-establishing tolerance [152], and although presenting different phenotypic and functional characteristics, both mechanisms have in common the expression regulation of some genes, such as *PD-1*, *ICOS*, *LAG3*, *CTLA-4*, *EGR2* [151], *GRAIL* [152, 153], *CBL-B*, and *ITCH* [154, 155].

The suppression of antigen-specific T cell responses either through the expansion of Tregs or the induction of anergy represents an attractive immunotherapeutic approach to target autoreactive T cells in autoimmune diseases [156]. The generation of Tregs has been of interest, but Tregs can exert unspecific regulation and may be prone to conversion into proinflammatory Th17 cells [157]. By contrast, the induction of a stable hyporesponsive state appears to be a promising strategy to specifically silence self-reactive T cells in autoimmune diseases without undesired adverse effects. *In vivo* anergy induction in autoreactive $CD4^+$ T cells has been demonstrated to control disease onset and progression in murine models of autoimmune diseases [158]. The possibility that anergic T cells can also acquire suppressive capacities supports their fundamental role in the control of immune responses. Thus, T cell anergy is an effective mechanism to eradicate aberrant T cell responses to "self" and for the reestablishment of self-tolerance in patients with autoimmune diseases [159, 160].

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Chapter 3

Physiology and Pathology of Cytokine: Commercial Production and Medical Use

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Additional information is available at the end of the chapter

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Abstract

Cytokines are small, short-lived proteins secreted by many different cell types. As signaling molecules, cytokines provide communication between cells and play a crucial role in modulating innate and adaptive immune response. The family of cytokines includes interferons, interleukins, chemokines, mesenchymal growth factors, tumor necrosis factor family and adipokines. Interferons (IFNs) are a multigene family of inducible cytokines with antiviral, antiproliferative, and immunomodulatory function. Recombinant DNA technology can be useful in the production of human IFNs. This process includes fermentation, purification, and formation of the final product. Interleukins are classified in families based on sequence homology, receptor-binding properties, biological function, and cellular sources. TNF and IL-1 are considered to be key mediators of inflammatory response, while IL-6 plays a key role in the transition from acute to chronic inflammation. The inhibition of TNF includes administration of anti-TNF antibody and TNF receptor (TNFR). The reduction of IL-1 level can be achieved by the administration of anti-IL-1 antibody or IL-1 receptor antagonist (IL-1Ra), and the reduction of IL-6 level in the treatment of chronic inflammatory diseases can be achieved by the administration of anti-IL-6 antibody and anti-IL-6 receptor antibody. Recombinant cytokines and cytokine antagonists (antibodies and receptors) can be used in treating many different diseases.

Keywords: cytokines, interleukins, interferons, TNF

1. Introduction

Cytokines are low molecular weight proteins or glycoproteins secreted by a number of cell types. The term cytokine is made up of two parts: *cyto* (cell) and *kine* (movement) [1]. As

signaling molecules, cytokines provide communication between cells and play a crucial role in modulating of the innate and adaptive immune response (**Table 1**).

Nomenclature of cytokines was created either according to the type of cells which secrete them (in this case named interleukins and adipokines) or biological activity (in this case

Family	Members	Functions
IL-1 family		
IL-1 subfamily	**Agonist activity**	
	IL-1α (IL-1F1)	Induction of proinflammatory response
		Th17 cell differentiation
	IL-1β (IL-1F2)	Induction of proinflammatory response
		Th17 cell differentiation
	IL-33(IL-1F11)	Acts as an alarmin
		Induction of Th2 response
		Activation of ILC2 cells
		Th1 cell differentiation dependent on IL-12
		Induction of Treg cells
	Receptor antagonists	
	IL-1Ra (IL-1F3)	Antagonism of IL-1
IL-18 subfamily	**Agonist activity**	
	IL-18(IL-1F4)	Induction of IFN-γ in presence of IL-12
		Enhances NK cell cytotoxicity
		Promoting Th1 or Th2 cell responses depending
		cytokine milieu
		Activation and cytokines release from neutrophils
	Antiinflammatory activity	
	IL-37 (IL-1F7)	Suppression the production of proinflamatory cytokines
		Inhibition of dendritic cells (DCs) function (foremost through stimulation of TGF-β)
IL-36 subfamily	**Agonist activity**	
	IL-36α(IL-1F6)	Promoting Th1 and Th17 cell responses (skin, lungs, kidneys)
	IL-36β (IL-1F8)	Promoting Th1 and Th17 cell responses (joints)
	IL-36γ(IL-1F9)	Promoting Th1 and Th17 cell responses (lungs)
	Receptor antagonists	
	IL-36Ra(IL-1F5)	Antagonism of IL-36-α, IL-36-β and IL-36γ
		Antiinflammatory brain action via induction of IL-4 expression in glia cells

Family	Members	Functions
	IL-38(IL-1F10)	Antagonism of IL-36-α, IL-36-β and IL-36γ
		Inhibits the production of IL-17 and IL-22
		Inhibits the production of IL-8 induced by IL-36γ
Common γ chain cytokine family		
	IL-2	Proliferation and differentiation into effector and memory T cells
		Development of Treg cells
		Proliferation of B cells
		Proliferation and differentiation of NK cells
	IL-4	Th2 cell differentiation
		IgE class switching
		Antagonise the effects of IFN-γ
		Alternative activation of macrophages (M2 phenotype)
		Upregulation of class II MHC molecules expression on B cells and monocytes
		Upregulation of FcεRII (CD23) and IL-4R
		Survival factor for B and T cells
		Role in tissue adhesion and inflammation
	IL-7	Proliferation of early T and B cell progenitors
		Naive and memory T cell survival
		Development of γδT cells (VDJ recombination of TCRγ)
		Induction of CTLs and LAK cells
	IL-9	$CD4^+$ T cells and mast cells growth factor
		Proliferation of $CD8^+$ T cells and mast cells
		Inhibition of Th1 cytokines
		Promotes Th17 cell differentiation
		IgE production
		Chemokine and mucus production in bronchial epithelial cells
	IL-15	Induction of Th1 and Th17 responses
		Activation of T cell (decreased TCR activation threshold)
		Survival and proliferation of memory $CD8^+$ T cells
		Loss of Treg cells- and TGF-β immunoregulation
		Suppression of IL-2 induced AICD of T cells
		Differentiation of γδT cells
		Proliferation and activation of NK cells
		Homeostasis of NK and NKT cells
		Induction of LAK cells

Family	Members	Functions
	IL-21	Regulation of B cell proliferation, differentiation and apoptosis
		Antibody isotype balance (increased IgG and decreased IgE)
		Generation of long-lived plasma cells and T cell-dependent antigen responses and memory
		T cell and NK cell proliferation
		Increases cytotoxic activity of NK cells and CTL cells
		Induces the differentiation of T follicular helper (T_{FH}) cells
		Expansion of Th17 cells
		Inhibits DC activation and maturation
IL-6 family		
	IL-6	Synthesis of acute phase proteins in liver
		Inducing secretion of chemokines: CCL2, CCL8, CXCL5, CXCL6
		Induction of neutrophil apoptosis
		Switching from neutrophil to monocyte recruitment
		Transition from innate to acquired immunity
		Direct mediator of T cell migration
		T cell differentiation, activation and survival
		B cell differentiation and production of IgG, IgM, IgA
		Survival of hematopoietic stem cells and early progenitors
		Proliferation and differentiation of myeloid, erythroid, megakaryocyte progenitors
		Survival factor for neuronal cells
	IL-11	Synthesis of acute phase proteins in liver
		Growth factor for myeloid, erythroid, megakaryocyte progenitors
		Bone remodeling
		Protects epithelial cells and connective tissue
		Inhibition of macrophage activity
		Inhibition of adipogenesis
		Promotion of neuronal development
	IL-31	Induction of IL-1β, IL-6, IL-8, GRO-α (CXCL1), MCP-1 (CCL2) and DC-CK1 (CCL18) production in eosinophils
		Increased chemokines mRNA expression (GRO-α (CXCL1), TARC (CCL17), MIP-3β (CCL19), MDC (CCL22), MIP-3 (CCL23), MIP-1β (CCL4)) in keratinocytes
		Antiapoptotic effect on eosinophils
		Expression of growth factors and chemokines in epithelial cells
		Inhibition of proliferation and apoptosis in epithelial cells

Family	Members	Functions
	Leukemia inhibitory factor (LIF)	Self-renewal and block in differentiation embryonic stem cell
		Embryonic implantation (receptive state of endometrial, the interaction between endometrial and embryo, stromal decidualization, the invasion of blastocyst, blastocyst development, infiltration of uterine leukocytes, synthesis of prostaglandins)
		Anti-inflammatory effect
		Neuronal development
	Oncostatin M (OSM)	Maintenance of erythroid and megakaryocyte progenitor pools in BM by regulation hematopoietic cytokine production in stromal cells and direct effect on erythrocytic and megakaryocytic progenitors
		Tumor suppression
		Neuronal development
IL-10 family		
	IL-10	Immune suppression
		Inhibition of expression IL-12, costimulators , and class II MHC molecules on macrophages and DCs
		Inhibition of proliferation $CD4^+$ T cells and production of IL-2, IFN-γ, IL-4, IL-5 and TNF-α
		Enhancing Treg cells function (suppressing autoreactive T cells)
		Reduces immune suppression of autoreactive B cells
		and enhances antibody production
IL-20 subfamily	IL-19	Enhances the production of Th2 cytokines
		Induces IL-6, IL-8 and IL-10 production in monocytes
		Alternative activation of macrophages (M2 phenotype)
		Induction of angiogenesis
		Role in skin inflammation (psoriasis)
		Production of antimicrobial peptides (S100A7 (also known as psoriasin), S100A8, S100A9, and β-defensins) and barrier function increase
	IL-20	Role in skin inflammation (psoriasis)
		Development of hematopoietic cells
		Production of antimicrobial peptides (S100A7 (also known as psoriasin), S100A8, S100A9, and β-defensins) and barrier function increase
		Inhibition of neutrophil phagocytosis, granule exocytosis,and migration

Family	Members	Functions
	IL-22	Controlling the intestinal microbiota
		Production of antimicrobial peptides (S100A7 (also known as psoriasin), S100A8, S100A9, and β-defensins) and barrier function increase
		Wound healing
		Tissue regeneration (intestine, liver, thymus, pancreas and kidneys)
		Role in skin inflammation (psoriasis)
	IL-24	Tumor suppression (loss of proliferative capacity)
		Production of antimicrobial peptides (S100A7 (also known as psoriasin), S100A8, S100A9, and β-defensins) and barrier function increase
		Role in skin inflammation (psoriasis)
	IL-26	Production of proinflammatory cytokines (IL-1β, IL-6, TNF and CCL20)
		Th17 cell differentiation
		Direct killing of bacteria
See Type III interferons	IL-28A (IFN-λ2)	
	IL-28B (IFN-λ3)	
	IL-29 (IFN-λ1)	
IL-12 family		
	IL-12	Th1 cell differentiation
		Increases cytotoxic activity of NK cells and $CD8^+$ T cells and production of IFN-γ
		Antiangiogenic effect
	IL-23	Th17 cell expansion, maintaining activation and secretion of IL-17A, IL-17F, IL-22 and GM-CSF
		Dependent pathogenicity of Th17 cells
		Stimulation of macrophages to produce TNF, IL-1
	IL-27	Pro- and anti-inflammatory effects (induction of Th1 response and suppressive effect on $CD4^+$ T cell production of IL-2, inhibition of Th2, Th17 and iTreg cells)
		Limits the intensity and duration of innate and adaptive immune responses
	IL-35	Induction of Treg cells proliferation
		Inhibition of Th17 response

Family	Members	Functions
IL-17 family		
	IL-17A	Induction of proinflamatory cytokines, chemokines, and
	IL-17F	metalloproteases (controlling bacterial and fungal infection)
		Modulation of viral infection
		Recruitment of neutrophils
	IL-17B	Induction of proinflamatory cytokines, chemokines, and
	IL-17C	
	IL-17D	metalloproteases
	IL-25 (IL17E)	Induction of Th2 response
Th2 like cytokines		
	IL-5	Differentiation and function of myeloid cells
		Increment of chemotactic activity and adhesion capacity on eosinophils
		Remodeling and wound healing
	IL-13	Switching to IgE
		Antagonises the effects of IFN-γ
		Upregulation of FcεRII (CD23) and class II MHC molecules expression on B cells and monocytes
		Alternative activation of macrophages (M2 phenotype)
		Activation of eosinophils and mast cells
		Recruitment and survival of eosinophils
		Defense against parasite infections (mucus production)
Chemokine activities		
	IL-8	Chemoattractant for neutrophils, NK cells, T cells, basophils, eosinophils
		Induces phagocytosis
		Mobilisation of hematopoietic stem cells
		Angiogenesis
	IL-16	Chemoattractant for cells with CD4 molecule
		Modulation of T cell response
Non-classified		
	IL-3	Induces maturation of all hematopoietic lineages
		Activation of basophils
		Activation and survival of eosinophils
	IL-14	Induces growth and proliferation of B cells and inhibits antibody secretion

Family	Members	Functions
	IL-32	Induction of TNF-α, IL-6 and IL-8
		Induction of apoptosis
	IL-34	Differentiation and viability of monocytes and macrophages
Type I interferons		
	IFN-α	Antiviral state
	(13 subtypes)	Increases the expression of class I MHC molecules
		Activation of NK cells
	IFN-β	Antiviral state
		Increases the expression of class I MHC molecules
		Activation of NK cells
	IFN-κ	Antiviral response
	IFN-ω	
	IFN-ε	
	IFN-δ (pigs)	
	IFN-ζ	
	IFN-τ (ruminant)	
	IFN-v	
Type II interferons		
	IFN-γ	Th1 cell differentiation
		Classical activation of macrophages (increased microbicidal functions)
		Promotes cytotoxic activity
		Isotype switching to opsonisation and complement-fixing IgG subclasses (established in mice)
		Upregulation of class I and class II MHC molecules
		Increases antigen processing and presentation to T cells
		Antiviral properties
		Inhibition of cell growth
		Proapoptotic effects
Type III interferons		
	IL-28A (IFN-λ2)	Antiviral response
	IL-28B (IFN-λ3)	Promotes cytotoxic activity
	IL-29 (IFN-λ1)	Antiviral response

Family	Members	Functions
	IFN-λ4	Antiviral response
		Impairs HCV antiviral program or clearance by impeding receptor binding of the other members of the IFN-λ family
TNF superfamily	TNF	Induction of inflammation (vasodilatation, edema, facilitation of the adhesion of leukocytes, production of reactive oxygen species)
		Induction of intravascular thrombosis
		Fever occurrence
		Induces secretion of IL-6 from leukocytes
		Loss of appetite, wasting of muscle and fat cells (cachexia)
		Induction of insulin resistance
		Inhibits myocardial contractility and vascular smooth muscle tone (low blood pressure)
		Stimulates capillary leak

Table 1. Characteristics of cytokines.

named interferons, chemokines, mesenchymal growth factors and tumor necrosis factor family). This nomenclature is still used [2]. There are several basic common properties of cytokines which are important in understanding their effect in the human body:

1. Synthesis of cytokine is mainly induced by various stimuli which act on cells. Also they can exist in preformed granules which are constitutively produced and secreted from cells.

2. Cytokines achieve their effects by binding with high affinity to specific membrane receptors on cells. Therefore, cells show a relatively small number of specific cytokine receptors (100–1000 per cell). In other words, very low concentrations of cytokines can trigger biological effects in cells. Cellular response to the effects of cytokines is well regulated and it is reflected in the changes of gene expression in target cells resulting in the expression of new functions.

3. Cytokines exert effects on different types of cells (the same cells express a variety of cytokine receptors), or one cytokine can exert many different biological effects. This cytokine action is called **pleiotropy**. Also, several cytokines share the same functional effects, and various cytokines can have the same or similar biological activity (various cytokines activate the same signaling pathways) which is called **redundancy** [3].

4. Cytokines affect the synthesis and the activity of other cytokines, acting antagonistically, additively or synergistically.

5. Cytokine activity can be autocrine (on the very cell that secretes it), paracrine (on surrounding cells) and endocrine (in distant cites from the production). Basic characteristics of cytokines suggest that their implementation achieves complex effects which are often accompanied by numerous side effects.

2. Interferons

Interferons (IFNs) consist of multigene family of inducible cytokines with predominantly antiviral activity [4]. Interferons were detected more than 50 years ago as a soluble substance which inhibits the replication of influenza virus. They were named after their ability to interfere (hinder) the replication in host cells protecting healthy cells from viral infection [5]. The family of interferons is now described as a key component of innate immune response and the first line of defense against viral infections. Interferons are proteins that synthesize and produce host cells in response to the presence of various pathogens: viruses, bacteria and parasites. As signaling molecules they provide communication between cells for the purpose of activating and directing the immune response in order to eliminate the pathogen in the most effective way. Due to the fact that interferons belong to the cytokine family, they influence the processes of proliferation, differentiation and apoptosis, and exhibit a number of immunomodulatory functions. According to the type of receptor complex via which they transmit the signal, all interferons are classified into three classes (Type I, II and III). This means that interferons can achieve their activity over three receptor complexes. Type I (IFN-α, IFN-β) and type III interferons have been identified as antiviral types, and type II (IFN-γ) is known as the immune interferon.

Antiviral and antiproliferative activity of interferons, as well as their ability to modulate immune and inflammatory responses, make them highly applicable in medical treatment [6]. There are many preparations of interferons which are approved for clinical use. Further clinical studies are conducted presently. It is expected that the obtained results will enable a wider application of interferons for medical purposes. Although it became clear in XX century that the possibilities for therapeutic application of interferons are huge, the following issues had to be resolved in order to find their wider application in medicine:

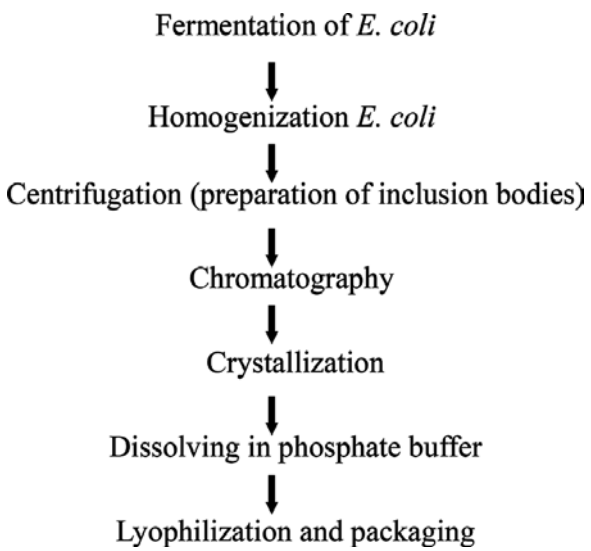
1. An extremely low level of interferons is produced in the human body.
2. Interferons show species specificity, which implies that only human interferons can be used for human clinical treatments.

Since interferons were grown from human leukocytes present in blood transfusion stocks until the 1970s of the last century, their massive production was virtually untenable. In fact, this method provided a mixture of various types and subtypes of interferons in different quantities. However, clinical studies conducted with modest amounts of insufficiently treated interferon preparations have yielded encouraging results. A significant production of interferons was achieved by mammalian cell lines used in the late 1970s. A variety of tumor cell lines was tested first and it was revealed that Namalwa cell line (a type of human

lymphoblastic cell) produced a large amount of interferons [7]. The exposure of these cells to certain viruses (typically Sendai virus) caused an increased production of IFN. Subsequent analysis showed that the final product contains thirteen subtypes of IFN-α, which is why it was necessary to introduce new production technologies. Nowadays, the recombinant DNA technology is used to obtain individual interferon (sub) types. The production of the desired interferon is achieved by insertion of specific genes using vectors for interferons (usually a virus) in mammalian cell lines (Chinese Hamster Ovary - CHO, monkey kidney, etc.), bacteria *Escherichia coli* (*E. coli*) or fungi. Although around 70% of pharmaceutical recombinant proteins currently used for medical purposes is produced in mammalian cells, interferons are produced mainly in *E. coli*. The purification of interferons aims to obtain a final product with 99% level purity, which is mainly achieved by using chromatographic techniques (metal-chelate affinity chromatography, exclusion chromatography (gel filtration), ion-exchange chromatography). Finally, the quality and quantity of interferons are checked by monoclonal antibodies and their effects are analyzed on biological material by different biological assays.

2.1. Production and medical use of IFN-α

Interferon production process includes the fermentation process, purification and the formation of the final product. Fermentation is conducted in specially designed vessels made of stainless steel. This process involves transfer and expression of genes in *E. coli* and the subsequent intracytoplasmic protein production of the inserted gene. Thus, recombinant proteins constitute intracytoplasmic accumulation of inclusion bodies. Homogenization *E. coli* and centrifugation, followed by a chromatographic purification process, need to be done in order to prepare inclusion bodies. The crystallization method is used for the final purification of interferons. The crystallized product is then dissolved in a phosphate buffer which contains glycine and human albumin (carriers of an active substance). Finally, lyophilization and packaging of the product is done.



There are several significant advantages achieved by the modification of interferons. Recombinant interferon modification is achieved by the process of "pegylation." This process was first described by Frank Davis and Abraham Abuchowski and his associates in 1977 [8]. In contrast to the modification process of drug formulation (using colloidal systems or osmotic pumps), the process of interferon pegylation involves covalently binding polyethylene glycol (PEG) polymer chain to the amino group of interferons, which significantly improves its pharmacokinetic and pharmacodynamic characteristics [9]. Many existing pharmacological limitations of interferons are overcome by the process pegylation. Native PEG is an inert, nontoxic polymer with two terminal hydroxyl groups. Chemically active form of PEG molecule is obtained by modifying the terminal hydroxyl group (substitution process of a hydroxyl group with reactive (functional) one). The incubation of purified interferon with methoxypolyethylene glycol (chemically active form of PEG) spontaneously forms covalent bonds [10]. There are different configurations of PEG molecules today, including linear and branched structures of various molecular weights. Formation of stable (covalent) bonds in the process of pegylation of interferon is necessary for a long-term preservation of incurred pharmacological changes. Resultant molecules of interferon are predominantly monopegylated with small impurities of di-, and non-pegylated molecules. Sodium phosphate, saccharose and polysorbate are used as carriers of the active substance for the pegylated product in lyophilized form.

After subcutaneous administration IFN-α is rapidly absorbed and the peak of serum concentration is reached 7–12 hours after which its concentration decreases rapidly. As half-life of IFN-α is 3–8 hours, its serum concentration falls below detection limit within 24 hours after administration [11]. Since the administration of IFN-α is conducted 3 times a week, the desired serum concentration is not maintained in most intervals between applications.

Different linear and branched structures of PEG molecules are used for the pegylation of IFN-α, so that the resulting preparations exhibit different pharmacokinetic and pharmacodynamic characteristics which are improved in comparison to standard IFN-α. PEG group protects IFN-α from enzymatic degradation, prolongs the absorption period at the administration site of subcutaneous injection and reduces its clearance from the body, so the period to reach maximum concentration is significantly prolonged (80 hours). Taking into consideration the above mentioned, it is clear that IFN-α concentration in blood is higher and more constant for a much longer period of time when compared to standard IFN-α. These pharmacokinetic properties enable once-a-week subcutaneous administration of pegylated IFN-α to achieve and maintain the concentration in blood that provides the desired effectiveness (desired effects are time and concentration dependent). Pegylation of IFN-α greatly improves its pharmacodynamic characteristics, and testing shows 100 times stronger antiviral activity and 20 times stronger antitumor activity. Nowadays, IFN-α is used in the treatment of hepatitis B and C, chronic myeloid leukemia, malignant melanoma, non-Hodgkin's lymphoma, Kaposi's sarcoma, and other diseases (**Table 2**).

2.2. Production and medical use of IFN-β

There are several preparations made of IFN-β used for therapeutic purposes. Some of them were made by recombinant DNA technology in *E. coli*, but nowadays CHO cell line is mostly

Generic name	Trade name	Treatment	Year of first FDA approval	Company
IFN-α – con-1	Infergen	Chronic hepatitis C	1997	Amgen
IFN-α – n3, leukocyte derived	Alferon-N	Condylomata acuminata	1989	Hemisperx Biopharma
IFN-α – 2a, pegylated	Pegasys	Chronic hepatitis C Chronic hepatitis B	2002	Roche
IFN-α – 2a, recombinant	Roferon-A	Chronic hepatitis C Hairy cell leukemia Kaposi's sarcoma Chronic myeloid leukemia	1986	Roche
IFN-α – 2b, pegylated	PEG-Intron	Chronic hepatitis C	2001	Schering-Plow
IFN-α – 2b, pegylated	Sylatron	Malignant melanoma	2011	Schering-Plow
IFN-α – 2b, recombinant	Intron-A	Hairy cell leukemia Kaposi's sarcoma Chronic hepatitis B/C Malignant melanoma Follicular lymphoma Condylomata acuminata	1997	Schering-Plow

Table 2. IFN-α approved by FDA for therapeutic use.

used in their production [12]. Recombinant human IFN-β produced in CHO cell line (rhIFN-β-1a) is glycosylated and it has the same amino acid sequence as natural IFN-β [13, 14]. Although *E. coli* does not generate glycosylation of IFN-β [15], this deficiency does not affect the efficiency of its therapeutic applications. IFN-β is presently used in the treatment of multiple sclerosis. However, in IFN-β glycans play an important role in the protein stabilization and thus enhance its biological activity [16] (**Table 3**).

Generic name	Trade name	Treatment	Year of first FDA approval	Company
IFN-β – 1a	Avonex	Relapsing multiple sclerosis High risk for MS	1996	Biogen IDEC
IFN-β – 1b	Betaseron	Relapsing multiple sclerosis High risk for MS	1993	Berlex
IFN-β – 1b	Extavia	Relapsing multiple sclerosis	2009	Novartis

Table 3. IFN-β approved by FDA for therapeutic use.

Generic name	Trade name	Treatment	Year of first FDA approval	Company
IFN-γ – 1b, bioengineered	Actimmune	Chronic granulomatous disease	1990	Intermune Pharma
		Malignant osteopetrosis		

Table 4. IFN-γ approved by FDA for therapeutic use.

2.3. Production and medical use of IFN-γ

IFN-γ preparations are made by recombinant DNA technology in bacteria *E. coli*. Despite the fact that recombinant human IFN-γ is not glycosylated [17], its biological activity is not affected. It is used for the treatment of chronic granulomatous disease (**Table 4**).

2.4. Side effects of interferon application

Administration of interferons can cause many side effects [18].

Application of **IFN-α** causes increased temperature, chills and headache. These reactions are often manifested a few weeks after the application and paracetamol is simultaneously applied to alleviate them. Severe cases develop anorexia, insomnia, cardiovascular complications and autoimmune reactions, which requires immediate termination of its application.

Application of **IFN-β** causes increased temperature, chills and headache. Severe cases develop hypersensitivity reactions, depression and menstrual disorders.

Application of **IFN-γ** causes increased temperature, chills and headache. Severe cases develop heart failure, metabolic disorders and disorientation.

3. Interleukins

More than 40 interleukins with different properties are known today. Interleukins are classified in families based on sequence homology, receptor-binding properties, biological function and cellular sources.

There are 38 interleukins which are designated by the abbreviation IL (from **I**nterleukin) and Arabic numbers [19]. Interleukins are produced by various types of body cells, wherein the specific interleukins (IL-1) can be secreted by up to 20 different cell types. Most cells capable of synthesizing one interleukin are capable of synthesizing several different. Today, the recombinant DNA technology is used for interleukin production, enabling quantities sufficient to meet demanding medical needs.

3.1. Production and medical use of interleukin 1 (IL-1)

IL-1 is a proinflammatory cytokine which stimulates the synthesis of substances involved in the induction of inflammation. Activated mononuclear phagocytes are the main cellular source

of IL-1. When secreted in small quantities, IL-1 acts as a paracrine mediator of local inflammation, while in larger amounts the endocrine effect can induce body temperature increase, synthesis of acute-phase proteins in the liver and the production of neutrophils and platelets in the bone marrow. In diseases with the elevated level of IL-1 it is important to decrease IL-1 level due to its effect in the induction of inflammation [20]. It has been shown that preparations which reduce the level of IL-1 are therapeutically useful when administered alone or, more preferably, in the combination with low doses of other therapeutic agents. In accordance with the above, the reduction of IL-1 level can be achieved by the administration of:

1. Anti-IL-1 antibody
2. The IL-1 receptor antagonist (IL-1Ra)

3.1.1. Anti-IL-1 antibody

Canakinumab is a human anti-IL-1ß monoclonal antibody which is administered subcutaneously for the treatment of syndromes associated with periodic cryopyrin (CAPS—**c**ryopyrin **a**ssociated **p**eriodic **s**yndromes). The main sign of CAPS is urticaria with neutrophilia, accompanied by high fever, headache and arthralgia.

3.1.2. The IL-1 receptor antagonist (IL-1Ra)

Anakinra/Kineret is a recombinant, nonglycosylated form of human interleukin-1 receptor antagonist (IL-1Ra). Anakinra is different from native human IL-1Ra as it is non-glycosylated and may contain an additional N-terminal methionine residue at its amino-terminus, as the result of its production by recombinant DNA technology in prokaryotic system (*E. coli*). In people suffering from rheumatoid arthritis an elevated level of IL-1 is present in the synovial fluid of joints affected. IL-1 shows negative effects on the joints and bones, which include degradation of cartilage and stimulates bone resorption. Therefore, in patients suffering from rheumatoid arthritis the subcutaneous injection of 100 mg (0.67 ml) of Anakinra is administered daily. The following substances are present as carriers of active substance in the final product: sodium citrate (1.29 mg), ethylenediaminetetraacetic acid (EDTA) (0.12 mg), sodium chloride (5.48 mg) and polysorbate 80 (0.70 mg). It is also approved to be applied in the treatment of Neonatal-Onset Multisystem Inflammatory Disease (severe form Cryopyrin-Associated Periodic Syndromes).

3.2. Production and medical use of interleukin 2 (IL-2)

As it was the case with most other cytokines, the use of IL-2 for medical purposes was initially impractical because of small production quantities. Some transformed cell lines, particularly cell line Jurkat (T cell leukemia), produced IL-2 in larger quantities [21]. The largest amounts of IL-2 used in initial studies were obtained from this source. The production of significant amounts of IL-2 is possible by the development of recombinant DNA technology. Today, complementary DNA (cDNA) to the IL-2 gene is expressed in many cell lines, while *E. coli* was used at the beginning (absence of glycosylation of the recombinant product does not alter biological activity of IL-2).

Basiliximab (anti-IL-2Rα (CD25); chimeric monoclonal antibody) is produced in a cell line Sp2/0 and is administered intravenously to patients after kidney transplantation. Basiliximab and cyclosporine are administered in the treatment of kidney transplant [22]. It is important to note that studies have shown that basiliximab slows cyclosporine elimination from the body (it is believed that cytochrome P450 plays an important role in this process).

Daclizumab (anti-IL-2Rα (CD25); humanized monoclonal antibody) was administered intravenously to patients after kidney transplantation. It was withdrawn from the market of the European Union and the United States of America in 2009.

Recombinant IL-2.

Proleukin (Aldesleukin) is indicated for the treatment of adults with metastatic renal cell carcinoma (year of first approval-1992) and metastatic melanoma (year of first approval-1998). Proleukin helps an increased production of several different components of the immune system found in the blood, including T lymphocytes and natural killer cells. Proleukin should be restricted to patients with healthy heart and lung function. Proleukin can cause capillary leak syndrome and the treatment is associated with a reduced neutrophil chemotaxis and with an increased risk of disseminated infection, including sepsis.

3.3. Production and medical use of interleukin 6 (IL-6)

IL-6 has pro- and anti-inflammatory properties and plays a crucial role during the transition from innate to acquired immunity. It has the ability to stimulate neutrophil production, promote expansion and activation of T cells, the differentiation of B cells, and the regulation of the acute-phase response [23]. Proteolytic shedding of IL-6Rα from invading neutrophils subsequently drives IL-6 *trans-signaling* in resident tissue cells, leading to a switch from neutrophil to monocyte recruitment by suppressing mainly neutrophil-attracting (CXCL1, CXCL8 and CX3CL1) and enhancing mainly monocyte-attracting chemokines (CCL2, CCL8, CXCL5 and CXCL6), and cellular adhesion controlled by the lymph node–homing receptor CD62L, and modulates expression of adhesion molecules ICAM-1 and VCAM-1. Besides its role in attracting monocytes, IL-6 *trans-signaling* has been shown to skew monocyte differentiation toward macrophages by upregulating M-CSF receptor expression. The finding that IL-6 induces neutrophil apoptosis contributes to the resolution of acute neutrophil infiltration. Increased IL-6 level is often a better predictor of disease activity in the context of infection, autoimmunity or cancer than C-reactive protein [24, 25]. Consistent with the early description of IL-6 as a lymphocyte-stimulating factor, IL-6 deficiency leads to impaired innate and adaptive immunity to viral, parasitic and bacterial infection. Indeed, children with inhibitory autoantibodies to IL-6 develop recurrent staphylococcal cellulitis and subcutaneous abscesses. While IL-6 has a protective role in many infections, the same activity can be key to the maintenance of chronic inflammation that includes rheumatoid arthritis and multicentric Castleman's disease. The reduction of IL-6 level (**Table 5**) as the treatment of chronic inflammatory diseases can be achieved by the administration of:

1. Anti-IL-6 antibody
2. Anti-IL-6 receptor antibody

International nonproprietary name (INN)	Target	Type	Year of first EMA approval	Year of first FDA approval	Cell line	Therapeutic indication
Tocilizumab	IL-6R	Humanized IgG1	2009	2010	CHO	Rheumatoid arthritis Juvenile idiopathic arthritis
Siltuximab	IL-6	Chimeric IgG1	2014	2014	CHO	Multicentric Castleman's disease
Sarilumab	IL-6R	Human IgG1	2017	2017	CHO	Rheumatoid arthritis

Table 5. Monoclonal antibodies target at IL-6/IL-6R approved by FDA and EMA for therapeutic use.

3.3.1. Anti-IL-6 antibody

Siltuximab is a chimeric immunoglobulin (IgG)1 monoclonal antibody that binds IL-6. IL-6 antagonist is indicated for the treatment of patients with multicentric Castleman's disease (MCD) who are human immunodeficiency virus (HIV) negative and human herpesvirus-8 (HHV-8) negative.

3.3.2. Anti-IL-6 receptor antibody

Sarilumab is a human immunoglobulin (IgG)1 monoclonal antibody which binds specifically to both soluble and membrane-bound IL-6 receptors (sIL-6Rα and mIL-6Rα) and inhibits IL-6-mediated signaling. It is administered subcutaneously in a dose of 200 mg once every 2 weeks for the treatment of rheumatoid arthritis. By binding to IL-6Rα, sarilumab prevents the formation of high-affinity complex of IL-6 with IL-6Rα and thus blocks IL-6 signaling. As sarilumab blocks both mIL-6Rα and sIL-6Rα, it has the potential to inhibit both intra-articular and systemic IL-6 signaling. Patients treated with Sarilumab are at increased risk for developing serious infections.

3.4. Production and medical use of tumor necrosis factor (TNF)

Tumor **n**ecrosis **f**actor (TNF) was originally described as a circulating protein which causes tumor necrosis (discovered in 1975 as endotoxin-induced glycoprotein which causes necrosis of sarcoma in mice). Human TNF was first produced in 1985 by recombinant DNA technology in *E. coli* [26]. Activated mononuclear phagocytes are the most important cellular source of TNF. TNF exerts a variety of effects on vascular endothelial cells and leukocytes. In response to TNF endothelial cells enhance the expression of different combinations of leukocyte adhesion molecules (including E-selectin, ICAM-1 and VCAM-1), which in combination with the secretion of chemokines (IL-8, including, MCP-1 and IP-10) from peripheral blood leukocytes and endothelial cells induces chemotaxis of different populations of leukocytes to the site of inflammation apart from the recognition of the antigen. In addition, TNF stimulates microbicidal activity of neutrophils and monocytes in order to eliminate the cause easier. Secreted in small amounts, TNF acts locally (autocrine and paracrine), primarily on vascular endothelial

cells and leukocytes. The effect of TNF on the endothelium and leukocytes is most likely crucial for a successful local inflammatory response to microorganisms. TNF is produced in large quantities in severe infections. It enters the bloodstream and affects distant cites (endocrine), causing pathological and clinical effects on the body. Systemic effects caused due to excessive TNF production or exogenous administration in high doses can induce increased body temperature, synthesis of acute-phase proteins in the liver, production of neutrophils and platelets in the bone marrow. TNF is presently considered a key mediator of inflammatory response which has a wide range of effects in inflammation, infection and response to tumors. In this respect, several approaches can be useful, and this includes the administration of:

1. Anti-TNF antibody
2. TNF receptor (TNFR)
3. TNF

3.4.1. Anti-TNF antibody

Infliximab is an anti-TNF-α antibody which binds TNF-α and blocks the inflammation induced by cytokine. Today it is successfully used in the treatment of rheumatoid arthritis, ankylosing spondylitis, Crohn's disease, ulcerative colitis, etc.

Adalimumab and **Golimumab** are human anti-TNF-α antibodies which bind to TNF-α by blocking its activity. As TNF is a primary mediator of inflammation, these antibodies are very powerful anti-inflammatory agents.

Adalimumab has been successfully used in the treatment of rheumatoid arthritis, ankylosing spondylitis, psoriatic arthritis, Crohn's disease, ulcerative colitis, etc. It is administered in the treatment of rheumatoid arthritis and ankylosing spondylitis as a subcutaneous injection in a dose of 40 mg twice a month (in severe conditions once a week). In the treatment of Crohn's disease starting dose is 80 mg, then 40 mg every other week (in severe conditions starting dose is 160 mg).

Golimumab has been successfully used in the treatment of rheumatoid arthritis, ankylosing spondylitis and psoriatic arthritis.

Certolizumab pegol is a humanized Fab fragment of a pegylated anti-TNF antibody produced by recombinant DNA technology in *E. coli*. It is used in the therapy of Crohn's disease and rheumatoid arthritis. Pegylation of Fab fragments significantly improves distribution in tissues and prolongs the half-elimination to 14 days. The lack of Fc fragment in cells prevents the antibody binding to the protective FcRn receptor (Neonatal Fc receptor).

3.4.2. TNF receptor (TNFR)

Etanercept/Enbrel is a recombinant human hybrid protein in which extracellular domain of TNFR2 fuses with Fc fragment of IgG (Soluble p75 TNF receptor-Fc fusion). It is produced by

recombinant DNA technology in a CHO cell line. After the purification and addition of the carrier (mannitol, saccharose, and trometamol), the product is lyophilized. It is used for the treatment of rheumatoid arthritis (RA) by subcutaneous injection of 25 mg dose twice a week or 50 mg once a week. It is also applied in the treatment of juvenile idiopathic arthritis, ankylozing spondylitis, psoriatic arthritis, plaque psoriasis and plaque psoriasis in children ages 4–17. Etanercept functions as a competitive inhibitor of TNF, because it prevents its binding to receptors present on cell surface. Side effects of etanercept include the development of infections and tumors, allergic reaction (hives, swelling of the face, etc.), headache and heart failure.

3.4.3. TNF

Tasonermin is a human TNF-α-1a produced by recombinant DNA technology in *E. coli*. The purified product is packed in tube vials (1 mg of active substance per bottle) as a lyophilizate. The carriers of active substance in the final product are sodium chloride, phosphate buffer and serum albumin. It is used in the treatment of soft tissue sarcoma on the limbs in order to prevent or postpone the amputation. Tasonermin is dissolved in physiological saline to a concentration of 0.2 mg/ml after which the tissue perfusion of the affected limb is done with 3–4 mg of the substance for a period of 90 minutes. Side effects of tasonermin could be local (edem, nerve damage) and systemic (arrhythmia, nausea, liver damage) if it enters the systemic circulation.

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Chapter 4

Physiology and Pathology of Drug Hypersensitivity: Role of Human Leukocyte Antigens

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Additional information is available at the end of the chapter

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Abstract

Drug Hypersensitivity reactions can be distinguished in adverse drug events and adverse drug reactions. They represent a major problem in the medical scheme, since they are often underestimated. Pharmacogenetic analysis demonstrated significant associations between emerging hypersensitivity reactions and distinct genes of the HLA complex. HLA-mediated hypersensitivity reactions particularly affect skin and liver, however, impairment of the bone marrow and kidney function could also be observed. These life threatening medical conditions can be attributed to the activation of autologous drug-specific T-cells. Severe drug hypersensitivity reactions that resemble acute GvHD are linked to certain specific HLA alleles. The most common hypersensitivity reactions occur after the treatment of HLA-B*57:01$^+$ HIV patients with abacavir and HLA-A*31:01$^+$ or B*15:02$^+$ epileptic patients with carbamazepine (CBZ).

Keywords: HLA, hypersensitivity, adverse drug reactions, T-cells, carbamazepine

1. Introduction

The administration of a drug can be accompanied by harmful adverse events such as gastrointestinal bleeding or skin rashes (**Table 1**). The classification of these adverse events is illustrated in **Figure 1**. Adverse events comprise all harmful reactions during drug application regardless of a causal link between the drug and the event. If the drug usage is causal for the symptoms, the condition is called adverse drug event (ADE) [1–3]. The term ADE comprises harm caused by the drug itself as well as harm caused by the use of the drug, for instance inappropriate dosages or premature discontinuation of the medication [1]. Mostly,

Drug	Function	Symptoms of an ADE	Mechanism	Classification
Immunosuppressive	Suppression of the immune system	Virus infections	Immune system is not able to properly cope with the virus	ADE
Morphine	Analgetic agent	Unconsciousness, hypoventilation, miosis	Overdose	ADE
Antihistamines of the 1st generation	H1-receptor antagonist	Sedation	Crossing of the blood-brain barrier and off-target binding	Type A ADR
NSAIDs	Inhibition of the synthesis of proinflammatory prostaglandins	Gastrointestinal bleeding	Production of protective mucous in the stomach is decreased	Type A ADR
		Asthma, rhinitis, angioedema	Synthesis of leukotrienes, activation of the innate immune system	Pseudoallergic type B ADR
Penicillin/β-lactam antibiotics	Antibiotic drug	Urticaria, anaphylaxis, hypotension, bronchospasm, angioedema	Hapten-model, type I reaction	Allergic type B ADR
Methyldopa	Antihypertensive drug	Hemolytic anemia	Type II reaction	Allergic type B ADR
Aminopyrine		Leukopenia	Type II reaction	Allergic type B ADR
Minocycline	Antibiotic drug	DRESS	Type III reaction	Allergic type B ADR
Allopurinol	Uricostatic drug	SJS/TEN	P-i model, type IV reaction	Pharmacologic type B ADR
Abacavir	Antiretroviral medication	Rashes, fever, gastrointestinal and respiratory symptoms, malaise, lethargy, arthralgia, myalgia	Altered repertoire model, type IV reaction	Pharmacologic type B ADR
Carbamazepine	Antiepileptic drug	Maculopapular exanthema, DRESS, SJS/TEN	Altered repertoire model, type IV reaction	Pharmacologic type B ADR

Table 1. Examples of drugs leading to adverse events.

medication errors do not cause any harm in patients, but in some cases ADEs are triggered by increased or decreased drug doses [1]. Opioid-intoxication, as for example a morphine overdose leads to unconsciousness, hypoventilation and miosis. The probability of an ADE differs from substance to substance. The antimitotic nystatin is very unlikely to cause unwanted effects, since it is directed against a cell wall component of fungi and mycoplasma. In contrast, immunosuppressive medication has a high risk of enabling virus infections and diminishing the surveillance of cancer development as the down regulated immune system is no longer able to properly cope with the virus or neoplastic cells [4].

However, certain drugs can cause the patient harm despite proper application. Those unintended and harmful reactions to drugs at therapeutic levels are termed adverse drug reactions (ADRs) (WHO 1972). They are triggered by the drug itself and not by inappropriate use of the drug. In contrast, side-effects are defined as predictable, but distinct from the intended effects. They comprise unwanted, as well as positive or irrelevant effects of a drug appearing at normal dosage [1, 4].

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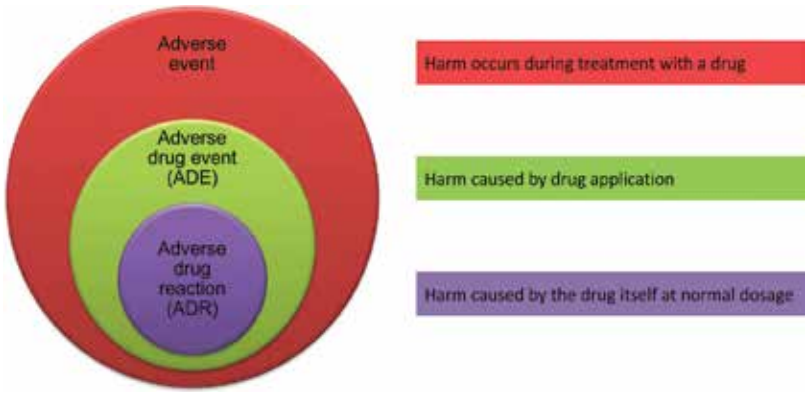

Figure 1. Classification of adverse events. Adverse events include all harmful events occurring during treatment with a drug without the necessity of a causal link between the drug and the reaction. If the use of medication is causal for the reaction, the condition is called adverse drug event. A subform of adverse drug events are adverse drug reactions that are triggered by the drug itself despite its appropriate dosage.

These noxious reactions to drugs are caused by distinct mechanisms, thus different forms of ADRs are distinguished as illustrated in **Figure 2**. Dose-dependent and predictable type A ADRs are explained by the pharmacological activity of the drug, whereas dose-independent type B reactions appear to be idiosyncratic [5].

With >80% the majority of all ADRs are classed among type A reactions that are rarely fatal [5, 6]. They are triggered by off-target binding to non-immune receptors, drug-drug interaction or toxicity; thus the clinical picture depends on the drug [7]. For example, nonsteroidal

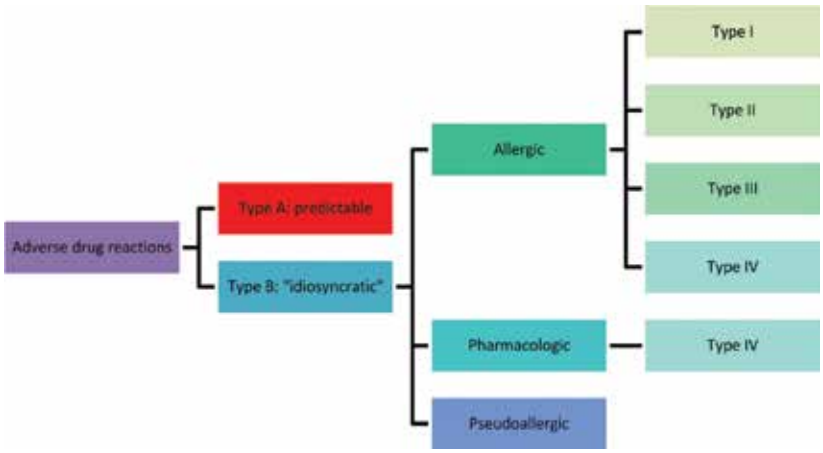

Figure 2. Classification of adverse drug reactions. The majority of all ADRs is dose-dependent and predictable type A reactions. Type B reactions occur less. The majority of all ADRs is dose-dependent and predictable type A reactions. Type B reactions occur less frequent and have a higher mortality. They are subdivided into allergic, pseudoallergic and pharmacologic reactions.

anti-inflammatory drugs (NSAIDs) are likely to cause gastrointestinal bleeding by inhibiting prostaglandin-synthesis, since prostaglandins do not only reduce inflammation, but also impede the production of protective mucus in the stomach. Another example are antihistamines of the first generation; being able to cross the blood-brain barrier the H1-receptor-antagonists also induce sedation by off-target binding.

Because these reactions to drugs are accounted for by their pharmacological mode of action, they are dose-dependent. Their emergence is comprehensible and predictable.

Type B ADRs are characterized by direct involvement of the immune system. They occur less frequently, but have an increased mortality rate [5, 7]. Type B reactions can affect almost every organ, but often feature involvement of the skin, liver and blood cells. The symptoms can be systemic as well as restricted to a single organ [8].

The main trigger of such drug hypersensitivity reactions are antibiotics, non-steroidal anti-inflammatory drugs and antiepileptics [9]. The nucleoside reverse transcriptase inhibitor abacavir utilized for treatment of human immunodeficiency virus type I patients leads to a severe and life-threatening hypersensitivity syndrome. Those affected individuals develop rashes, fever, gastrointestinal symptoms, lethargy, malaise, arthralgia, myalgia or respiratory symptoms in the first weeks after initiation of the intake of the drug. Abacavir hypersensitivity is highly associated with the human leukocyte antigen (HLA) allele HLA-B*57:01 [10, 11]. Another example of a type B adverse reaction is the allergy against penicillin. Symptoms include sudden anaphylaxis, hypotension, bronchospasm, angioedema and urticarial [12].

Drug hypersensitivity reactions often occur as skin exanthemas [9]. Several clinical pictures can be distinguished. Drug reaction with eosinophilia and systemic symptoms (DRESS) is known under various names including drug induced delayed multiple organ hypersensitivity syndrome (DHDMOHS), drug-induced hypersensitivity syndrome (DIHS), drug hypersensitivity syndrome (DHS) and hypersensitivity syndrome (HSS). It is characterized not only by cutaneous exanthema, but also by organ involvement, for example hepatitis, arthralgia and lymphadenopathy [13]. Danger signs indicating a DRESS are changes in blood count revealing eosinophilia or atypical lymphocytes and signs of organ involvement, namely high liver enzymes, high kidney values or lymphnode enlargement. At first, DRESS might resemble maculopapular exanthema, but in the course of the reaction it spreads over more than half the body [13]. Some drugs are highly suspected to induce DRESS: the antiepileptics carbamazepine, oxcarbazepine, lamotrigine phenytoin and phenobarbital, sulfonamides, as well as the uricostaticum allopurinol [13].

Other disease patterns are Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN). In these conditions skin blisters and bullae rise, the skin detaches and erosions of mucous membrane are found [14]. The patients develop high fever, hypovolemia and complications with lung involvement are possible [15]. In SJS, the detachment of skin affects less than 10% of the body surface, whereas in TEN more than 30% of body surface detaches [16]. Approximately 48% of all TEN patients die due to the disease, for the elderly the mortality is 70%. SJS, SJS/TEN and TEN together have an overall mortality of 20–25% [15, 17]. In early stages tiny vesicles or crusts and painful or burning skin and mucosa

point towards SJS/TEN. Patients are positive for Nikolsky's sign, but specific laboratory parameters do not exist [18]. Medications with a high risk to induce SJS/TEN are the antiepileptics carbamazepine, lamotrigine, phenytoin, phenobarbital, some sulfonamides, the uricostaticum allopurinol, oxicam-NSAIDs, sulfasalazine and the antiretroviral drug nevirapine. The algorithm of drug causality for EN algorithm (ALDEN) helps to exclude or confirm the suspicion of SJS/TEN [19]. Currently, 67% of SJS/TEN-cases in Europe are drug induced with allopurinol being the main trigger [20].

Acute generalized exanthematous pustulosis (AGEP) has an acute onset with fever, large erythema and sterile, non-follicular pinhead-sized rapidly appearing pustules. Desquamation starts 4 to 10 days later. In AGEP neutrophilia also occurs; usually other internal organs are not, while the mucosa is little involved. Drugs associated with AGEP are for example aminopenicilins, quinolones and pristinamycine [21].

The first step for diagnosis of a drug hypersensitivity reaction is the analysis of the medical history of the patient [9]. Therefore, the symptomatology, the chronology of the symptoms, additional drug administration and the medical background are parameters to consider for a correct assessment [14]. A differential blood count is considered for confirmation of eosinophila in DRESS or neutrophilia in AGEP [9, 13, 22]. The involvement of other organs (liver, kidney, heart) is evaluated by investigation of laboratory parameters [13].

Skin tests and drug provocation tests enable in vivo identification of the drug responsible for the reactions. Patch tests are a safe method to identify the accountable drug in DRESS mediated by antiepileptics [9]. Nevertheless, patch, prick and intracutaneous skin tests are often insensitive, especially in case of non-immediate reactions to beta-lactam antibiotics as penicillin [23]. For drug provocation tests, only performed at specialist centers with resuscitative equipment, the administration of the suspected drug takes place under controlled conditions [24]. They are controversial, since severe reactions can be triggered [9, 24]. Likewise, provocation tests are not standardized for delayed reactions [9].

An advantage of in vitro tests is the safety of the patient who is not exposed to the drug. Additionally, they enable valuable insight into the pathomechanism of the drug allergy. However, in vitro tests are not standardized for all drugs and are not suitable to detect all types of drug hypersensitivity [9]. The lymphocyte transformation test (LTT) enables simultaneous testing of many drugs and drug concentrations [9]. Measurement of proliferation of drug-specific T-cells stimulated with the drug in question is enabled by incorporation of ^{3}H-thymidine [25]. The sensitivity of LTTs varies depending on the clinical manifestation and the drug, in AGEP and DRESS it is higher, as well as for beta-lactam antibiotics and antiepileptics. LTTs for SJS should be performed in the acute phase, whereas for DRESS the resolution phase has highest sensitivity. The number of cells releasing cytokines upon stimulation with the suspected drug can be determined by enzyme-linked immunosorbent spot assay (ELISpot) [25]. Upregulation of CD69 can be observed via flow cytometry, but this procedure is difficult to standardize [9]. When considering transitory peaks and degradation, cytokine synthesis and secretion can indicate hypersensitivity reactions. Measurement is possible via enzyme-linked immunosorbent assay, ELISpot and flow cytometry. Cytotoxicity can be determined equally [9]. Another test is

the basophil activation test (BAT) that can identify IgE-mediated reactions [26]. Combinations of these tests are currently best in order to diagnose a drug hypersensitivity reaction.

There are several approaches to divide drug hypersensitivity into classes depending on the time when first symptoms emerge, the type of immune mechanism or drug or the mode of drug action with immune cells [7]. The latter is composed of three groups (see **Figure 2**): Allergic reactions involve the innate and the adaptive immune system, pharmacologic reactions are exclusively triggered by T-cells, whereas pseudoallergic reactions are mediated by the innate immune system [7].

In detail, an allergic reaction to drugs is explained by the hapten/prohapten model where the drug itself or a reactive metabolite bind covalently to a high molecular weight protein. Thus, even small molecules that should not be recognized by the immune system become immunogenic [27–32]. The drug-carrier molecule can either activate the innate immune system via pattern recognition receptors or cells of the adaptive immune system react to the newly formed antigen after processing and presentation on HLA molecules [7]. Because of the hapten binding to multiple proteins, these allergic reactions are very heterogeneous [7, 29, 33]. A typical characteristic of allergic reactions is the immediate reaction of the patient due to the IgE-meditated urticaria, angioedema, rhinitis, bronchospasm and anaphylactic shock [34]. Drug allergies can also be triggered by IgG or T-cells [7]. Allergy against penicillin for example can manifest as IgE-mediated hypersensitivity reaction or as a delayed T-cell response [33, 35, 36].

Pseudoallergic type B reactions include mast cell and granulocyte activation, as well as involvement of enzymes and co-factors. Hence, the basis of those reactions is not a drug- or antigene-specific sensitization [7], but direct stimulation of effector cells [9]. NSAIDs do not only inhibit prostaglandin-synthesis, but also lead to increased amounts of leukotrienes that mediate inflammation. Therefore, the intake of NSAIDs can also result in asthma and rhinitis or angioedema [37].

Pharmacological reactions are characterized by noncovalent off-target binding of the drug or a metabolite to immune receptors. This excludes binding to the peptides presented by HLA molecules. Instead, the drug binds to either the T-cell receptor (TCR) or an HLA molecule, both are extremely polymorphic [7]. Abacavir hypersensitivity reactions belong to this category, since the drug binds to the peptide binding groove of HLA-B*57:01 [38].

2. The relevance of ADEs and ADRs

In consequence of the thalidomide disaster the world health organization (WHO) started the Program for International Drug Monitoring with the objective to improve the safety of medications [39]: In the early 60s of the last century the drug thalidomide that was sold under various names all over the world made history [40]. It was advertised as a sedative, tranquilizer and antiemetic without side-effects especially suited for pregnant women [41, 42]. By the end of 1960 first doubts emerged concerning toxic effects of the drug [43] but it was not until 1961

that its teratogenicity was stated [44–47]. Over time several adverse effects became apparent with peripheral neuropathy being the most frequent in patients taking thalidomide for long-term [48]. Also rashes and constipation turned out to be unexpected effects of the drug [40, 49].

When thalidomide was withdrawn in most countries in 1961–1962, more than 10,000 children with partially severe malformation had already been born [41, 42]. The number of serious cases was boosted by the demeanor of the manufacturer Grünenthal favoring the continued sale of the drug over informing the public of the toxic effects the company was aware of since 1959 [50]. This led to more consciousness about ADRs and other drug-related problems as for example medication errors or misuse/abuse of medicines and the raise of pharmacovigilance.

ADRs are an expensive burden on public health, they are under-diagnosed and under-reported [6, 51]. Already 30 years ago people began to wonder about unintended reactions to medication in hospitals. Initially, the question arose which method might be most successful in detecting such event [52]. The studies spotted remarkable observations: It was revealed that 86% of cases went unreported in Sweden [53], whereas in Canada under-reporting reached as much as 96% [54]. This might be due to the methods used to identify ADEs or due to unawareness of reporting systems [39]. There are different approaches to improve patient safety by early recognition and prevention of ADEs including voluntary reports and computer-based monitoring. Traditional detection methods as voluntary reporting are inconvenient and have the disadvantage of relying on the commitment of physicians and nurses [55]. Already in 1991 Classen, Pestotnik [55] reported that their computerized surveillance of ADEs drastically elevated their detection and reporting. Based on information about abrupt discontinuation of drugs, antidote ordering and anomalous laboratory values, the computer program recognized 641 of 731 ADEs in 36,653 patients, whereas only 9 of those ADEs were revealed by the traditional detection methods [55]. This result is coincident with other publications reporting that physicians only identified a third of ADRs notified by automatic signals generated from laboratory signals [56] and that half of true-positive alerts were unrecognized prior to the warning [57].

Evans, Pestotnik [58] stated that the type and the intensity of an ADE had implications on the length and costs of the stay in hospital. While patients without ADEs stayed for an average of 5 days, patients experiencing a type A or type B reaction had prolonged stays of 14 or 17 days, respectively. Hence, the costs of hospitalization increased by 3.7- or 4.8-fold for these patients. Moderate ADEs led to extended stays of 13 days and a 3.6-fold increase in costs; severe ADEs prolonged the stay to an average of 20 days and caused a 6-fold increase in costs.

Different studies considered 30–50% of all ADEs [2, 59] to be preventable, whereas others appraised 50–80% of all ADRs to be avoidable [6, 51, 56, 60, 61]. Interestingly, severe reactions were more frequently classified as preventable than mild reactions [2, 59]. Nevertheless, about 3% of all deaths and approximately 6.4% of hospital-fatalities in the UK are caused by ADRs [62]. There are several reasons for those preventable ADEs to happen. Too high doses of drugs in relation to the patient's age, renal function, weight and underlying disease were identified by Evans, Pestotnik [58] as a main reason for the moderate reactions. Errors during ordering and administration were found causal for most ADEs by Bates, Cullen [59]; other

studies claimed ADEs to emerge more likely due to errors while ordering and monitoring, whereas dispensing and administration of the drugs rarely caused the reactions [2].

As a possible strategy to improve patient safety, several authors have demonstrated significant prevention of ADEs by the application of pharmacy alerts for known drug allergies [57, 58], as well as presence of pharmacists on ward rounds, improved monitoring and education of prescribing [6, 63].

3. Mechanisms of type B ADRs

Most drugs are not antigenic due to their small size (<1000 Da), however, by forming a hapten or prohapten through covalent binding to carrier proteins the drug-protein complex becomes chemically reactive and can subsequently trigger an immune response. Prohaptens are precursor haptens that become reactive by metabolizing the drug to generate active haptens. In order to cause an allergic reaction, these hapten complexes have to be processed by antigen presenting cells (APC). After migration to the local lymphoid tissue, sensitization of naïve T-cells or stimulation of B cells can occur. Primed T-cells proliferate and act as effector T-cells and may also aide the differentiation of B cells to plasma cells that produce drug-hapten specific IgE or IgG antibodies, depending on the presence of either Th1 or Th2 helper cells. Accordingly, allergic hypersensitivity reactions are categorized into four types (Type I–IV) based on the classification system established by Gell & Coombs [7].

4. Antibody mediated hypersensitivity reactions (Type I–III)

Type I–III reactions (**Figure 3**) occur if drug-specific B cells differentiate into antibody producing plasma cells through $CD4^+$ Th2 cell stimulation. In the case of Type I reactions these plasma cells produce IgE antibodies. Many of these reactions are caused by antibiotics of the β-lactam family (e.g. penicillin and its derivatives) that can lead to symptoms ranging from mild skin reaction to the life threatening anaphylactic shock. In the case of penicillin, the antibiotic binds covalently to high-molecular weight proteins such as albumin [64] thus forming a molecule complex that can be recognized by IgE antibodies. During sensitization, these IgE antibodies bind to mast cells in tissues and basophiles in the blood *via* the FcεRI receptor. Subsequent cross-linking of the IgE antibody with the antigen elicits the type I reaction resulting in the release of histamines, leukotrienes and serotonin as well as prostaglandin causing allergic symptoms [14]. Type I reactions are immediate reactions that take place directly after administration of the drug or up to 2 hours later. Typically, clinical manifestations contain symptoms such as urticaria, mild skin rashes and anaphylactic shock.

In non-immediate type II and type III reactions symptoms emerge 5 to 21 days after administration of the drug [14], however, first symptoms are usually observed after 24 to 48 hours. Both types are primarily IgG-mediated. Damage mediated by tissue-specific IgG or IgM antibodies is the basis for type II reactions: On exposure, the drug forms a hapten with a

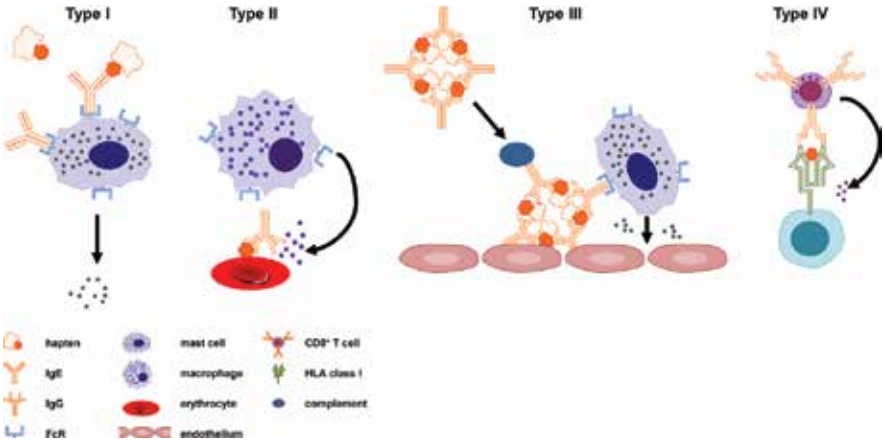

Figure 3. Type I – IV drug-related hypersensitivity reactions.

self-protein thus creating a modified self-protein. Binding of IgG or IgM to the modified self-tissue is followed by activation of normal immunoglobulin effectors. Drug specific type II reactions are mostly associated with the destruction of red blood cells and platelets, where the respective drug bound to the cell surface serves as an antigenic target for IgG antibodies leading to antibody-dependent cell-mediated cytotoxicity (ADCC). Consequently, the cell bound antibody then triggers clearance of the cell from the circulation by macrophages or NK cells that recognize the Fc part of the IgG antibodies *via* the FcγRIII (CD16) surface receptor. Examples are hemolytic anemia as an adverse reaction to methyldopa or leukopenia in the case of aminopyrine.

Type III hypersensitivity reactions are caused by soluble drug-haptens that form immune complexes with IgG antibodies [14]. Larger aggregates are fixed by complement und consecutively cleared by phagocytes, however, smaller immune complexes deposit at local tissue sites where FcR binding on leukocytes and mast cells induces an inflammatory response leading to increased vascular permeability. Conditions that arise from type III reactions are serum sickness (especially β-lactams), drug-induced lupus erythematosus and thrombocytopenia (quinidine) or vasculitis or even DRESS (minocycline).

5. T-cell-mediated drug hypersensitivity (type IV) without prior drug exposure

Type IV reactions (**Figure 3**) take the longest time to develop, ranging from 2 days up to 20 days until first symptoms emerge. Symptoms include mild conditions such as MPE to more severe conditions such as TEN or SJS. Type IV ADRs are T-cell mediated drug hypersensitivity reactions based on the erroneous T-cell activation through HLA molecules on the surface of endogenous cells. Different modes of activation can be distinguished, whether the antigen is formed by binding of the drug to a self-protein, thus creating a foreign antigen for T-cell recognition

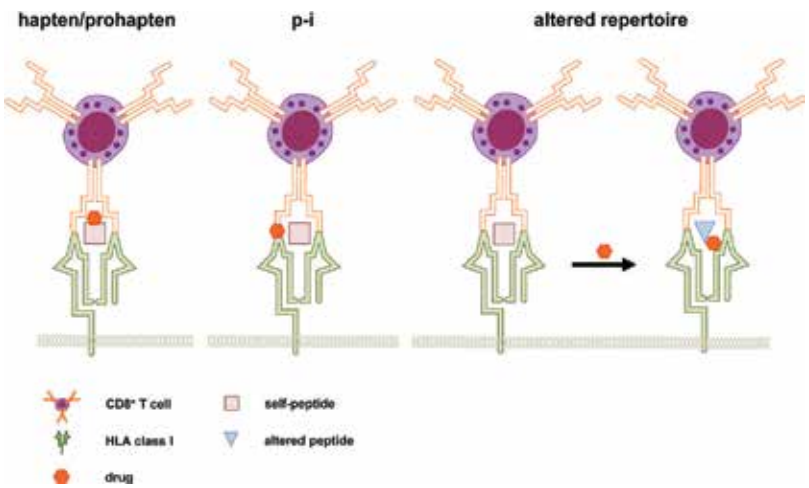

Figure 4. Overview of the models explaining T cell-mediated hypersensitivity.

(allergic), or the drug interfering directly or indirectly with the interaction between T-cell receptor (TCR) and HLA (pharmacological). The processing and interference of presentation by APCs leads to an immunostimulatory potential that manifests in delayed hypersensitivity reactions [65] and although direct recognition of the drug as an immunogen is more common with non-human protein therapeutics [66], most small molecules are not direct immunogens. Their potential for eliciting a hypersensitivity reaction is explained by either of the following models (**Figure 4**): Hapten model, p-i model and altered peptide repertoire model [7].

6. The hapten/prohapten model

The hapten model is based on the binding of small chemicals to proteins or peptides and thus generating new antigenic determinants. These complexes are processed by APCs in lymphoid tissues and generate antigenic hapten-peptides that have the ability to stimulate T-cells in an HLA dependent manner. Examples are sensitive reactions to β-lactam antibiotics. Penicillin, for instance, is known to bind extracellular proteins, in particular to lysine residues of serum albumin [67]. In the case of penicillin, haptenated peptides are presented to $CD4^+$ T-cells by HLA-DRB1 [68]. Chemically inert drugs may also produce delayed hypersensitivity reactions if the metabolite of the otherwise non-reactive drug becomes active. An example here is sulfamethoxazole. In the liver CYP2C9 modifies sulfamethoxazole into hydroxylamine metabolite that is reactive, converts spontaneously to nitroso sulfamethoxazole that readily binds protein cysteine residues of extracellular and cellular proteins [69].

7. The p-i model

After it became apparent that the hapten model is not sufficient to explain the diversity of different hypersensitivity reactions, the pharmacological interaction with immune receptors

(p-i) model and the altered peptide repertoire model were proposed. The p-i model postulates that binding of the drug itself to either the TCR or the HLA molecule may elicit the hypersensitivity reaction [70]. Such binding is independent from metabolites and processing by APCs and additionally, the binding is non-covalent and therefore potentially weak [71]. This means the p-i mechanism is reversible and binding can occur at the interaction sites of the TCR-HLA complex as well as outside of the binding regions. In either case, the drug-binding interferes with the interaction between the HLA molecule and the TCR. In general, as part of the p-i concept, the drug binding has to induce functional changes and the mechanism is immediate because it directly interferes with the already present system. Also, the innate immune system and B cells are not involved because antigen processing is not involved in the p-i concept. Because these structures are allele specific and therefore specific to highly polymorphic regions, this immune response is only observed for carriers of certain HLA alleles. Additionally, the drug can bind in the groove and change the features of the pockets in the peptide binding groove, so that even though a correct peptide is presented the change in overall conformity can lead to T-cell activation. Examples for the p-i concept are the interaction of allopurinol with HLA-B*58:01 where binding of the drug leads to immediate T-cell activation that was not limited to a specific TCR Vβ pattern [72].

8. The altered peptide repertoire model

The relation between delayed hypersensitivity reactions and HLA associations is further explained by the altered peptide repertoire model. This concept is based on the binding of the drug inside the peptide binding groove during HLA assembly in the ER [38]. However, binding in the peptide binding groove leads to altered peptide specificity and thus changes the presented self-repertoire. Consequently, an erroneous T-cell response is triggered because the TCR does not recognize these altered peptides as self anymore. This model was first based on findings that were made from the peptide elution studies and crystal structure of HLA-B*57:01 with abacavir [38, 73]. The structure demonstrated that Abacavir resides within the C, D, E and F pocket of the peptide binding groove influencing the peptide binding capacity of HLA-B*57:01 leading to a shift in the presented repertoire. For endogenous T-cells, this poses an allogenic antigen prompting an immune response similar to the mechanism of allograft rejection and graft versus host disease (GvHD).

9. HLA-mediated ADRs

Through genome-wide association studies an increasing number of associations between certain allelic HLA variants and drug-hypersensitivities could be identified [74]. In order to secure safer treatment of patients, it is essential to understand the underlying mechanisms [75]. The discovery of an association between ADRs and certain HLA alleles represented an important medical step towards the prediction and prophylaxis of Type B ADRs. These particular HLA-mediated hypersensitivity reactions are highly specific; hence HLA subtypes that are linked to ADRs represent biomarkers for the determination of individual medications. HLA molecules bind and present peptides of the intracellular proteomic content; their origin

is determined by the health status of the cell. During pathological conditions, HLA molecules can bind peptides of non-self origin and display targets for effector cells that scan peptide-HLA complexes on the cellular surface for self- / non-self discrimination. The HLA-system is extremely polymorphic. For most HLA genes several allelic variants exist, most of them are distinguished by amino acid (AA) exchanges within the peptide binding region (PBR). Structural alterations within the PBR result in the selection and binding of peptides exhibiting differential features (origin, sequence, length). Every single peptide alters the accessible surface of a given peptide-HLA complex for recognition by an effector cell receptor. T-cell responses can be triggered through the recognition of single AA mismatches that alter the biophysical state of the PBR and thus the features of the bound peptides, the heavy chain and hence the mode of peptide loading and/or the half life time of the pHLA complexes.

The first discovered and most prominent example is the association between the antiretroviral drug abacavir and HLA B*57:01 [10]. Abacavir is a nucleoside analogue of guanosine, it inhibits competitively the reverse transcriptase of the retrovirus HIV. 5–8% of treated patients develop hypersensitivity reactions, comprising fever, fatigue, gastrointestinal symptoms up to life threatening, multiorgan diseases. HLA-restricted hypersensitivity reactions triggered by abacavir are verified to be $CD8^+$ T-cell-mediated [76], using a broad repertoire of TCR clonotypes [75, 77]. Thereby, Abacavir-induced $CD8^+$ T-cell activation is elicited by an altered repertoire of self-peptides, presented by HLA B*57:01 [78]. Due to the high incidence of HLA B*57:01, all patients are typed for HLA class I molecules prior to therapy in order to protect patients from hypersensitivity syndrome and the pharmaceutical industry from its associated costs [79, 80].

Another example is the HLA-associated ADR induced by Allopurinol. This inhibitor of xanthine oxidase, applied in gout and hyperuricemia, causes severe cutaneous adverse reactions in patients carrying the HLA B*58:01 gene [81].

CBZ-induced ADRs are strongly associated with two HLA genotypes, HLA B*15:02 in Han Chinese [82, 83] and HLA A*31:01 in Caucasian and Japanese population [84, 85]. Both HLA alleles differ substantially in their AA composition and their immune function. However, a strong discrimination between the clinical outcome of HLA-B*15:02 or A*31:01 positive patients following CBZ administration can be observed. The anticonvulsive drug CBZ is commonly used to treat epilepsy, trigeminal neuralgia, bipolar disorder or chronic pain. In 5% of cases, therapy with CBZ is discontinued because of adverse drug reactions. Nevertheless, CBZ is commonly applied due to its therapeutic success and its comparable tolerability. CBZ-induced ADRs vary in their severity from mild maculopapular exanthema (MPE) or hypersensitivity syndrome (HSS) to life-threatening Stevens-Johnson syndrome (SJS) or toxic epidermal necrolysis (TEN) [86, 87]. The ADR-causing mechanism, triggered by CBZ, is not yet completely discovered. SJS and TEN are caused by cytotoxic $CD8^+$ T-cells [88], MPE and HSS also involve skin-infiltrating $CD4^+$ T-cells. These $CD4^+$ T-cells damage the skin by secreting inter alia perforin and granzyme B [89]. Interestingly, HLA B*15:02 is associated with SJS/TEN, but not with MPE or HSS [86]. In contrast, HLA A*31:01 is associated with HSS, MPE and SJS/TEN [90]. Associations are detected in Japanese, Han Chinese as well as in European ancestry [84–86]. The prevalence of this allele is 2–5% in Northern European population, 2% in Han Chinese population and 9% in Japanese population [84]. According to the broad range

of HLA A*31:01-restricted CBZ-induces ADRs, different types of CBZ-specific T-cells were isolated of patient's peripheral blood: $CD3^+/CD4^+$, $CD3^+/CD8^+$ as well as $CD3^+/CD4^+/CD8^+$ T-cells [91, 92].

Thereby, further association between CBZ-specific $CD4^+$ T-cell response and the HLA class II molecules HLA DR and DP could be detected. Especially, HLA-DRB1*04:04 seems to be associated with CBZ-induced ADR driven by $CD4^+$ T-cells. This HLA allele occurs commonly in a haplotype block with HLA A*31:01 in Caucasians [92, 93]. While HLA A*31:01-restricted CBZ-induced ADRs are widely unexplained [90], there are several suggestions about the mechanism of HLA B*15:02-restricted CBZ-induced ADRs. STS/TEN are triggered by cytotoxic T-cells inter alia via perforins, granzyme B and granulysin [94]. There are reasonable presumptions, that T-cell activation occurs via direct interaction of CBZ with the immunoreceptor [95], in accordance with the p-i model [96, 75]. Confirmed T-cell activation independently of metabolism of CBZ and intracellular antigen processing, supports this hypothesis [97, 95]. In contrast, presentation of an altered self-peptide repertoire by HLA-B*15:02 due to CBZ-exposure is reported, leading to the presumption, the T-cell receptor is activated according to altered repertoire model [38]. Additionally, the presence of HLA B*15:02 is not a sufficient characteristic to elicit $CD8^+$ T-cell response, since not all carriers are responders [35, 98]. However, restricted usage of TCR clonotype is required for immune activation [99]. Thus, it could be illustrated that only HLA B*15:02-typed patients with T-cells, expressing the TCR Vβ 11-ISGSY, react hypersensitive to CBZ [100].

The mechanism of HLA-meditated hypersensitivity reactions to drugs are not completely understood, yet. Polymorphic residues within a given HLA molecule affect their conformation and their bound peptides. Open questions remain i) how does the drug interact with selected residues of the HLA-molecules heavy chain?, ii) is the reaction triggered by the drug itself or by a metabolite?, iii) can non-responders to a drug be attributed to the presence or a lack of given TCRs and their immunological vitality?

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Chapter 5

Physiology and Pathology of Autoinflammation: NOD like Receptors in Autoinflammation and Autoimmunity

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Additional information is available at the end of the chapter

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Abstract

Immune regulation is an essential feature of immune responses. The failure of such regulation results in allergic reactions and debilitating autoimmune diseases that can be fatal. Furthermore, the recent increase in the prevalence of the latter as well as the medical severity makes this a subject of great medical interest. Autoimmunity results from a breakdown in or the failure of the self-tolerance mechanisms. Many genes have been identified in which mutations cause the predisposition to autoinflammation and autoimmunity in human and in animal models. The relatively small number of genes explored to date unquestionably shows the challenges of identifying the associated genes in outbred populations of humans. One chief contributing gene family to both autoinflammatory and autoimmune diseases is the nucleotide-binding and oligomerization domain (NOD)-like receptor (NLR) family. Ever since their discovery, NLRs have drawn considerable attention for their ability to form multiprotein complexes called inflammasomes and also for their roles as NLRs, independent of inflammasome complexes. We herein first revisit general characteristics of NLRs and inflammasomes. We then couple this knowledge with the most recent findings related to autoinflammatory and autoimmune diseases, while highlighting some unanswered questions and future perspectives in elucidating NLR roles in health and disease.

Keywords: NOD-like receptor signaling, inflammasomes, PAMPs, DAMPs, HAMPs, SAMPs, autoinflammation, autoimmunity

1. Inflammasomes and NOD-like receptors (NLRs)

Inflammasomes are nucleotide-binding and oligomerization domain (NOD)-like receptor (NLR) multiprotein complexes that activate the cysteine protease caspase-1 (IL-1 beta-converting enzyme) and then lead to the maturation of pro-IL-1β and IL-18. Even though they

are the component of the innate immune system, their ability to regulate the adaptive immune system have been previously suggested (**Figure 1**) [1]. The immune system in mammals comprises a germline-encoded innate immune system and an acquired adaptive immune system that is able to eradicate pathogenic microorganisms with a sophisticated specificity and a long-term memory. The innate immune system is a primary role player in shaping host resilience. This system is armed with a broad portfolio of pattern recognition receptors (PRRs) that convert microbial and danger recognition into rapid host defenses as well as convey signals to prime the adaptive immune responses for a long-lasting protection. Nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs) are a class of evolutionarily conserved intracellular PRRs that play an important role in innate immunity and host physiology and also most recently in regulating and shaping adaptive immunity as predicted by their prevalence in organisms [2]. To date, there are 22 known NLRs in humans, and the single nucleotide polymorphisms (SNPs) in their genes as well as the association of mutations with human diseases emphasize their critical role in host defense.

Of the number of genes involved in the development of autoinflammatory and autoimmune diseases, some affect the cells of the immune system directly, changing the immunoreactivity of their host. These genes are mostly not disease-specific. This type of genes has been identified in mouse models as well. An excellent example for such a gene family is NLR-encoding gene family. NLRs are a special group of cytosolic proteins that play an important role in the regulation of host innate immune responses. They are expressed in lymphocytes, macrophages, dendritic cells (DCs) as well as in some non-immune cells such as epithelium [3]. In the most general terms, NLRs are classified into four subfamilies based on the structural similarities of their proteins: NLRA, acidic domain containing; NLRB, baculoviral inhibitory repeat (BIR) domain containing; NLRC, caspase activation and recruitment domain (CARD)

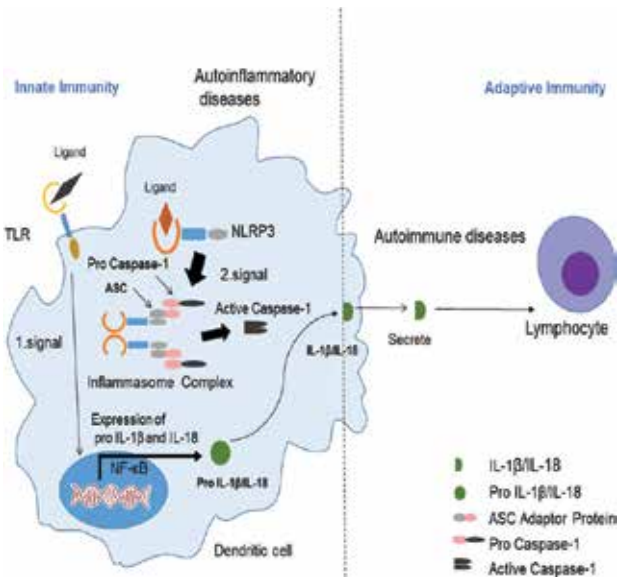

Figure 1. Activation of the inflammasome and its connections between the innate and adaptive immune system.

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containing; NLRP, pyrin domain (PYD) containing; NLRX, with no strong homology to the N-terminal domain of any other NLR subfamily member [4]. After describing the subfamilies of NLR family members, based on the N-terminal region domain, we now describe the other essential domains. A typical NLR protein is composed of three domains. These domains are effector domains in N-terminal (PYRIN, CARD or BIR domains) as just been discussed, a central nucleotide-binding domain (NACHT or NOD domain) and a C-terminal leucine-rich repeats (LRRs). N-terminal effector domains are responsible for interacting with signaling molecules in downstream pathway [5]. The NACHT or NOD domain is responsible for oligomerization of protein and LRRs are required for identification of ligand molecules when there is a potential ligand. LRR domain, on the other hand, acts as a suppressor of NLR activation by preventing activation of the N-terminal domain when no ligand present in the environment, therefore playing a role in the autoregulation of these proteins [6]. Following ligand binding, the auto-regulatory LRR undergoes a conformational change, which then exposes the N-terminal domain, therefore, its interaction with downstream signaling adaptor proteins or effectors and finally the multiprotein complex formation [7, 8].

NLRC4, NLRP3, NLRP6, NLRP1, NLRP12, NLRP7 and the PYHIN family member AIM2 have been shown to form inflammasomes that play a critical role in recognizing pathogen-associated molecular patterns (PAMPS) and danger-associated molecular patterns (DAMPs) and most recently homeostasis altering molecular processes (HAMPs) triggering the immune response [9–11]. Caspase-1 is necessary for the maturation of inflammatory cytokines IL-1β and IL-18 from their pro-forms and eventually the induction of a cell death called as pyroptosis [9, 12]. During activation, the NLR triggers caspase-1 activation either directly by CARD-CARD interaction or indirectly through the adaptor molecule apoptosis-associated speck-like protein containing a caspase recruitment *domain* (ASC). Caspase-1 then cleaves pro-IL-1β and pro-IL-18 leading to their activation and secretion [13]. Although NLRs, including NLRC4, NLRP3, NLRP6, NLRP1, NLRP12, NLRP7 and the PYHIN family member absent in melanoma 2 (AIM2) are suggested to function by forming inflammasomes, other NLRs such as NOD1, NOD2, NLRP10, NLRX1, NLRC5 and CIITA do not function through the formation of inflammasomes but act via the activation of nuclear factor-kB (NF-kB), interferon (IFN) regulatory factors (IRFs) and mitogen-activated protein kinases (MAPKs) to induce innate immune responses [3].

As we will further discuss in the next topic, NLRs have the ability to recognize PAMPs and DAMPs which makes them remarkable molecules to set the activation threshold in case of an infection. Added to these mechanisms of recognition, HAMPs have strikingly, been postulated to have roles in the regulation of inflammasomes. According to the HAMP hypothesis, the pyrin domain (PYD) of a NLR protein is kept inert by a molecular pathway wherein the small GTPase RAS homologue gene family member A (RHOA) activates serine/threonine protein kinase N1 (PKN1) and PKN2, resulting in the subsequent phosphorylation of pyrin on serine 242 [14]. 14-3-3 proteins are a conserved protein family that play roles in many different cellular signaling pathways. They bind pyrin following its phosphorylation, maintaining its inactivated state. In the presence of a PAMP, such as *Clostridium difficile* toxin B (TcdB) pyrin is activated; however, this activation does not result in the activation of the immune system, because pyrin activation is dependent on the function of the toxin, not its structure. TcdB disrupts the RHOA phosphorylation pathway thereby leads to the removal of the 14-3-3, allowing the activation of pyrin (dephosphorylated state). By this mechanism, pyrin can respond to any

microbe infection that changes the RHOA, PKN1 and PKN2, as well as 14-3-3 activity. On the basis of pyrin's ability to sense the alterations in phosphorylation balance which is an altered homeostasis, pyrin is proposed to function not only as a universal sensor of extensive cellular changes, but also as a sensor for a single PAMP or DAMP [9]. This toxin function-based detection mechanism overrides the structural restrictions of the conventional PAMP recognition model. On the other hand, the model of HAMP recognition has some ramifications. A non-pathogenic agent might also alter the cellular phosphorylation processes which will lead to pyrin activation. Defective prenylation causes the inactivation of RHOA and therefore pyrin activation. These individuals with deficient protein prenylation develop hyper-IgG syndrome, which is considered as an auto-inflammatory disease [15, 16]. Sensing HAMPs through pyrin constitutes an example for the ability of NLRP1, NLRP3 and NLRP6 to respond to broad and diverse molecular stimuli. Although the most studied of these sensors is NLRP3, the complete molecular mechanism of action for NLRP3 activation is largely unknown. One of the most recent report demonstrated that NLRP3 is phosphorylated in a similar way to pyrin, suggesting that NLRP3 activation might require the detection of phosphorylation [17]. Furthermore, IL-1β plays a role as an effector molecule as well as a HAMP sensor. Inactive forms of IL-1β is cleaved by caspase-1 after the inflammasome assembly is complete. However, it should be noted that IL-1β can also be cleaved by bacterial proteases. This notable adaptation aids in the efficient clearance of the bacteria. However, the mutations that were acquired by the pathogens can hinder the maturation of IL-1β via cleavage by bacterial proteases [18], therefore they might enhance the invasion by bacteria. The use of IL-1β-inhibiting drugs during infections, does not let the rise of such mutations. In this case, it is clear that activation of innate immune response depends on the detection of protease activity, meaning that the function but not the structure is the determinant of the inflammatory responses, another supporting evidence for the HAMP model. In contrast to the non-mammalian-derived PAMP detection point of view, HAMPs and DAMPs would most likely be generated in the absence of a pathogen, hence would increase the risk of inflammatory diseases and may theoretically contribute to the pathophysiology of inflammatory diseases. The nutrients, growth factors, oxygen and neighboring other cells, surrounding extracellular milieu maintain the homeostasis of cells. The alteration in the components of the environment such as pH, oxygen levels, temperature, concentration of certain molecules disturbs the physiological basal state of cells (i.e. the homeostatic balance) [19]. Altered homeostasis triggers a cellular stress response, resulting in the release of DAMPs as well as HAMP detection by pyrin. Recognition of stress by tissue macrophages activates signaling pathways, including inflammasomes, inducing an inflammatory response to recover tissue functionality during homeostatic imbalance. The inflammation dependent on the tissue-resident macrophages that induces an adaptive response is termed "para-inflammation" [20]. It has been proposed that the para-inflammation is of great importance to the chronic inflammatory responses that are associated with modern human diseases, like autoinflammatory and autoimmune diseases as well as the acute inflammatory responses that will damage the tissue [21]. Although the development of inflammatory diseases resulting from HAMP detection as a pathogen recognition system require more experimental data, ER stress-induced NLRP3 inflammasome activation in chronic liver diseases has been reported [22]. The new discoveries of inflammasome associations with inflammatory diseases remain to be of great interest, however; despite the incremental data, negative

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regulation of inflammasome activation is still poorly understood. As the controlled inflammation is crucial to health, the mechanisms of inflammasome inactivation was evaluated and reported that NLRP3 inflammasome activation was dampened by protein kinase A (PKA), which phosphorylated NLRP3 and hindered its ATPase function. PKA phosphorylation was mediated by prostaglandin E_2 (PGE_2) signaling upon binding the PGE_2 receptor E-prostanoid 4 (EP4) [17]. In the negative regulation of NLRP3, Ser295 in human NLRP3 was found to be significant for immediate inhibition and PKA phosphorylation. The NLRP3-S295A mutation displayed a phenotype similar to the human cryopyrin-associated periodic syndrome (CAPS, an autoinflammatory disease) mutants. These data suggest that negative regulation at Ser295 is essential and important for restricting the NLRP3 inflammasome and define a molecular basis for NLRP3 mutations associated with CAPS [17]. Mutations and variations of NLR proteins are found to be significantly associated with autoinflammatory and autoimmune diseases (**Figure 2**). Another inflammasome is absent in melanoma 2 (hereafter AIM2). AIM2 recognizes dsDNA in a way that does not require a specific sequence. However, to be able to recognize the dsDNA, its length should be at least 80 base pairs [23]. Following DNA binding, AIM2 forms an inflammasome complex with ASC adaptor molecule and caspase-1, resulting in the maturation of pro-IL-1β and pro-IL-18. Uncontrolled recognition of self dsDNA contributes to the development of autoinflammatory and autoimmune diseases such as psoriasis and dermatitis [24]. Importantly, polymorphisms or changes in expression of AIM2 have been associated with systemic lupus erythematosus (SLE) in humans [25]. In mice prone to lupus, inefficient degradation of self-DNA immune complexes in the lysosome let DNA enter the cytoplasm, which then activates the AIM2 inflammasome in macrophages [26, 27]. Vascular damage is one symptoms of SLE, and expression of AIM2 and IL-18 have been reported to increase in endothelial cells from patients with SLE as well as in a mouse model of SLE [28].

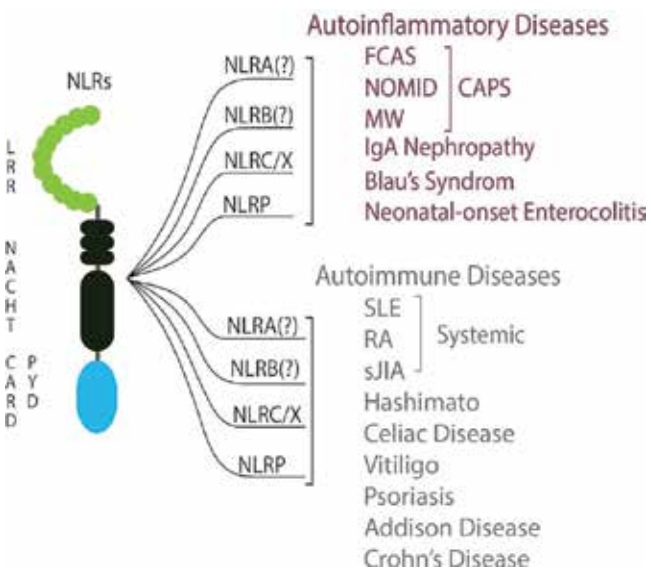

Figure 2. NOD-like receptor subfamilies associated with autoinflammatory and autoimmune diseases.

2. Pattern-associated molecular patterns (PAMPs)

The molecular characteristics of antigen recognition are remarkably different between adaptive and innate immune systems. In the adaptive immune system, random genomic recombination generates antigen receptors that recognize a wide range of antigens, while the innate immune system recognizes pathogens via a set of 20–40 pattern recognition receptors (PRRs) that are germline encoded. Each of these PRR proteins is specialized to recognize a relatively limited collection of pathogen-associated molecular patterns (PAMPs). The PRRs include the toll-like receptor (TLR), NOD-like receptor (NLR), RIG-I-like receptor (RLR) and C-type lectin receptor (CLR) families. Therefore, they are fixed and their ability to recognize rapidly evolving pathogens is quite limited [29, 30]. Sole reliance on recognition of the highly conserved PAMPs by PRRs constitutes a dangerous situation for the host. The past decade has seen a remarkable refocusing in immunology on the cells of the innate immune system, especially macrophages and dendritic cells. A preponderance of evidence suggests that the innate immune system holds more sophisticated recognition mechanisms than originally predicted. In addition to PAMPs, the alternative recognition system involves danger-associated molecular patterns (DAMPs); however, the DAMP molecules, such as ATP, uric acid crystals [31] and extracellular ATP, originate from self. This mechanism basically allows the innate immune system to sense cell death, bypassing the PAMPs [32]. Homeostasis-altering molecular processes (HAMPs) [9] are a newly emerging mechanism, distinct from DAMPs, proposed by Liston et al. Even though both DAMPs and HAMPs are specific to the host's own cells, DAMPs are recognized by PRRs in the same manner as the PAMPs. One important distinction of HAMPs is that, unlike PAMPs and DAMPs, they are not recognized by PRRs. They are the output of an alteration in homeostasis in a living cell, in which case, the innate immune system detects a cellular imbalance rather than a pattern. Intracellular inflammasome complexes provide excellent examples of this mechanism in action, as we discuss later in this chapter.

In contrast to foreign pathogen recognition through PAMPs by PRRs, there is an alternative mechanism for HAMPs (or DAMPs) that can cause inflammation in a sterile manner, resulting in tissue injury in the absence of a pathogen. This generates a potential link between HAMPs and (auto)-inflammatory diseases.

In addition to PAMPs, DAMPs and HAMPs, the term "SAMP" was introduced for self-associated molecular patterns, which could be sensed by innate inhibitory receptors to maintain a steady state level of immune cells and mitigate responses to self-molecule recognition (**Figure 1**) [12]. Host cells produce many different types of plasma membrane molecules that preclude complement reactions from occurring on their cell surfaces. The most important of these molecules is the carbohydrate moiety sialic acid, a common component of cell-surface glycoproteins and glycolipids. Given that they are abundant on cell surfaces and in the extracellular matrix, sialic acid as a self-glycan is the best candidate that fulfills the requirement to be a SAMP molecule. Other candidate SAMPs are glucose amino glycans (GAGs) such as sulfate heparin and dermatan sulfate [33]. As pathogens lack sialic acid, they are selected for destruction by complement pathway, while host cells are protected in the process. Some pathogens, including the bacterium *Neisseria gonorrhoeae* that causes the sexually transmitted disease gonorrhea, cover themselves

with a sialic acid layer to evade from the complement system. Hence, to recognize SAMPs, there might be self-PRRs (SPPRs). One suggested example is an innate component that inhibits the alternative complement pathway, called factor H (FH), which is a serum protein. FH inhibits the alternative complement pathway activation on host cell surfaces by detecting "self" in the form of sialic acid-bearing patterns on cell surfaces. Important residues in the sialic acid binding site are conserved from mouse to man, proposing a potential role for sialic acid as a host marker also in other mammals and a key role in human complement homeostasis [34]. FH recognizes heparin/heparin sulfate GAGs as well as sialic aiding host-non host discrimination by complement pathway [35]. Mutations in the critical residues that are involved in the binding of FH to the sialic acid have been shown to result in the unintended innate immune reactivity [36]. Besides FH, Siglecs (sialic acid recognizing Ig-like lectins) are considered second class of SPRRs for their ability to recognize sialic acid and sending inhibitory signals to innate immune cells. In concert with this observation, Siglec-G deficient mouse displayed overly activated response to DAMPs and PAMPs [37] and mouse eosinophils with deficient Siglec-F gave a hyperactive response [38]. Abundance and dominance of PAMPs and DAMPs indicate that there will most likely be more examples of SAMPs and SPRRs that are evolving to maintain self-glycan recognition.

3. Autoinflammatory diseases

The prevalence of a large group of autoimmune diseases is estimated 3–5% of the general population [39, 40]. The immunological deficiencies are fundamentally driven by a broad spectrum of genes and dysfunctional proteins that are not only limited to NLRs. According to the current literature, immune system encompasses perplex and highly specific interactions between numerous different cell types and molecules. Numerous events must occur prior to a cell-mediated or a humoral immune response is activated, which make these series of events vulnerable to disruptions at several stages by number of factors. Therefore, a broad definition of immune system would be "vast communication network of cells and chemical signals distributed in blood and tissue throughout the human body, which regulates normal growth and development of the organism while protecting against disease".

Immunology emerged from the field of microbiology; hence, generations of immunologists were trained by microbiologists and historically, research in both these fields has addressed the relationship between host and microbe [41]. More than a century ago, Metchnikoff postulated that the primary task of the immune system is not attacking non-self but rather "co-existing with self" or even generation of a multi-cellular organism, despite the internal inconsistencies of its components. When functioning properly, the immune system detects numerous external threats including viruses, bacteria, parasites and stress as well as internal threats, such as tissue injuries, reactive oxygen species (ROS), uric acid crystals distinguishes them from the body's own healthy tissue. Hence, the deregulation of the immune system may result in autoimmune diseases, inflammatory diseases and cancer. In humans, immunodeficiency can result from a genetic disease such as severe combined immunodeficiency (SCID) or can be an acquired condition such as acquired immunodeficiency deficiency syndrome (AIDS), or else the use of immunosuppressive medication can cause immunedeficiencies. The

other end of the spectrum includes autoinflammatory and autoimmune conditions. Michael F. McDermott coined the term "autoinflammatory" at the end of the twentieth century to explain a group of genetic disorders identified by ambiguous, repeated episodes of fever and abnormal chronic inflammation which generally affect skin, eyes, joints, and gut [42]. In autoinflammatory diseases, the innate immune system is the main player, whereas in autoimmune diseases the adaptive immunity is suggested to be the main effector [43]. However, a growing body of evidence shows that this comparison seems to be an over simplification of the differences. A broader and more accurate definition suggested by Wekell and his colleagues is that "*autoinflammatory diseases are defined by abnormally increased inflammation, driven by dysregulation of molecules and cells of the innate immune system with a host predisposition as necessary and sufficient criteria, frequently associated with activation of the adaptive immune system and potentially with immune dysfunctions such as susceptibility to infections, autoimmunity or uncontrolled hyper inflammation*" [44]. The host's genetic background is critical in severe inflammation, immune system-mediated tissue damage and even in recurrent episodes of fever. New genes and proteins have been identified and the list of autoinflammatory diseases is continually growing. Mutations in inflammasome-related proteins, especially in NOD-like receptor (NLR) genes, have been reported to be significantly associated with autoinflammatory diseases. Autoinflammatory diseases would be classified into monogenic and polygenic diseases depending on the genes involved [45]. The examples of monogenic autoinflammatory diseases with inflammasome-related proteins and/or NLR gene associations are Familial Mediterranean Fever (FMF), cryopyrin-associated periodic syndrome (CAPS), familial cold auto-inflammatory syndrome (FCAS), Muckle-Wells syndrome (MWS), Neonatal onset multisystemic inflammatory disorder (NOMID), NALP12-associated periodic fever, Blau's syndrome and Crohn's disease is an example of a polygenic autoinflammatory disease with a NLR association [45, 46]. Therapeutic approaches to treat autoinflammatory diseases include glucocordicoids and non-steroid anti-inflammatory drugs such as colchicine chloroquine, cyclophosphamide, azathioprine, methotrexate, and more recently mycophenolate mofetile. Especially, IL-1 targeting drugs are effective for many of these diseases [47]. The examples of the IL-1 drugs are anakinra, rilonacept and canakinumab. Lastly, the exploration of the multiple steps in the upstream of IL-1β release reveals a number of potential targets at different steps in the pathway. These drugs could be very effective at blocking several common inflammasome-mediated disorders but may not be used in the treatment of autoinflammatory disorders due to mutations in the inflammasome pathway resulting in hyperactive or constitutive activation that is independent of upstream effectors.

4. Autoimmune diseases

Autoimmunity can be result of a hyperactive immune response, fighting against healthy tissues by losing the ability to distinguish the foreign from self. Autoimmune diseases are a large group of at least 80 chronic disorders in which the immune system mounts an immune response against self-tissues and cells [48]. The concept of autoimmunity goes back to the early twentieth century. Paul Ehrlich initially proposed this concept of *horror autotoxicus*,

meaning that a "normal" body does not generate an immune response against its own tissues. In retrospect, Ehrlich was proven wrong, as the presence of autoantibodies and autoreactivity has become clear [49, 50]. Theoretically, autoimmunity is considered as a deficiency of B or T cell selection, with abnormal cell responses to self-antigens [45, 51]. Autoimmune diseases are regulated by a combination of host genes and environmental factors. Both these can contribute to the predisposition to autoimmunity by altering the sensitivity and behavior of the immune system cells. Therefore, it is reasonable to argue that antigen specificity, recognition, expression, as well as the state and the response of the target tissues are influential in the occurrence of autoimmune diseases [48]. There are many ways to classify the autoimmune diseases. However, the most definitive and helpful way to group them would be according to the target tissue or organs that are damaged by the immune system. A few examples of these autoimmune diseases are listed: ((*) NLR-associated diseases)

1. Organ-specific autoimmune diseases: Liver (autoimmune chronic active hepatitis [52]), muscle (myasthenia gravis [53]), blood (autoimmune hemolytic anemia*, autoimmune leukopenia [54, 55]), Gastrointestinal (Crohn's disease* (IBD-C)), food protein intolerance enteropathies (such as gluten sensitive enteropathy celiac disease* [27]), atrophic gastritis of autoimmune type [56] which leads to pernicious anemia [57], Nervous system (multiple sclerosis*, amyotrophic lateral sclerosis [58]), kidney (immune complex glomerulonephritis [59], skin (vitiligo* [60]).
2. Endocrine organ-specific autoimmune diseases: Adrenal gland (Addison's disease [27]), ovaries (premature ovarian failure [61]), thyroid gland (Hashimoto's autoimmune thyroiditis [62]), Graves' disease [63], pancreas (Type I diabetes* [64]).
3. Systemic autoimmune diseases (the "lupus group"): Lupus erythematosus*, rheumatoid arthritis* [27]).
4. Other autoimmune diseases: Wegener's granulomatosis, spontaneous male infertility [65].

In *organ-specific autoimmune diseases*, almost any organ in the body can be the specific target for immune response because of the antigen expressed only in that organ. Likewise, in *endocrine organ-specific autoimmune diseases*, immune system directs its response at the organs that are part of the endocrine system. However, in *systemic autoimmune diseases*, such as systemic lupus erythematosus (SLE), immune response targets antigens broadly expressed throughout the body including the central nervous system, kidneys, and heart. The sera from SLE patients contain antibodies directed against various components in the nuclei of cells, including small nuclear ribonucleoproteins (snRNPs); proteins of the chromosomes' centromeres; and, most markedly, double-stranded DNA. Of all the autoimmune disease categories we discuss in this chapter, there are notable commonalities at each end of the spectrum. As such, thyroid autoantibodies are observed at high frequency in pernicious anemia patients who suffer from stomach autoimmunity. These individuals have a higher prevalence of thyroid autoimmunity than the healthy individuals. The group of rheumatologic diseases also display remarkable common features at the other end of the spectrum. Characteristics of rheumatoid arthritis have a number of resemblances with the clinical features of SLE. In these diseases, immune

complexes are accumulated consistently in the kidneys, joints, and skin. Finally, *other autoimmune diseases* include the diseases that do not belong to any of the aforementioned groups. This is by no means an entire listing of autoimmune diseases, and whether some diseases are completely or partly autoimmune is controversial. Most of these diseases are either is a result of serum antibody increase in host, immune-complex deposition in host tissues, high frequency of tissue eosinophils, or elevated infiltration of lymphocytes to target tissues. Because of these immune reactions that take place in host, target tissues are injured in way that may or may not be preventable and moreover may or may not be reversible. Currently used therapies involve glucocorticoids and non-steroid anti-inflammatory drugs. Chloroquine, cyclophosphamide, azathioprine, methotrexate, as well as mycophenolate mofetile, anti-TNF agents (anti-TNF monoclonal antibody), and anti-inflammatory cytokines such as IL-10 and TGF-beta are listed as autoimmune disease treatment approaches [66].

5. Autoinflammatory mechanisms in autoimmune diseases

Approximately 500-million-year-old adaptive immune system recognizes "non-self" substances through the immunoglobulins that are produced by plasma cells and/or T cell receptor interactions with major histocompatibility complex (MHC)/peptide complexes. Cells of the more ancient innate immune system carry receptors that recognize foreign glycans, certain motifs from pathogens [67]. The adaptive immune system is signaled into action by the innate immune system for the optimal host defense [68], therefore it is reasonable to consider the involvement of autoinflammatory mechanisms in autoimmune diseases. By and large, in autoinflammatory diseases, tissue and organ destructions are mediated by cytokine production by macrophages and granulocytes such as neutrophils, whereas in the pathogenesis of autoimmune diseases, tissue and organ damage is mediated by hyper-activation of T and B lymphocytes, and the production of autoantibodies. However, the innate immune system has an effect on the differentiation of immune cells of the adaptive system. The inflammasome-driven innate cytokines Interleukine-1beta (IL-1β) and IL-18 play roles in the differentiation of T helper subsets Th1 or Th17 by the upregulation of receptors like the IL-2 receptor, expands the lifespan of T cells, and also augmentation of B cell proliferation and antibody production. In the classical autoimmune disease systemic lupus erythematosus (SLE), autoinflammatory reactions have roles in a subset of SLE patients. TREX1 endonuclease gene mutations leads to an increase in the levels of cytosolic DNA which is then recognized by toll-like receptor 7 (TLR7) and TLR9, resulting in the expression of interferon-alpha (IFN-α) [69, 70]. IFN-α enhances the dendritic cell (DC) maturation and activation which causes the subsequent activation of B cells and antibody production [41]. Most recent studies underlie the control of adaptive immunity by innate immune responses that activated DCs have been shown to favor the Th17 cell differentiation from naive T helper cells through the activation of NLRP3 inflammasome complex [71]. Our understanding of immune deficiencies that share the prefix "auto-"resulting from dysfunctional NLRs and inflammasomes has broadened considerably over the past decade. In the next section, NLRs and inflammasomes will be discussed in detail due to their involvement in the progression of autoinflammatory and autoimmune diseases.

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6. NLRs in autoinflammatory diseases

Numerous autoinflammatory diseases have been strongly linked with gain-of-function mutations or variations in inflammasome-forming NLRs (NLRP1, NLRP3, NLRP6, NLRP7, NLRP12, NLRC4, and NAIPs) and non-inflammasome-forming NLRs (NOD1/2, NLRP10, NLRX1, NLRC5, and CIITA) [72]. Here, we are going to examine NLR proteins individually for the autoinflammatory diseases in which they are involved.

1. NLRA subfamily:

    a. Class II transactivator (CIITA): CIITA is a human gene which encodes class II, major histocompatibility complex (MHC), transactivator. MHC CIITA was discovered in 1993 as the gene associated with hereditary major histocompatibility complex Class II deficiency, also mutations in CIITA gene were found to be responsible for the bare lymphocyte syndrome in which the immune system is highly compromised and cannot effectively mount a counterattack against the infection [73]. Mainly lymphocytes, dendritic cells, macrophages, and other professional antigen presenting cells are known to express CTIIA. To date, a number of autoimmune diseases *but not* autoinflammatory diseases have been reported to be linked to CIITA gene. Later in this chapter, we will revisit the CIITA involvement in the development of autoimmune diseases.

2. NLRPB subfamily:

    a. Neuronal apoptosis inhibitory protein (NAIPs): The first discovered inhibitor of apoptosis protein (IAP) in mammals was NAIP. Mutations and deletions of the NAIP gene have been associated with the spinal muscular atrophy (SMA) phenotype [74]. Like CIITA protein, NAIP was speculated to be involved in autoimmune reactions rather than autoinflammation. In mice different paralogues of NAIP determine the specificity of the NLRC4 inflammasome assembly for distinct bacterial ligands. Innate immune recognition of bacterial ligands by NAIPs determines inflammasome specificity, therefore NAIP has important contributions to the inflammatory reactions. Yet, the involvement of NAIPs in autoinflammation requires further research.

3. NLRC/X subfamily:

    a. NOD1/2: NOD1 and NOD2 are the protein products of CARD4 and CARD15 genes, respectively. The studies focusing on NOD1 and NOD2 primarily involves their signaling activities. The peptidoglycan components diaminopimelic acid (DAP) and muramyl dipeptide (MDP) from Gram-negative and Gram-positive bacteria are recognized by NODs [75]. NOD1 and NOD2 have been associated in a multitude of inflammatory diseases. Especially mutations and SNPs in CARD15 have been associated with Blau Syndrome which is characterized by arthritis, uveitis, and skin rash [76, 77]. It is plausible to suggest that a gain of function mutation of NOD2 in Blau's syndrome is leads to a continuous pro-inflammatory state. Patients are treated with oral steroids and immunosuppressive drugs such as cyclosporine, methotrexate with variable results [46, 78].

b. NLRC3, 5, and NLRX1: Although, they are listed in this subfamily, their associations or their functional contributions to the pathogenesis of autoinflammatory diseases have not been reported yet. NLRC5, as one of the newest additions to the NLR family; NLRX1 as a unique NLR in that it carries an N-terminal mitochondrial targeting sequence [79], are known to be involved in inflammatory processes and the latter enhances the reactive oxygen species (ROS) production. However, their effect on human health and diseases remains to be elusive.

c. NLRC4: Interestingly, a de novo gain-of-function mutation in NLRC4 was found to co-segregate with a disease. The disease is characterized by neonatal onset enterocolitis, periodic fever, and fatal or near-fatal episodes of autoinflammation Over activating mutation in NLRC4 leads to the constitutive production of IL-1FC and macrophage cell death through pyroptosis. These results suggested a novel role for NLRC4 inflammasome in causing a debilitating but treatable autoinflammatory disease [80].

4. NLRP subfamily:

a. NLRP1: The NLRP1 protein has a distinct structure as compared to other NLRs. Human NLRP1 has a PYD on the N terminus and a CARD on the C-terminus, with ZU5 and UPA domains in the internal region which is attributed to proteolytic activity [81]. Most recently, it was demonstrated that cytosolic double-stranded (ds) DNA triggered the activation of caspase-5 in keratinocytes and subsequent release of IL-1β. Moreover, interleukin-17A enhanced caspase-5 function through priming of NLRP1-inflammasome. In the study, anti-inflammatory vitamin D have been shown to prevent the IL-1β release and to suppress caspase-5 in keratinocytes and in psoriatic skin lesions. The NLPR1-dependent caspase-5 activity in psoriasis was suggested by exploring potential therapeutic targets in Th17-mediated skin autoinflammation [82]. Furthermore, another group has recently demonstrated that human NLRP1 is involved in a novel autoinflammatory disorder that researchers propose to call NAIAD for NLRP1-associated autoinflammation with arthritis and dyskeratosis. This disease could be a novel autoimmuno-inflammatory disease having both autoinflammatory and autoimmune characteristics [83].

b. NLRP3: Among all the NLRs, NLRP3 by far the most studied inflammasome. It is mostly expressed in the cells of innate immunity such as splenic neutrophils, macrophages, monocytes, and dendritic cells [84]. NLRP3 has been linked to autoinflammatory diseases by several research groups. Gain-of-function mutations in the NLRP3 inflammasome lead to the increased production of IL-1β and cause (CAPS) [85]. CAPS are a large arsenal of diseases classified as familial cold auto-inflammatory syndrome (FCAS), Muckle-Wells syndrome (MW) and neonatal onset multisystem inflammatory disorder (NOMID). These three CAPS are distinguished from one another based on their phenotypic severity. These diseases are basically identified by inflammation affecting skin, joints, eyes, bone, muscles, and central nervous system as a result of increased IL-1β production. There have been over 50 different NLRP3 mutations identified and supression of IL-1β by anakinra, rilonacept, or canakinumab help mitigate clinical symptoms [27]. IgA nephropathy is another disease which is characterized by leukocyte and lymphocyte infiltration in the glomerulus. It is demonstrated that NLRP3 inflammasome localization to mitochondria in tubular epithelium has a crucial role in the progress of this pathology [86].

c. NLRP12: NLRP12 gene mutations have been found in a group of patients with clinical manifestations identifiable with CAPS, such as recurrent fever and cold sensitivity associated with added symptoms such as neuronal hearing loss, lymphadenopathy, abdominal pain, and acute phase response. These patients did not have mutations at the NLRP3 locus [87].

7. NLRs in autoimmune diseases

To date, many genes have been reported to operate in the development of autoimmunity and modification of inflammation of specific tissues; however, we will continue to focus on the NLR family members that are significantly associated with autoimmune diseases. As discussed in the previous section, it is essential to note that there are overlapping NLRs in the development of both autoinflammatory and autoimmune diseases.

1. NLRA subfamily:

a. CIITA: Genome-wide association studies (GWAS) and whole exome sequencing studies have found SNPs in CIITA that are linked to celiac disease [88], which is characterized by destruction of the lining of the small intestine by T cells reactive to certain dietary molecules [27]; rheumatoid arthritis which is caused by chronic inflammation of the synovial membrane in the joints [89]; multiple sclerosis (MS) in which autoreactive T cell infiltration in central nervous system results in the destruction of myelin sheaths covering the nerve cells [90]; SLE, a disease where immune response (autoantibodies) against self-antigens affect multiple organs and tissues [91], and type-1 diabetes which is characterized by infiltration of T cells to the pancreatic islets resulting in the destruction of β cells that are responsible for insulin production [92]. Despite the presence of several studies on the association of CIITA gene to a variety of autoimmune diseases, these results were not always reproducible. These variations among the studies were suggested to be related to the age-dependent variation in CIITA gene [92, 93].

2. NLRB subfamily:

a. NAIP: It is a critical component of the NLRC4 inflammasome and important for the detection of bacterial components, as well as the scaffolding of the NAIP-NLRC4 inflammasome. The expression of the IAP family of anti-apoptotic protein encoding genes in peripheral blood samples and brain tissues from MS patients suggest a role for differential regulation of these proteins in the pathology of MS. As a member of IAP family, NAIP mRNA was found to increase in whole blood [94].

3. NLRC/X subfamily:

a. NOD1/2: The most common mutation of NOD2 is a frameshift mutation in the LRR region of the receptor that causes the Crohn's disease [88]. The disease is caused by autoreactive T cells against intestinal flora antigens, while the mutations conferring susceptibility to Blau syndrome were reported in the NOD region of the same receptor [76].

b. NLRC3 and NLRX1: Despite the absence of reports on the association of NLRC3 and NLRX1, there are studies that focused on these 2 NLRs in the context of SLE. The

mitochondrial anti-viral signaling protein (MAVS) is required for anti-viral defense of innate immunity. Melanoma differentiation-associated protein 5 (MDA5) is a retinoic acid inducible gene-I (RIG-I) receptor that recognizes viral dsRNA and undergoes a conformational change which then induces the activation of MAVS, resulting in the type I interferon production [95]. A considerable fraction of patients who suffer from SLE display MAVS aggregation in their peripheral blood cells and that the type-I interferon production contributes to the SLE development. It has been suggested that NLRC3 plays inhibitory roles during inflammation and it may interact with the RIGI-MAVS pathway through stimulator of interferon genes (STING) [96]. Thus, the authors compared and found the same levels of NLRC3 and NLRX1 in the aggregates-positive and aggregates-negative groups of SLE patients, suggesting no involvement of NLRC3 and NLRX1 in SLE development [97].

4. NLRP subfamily:

a. NLRP1: GWAS and candidate gene analysis studies provided data regarding the association of *NLRP1* variants with vitiligo alone and vitiligo-associated multiple autoimmune diseases. This disease is characterized by the absence of melanocytes in the epidermis which is observed as white patches on the skin. Mutations of NLRP1 were detected in the promoter and/or coding regions of NLRP1 [98]. The functional role of SNPs in NLRP1 is not clear, so the processes linking NLRP1 variations and vitiligo remains unclear. However, the expression of NLRP11 in T cells and Langerhans cells suggest a role for NLRP1 in skin autoimmunity [99]. In addition to vitiligo, NLRP11's involvement in other autoimmune diseases has been noted, including Addison's disease that is characterized by destruction of adrenal cortex and type-1 diabetes [100], celiac disease [101], autoimmune thyroid disorders (aka Hashimoto's Thyroiditis) results from the destruction of thyroid tissue that leads to hypothyroidism [62, 102], systemic lupus erythematosus (SLE), and rheumatoid arthritis [103].

b. NLRP3: Given the abundance of studies conducted to decipher the roles of NLRP3, more evidence-based report is available for the associations of NLRP3 both in autoinflammatory and autoimmune diseases. SNPs in the NLRP3 gene have been linked to a wide variety of autoimmune diseases among which are type-1 diabetes and celiac disease [104], psoriasis [105].

c. NLRP2, 9, 11: SNP array analysis in 50 patients with systemic Juvenile Idiopathic Arthritis (s-JIA) showed many disease-related copy number variations (CNVs). Notably, most of them were inherited from either of normal-phenotype parents. In one patient, authors were able to identify two de novo micro-duplications at 19q13.42. The duplications span NLRP2, NLRP9, and NLRP11, also IL-11 and HSPBP1, all of which function in inflammatory pathways. These genes have been suggested be involved in the pathogenesis of s-JIA11 [106].

8. Concluding remarks and future perspectives

Our understanding of how NLRs drive autoimmunity has advanced tremendously in the last decade. Even so, many questions remain unaddressed, mainly because a plethora of different parameters are responsible for the predisposition to autoimmune diseases and the precipitation of such illnesses. In this chapter, we discussed the subject of autoimmunity with respect to NLRs in an attempt to clarify their connection to autoimmunity. The molecular genetics of inflammasomes have been intensively studied in both autoinflammatory and autoimmune diseases and these studies identified mutations in genes encoding NLRs or polymorphisms that cause the development of such diseases. With the advent of new technologies such as genome-wide screening and next generation sequencing, we can now evaluate the pathogenesis of autoinflammation-related diseases from a more holistic point of view. The potency of NLRs in mounting an immune response is crucial for the host, but can also be the reason for life-threatening health problems when inappropriate responses occur. Ever increasing new data from large scale studies deepen our current knowledge on the roles of NLRs, however the function of several of the NLRs remains unclear. In particular, a long-standing question is how NLRs interact with a variety of structurally different ligands. Furthermore, the presence of layers of regulatory pathways and different binding partners make it even more perplexing. Our hope and expectations are that discovery of the complete portfolio of hidden cellular activities that NLRs mediate will tell us how these innate immune molecules function to regulate immunity and will ultimately lead to new, more effective life-saving therapeutic drugs for treatment of autoinflammatory and autoimmune diseases.

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Chapter 6

Physiology and Pathology of Innate Immune Response Against Pathogens

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Additional information is available at the end of the chapter

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Abstract

Pathogen infections are recognized by the immune system, which consists of two types of responses: an innate immune response and an antigen-specific adaptive immune response. The innate response is characterized by being the first line of defense that occurs rapidly in which leukocytes such as neutrophils, monocytes, macrophages, eosinophils, mast cells, dendritic cells, etc., are involved. These cells recognize the pathogen-associated molecular patterns (PAMPs), which have been evolutionarily conserved by the diversity of microorganisms that infect humans. Recognition of these pathogen-associated molecular patterns occurs through pattern recognition receptors such as Toll-like receptors and some other intracellular receptors such as nucleotide oligomerization domain (NOD), with the aim of amplifying the inflammation and activating the adaptive cellular immune response, through the antigenic presentation. In the present chapter, we will review the importance of the main components involved in the innate immune response, such as different cell types, inflammatory response, soluble immune mediators and effector mechanisms exerted by the immune response against bacteria, viruses, fungi, and parasites; all with the purpose of eliminating them and eradicating the infection of the host.

Keywords: innate immune response, eosinophils, mast cells, cytokines, inflammatory response, bacteria, fungi, viruses, parasites

1. Introduction

The immune system consists of a series of effector mechanisms capable of destroying pathogenic organisms such as bacteria, fungi, viruses, and parasites [1]. The immune system consists

of two types of responses: an antigen-specific adaptive immune response and an innate immune response, also called natural, which recognizes pathogen-associated molecular patterns (PAMPs) [2]. These PAMPs are recognized by pattern recognition receptors (PRRs), mainly expressed in the innate immunity cells. PRRs can also recognize host molecules containing damage-associated molecular patterns (DAMPs), molecules that are often released from necrotic cells damaged by invading pathogens [3].

The innate immune system is composed mainly of physical barriers, such as skin and mucous membranes, chemical barriers, through the action of antimicrobial peptides and reactive oxygen species [4], innate immune cells, and soluble mediators such as the complement system, innate antibodies, and associated cytokines [2].

The main purpose of the innate immune system is: (1) to prevent the entry of pathogens into the body through physical and chemical barriers [4]; (2) to avoid the spread of infections through the complement system and other humoral factors; (3) to remove pathogens through phagocytosis and cytotoxicity mechanisms [5]; and (4) to activate the adaptive immune system through the synthesis of several cytokines and antigen presentation to T and B cells [6].

2. Innate immune system cells

The cells of the innate immune system have several functions that are essential for defense against pathogens. Some cells form physical barriers that impede infections. Several cell types express the various PRRs that recognize PAMPs and DAMPs, which respond by producing inflammatory cytokines to kill microbes or infected cells. These cells include nonmyeloid cells, myeloid cells, and some lymphoid cells.

2.1. Nonmyeloid cells

Nonmyeloid cells include epithelial cells, fibroblasts, etc., that basically form a barrier between the internal and external environment. These cells produce antimicrobial substances that hinder the entry of pathogens [1, 2]. These antimicrobial substances are called antimicrobial peptides (AMPs), and they are essential components of the innate immune response, which contribute to the first line of defense against infections [7]. In humans, AMPs are classified into three main families: defensins (α and β), cathelicidin, and statins. AMPs have a wide spectrum of antimicrobial activity, exerting their functions through electrostatic interactions between their positive charge and the negative charge that certain pathogens have on their cell wall. AMPs mediate the inflammatory response allowing cytokine release, cell proliferation, angiogenesis, wound healing, and chemotaxis [8]. Currently, their synergistic activity with antibiotics used in the clinic has been demonstrated. Therefore, their study on potent adjuvants in the eradication of bacterial infections continues to be studied [9].

2.2. Myeloid cells

Myeloid cells include monocytes, macrophages, dendritic cells (DCs), neutrophils, eosinophils, basophils, mast cells, and platelets. All these cells have specialized functions for defense against invading pathogens [2, 10].

2.2.1. Monocytes

Monocytes are cells that develop in the bone marrow, and they are released into the bloodstream to circulate for approximately 72 hours and then emigrate to different tissues where they differentiate into macrophages or DCs. They represent the major type of mononuclear phagocytes found in blood and are members of the myeloid cell family [11]. In humans, monocytes are classified into classical and nonclassical depending on their surface expression of cluster of differentiation (CD)-14 and CD16. Classical monocytes with phenotype $CD14^{+}CD16^{-}$ are considered inflammatory cells representing more than 92% of total monocytes. In contrast, nonclassical monocytes with $CD14^{+}CD16^{+}$ phenotype can eliminate debris from the vascular system and produce low levels of proinflammatory cytokines, as well as high levels of anti-inflammatory factors. Several studies have shown both subpopulations under inflammatory conditions; the inflammatory response is a gradual process which starts with the main appearance of classical monocytes, and a few days later, nonclassical monocytes appear [12]. Among the main monocyte functions, is their involvement in the innate immune response against pathogens and during inflammatory processes, in which blood monocytes migrate to the infection site, where the process occurs, and they mature into macrophages or DCs to participate as phagocytes as either by digesting pathogens or cellular debris [13]. In addition, monocytes are antigen-presenting cells (APCs) known for their participation in the antigenic presentation through major histocompatibility complex (MHC) to T cells, also cooperating in the activation of the adaptive immune response [14].

2.2.2. Macrophages

Monocytes are precursor cells that are produced in the bone marrow, which are mobilized into the bloodstream and then differentiate into macrophages at the site of inflammation [15]. Macrophages are a very heterogeneous cell population, such as effector cells of the innate immune system, which play an important role in a host's defense and inflammation. In general, macrophages can be divided into two populations: resident and inflammatory macrophages [16]. Resident macrophages are found in almost all tissues and contribute to their development, as well as immunological surveillance, homeostasis, and tissue repair [17, 18]. On the other hand, inflammatory macrophages are derived from circulatory monocytes and rapidly infiltrate tissues compromised by injury or infection. In response to several signals from the microenvironment, macrophages can be activated and adopt different functions: M1 macrophages (classically activated macrophages) and M2 macrophages (alternatively activated macrophages) [19, 20]. M1 macrophages have proinflammatory functions and participate in a host's defense against pathogens and tumoral cells [21], and it is considered that

they promote the Th1 immune response. When M1 macrophages are activated by interferon (IFN)-γ, granulocyte macrophage colony-stimulating factor (GM-CSF), or other ligands of Toll-like receptor, these macrophages produce proinflammatory cytokines such as interleukin (IL)-1β, IL-12, and tumor necrosis factor (TNF)-α, chemokine (C–C motif) ligand (CCL)-15, CCL20, C-X-C motif chemokine (CXC)-8-11 and CXCL13 and reactivate species of nitrogen and oxygen [22], increase the complement-mediated phagocytosis as their main purpose is to kill intracellular pathogens. In contrast, M2 macrophages are associated with tissue remodeling and tumor progression and have an immunoregulatory effect. M2 macrophages express IL-10, IL-1 receptor antagonist, chemokines (e.g., CCL22 and CCL17), transforming growth factor (TGF)-β, mannose, and galactose receptors and possess efficient phagocytic activity. M2 macrophages are considered to promote the Th2 immune response and antagonize the inflammatory response and its mediators [23, 24].

Macrophages possess a wide range of surface receptors, which gives them an ability to recognize a wide range of endogenous/exogenous ligands to respond adequately, which is critical in these cells. These receptors include Toll-like receptors (TLRs), NOD-like receptors, retinoic acid-inducible gene (RIG)-I family, lectins, and scavenger receptors, which recognize PAMPs, DAMPs, foreign substances, and dead or damaged cells [25–27]. During the inflammatory response by pathogens, macrophages activated with an inflammatory phenotype produce several inflammatory mediators, such as TNF-α, IL-1, IL-6, and INF-γ, which are involved in the activation of microbicidal mechanisms contributing to the pathogen elimination. The inflammatory response of macrophages comprises mainly four stages: (1) recognition of the infectious agent through the macrophages PRRs; (2) in situ recruitment and proliferation of macrophages into infected tissue; (3) elimination of the infectious agent; and (4) the conversion to M2 macrophages to restore damaged tissue [28].

2.2.3. Dendritic cells

Monocytes circulate in the blood, bone marrow, and spleen [29, 30] and represent immune effector cells equipped with chemokine and adhesion receptors that mediate cell migration from blood to tissues during infection. Monocytes produce inflammatory cytokines and phagocyte, both cells and toxic molecules. Monocytes can differentiate into inflammatory DCs during inflammation. Migration to tissues and differentiation to inflammatory DCs depend on the inflammatory environment and PRRs [31]. These PRRs, including the TLR family, are capable to recognize PAMPs, on the surface of bacteria, viruses, fungi, and parasites [29].

DCs represent an important link between innate and adaptive immunity [2]. DCs are heterogeneous population of antigen-presenting cells that are crucial to initiate and polarize the immune response. Although, all DCs are capable of capturing, processing, and presenting antigens to T cells, DCs subtypes differ in origin, location, migration patterns, and specialized immunological roles [32]. There are mainly two subtypes of DCs: classical DCs and plasmacytoid DCs. The classical DCs are cells specialized in the processing and presentation of antigens, with high phagocytic activity as immature cells and high cytokine-producing capacity as mature cells [26]. Classical CDs are highly migratory cells that can move from tissues to the T cell and B cell zones of lymphoid organs. Classical DCs regulate T cell responses both at steady state and during infection. They are usually short-lived and replaced by blood-borne precursors [33, 34].

On the other hand, plasmacytoid DCs differ from classical DCs in that they are relatively long-lived [35]. Plasmacytoid DCs are present in the bone marrow and in all peripheral organs, and they are specialized to respond to viral infection with massive production of type I interferons (IFNs). However, they can also act as antigen presenting cells and control T cell responses [36].

2.2.4. Neutrophils

In humans, about 100 billion neutrophils enter the bloodstream each day [37]. Neutrophils originate from hematopoietic stem cells in response to both extracellular stimuli and intracellular regulators. They come from the myeloid cell line in the formation of granulocytes. The granulopoyesis that occurs in the bone marrow is initiated when the neutrophils myeloblasts (MB) develop in promyelocytes (PM), characterized by a round nucleus and presence of azurophil granules. Subsequently, they mature into myelocytes with specific granules, maturing to metamyelocytes (MM), cells composed by a nucleus with kidney form. Metamielocitos mature to band cells (CB) and in segmented cells (CS) also known as polymorphonuclear cells (PMNs). The PMNs are then called from their segmented nucleus, which are finally released into the bloodstream [38, 39]. Neutrophils play a major role in the resolution of microbial infections. After pathogens break into epithelial barriers, neutrophils are the first cell line of defense for the innate immune response, which are recruited from the bloodstream to the site of infection. Neutrophils cross the blood vessels and migrate to the infection site with the help of chemotactic factors and cytokines, which are produced as inflammatory signals during the tissue damage caused by the invading pathogens. Neutrophils reach the infection site and initiate the phagocytosis process through recognition of PAMPs by their receptors such as TLRs. Neutrophils exert their antimicrobial actions through the release of reactive oxygen species and cytotoxic components contained in their granules such as AMPs [40]. Likewise, neutrophils using a mechanism called extracellular traps (NETs) composed of DNA fibers, which are formed and released into the extracellular space, are used by the innate immune system to destroy and eliminate pathogens [41]. However, studies have shown that neutrophils NETs are involved in the development of several pathologies [42–44]. Finally, neutrophils can also regulate the adaptive immune response, as they mediate suppression of T cells proliferation as well as their activity. Neutrophils can also stimulate and activate splenic B lymphocytes [45].

2.2.5. Eosinophils

Eosinophils are produced in the bone marrow from pluripotent stem cells, which first differentiate into a precursor for basophils and eosinophils and then differentiate into an eosinophilic lineage [46]. IL-3, IL-5, and GM-CSF are particularly important in regulating the eosinophils development [47–50]. Of these three cytokines, IL-5 is the most specific for the eosinophilic lineage and is responsible for the selective differentiation [51] and release of eosinophils from the bone marrow into the peripheral circulation [52]. IL-5 plays a critical role in the eosinophils production, as the overproduction [53, 54] and neutralization [55–57] of this cytokine are associated with a significant increase or decrease in eosinophilia, respectively.

Eosinophils are multifunctional leukocytes involved in the pathogenesis of numerous inflammatory processes [58], including parasitic helminths infections and allergic diseases [59–61]. Under basal conditions, most eosinophils traffic into the gastrointestinal tract where

they normally reside within the lamina propria, whose production is independent of lymphocyte production [62]. Recruitment of gastrointestinal eosinophils is regulated by the constitutive expression of eotaxin-1 [63], a chemokine involved in allergen-induced eosinophil responses [64].

In response to several stimuli, such as immunoglobulins, cytokines, and complement system, eosinophils are activated and recruited from the circulation to the site of inflammation [65]. The trafficking of eosinophils into inflammatory sites involves various cytokines derived from a Th2 immune response such as IL-4, IL-5, and IL-13 [66, 67], adhesion molecules (e.g., β1, β2, and β7 integrins) [68] and chemokines (e.g., eotaxins) [69]. Once at the site of inflammation, eosinophils can modulate the immune response through the secretion of several proinflammatory mediators such as IL-2, IL-6. IL-8, TGF-α/β, GM-CSF, TNF-α, INF-γ, as well as chemokines and lipid mediators, such as platelet-activating factor (PAF) and leukotriene (LT)-C4 [70], which exert proinflammatory effects as positive regulation of adhesion systems, modulation of cellular trafficking, activation and regulation of vascular permeability, mucus secretion, and smooth muscle constriction. In addition, eosinophils can serve as effector cells, which can induce tissue damage by releasing a diverse of cationic proteins from their cytotoxic granules, major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), and neurotoxin derived from eosinophils (EDN) [59]. These proteins are very important, because they are directly related to the effector functions of eosinophils. For example, ECP is involved in the suppression of T cell proliferative responses, and the synthesis of immunoglobulins by B cells induces mast cell degranulation and stimulation of mucus secretion in the airways, as well as the production of glycosaminoglycans by human fibroblasts [71], while EPO is associated in the formation of reactive oxygen species and reactive nitrogen metabolites. These molecules promote oxidative stress and subsequent cell death by apoptosis and necrosis [72–74].

In addition to the multiple effector actions of eosinophils, these cells can initiate antigen-specific immune responses by acting as APCs [75, 76], as they can process and present a variety of bacterial [77], viral [78], and parasitic [79] antigens. Although investigations demonstrated a direct association of eosinophils with parasitic helminths infections, establishing the hypothesis that eosinophils are the classic effector cells in a host's defense [80]. Several studies have also shown that the eosinophils absence during parasitic helminths infections protects the host [81], so that eosinophils may influence the immune response in a manner that supports chronic infection and ensures survival of the parasite in the host [82–84].

2.2.6. Basophils

Basophils are cells derived from the myeloid hematopoietic progenitors in the bone marrow, and they are phenotypically and functionally distinct from other leukocytes, including mast cells, since mast cells reside in tissues while basophils reside in the circulation and can be recruited to the tissues [85–89]. Basophils have the ability to bridge innate and adaptive immunity, including the capacity to induce and propagate Th2 immune responses [90]. Basophils are important in all allergic diseases, including anaphylaxis, allergic rhinitis, asthma, urticaria, and food allergies. Basophils rapidly release histamine and synthesize LTC4 after that immunoglobulin (Ig)-E binds to their receptor FcεRI and subsequently produces Th2

cytokines such as IL-4 and IL-13 [91–95], causing the clinical symptoms of immediate hypersensitivity, also promoting delayed hypersensitivity reactions [96–99]. The role of basophils in protective immunity against helminths is well known [96, 100]. However, recently, basophils have also been implicated in the initiation of immune responses against bacterial respiratory infection [101].

2.2.7. Mast cells

Mast cells are granulated tissue-resident cells from $CD34^+$ hematopoietic progenitor cells [102, 103]. Mast cells circulate as immature cells and migrate to vascularized tissues, where they complete their differentiation. Mast cells represent, together with dendritic cells, the first immune cells that interact with environmental antigens, pathogens, and toxins. Therefore, they can be considered "sentinels" of the innate immune system [104]. Mast cells are activated by danger stimuli, which they react by rapidly releasing a wide range of mediators, both preformed and newly produced. Some of these mediators (e.g., histamine, TNF-α, vascular endothelial growth factor, VEGF) contribute to local vascular permeability and edema at the site of inflammation [105], while chemokines (e.g., IL-8/CXCL8, eotaxin) induce the recruitment of other immune cells [106], such as neutrophils, natural killer (NK) cells, and eosinophils. It is important to note that mast cells may also be involved in the defense against pathogens by different mechanisms, such as phagocytosis, antimicrobial peptide release, or the production of extracellular traps similar to those described in neutrophils [107, 108]. Mast cells detect these invading pathogens through PRRs, such as TLRs [109]. Investigations have shown that bacterial and viral proteins can activate mast cells through specific receptors [110, 111].

Mast cells express the high affinity receptor for IgE (FcεRI) [90, 112]. Cross-linking of the FcεRI by IgE-antigens and/or allergens complexes induces mast cell activation and rapid release of proinflammatory mediators via degranulation. Due to this property, together with circulating basophils, mast cells are known primarily as effector cells for IgE-mediated (Th2-like) responses [113], an arm of the adaptive immune system against helminths infection [114], and as primary effector cells in hypersensitivity reactions [115]. In addition to their functions as effector cells, recent evidence suggests that mast cells are capable to modulate both the innate and adaptive immune response, acting as immunomodulatory cells [116, 117].

2.2.8. Platelets

Platelets are cytoplasmic fragments (1 to 4 μm in diameter) produced as a result of fragmentation from megakaryocytes that are cells from bone marrow. Platelets are non-nucleated organelles that have functional characteristics like complete cell, since they possess cytoskeleton, mitochondria, Golgi residues, and endoplasmic reticulum involved in the synthesis of enzymes, storage of calcium ions, as well as storage granules [118, 119]. These storage granules are δ-granules [120], α-granules, and lysosomal granules [121], which play an important role in homeostasis, inflammation, wound healing, and cell-matrix interactions. During the inflammatory response, platelets can be activated through their receptors, which act as adhesion molecules that interact with damaged endothelium, other platelets and leukocytes,

playing an important role in the coagulation process for repairing the damaged blood vessel and restoring its integrity [122–124].

2.3. Lymphoid cells

Lymphoid cells include the NK cells, natural killer T (NKT) cells, and innate lymphoid cells (ILCs). ILCs are a novel family of hematopoietic effectors that serve protective roles in innate immune responses to infectious microorganisms, in lymphoid tissue formation, in tissue remodeling after damage inflicted by injury or infection and in the homeostasis of tissue stromal cells [125].

2.3.1. Innate lymphoid cells (ILCs)

ILCs represent the innate version of helper and cytotoxic T cells as part of the innate immune system, which play essential roles in the early immune response [126, 127]. All members of the ILCs family are characterized by a classical lymphoid cell morphology and the expression of IL-7Ra (CD127) and CD161, but they lack the expression of cell surface molecules that characterize other types of immune cells such as T cells (CD3, TCR$\alpha\beta$, and TCRδ), B cells (CD19), NK cells (CD16 and CD94), myeloid cells (CD1a, CD14 and CD123), granulocytes (FcεR1α and CD123), stem cell hematopoietic (CD34), and plasmacytoid dendritic cells (BDCA2 and CD123), so they are defined as cells that do not express lineage markers (Lin-) [128]. ILCs can be classified based on their phenotypic and functional characteristics in three groups: Group 1 (ILC1) comprises cells that have the ability to produce IFN-γ as their major effector cytokine and express the T-bet transcription factor. The prototype cell of this group is the NK cell. Group 2 (ILC2) are cells that require IL-17 for their development. These cells are characterized by cytokine production associated with the Th2 immune response, in response to stimulation with IL-25, IL-33 and thymic stromal lymphopoietin (TSLP) and shows a GATA3 and RORα phenotype for their development and function. Group 3 (ILC3), includes cell subtypes that produce IL-17 and/or IL-22 and IFN-γ, and these cells depend on the RORγt transcription factor for their development and function [129]. Recent studies have identified various functions of ILCs cells: (1) ILCs promote a host's defense against infections and regulate interactions with the microbiota; (2) as well as orchestrate wound healing and tissue repair and (3) in other circumstances, ILCs may promote inflammation and tumor progression [130]. ILCs are poorly represented in lymphoid tissues, but they are found to be important in parenchymal tissues, especially mucosal surfaces. Therefore, the subtypes of ILCs play an important role in the innate immune response to viruses, bacteria, fungi, and intracellular and extracellular parasites in this type of tissue, and they have a rapid activation through cytokines and growth factors [125, 131].

2.3.2. Natural killer cells

NK cells are derived from cellular lymphoid progenitors. However, they do not mediate the conventional adaptive immune response because they lack antigen-specific receptors such as T and B lymphocytes [132]. Previously, it was believed that the development of NK cells

in humans occurred exclusively in the bone marrow. However, recent studies have shown that NK cells also develop in secondary lymphoid organs [133]. The dominant population of the NK cells in blood circulation has a $CD56^{dim}CD16^{+}$ phenotype corresponding to its final maturation stage, whereas the NK cells with phenotype $CD56^{bright}$ are considered as relatively immature cells [134]. NK cells are important effector lymphoid cells of the innate immune system, since they represent a key element in the rapid recognition and death of both infected or tumorigenic cells, which can cause damage to the integrity of host tissues. NK cells identify target cells (cells that have some damage) through complex combinations of signals from the activation or inhibition of receptors, which interact with ligands that are expressed on the surface of stressed or normal cells, respectively [135]. The decision to eliminate or not eliminate these cells depends on the result of the balance between positive (activation) and negative (inhibition) signals. Also, the activation of NK cells is regulated through cooperation with other immune cells, including DCs [136], which allows that NK cells to acquire potent cytotoxic activity, the ability to produce cytokines such as IFN-γ and contribute to the adaptive immune response by triggering the T cell–mediated response [137].

2.3.3. Natural killer T cells

NKT cells constitute a small subpopulation of lymphocytes that are characterized by the markers expression of the NK cell lineage, as well as receptors of the $\alpha\beta$ T lineage. NKT cells develop in the thymus and have the same common lymphoid precursor of conventional T cells, but they have phenotypic and functional characteristics different of T cells [138]. Four subpopulations of NKT cells $CD4^{+}$, $CD8^{\alpha\beta+}$, $CD8^{\alpha\alpha+}$, and double negatives ($CD4^{-}CD8^{-}$) were identified in human peripheral blood [139], which differ in the cytokine secretion profile and the expression of chemokines receptors, integrins, and NK receptors [140]. In addition, NKT cells recognize glycolipid antigens that are presented through CD1d molecules, MHC-like molecules that are constitutively expressed by antigen presenting cells such as DCs, B cells, and macrophages. NKT cells also have the ability to respond to cells participating in innate immunity with minimal involvement of the T cell receptor (TCR), and memory cells through a portion of the TCR, which makes them capable to be a bridge between the innate and adaptive immune response [141].

3. Pattern recognition receptors in innate immunity

Pathogens that invade a human host are controlled by the immune system, both innate and adaptive. The adaptive immune system, which is mediated by T and B cells, recognizes pathogens with high affinity through the rearrangement of certain receptors. However, the establishment of this adaptive immune response is often not fast enough to eradicate pathogens, and it also involves cell proliferation, genetic activation, and protein synthesis [142]. Thus, the fastest defense of a host mechanism is provided by the innate immune system, which has developed the ability to recognize invading pathogens and thus effectively eliminate them so that they do not cause damage to host cells.

The recognition of pathogens occurs through cells involved in the innate immunity response by nonspecific molecules that are commonly shared by most pathogens called PAMPs. PAMPs are highly conserved products and are produced by numerous microorganisms. These PAMPs do not show specific structures with antigenic variability, and host cells do not share the same molecular patterns with pathogens, resulting in recognition of the immune system, capable to discriminate between self and nonself [143]. Among the PAMPs that present the pathogens are lipopolysaccharide (LPS), peptidoglycan (PGN), lipoteichoic acid, unmethylated cytosine phosphor-guanine (CpG) motifs, double-stranded RNA virus, and the cell wall component of yeast called manan. LPS represents the major component of Gram-negative bacteria, as PGN represents the major component of Gram-positive bacteria [144]. Recognition of these PAMPs is mediated through PRRs, primarily attributed to the family TLRs [142].

However, pathogens are not the only cause of cell and tissue damage. A trauma, a vascular event, even in physiological states as well as in disease states, are other causes of damage, and when this occurs, intracellular proteins called "alarminas" are released, which are considered in a subgroup of a large quantity of DAMPs [145]. This occurs by identifying changes in the host's own structures that show signs of damage and then repairing and removing damaged tissue. DAMPs include any endogenous molecule that experiences a change of state in association with a tissue injury, which allows the immune system to be informed that any damage has occurred [146].

When these DAMPs are released from damaged or necrotic cells, together with PAMPs, are recognized by certain PRRs for their subsequent activation and induction of a potent acute inflammatory response [147]. These PRRs include Toll-like receptors (TLRs), nucleotide-binding domain and leucine-rich repeat containing receptors (NLRs), and retinoic acid-inducible gene-I (RIG-)-like receptors (RLRs).

TLRs are evolutionarily conserved proteins that detect PAMPs. They were originally identified in the *Drosophila* fly as an important gene for its ontogenesis and its immunological resistance against fungal infections. In addition, it was found that during microbial infections of flies, Toll receptors induce the production of antimicrobial peptides [148]. In humans, the first protein structurally related to the *Drosophila* Toll receptor was identified and called the Toll-1 receptor (TLR-1). These proteins are characterized by the presence of an extracellular domain formed by leucine-rich repeats, in which the recognition of the PAMPs is given; and an intracellular region called intracellular Toll/IL-1R (TIR), which is responsible for the signals transmission that culminates in the activation of nuclear factor (NF)-κB, which induces the synthesis of proinflammatory cytokines [149]. Currently, 10 TLRs have been identified (TLR-1 to TLR-10), the TLR-1, TLR-2, TLR-4, TLR-5, and TLR-6 expressed on the cell surface; while TL-3, TLR-7, TLR-8, and TLR-9 are found intracellularly in endosomes [150].

Different TLRs specifically recognize distinct PAMPs and DAMPs [151]. TLR-2 forms heterodimers with TLR-1 or TLR-6. The TLR-1/TLR-2 complex mainly interacts with lipopeptide triacyl ligands in contrast to the TLR-2/TLR-6 complex, which binds only to diacyl lipopeptides. TLR-3 recognizes double-stranded RNA ligands, which are produced by most viruses in replication stages. TLR-4 requires binding with the MD-2 co-receptor and is specific for

interacting with LPS ligands, which comes from Gram-negative bacteria. TLR-5 responds to bacterial flagellin ligands. Both TLR-7 and TLR-8 recognize single-stranded ARN. TLR-9 binds to ligands containing CpG motifs [152]. TLRs are a family of transmembrane receptors that are key in the response and regulation of both innate and adaptive immunity [151], since they recognize diverse pathogens and help to eliminate them.

There are other receptors such as NLRs, which are a family of 23 members that have been identified in humans. They are intracellular receptors that are structurally composed of caspase recruitment domains (CARDs), as in the case of members called NODs, a pryin domain, as in the case of NLRP members. Among the most important members of these receptors are NOD1 and NOD2, which recognize specific ligands from various pathogens. This family is involved in increasing the proinflammatory events caused by cell death, pyroptosis and pyronecrosis, and several more proinflammatory processes [153].

Another family of receptors is the RIGs. They are intracellular recognition receptors for patterns involved in the recognition of viruses by the action of the innate immune system. There are three members: RIG-1, MDA-5, and LGP2. They act as sensors for viral replication within human host cells necessary to mediate antiviral responses [154].

4. Soluble mediators of the innate immune system

In innate immunity, a large number of soluble mediators such as cytokines, chemokines, and the complement system participate. All these mediators provide protection in the initial phase of contact with pathogens and are responsible for preventing potentially harmful infections.

4.1. The complement system

The complement system has been considered as an effector response of the innate immune system capable of eliminating a great diversity of pathogens including bacteria, viruses, and parasites [155]. The complement system is composed of plasma proteins, which are present as inactive proteins [156]. After activation, the products that are generated from the complement system facilitate the recruitment of cells from the immune system to the site of damage to eliminate the pathogen through opsonization or direct destruction [157]. Activation of the complement system occurs through three pathways: (1) the classical pathway for the antigen–antibody complex; (2) the alternating pathway through the spontaneous hydrolysis of C3; and (3) the lectin pathway where certain sugars are recognized on the surface of the pathogens through mannose-binding lectin (MLB). Once activated, the pathway of the complement system generates a multimolecular enzyme complex that cuts to C3 and forms C3a and C3b. The C3b fragment that is generated binds to C3 convertase to form the C5 convertase, and once formed, this complex cuts to C5 to form C5a and C5b [155]. Then, C5b begins to recruit complement components C6, C7, C8, and C9 to form the membrane attack complex which is a lytic pore inserted into the membrane of the pathogen [158]. Since the complement system uses multiple activation pathways, it has the ability to maximize the number of pathogens that it

can recognize and thus eliminating a great diversity of these. In addition, it is responsible for eliminating apoptotic cells, this occurs through depositing a low amount of C3b molecules which facilitates the removal of these cells by macrophages [159].

4.2. Cytokines

Cytokines form a molecular network that is synthesized and released by different cell types. These molecules act in a paracrine and endocrine way through their receptors that express the target cell. These molecules are synthesized and released in response to some damage or recognition of specific structures of the pathogens through their receptors (e.g., PAMPs and TLRs) [160]. Initially, the cytokines were defined based on the activity they performed, among these activities are regulating the immune system but also exerting an effector function on the cells, these effects not only occur at local level but also occur through the tissues or systems. Cytokines are involved in regulating the homeostasis of the organism but when its production or its signaling pathway in the cell is not regulated, this homeostasis is altered, which can trigger in a pathology [161, 162]. Cytokines can be classified into five groups: type I cytokines (include cytokines from IL-2 to IL-7), type II cytokines (interferons and cytokines of the IL-10 family), type III cytokines (the TNF family), type IV cytokines (IL-1 family, such as IL-1, IL-18, IL-36, IL-37, and IL-38), and type V cytokines (the IL-17 family that includes IL-17E) [162]. Cytokines may increase systemic level during some pathological condition, either acute or chronic, these molecules exert their effect by binding to their receptors, where the signal translation is given, which leads to the gene expression and finally can regulate the function of the target cell. The cytokine pattern that is released from the cell depends primarily on the nature of the antigenic stimulus and the type of cell being stimulated. Cytokines compromise leukocytes to respond to a microbial stimulus, through regulating positively the expression of adhesion molecules on endothelial cells and amplifying the release of molecules such as reactive oxygen species and nitrogen, histamine, serotonin, as well as arachidonic acid derivatives, which regulate the release of the cytokines. On the other hand, cytokines can promote apoptosis by binding to receptors that contain death domains, for example TNF receptor 1(R1) [163].

4.3. Chemokines

Chemokines or chemotactic cytokines are small molecules which constitute a large family of peptides (60–100 amino acids) structurally related to cytokines. Their main function is to stimulate leukocyte migration. They are secreted in response to some signals such as proinflammatory cytokines, where they play an important role in selectively recruiting monocytes, neutrophils, and lymphocytes [164, 165]. These molecules are defined by the presence of four conserved cysteine residues that form two disulfide bonds (Cys1-Cys3 and Cys2-Cys4) and are classified into four families based on the number of amino acids between the first two cysteines: CXC-(α), CC-(β), CX3C-(δ), and C-(γ) according to the systematic nomenclature [166]. The chemokines CXC and CC are distinguished according to the position of the first two cysteines, which are adjacent (CC) or separated by an amino acid (CXC) [167]. The CC chemokine family is the largest and can be subdivided into several subfamilies. One is monocyte chemotactic protein (MCP), this subfamily is characterized by recruiting monocytes to damaged tissue after

ischemia, which is conformed for five members: CCL2 (MCP-1), CCL8 (MCP-2), CCL7 (MCP-3), CCL13 (MCP-4), and CCL12 (MCP-5). Another chemokine in this group is the macrophage inflammatory protein (MIP)-1α (CCL3), MIP-1β (CCL4), and RANTES (CCL5) [168]. The second family consists of CXC chemokines; the prototype of these chemokines is IL-8 (CXCL8); mainly this chemokine attracts polymorphonuclear cells to the site of acute inflammation. Also, CXCL8 activates monocytes and can recruit these cells to vascular injury. The third family, consisting of a single member is Fraktalkine (CX3CL1) which is one of the two transmembrane chemokines and has two isoforms, one binds to the membrane and the other is a soluble form. According to its isoform, it may have different functions, the form that is anchored to the membrane serves as adhesion molecule for cells expressing CX3CR1, while the soluble form possesses a potent chemotactic activity [169]. The fourth family has only one member lymphotoxin (XCL1); this chemokine is similar to members of the CC and CXC families, but the lack of two of the four cysteine residues are characteristic of this chemokine. Its chemotactic function is for lymphocytes and not for monocytes and neutrophils as do other chemotactic chemokines [170].

5. Immune response against pathogens

Inflammation is a protective response to extreme challenges to homeostasis, such as infection, tissue stress, and injury [171], which is characterized by its cardinal signs: redness, swelling, heat, pain, and disrupted function [172]. A typical inflammatory response consists of four components: (1) inflammatory inducers: depending on the type of infection (bacterial, viral, fungi or parasitic) [173]; (2) sensors that detect the inflammatory inducers: these sensors are receptors of the innate immune system such as TLRs, NLRs and RLRs [153, 174]; (3) inflammatory mediators induced by the sensors, such as cytokines, chemokines and the complement system [175]; (4) target tissues that are affected by the inflammatory mediator. Each component comes in multiple forms and their combinations function in distinct inflammatory pathways.

The inflammatory reaction is characterized by successive phases: (1) silent phase, where cells reside in the damaged tissue releases in the first inflammatory mediators, (2) a vascular phase, where vasodilation and increased vascular permeability occur, (3) cellular phase, which is characterized by the infiltration of leukocytes to the site of injury [176], and (4) resolution of inflammation, which is the process to return tissues to homeostasis [177, 178].

5.1. Immune response against bacteria

In an infection by extracellular bacteria, the host triggers a series of responses to combat the pathogen and prevent its spread. The main mechanism of the innate immune response to eradicate bacteria is activation of the complement system, phagocytosis, and inflammatory response (**Figure 1**). Both the alternative and the lectin pathways of the complement system participate in the bacteria opsonization and potentiate their phagocytosis. To perform the correct phagocytosis, activation of several surface receptors in phagocytes, including scavenger receptors, mannose, Fc, and mainly TLRs is required. Activation of these receptors

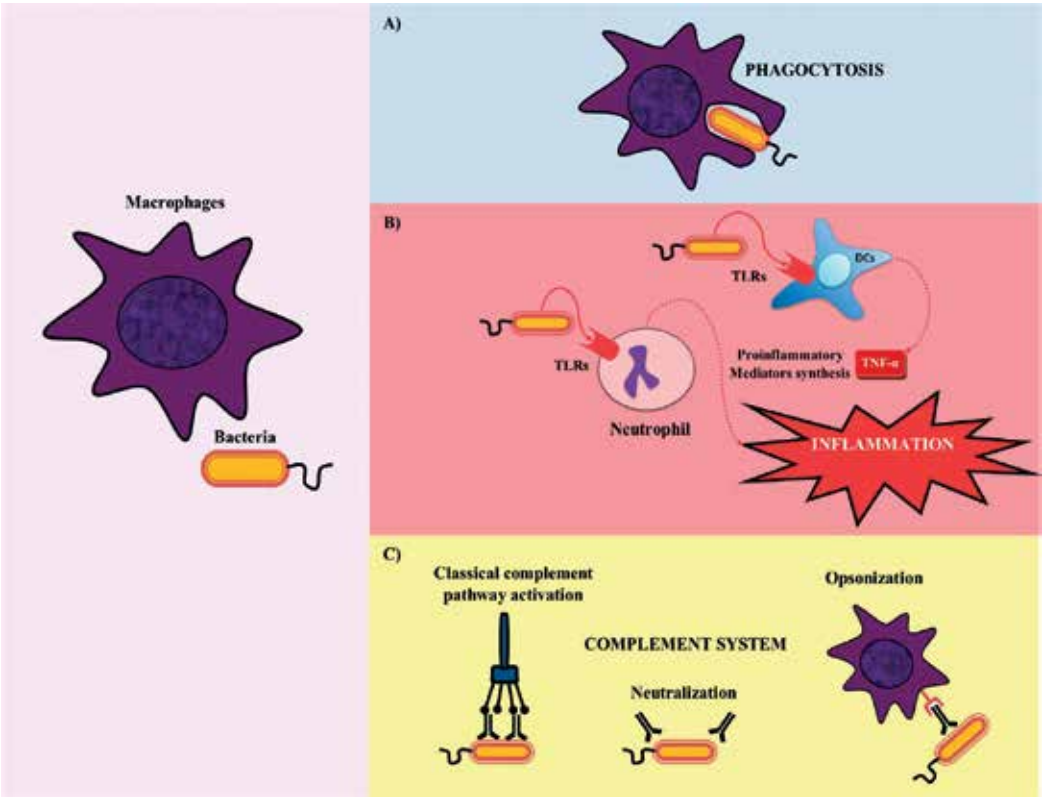

Figure 1. Immune response against bacteria. Mechanisms of the innate immune response to eradicate bacteria are (A) phagocytosis, (B) inflammatory response, and (C) participation of the complement system. Description in the text.

results in inflammation, by recruiting leukocytes to the site of infection [152]. On the other hand, the humoral adaptive immune response is the main protective against extracellular bacteria. Its primary function is to block infection, through the release of antibodies that are directed against the antigens of the bacterial cell wall, as well as of the toxins secreted by certain extracellular bacteria. The effector mechanisms used by the antibodies include neutralization, opsonization, and classical complement pathway activation, which allow bacteria phagocytosis. In the case of neutralization, IgG, IgM, and IgA participate; while in the opsonization, the IgG participates; and in complement activation, the IgM and some subclasses of IgG participate. Protein antigens from extracellular bacteria also activate the cellular adaptive immune response, which is mediated by $CD4^+$ T cells. These $CD4^+$ T cells produce cytokines that induce local inflammation, increase phagocytosis, as well as microbicidal activities of macrophages and neutrophils. The Th17 cells are also involved in recruiting monocytes and neutrophils, promoting local inflammation. Similarly, there is an induction of the Th1 immune response that contributes to the macrophages activation with ample phagocytic capacity and the production of the cytokines, such as IFN-γ [179].

In the case of infection by intracellular bacteria, they have the ability to survive and replicate within phagocytic cells, which causes the circulating antibodies to be inaccessible to intracellular

bacteria. The innate immune response against these bacteria is mediated primarily by phagocytes and NK cells [180]. Among the phagocytes involved are neutrophils and then macrophages. However, these pathogens are resistant to degradation, but their products are recognized by TLRs and NLR receptors that are responsible for activating more phagocytes. NK cells are also activated in this type of infections and participate by stimulating the production of cytokine IL-12 by DCs and macrophages. Also, the NK cells produce IFN-γ, which promotes the death of phagocytic intracellular bacteria. But usually this immune response is ineffective against infection. In contrast, the adaptive immune response against infections by intracellular bacteria is mediated by $CD4^+$ T cells that help recruit and activate phagocytes that kill the pathogen, and the response of cytotoxic $CD8^+$ T cells that kills the infected cells. Both subpopulations of T cells respond through the antigen presentation by MHC type I and II. All this to eradicate the infection of the host [181].

5.2. Immune response against fungi

Most fungi are present in the environment, so animals including humans are exposed and then can inhale spores or yeasts [182]. The mechanisms for defense against the fungi comprise of both innate and adaptive immune responses. TLRs recognize several PAMPs, so that TLR1, TLR2, TLR3, TLR4, TLR6, and TLR9 have been implicated in the recognition of PAMPs from fungi. Activation of TLR4 and CD14 by recognition of conidia derived from some fungi has been shown to increase the production of inflammatory molecules such as TNF-α. Meanwhile, the TLR2 may recognize conidia and hyphae, as well as β-glucans from pathogenic fungi *Coccidioides*. TLR2 activation induces oxidative pathways in polymorphonuclear (PMN) cells with the release of gelatinases and inflammatory cytokines. TLR6 is involved in the recognition of *Candida albicans*, which is involved in the production of IL-23 and IL-17A, which promote Th17 responses. TLRs can be combined to recognize a large number of fungal structures and thus generate a broader response against the various fungal structures [183, 184].

The NLRs are involved in detection of fungal structures, such as *Aspergillus fumigatus* hyphal fragments, and once activated the production of IL-1β and IL-18 is induced by the formation of a multimeric complex known as inflammasome [182, 185].

Type C lectin receptors (CTLRs) make up a receptors family that can recognize several molecules like proteins, carbohydrates, and lipids. Among these receptors, the best studied are dectin-1, dectin-2, dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN), macrophage inducible C-type lectin, and mannose receptor (MR) involved in the recognition of some structures of the fungi [186]. Dectin-1 recognizes β-glucan and promotes its phagocytosis, it can also interact with TLR2 to induce the activation of NF-κB and the production of reactive oxygen species [187]. Dectin-1 activation can also induce mast cells to produce proinflammatory and TH2-polarizing cytokines, such as IL-4 and IL-13. Dectin-2 also activates NF-κB. In addition, dectin-2 promotes Th17 polarization by inducing IL-17A, which is crucial in neutralizing some fungi. The MR recognizes mannose, fucose, or N-acetylglucosamine residues present in fungi. MR generates a Th17 response and promotes fungi phagocytosis [183]. The response that occurs through the activation of these receptors includes the binding to fungi and their phagocytosis, the induction of antifungal effector mechanisms and the production of soluble mediators such as cytokines, chemokines, and inflammatory lipids [187].

The immunity against fungi requires the recruitment and activation of phagocytosis, which is mediated through factors that induce inflammatory molecules such as proinflammatory cytokines and chemokines. The PRRs interaction with fungal structures plays an important role in the control of infections against these pathogens, since this interaction is determinant for the generation of the profile of cytokines or chemokines that influence the immune response. For example, the interaction of *Candida albicans* with TLR4 or TLR2 generates a Th1 or Th2 response, respectively. Therefore, these interactions of the different fungal structures and the PRRs generate different responses polarizing toward one or the other depending on the cytokine profile that could be generated after these interactions (**Figure 2**) [188].

5.3. Immune response against viruses

In an infectious process, the most common host response is to generate inflammation. Viruses in the absence of cytopathologic damage at early stages of infection inhibit the induction of acute phase protein response because early monocytes are not activated. By contrast, the participation of NK cells against the virus play an important role in the host's defense, they recognize cells infected by viruses in an antigen-independent manner, exert cytotoxic activities and rapidly produce large amounts of IFN-γ that participate in the activation of the adaptive immune cell [5]. Type I interferons are the major cytokines responsible for defending the human host against viral infections. It has been shown that interferons

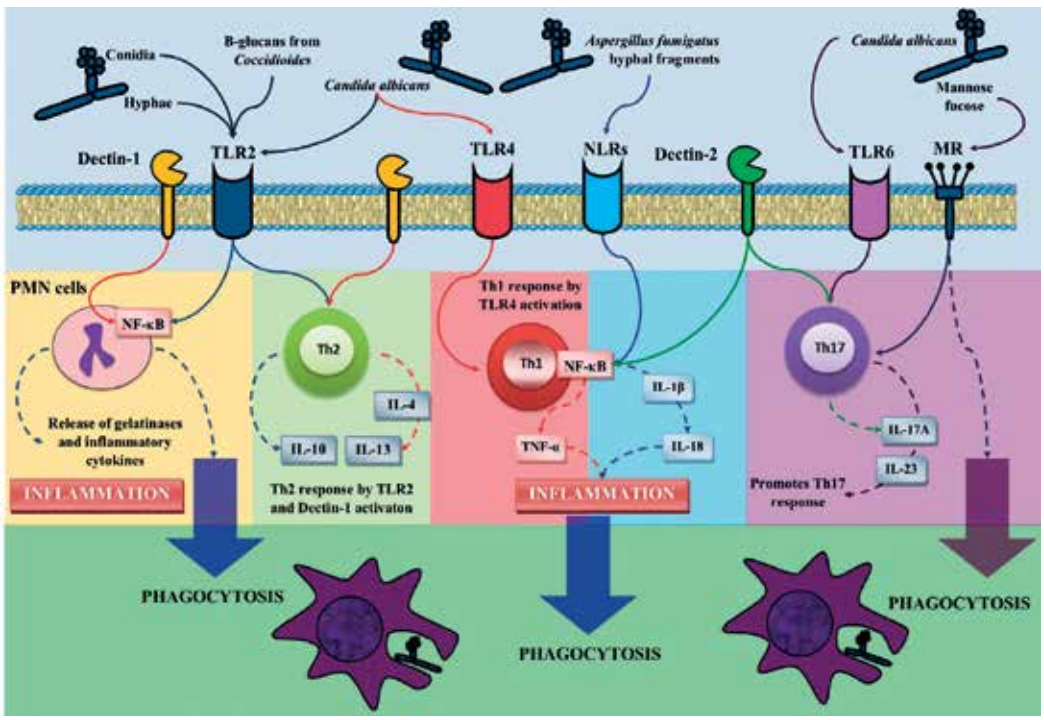

Figure 2. Immune response against fungi. PRRs, such as TLR2, 4, 6, NLRs, dectins-1 and 2, and RM, are involved in the recognition of some structures of the fungi. The activation of these receptors includes the binding to fungi and their phagocytosis. Description in the text.

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do not exert their antiviral effects by direct action on viruses, but they help in the gene activation that results in the production of antiviral proteins, which participate as mediators in the inhibition of viral replication, as well as mediating the effects of suppressor T cells [189].

The adaptive immune response against this type of infection is primarily composed of the humoral immune response with the antibody production directed against viral antigens. However, the cellular immune response is the most important for virus eradication. T CD4$^+$ cells recognize antigens presented by MHC-II molecules on the surface of APCs [190]. Subsequently, T CD4$^+$ cells perform multiple effector functions including direct activation of antigen-specific macrophages and B cells, as well as cytokine-dependent activation of T CD8$^+$ cells. T CD8$^+$ cells eliminate virus-infected cells and secrete cytokines such as TNF-α and IFN-γ, which also participate in the inhibition of viral replication. Thus, both the innate immune response and the adaptive immune response in their cellular and humoral involvement eradicate viral infections in most cases (**Figure 3**). However, certain viruses have developed mechanisms of immune evasion to survive longer and thus be able to replicate without any problem until causing serious damage to the host [191].

5.4. Immune response against parasites

Due to there being a large variety of parasites and that each of their life cycles are very complex, in this section, we will focus on the immune response against helminth parasites. This is

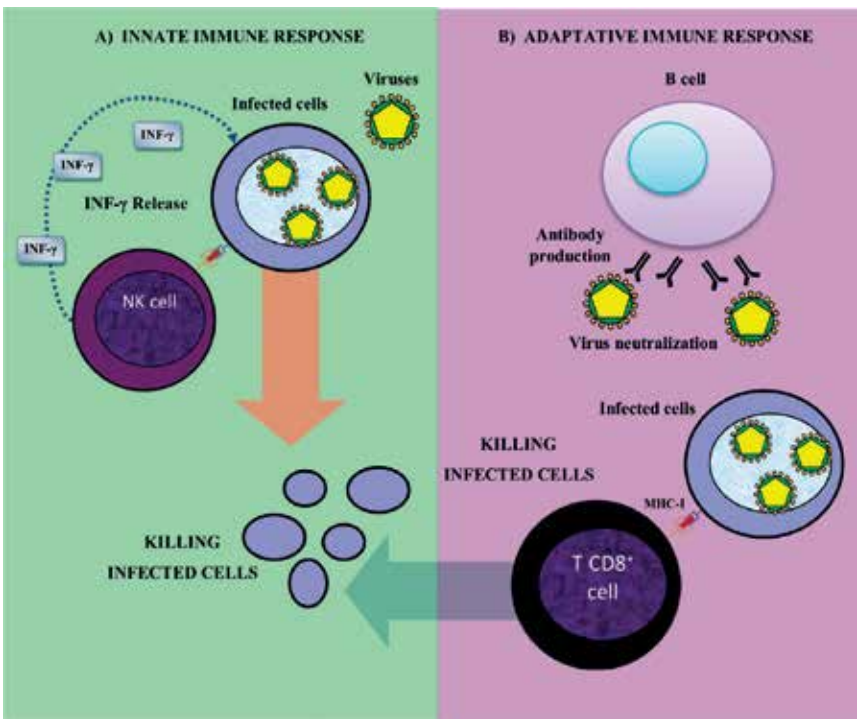

Figure 3. Immune response against viruses. (A) Innate immune response: NK cells recognize cells infected by viruses in an antigen-independent manner, exert cytotoxic activities and rapidly produce large amounts of IFN-γ to eliminate infected cells. (B) Antibody production directed against viral antigens. T CD8$^+$ cells eliminate virus-infected cells and secrete cytokines such as TNF-α and IFN-γ. Description in the text.

because more than 1 billion people are currently infected with helminth parasites worldwide [192], making them one of the most prevalent infectious agents responsible for many diseases in both animals and humans [193]. The investigation of these parasitic infections is not only of direct relevance to human and animal health but also because they present a constant and important challenge to the host immune system, since both in humans and animals, helminth parasites establish chronic infections [194] associated with a significant downregulation of the immune response.

The first defense barrier during intestinal helminth parasites infection is the mucus layer secreted by the host's intestine, either in a larval stage during the early infectious process or as adult parasites during the reproductive phase of infection. Thus, helminth parasites will interact with the mucus layer and in many cases will have to cross it to reach the epithelial layer and thus thrive and reproduce within it [192].

The immune response against helminth parasites involves both the innate and adaptive immune response [195, 196]. Helminth parasite antigens are capable of inducing the DCs maturation, leading to the expression of MHC class II [197, 198], promoting the development of a Th1 type cellular immune response (**Figure 4A**) [199]. Several studies have shown that during intestinal infection by helminth parasites, there is an increase in the levels of

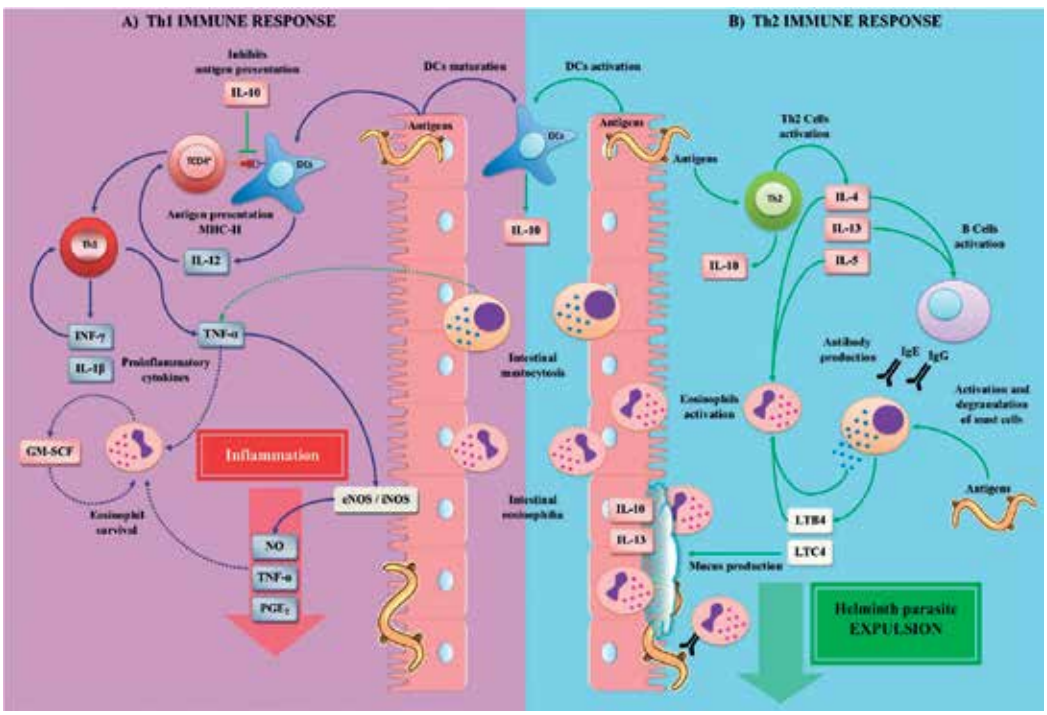

Figure 4. Immune response against parasites. (A) Th1 immune response: helminth parasites antigens induce maturation of DCs by polarizing a Th1 immune response, which is mainly characterized by the release of IL-12, INF-γ, GM-SCF, NO, PGE_2, IL-1β, and TNF-α, which together with eosinophilia (derived from the Th2 immune response) enhance intestinal inflammatory response, resulting in the development of intestinal pathology, creating a favorable environment for the helminth parasites survival. (B) Th2 immune response: helminth parasites antigens activate T cells that together with IL-10 induce a Th2 immune response characterized by the release of IL-4, IL-5, IL-10, and IL-13 favoring helminth parasites antigens expulsion.

gene expression of TLR4 and TLR9 [200], with a significant increase of proinflammatory cytokines such as IL-12, INF-γ, IL-1β, TNF-α, nitric oxide (NO), and prostaglandin (PG)-E_2 [201–207].

Helminth parasite antigens also induce Th2 immune response (**Figure 4B**) trough $CD4^+$ T cells [208], and DCs activation, leading to the secretion Th2 cytokines, such as IL-10 [209], IL-4, IL-5 [210], and IL-13 which stimulate IgE synthesis, inducing mast cell and eosinophil hyperplasia, triggering immediate hypersensitivity reactions, promoting the helminth parasites expulsion from the intestine [197, 208, 211–213]. However, mast cells rapidly expand in the mucosa, where helminth parasites antigens can directly induce their degranulation, releasing effector molecules such as histamine, serine proteases [197], TNF-α, LTC4, LTB4 [213], IL-4, IL-13 [201], which together with the eosinophils contributes to the intestinal inflammation development [214, 215].

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Chapter 7

Physiology and Pathology of Infectious Diseases: The Autoimmune Hypothesis of Chagas Disease

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Additional information is available at the end of the chapter

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Abstract

Infectious pathologies are a group of diseases that contribute with great impact on public health worldwide. Among the various diseases, some have a higher epidemiological importance, since their morbidity and mortality are very significant. In addition to the usual immune response, mounted against noxious agents, there is still the concept of infection-induced autoimmunity. Autoimmune diseases are defined as illnesses in which the evolution from benign to pathogenic autoimmunity takes place. However, proving a disease to be of autoimmune etiology is not a simple task. It is well known that both genetic influences and environmental factors trigger autoimmune disorders. However, some theories are still under great discussion. One of the most intriguing self-induced disorders is the hypothesis of autoimmunity during Chagas disease. Since the mid-1970s, the Chagas autoimmunity hypothesis has been considered an important contributor to the complex immune response developed by the host and triggered by *Trypanosoma cruzi*. New ideas and findings have strengthened this hypothesis, which has been reported in a series of publications from different groups around the world. The aim of this chapter is to discuss the mechanisms involving autoimmunity development during Chagas disease.

Keywords: Chagas disease, autoimmunity, *T. cruzi*, autoantibodies, immunology, cardiomyopathy

1. Introduction

Autoimmune diseases are chronic disabling disorders described as immune responses against self-antigens that comprise cells, tissues, and organs, resulting in devastating consequences for patients [1]. For over decades, the concept of autoimmune disease has been studied, and the relationship between infectious diseases and autoimmunity has been established. Among all illnesses, Chagas disease has caught the attention of many researchers worldwide. Along with all the possible pathogenic mechanisms involved in *Trypanosoma cruzi* infection, the autoreactive hypothesis has been discussed over the years and plays an important role in the cardiac damage presented by Chagas patients. This chapter intends to explore the *T. cruzi*-induced autoimmunity hypothesis by discussing its pathogenic mechanisms and its potential role in the tissue aggravation during Chagas disease.

2. Autoimmunity: an obscure immunological path

In order to understand autoimmunity, it is essential to go back into the first steps of T- and B-cell development, differentiation, and maturation. During the process of generation of new lymphocytic clones derived from a stem cell, a novel lymphocyte carrying a specific pattern of B-cell receptor (BCR) or T-cell receptor (TCR) is formed as revised recently [2, 3]. While B-lymphocytes may undergo full maturation in the bone marrow, T lymphocytes need thymic education, as can be observed in **Figure 1**.

After egressing the bone marrow, T lymphocytes undergo differentiation and maturation in the thymus in which they pass through a process called negative and positive selection [4, 5].

Positive selection occurs during classical thymic differentiation, in which thymocytes may generate naive conventional T lymphocytes. These cells migrate to peripheral tissues and further differentiate in response to encounter with nonself-antigens. The negative selection is based on the elimination or inhibition of "self-reactive" cells. Nevertheless, some of these self-reactive T cells could escape negative selection and might become activated during the inflammatory process. On the other hand, a second or agonist-driven thymic selection, was proposed in which specialized T-cell subsets are generated from thymocytes that bind with high avidity to self-antigens. In this case, these cells leave the thymus as antigen-experienced activated T cells, such as double-negative TCR$\alpha\beta$+ intestinal T cells, CD8$\alpha\alpha$+, invariant iNKT, Foxp3+ nTreg cells, TH17 cells [6] and possibly mucosal associated invariant T cells (MAIT) [7]. Two barriers for self-reactive cells exist, which are the central and peripheral tolerance [5]. Failure in these processes may allow the proliferation of self-reactive clones, thus establishing an autoimmune pathogenic disease. Genetic disorders as well as environmental conditions can trigger the failure on central or peripheral tolerance, [8–12]. In addition, other pathways can lead to autoimmunity such as molecular similarity or mimicry, specific epitope spreading, indirect or bystander activation, B- and T-cell polyclonal activation, infections, and self-inflammatory activation that trigger innate immunity [8, 13].

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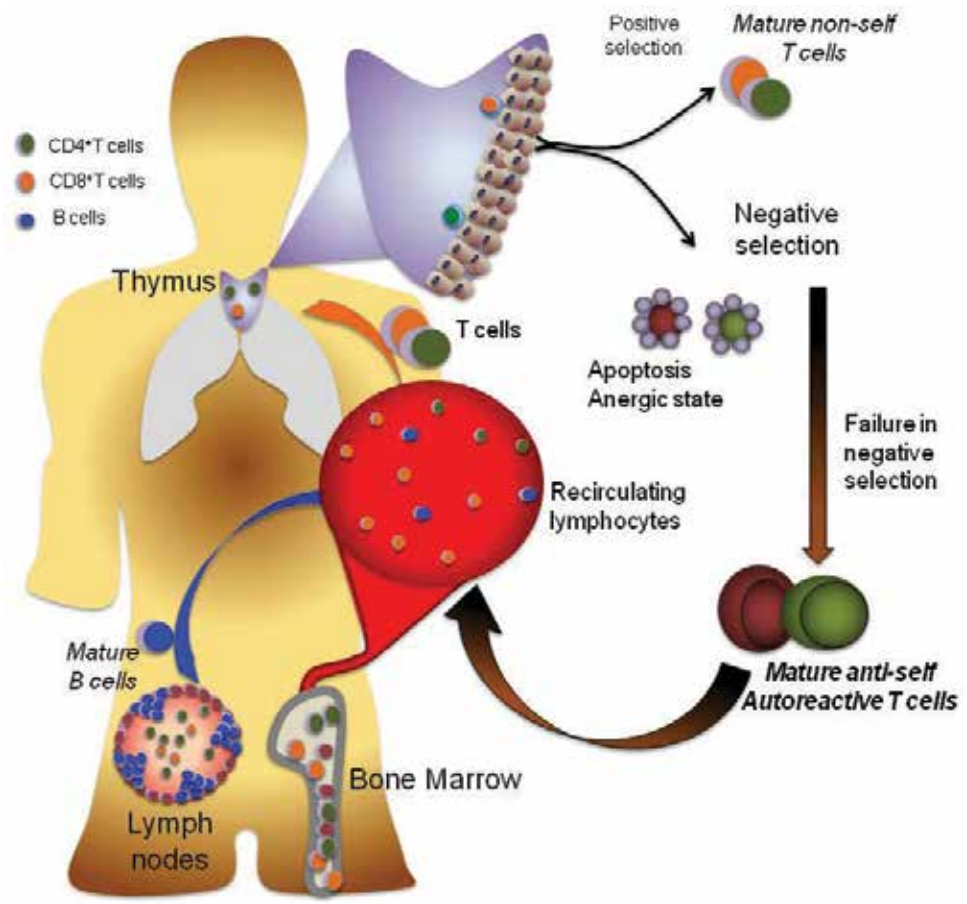

Figure 1. Dynamics of hematopoiesis, maturation and recirculation of newly generated lymphocytes as well as thymic selection of T-cell clones.

3. Chagas disease: an outburst of inflammatory events that may lead to autoimmunity

T. cruzi is a haemoflagellate parasite transmitted by several species of triatomine bugs. The best known mood of transmission is the vetorial via, which consists of vector insects ingesting contaminated blood meal containing bloodstream trypomastigotes. Later on, inside the insect's gut, the parasite differentiates into epimastigotes and replicates. The cycle is complete once the insect defecates, and releases metacyclic trypomastigotes forms which invade the host through the bite wound or mucosal membranes. Chagas disease progresses from a somewhat asymptomatic short acute phase to a chronic phase. Most individuals that progress to chronic phase remain asymptomatic, and the disease is detected by serological tests, but no clinical, radiologic, electrocardiographic, or echocardiographic evidenced. Overall, 20–40% of asymptomatic individuals develop clinically relevant Chagas heart disease, while approximately 10%

of the cases progress to digestive problems [14, 15]. In fact, the disease outcome toward differential clinical forms is related to many factors including the host-parasite interaction. Several studies have been proposed that the host immunological response plays a major role in the pathogenesis of Chagas disease [16]. Indeed, the *T. cruzi* can trigger an immune response, since parasite antigen has been consistently found in heart tissue infiltrated from cardiac patients [17, 18]. Nevertheless, the divergence between low parasite load in the tissue and severity of the lesions observed during the chronic phase reinforces the hypothesis that other factors than the immune response developed against the parasite might be involved in the development of Chagas pathology [19]. In this context, humoral and cellular autoimmune mechanisms are developed during Chagas disease [20–23]. Autoimmune mechanisms triggered during infection by *T. cruzi* may occur after bystander activation, parasite-cardiomyocyte harm, or molecular mimicry [24]. The mechanisms discussed in this chapter are schematically summarized in **Figure 2**.

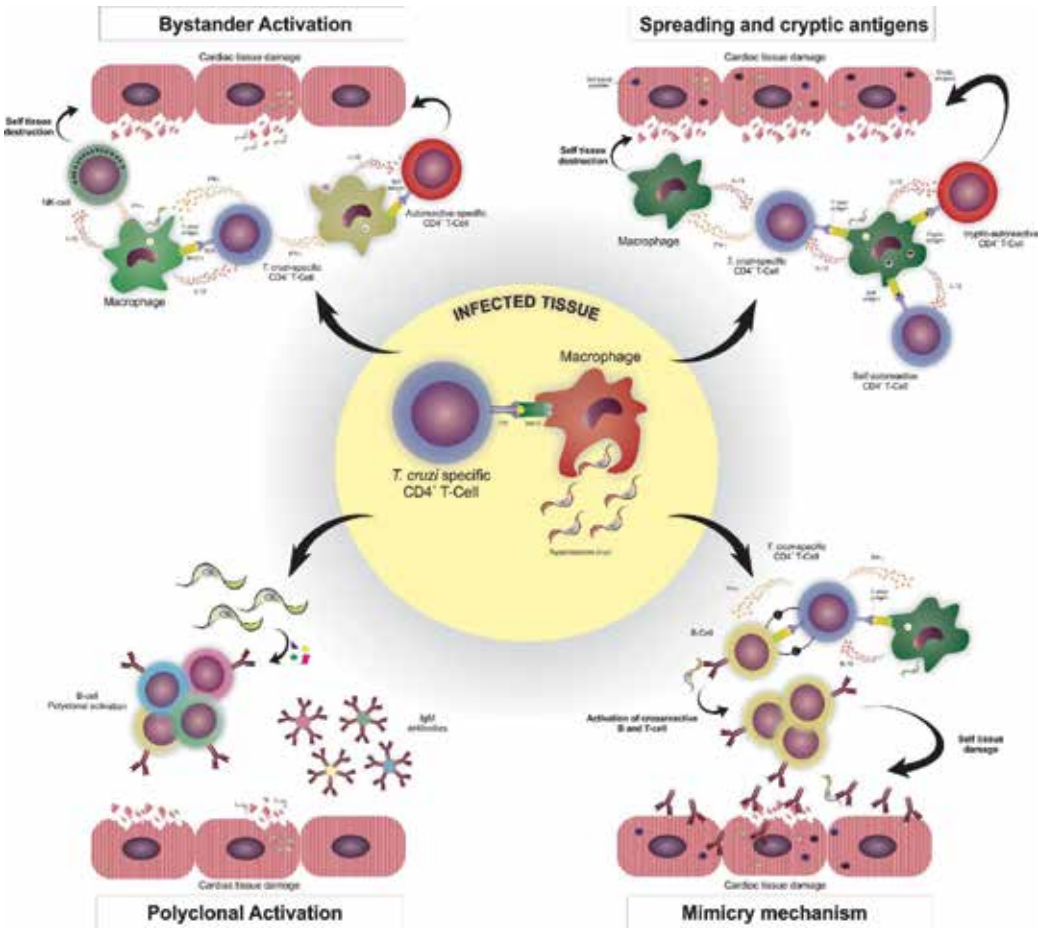

Figure 2. Scheme of the probable mechanisms involved in the autoimmune pathogenesis of Chagas disease.

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Since *T. cruzi* presents a high variability of surface antigens, it is possible that the parasite causes polyclonal activation, which involves the T-independent stimulation of self-reactive B lymphocytes [25]. The lipopolysaccharide has been described as polyclonal activator, which induces the hypergammaglobulinemia phenomenon with higher secretion of autoantibodies, mainly IgM isotype [26]. Furthermore, *T. cruzi*-derived antigens can activate B1 lymphocytes before the development of T-cell-mediated immune response during the early stages of Chagas disease. [27, 28]. High percentage of B1 cells are found in the peripheral blood of chronic Chagas patients, as well as a significant decrease in the percentage of $CD3^+$ T cells [27, 28]. Additional studies have demonstrated that auto-anti-idiotypic antibodies (Ids) from chagasic cardiac disease patients preferentially stimulate B1 cells and $CD8^+$ T cells in magnified proportion as compared to indeterminate patients [29, 30]. The lower levels of T cells in the peripheral blood of chagasic cardiac patients suggest that T-cell-mediated immunity should be restricted to inflammatory foci, considering previous reports of the presence of T cells, mainly $CD8^+$ T lymphocytes, in the inflammatory infiltrate of cardiac tissue from individuals infected by *T. cruzi* [16]. A study using murine experimental model based on infection by *T. cruzi* demonstrated that the onset of cytokine production by T cells in the cardiac tissue is correlated with the local increase in the expression of cell adhesion molecules that is consistent with the T-lymphocyte migration to the inflammatory milieu [31]. In this study, the authors propose that chronic inflammation in the cardiac tissue from Chagas disease patients is highly active and is related to a permanent proinflammatory immune pattern that extends from the recent acute phase to the late stages of the chronic phase. Indeed, it is well accepted that the absence of pathology in individuals infected by *T. cruzi* is associated with the individual's ability to regulate the anti-*T. cruzi* response, which is responsible for the control of persistent parasitemia and tissue inflammatory damage, characteristic of Chagas disease [16, 32–34]. Certainly, the tissue inflammatory damage should be more severe in the absence of regulatory mechanisms involving both innate and adaptive immune responses. Indeed, our group has reported that in the indeterminate clinical form of Chagas disease, there is a higher frequency of $CD4^+CD25^{High}$ T cells and NKT lymphocytes than individuals with cardiac disease [35, 36]. As $CD4^+CD25^{High}$ T cells and NKT lymphocytes showed an important role in modulating the activation of $CD8^+$ T cells via apoptosis, as well as throughout the secretion of regulatory cytokines, it is possible that these cells during indeterminate clinical state further contribute to the control of the cytotoxic activity and deleterious events mediated by $CD8^+$ T cells [37, 38]. It is worth to mention that a fine equilibrium between inflammatory and regulatory cytokines represents a crucial element in the establishment of distinct clinical forms of chronic Chagas disease [33, 38]. It has been demonstrated that in the severe cardiac clinical status, leucocytes produce more IFN-gamma, while IL-10 is predominantly produced by PBMCs from indeterminate [38, 39].

In this regard, the functional role of peripheral blood leukocytes in patients infected by *T. cruzi*, after antigen stimulation has demonstrated that the main source of IFN-γ in cardiac patients is $CD4^+$ T lymphocytes, while monocytes are responsible for the production of high levels of IL-10 in patients with indeterminate status, favoring the regulation of the immune response and the control of disease morbidity [40]. It is possible that regulatory T cells are involved in this process, since they can inhibit the synthesis of IFN-γ, which could explain the low levels of

this cytokine produced by T cells from indeterminate Chagas disease patients. In this context, it is important to reinforce that the inflammatory environment and cell destruction induced by *T. cruzi* infection could alter and induce antigen processing/presentation in such a way that novel self-epitopes are generated and recognized by the immune system, namely, cryptic epitope [41]. In steady state, the cryptic epitope displays low affinity for MHC molecules and is rarely presented by somatic cells, while dominant peptides show a high affinity for MHC molecules and are frequently presented by somatic cells. Furthermore, $CD8^+$ T cells specific for cryptic epitope could escape from the negative selection process in central tolerance [42]. Despite the fact that this process during *T. cruzi* infection is not yet clearly understood, this mechanism has been demonstrated to be involved in the pathogenesis of other autoimmune diseases [43]. Moreover, it has been shown that antigen processing and presentation were altered after the in vitro IFN-γ treatment, strengthening the hypothesis that the higher levels of IFN-γ in cardiac patients may favor the establishment of autoimmune mechanisms during Chagas disease. Indeed, the bystander activation mechanism caused by massive host antigens released in a proinflammatory environment may stimulate autoimmunity during *T. cruzi* infection.

4. The autoimmunity during Chagas disease: theories, concepts, and mechanisms triggered by *Trypanosoma cruzi*

Starting from the innate response, the human organism defends itself in a variety of ways. Physical barriers, phagocytic cells, (such as macrophages and dentritic cells), natural killer cells, neutrophils and the complement system are just a few examples of how precise and efficient our body reacts. Besides the innate response, the immune system is comprised of the adaptive response, a complex compartment of defense. Leucocytes (T and B lymphocytes), antibodies, and many other molecules are constantly working in order to keep the homeostasis. Furthermore, another important mechanism that is part of the immunological system is the autoimmunity concept. The autoimmunity concept started when researchers proposed that *T. cruzi* infection promoted rejection of allogeneic heart cell transplants and that T lymphocytes from *T. cruzi*-infected animals rapidly destroyed embryonic cardiomyocytes in culture [44]. However, reports that protective *T. cruzi*-specific T-cell-mediated immunity could be induced without eliciting pathogenic autoimmunity were soon published.

The debate about a role for autoimmunity in Chagas disease continued. Autoreactive T cells and antibodies were identified in individuals with chronic Chagas disease, and several specific antigens abundant in the myocardium were identified as targets of autoreactive responses [22, 45, 46]. Inflammatory factors present in the local environment, such as cytokines and nitric oxide, were also found to promote the activation of potentially autoreactive T cells encountering major histocompatibility complex-bound cognate antigen [21, 47, 48]. In this context, the autoimmune hypothesis plays a crucial role in the pathogenesis of many infectious diseases, including Chagas. The mechanisms proposed in this section for the generation of autoimmunity during Chagas disease are mimicry, indirect or bystander activation, and epitope spreading.

Mimicry is defined by the development of immune responses against foreign antigens that share sequence or structural similarities with self-antigens. This is due to the fact that immune responses can be directed against peptides with similar charge distribution and shape [8].

This mechanism suggests that *T. cruzi*-induced cardiac damage and/or molecular mimicry between parasite and host antigens leads to a breakdown in self-tolerance, resulting in eventual tissue damage [49]. Two cardiac molecules that have been related to the induction of Chagas autoimmune responses are myosin and troponin I [50, 51]. Troponin I have been recently described in *T. cruzi*-naturally infected macaques, which present high titers of troponin I autoantibodies in their circulatory system. In regards to myosin, it is the most abundant protein in the heart and may represent a significant cardiac antigen. Several studies have demonstrated the presence of autoantibodies against this protein circulating in the sera of Chagas disease experimental models [41, 52, 53]. Its potential to become an antigen mimicry candidate has been under discussion for over decades, especially considering several studies, which have linked it to the autoimmune hypothesis. Furthermore, a robust myosin-specific autoimmunity as well as immune tolerization to myosin suppresses parasite-specific immunity [41, 53]. However, it seems that myosin-specific autoimmunity itself is not essential to establish an inflammation [54].

In addition, other host antigens have been studied and proved to cross-react with *T. cruzi* proteins, such as B1 and B2 adrenoreceptors, lymphocyte, neuronal tissues, muscle antigens, m2 muscarinic acetylcholine receptors, small nuclear ribonucleoprotein, and Cha, a novel autoantigen [22, 55]. Moreover, some *T. cruzi* proteins have also been identified as potential antigen mimicry candidates, like epitopes of B13, 24-kDa, 36-kDa, 38-kDa, ribosomal P1 and P2 proteins, the shed acute-phase antigen (SAPA), and the *T. cruzi* cysteine protease cruzipain [22, 51, 55–59]. Worthy of mentioning was the identification of three regions of homologous linear sequence among cruzipain and myosin as well as the partial homology displayed in ribosomal P protein internal peptide sequence between *T. cruzi* and humans. These findings provide more evidence that the molecular mimicry mechanism may stimulate autoimmunity and strengths the hypothesis.

An additional concept of great discussion is the bystander activation. During microbial infection, toll-like receptors (TLRs) and pattern recognition receptors present on antigen-presenting cells (APCs) are stimulated leading to the synthesis and release of proinflammatory mediators. Together with self-antigens, coming from tissue destruction and creating a milieu of proinflammatory factors, all of these mediators may defeat self-tolerance by decreasing the threshold of activation enough to activate potentially autoreactive T cells and trigger autoimmunity. Furthermore, $CD8^+$ T cells may also initiate bystander activation by proliferating in response to self-antigen presented by APCs [21, 49, 60]. Hyland et al. [61] have shown that a reduction in parasitemia via treatment with Benznidazole, decreased or eliminated myosin-specific autoimmunity. They hypothesized that reduction of parasitemia consequently reduces release of host antigens and also dampens the inflammatory environment lessening bystander activation. Altogether, these data bring great perspectives to elucidate the autoimmune hypothesis of Chagas disease.

Subsequent to bystander activation, there is the development of autoimmune responses to endogenous epitopes secondary to the release of self-antigens, the so-called epitope spreading. It results from a change in protein structure, for example, changing of an amino acid from arginine to citrulline, which may succumb in an immune reaction against either the original protein or the citrullinated protein. Some of the mechanisms involved in epitope spreading are endocytic processing, antigen presentation, and somatic hypermutation, all culminating

in broadening the immune response in autoimmune pathologies. Several authors have demonstrated the epitope spreading against many cardiac proteins, such as myosin, Cha protein, desmin, actin, myoglobin, tubulin, and B1 adrenergic receptor [49, 55, 62]. Still, more studies must be conducted in order to prove that this mechanism fully contributes to the propagation of autoimmunity in Chagas disease.

5. Final remarks

Chagas disease afflicts millions of people each year worldwide. Some individuals present mild to moderate symptoms during the chronic phase, while others develop severe and life-threatening cardiac and digestive conditions. There have been great advances in understanding the physiopathology of Chagas heart disease, which may contribute to the evolution in the field of drug development and therapy approaches. Apparently the auto-reactive cells are great responsible for destruction of the cardiac tissue leading to cardiac failure, the most severe consequence of the disease. Although the data describing the existence of *T. cruzi*-induced autoimmunity continues to grow, there is still lack of direct evidencere ported in the literature. Elucidating the autoimmune hypothesis of Chagas disease may help to solve potential complications for Chagas disease treatments involving autoimmunity, as well as to better understand many questions that remain unanswered about Chagas disease pathogenesis. Moreover, this hypothesis may serve as a model for studying infection-induced autoimmunity, which may be applicable to proposing and investigating new immune therapies for autoimmune diseases.

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Chapter 8

Physiology and Pathology of Autoimmune Diseases: Role of CD4+ T cells in Rheumatoid Arthritis

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Additional information is available at the end of the chapter

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Abstract

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterised by synovial inflammation leading to bone erosion and to systemic manifestations in patients with long RA duration. Although the aetiology is unknown, several observations make currently clear that CD4 T cells play a key role in the pathogenesis: (1) RA associates with certain polymorphisms of HLA class II molecules, and (2) the repertoire and aging of CD4 T cells as well as the intracellular signalling mediating CD4 T cell activation are altered in RA patients. We describe herein the alterations found in CD4 T cells and the role of these cells in the development and progression of RA.

Keywords: autoimmunity, lymphocytes, synovitis, T cell signalling, T cell aging

1. Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease, which affects 0.33 to 2.65% of the population, showing differences between countries and studies [1–7]. It is more frequent in North America than Northern Europe, with Southern Europe having the lowest rate of incidence [8]. As other autoimmune diseases, RA is more prevalent in women than in men, suggesting that hormonal [9] and gender-related genetic factors [10] contribute to the development of the disease. RA is also more frequent in the elderly, consistent with a key role of immune system aging in this disease [11, 12].

RA physiopathology is characterised by persistent synovial inflammation that leads to joint deformity, stiffness and bone erosion. Consequently, patients suffer pain and progressive disability. Although the most evident feature of RA is synovitis, extra-articular manifestations of

RA (ExRA) such as cardiovascular disease can be present in long-duration disease, raising the risk of early death [13, 14].

RA is associated to certain alleles of the major histocompatibility complex class II (MHC-II), and CD4 T cells of RA patients show abnormalities in intracellular signalling, repertoire and aging. It is then conceivable that CD4 T cells could be essential mediators in the development of the chronic inflammation occurring in RA. These cells are key regulators of the immune response secreting pro-inflammatory cytokines and cooperating with B cells for secreting antibodies. In fact, certain RA patients develop autoantibodies such as anti-citrullinated protein antibodies (ACPA) or rheumatoid factor (RF, which recognises the Fc portion of IgG), while other patients do not, indicating that RA comprises at least two different pathologies, seropositive and seronegative [15].

The study of CD4 T cell population has changed our understanding of RA: from the traditional paradigm, which considered that a small set of joint antigens causes the selective expansion of few antigen-specific cells, to a new model in which RA would be a systemic disease caused by alterations in T cell homeostasis and aging. In this chapter, we will describe the role of CD4 T cells in the development of RA and the abnormalities that these lymphocytes show in diseased individuals.

2. Aetiology of rheumatoid arthritis

Although the aetiology of RA remains elusive, genetic and environmental risk factors have been described [16, 17]. MHC-II genes, particularly HLA (human leukocyte antigen) -DRB1 alleles (the so-called shared epitope [18, 19]), constitute the strongest genetic risk factor, accounting for 50% of the genetic contribution to RA [20]. Association with HLA-DRB1 has been established in different populations across the world [21–25], especially in ACPA-positive pathology, and different haplotypes of HLA-DRB1 associate with distinct RA severity and treatment response [26]. Single-nucleotide polymorphisms (SNPs) in other genes have also been linked to RA [16], including genes coding for molecules that regulate T cell activation, which will be discussed below. These genetic associations strongly indicate a decisive role of helper T lymphocytes in the pathology.

The major environmental risk factor is smoking habit, which seems to alter citrullination of mucosal proteins [27]. Genetic and environmental risk factors work together in promoting the disease. For example, smoking habit alters methylation of the HLA-DRB1 region, increasing the chance of developing ACPA-positive RA [28, 29].

Some infectious agents might also be risk factors of RA. For example, there is a positive association between the prevalence of periodontitis and RA [30]. *Porphyromonas gingivalis*, the major causative agent of periodontitis, produces an enzyme that induces aberrant citrullination of host proteins [31]. This generates neoantigens that can then be recognised by the immune system of the host, triggering ACPA production. In addition, it has been shown that ACPA from RA patients cross-react with various autoantigens and microbial and plant-citrullinated proteins [32]. This suggests that environmental factors such as infections and diet may trigger

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the production of ACPA in individuals with genetic predisposition. ACPA can then cross-react with self-proteins through molecular mimicry, inducing RA.

3. Pathophysiology of rheumatoid arthritis

A healthy joint (**Figure 1A**, left side) is composed of two adjacent bony ends covered with a layer of cartilage. The space between ends is called articular cavity, which is delimited by the synovial membrane on both sides and contains synovial fluid. The synovial membrane is a thin layer of cells, formed by two types of synoviocytes: type A or macrophage-like synovial cells

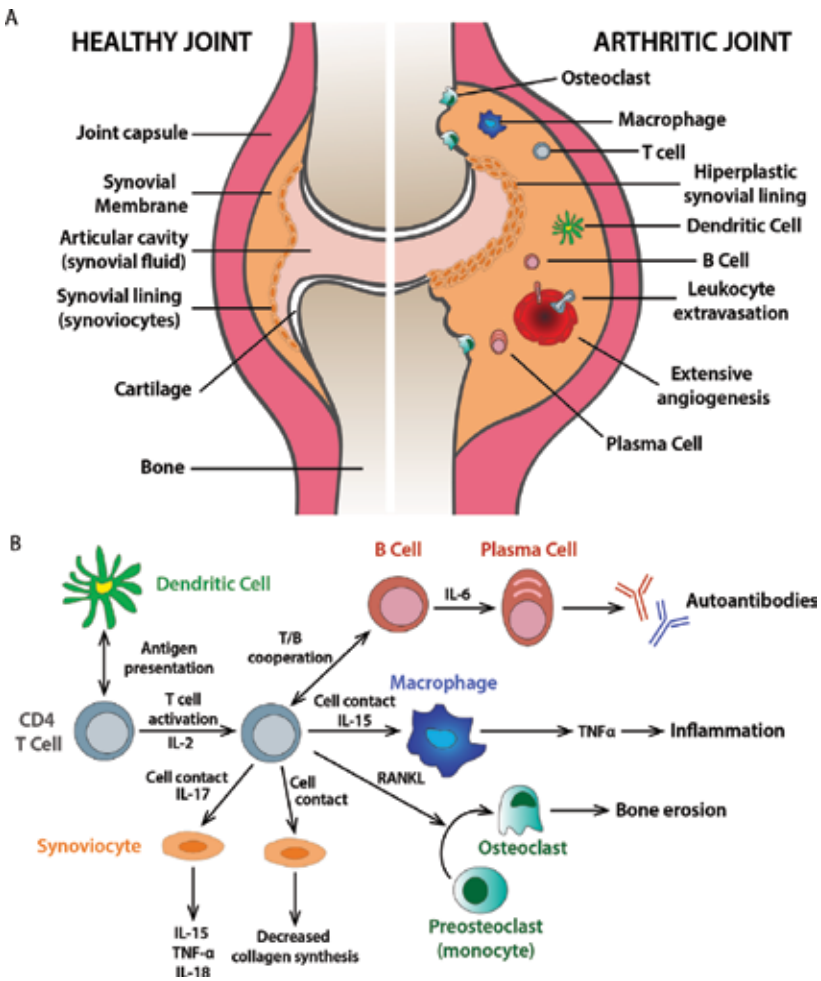

Figure 1. Role of CD4 T cells in rheumatoid synovitis. (**A**) In a healthy synovial joint (left), a thin layer of synoviocytes delimits the joint capsule. By contrast, in RA (right), synoviocytes form an invasive synovial lining and leukocytes infiltrate the synovial membrane. (**B**) Activated CD4 T cells play a central role in inflammatory responses in the synovial membrane, including autoantibody production by plasma cells, secretion of inflammatory cytokines by macrophages and synoviocytes, bone erosion by osteoclasts and inhibition of collagen secretion by synoviocytes.

and type B or fibroblast-like synoviocytes (FLSs). The synovial membrane produces synovial fluid and due to its porous organisation allows diffusion of the nutrients in serum to the avascular cartilage.

The confluence of genetic susceptibility and environmental factors determines the development of an autoimmune response that precedes clinical arthritis. For reasons poorly understood, this autoimmune response exacerbates in the synovium, where leukocytes infiltrate causing synovial membrane inflammation (rheumatoid synovitis) (**Figure 1A**, right side). Synovial infiltrate includes both innate and adaptive immune cells [33, 34] and creates a microenvironment where FLSs acquire an invasive and inflammatory phenotype, leading to hyperplasia of the synovial lining [35, 36]. FLSs secrete matrix metalloproteinases (MMPs) and collagenase, promoting cartilage destruction [37]. Leukocyte infiltration and secretion of pro-inflammatory cytokines favour maturation of pre-osteoclasts to osteoclasts, which leads to bone erosion [38–40]. Cytokines and growth factors released by infiltrated cells, together with the hypoxia resulting from synovial hyperplasia, trigger angiogenesis [41–43], establishing a feedback loop that favours continuous leukocyte infiltration and chronic inflammation.

Inflammation initiated in the synovium gives way to systemic inflammation that alters the function of distant tissues and organs, such as vascular endothelium, adipose tissue, liver and lungs. As a result, ExRA is present in RA patients, such as cardiovascular disease (CVD), anaemia or rheumatoid lung, among others [44].

Although different immune cells infiltrate the inflamed joint, we will focus on CD4 T cells, which, as mentioned above, seem to be central in the pathophysiology of RA by secreting cytokines and by cooperating with synovial cells.

4. Pathogenic role of CD4 T cells in rheumatoid arthritis

4.1. CD4 T cell activation and function in synovitis

CD4 T cells are the most abundant lymphocyte in the synovial infiltrate [45], where they regulate other cell types in the synovium and play a central role in the pathological immune response leading to the joint damage (**Figure 1B**).

4.1.1. CD4 T cell activation by DCs

Dendritic cells (DCs) are key initiators of adaptive immune responses, since they are professional antigen-presenting cells (APCs), able to present to T cell antigenic peptides in the context of the MHC-II. Initially, infiltrated CD4 T cells interact with synovial DCs, resulting in T cell stimulation (**Figure 1B**). Activation of CD4 T cells requires the engagement of the T cell receptor (TCR) by antigen-MHC-II complexes on the surface of the APC. In addition, full T cell activation requires interaction between the molecule CD28 on the T cell and its ligands CD80 and CD86 expressed by APCs, which provides costimulatory signals. Activated CD4 T cells upregulate the expression of the inhibitory molecule cytotoxic T lymphocyte antigen-4 (CTLA-4), which binds CD80 and CD86 with higher affinity

than CD28 [46]. During consecutive contacts with APCs, CTLA-4 will compete with CD28 for CD80/CD86, and binding of CTLA-4 to these ligands will result in inhibition of T cell activation [47]. The importance of APC-mediated T cell costimulation for the progression of RA has been proved by therapy with the CTLA-4-immunoglobulin fusion protein abatacept. This molecule binds to CD80/CD86 on the APC, impeding binding of CD28 and, therefore, blocking T cell costimulation [48]. Treatment with abatacept reduces disease activity and radiographic progression of RA [49, 50].

4.1.2. Cooperation between CD4 T cells and B cells

B cells play a fundamental role in seropositive RA, in which patients develop autoantibodies contributing to inflammation and tissue damage. Autoantibodies are synthesised by plasma cells, which differentiate from B cells after cooperation with CD4 T cells. Upon activation, T cells upregulate the surface expression of CD40 ligand (CD40L or CD154), which interacts with CD40 expressed by B cells. During T/B cooperation, stimulation through CD40 together with IL-6 signalling favours isotype switching, differentiation of B cells into plasma cells and synthesis of antibodies such as ACPA (**Figure 1B**) [51]. CD4 T cells, B cells and DCs found in joints of RA patients range from diffuse infiltrates to follicular structures, forming ectopic germinal centres (EGCs) in some patients [52]. Formation of EGCs favours the formation of high affinity autoantibodies, increasing the severity of the disease [53]. EGCs and B cells seem to be critical for T cell activation in the synovium [54].

4.1.3. Regulation of FLSs by CD4 T cells

As mentioned before, FLSs are an important component of joint architecture. In a healthy joint (**Figure 1A**, left side), FLSs form the synovial lining and produce synovial fluid. FLSs acquire an invasive phenotype in RA, causing hyperplasia of the synovial lining (**Figure 1A**, right side). This hyperplasia originates a hypoxic environment where angiogenesis is activated, favouring perpetuation of inflammation. In addition, RA FLSs secrete high amounts of proteases, which trigger cartilage destruction, and pro-inflammatory cytokines.

Antigen-experienced CD4 T cells affect the function of FLSs by direct cell-cell interaction. For example, CD4 T cells induce the production of the pro-inflammatory cytokines IL-15, TNF-α and IL-18 by FLSs (**Figure 1B**). This is dependent on CD40L-CD40 engagement as demonstrated by a blocking agent [55]. Collagen synthesis by FLSs is also decreased by CD4 T cells, a process mediated, at least in part, by T cell membrane-associated IFN-γ, TNF-α and IL-1α [56].

4.1.4. Regulation of macrophages/monocytes by CD4 T cells

Macrophages infiltrate the RA joint, where they interact with synovial cells and produce the pro-inflammatory cytokine TNF-α. CD4 T cells regulate macrophages in the synovium, as shown by the finding that freshly isolated synovial T cells can induce the expression of the pro-inflammatory cytokine TNF-α by macrophages in an IL-15-dependent manner (**Figure 1B**) [57]. Resembling the behaviour of T cells in RA patients, T cells of healthy donors stimulated with an inflammatory cytokine cocktail can induce the production of TNF-α by

resting monocytes [58]. It should be noted that TNF-α production by myeloid cells is also induced by IL-15-stimulated NK cells [59]. Due to the central role of TNF-α in the progression of RA, as demonstrated by the succeeded neutralising therapy [60], it will be needed to further investigate this complex regulation of immune cells in the inflamed joint.

Monocytes are the progenitors of osteoclasts, which constitute the only cell type that is able to degrade bone. In health, bone resorption by osteoclasts and bone generation by osteoblasts are tightly regulated to maintain skeletal integrity and homeostasis. In RA, osteoclast activity in the joint is increased, resulting in an unbalanced bone erosion. Synovial CD4 T cells from RA patients, as well as activated peripheral blood T cells from healthy donors, express receptor activator of nuclear factor κB ligand (RANKL), which engages RANK expressed on monocytes, inducing their differentiation to osteoclasts [61, 62] and, consequently, triggering bone erosion (**Figure 1B**).

4.1.5. Role of IL-17 secretion by T cells

Synovial CD4 T cells produce pro-inflammatory cytokines themselves (**Table 1**). Among these, IL-17 expression is increased in the synovial tissue of RA patients [63], its levels correlate with disease activity [64] and it has a predominant role in rheumatoid pathology [65]. This cytokine is produced by Th17 cells that are critical drivers of synovitis [66]. In the synovium, IL-17 stimulates the production of pro-inflammatory cytokines by rheumatoid synovial cells [67, 68], triggers osteoclastogenesis [69] and impairs cartilage repair [70]. Methotrexate, a first-line conventional therapeutic agent in RA, attenuates IL-17 production by peripheral blood mononuclear cells in vitro [71], supporting the pathogenic role of this cytokine.

Interestingly, the balance between Th17 and regulatory T cells (Treg), which exert anti-inflammatory functions, is shifted towards the Th17 subset in RA [72]. The first hypothesis explaining the excessive Th17 response in RA is that it might be an enhanced Th17 differentiation due to the inflammatory environment. Th17 cells differentiate in the presence of IL-1β, IL-6 and IL-23 [73], which are secreted by activated macrophages and dendritic cells in inflammatory conditions [74]. Supporting this hypothesis, both IL-23 and IL-6 levels are increased in patients with RA [75, 76]. IL-23 levels correlate with the activity of early arthritis [77]. A second hypothesis would be that intrinsic alterations in naïve CD4 T cells might prone Th17 rather than Treg differentiation. Supporting this hypothesis, naïve RA T cells overexpress glucose-6-phosphate dehydrogenase (G6PD), which causes insufficient activation of ataxia telangiectasia mutated (ATM), leading to biased differentiation of CD4 T cells towards Th17 and Th1 subsets (**Table 2**) [78].

4.2. Abnormalities in CD4 T cell activation and signalling

As mentioned in the previous sections, CD4 T cell activation in the synovium is a key event in RA pathology. CD4 T cell activation is initiated by interaction of the TCR with the antigen-MHC-II expressed on the surface of an APC. Engagement of TCR/MHC-II-antigen complex triggers the activation of intracellular signalling networks in which phosphorylation plays a decisive role. The kinases Lck and ZAP70 are rapidly activated after TCR stimulation and activate downstream effectors such as extracellular signal-regulated kinase (ERK) to induce

Cytokine	Pathogenic role
TNF-α	• Activates leukocytes, synovial fibroblasts, endothelial cells and osteoclasts • Induces production of inflammatory cytokines • Enhances metalloproteinase expression • Suppresses Treg cells
IFN-γ	• Increases antigen presentation • Activates macrophages • Increases chemokine secretion
IL-1	• Activates leukocytes, synovial fibroblasts, endothelial cells and osteoclasts • Induces production of matrix proteinases
IL-6	• Activates leukocytes and osteoclasts • Stimulates antibody production
IL-17	• Induces production of inflammatory cytokines • Activates innate immune cells • Increases osteoclastogenesis • Stimulates neutrophil recruitment
IL-21	• Activates Th17 and B cells

Table 1. Pathogenic role of cytokines secreted by CD4 T cells in the RA synovium.

gene expression and cell proliferation. In physiologic conditions, signalling downstream the TCR is tightly regulated by proteins such as phosphatases. In T cell-mediated autoimmune pathologies, such as RA, intracellular signalling is deregulated, leading to alterations in T cell responses.

Another physiological mechanism regulating T cell responses and preventing autoimmunity is the elimination of self-reactive T cells. This mechanism is called tolerance and occurs both on immature T cells in the thymus (central tolerance) and on mature circulating T cells (peripheral tolerance). In RA, activation of CD4 T cells by self-antigens seems to be permitted by losing peripheral or central tolerance and promoted by enhanced sensitivity to self-antigens due to alterations in signalling networks integrating extracellular stimuli.

Several observations indicate that peripheral blood, and not only synovial-infiltrating T cells, show hyper-activation in RA patients [79, 80]. An aberrant function or expression of signalling molecules, some of them regulating T cell responses, has been found in CD4 T cells of RA patients (**Table 2**) and will be discussed below.

Protein	Alteration	Consequence in CD4 T cells	Reference(s)
G6PD	Overexpression	• Insufficient ATM activation • Hyperproliferation • Increased Th1/Th17 differentiation	[78]
LYP (rs2476601 SNP)	Gain of function mutation	• T cell hyporesponsiveness	[88–92]
TC-PTP (rs1893217(C) SNP)	Reduced expression	• Decreased STAT5 phosphorylation • Decreased FOXP3 expression upon activation	[95, 96]
CDC25B	Reduced expression	Not reported	[99]
DUSP7	Reduced expression	Not reported	[99]
B-RAF K-RAS	Overexpression	• Increased ERK phosphorylation and signalling • Autoreactive response to citrullinated peptides	[101]
PD-1	Reduced expression	Not reported	[91–95]
Telomerase	Insufficient induction	Susceptibility to apoptosis	[12]
MRE11A	Reduced expression	• Telomeric damage • Senescence	[11]

G6PD, glucose-6-phosphate dehydrogenase; ATM, ataxia telangiectasia mutated; LYP, lymphoid-specific tyrosine phosphatase; TC-PTP, T cell protein tyrosine phosphatase; STAT5, signal transducer and activator of transcription 5; FOXP3, forkhead box P3; CDC25B, cell division cycle 25 B; DUSP7, dual-specificity phosphatase 7; ERK, extracellular signal-regulated kinase; PD-1, programmed death 1; MRE11A, meiotic recombination 11 homolog A

Table 2. Alterations in gen/protein expression or activity found in CD4 T cells from RA patients and their phenotype.

4.2.1. PD-1

Programmed death-1 (PD-1) receptor is inducibly expressed on CD4 T cells upon activation through the TCR [81]. Upon binding to its ligands during TCR stimulation, PD-1 delivers inhibitory signals that suppress T cell activation and proliferation and impair T cell survival [82]. A set of SNPs in the gene coding for PD-1 are linked to RA [83–85], and PD-1 expression is decreased in T cells from RA patients [86]. This reduced expression would lead to a defect in peripheral tolerance, favouring autoimmunity.

4.2.2. LYP

The lymphoid-specific tyrosine phosphatase (LYP) is encoded by the gene *PTPN22*. This protein is exclusively expressed in cells of the immune system and in T cells negatively regulates TCR signalling by inactivating the kinases Lck and ZAP70 [87]. Therefore, LYP is an important inhibitor of signalling downstream the TCR. The SNP rs2476601 in *PTPN22* is associated with RA [88, 89]. The pathological function of this SNP, which results in the LYP mutant R620W, remains controversial. Various reports show that the LYP R620W variant is more effective in

downregulating TCR signalling than the LYP WT [90, 91]. In this situation, LYP R620W would trigger autoimmunity because it would suppress TCR signalling of autoreactive T cells during negative selection in the thymus, promoting their survival and compromising central tolerance [92]. Molecular mechanisms leading to autoimmunity in the presence of this polymorphism should be further studied.

4.2.3. TC-PTP

The T cell-phosphotyrosine phosphatase (TC-PTP) is encoded by the gene *PTPN2*. This tyrosine phosphatase negatively regulates TCR and JAK-STAT signalling, being an inhibitor of T cell activation [93, 94]. The SNP rs1893217(C) in *PTPN2* is associated with juvenile idiopathic arthritis and results in decreased gene expression [95]. Strikingly, decreased phosphorylation of STAT5 and reduced FOXP3 expression are found in cells carrying this mutation [96]. Because FOXP3 is the master regulator of Treg differentiation [97], this SNP might cause abnormalities in Treg functions, resulting in increased inflammation. The mechanism for this phenotype should be investigated.

4.2.4. CDC25B

The dual-specificity phosphatase cell division cycle 25 B (CDC25B) positively regulates cell proliferation by promoting G2/M transition [98]. Recently, our group has found a reduced expression of this phosphatase in CD4 T cells of patients diagnosed with early arthritis [99]. Importantly, altered CDC25B levels associate to the activity of the disease. Whether this alteration causes or is a consequence of the inflammatory environment characteristic of RA, and its effect in T cell responses will need further investigation.

4.2.5. Regulators of ERK signalling

As mentioned before, ERK is a key effector molecule downstream TCR activation. Hence, defective regulation of ERK phosphorylation levels could lead to aberrant T cell responses. The expression of some ERK regulator is altered in T cells of RA patients.

The dual-specificity phosphatase 7 (DUSP7) negatively regulates ERK phosphorylation and activity [100]. Although its role in T cells has not been addressed, it is conceivable that DUSP7 could be a negative regulator of MAPK signalling in T cells being activated. CD4 T cells of patients with seropositive early arthritis have reduced expression of DUSP7 [99]. The fact that defective expression is restricted to seropositive patients could indicate a role of this phosphatase in T/B cooperation. Further investigation is needed to determine the functional significance of DUSP7 in T cells.

The GTPase K-RAS and the kinase B-RAF are positive regulators of ERK signalling upon TCR stimulation. A higher TCR-induced ERK phosphorylation results in a lower T cell activation threshold, contributing to autoimmunity. K-RAS and B-RAF are overexpressed in T cells of RA patients [101]. Interestingly, overexpression of B-RAF and K-RAS increases the activation of CD4 T cells of healthy donors by a citrullinated vimentin peptide. This finding provides support to the notion that higher CD4 sensitivity could cause loss of peripheral tolerance in RA patients.

4.3. Abnormalities in CD4 T cell repertoire and aging

The ability of the adaptive immune system to respond to the large diversity of pathogens found throughout life depends on the generation of a wide TCR repertoire. This repertoire is generated in the thymus, where the V, D and J segments of the TCR rearrange randomly. Newly generated naive T cells migrate from the thymus to the periphery to exert their functions. The thymic output, however, declines throughout life. In the elderly the thymus no longer functions as a source of new naïve T cells, which have to be produced by replication of mature peripheral T cells, a process called homeostatic proliferation [102]. The expansion of peripheral T cell clones generates a contraction in T cell repertoire and induces a phenotype of replicative stress that is characteristic of aged people [103]. Clone expansion of peripheral cells might favour an increased presence of autoreactive clones. Consistent with this idea, autoimmune signs such as autoantibody production are higher in elderly individuals [104].

Repertoire contraction and clonally expanded populations in the CD4 compartment have been reported in RA [105]. Clonal expansion was initially interpreted as a consequence of specific responses to synovial self-antigens, but this hypothesis is unlikely. Contraction in CD4 T cell diversity is not limited to the memory compartment, but involves also naïve T cells [106]. This seems to be due to an accelerated aging of the immune system in RA patients, in which the thymus function is lost earlier than in healthy people [107].

A hallmark of immune aging is the accumulation of end-differentiated effector CD4 T cells that lack expression of the costimulatory receptor CD28 [108]. Indeed, the frequency of CD4+ CD28− lymphocytes is higher in RA patients [109, 110]. These cells are producers of IFN-γ, display cytotoxic functions and are autoreactive [109, 111, 112]. Such phenotype could be mediated, at least in part, by increased expression of the NK cell-activating receptor NKG2D. Ligands of NKG2D are highly expressed in inflamed synovium [113].

Another hallmark of cellular aging is telomere shortening [114], and lymphocytes from RA patients show premature telomeric loss [115]. In naïve CD4 T cells, this is due to insufficient upregulation of telomerase activity (**Table 2**), which in addition promotes apoptosis in these cells [12]. Excessive loss of naive T cells will further stimulate homeostatic proliferation of effector T cells, providing a positive feedback loop of replicative stress.

Recently, another alteration in DNA repair machinery was found in CD4 T cells from RA patients [11]. The expression of repair nuclease MRE11A is decreased in these cells, leading to telomeric damage and upregulated senescence markers (**Table 2**).

4.4. CD4 T cells in extra-articular disease

Although the main site of inflammation in RA is the synovium, pro-inflammatory cytokines and activated cells are released to the bloodstream, leading to systemic inflammation. This inflammatory state has multiple ExRA on distant organs, such as skin, lungs, heart, blood or bone [116]. Smoking habit and autoantibodies predispose to severe ExRA [117]. Several systemic

pathologies are frequent in RA patients, such as systemic vasculitis, interstitial lung disease and pericarditis, which is the most common cardiac complication [116]. We focus here on CVD.

Chronic inflammation generates a pro-atherogenic environment in RA. Indeed, RA patients have increased risk of cardiovascular death [118] and higher incidence of atherosclerotic heart disease [119]. Atherosclerosis is an inflammatory process in which the plaque, constituted by lipid accumulation on arterial walls, causes endothelial injury and activation. This promotes the recruitment of leukocytes, which culminates in the disruption of the plaque and thrombosis. Vascular inflammation in atherosclerosis and synovial inflammation in RA share features of immune activation, including accumulation of inflammatory macrophages and T cells, production of inflammatory cytokines and degradation of the extracellular matrix. High levels of soluble factors such as C-reactive protein, TNF-α and IL-6 are associated with coronary artery disease [120–122]. These cytokines are also elevated in chronic inflammation, which renders lipoproteins more atherogenic, reduces the repair of injured endothelium and upregulates the expression of endothelial adhesion molecules, which enhance leukocyte recruitment [123]. Consistent with a role of systemic inflammation in atherosclerosis, RA therapies based on methotrexate and TNF-α antagonists decrease CVD rates [124, 125].

As mentioned before, the CD4+CD28− T cell subset is expanded in RA [109, 110]. This T cell subset is also expanded in patients with unstable angina (UA) [126], a pathology in which the atheroma plaque is disrupted causing thrombosis. The percentage of CD4+CD28− cells correlates with recurrence of UA, pointing to a direct role of these cells in the progression of the pathology [127]. In addition, expanded CD4+ CD28− found in the atherosclerotic lesion includes large monoclonal populations, suggesting that these cells can recognise antigens in the atheroma plaque [128]. Consistently, RA patients with expansion of circulating CD4+CD28− cells show preclinical atherosclerotic changes, including endothelial dysfunction [129]. The implication of CD4+CD28− cells in atherosclerosis is further supported by anti-TNF therapy, which normalises CD28 expression [130] and decreases CVD rates [125].

5. Conclusion

RA is a chronic inflammatory disease characterised by synovitis and systemic features, such as exacerbated atherosclerosis. CD4 T cells are key mediators of tissue damage, both in the joint and in extra-articular lesions, through a variety of mechanisms. Certain alleles of the MHC-II as well as different alterations of signalling molecules and checkpoints for activation seem to favour self-antigen recognition, activation and break of tolerance. Besides, abnormalities found in CD4 T cell repertoire and phenotype in patients with RA strongly suggest that in these patients there is an accelerated aging of the immune system that leads to oligoclonality and senescence of T cells, making these lymphocytes autoreactive. Understanding the mechanisms underlying these systemic alterations will be essential for the development of more effective therapies for RA treatment.

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Chapter 9

Physiology and Pathology of Neuroimmunology: Role of Inflammation in Parkinson's Disease

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Additional information is available at the end of the chapter

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Abstract

Parkinson's disease (PD) is a neurodegenerative disease that affects 1% of the population aged 65 and over and is the second most common neurodegenerative disease next to Alzheimer's disease. Interneuronal proteinaceous inclusions called Lewy bodies (LB) and a selective degeneration of dopaminergic neurons of the substantia nigra pars compacta (SNPC) are the main features of PD pathology. The most common clinical manifestations are rigidity, tremor, bradykinesia, postural instability, sleep disorders, alterations in gait, smell, memory, and dementia. Genetic and environmental factors are involved in PD, and, recently, oxidative stress, proteasome-mediated protein degradation, and inflammation have acquired relevance as major mechanisms of neuronal dysfunction. Increased levels of reactive oxygen and nitrogen species in the brain contribute to greater vulnerability of proteins to nitro-oxidative modification and to greater degrees of aggregation. These protein aggregates contain a variety of proteins of which α-synuclein appears to be the main structural component. Interestingly, α-synuclein can be secreted by neuronal cells and may lead the initiation and the maintenance of inflammatory events through the activation of microglia, which contributes to dopaminergic neuron depletion. New evidence also suggests that PD may be the result of an autoimmune response in which the immune cells recognize the neurons as foreign elements and would act against them, causing their death.

Keywords: Parkinson's disease, physiology, pathology, neuroimmunology, inflammation

1. Introduction

The central nervous system (CNS) has traditionally been considered immunologically privileged due to the protection conferred by the blood-brain barrier; it lacks lymphatic vessels and is devoid of dendritic cells, and the parenchyma cells do not express major histocompatibility complex (MHC) class-I antigen-presenting molecules. However, the CNS can modulate the immune response and limit inflammation-induced tissue damage [1]. Neurons of the CNS are actively involved in control of the immune response by modulating the function of glial cells and T lymphocytes. There are mechanisms involved in the control of the immune response: the direct contact through membrane glycoproteins (CD22, CD47, CD200), neural cell adhesion molecules (NCAM or CD56), intercellular cell adhesion molecule-1 (ICAM-1), semaphorins and cadherins, and the mechanism independent of cell-cell contact that involves the expression of the Fas ligand or CD95L, which promote apoptosis of microglial cells and T lymphocytes. The immune system is not a completely autonomous system since the lymphoid organs are innervated by cholinergic, catecholaminergic, and peptidergic neurons and other neurons [2]. Thus, the nervous system and the immune system can interact not only through the hypothalamic-pituitary-adrenal axis, whose activation leads to the synthesis of corticosteroids that inhibit the immune response, but can also do so through neuronal circuits at the central level through the autonomic nervous system (ANS), both sympathetic and parasympathetic, which, through sensory and effector circuits, transmit impulses that reflexively induce the implementation of an anti-inflammatory response. In physiological conditions, the sensory and afferent fibers of the ANS travel in the vagus nerve from the peripheral tissues to the CNS to provide information about tissue function or, on the contrary, about the existence of injury within tissues that leads to the development of a cytokine-induced inflammatory process. The afferent sensory stimulus triggers a response in the CNS that includes the signs and symptoms of the disease and the efferent sympathetic pathway, called the cholinergic anti-inflammatory reflex, which, through the vagus nerve, inhibits the synthesis of pro-inflammatory cytokines and thus limits or prevents tissue damage produced by these mediators.

Pro-inflammatory cytokines such as tumor necrosis factor (TNF), interleukin (IL)-1, and IL-6, produced during the activation of innate immunity cells in peripheral tissues, are able to modulate the activity of CNS neuronal circuits through specific receptors expressed by neurons of the hypothalamus and other regions of the brain. In this way, a response is characterized by the transmission of action potentials that trigger local and systemic symptoms and signs of the disease syndrome, which are then controlled by the cholinergic and anti-inflammatory vagal route. This CNS response leads not only to control the progression of the inflammatory process in the peripheral tissue but also to prevent eventual immune-mediated tissue damage. Thus, the immunological activation of this neuronal circuit confers protection against tissue damage by inhibiting the release of cytokines during infection, autoimmunity, shock, and other inflammatory syndromes in the CNS.

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Parkinson's disease is a neurodegenerative disease characterized by an early loss of dopaminergic neurons in the substantia nigra pars compacta, located in the basal ganglia. The resulting deficiency of dopamine leads to a movement disorder characterized by classic motor symptoms (rigidity, resting tremor, bradykinesia, and postural instability), as well as non-motor symptoms which may often appear even years before the diagnosis of the disease. The gold standard for the diagnosis of PD is still an autopsy demonstrating degeneration of the substantia nigra and, in most cases, evidence of Lewy bodies (abnormal aggregates of α-synuclein protein). The association between Lewy pathology and the pathogenesis of the disease is poorly understood and is not limited to the brain, but it can also be found in the spinal cord and peripheral nervous system (including the vagus nerve, sympathetic ganglia, cardiac plexus, enteric nervous system, salivary glands, adrenal medulla, cutaneous nerves, and sciatic nerve). Moderate loss of neurons of the substantia nigra is also present in early stages of the disease. In addition, neuronal loss in PD occurs in many other brain regions including the locus coeruleus, Meynert's basal nucleus, pedunculopontine nucleus, raphe nucleus, dorsal motor nucleus of the vagus, amygdala, and hypothalamus [3]. Hence, the varied symptomatology of the disease is now conceptualized more like a syndrome than as a disease itself.

Commonly, deterioration of the sense of smell is one the earliest symptoms of PD. It usually manifests as a partial reduction in the ability to discriminate or perceive odors, and this occurs due to changes in the α-synuclein protein of the dorsal motor nucleus of the vagus and olfactory bulb [4, 5]. On the other hand, a prospective study showed that 40 of 78 relatives of patients with PD had hyposmia at the start of the study, and 4 of them developed the disease after 2 years [6].

Mood disorders such as anxiety, depression, and personality changes have often been linked to early stages of PD. In fact, depression is a major contributor to poor quality of life, future disability, and average survival to the disease [7]. Depression has been linked to multiple neurotransmitter dysfunctions, including dopamine, serotonin, and noradrenaline. About 35% of the patients with PD had clinically significant symptoms of depression over the course of the disease, and depressive symptoms precede motor symptoms in 30% of the patients. The incidence of depression appears to increase during the last few years prior to the diagnosis of PD. In addition, more than 30% of the patients have cognitive impairment in the early stages of the disease. In early stages, the cognitive impairment is mild, is non-amnesic, and has a frontal subcortical pattern, whereas the progress toward dementia is due to the damage to the posterior cortical areas. Alterations of working memory and difficulties understanding complex grammatical structures may also be present [8].

Sleep disorders such as excessive daytime sleepiness or rapid eye movement (REM) sleep behavior disorder (RBD) are commonly identified even many years before the diagnosis of the disease has been made. In RBD, the subject loses the characteristic atony of the REM phase in which all body muscles except for the ocular muscles are paralyzed. These patients make body movements in apparent response to the content of their dreams. It has been demonstrated that over 50% of the people with RBD will develop the disease in a period of 10–15 years [9]. Therefore, many authors argue that RBD is by far the strongest available clinical predictor of neurodegenerative disease associated with α-synuclein accumulation in the brain. Other early symptoms are gastrointestinal problems such as reduced intestinal transit, constipation, and changes in the intestinal microbiota. These symptoms have been observed as early as 20 years before the onset of the motor symptoms [10, 11].

Excess saliva, which often causes drooling, has been recognized as a feature of the disease since James Parkinson initially described the syndrome in 1817. Although it is not a dangerous symptom, it can be embarrassing in the social context and thus became very annoying for the patient and the caregivers. Interestingly, this problem is not due to saliva overproduction, since people with PD usually generate less saliva than normal [12]. Bladder control dysfunction is another autonomic dysfunction found in PD patients, probably caused by brain stem deterioration.

2. Physiology of the basal nuclei

The functional organization model of the basal nuclei assumes that the connections between the basal nuclei (BN), the cerebral cortex, and the thalamus form parallel and separate circuits. The sensory-motor and association areas of the cerebral cortex send glutamatergic excitatory projections to the sensory-motor and associative regions of the striatum, which projects through two striatal pathways to the exit of BN (globus pallidus internus (GPi)/globus pallidus externa (GPe) and substantia nigra pars reticulata (SNR)). The direct pathway, which facilitates the initiation and execution of voluntary movement, originates from the inhibition through gamma-aminobutyric acid (GABA) and substance P (SP) of the inhibitory striatal neurons, thus triggering the thalamus disinhibition. In the indirect pathway, the activation of the inhibitory striatopallidal projections, through GABA and enkephalins, suppresses the activity of GPe neurons, thus inhibiting the subthalamic nucleus (STN). The STN reaches the thalamus through glutamatergic projection. Therefore, when activated, it produces an excitatory action. Its inhibition through the GPe in the indirect pathway in turn increases its inhibition over the thalamus. In addition, the high discharge frequency of most pallidal neurons exerts a tonic inhibition on the STN.

During the execution of a specific motor act, movement-related neurons in the GPi/GPe and SNR present a phasic increase or decrease in their spontaneous discharge frequency. The phasic decrease plays a crucial role in motor control by inhibition of the ventral lateral (VL) nucleus of the thalamus, facilitating cortical initiated movements, and the phasic increase seems to have the opposite effect. The direct and indirect pathway inputs on the GPi/GPe and the SNR neurons are not fully described. However, it is possible that the direct and indirect inputs that are selectively and cooperatively activated, in relation to a cortically initiated movement, can be directed to the same group of neurons. This enables the entrances of the indirect pathway to downregulate a movement that was reinforced by the direct pathway. Another possibility is that the inputs of the direct and indirect pathways associated to a specific movement are directed to different neuronal groups, playing a double role in the cortical modulation of movement by reinforcing a selected motor model by the direct route and suppressing a conflictive one by the indirect pathway. The nigrostriatal dopaminergic projection exerts opposing effects on the striatal efferent pathways. It seems to have an excitatory effect on the striatal neurons of the direct pathway and an inhibitory effect on the indirect pathway. Thus, the action of the DA on the striatum reinforces the cortical activation of the circuit, facilitating the conduction through the direct pathway which has

an excitatory effect on the thalamus and suppressing conduction through the indirect pathway that has an inhibitory effect on the thalamus (**Figure 1**) [13].

2.1. Basal nuclei and the movement control

Basal nuclei are part of the cortico-subcortical circuits involved in the programming and execution of movement, as evidenced by the profound alterations in movement in the diseases in which BN are affected [14]. A number of studies have been published on the action of the various neurotransmitters integrated in the BN and their specific role. These studies have helped to understand the role of neurotransmitters involved in the organization of movement as well as the interactions of each of the nuclei that form part of the BN [15–18].

The striatum is one of the main structures involved in the rotational behavior; it receives an important afferent projection of the neurons and through the nigrostriatal pathway reaches

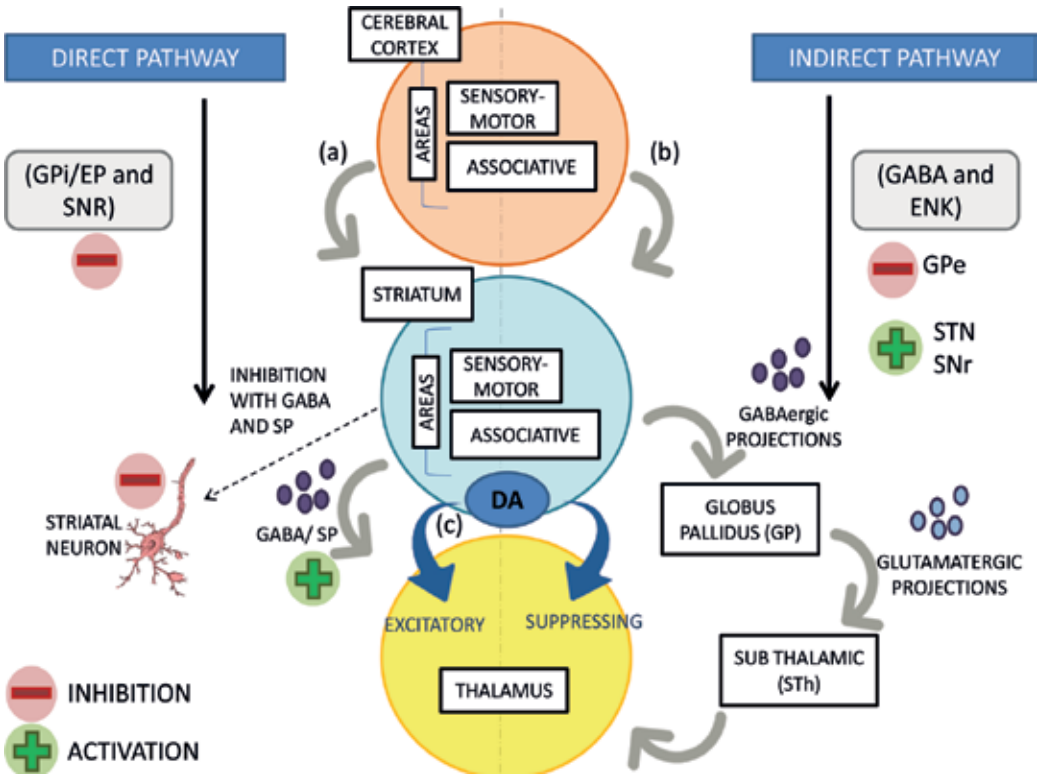

Figure 1. Physiology of basal nuclei (direct and indirect pathways). Functional organization of the basal nuclei has lead to the postulation of a model assuming connections between cerebral cortex, thalamus and basal nuclei. (a) direct pathway is originated by inhibition of striatal neuron with GABA and SP, their activation leads to a disinhibition of the thalamus. (b) the indirect pathway (inhibitory with GABA and ENK) reaches first to (GP) through an GABAergic projection then to STh through a glutamatergic projection. (c) the action of the DA on the striatum reinforces the cortical activation of the circuit, facilitating the conduction of the "direct path", which has an excitatory effect on the thalamus or suppressing conduction through the "indirect pathway" that has an inhibitory effect on the thalamus.

the GABAergic neurons of the SNR. The rotational behavior is influenced by the CNS dopaminergic and the SNR GABAergic neurons [19]. Posture control is also attributed to the nigrostriatal dopaminergic neurons of the CNS and the non-dopaminergic neurons of the SNR. The unilateral lesion of the nigrostriatal projection with 6-hydroxydopamine produces a dramatic asymmetry with a tendency of the animal to rotate toward the injured side (homolateral rotation). On the other hand, unilateral electrolytic lesions of the SNR induce a rotation preference toward the uninjured side (contralateral rotation), indicating the existence of non-DA neurons that originate from or across the SNR. Unilateral injection of SNR with kainic acid produces spontaneous contralateral rotation, maintaining a relative integrity of the CNS and a low reduction of serotonin but a marked decrease of glutamic acid decarboxylase and catalase in the striatum, which suggests that kainic acid damages the non-dopaminergic (GABAergic and cholinergic) neurons of the SNR. Unilateral intra-nigral injection of ethanolamine-O-sulfate, which produces an endogenous GABA accumulation within the neuron by blocking the enzyme GABA transaminase, also produces contralateral rotations similar to those produced by kainic acid. This suggests that the destruction of GABAergic neurons of the SNR would control rotations in a manner opposite to nigrostriatal dopaminergic neurons. The unilateral lesion of dopaminergic nigrostriatal neurons with kainic acid produces contralateral rotations independent of the action of nigrostriatal dopaminergic neurons which produces a decrease in neurons of the SNR. Thus, the non-dopaminergic neurons of the SNR control rotations and posture in a manner opposite to dopaminergic neurons [19].

Unilateral pedunculopontine tegmental nucleus (PPTg) injury is associated with rotational movement. The unilateral injection of GABA agonists into PPTg triggers rotation and contralateral postural asymmetry. Conversely, injection of GABAergic antagonists has the inverse effect. The stimulation of PPTg with kainic acid produces homolateral rotations which can be blocked by haloperidol (DA antagonist), α-methyl tyrosine (TH blocker that reduces neuronal dopamine and norepinephrine), and bilateral atropine injections. These data suggest cholinergic-dopaminergic interactions. In unilateral kainic acid lesions in PPTg, slow rotations occur in response to systemic amphetamine: unilateral quinolinate lesions in the PPTg produce a slight homolateral inclination in response to systemic amphetamine. However, bilateral quinolinate lesions have no effect on locomotor activity. On the other hand, lesions of ibotenate produce a slight contralateral inclination with amphetamine. These effects may be due to a loss of a large number of cholinergic and a smaller number of non-cholinergic PPTg neurons after injury with ibotenate [20].

2.2. Basal nuclei and Parkinson's disease

The observation that Parkinsonian patients have difficulties initiating movement led to the hypothesis that BN are involved in the automatic execution of learned movements [21]. There are two categories of motor disorders produced by BN alterations: hyperkinetic and hypokinetic. The hypokinetic motor disorders include bradykinesia, akinesia, and/or rigidity. PD is the prototype of the hypokinetic disorders since it is characterized by bradykinesia, increased muscle tone, and slow spontaneous movements [21]. Parkinson's disease is a variable combination of certain signs attributable to BN dysfunction, for which there is no apparent etiology

[22, 23]. The main pathophysiological findings in PD are the degeneration of neuronal bodies (greater than 80%) and an anterograde loss of ascending nigrostriatal axons and of its terminal ramifications reaching the putamen and caudate, which causes a reduction of DA and a significant loss of the dopaminergic neurotransmission. Therefore, the signs of PD are due to a deficiency of DA in BN. Although there are other biochemical alterations, their contribution to the signs and symptoms of PD is unknown [22–25]. But, what causes those neurons to die? Currently, four possible culprits are involved in neuronal loss: (1) excessive free radical production, (2) environmental toxins, (3) premature aging of neurons, and (4) hereditary factors (**Figure 2**).

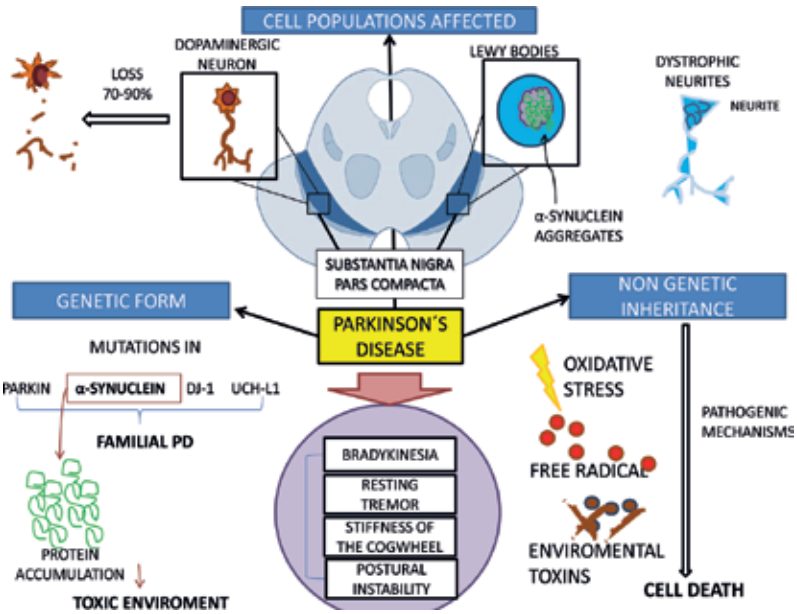

Figure 2. General pathophysiology of Parkinson´s disease. Classic symptoms of PD includes bradykinesia, resting tremor, stiffness of the cogwheel and postural instability manifested only when 70–90% of the dopaminergic neurons have been lost in the pars compacta, also the presence of Lewy bodies (containing eosinophil inclusions containing an aggregated α-synuclein center, along with other proteins and an area of radiated fibers) and dystrophic neurites are associated with pathological mark of PD. Approximately 10% of patients have a familial PD, with a defined genetic component. mutation in genes, α-synuclein, parkin (a ubiquitin E3 ligase involved in the degradation of multiple compounds), and DJ-1 (its role is not clearly defined but would be compensatory during oxidative events) are associated with early onset PE, and mutations in UCH-L1 (carboxyl-terminal ubiquitin hydrolase L1, with beneficial activity as hydrolase, but also with potentially harmful ligase activity). in patients without a clear genetic inheritance, pathogenic mechanisms have been more difficult to understand, and a number of factors, including environmental toxins, oxidative stress, and mitochondrial dysfunction.

3. General pathophysiology of Parkinson's disease

The classic symptoms of PD (bradykinesia, resting tremor, cogwheel stiffness, and postural instability) are manifested only when 70–90% of the dopaminergic neurons have been

lost in the substantia nigra or when 50% of the nigrostriatal synapses are lost. In addition, extensive extranodal pathology is also observed indicating that other cell populations are also susceptible to neurodegeneration. The presence of Lewy bodies and dystrophic neurites is associated with neurodegeneration and constitutes a pathological distinguishing feature of PD. Lewy bodies consist of rounded eosinophil inclusions which contain an aggregated α-synuclein center surrounded by other misfolded proteins and an area of radiated fibers. The distribution pattern of these structures correlates with the severity of neurodegeneration. However, not all forms of PD contain Lewy bodies, and, as mentioned later, mutations that affect other proteins such as Parkin generally lack them.

Although only 10% of the patients have familial PD in which a defined genetic dysfunction is identified, this group of patients has allowed us to study the specific risk factors associated with the disease. Mutations in three genes: α-synuclein, parkin (a ubiquitin E3 ligase involved in the degradation of proteins), and DJ-1 (inhibits the aggregation of α-synuclein via its chaperone activity and thus protects neurons against oxidative stress) are associated with early onset PD [26–29]. Mutations in the ubiquitin carboxy-terminal hydrolase (UCH-L1) gene are implicated in the pathogenesis of PD. The UCH-L1 protein has hydrolase activity that is protective against neuronal degeneration but also has a potentially harmful ligase activity [30].

α-Synuclein appears to be strongly related to the etiology of PD. The expression of mutant α-synuclein produces the accumulation of aberrant protein that causes severe neuronal toxicity. However, an elevation of the normal "wild" α-synuclein protein is sufficient for the development of PD [31]. This suggests that aberrant metabolism of wild-type α-synuclein could be the cause of the loss of dopaminergic cells in patients who have the nonfamilial form of PD. Although, the idea of establishing α-synuclein as the main etiologic factor implicated in PD is attractive, caution should be taken because in the studies that have been done that the α-synuclein region also contained another 17 additional genes that could have certain participation in the pathogenesis of PD [32].

In vitro studies suggest that prefibrillar assemblies represent toxic species of α-synuclein and that a homogenous population of fibrils, rather than their precursor on-assembly pathway oligomers, is highly toxic to cells. Fibrils have been shown to permeably membrane vesicles and to alter calcium homeostasis. Moreover, cells exposed to increasing concentrations of fibrils resulted in the activation of caspase-3 in a concentration-dependent manner and cell death [32, 33]. These inclusions, especially if they are large, may potentially alter intracellular traffic or other functions, leading to cell death. It has been shown that the protofibrillar form of α-synuclein transiently makes the membranous vesicles permeable and thus alters intracellular homeostasis, which predisposes to cell apoptosis [34]. In experimental models of PD, overexpression of α-synuclein can kill selectively dopaminergic neurons. Studies using α-synuclein viral transfection have shown that dopaminergic neurons are considerably more vulnerable to cellular apoptosis than non-dopaminergic neurons in substantia nigra [35, 36]. It has been shown that α-synuclein toxicity is increased by the generation of oxygen radicals in the presence of dopamine [37] and that dopamine, in vitro, favors the formation of α-synuclein adducts [38].

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4. Parkinson's disease and proteasome

In PD, α-synuclein-rich Lewy bodies are almost certainly the result of inefficient removal of α-synuclein. The formation of Lewy bodies would depend on the balance between the tendency of α-synuclein to aggregate spontaneously and the ability of cells to remove the protein before it reaches its critical concentration [39]. It should be borne in mind that Lewy bodies can represent a defensive response of the organism, whose aim is to avoid the inherent cytotoxicity of the compounds that accumulate in them. Although the connection between poorly folded protein aggregates and neuronal damage is still incomplete, there is evidence in PD, and in other neurodegenerative pathologies, that alteration in the removal of damaged proteins is part of the pathological process. Under physiological conditions the cellular proteins are destined to be destroyed through of the heat-shock proteins (HSPs) and the ubiquitin-proteasome system [40]. These two systems ensure that poorly folded proteins are quickly eliminated. The HSPs targets the proteins to be degraded both by the lysosomal pathway and the proteasome pathway, whereas ubiquitin represents the major proteasome pathway. The ubiquitination is a highly ordered process in which ubiquitin molecules are attached to the lysine residues of a protein through a three-stage enzymatic process (E1–E3). The ubiquitin-tagged proteins are then degraded by the proteasome. Interestingly, proteasome activity in the CNS is reduced in patients with PD [41, 42], and α-synuclein inhibits proteasome activity in a concentration-dependent manner [42]. It is proposed that an altered ubiquitin-proteasome system can sensitize specific cellular populations to exogenous stress. Studies in cells with alterations in protein folding suggest that dysregulation at the endoplasmic reticulum would be the downstream path responsible for cell death [43]. A reduced proteasome function can affect many cellular functions that normally depend on adequate protein degradation. Also, as previously mentioned, a reduction in the removal of protofibrillary α-synuclein can be directly toxic since it could alter dopamine homeostasis and increase oxidative stress. In fact, experimental inhibition of the proteasome affects more the dopaminergic neurons than the GABAergic neurons [44].

5. Is inflammation responsible for neurodegeneration in Parkinson's disease, or is it a simple response to neuronal death?

Recent studies demonstrate that excessive inflammation and overactivation of immune cells could play an important role in the onset and progression of this pathology [45]. One of the most striking aspects of neurodegenerative diseases (including PD) is the selective vulnerability of specific neuronal populations. For example, although α-synuclein is expressed in extensive regions of the CNS, neurodegeneration is mainly restricted to the substantia nigra. Dopaminergic neurons are particularly exposed to oxidative stress because the metabolism of dopamine produces dopamine-quinone species, super oxide radicals, and hydrogen peroxide [46]. Dopamine can also be enzymatically deaminated by monoamine oxidase (MAO) into the nontoxic metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) and hydrogen peroxide [47]. Therefore, metabolism of dopamine can activate apoptotic cascades and neuronal death (**Figure 3**) [47].

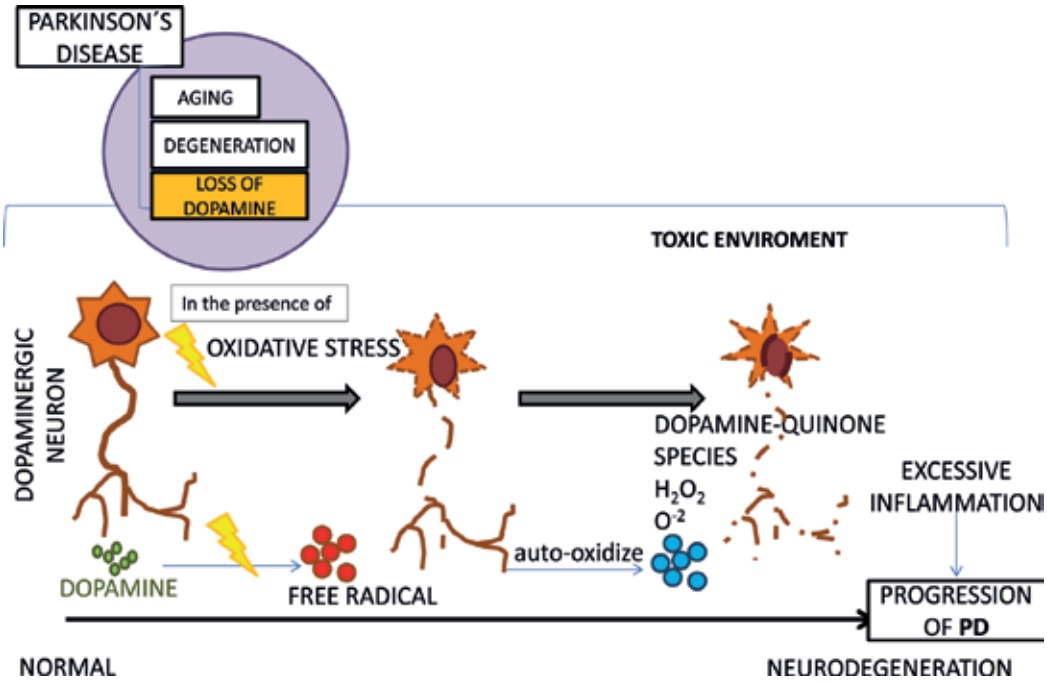

Figure 3. Inflammation in Parkinson´s disease. Dopaminergic neurons are particularly exposed to oxidative stress because the metabolism of dopamine causes a number of molecules that are potentially toxic if not properly removed. Dopamine behaves as a free radical generating compounds that can auto-oxidize to physiological pH forming toxic dopamine-Quinone species, super oxide radicals and hydrogen peroxide. This excessive toxic environment and inflammation can lead to the neurodegeneration and progression of the disease.

Accumulation of reactive oxygen species (ROS) is toxic per se due to the depletion of cellular antioxidants (e.g., vitamin E and reduced glutathione) which increases membrane lipid peroxidation, DNA damage, and alteration in protein folding [47]. In addition to general oxidative damage, there is evidence that the interaction between α-synuclein and dopamine metabolites determines the preferential neurodegeneration of dopaminergic neurons. Abnormal protein aggregates may produce a chronic inflammatory reaction capable of inducing synaptic changes and neuronal death [48]. In fact, the existence of a chronic inflammation process includes the presence of astrocytes and microglial activation in the brain biopsies of PD patients, especially in the vicinity of the protein aggregates. Furthermore, the compounds released by damaged neurons can induce the production of neurotoxic microglial factors aggravating the neurodegeneration [49]. Neuromelanin is a secreted compound that forms neuromelanin-iron complexes which activate the microglia in vitro, resulting in the release of TNF-α, IL-6, and nitric oxide. An increase in total iron concentration in the substantia nigra has been reported in PD, although the underlying mechanism is not understood [49]. Accelerated α-synuclein aggregation in turn may induce the formation of more ROS, and when the dopaminergic neurons are within an oxidative environment, the α-synuclein accumulation is increased, thereby generating a vicious cycle that leads to neuronal death [50, 51].

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Inflammation is a complex cascade of physiological responses to a harmful stimulus from the environment, and the CNS has a specialized immunity through the action of glial cells. Glial cells regulate the innate immunity, constituting the first line of defense whenever an injury or illness occurs. The activation of glial cells can be detected in a wide range of stimuli (**Figure 4**) [52]. Inflammation present in both acute injuries and chronic neurodegenerative diseases occurs in response to an alteration of the CNS, which triggers an innate immune response that activates glial cells (astrocytes and microglia) and stimulates the release of cytokines, chemokines, prostaglandins, complement cascade proteins, ROS, and RNS. An excessive and uncontrolled inflammatory response may be an additional source of damage to the integrity and function of neurons. Neural tissue has very restricted cellular regeneration which makes the CNS extremely vulnerable to immune and inflammatory processes. Inflammation contributes to neuronal loss in neurodegenerative diseases, but it is unknown how inflammation decisively contributes to the chronic progression of these diseases [49–52]. The involvement of glial cells in the inflammatory process and the processes that derive from the activation of these cells are described below.

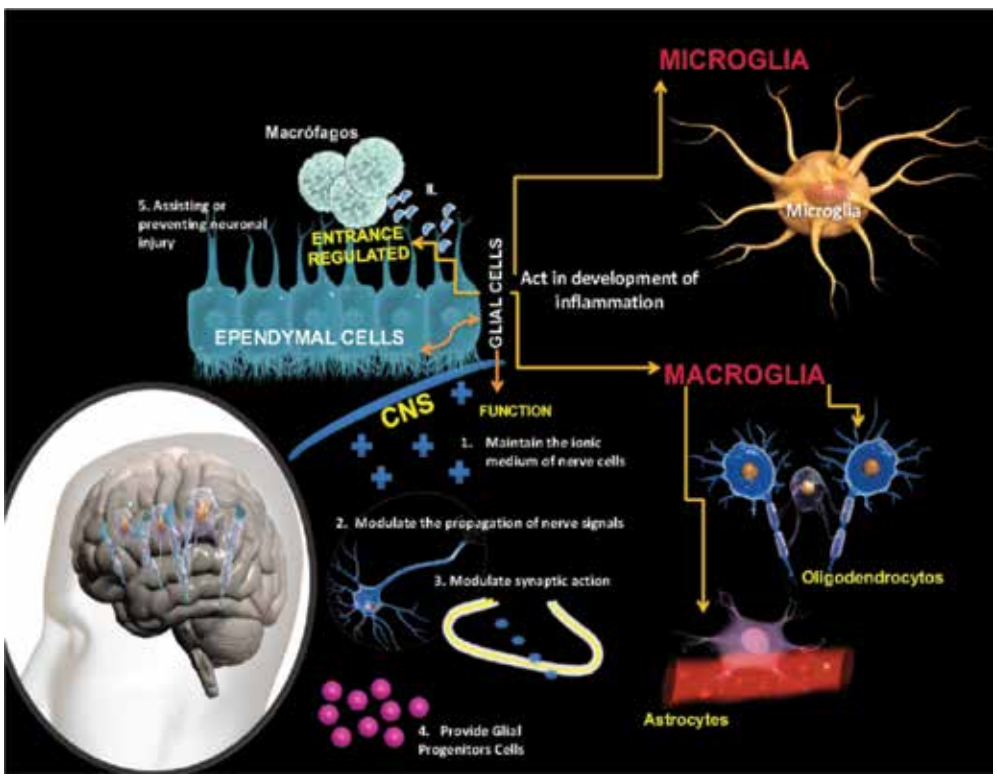

Figure 4. Role of Glia in the inflammatory response. The classification of glia is divided into macroglia and microglia. Their functions are listed from 1 to 5, IL, interleukins; CNS, central nervous system. Pathway builder online tool was used to draw the figure. The original image may be found at www.QIAGEN.com/es/shop/genes-and-pathways in conjunction with any use of the IMAGES, either on the IMAGES themselves or in close proximity to the IMAGES, such that QIAGEN's right in the original IMAGES shall be conspicuous.

5.1. The role of the glia in the inflammatory response

Glial cells react energetically to any immune stimulus or neuronal damage and play an active role in the development of inflammation. In general, glial cells are generally classified into two groups: microglia (astrocytes and oligodendrocytes) and microglia which have a mesodermal origin. Glial cells differ, do not have synaptic contacts, and have the ability to divide over a lifetime. The main functions of glial cells are to:

1. Maintain the ionic medium of neurons.
2. Modulate the rate of propagation of nerve signals.
3. Modulate synaptic action by controlling the uptake of neurotransmitters.
4. Provide a foundation for neural development.
5. Assist in (or prevent, in some cases) recovery from a neuronal injury [53].

5.2. Microglia

Microglia are specialized macrophages that represent about 20% of the total population of non-neuronal cells and are especially important to protect the integrity and homeostasis of the brain. Microglial cells are activated after an injury or infection (**Figure 5**). Once activated, it is subjected to maturation into two different states: the active and the reactive. The active microglial cells are characterized by being swollen and branched with a large cell body and short projections. They express CR3 complement receptors and histocompatibility complex class-1 (MHC-1). The reactive microglial cells are smaller, are spherical, and lack ramifications. The reactive cells, like macrophages, express the MHC-I and MHC-II and have the ability to present antigens to T. Under normal conditions, the expression of MHC-I and MHC-II is very low, but in almost all neurodegenerative diseases, its expression is increased [54].

The mechanism that regulates the function of microglia in PD is poorly understood. In the early stages of inflammation, the microglia promote secretion of neuronal survival factors such as glial-derived neurotrophic factor (GDNF), in order to limit damage and protect the population of vulnerable neurons of the central nervous system (CNS) and to stimulate the repair of damaged tissue [54]. Moreover, microglia promote neurotoxic activities by producing ROS, RNS, prostaglandin, chemokines, and cytokines. If microglial activation persists for long periods, it could lead to a lack of control of the inflammatory response that gives rise to a cycle of chronic inflammation [55]. Therefore, the microglial activation influences the extent of brain injury following an uncontrolled inflammatory stimulus. Chronic microglial activation is involved in the development and progression of PD [56].

5.3. Microglial cells and inflammation

Microglial cells release other inflammation mediators such as galectin-3, a protein which triggers an inflammatory cascade by binding to TLR3 receptor [57]. Under physiological conditions

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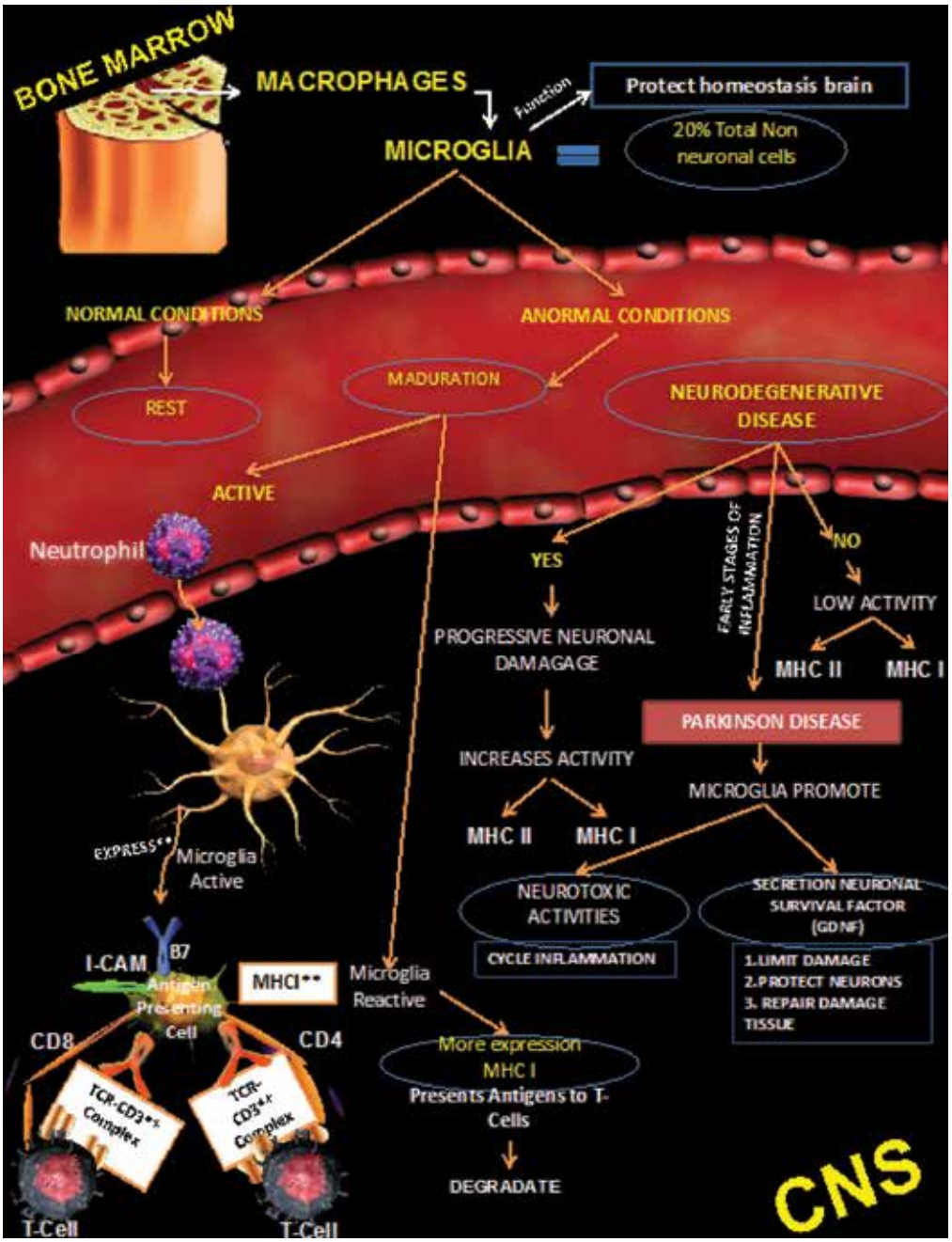

Figure 5. Parkinson and microglia. Activation and inactivation of microglia and its effects on Parkinson's disease. MHC, major complex histocompatibility; CD-8, cytotoxic T-cells; CD-4, helper T-cells; TCR-CD3, T-cell receptor of CD3; B7 ,protein ; I-CAM, intercellular adhesion molecule; CNS, central nervous system. Pathway builder online tool was used to draw the figure. The original image may be found at www.QIAGEN.com/es/shop/genes-and-pathways in conjunction with any use of the IMAGES, either on the IMAGES themselves or in close proximity to the IMAGES, such that QIAGEN's right in the original IMAGES shall be conspicuous.

damage-associated molecular patterns (DAMPs) are intracellularly sequestered molecules and are hidden from recognition by the immune system. However, under certain cellular stress or tissue injury, DAMPs can either be actively secreted by stressed immune cells or exposed on stressed cells, or they can be released into the extracellular environment from dying cells or the damaged extracellular matrix. DAMPs are recognized by pattern recognition receptor (PRR)-bearing cells of the innate immune system to promote pro-inflammatory pathways [56]. Neuromelanin and α-synuclein are examples of DAMPs that activate microglia [57]. Recently, it has been demonstrated that α-synuclein triggers Toll-like receptor (TLR) two in rat microglia and in human monocytes, causing interleukin-1β (IL-1β) production (**Figure 6**) [58].

Monocytes are a heterogeneous cell population that can be characterized according to CD14 and CD16 expression [59]. In general, $CD16^{+}$ monocytes present a more pro-inflammatory profile than $CD16^{-}$ monocytes. The $CD14^{+}CD16^{+}$ monocytes are increased in inflammatory diseases, indicating that imbalance in proportions of monocyte subsets can contribute to their pathogenesis [59]. Indeed, in patients with PD, alterations in cytokine receptor expression in $CD16^{-}$ monocytes suggest a preferential recruitment of this monocyte subset into the inflamed brain. Since DAMPs can trigger immune responses in the brain and in peripheral blood cells, circulating monocytes arise as important precursors of microglial cells [59].

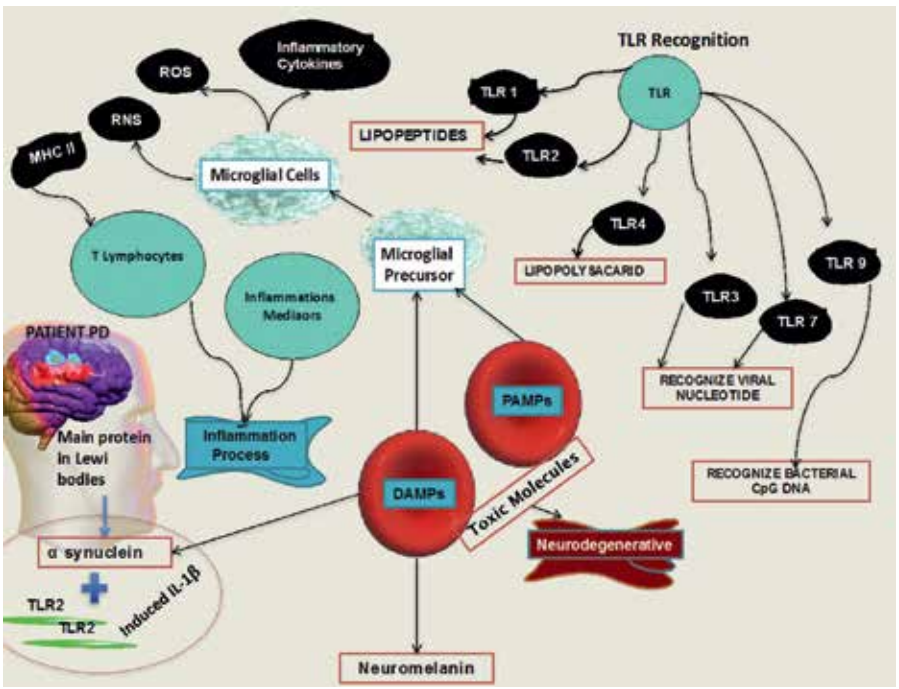

Figure 6. Microglia cells and PAMPs-DAMPs. The effect of PAMPS and DAMPS on neurodegeneration, its relation with toxic molecules, inflammation and tissue damage. PAMPs, pathogen associated molecular patterns; DAMPs, damage associated molecular patterns; MHC II, major complex histocompatibility II; RNS, reactive nitrogen species; ROS, reactive oxygen species; TLR, toll receptor 1 to 9; PD, Parkinson disease; IL-1β, interleukine-1β. Pathway builder online tool was used to draw the figure. The original image may be found at www.QIAGEN.com/es/shop/genes-and-pathways in conjunction with any use of the IMAGES, either on the IMAGES themselves or in close proximity to the IMAGES, such that QIAGEN's right in the original IMAGES shall be conspicuous.

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6. Lack of control in inflammation: the unresolved role of glial cells

The lack of control of the inflammatory cascade generates a vicious cycle that damages the neurons and is partially responsible of the progression of PD. Acute damage to the CNS can lead to neuronal degeneration. How does this initial damage to the neurons transform into a chronic and progressive neurodegeneration? It is postulated that damage to neurons triggers an uncontrolled signal in the glia to induce reactive gliosis, which further aggravates neuronal damage by releasing inflammatory and neurotoxic factors. Despite this, it remains unclear that it could boost inflammation in patients with Parkinson's disease—Parkinsonism. As a result of cellular damage, neurons consistently produce harmful compounds that are released into the extracellular medium that may be responsible for inducing the reactive gliosis. These compounds include membrane degradation products; processed, modified, or abnormally aggregated proteins; and altered or increased molecules such as the excitatory neurotransmitter glutamate which initiates the excitotoxicity cascade. These endogenous compounds activate the pattern of recognition receptors expressed in glial cells to activate an auto-amplifying inflammatory response. Therefore, the strict control of inflammation is lost, and, consequently, a vicious cycle is generated between the injured neurons and the uncontrolled inflammation (**Figure 7**).

One common pathway of these molecular and cellular events is the activation of microglial cells and astrocytes in specific regions of the brain. If protein aggregates cannot be removed, chronic activation of glial cells results in chronic neuroinflammation and oxidative stress [56].

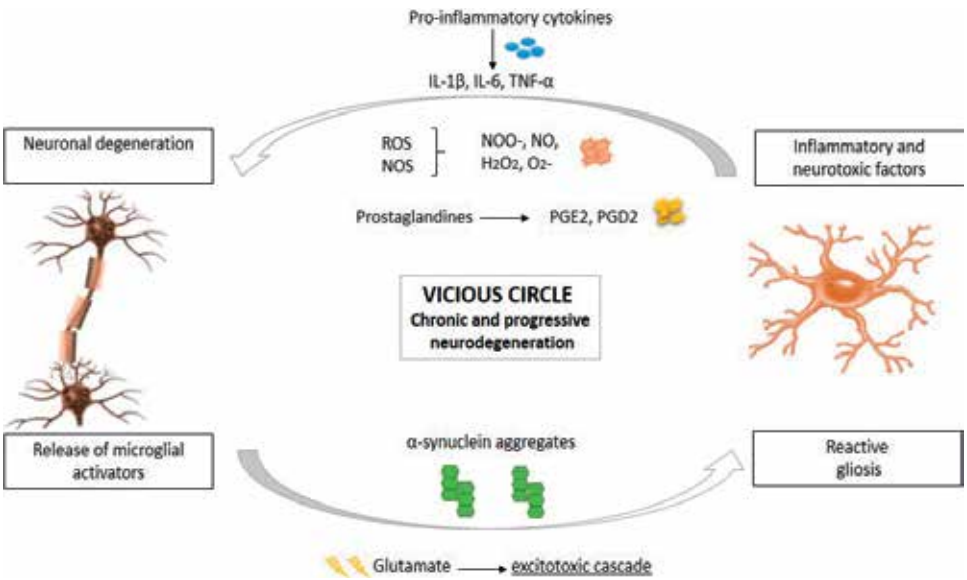

Figure 7. Inflammation: a vicious cycle in Parkinson's disease. Chronic neuroinflammation is associated with the pathophysiology of Parkinson's disease. Neurotoxicity can generate a vicious cycle of cytotoxic and stimulatory factors that leads to microglial activation. Microglia enters in an overactive state in specific regions of the brain and release inflammatory and neurotoxic factors such as pro-inflammatory cytokines, reactive oxygen species (ROS) and reactive nitrogen species (NOS) that leads to gradual oxidative neurodegeneration of dopaminergic neurons and progressive neuronal loss over time.

Inflammation in PD causes a progressive degeneration of dopamine-secreting nigrostriatal neurons. Interestingly, chronic anti-inflammatory treatment with nonsteroidal anti-inflammatory drugs and/or dexamethasone significantly reduces the risk of developing PD [60].

7. Cytokines in Parkinson's disease

Pro-inflammatory cytokines are low-molecular-weight mediators produced by both immunological and non-immunological cells, and they are key regulators of the innate and adaptive immune response [52]. In the brain, the main cytokines are TNF-α, IL-1β, IL-17, IL-6, transforming growth factor-beta (TGF-β), and the interferon-gamma family (IFN-γ) (**Table 1**). All of them act in a coordinated manner to modulate the inflammatory processes that affect the permeability of the blood-brain barrier [52, 61]. The IL-1β plays an important role in the development of acute neuronal lesions, since increased expression of IL-1β in the CNS is observed after brain damage [52, 60, 61]. Conversely, neuronal death is significantly reduced by IL-1 receptor antagonist [60].

Monocytes express TLR and produce pro-inflammatory cytokines (TNF, IL-1β, IL-6, and IL-12p70) and anti-inflammatory cytokines (IL-10) when TLR is triggered [56]. These cytokines seem to have a role in neuroinflammation in patients with PD. For example, increased levels of serum IL-6 and TNF receptor 1 have been found in patients with PD. It is known that monocytes are very sensitive to stimulation, and because of this whole-blood cell, cultures have been extensively used to evaluate their functions, especially regarding cytokine production. Due to a cross talk between immune cells in the CNS and the peripheral blood

Cytokine	Source	Function
Tumor necrosis factor-alpha (TNF-α)	Microglia, astrocytes, T lymphocytes	Endothelial cells activation, coagulation, inflammation, synthesis of acute phase proteins, endogenous pyrogen, apoptosis of many types of cells
Interleukin-1-beta (IL-1β)	Microglia, astrocytes, T lymphocytes	Endothelial cells activation, endogenous pyrogen, synthesis of acute phase proteins, neuronal death and damage
Interleukin-17 (IL-17)	Macrophages, endothelia, Epithelia, T lymphocytes	Induce and mediate pro-inflammatory responses, induces the production of other pro-inflammatory cytokines
Interleukin-6 (IL-6)	Microglia, astrocytes, Endothelial cells	Stimulatory of acute phase, increase proliferation of B lymphocytes
Interferon-gamma (IFN-γ)	Macrophages, T lymphocytes	Activation of macrophages, Activates inducible nitric oxide synthase, promotes Th1 lymphocytes differentiation, antiviral effects
Interleukin-12 (IL-12)	Macrophages, dendritic cells	Increase differentiation of lymphocytes Th1, synthesis of IFN-γ, increase cytotoxic activity

Table 1. Cytokine function in innate and adaptive immune response.

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cells, the evaluation of the status of immune cell function in peripheral blood could unravel the participation of peripheral leukocytes in neuroinflammation. Thus, systemic immune alterations could be biomarkers of the level of neuroinflammation/neurodegeneration in PD, since DAMPs released during brain damage can modulate peripheral blood cell functions. Active astrocytes produce a variety of molecules (chemokines, eicosanoids, prostaglandins, and thromboxanes) [60] and nitric oxide. Therefore, astrocytes also play an important role in neurological disorders [62]. DJ-1, an abundant protein in the brain, is expressed primarily in astrocytes and has the following functions: transcriptional regulation, antioxidative stress reaction, chaperone, protease, and mitochondrial regulation, and its activity is regulated by its oxidative status. For example, the expression level of DJ-1 is increased under an oxidative stress condition, and excess oxidation of DJ-1, which renders DJ-1 inactive, has been observed in patients with sporadic PD [63].

8. Role of mitochondria in Parkinson's disease

An oxidative stress sensor in the cells [64–75] is located within the mitochondria; therefore, the involvement of this organelle is crucial in the pathogenesis of PD. For instance, in sporadic forms of PD, the activity of the oxidative phosphorylation pathway, especially the complex I, is strongly reduced [72]. The 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine and rotenone are two environmental toxins which have shown selective degeneration of the nigrostriatal pathway through the inhibition of complex I in the mitochondria [73]. Its acidic isoform accumulates after oxidative stress indicating that DJ-1 limits cellular toxicity [74].

On the other hand, leucine-rich repeat kinase 2 (LRRK2), a large multifunctional (286 kDa) protein which appears to be expressed in most of the brain regions, is very abundant in the outer membrane of the mitochondria [76]. The LRRK2 kinase domain belongs to the mitogen-activated protein kinase subfamily. It has multiple functions which include binding to substrates, protein phosphorylation, and regulation of protein-protein interactions. The mutations most frequently observed in the family study of Parkinson's disease within the Roc and kinase domains are the substitutions affecting the microarrays of high density of SNP's 40 codons R1441, G2019, and I2020. From epidemiological studies it has been deduced that the mitochondria are at the epicenter of the complex pathophysiological pathway of PD [77, 78]. An approximate 35% deficiency was found in the activity of complex I in the CNS [79], and this enzymatic defect was also identified in the platelet mitochondria of patients with PD [80–82]. Mitochondria are the energy powerhouse of the cells, producing through the oxidative phosphorylation system the adenosine triphosphate (ATP). The functions of mitochondria as energy producers lie in their ability to keep their inner membrane polarized. Such electrochemical potential difference is exploited by mitochondrial ATP synthase to produce ATP. When mitochondria age, or are affected by certain toxins, their inner membrane is depolarized and thus incapable of generating energy. Then, the cells eliminate these mitochondria (**Figure 8**).

Phosphatase and tensin homolog-induced putative kinase 1 (PINK1) protein is mutated in some forms of familial PD. It is usually located on the outer membrane of the mitochondria. Following

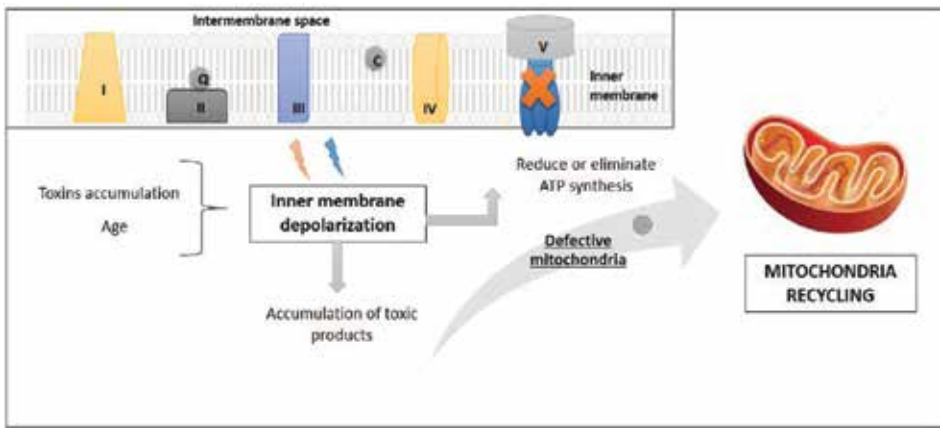

Figure 8. Mitochondrial cycle of renewal. Mitochondria is critical for cell survival due to their role in energy metabolism. Accumulation of toxins and aging leads to changes of the inner membrane permeability, causing depolarization, uncoupling of oxidative phosphorylation, release of intramitochondrial ions and metabolic intermediates. Mitochondria's are incapable of generating energy and they have to be eliminated and replaced by new ones.

depolarizing treatment, the cell responds by increasing the amount of PINK1 in the mitochondria. PINK1 seems to play an essential role in mitochondrial turnover because if cells do not produce PINK1, or produce mutated PINK1, they are unable to remove the depolarized mitochondria. Accumulated damaged mitochondria can generate or leak molecules such as ROS. It is also known that there is a relationship of PINK1 with parkin, a protein that has also been associated with PD. Parkin is a protein of the cytoplasm that is carried to the mitochondrial membrane when this organelle is depolarized. This protein is only recruited when there is non-damaged PINK1 within the mitochondria, which indicates that there is a link between PINK1 and parkin in the maintenance of healthy mitochondria. There is a cooperative work between PINK1 and parkin in the molecular tagging of the mitochondria that must be eliminated. In fact, it has been shown that mutated forms of parkin prevent the translocation of PINK1 protein to the mitochondria, which limits the initiation of autophagy, once again demonstrating the relationship of the tandem PINK1-parkin in the mitochondrial recycling [83]. That recycling could be done through macro-autophagy, a cellular catabolic process in which cytosolic components are degraded and take place in situations of nutrient shortage or toxic stress.

Experimental models offer an explanation capable of including many of the potential causes for the development of Parkinson's disease, including the mitochondrial failure, the presence of environmental toxins, the genetic load, and the processes of oxidative stress and inflammation associated with aging. These models offer possible therapeutic targets that can significantly improve the prognosis and treatment of PD.

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Chapter 10

Physiology and Pathology of Multidrug-Resistant Bacteria: Antibodies- and Vaccines-Based Pathogen-Specific Targeting

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Additional information is available at the end of the chapter

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Abstract

Multidrug-resistant bacteria (MDR) are increasing rapidly and posing a global threat to mankind. Alternative strategies other than antibiotics have to be explored urgently. In this chapter, we review the current status of nonantibiotics strategies including antibody-based therapy and vaccine development for targeting Gram-positive strains (methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecium*) and MDR Gram-negative strains (*Acinetobacter baumannii* and *Pseudomonas aeruginosa*). Biologics-based clinical progress against these bacterial infections is updated.

Keywords: multidrug-resistant bacteria, MDR, MRSA, VRE, *A. baumannii*, *P. aeruginosa*, infection, biologics, antibody, vaccine

1. Introduction

Antibiotics treatment for bacterial infections has been extensively used for over half century. This is coupled with increasing reports of bacteria drug resistance to almost all available classes of antibiotics.

The antibiotics multidrug resistance (MDR) situation is particularly severe in clinics and community for the designated ESKAPE notorious bugs (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* spp.) [1, 2].

Given the prevalence of antibiotic resistance to these bacteria-associated infections, alternative strategies are urgently needed. This chapter reviews the current status of nonantibiotics-based

strategies including antibody-based therapy and vaccine development for Gram-positive strains methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus faecium* (VRE) and MDR Gram-negative strains (*A. baumannii* and *P. aeruginosa*). **Figure 1** shows the basic

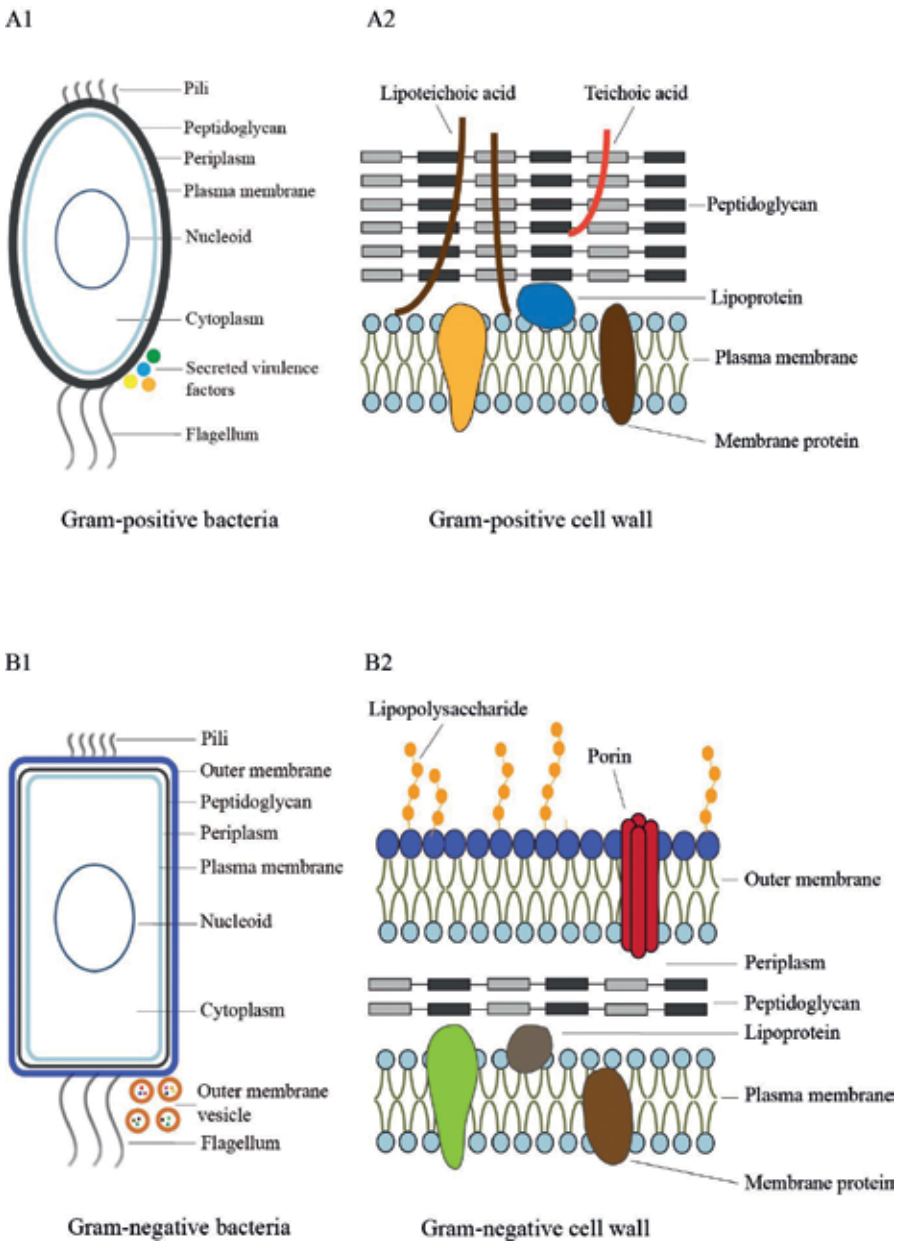

Figure 1. Bacterial cell and detailed cell wall architecture. Gram-positive bacterial cell (A1), the detailed Gram-positive bacterial cell wall (A2), Gram-negative bacterial cell (B1) and the detailed Gram-negative bacterial cell wall (B2) are shown.

Drug name	Sponsor (s)	Target	Product class	Indication	Development stage	Ref.
Altastaph	Nabi Biopharmaceuticals	CP5/CP8	*S. aureus* antibody	Treatment of bacteremia and continuing fever	Phase I/II	[48]
Aurexis (Tefibazumab)	Bristol-Myers Squibb	ClfA	*S. aureus* antibody	Treatment of bacteremia	Phase II	[68]
Aurograb	NeuTec Pharma	ABC transporter GrfA	*S. aureus* antibody	Treatment of severe, deep-seated infections	Phase III, failed	[189]
Pagibaximab	Biosynexus Inc./ GlaxoSmithKline	Lipoteichoic acid	*S. aureus* antibody	Prevention of staphylococcal sepsis in very low birth weight infants	Phase II/III, failed	[190]
MEDI4893	MedImmune LLC	α-toxin	*S. aureus* antibody	Prevention of pneumonia	Phase II	[36]
SAR279356 (F598)	Sanofi	PNAG	*S. aureus* antibody	Prevention of pneumonia	Phase II, terminated	[191]
Veronate	Bristol-Myers Squibb	ClfA and SdrG	*S. aureus* antibody	Prevention of infections in neonates	Phase III	[192]
SA3Ag	Pfizer	CP5/CP8/ClfA	*S. aureus* vaccine	Prevention of infections	Phase I/II	[92]
StaphVAX	Nabi Biopharmaceuticals	CP5/CP8	*S. aureus* vaccine	Prevention of infections	Phase III, failed	[50]
STEBVax	National Institute of Allergy and Infectious Diseases	SEB	*S. aureus* vaccine	Treatment for toxic shock syndrome	Phase I	[193]
V710	Merck	IsdB	*S. aureus* vaccine	Prevention of infections	Phase III, failed	[194]
SA4Ag	Pfizer	CP5/CP8/ClfA/ MntC	*S. aureus* vaccine	Prevention of infections	Phase I, II, IIb	[195]
4C–Staph	GSK	HlaH35L/EsxAB/ FhuD2/Csa1A	*S. aureus* vaccine	Prevention of infections	Phase I	[196]
MEDI3902	MedImmune LLC	PcrV/Psl	*P. aeruginosa* antibody	Prevention of pneumonia	Phase II	[197]
KB001-A	KaloBios Pharmaceuticals	PcrV	*P. aeruginosa* antibody	Prevention of infections	Phase II, failed	[198]

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Drug name	Sponsor (s)	Target	Product class	Indication	Development stage	Ref.
PseudIgY	Immunsystem AB	Unknown	*P. aeruginosa* antibody	Prevention of infections	Phase I/II	[199]
KBPA-101	Kenta Biotech Ltd	O-polysaccharide	*P. aeruginosa* antibody	Treatment of infections	Phase I/II	[200]
IC43	Valneva Austria GmbH	OprF/OprI	*P. aeruginosa* vaccine	Prevention of infections	Phase II/III	[201]
Aerugen	Crucell	O-polysaccharide	*P. aeruginosa* vaccine	Prevention of chronic CF infection	Phase III, failed	[140]
Flagella		Subtype-a and subtype-b flagellin	*P. aeruginosa* vaccine	Prevention of chronic CF infection	phase III	[131]
MEP	Univax Biologics	MEP antigen	*P. aeruginosa* vaccine	Prevention of chronic CF infection	Phase I	[142]
Pseudostat	Provalis PLC	Inactivated *P. aeruginosa* strain 385	*P. aeruginosa* vaccine	Prevention of chronic CF infection	Phase I	[145]

CP5/8: serotype 5/8 capsular polysaccharides; ClfA: clumping factor A; PNAG: poly-N-acetyl glucosamine; SdrG: serine-aspartate repeat-containing protein G; SEB: Staphylococcal enterotoxin serotype B; IsdB: iron-regulated surface determinant protein B; MntC: manganese transport protein C; HlaH35L: α-Hemolysin H35L; EsxAB: ess extracellular A/B; FhuD2: ferric hydroxamate-binding lipoprotein; Csa1A: conserved staphylococcal antigen 1A; PcrV: Low calcium response locus protein V; OprF/OprI: Major outer membrane porin F/I; MEP: mucoid exopolysaccharide.

Table 1. Antibodies and vaccines for *S. aureus* and *P. aeruginosa* in clinical development.

structures of Gram-positive and Gram-negative bacteria that are a key for design and development of antibodies and vaccines to target against these MDR bacterial infections.

Monoclonal antibodies (mAbs) have advantages over traditional chemotherapy in that (1) mAbs can bind target antigen specifically and thus reduce off-target side effects associated with traditional chemotherapy; (2) through Fc neonatal receptor (FcRn) recycling mechanism, mAbs have long serum half-life (ranges in days to weeks) when compared to chemotherapy (ranges in minutes to hours); (3) mAbs can recruit effectors for antibody-dependent cell-mediated phagocytosis (ADCP), antibody-dependent cellular cytotoxicity (ADCC), and complement-dependent cytotoxicity (CDC) through its Fc region, which functions are missing in chemotherapy [3]. By 2015, more than 60 monoclonal antibodies (mAbs) have been approved by the United States Food and Drug Administration to treat cancer, autoimmune disorders, and infections [4].

To conquer the serious antibiotic resistance from bacterial pathogens, passive immunization (mAb treatment against bacterial pathogen) and active immunization (vaccine against bacterial pathogen), as alternative strategies, are being actively explored.

In this chapter, we focus on the current status of antibody and vaccine development against Gram-positive strains (*S. aureus* and *Enterococci*) and Gram-negative strains (*P. aeruginosa* and *A. baumannii*). Antibodies and vaccines under clinical trials are summarized in **Table 1**.

2. Antibody and vaccine development against *S. aureus*

S. aureus establishes infection through a variety of complicated mechanisms. *S. aureus* produces cell envelope-associated proteins, nonprotein glycopolymers, a collection of secreted toxins that mediate host-microbe adhesion, host cell lysis, antibody function interference, complement activation inhibition, and invasion of immune nonprofessional phagocytes [5, 6].

2.1. Antibodies against staphylococcal-secreted virulent factors

2.1.1. Staphylococcal superantigens as antibody targets

S. aureus is a round-shaped, facultative anaerobe, which can produce an array of superantigens (SAgs), including staphylococcal exotoxins, enterotoxins, and toxic shock syndrome toxin 1 (TSST-1). These toxins exert their hyper-stimulatory properties and cause food poisoning, toxic shock syndrome, acute lung diseases, and autoimmune diseases [7–10]. The superantigenicity of SAgs is largely achieved by the activation of APCs and T cells, leading to a massive release of cytokines, including IL-1β, IL-6, and TNFα [11].

Staphylococcal enterotoxin serotype B (SEB) was classified as a category B select agent by the Centers for Disease Control and Prevention (CDC) due to its high toxicity to human and potential use as a biological weapon [12]. Several mAbs targeting on SEB are under investigation. A high-affinity SEB-specific mouse mAb, 20B1, was investigated in mouse models with superficial skin, sepsis, or deep-tissue infections [13]. Treatment of 20B1 significantly increased the

survival in the sepsis model, whereas it reduced bacterial burden and dissemination of bacteria in the superficial skin model. Moreover, 20B1 was shown to decrease pro-inflammatory cytokine levels and T cell proliferation. Remarkably, their following work further showed that isotype switching from original IgG1 to IgG2a, without changing of SEB binding affinity, greatly enhanced the protective ability in *S. aureus* sepsis models [14]. This is consistent with a recent report in which humanized anti-SEB mAbs attenuated virulence of exogenous SEB expressing *S. aureus* in a mouse pneumonia model [15].

In addition, Tilahun and colleagues explored the use of combined mAbs targeting on different epitopes of SEB, as well as co-administration of mAb and antibiotic, both of which showed synergistic protection in *S. aureus* infection mouse model [16, 17]. This strategy seems promising as synergistic protection by co-administration of two mAbs recognizing distinct SEB epitopes was also observed independently in another study [18]. To date, there are not any anti-SEB mAbs being tested in clinical trials. Of note, a phase I clinical study of safety of a recombinant SEB vaccine (STEBVax) against toxic shock syndrome has been completed [19].

TSST-1 is a 22 kDa monomeric protein, of which the N-terminal domain binds to the MHC-II on APCs and the C-terminal domain is implicated in β-chain variable region of TCR (TCR-Vβ) interaction [20, 21]. In a recent report, human single chain variable fragments (scFvs) against recombinant TSST-1 were panned out from synthetic human scFv library by phage display technology [22]. The scFvs were demonstrated to be able to inhibit TSST-1–mediated T cell activation and pro-inflammatory cytokine production. Besides, a recombinant TSST-1 vaccine (Biomedizinische Forschungs gmbH) has been completed in phase I clinical study and proved to possess a good safety profile with no observable severe adverse events occurred [23, 24].

2.1.2. α-Hemolysin as antibody target

S. aureus releases a number of cytolytic toxins, among which the pore-forming α-hemolysin (Hla, α-toxin) is the most studied one. Hla is secreted as a 33 kDa monomer consisting almost entirely of β-strands by circular dichroism [25]. It exerts cell lytic activity through a membrane perforating mechanism, which is initiated through binding to membrane lipid or/and its proteinaceous receptor, a disintegrin and metalloprotease 10 (ADAM10) [26]. In detail, Hla monomers assemble into a heptameric structure on susceptible host cell membrane and form a central pore of approximately 1–3 nm in diameter [27, 28]. This allows rapid egress of Ca^{+}, ATP, and low molecular weight molecules through the pore, resulting in alteration of cellular signaling pathways and cell lysis [29–31].

Therapeutic anti-Hla mAbs have been actively developed due to the key role of Hla in Staphylococcal pathogenesis. In a study in which a recombinant Hla, AT62, was used as a vaccine, the study also showed that passive immunization of anti-AT62 IgG reduced wound infection and tissue damage in a mouse model [32]. In a *S. aureus* dermonecrosis model, combined administration of Hla-targeting mAb, MEDI4893*, with frontline antibiotic linezolid or vancomycin, exhibited enhanced protection by reduced lesion size, reduced tissue damage, and accelerated healing in a synergistic manner [33]. Furthermore, MEDI4893 (MedImmune) was generated from MEDI4893* by introducing three amino acids substitution (M252Y/S254 T/T256E) [33]. The YTE mutation

has been shown to extend half-life by two- to fourfold without affecting distribution properties [34]. MEDI4893 not only abrogated Hla-host cell interaction but also potentially blocked oligomer formation due to steric hindrance [35]. Recently, a phase I clinical trial was completed by evaluating the safety, tolerability, and pharmacokinetics of MEDI4893 in healthy adult subjects [36]. Currently, a phase II study is ongoing to evaluate the safety and efficacy of MEDI4893 in the prevention of *S. aureus* pneumonia [37].

2.2. Antibodies against staphylococcal surface-associated components

2.2.1. Capsular glycopolymer as antibody target

Bacterial capsule is a polysaccharide layer lying outside of the cell wall found in both Gram-positive and Gram-negative bacteria. Capsule produced by pathogens has been involved in promoting adherence, resisting bacterium from host immune attack, and mediating release of virulent factors [38]. Encapsulation of *S. aureus* prevents bacterial phagocytosis by interfering with effective opsonization [39].

Serotype 5 (CP5) and serotype 8 (CP8) capsular polysaccharides predominate among *S. aureus* clinical isolates, representing 75–80% of total isolates [40]. While several CP5 or CP8-specific mAbs were studied [41, 42], serum containing antibodies that recognize the shared epitope of CP5 and CP8 were recently developed [43, 44]. The cross-reactivity was confirmed *in vitro* and the sera were demonstrated to promote opsonophagocytic killing of both CP5 and CP8 *S. aureus* strains. There are no reports on therapeutic antibodies targeting staphylococcal polysaccharide in clinical trials. However, two vaccines, StaphVAX and Altastaph (Nabi Biopharmaceuticals), have been completed for their clinical studies for safety and immunogenicity evaluation [45–48]. Although Altastaph was able to induce significant elevation of anti-CP5 and anti-CP8 antibody levels, unfortunately, it failed to show efficacy in a phase II clinical trial [49]. StaphVAX also showed ineffectiveness in the reduction of *S. aureus* in patients on hemodialysis and thus failed in a phase III trial [50].

Bacterial poly-N-acetyl glucosamine (PNAG) is another major class of surface polysaccharide that has been evaluated as a vaccine. PNAG, which is synthesized by enzymes encoded in intercellular adhesin (ica) locus, contributes to biofilm formation, colonization in host tissue, and immune evasion [51, 52]. Recent work showed that deacetylation of PNAG (dPNAG) by surface protein, IcaB, is a critical step for PNAG association to cell wall and plays key roles in colonization and resistance to host immune defense [53]. Indeed, antibodies specific to dPNAG were better in opsonic killing than that specific to PNAG [54]. In consistence, passive immunization of mice with antisera raised to dPNAG showed efficient clearance of *S. aureus*, compared with that raised to acetylated form [55].

2.2.2. Staphylococcal protein A as antibody target

Staphylococcal protein A (SpA) is anchored to *S. aureus* cell wall by sortase A through amide linking of its C-terminal threonine of LPXTG motif to pentaglycyl crossbridge within peptidoglycan [56]. SpA interferes with immunoglobulin (Ig) function by binding to Fcγ domain

of Ig and prevents the bacterium from opsonophagocytic killing [57]. It also interacts with B cell receptor through binding with VH3-clan of antigen-binding fragment (Fab) region and induces supraclonal B cell responses, resulting in insufficient adaptive responses against infection [58–60].

Based on the mechanistic studies, a mutated form of SpA, SpA(KKAA), was generated to abolish both Fcγ and Fab binding abilities [61]. Vaccination of SpA(KKAA) was able to elicit robust antibody responses against multiple staphylococcal antigens in a MRSA-infection mouse model. In their following studies, passive immunization of antibodies specific for SpA(KKAA) significantly promoted opsonophagocytic clearance, reduced abscess formation, and decreased the mortality [62]. Furthermore, a humanized version successfully conferred protection against *S. aureus* sepsis in neonatal mice [63].

2.2.3. Clumping factor A as antibody target

Microbial adhesion to host tissue is crucial to infection initiation in most of the bacterial infections. Microbial surface component recognizing adhesive matrix molecules (MSCRAMM), such like clumping factor A (ClfA), plays a vital role in this process [64]. ClfA, a fibrinogen-binding protein, is required for establishing early infection, abscess formation, protection against phagocytosis, as well as bacterial persistence in host [65, 66].

Tefibazumab, a humanized anti-ClfA mAb, was developed and exhibited high affinity and specificity for ClfA [67]. *In vitro* study showed that tefibazumab inhibited fibrinogen-binding ability of ClfA and protected against MRSA infection in murine septicemia and rabbit infective endocarditis models. Safety and pharmacokinetic profile of tefibazumab were evaluated in phase II clinical trial [68]. Unfortunately, it failed to show significant differences between treatment and placebo groups in overall adverse clinical events. A detailed analysis of ClfA-fibrinogen structure observed a modest IC50 value of binding between ClfA and tefibazumab, which might partly explain the unsatisfactory clinical outcome [69].

2.2.4. Autolysin as antibody target

Autolysin (Atl) is a cell wall-associated enzyme with various functions. The major *S. aureus* autolysin (AtlS) contains two distinct domains, amidase and glucosaminidase, which are responsible for enzyme localization to cell wall and peptidoglycan hydrolysis, respectively [70, 71]. Atl participates in biofilm formation, separation of daughter cells after cell division and attachment to host matrix [72]. Moreover, AtlS is highly conserved among strains of *S. aureus* and other *Staphylococci*. These features together make AtlS an attractive target for anti-*S. aureus* mAb and vaccine investigation.

To test it, a mAb, 1C11, was generated to inhibit AtlS glucosaminidase domain and its effect in animal model was assessed [73, 74]. The mAb was shown to impair cell growth and cause cell aggregation and sedimentation in *in vitro* assay. Following this study, administration of 1C11 reduced severity of implant-associated osteomyelitis in a mouse model by decreased abscess numbers and efficient internalization of antibody-opsonized *S. aureus*.

Immunodominant staphylococcal antigen A (IsaA) is another highly conserved Atl. Similarly, protection was conferred by a mAb specific to IsaA in a mouse model [75]. The mode of action of mAb is mainly through activation of professional phagocytes and induction of oxidative burst activity of neutrophil.

2.3. Antibodies against staphylococcal cell wall components

2.3.1. Lipoteichoic acids as antibody target

Most Gram-positive bacteria produce teichoic acids (TAs) to facilitate their survival under disadvantageous conditions. Teichoic acids covalently link to either peptidoglycan or cytoplasmic membrane, known as wall teichoic acids (WTA) and lipoteichoic acids (LTA), respectively [76]. The roles of TAs in pathogenic bacteria include adherence to host cells [77], activation of complement [78], and cytokine induction [79].

Since structures of LTA are highly conserved across many clinical isolates, including *Enterococci*, *Staphylococci*, and several *Streptococci*, LTA is considered as a promising target for vaccine and therapeutic antibody development [80]. In a recent report, antibodies against *E. faecalis* LTA were used to test cross-activity with other Gram-positive bacteria, including *S. aureus* [80]. The *in vitro* data showed that the antibodies were also able to bind with LTA purified from *S. aureus*. Remarkably, the antibodies exhibited 60–90% opsonophagocytic killing activity across a variety of *S. aureus* strains, and great protection against MRSA infection in a mouse peritonitis model. In accordance with the observation, immunization with a BSA-conjugated LTA fragment, containing a conserved minimal structure in majority of Gram-positive bacteria, was able to induce opsonic killing of *E. faecium* E1162 and *S. aureus* MW2 [81]. Besides, immunization of WTA also elicited an anti-WTA immune response, illustrated by complement-dependent opsonophagocytosis [82, 83].

2.3.2. Peptidoglycan as antibody target

Peptidoglycan (PG) is composed of cross-linked polysaccharide and peptide chains, which forms the backbone of bacterial cell wall. So far, reports on therapeutic antibody or vaccine targeting on PG are scarce. A mAb against deacetylated peptidoglycan, ZBIA5H, was screened with best protective property in mouse models [84]. Surprisingly, ZBIA5H did not show the highest affinity to PG, compared with other mAbs. The superior property of ZBIA5H may be attributed to the unique epitope it recognizes. This study highlights that besides antigen binding affinity, other factors, such as epitope, should also be taken into consideration in therapeutic antibody discovery.

2.4. Antibodies against nutrient transporter proteins

Nutrient acquisition is one of the most basic and essential process virtually in all forms of life. Vertebrate host has evolved powerful strategy, termed nutritional immunity, to limit proliferation of invading pathogens by sequestering essential nutrients [85]. One of the best characterized examples of nutritional immunity is transition-metal-ion sequestration in which

metal ions are predominantly trapped by host metal-binding proteins [86]. To combat with host defensive system, microorganism employs mechanisms to maintain intracellular metal homeostasis. Therefore, these mechanisms could be suitable targets for therapeutic antibody development. For example, an Fab was screened to inhibit acquisition pathway for Mn(II), which is essential for detoxification of reactive oxygen species (ROS) [87, 88]. The mAb is bound to manganese transporter C (MntC) of an ATP-binding cassette (ABC) transporter system and thereby blocks the metal delivery to the channel. *In vitro* assay showed that the Fab increased the sensitivity of *S. aureus* to ROS by over 10-fold.

An earlier report identified ABC transporter as the most commonly associated protein with IgG from the sera of 26 patients suffered with septicemia [89]. ScFvs against the conserved peptides from the ABC transporter were then panned from a phage display library and were shown to reduce the bacterial burden in a mouse model.

2.5. Multicomponent vaccines

So far, neither passive nor active immunization has shown potent efficacy on humans. The failure from basic research to clinical practice could partly be attributed to the limited understanding of the sophisticated events associated with every stage of infection. Prior strategies targeting on single virulent factor showed efficacy only in certain experimental settings. In this regard, novel vaccine formulations targeting on multiple pathogenic components are proposed to offer protection from distinct aspects through a synergistic working mode.

Recently, efficacy of a combination vaccine, 4C–Staph (four-component *S. aureus* vaccine), was evaluated [90]. 4C–Staph is composed of detoxified α-Hemolysin, a fusion of ess extracellular A (EsxA) and ess extracellular B (EsxB), two staphylococcal surface proteins, which are ferric hydroxamate-binding lipoprotein (FhuD2) and conserved staphylococcal antigen 1A (Csa1A). 4C–Staph induced broad and synergistic protection against several *Staphylococcal* clinical isolates in different models. In addition, mechanistic study showed that the protection was mainly antibody dependent.

SA3Ag (Pfizer), a tri-component vaccine, consists of CP5 and CP8 individually linked with a nontoxic form of diphtheria toxin, and a recombinant mutant form of clumping factor A (rClfAm) [91]. A phase I clinical trial was completed to evaluate safety, tolerability, and effect of SA3Ag [92]. This vaccine showed a relatively safe profile among older and young adults.

In order to further enhance protection against *S. aureus*, another component, MntC, was added to SA3Ag to form a four-component vaccine SA4Ag (Pfizer) [93]. In phase 1/2 clinical trials, single-dose administration of SA4Ag was well-tolerated among young and older adults, shown by mild or moderate local reactions and comparable systemic events with placebo control [94, 95]. More excitingly, SA4Ag induced a rapid, robust, and sustained functional antibody response.

2.6. Antibody-antibiotic conjugate

While *S. aureus* has classically been considered as an extracellular pathogen, a growing body of evidence reveals that it is capable to survive and persist within host cells, including phagocytic cells, which are responsible for bacterial clearance [96, 97]. Although phagocytic cells,

particularly neutrophils and macrophages, can efficiently kill majority of invading bacteria, a small population of persisters can however turn the circulating phagocytes to "Trojan horses" to facilitate bacterial dissemination via bloodstream [98]. Meanwhile, intracellular persistence allows bacteria to escape from antibiotic and immune attack. Indeed, most of the current antibiotics are less efficient in intracellular *S. aureus* killing, which may partly explain the poor response to treatment and the high frequency of recurrence in clinical practice [99, 100].

Based on these findings, therapies specifically targeting on intracellular pathogen may promote clinical outcome. Similar to antibody-drug conjugate (ADC), which has been successfully applied for cancer therapy, antibody-antibiotic conjugate (AAC) was first proposed and evaluated by Lehar and his colleagues in 2015 [101]. The AAC is composed of three building blocks: an antibody to target on bacteria, a highly bactericidal antibiotic payload, and a linker to attach antibiotic payload to the antibody. The AAC was designed with no antibacterial activity as antibiotic serves as a prodrug when covalently linked. However, when planktonic AAC-tagged bacteria are internalized by host cells, the antibiotics can be efficiently released in their active form by cleavage from host protease. Thus, the AACs take bacteria as "Trojan horses" to deliver potent antibiotics to cytoplasmic compartment and resulting in intracellular antibacterial effect. To their anticipation, the AAC was shown to efficiently restrict intracellular *S. aureus* growth when treatment was initiated several hours after intravenous infection. In contrast, poor efficacy was observed by delayed treatment of vancomycin. This result is particularly interesting as majority of bacteria were found to associate with neutrophils within 10–15 minutes [97]. Moreover, the AAC was able to limit metastasis of *S. aureus* to brain in an intravenous infection model.

3. Antibody and vaccine development against *E. faecium*

Different from *S. aureus,* which produces an array of virulent factors, pathogenesis of *Enterococci* is largely determined by their adherence to host tissue mediated by surface adhesion components. Several most-studied components include aggregation substance proteins, collagen adhesins, enterococcal leucine-rich repeat-containing proteins, pili, polysaccharides, and glycolipid [102], which are potential targets for antibody and vaccine development.

3.1. Enterococcal pili as antibody target

Enterococcal surface pili are filamentous proteins with Ig-like folds and LPXTG motifs, which have been implicated in biofilm formation, endocarditis, and catheter-associated urinary tract infections (CAUTIs) [103, 104]. Endocarditis and biofilm-associated pilus A (EbpA), one of the most-studied pili in *Enterococci*, is widely present among *Enterococcal* species and highly conserved in N-terminal domains [105]. In detail, N-terminal domain of EbpA (EbpANTD) binds to host fibrinogen deposited on urinary catheter to facilitate Enterococcal colonization [106]. Sera against EbpANTD was recently shown to provide universal protection in a murine model by reducing bacterial titers of a broad spectrum of Enterococcal isolates, including *E. faecalis*, *E. faecium*, and VRE [105]. Consistently, vaccination of EbpA or EbpANTD, but not its carboxyl-terminal domain, diminished biofilm formation and prevented CAUTIs in *E. faecalis* infection model [106].

3.2. Polysaccharide antigens as antibody targets

Based on a previous serotyping analysis, about 60% of *E. faecalis* isolates fall into four serotypes from CPS-A to CPS-D [107]. CPS-C and CPS-D can express capsular polysaccharide, whereas CPS-A and CPS-B are nonencapsulated due to deficiency of essential gene locus [108]. In an early study, antibodies raised against LTA from CPS-A strain only opsonized acapsular CPS-A and CPS-B strains, but not encapsulated ones [109, 110]. To develop antibodies against capsule-bearing CPS-C and CPS-D strains, a novel diheteroglycan was identified from capsular polysaccharide [110]. As a result, passive immunization of anti-diheteroglycan antibodies successfully protected CPS-C and CPS-D *E. faecalis* bacteremia mouse model. However, it was observed that considerably lower susceptibility of CPS-C and CPS-D strains to opsonic killing by naturally acquired antibodies was present in healthy human sera as compared with CPS-A and CPS-B [111]. Therefore, capsule may be a natural barrier to access therapeutic antibody by masking antigens underneath.

3.3. Lipoproteins as antibody targets

A transcriptomic analysis from an *E. faecalis* infection mouse model identified two ABC transporter substrate-binding lipoproteins upregulated upon infection: PsaAfm for manganese transport and AdcAfm for zinc transport [112]. Treatment of antibodies raised from recombinant proteins showed increased opsonic killing *in vitro* and reduced colony counts in a mouse bacteremia model. Protective role was also seen in treatment with antibodies against distinct ABC transporter proteins [113], suggesting the potential of ABC transporter as a therapy target in enterococcal infection.

4. Antibodies and vaccines against *P. aeruginosa*

Effective control of *P. aeruginosa* infections remains a challenging problem due to its remarkable ability to evolve resistance to many antibiotics. Antibodies and vaccines are considered to be a promising and alternative strategy to treat or prevent *P. aeruginosa* infections in susceptible populations. The identified *P. aeruginosa* antibody and vaccine targets include the lipopolysaccharide (LPS) O-antigens, pilus, flagella, alginate, outer membrane proteins (OMPs), mucoid exopolysaccharide (MEP), and antigens from the type III secretion system (T3SS) [114].

4.1. Antibody and vaccine development against T3SS translocation protein PcrV

Type III secretion system (T3SS), as a key virulence determinant in *P. aeruginosa*, is encoded by at least 42 genes and assembled as a needle-like apparatus that can directly inject bacterial effector proteins into host cell to elicit pathological response [115]. PcrV is located at the tip of needle-like apparatus and closely involved in translocation of effector proteins from *P. aeruginosa* to host cell [115].

Fab 1A8, a human Fab antibody fragment, can specifically target against *P. aeruginosa* PcrV antigen and elicit protective effects for mice with lethal pulmonary *P. aeruginosa* challenge

[116]. KB001, a PEGylated anti-PcrV Fab fragment in clinical phase-2a trial for ventilator associated and *P. aeruginosa* colonized but not for infected patients in intensive care units (ICUs), showed good safety, tolerability, and pharmacokinetic profile. Although statistical significance was not observed for patients with KB001 treatment and placebo treatment, incidence of *P. aeruginosa* pneumonia was decreased in KB001 treatment group (31%) as compared to that of placebo treatment group (60%) [117]. Identification of anti-PcrV IgG from human sera confirms that PcrV is a vaccine target [118]. Moreover, human high titer anti-PcrV sera clearly have prophylactic effect for mice with lung *P. aeruginosa* infection [118].

4.2. Antibody and vaccine development against PsI

By construction and phenotypic screening of human scFv phage display libraries from peripheral blood B cells of healthy individuals and patients recovered from recent *P. aeruginosa* infections, mAbs against one epitope of Psl, the exopolysaccharide important for *P. aeruginosa* attachment to host cell and biofilm maintenance, was identified to show potent protection in several animal *P. aeruginosa* infection models [119]. Also, this finding suggests that PsI can be used as a vaccine target. However, most patients suffered from *P. aeruginosa* bloodstream infection (BSI) had low anti-PsI titer that showed nonprotective to *P. aeruginosa* BSI infection [120]. MEDI3902, the combination of anti-PsI and anti-PcrV in a bispecific format, showed synergistic protection against *P. aeruginosa* murine pneumonia models as compared with each parental mAb [121]. Moreover, MEDI3902 can synergize several classes of antibiotics for the treatment of clinical antibiotics resistant isolates [121].

4.3. Antibody and vaccine development against outer membrane proteins (OMPs)

OMPs form porins and other structural and functional components on the bacterial cell surface. CFC-101, a mixture of OMPs from *P. aeruginosa*, was used to immunize healthy human volunteers in a phase I/IIa clinical trial [122]. CFC-101 was safe and immunogenic in eliciting human mAbs after immunization that can passively protect mice from lethal *P. aeruginosa* challenge [122].

OprF and OprI are the major OMPs that are surface-exposed and conserved in wild-type strains of *P. aeruginosa* [123]. In phase I human trials, OprF-OprI vaccine (IC43) conjugating with aluminum hydroxide was safe and induced specific antibodies in healthy volunteers and burn patients by intramuscular administration [124, 125]. Intranasal immunization of OprF-OprI vaccine followed by systemic boost elicited a long-lasting systemic and local lung mucosal antibody response in patients with chronic pulmonary diseases [126]. Recently, phase II study on ICU *P. aeruginosa* infection showed that IC43 also produced a significant immunogenic effect without mortality or safety concerns [127].

4.4. Antibody and vaccine development against flagellins and pilins

Flagella are essential for motility, chemotaxis, invasiveness, and adhesion of *P. aeruginosa* to activate host inflammatory responses [128]. Flagellin is the primary protein component of flagella and consists of subtype a and subtype b [129].

A monovalent *P. aeruginosa* flagella vaccine was safe and immunogenic in healthy human adults by intramuscular immunization and showed high and long-lasting serum antibody (IgG and IgA) titers against flagella positive *P. aeruginosa* [130].

Then, a bivalent flagella vaccine, containing some of the flagella subtype antigens (a0a1a2 and b), was evaluated over a 2-year period on cystic fibrosis (CF) patients not colonized with *P. aeruginosa* in phase III trial. The vaccine lowered the risk of patients for initial infection as compared with that from the placebo group, though not statistically significant. Therefore, multivalent vaccine against *P. aeruginosa* flagella subtypes a and b is needed to improve overall efficacy of vaccine to more flagella subtypes [131]. A multivalent protein fusion vaccine consisting of flagellin subtype a and b, Oprl and OprF epitope 8, was used to immunize mice that induced specific IgGs against each individual antigen [132]. Although these IgGs elicited potent ADCC and increased clearance of nonmucoid *P. aeruginosa*, which reflect the initial colonization of *P. aeruginosa*, they were less effective for mucoid *P. aeruginosa*, which represent the colonized and chronic *P. aeruginosa* biofilm formation [132]. Conjugation vaccine of flagellin subtype a (FLA) with polymannuronic acid (PMA) built from mannuronic acid, the major component of alginate and biofilm, induced protection against mucoid *P. aeruginosa* in mice and rabbits [133].

Pili, as one key virulent factor, are filaments of pilin polymers located at the pole of *P. aeruginosa* and are responsible for adhesion of *P. aeruginosa* to host epithelial surfaces and twitching motility [134, 135]. A disulfide loop (DSL) at the C-terminal of pilin is the major epitope in bridging adherence of *P. aeruginosa* to host cell [134, 135]. Single copy of DSL was not an effective immunogen in mice, whereas multi-copy of DSL peptides increased IgG response 1000 times [136]. Immunization of mice with full length pilin of *P. aeruginosa* induced mAbs that inhibited pili-mediated epithelial cell adhesion [137].

4.5. Antibody and vaccine development against LPS

LPS is the major component of the outer membrane of *P. aeruginosa*. LPS has two types, smooth or S-type and rough or R-type. S-type LPS consists of O-polysaccharide (O-antigen) repeats linked with a core-conserved oligosaccharide and a lipid A moiety, while R-type LPS lacks O-antigen and only contains the core oligosaccharide [138]. The S-type LPS is involved in nonmucoid and in early stage of *P. aeruginosa* infection in CF patients, whereas the R-type LPS is associated with mucoid and late stage of *P. aeruginosa* infection in CF patients [139]. The O-antigen is immunogenic in the host for the induction of protective antibodies, whereas lipid A is the core endotoxic component for induction of inflammatory responses [138]. More than 20 serotypes of O-antigens have been identified [138].

Pseudogen, a heptavalent O-antigen vaccine, showed efficacy in nonrandomized trials among adult cancer and burn patients in preventing fatal *P. aeruginosa* infections but no benefit in leukemia and CF patients [139]. Furthermore, Aerugen, an octavalent vaccine, was developed by conjugating purified O-antigens from eight *P. aeruginosa* strains with exotoxin A. This vaccine induced high levels of specific opsonizing antibodies in CF patients and significantly reduced the frequency of chronic infection for 10 years without apparent adverse effects in a nonblind trial. However, a subsequent double blind, randomized, placebo-controlled phase III trial failed to confirm the initial positive results and the further development of this vaccine was suspended [140].

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4.6. Antibody and vaccine development against alginate

Alginate or mucoid exopolysaccharide (MEP), a linear polymer of partially acetylated D-mannuronic acid and L-guluronic acid, is the major component of the *P. aeruginosa* biofilm matrix and thus critical in persistence of the bacteria in the CF lung [141]. MEP is relatively conserved between strains, which makes it an attractive vaccine antigen for CF patients. A high molecular weight MEP vaccine elicited long-lived opsonic antibodies in 80–90% of the volunteers in phase I trial [142]. MEPs conjugated to various carrier proteins successfully enhanced the MEP-specific immune responses and elicited opsonizing antibodies against heterologous MEPs in mice and rabbits [143]. However, a successful clinical product has not yet been developed, indicating that vaccine of MEP alone may not be sufficient for potent immunization in human and conjugation with other vaccine targets may be considered.

4.7. Inactivated whole-cell vaccine and antibody development against *P. aeruginosa*

Whole cell-inactivated vaccines contain multiple bacterial antigenic components and thus can potentially induce diverse immunologic responses against various targets of *P. aeruginosa*. Oral immunization of bronchiectasis patients with an enteric-coated whole-cell killed vaccine resulted in significant reduction of *P. aeruginosa* in the sputum by specific lymphocyte responses [144]. Oral immunization of healthy volunteers with killed *Pseudomonas* vaccine was safe and increased *Pseudomonas*-specific serum antibodies, most notably IgA, and promoted phagocytosis elimination of *P. aeruginosa* [145]. Whole cell inactivation by X-ray irradiation kept antigen expression functional but inhibited replication in *P. aeruginosa* [146]. Mice immunized with this vaccine showed statistically significant protection against *P. aeruginosa* challenge in acute pneumonia model via opsonic killing, recruitment of CD4+ T lymphocytes and neutrophil cells [146].

4.8. Antibody and vaccine development against exotoxin

Exotoxin A is a key virulence factor secreted by around 90% *P. aeruginosa* clinical isolates and around 10,000 times more lethal than LPS [147, 148]. Exotoxin A is an ADP-ribosyltransferase and can kill macrophages, polymorphonuclear leukocytes, and other immune-related cells by receptor-mediated endocytosis and inhibition of protein synthesis elongation factor 2 [148].

mAbs against two epitopes of exotoxin A after immunization of rabbits showed potent inhibition of exotoxin A-induced cytotoxic activity *in vitro* [149]. Furthermore, these mAbs showed protective effects against *P. aeruginosa* infection for mice after immunization and enhanced the survival rate of mice model when antibiotic amikacin was combined [150]. Similarly, immunization of mice with exotoxin A showed 93.8% protection efficacy against mice burn and *P. aeruginosa*-challenged models when compared with unimmunized mice group that all died within the 70-day period [151].

Chimeric vaccine composed of a nontoxic (active-site deletion) exotoxin A and a key pilin fragment sequence was used to immune rabbits subcutaneously [152]. The produced antibodies could target against both pilin to weaken *P. aeruginosa* adherence and exotoxin A to neutralize its cytotoxic activity *in vitro* [152]. Intranasal immunization of chimeric vaccine (pilin and exotoxin A) in

mice elicited serum and saliva immune responses [153]. Moreover, saliva samples contain antibodies that can inhibit pilin-dependent *P. aeruginosa* adherence and neutralize exotoxin A [153]. This approach of immunization may be useful to provide protection against *P. aeruginosa* early-stage adhesion and infection via oropharyngeal airway [153].

5. Antibody development against *A. baumannii*

5.1. Iron-regulated outer membrane proteins (IROMP) as antibody and vaccine target

Iron is essential for bacteria to survive within host. Bacteria have evolved several ways to compete with host for iron uptake. Expression of iron-regulated outer membrane proteins (IROMPs) in bacteria is one such way. IROMPs, with molecular weight ranging from 77 to 88 kDa, are a class of specific cell surface receptors that can bind iron chelator siderophore with high affinity and subsequently lead to the internalization of iron-loaded siderophore and iron assimilation in *A. baumannii* [154, 155]. Goel et al. [155] used IROMPs from *A. baumannii* to immunize BALB/c mice and identified several mAbs of IgM isotype that can block interaction of siderophore with IROMPs and induce bactericidal and opsonizing activity *in vitro*.

5.2. Inactivated whole cell, outer membrane complexes (OMCs), and outer membrane vesicles (OMVs) as vaccine and antibody target

Immunization of mice with inactivated whole *A. baumannii*, prepared from formalin-treatment, elicited protective antibody response against *A. baumannii* post-infection challenge in mice sepsis model [156]. Subsequently, these antibodies separated from immunized mice sera also showed passive protection against mice with *A. baumannii* infection [156]. As inactivated whole *A. baumannii* vaccine contains LPS (endotoxin) that may complicate immune responses after immunization, LPS-deficient and inactivated whole *A. baumannii* cell was used to immune mice [157]. Similar humoral and cellular immune responses was observed as compared with wild-type inactivated whole *A. baumannii* vaccine in protection against different mouse models with disseminated *A. baumannii* infections of various strains [157].

Vaccine made of outer membrane complexes (OMCs) from *A. baumannii* induced protective humoral and cellular immune responses against murine sepsis model [158]. Similarly, passive transfer of antiserum from immunized murine to naive mice rescued these mice from *A. baumannii* infection [158].

Outer membrane vesicles (OMVs), released from Gram-negative outer cell wall surface, have a diameter within the range of 50–250 nm and contain all constituents as Gram-negative outer cell wall, such as proteins, LPS, phospholipids, DNAs, and RNAs [159–161]. OMVs play important pathological roles by delivering virulence factors into host cell and coordinate group communications known as quorum sensing [160, 161]. High-dose challenging of mice with OMVs (200 μg) triggered a strong pro-inflammatory cytokine release that may be pathological to host [162].

Interestingly, immunization of mice with low dose OMVs (10 μg) from one clinical MDR *A. baumannii* isolate induced clear protection against mice pneumonia and sepsis models after *A. baumannii* challenge [163]. The protective mechanism is in part from specific anti-OMV antibody induced opsonophagocytic activity and suppressed pro-inflammatory cytokine release [163].

Recently, OMVs were engineered as a delivery vehicle to package and display Omp22 at the OMV surface [164]. The displayed Omp22-OMV can induce high-titer anti-Omp22 specific antibodies and protect mice from sepsis after lethal *A. baumannii* challenge [164].

5.3. Targeting outer membrane protein A (OmpA)

Outer membrane protein A (OmpA), previously known as Omp38, is a lethal and most abundantly expressed surface virulence factor in *A. baumannii* [165, 166]. OmpA belongs to the porin family with low permeability that may be a key factor contributing to its multidrug resistance [167]. OmpA can bind with host cell directly, internalize within mitochondria and nuclei compartments of host cell, and induce host cell death [165, 166]. Moreover, OmpA is highly conserved within six clinical isolates (99% protein sequence identity) and 14 other NCBI GenBank deposited sequences from different isolates of *A. baumannii* (89% protein sequence identity), while OmpA shows no homology to human proteins [168].

Thus, OmpA from *A. baumannii* is a potentially ideal vaccine and antibody target.

In agreement with the sequence identity analysis, immunization of diabetic mice subcutaneously with recombinant OmpA induced markedly protective effect upon lethal, extreme drug resistant-*A. baumannii* challenge; use of antibodies against OmpA also elicited similar protective effect on diabetic mice with lethal *A. baumannii* infection [168]. Interestingly, dosage of *A. baumannii* rOmpA vaccine correlates with various B cell epitopes and immunodominant T cell epitopes, emphasizing dosage needs to be taken into account for vaccine development [169]. Recently, intranasal immunization of mice with OmpA can trigger both mucosal and systemic protective antibodies against MDR *A. baumannii* infection [170].

Omp22 is an outer membrane protein with molecular weight of 22-kDa. Omp22 is more than 95% conserved within 851 reported *A. baumannii* strains [171]. In contrast, there is no homology with human proteins. This unique and conserved sequence makes Omp22 an ideal vaccine candidate. Immunization of mice with recombinant Omp22 induced clear protection from MDR *A. baumannii* infections, showing a potential vaccine candidate [171].

FilF is a highly conserved outer membrane protein predicted to be involved in pilus assembly in *A. baumannii* [172]. Immunization of mouse pneumonia model induced high titer of antibody, decreased the bacteria lung burden, and rescued around 50% of mice from lethal *A. baumannii* infection [172]. These promising results may suggest that FilF is a promising vaccine candidate for further evaluation [172].

5.4. Biofilm related proteins as vaccine and antibody target

Biofilms are bacterial communities connected by a surface of extracellular matrix with complicated compositions that may vary based on different bacteria and different living

environments [173]. Identified biofilm components contain polysaccharides, proteins, and extracellular DNAs and play essential pathological roles in bacterial adhesion to host cell and shielding bacteria from nearby pressures such as antibiotics [173, 174].

Surface polysaccharide poly-beta-(1-6)-N-acetylglucosamine (PNAG), as a major component of biofilm, is a key virulence factor in *A. baumannii* [175]. Immunization of rabbit with conjugation of a synthetic oligosaccharide, mimicking PNAG, with tetanus toxoid induced antibodies that can opsonize clinical isolates of *A. baumannii* with surface expression of PNAG *in vitro* and protect *A. baumannii* challenged mice [176].

Biofilm-associated protein (Bap) in *A. baumannii*, 8620 amino acids in length, is one of the largest proteins identified within bacterial proteins and plays a vital role in biofilm formation [177]. Bap, containing seven tandem repeats of modules, is 41–66% conserved among clinical isolates and its expression is induced by low iron concentration [177, 178]. Immunization of mice with one region of Bap from *A. baumannii* elicited protective immunity against *A. baumannii* of different strains, suggesting that Bap is conserved and can be used as a potential vaccine candidate [179].

Ata, a trimeric transporter and a key virulence factor in *A. baumannii*, is essential in biofilm formation [180]. Rabbit sera from Ata vaccination can opsonize *A. baumannii* isolates effectively in complement and polymorphonuclear cells dependent manners [181]. Moreover, the rabbit sera can significantly lower the burden of mice lung infection from MDR *A. baumannii* strains, showing that Ata is one more potential vaccine target [181].

5.5. Targeting K1 capsular polysaccharide

K1 capsular polysaccharides are an important virulence factor that helps *A. baumannii* to establish infections within host [182]. Immunization of mice with sub-lethal and K1 capsular polysaccharide positive *A. baumannii* induced generation of specific anti-K1 capsular polysaccharide IgM monoclonal antibody (13D6) [183]. Moreover, 13D6 can induce efficient neutrophil-mediated in vitro opsonization and in vivo passive protectivity in rat soft tissue infection model [183]. However, only 13% of 100 collected *A. baumannii* strains were positive against 13D6, suggesting other capsular polysaccharide serotypes that may be unexplored. Additionally, lack of immunoglobulin class switch from IgM to IgG may not effectively trigger adaptive long-term immune memory response. Failure of class switching may be the inherent property of most capsular polysaccharides that only elicits a T cell independent immune response after immunization [184]. Thus, to target more *A. baumannii* strains effectively, identification of more capsular polysaccharide serotypes and conjugation of capsular polysaccharide with carrier proteins may be needed. As a matter of fact, this strategy has been successfully applied in clinics for the prevention of *Streptococcus pneumoniae* infection by the introduction of 23-valent nonconjugated and 13-valent conjugated capsular polysaccharide vaccines [185].

6. Concluding remarks

Antibody and vaccine are important treatment options in the mobilization of human immune system passively or actively to recognize, kill bacteria enemies, and moreover memorize these

enemies for the long-term protection. Antigen selection is the key for antibody and vaccine development, which needs to be immunogenic and conserved. Initially, antibody and vaccine development mainly focused on individual antigen. It is now clear that multivalent antigens should be more potent in eliciting immune responses against bacteria. Combination of pan-genomics, proteomics, and reverse vaccinology analysis of bacteria revealed a list of conserved antigens as potential vaccine or antibody targets and some of these antigens are already known as virulence factors of related bacteria [186, 187]. These bioinformatics-based "omics" analysis will undoubtedly facilitate effective vaccine and antibody target identification and development.

Other alternatives to antibiotics, including short antimicrobial peptides, antibiofilm peptides, and host defense peptides, are not covered in this chapter; readers can refer to a recent excellent review and references therein for further information [188].

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Chapter 11

Physiology and Pathology of Multidrug-Resistant Bacteria: Phage-Related Therapy

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Additional information is available at the end of the chapter

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Abstract

Multidrug-resistant bacteria (MDR) are spreading rapidly across the world that outpace development of new antibiotics. Options other than antibiotics treatment are urgently needed. In this chapter, we review the current status of nonantibiotics-based strategies including phage therapy and phage-derived protein therapy for targeting Gram-positive strains (methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecium*) and MDR Gram-negative strains (*Acinetobacter baumannii* and *Pseudomonas aeruginosa*).

Keywords: multidrug-resistant bacteria, MDR, MRSA, VRE, *A. baumannii*, *P. aeruginosa*, infection, biologics, bacteriophage, lysin, endolysin, phage therapy

1. Introduction

Host-pathogen battle is a never ending theme regarding infection and immunity. Human innate immune defense is triggered at early stages of bacterial infections. As the major players of innate immunity, macrophages, neutrophils, dendritic cells (DCs) and natural killer cells recognize pathogen-associated molecular patterns (PAMPs) and damage (or danger)-associated molecular patterns (DAMPs) through their pattern recognition receptors (PRRs) [1–3].

Known PRRs consist of Toll-like receptors, C-type lectin receptors, Nod-like receptors (targeting intracellular pathogens via inflammasome), AIM2-like receptors, RIG-I-like receptors and microbial nucleic acid sensors [1, 4, 5]. Identified PAMPs include lipopolysaccharide (LPS or endotoxin), peptidoglycan, lipoteichoic acid, exotoxin, effector protein, lipoprotein, porin, flagellin, pilin, glycoprotein, glycosylphosphatidylinositol, microbial nucleic acid and outer

membrane vesicle (extracellular vesicle or exosome) [1, 4, 6, 7]. Activation of innate immune systems through interactions of PAMPs and DAMPs with PRRs induces antigen presenting cells (APCs, mainly macrophages and DCs) to phagocytose bacterial pathogens and cleave pathogen-related proteins to peptides within endosomes and lysosomes [8]. The cleaved peptides can be recognized by major histocompatibility complex (MHC)-II and presented to the surface of APCs. MHC-II-peptide complex is the natural ligand of T-cell receptor (TCR) from $CD4^+$ T cell that can stimulate cytokine and chemokine secretion, inflammatory signaling cascade and activate adaptive immune responses from both T lymphocytes and B lymphocytes for protective immunity and elimination of pathogens [1, 4, 8].

On the other hand, bacteria evolve strategies to compromise, manipulate or evade host immune system that can lead to host cell autophagy and pyroptosis and thus enhance bacteria adhesion, colonization and chances of survival within the host [9, 10]. Moreover, excessive or chronic inflammations induced by bacterial infections are closely related with pathogenesis of autoimmune disorders [11]. Thus, effective treatment of bacterial infections is urgent. However, multidrug-resistant bacteria (MDR) appear to outpace current development of new antibiotics, especially to six frequently reported MDR bacteria, designated as *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* spp. (ESKAPE) [12, 13].

Staphylococcus aureus (*S. aureus*) is a Gram-positive, leading nosocomial pathogen that can cause many types of infections, ranging from surgical site infections from intensive care units (ICUs) to community-acquired skin and soft tissue infections. Methicillin-resistant *S. aureus* (MRSA) became endemic in hospitals by the 1980s and in some areas, more than 50% of *S. aureus* isolates are now resistant to methicillin [14]. In the United States, an estimated 80,000 invasive MRSA infections and 11,000 related deaths occur annually [15].

Enterococci are Gram-positive, facultatively anaerobic cocci that often occur in chains of various lengths. *Enterococci* are generally considered as low virulent as evidenced by their natural presence in human gastrointestinal tract and long being used as probiotics in human. They have attracted more attention since increasing number of patients who are immunosuppressed or receiving antimicrobial agents have been reported to suffer from MDR *Enterococci* infections [16]. In fact, an estimated number of 20,000 cases and 1300 deaths are caused by vancomycin-resistant *E. faecium* (VRE) infection annually in United States [17].

Pseudomonas aeruginosa (*P. aeruginosa*) is a Gram-negative, leading cause of nosocomial infections and shows potential of rapid evolution of antibiotics resistance during therapy [18]. Susceptible individuals include victims of cystic fibrosis and those with an impaired immune system caused by HIV infection, organ transplantation, cytotoxic drugs or burns with vascular damage [19].

Acinetobacter baumannii (*A. baumannii*) is a Gram-negative, drying and disinfectant-resistant bacterium that can evade human immunity and develop drug resistance to almost all classes of antibiotics [20, 21]. MDR *A. baumannii* infection is mainly restricted within hospitals for patients with mechanical ventilation, burns, wounds, sepsis, meningitis and often leads to high morbidity and mortality [20, 22, 23].

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Bacteriophage (short for phage), as its name indicates, is a natural virus that only infects bacteria and this unique property makes bacteriophage an attractive alternative for bacterial infection treatment, especially for the current MDR bacteria spreading worldwide. This chapter reviews the current status of phage therapy and phage-derived protein therapy for Gram-positive strains including MRSA and VRE and MDR Gram-negative strains (*A. baumannii* and *P. aeruginosa*).

2. Lytic bacteriophage structure

Phages are estimated to be the most diverse and abundant entity on earth that exist in every ecosystem with the range of 10^{30}–10^{31} and are about 10 times more than their bacterial hosts [24]. For instance, agricultural soils usually harbor a phage count of approximately 10^{8}–10^{9} per gram of dry soil and aquatic environments contain a phage titer of 10^{4}–10^{8}/mL [25–28].

The basic phage structure is made up of a hexagonal head, which harbors the phage double-stranded DNA (dsDNA), together known as capsid, a tail and a connector between head and tail (**Figure 1A**) [29]. The head is anchored to a tail sheath via a neck and a collar and ends into a hexagonal base plate. Tail fibers/spikes emerge from the base plate and the tail sheath tapers into a tail tube.

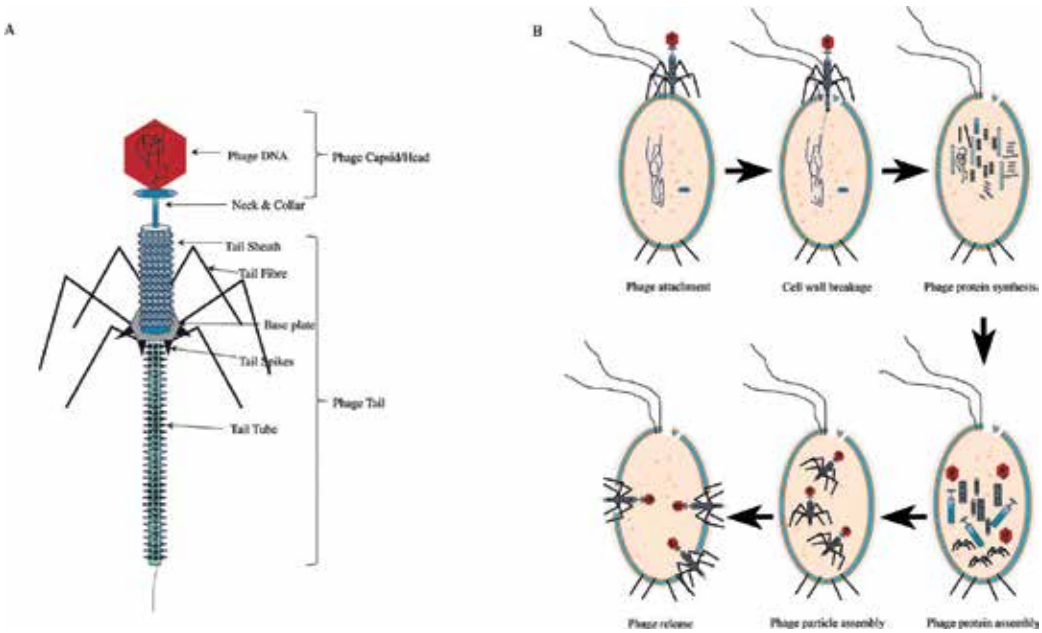

Figure 1. Structure and life cycle of a lytic bacteriophage. (A) The structure of a typical lytic bacteriophage is shown. (B) Lytic phage life cycle is shown starting with attachment on bacterial cell surface and proceeding to phage release by intermediate steps.

Phages are classified into two categories (lytic and nonlytic or temperate) and 13 families based on certain criteria including its host specificity, morphology, genotype, infective mode, with or without envelope and lipid [30]. Currently, over 5500 different bacteriophages have been sequenced and 96% of them, including most of therapeutic phages, belong to the order *Caudovirales* [31]. The order *Caudovirales* comprises three families according to the morphological features of the tail: *Myoviridae* (with long, rigid, contractile tails, e.g. T4), *Siphoviridae* (with long, flexible, noncontractile tails) and *Podoviridae* (with short, noncontractile tails).

3. Lytic bacteriophage life cycle

Lytic bacteriophages are of special interest in phage therapy of bacterial infections. Lytic phage life cycle typically consists of (1) Attachment/adsorption to the host cell—it involves the contact between tail fibers and the host cell receptors like lipopolysaccharide (LPS), peptidoglycan (PG), outer membrane (OM), fimbriae, flagellum or sex pilus; (2) Injecting phage DNA—the phage secretes specialized enzymes that destroy the LPS, PG and OM to inject the phage DNA through the tail tube into the host cell; (3) Phage DNA replication—after phage DNA injection, phage early genes are expressed which take the control of host cell machinery to replicate phage DNA. The replicated phage DNA then expresses phage late proteins necessary for virion assembly; (4) Assembly and packing of phage particle—once the assembly proteins are expressed, capsid assembles by encapsulating the phage genetic material and later a separately assembled tail joins the capsid to make a full phage particle; and (5) Host cell lysis and phage release—the phage late proteins comprise endolysins and holins which together break the PG layer, lyse the bacteria and burst out the fully formed bacteriophage into the environment [32, 33]. **Figure 1B** illustrates a cartoon process of how bacteriophage infects, lyses bacteria and releases progeny. Since the lytic phage kills the bacterial host cell after completing the lytic life cycle, they are seen as potential antibacterial agents.

4. Phage therapy against MRSA

More than 200 lytic phages against *S. aureus* have been characterized [31]. Most of *S. aureus* phages belong to the *Siphoviridae*, such as lytic phage ϕMR11 [34] and lytic phage phiIPLA35 [35]. A small number of *S. aureus* phages belong to the *Podoviridae* like SAP-2 phage [36] and *Myoviridae* like Stau2 [37] and well-known phage K [38].

Development of phage resistance to host-pathogen and cross-resistance with antibiotics are seldomly observed [39]. Thus, MRSA pathogens can be targeted by the anti-*S. aureus* phages such as phage K and φMR11 [34, 40]. φMR11, administrated intraperitoneally, appeared rapidly in the circulation of mice challenged with fatal *S. aureus* infection and successfully protected mice without any adverse effects [34].

S. aureus-specific phage MR-10, when combined with Mupirocin, can not only significantly reduce the *in vitro* adherence, invasion and cytotoxicity of MRSA on murine nasal epithelial

cells and effectively eradicate MRSA population from mouse nares but also decrease the frequency of mutation coupled with Mupirocin treatment alone to negligible levels [41]. Similarly, synergistic effect on anti-*S. aureus* was observed when combination of *S. aureus* phages with gentamicin or linezolid was used [42, 43].

Biofilms play a key pathological role in *S. aureus*-associated chronic infections [44]. Bacteriophage cocktail NOV012 containing two different phages, P68 and K710, showed high protection against MRSA-related chronic rhinosinusitis [44]. Moreover, Poland scientists demonstrated that efficient phage therapy was an alternative to antibiotics for treating chronic MRSA infections with significant savings in healthcare costs [45].

Interestingly, researchers found that some *S. aureus*-specific lytic phages, identified from natural sewage, showed higher protective efficiency against MRSA in mice than antibiotic or conventional phage and antibiotic combined treatment [46, 47].

To overcome the rapid release of toxics arising from lytic phage induced *S. aureus* lysis, the endolysin gene controlling the release of phage progeny was inactivated in *S. aureus* phages. These lysis-deficient phages successfully induced MRSA death in mice infection model without lysis induced side effects such as septic shock or toxic shock syndrome, possibly based on the sole activity of holin [48, 49].

Phage can be used as an efficient carrier to bring photosensitizers (light-activated antimicrobial agents) to *S. aureus* by chemical conjugation which then resulted in enhanced and selective killing of MRSA when exposed to low-dose red light [50]. Moreover, as the carrier for photosensitizers, the ability to selectively kill MRSA is independent of phage's ability to infect *S. aureus* [51].

5. Phage therapy against VRE

More than 27 phages have been isolated and tested for their protective efficacy VRE infection [52]. Most of these phages belong to the *Myoviridae* or the *Siphoviridae* families [52]. Phage ENB6, isolated from raw sewage, has lytic activity against a wide range of clinical VRE isolates and single dose of intraperitoneal injection was sufficient to rescue 100% of the fatally infected mice [53]. The authors also demonstrated that the ability of this phage to rescue bacteremic mice was not due to a nonspecific immune effect but due to the ability of phage ENB6 itself [53]. Similarly, *in vivo* therapeutic potential of virulent phage phiEF24C, evaluated in a sepsis BALB/c mouse model, proved to be effective against lethal VRE infection at a low concentration following a single or repeated phage exposure [54]. *Enterococcus faecalis* phage IME-EF1 was isolated from hospital sewage; when administrated intraperitoneally in a murine sepsis model, one dose of IME-EF1 or its endolysin was found to reduce the bacterial blood count and protect the mice from a lethal challenge of *E. faecalis* [55]. Biofilm-associated VRE infections are challenging for treatment. EFDG1, isolated from sewage water, was efficient not only in nearly eliminating 2-week old *E. faecalis* biofilms of around 100 μm thickness but also in prevention of *E. faecalis* root canal infection [52, 56].

6. Phage therapy against MDR *P. aeruginosa*

More than 110 phages specifically target *P. aeruginosa* and around 60% are lytic phages, which are frequently isolated from hospital wastewater and sewage wastewater [57]. Fu et al. [58] used an *in vitro* model to investigate the effect of lytic phages in the prevention of *P. aeruginosa* biofilm formation in hydrogel-coated catheters and found that catheters, when pretreated with single phage, presented high reduction of biofilm formation at early inoculation while cocktail phage treatment in keeping high reduction of biofilm formation lasted over 48 hours post treatment.

Torres-Barceló et al. [59], Knezevic et al. [60], Zhang and Hu [61] and Oechslin et al. [62] explored the combinatorial effect of phages with different antibiotics against *P. aeruginosa* and found that certain combination can lead to synergistic effect than single treatment alone. Moreover, Torres-Barceló et al. found that long-term combination of phages with antibiotics not only showed synergistic benefit but also weakened antibiotics-induced resistance for *P. aeruginosa* when used alone [63].

A mouse lung infection model was used to evaluate therapeutic and prophylactic efficiency of phage PAK-P1 against MDR *P. aeruginosa* by nasal. The curative treatment of one single dose 2 hours after bacterial infection allowed over 95% survival and preventive treatment with single dose 4 days before infection resulted in 100% survival whereas untreated mice all died within 2 days after infection [64].

To evaluate efficacy and safety of bacteriophage therapy in human, Wright et al. used a phage cocktail named as Biophage-PA to carry out the first controlled clinical trial phase I/II for treating MDR *P. aeruginosa* that caused chronic otitis in 2009 [65]. Encouragingly, Biophage-PA-treated patients showed significant clinical improvements and no related side effects or local systemic toxicities when compared with placebo control individuals [65].

7. Phage therapy against MDR *A. baumannii*

Phage AB1 and phageφAB2, as the early characterized phages in detail against MDR *A. baumannii*, were reported in 2010 [66, 67]. Phage AB1 belongs to the *Siphoviridae* family and harbors a narrow host range, a latent period of 18 minutes and a burst size of 409, whereas phage φAB2 is from the *Podoviridae* family, showing rapid adsorption (more than 99% absorbed in 6 minutes), a latent period of less than 10 minutes, a burst size of around 200 and a broad host range [66, 67]. Furthermore, phageφAB2 was shown to be used potentially as an anti-MDR *A. baumannii* hand wash [68]. Two *A. baumannii*-specific lytic phages, AB7-IBBI and AB7-IBB2, belonging to the *Siphoviridae* family and the *Podoviridae* family, respectively, demonstrated the ability to remove approximately 75% of preformed biofilms of MDR *A. baumannii* and showed potential application in hospital as environmental biocontrol agent [69, 70]. vB_AbaM-IME-AB2, a novel lytic *A. baumannii* phage, belongs to the *Myoviridae* family with a latent period of 20 minutes and a burst size of 62 and can infect MDR clinical isolates of *A. baumannii* [71].

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Mouse infection model-based studies showed that BS46, a specific *A. baumannii* phage, could protect mice infected intraperitoneally with five times the lethal dosage 50 (LD50) of a highly virulent *A. baumannii* strain [72]; and a five-membered *A. baumannii*-specific phage cocktail demonstrated therapeutic efficacy against MDR *A. baumannii* pathogen in an infected wound model [73].

Eight lytic phages, isolated from hospital sewage, can lyse 34 clinical *A. baumannii* strains with various spectrums [74]. One phage named as φkm18p, belonging to the *Podoviridae* family, showed potent lysis of 15/34 clinical *A. baumannii* strains, of which many were "extensively drug resistant" *A. baumannii* strains [74]. The authors suggested that a cocktail of φkm18p with other lytic phages has potential to treat all MDR *A. baumannii* strains [74].

Recently, other lytic phages or phage cocktails have been reported to have potentials for treatment of *A. baumannii* infections in ICUs including vB_AbaM_Acibel004 and podovirus vB_AbaP_Acibel007 [75], phage Bφ-C62 [76], vB-GEC_Ab-M-G7 [77] and vB_AbaM-IME-AB2 [78]. Of note, cleaning of ICUs with addition of active phage aerosol significantly reduced *A. baumannii* infection rate and consumption of antimicrobials [79], highlighting the potential of phage-based prevention and therapy against MDR *A. baumannii* in the near future.

8. Bacteriophage derived proteins as antibacterial biologics

Bacteriophage encodes specialized proteins that mediate the phage entry into and exit out of the bacterial host during the lytic cycle. These phage proteins/enzymes are critical for both disintegration of the physical barrier and exploiting physiological pathways to establish an infection. The bacterial cell wall comprises an outer membrane exopolysaccharides (OM-EPS) and inner membrane peptidoglycan (IM-PG), which serves as the target of various phage enzymes. Therefore, phage enzymes are perceived as "natural antibiotics" but the idea itself has remained in its infancy due to the largely popular and broadly effective antibiotic drugs. However, a growing number of MDR bacterial pathogens have rung the alarm and triggered a renewed interest in employing phage-derived proteins to treat bacterial infections. In this section, we will focus on the phage lysins, which enzymatically cleave the linkages in the peptidoglycan (PG) layer of the bacterial cell wall. The PG layer is made up of alternating N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) units in which the MurNAc residues are covalently linked via amide bonds to the L-alanine of the stem peptide [80]. The PG layer provides the structural integrity and rigidity to bacterium and its breakdown is essential for phage to enter and exit its host cell (**Figure 2**). Based on their temporal expression, phage lysins can be divided into two types: (1) virion-associated peptidoglycan hydrolases (VAPGHs) and (2) endolysins. **Figure 3** shows overall architecture of VAPGHs and endolysins. We present an overview of phage lysin function and therapeutic potential in treating bacterial infections. We also present the protein engineering strategies employed to enhance bacteriolytic property and tropism of such proteins.

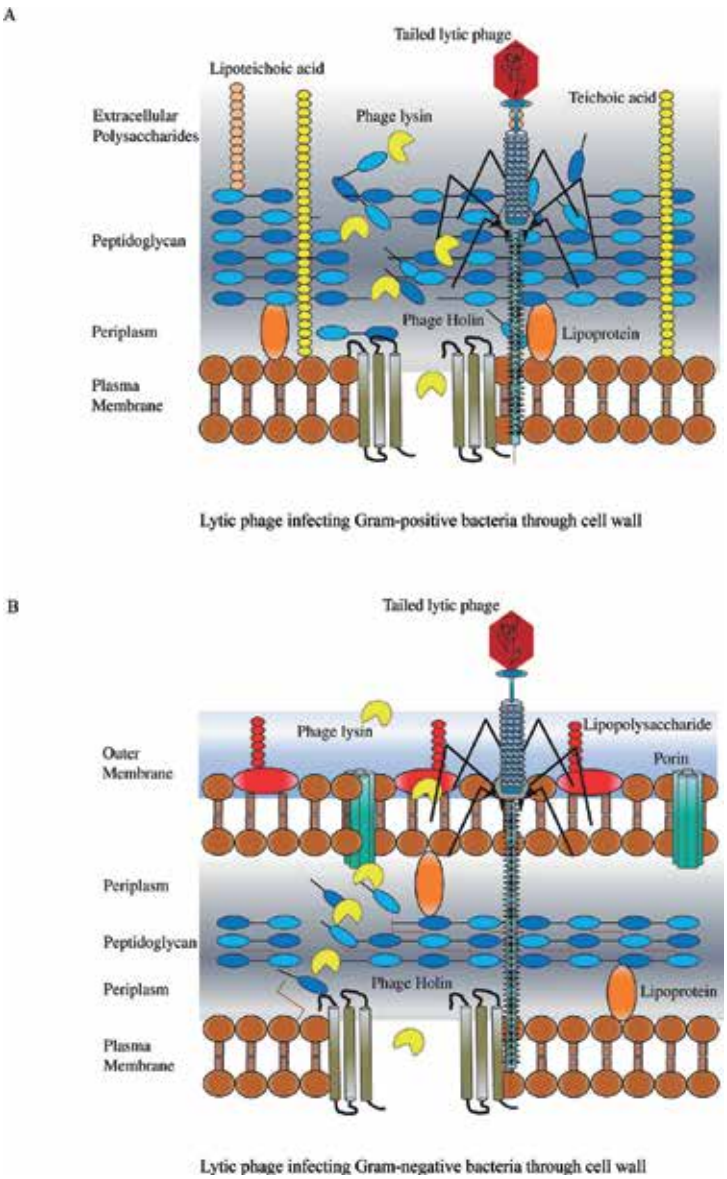

Figure 2. Bacterial cell wall degradation by phage enzymes. The bacteriophage enzymes comprising endolysin and holin together facilitate the degradation of the host bacterial cell-wall lysis by cleaving specific linkages in peptidoglycan layer and plasma membrane of (A) Gram-positive bacterial and (B) Gram-negative bacterial cell wall.

8.1. Virion-associated peptidoglycan hydrolases (VAPGHs)

VAPGHs are phage encoded hydrolytic enzymes which specifically degrade the PG layer of both Gram-positive and negative bacteria. VAPGHs are expressed early in the phage life cycle to degrade the OM for phage attachment and subsequent adsorption. These enzymes can have wide occurrence since the PG layer is common to both Gram-positive and Gram-negative bacteria.

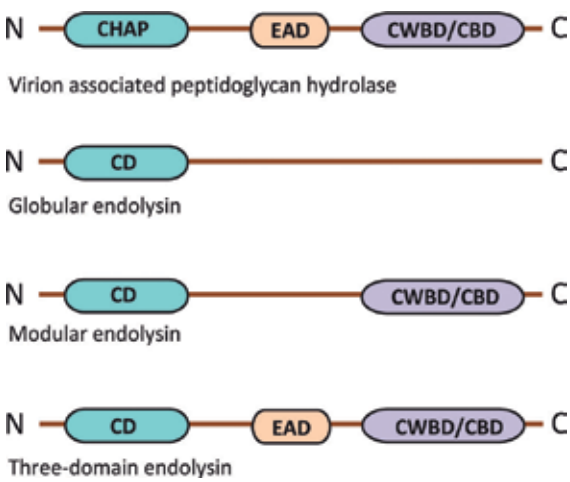

Figure 3. Domain architecture of phage muralytic enzymes. CHAP: cysteine/histidine-dependent amidohydrolases/peptidase; CD: catalytic domain; EAD: enzymatically active domain; CBD/CWBD: cell/cell wall binding domain.

However, it is thicker in Gram-positive organisms (20–80 nm) when compared to Gram-negative bacteria (10 nm) [80]. Different phage proteins can have various muralytic activities and their locations vary on the phage structure [81–83]. The VAPGH genes, although are not critical for phage multiplication, can ease out the phage infection process during suboptimal conditions [84, 85], against bacteria possessing an extensively cross-linked PG layer [83, 86] or if phage lysozyme activity is inhibited [87].

The muralytic activity of VAPGHs resides in the N-terminal and the cell wall/cell binding domain (CWBD/CBD) is present at the C-terminal (e.g. in a broad-spectrum staphylolytic phage P68 VAPGH P17) [88]. Owing to their modular architecture, VAPGHs can be engineered to enhance lytic activity and increase the tropism. A chimeric VAPGH P16-17 with N-terminal endopeptidase domain of Lys16 and the C-terminal CWBD of VAPGH P17 exhibits staphylolytic activity [89]. Chimeric versions of VAPGH HyDH5 and Lys16, produced by C-terminal fusion of bacteriocin lysostaphin SH3bCBD or a direct fusion of cysteine/histidine-dependent amidohydrolases/peptidase (CHAP) domain to SH3bCBD in the absence of enzymatically active domain (EAD), improve the lytic activity against *S. aureus* including MRSA, *S. epidermis and S. carnosus* [90–92]. This increased staphylolytic activity and tropism are attributed to the dual enzymatic activities targeting distinct linkages within the PG layer [90]. Similarly, *S. aureus*-specific temperate phage DW2 codes a hydrolytic VAPGH THDW2. This enzyme has a modular structure with N-terminal CHAP domain and an EAD at the C-terminal but lacks the CWBD/CBD [93].

8.2. Endolysins—phage enzymes degrading peptidoglycan

Endolysins are also muralytic enzymes like VAPGHs coded by dsDNA phage. Unlike VAPGHs, which act to degrade PG layer for phage DNA entry, endolysins are responsible for the release of the progeny phage late during the lytic phage cycle [94, 95]. Endolysins can be structurally divided into (1) globular endolysins which are constituted by a single catalytic domain (CD)

[83, 96]; (2) modular endolysins which are constituted by an N-terminal CD and a C-terminal CWBD/CBD [83, 97] and (3) three-domain endolysins which are constituted by CD, CWBD/CBD and an additional EAD in between CD and CBD [98]. The CD of different endolysins may have different enzymatic activities to cleave distinct linkages in the PG layer, whereas the CWBD is mainly responsible for imparting specificity of the interaction which can even be restricted to a particular serovar [99]. They can be further classified according to their functionality into (1) N-acetyl-β-D-muramidases with activity against MurNAc-GlcNAc linkages; (2) lytic transglycolases, which cleave N-acetylmuramoyl-β-1,4-N-acetylglucosamine bond; (3) N-acetyl-β-D-glucosaminidase cleaving the N-acetylglucosaminyl-β-1,4-N-acetylmuramine; (4) N-acetylmuramoyl-L-alanine amidases, which break amide bond between sugar and peptide and (5) endopeptidases, which cleave the peptide bond between two amino acid residues of the stem [94, 100].

The bactericidal property of the endolysins makes them attractive drug candidates to treat bacterial infections [101]. Artificial inoculation of *S. aureus* in human nares was shown to be completely cleared by intranasal administration of MV-L endolysin of phiMR11, a phage specific for *S. aureus* [102]. Similarly, nasal/oral administration of CHAP domain of endolysin LysK eliminated *S. aureus* from nares of the infected mice [103]. The modular structure of the endolysins targeting Gram-positive bacteria is appropriate to evolve into efficacious drugs. A chimeric lysozyme ClyS, developed by fusing Ply Twort endolysin EAD and phi13 phage NM3CBD, reduced MRSA from nasal passage and showed better effect than mupirocin treatment [104, 105]. Furthermore, combination therapy of endolysin and antibiotics can be more effective in relieving MRSA infection [104]. Domain swapping strategy has also been used to replace the CBD of a phage endolysin PlyPSA and Ply187 to enhance the lytic ability when compared to their parental proteins [106]. Even the lytic spectrum of these enzymes can be broadened by engineering CBDs from different endolysins [106]. A *Staphylococcus* phage endolysin P128 is currently being investigated for intranasal administration against *S. aureus* in phase III clinical trials [107] and PlySs2 endolysin (CF-301) is also being tested against *S. aureus* for safety in phase I [108]. Recently, SAL-200 endolysin, derived from staphylococcal phage SAP-1, is the first intravenously administered lysin, which showed good tolerance with no serious adverse effects in phase I safety studies [109, 110].

In 2012, Lukacik et al. showed that the fusion of FyuA-binding domain of pesticin and T4 lysozyme utilizes FyuA for transport across the OM of Gram-negative *Yersinia pestis* [111]. This hybrid toxin killed *Yersinia* and *Escherichia coli* strains and also bypassed the pesticin immunity (PIM) [111]. Furthermore, Ply187-derived CD, when fused to non-SH3b CBD from phage phi13 endolysin NM3, protects mice against MRSA [112]. Endolysins harboring SH3b CBD were reported to ensure 100% survival when compared to oxacillin and vancomycin in bacteremia model [113]. An important vision harming disease called endophthalmitis, in which *S. aureus* colonizes the eye, was treated effectively in mouse model by chimeric endolysin Ply187AN-KSH3b [114]. Native CD and CWBD were joined via a linker to develop chimeolysins (e.g. Lys168-87, Lys170-87, B30-182-lyso, Ply187N-V12C and ClyR), which showed broad antibacterial spectrum [112, 115]. One such chimeolysin, ClyR, effectively lyses *Streptococcal* spp. (*S. pyogenes*, *S. agalactiae*, *S. dysgalactiae*, *S. equi*, *S. mutans*, *S. pneumoniae*, *S. suis* and *S. uberis*), *E. faecalis* and *S. aureus*, including MRSA [115]. The chimeolysin ClyR is also effective in killing *Streptococcus* mutans, which colonizes as biofilm on tooth surface [115].

A shortcoming of natural endolysins is their inability to cross the OM of Gram-negative sp. (e.g. *P. aeruginosa*, *Salmonella typhimurium*, *Salmonella enterica*, *A. baumannii, E. coli, S. aureus* and *Bacillus subtilis*) [96, 97, 116]. Gram-negative bacteria have OM which is composed of lipopolysaccharide (LPS) and is only permeable to molecules smaller than 600 Da [117]. But recently, a Gram-negative endolysin SPN1S has shown to carry muralytic and glycosidic hydrolase activities in its alpha-helical structure [118]. To effectively penetrate OM of Gram-negative bacteria, endolysins have been fused to LPS-destabilizing polycationic peptides (PCNPs) to generate "Artilysins" [119]. The polycationic peptides can be fused to either N- or C-terminal of the endolysins but artilysins with N-terminal peptide are generally more effective [119]. Different LPS-destabilizing peptides, providing varying degrees of effectiveness, have been tested in artilysin constructs with polycationic peptide (PCNP) being the most effective one [119, 120]. The polycationic peptide punctures the LPS layer and facilitates the endolysin penetration into the OM, which subsequently degrades the PG layer [119]. An artilysin "Art-175" is a fusion product of KZ144 endolysin of *P. aeruginosa* phage phiKZ and sheep myeloid 29 amino acid peptide (SMAP-29) which can kill *P. aeruginosa* by more than 5 log in 30 minutes [119, 120]. Similarly, N-terminal fusion of PCNP to OBPgp276 endolysin (LoGT-001) of *P. fluorescens* phage OBD or PVP-SE1gp146 endolysin (LoGT-008) of *S. enterica* phage PVP-SE1 reduces *P. aeruginosa* by 4–5 log in 30 minutes [119]. Fusion of PCNP via C-terminal extended linker to OBPgp276 endolysin (LoGT-02) was as effective as N-terminal PCNP fusion [119]. Artilysin (Art-240; PCNP-λSa2lys endolysin) activity of greater than 3 log has also been demonstrated against Gram-positive bacteria (*S. agalactiae*, *S. dysgalactiae*, *S. pyogenes*, *S. uberis*, *S. suis*, *S. porcinus*, *S. gordonii*, *S. sanguinis*, and *S. viridans*) [121].

In addition, the protein transduction domains (PTDs), which facilitate protein transport across the eukaryotic membrane, have been used to engineer endolysin [94, 122, 123]. Fusion of endolysin with PTD is highly effective in reducing *S. aureus* burden in epithelial cell lines when compared to non-PTD endolysins [94, 123]. Catalytic peptides can also enhance the properties of a lysin as shown by fusing the Cecropin A peptide (residues 1–18) to the OBPsp279 lysin, which targets *A. baumannii* and *P. aeruginosa* during the phage growth [124]. More clinical trial data are needed to assess the safety and efficacy of these lysins.

9. Concluding remarks

Given the wide spread MDR bacteria and scarcity of new antibiotics in drug development pipeline, alternative options have to be explored urgently. As an alternative option, phage therapy is reattracting worldwide attentions. It is clear that phage therapy has several advantages in targeting against bacterial infections over conventional antibiotics [39, 125]: (1) phage is natural killer of bacteria that dictates its unique target specificity; (2) phage multiplies within bacteria host until host is lysed in a self-dosing manner; (3) phage shows efficacy to bacteria of MDR and (4) phage is environmentally friendly. Moreover, co-administration of phage or phage cocktail with antibiotics demonstrates synergistic effect over each individual treatment and increases antibiotics sensitivity from previous reports as reviewed in this chapter. However, as live virus, safety concern of phage therapy, due to the gap of deep understanding of phage-bacteria-human interaction network, is not easily cleared in the Western countries though former Soviet Unions accumulated a lot of empirically successful clinical reports in the nearly past 50 years.

Quality control of phage therapy based on Western medicine criteria has to be met. A small scale and strict quality control of a phage cocktail for treatment of *P. aeruginosa* and *S. aureus* infections was conducted in Belgium that included sequencing of whole phage genomes to verify the lack of toxin-encoding genes, confirmation of lytic phage property, lack of temperate phage, stability, removal of pyrogen, sterility and cytotoxicity [126]. This small-scale pilot study may set a foundation of standard in the Western countries for large-scale controlled clinical trials for phage therapy. Phage engineering can be employed to keep bacterial killing property but bypass lysis induced endotoxin release and related side effect [127]. More recently, human humoral immune response against phage therapy showed that anti-phage antibodies (Abs), including IgM, IgG and IgA, were detected in patient sera when staphylococcal MS-1 phage cocktail was used for treatment [128]. Interestingly, these anti-phage Abs did not compromise the final efficacy [128].

However, it seems that big pharmaceuticals are currently not interested in phage therapy, investment cost burden and patent filing may be another key considerations besides safety concern. To remove the worries from live virus-based therapy, phage-derived proteins (VAPGHs and endolysins) may become an option as these proteins also show the specificity and lytic efficiency against Gram-positive bacteria, albeit less efficient against Gram-negative bacteria due to the presence of OM cell wall.

Author contributions

DW conceived the topic of the study. All authors wrote the manuscript. LJ and AS contributed equally in writing the manuscript. DW revised the manuscript.

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