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New Insights into Inflammatory Bowel Disease

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Contributors

Takuya Inoue, Mohamed Oukka, Ahmet Eken, Jingmei Lin, Kurt Fisher, Hai-Lin Kurahara, Keizo Hiraishi, Kunihiko Aoyagi, Yaopeng Hu, Miho Sumiyoshi, Ryuji Inoue, Cathy Lu, Karen Kroeker, Sumant Arora, Talha Malik, Misbahul Arfin, Tsvetelina Velikova, Zoya Spasova, Ekaterina Ivanova-Todorova, Dobroslav Kyurkchiev, Iskra Altankova, Spaska Stanilova, Chafia Touil-Boukoffa, Ryma Toumi, Imene Soufli, Hayet Rafa, Neville Azzopardi, Valerie Fenech, John Schembri, Pierre Ellul, Godfrey Grech, Silvia Melgar, Mònica Aguilera, Fernando De La Portilla, Ana M García-Cabrera, Rosa M Jiménez-Rodríguez, Maria L Reyes, Damian García-Olmo

Notice

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Meet the editor

Dr. Samuel Huber's background is in the field of gastroenterology/immunobiology. He graduated in April 2006 and obtained the Board Certification for Medicine. Dr. Huber did his residency in the Department of Gastroenterology, University Medical Center Hamburg-Eppendorf, Germany (chairman, Prof. A.W. Lohse). From 2008 to 2012, Dr. Huber did his postdoctoral training at Richard A. Flavell's lab in the Department of Immunobiology, School of Medicine, Yale University, USA. Currently, he is leading the Section of Molecular Gastroenterology and Immunology at the I. Medical Department, University Medical Center Hamburg-Eppendorf, Germany. The main focus of the lab is to study the mechanism controlling the homeostasis in the intestine in order to identify new therapeutic targets for the treatment of inflammatory bowel disease and colorectal cancer.

Contents

Preface

The incidence of chronic inflammatory and autoimmune diseases is steadily increasing. Inflammatory bowel disease (IBD) is one example of a chronic inflammatory disease, which primarily affects the intestine but may also affect extraintestinal organs. The exact pathogenesis of IBD is currently unknown. However, it is clear that the pathogenesis is complex, involving barrier defects, changes in the intestinal microbiome, and chronic immune dysregulation. Of note, there is currently no cure for IBD. However, several therapies have evolved in the last years, which are overall able to—at least temporarily—suppress IBD.

Reported here are the current opinions on basic aspects of IBD: genetic mutations and environmental factors. Furthermore, complex interactions between barrier function, microbiome, and the immune system are discussed.

This book integrates knowledge about the underlying mechanism of IBD and how these might be target in future therapeutic approaches.

The search for effective IBD therapies with fewer side effects has been intensified in recent years. The present volume aims to introduce some of these therapeutic agents and the mechanism of action.

I would herewith like to thank the publishing team and all the authors for their contribution.

I am sure the chapters of this book will be of interest to many medical researchers and clinicians in the field.

Prof. Dr. med. Samuel Huber
Research group leader, I. Medical Department,
University Medical Center
Hamburg-Eppendorf
Hamburg, Germany

Section 1

Epidemiology and Genetics of Inflammatory Bowel Disease

Chapter 1

Inflammatory Bowel Disease: Epidemiology

Sumant S. Arora and Talha A. Malik

Additional information is available at the end of the chapter

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Abstract

Inflammatory bowel disease (IBD) is characterized by two partially distinct alimentary disease processes, namely Crohn's disease (CD) and ulcerative colitis (UC), affecting genetically predisposed individuals. CD and UC were first described in 1932 and 1859, respectively. It is estimated that 1.5 million in North America and 2.5 million persons in Europe have IBD. The peak incidence of CD and UC is between 20–30 years and 30–40 years of age, respectively. Both incidence and prevalence of CD and UC are similar across males and females. However, several studies suggest a female predominance in CD and a male predominance in UC. The pathogenesis of IBD is attributed to an uncontrolled immune-mediated inflammatory response to an unrecognized environmental trigger that interacts with the intestinal flora. Various determinants of IBD include the following: peculiar environmental triggers, intestinal immune mechanisms, heritable factors, gut flora, diet, mesenteric fat, medications, nicotine, infectious agents, immunization, hygiene, pregnancy, breastfeeding, stress and lifestyle. Predominant complications in IBD are surgery, malnutrition, disease exacerbations and cancer. Patients with CD have a higher mortality compared to general population. Epidemiological studies continue to expand our understanding of the distribution, determinants and mechanisms of IBD. This has enabled us to recognize safer and effective approaches to management.

Keywords: inflammatory bowel disease, Crohn's disease, ulcerative colitis, epidemiology, incidence, prevalence

1. Introduction

Inflammatory bowel disease (IBD) is an idiopathic chronic inflammatory disorder of the alimentary tract that encompasses two major closely related yet heterogeneously distinct disease entities—Crohn's disease (CD) and ulcerative colitis (UC). IBD is characterized by

chronic or relapsing uncontrolled immune activation and inflammation in genetically predisposed individuals to a yet unknown environmental trigger that interacts with the gut flora and primarily affects the digestive tract [1–7]. Historically, Dr. Burrill Crohn, Dr. Leon Ginzburg and Dr. Gordon Oppenheimer first described CD in 1932 as regional or terminal ileitis—inflammation of terminal ileum [8–10]. In 1859, Dr. Samuel Wilks recognized UC as a discrete entity, but it was Sir Arthur Hurst, who described its endoscopic pattern and distinguished it from the more common bacillary dysentery [8, 9, 11]. Pathologically, CD usually consists of transmural inflammation (all layers from mucosa to serosa) and may discontinuously involve any part of the alimentary tract from mouth to anus, whereas UC is characterized by submucosal inflammation limited to the colon [6]. Approximately, one and a half million residents in the USA and two and a half million in Europe have IBD, with about half represented in each of the two discrete IBD subgroups [2, 12].

2. Incidence, prevalence and distribution

Though now recognized worldwide, traditionally IBD was considered a condition that primarily affected Caucasians across Europe, North America and Australia [1]. Hence, most of the available epidemiologic data on CD and UC have been derived from population-based studies conducted in these geographic regions [1]. The incidence and prevalence of CD and UC have stabilized in the aforementioned regions; however, it is still higher than in the rest of the world [1]. Further, the incidence and prevalence of IBD, predominantly CD, have increased in the developing world particularly in the Middle East, Southeast Asia and the Asia Pacific Region [7, 12, 13]. Meanwhile, South America and Africa have significantly low incidence and prevalence rates, albeit anecdotal reports have hinted an increase in incidence [14, 15].

Even in the West, IBD has become increasingly recognized among minority populations [1]. The most significant rise in incidence has occurred in second-generation immigrants from low-risk geographic regions to Western countries, that is, high-risk regions. This supports the concept of an equal if not higher contribution from environmental influences compared to genetic predisposition [1, 16]. Also, has been noted a higher incidence of IBD among immigrants and their families who migrated from socioeconomically backward regions [1]. Moreover, compared to minorities in the West, recent immigrants tend to have a milder disease course [1].

Globally, there remains a paucity of accurate epidemiologic data due to clinical overlap of the IBD entities with conditions such as infectious colitis and differences in the health care systems precluding reliable case estimation. The recognized IBD cases may further only represent a fraction of the actual disease burden due to diagnosis requiring invasive and expensive modalities. Moreover, at times, CD cannot be clearly distinguished from UC, especially early in the disease course before distinctive characteristics have manifested, often requiring reassignment of the IBD subgroup diagnosis [17]. Despite the aforementioned limitations, the incidence and prevalence of both CD and UC have demonstrated a distribution trend. The

incidence and prevalence data vary across the globe depending upon geographic region, environment, immigration trends, ethnicity [1–3] and even differ within the same geographic region. Moreover, a north-south distribution gradient has been observed for IBD risk across the world [18]. This has been attributed to regional differences in sunlight and vitamin D exposure with high levels of exposure inversely correlated with risk of IBD [19, 20].

The annual incidence rates of CD are comparable across most of the developed world. It is estimated to be 20.2 per 100,000 person-years, 12.7 per 100,000 person-years, 29.3 per 100,000 person-years and 16.5 per 100,000 person-years in North America, Europe, Australia and New Zealand, respectively [21–23]. In contrast, Asia has a low incidence rate of approximately 0.54 per 100,000 person-years [24]. Similarly, the incidence rates for UC in North America, Europe and Asia range from 7.6 to 19.5 per 100,000 person-years, 1.7 to 13.6 per 100,000 person-years and 0.3 to 5.8 per 100,000 person-years, respectively [4]. In the past, UC was considered to be slightly more prevalent; however, an increased incidence of CD in the past few decades has resulted in a trend reversal. Most recent estimates of prevalence of CD in North America are 25–300 per 100,000 person-years and that for UC are 170–250 and 43–294 per 100,000 person-years, respectively, in North America and Europe [21, 25, 26]. Overall, both the incidence and prevalence of CD and UC are increasing with time. This can be attributed to a number of factors including improved sanitation, diet and medication exposures, increased IBD awareness among patients and clinicians, use of improved endoscopic and radiologic diagnostic modalities and widened health care access [21, 27].

2.1. Age and gender disparity

Although IBD can occur at any age, the peak age of onset for CD and UC is generally between 20–30 years and 30–40 years of age, respectively [1, 4, 6, 21]. However, some European cohorts have suggested a second peak between 60–70 years of age, especially for UC. The most plausible explanation for this additional peak is ascertainment bias due to increased health care access and more frequent evaluation of older patients. Majority of North American population-based study has shown that the median and mean age of diagnosis of CD and UC range between 30–45 years and 40–45 years, respectively [28, 29]. Additionally, these studies especially in adults have suggested a female predominance in CD and male predominance in UC [1, 30]. This gender-based disparity may be attributed to hormonal or life-style factors. However, the variation is inconsistent, particularly in low IBD incidence regions, where CD may be more prevalent among men [25, 31]. Men tend to be diagnosed with IBD, especially UC at a later age than their female counterparts [6]. On the other hand, in the pediatric population, the trend in gender distribution is reversed with more boys having CD than girls [32].

2.2. Racial and ethnic disparity

There appears to be a marked ethnic and racial variation in the incidence of IBD. Early studies from the 1960s reported a lower incidence of IBD, specifically UC among African-Americans [33]. However, these studies were conducted in regions with predominant white populations, and more recent studies from 1990s have challenged these findings with comparable incidence

rates among Whites and non-Whites [34, 35]. Further, CD was proposed to be more aggressive with earlier age of onset in African-Americans. A recent systematic review, however, suggested that the variance in IBD severity extrapolates from socioeconomic inequalities such as health care affordability and accessibility, rather than inherent biologic or genetic dissimilarities [36, 37]. Ethnically, the Jews in particular are vulnerable to develop IBD, with incidence rates being several fold higher than in the general population across the globe. Further, IBD is more common among the Ashkenazi Jews than the Sephardic Jews in the Middle East, but this trend is reversed in the United States and northern Europe, indicating influence of environmental factors [38].

3. Pathogenesis and risk factors

Pathogenically, IBD is believed to be due to uncontrolled immune activation and inflammation of the alimentary tract in genetically predisposed individuals. It is triggered by the interaction of an unknown environmental agent with the autoantigens believed to reside on nonpathogenic commensal bacteria of the intestinal microbiota (**Figure 1**) [7]. The primary mechanism of inflammatory insult in IBD is immune mediated. Intestinal epithelial cells in active IBD express HLA class II molecules that activate macrophages to secrete pro-inflammatory cytokines (IL-1, IL-6, IL-8, and TNF-α) and suppress the downregulatory cytokines (IL-2, IL-10, and TGF-β) in the lamina propria, thereby fostering chronic inflammation [5, 7, 12].

Figure 1. Factors implicated in the etiopathogenesis of IBD.

Various environmental triggers have been attributed to IBD causation. They include external antigens such as infectious pathogens (bacteria and viruses), dietary agents and autoantigens residing on the microbial gut flora [1, 6, 12]. In addition, both CD and UC tend to have genetic predisposition in about 15% cases. In regard to first-degree relative for CD and UC, the lifetime risk of developing IBD is approximately 5 and 2% among non-Jewish populations and 8 and 5% among Jewish populations, respectively [39]. The genetic predisposition is stronger for CD

than UC based on higher concordance rates (50 and 10% vs. 16 and 4%) among monozygotic and dizygotic twins, respectively [40, 41].

Dietary factors more pronounced in typical western diet have been implicated in the pathogenesis of IBD. Comprehensive review of studies involving patients with CD has suggested possible association between increased consumption of refined sugars and animal meat and risk of development of IBD [42, 43]. The aforementioned dietary components are believed to interact with intestinal flora and produce pro-inflammatory agents [44]. Individuals who consume less dietary fiber, raw fruits and vegetables tend to have higher predilection for IBD [44]. Meanwhile, molecular studies have linked adipose tissue to intestinal inflammation [45, 46]. However, it remains unclear if this translates into a causal or clinically meaningful association between obesity and CD. Regardless, obese patients with CD tend to have a rapid disease progression compared to their underweight counterparts [47, 48]. Moreover, sedentary lifestyle is associated with overall higher IBD incidence [49].

Among environmental factors, smoking has a pivotal role in IBD with divergent effects in UC and CD [1]. Both current as well as former smoking, including exposure to passive smoking during childhood, is associated with twofold increase in the risk of CD [50, 51]. Smokers with CD tend to have an earlier age of onset, more aggressive (stricturing or penetrating) disease phenotype, heightened need for steroids and immunosuppressants and overall more surgical interventions as well as higher risk of postresection recurrence [52, 53]. In contrast to CD, smoking safeguards against UC and even indeterminate colitis, with an estimated 50% risk reduction in current smokers. However, this protective effect is less pronounced in females. Further, smokers with UC tend to have milder disease course, with less frequent proximal extension of disease and decreased need for immunosuppression and surgery [53, 54].

The precise mechanisms driving these contrasting effects of smoking on the two IBD subtypes remain unclear. It is hypothesized that smoking causes polymorphisms in genes regulating nicotine metabolism and decreases heat shock protein-70 resulting in reduced protection against cellular oxidative stress, which in turn impairs endothelial function in the intestinal mucosal barrier and promotes inflammation [55–58]. On the other hand, it is proposed that smoking alters the gut flora to reduce predisposition to UC [59].

Recent studies have suggested that infectious agents, such as *Salmonella* and *Campylobacter*, impart heightened risk for IBD development [60], while *Clostridium difficile* and cytomegalovirus have been linked with IBD exacerbations [61, 62]. However, no definite causal association has been identified.

Meanwhile, poor hygienic conditions, including large family size, lack of access to running water, consumption of unpasteurized milk, early exposure to farm animals and pets, have been suggested to protect against IBD development [1, 30, 63–65]. However, these associations are derived from studies conducted in the West and they failed to be replicated in the developing world [1, 66]. On the other hand, there is no definite association between immunization and risk of IBD. Early studies have linked attenuated live measles virus vaccine with IBD occurrence; however, recent studies support the contrary thereby suggesting a protective role [67].

Several pharmacologic agents have also been implicated as potential risk factors for IBD. They include NSAIDs, oral contraceptives, hormonal replacement therapy and antibiotics [68–73]. On the contrary, studies suggesting role of nutritional factors such as vitamin D in IBD development remain equivocal [1].

With regards to pregnancy, there is no definite association between the mode of childbirth (caesarian vs. vaginal delivery) and risk of IBD [74]. However, breastfeeding may play a protective role against IBD development later in life [1]. Meanwhile, depression and anxiety have not only been linked to higher risk of development of IBD but also to increased disease severity, need for surgical intervention, reduced quality of life and diminished response to immunosuppresants [75].

4. Classification

The heterogeneity of demographic, anatomic and disease behavior characteristics in IBD warranted a systematic grouping scheme to place its various phenotypes into simple categories. The first attempt was made by the Working Party of the World Congress of Gastroenterology that met in Vienna in 1998. Their report known as the "Vienna Classification" was published in the Journal of Inflammatory Bowel Diseases in 2000. This classification attempted to stratify CD into 24 disease clusters based on age at diagnosis, disease location and disease behavior (**Table 1**) [76]. Subsequently, the Vienna classification was critiqued owing to lack of universal clinical applicability [77].

	Vienna classification	**Montreal classification**
Age at diagnosis	A1: Below 40 years	A1 Below 16 years
	A2: Above 40 years	A2 Between 17 and 40 years
		A3 Above 40 years
Location	L1 Ileal	L1 Ileal
	L2 Colonic	L2 Colonic
	L3 Ileocolonic	L3 Ileocolonic
	L4 Upper	L4 Upper disease modifier or isolated upper disease
Behavior	B1 Nonstricturing, nonpenetrating	B1 Nonstricturing, Nonpenetrating
	B2 Stricturing	B2 Stricturing
	B3 Penetrating	B3 Penetrating
		p Perianal disease modifier

Table 1. Vienna and Montreal classification of Crohn's disease.

The Working Party of the Montreal World Congress of Gastroenterology then met in 2005 and put forth the Montreal classification of IBD (**Tables 1** and **2**) [78]. This new scheme grouped

CD primarily based on the same variables proposed by the experts at Vienna including patient's age at diagnosis (A1, 16 years and younger; A2, 17–40 years; A3, >40 years), disease location (L1, ileal: L2, colonic; L3, ileocolonic) and disease behavior (B1, nonstricturing, nonpenetrating; B2, stricturing; B3, penetrating). In addition, it introduced modifiers for upper tract disease location (L4) and for perianal disease (*p*). Further, it extended the classification to stratify UC based on the extent and severity of the disease (**Table 2**) [78].

Class	Extent	Description
E1	Ulcerative proctitis	Proximal extent of inflammation distal to rectosigmoid junction
E2	Left-sided UC (distal UC)	Involvement limited to proportion of colorectum distal to the splenic
E3	Extensive UC (pancolitis)	Involvement extending proximal to splenic flexure

Table 2. Montreal classification of ulcerative colitis.

5. Disease course

Based on phenotype by location, of all patients with CD at the time of diagnosis, one-third of patients have ileal involvement, one-third of patients have colonic involvement and the rest have ileocolonic disease. While with regard to disease behavior, 80% of all patients with CD at the time of diagnosis have nonpenetrating/nonstricturing disease with the remaining 20% having stricturing or penetrating disease [79]. As CD evolves, of all with nonpenetrating/nonstricturing disease, up to one-third of patients progress to penetrating or stricturing complications at 5 years and about half at 20 years from diagnosis [79]. Further, in terms of disease activity, based on data from prebiologic era, about two-thirds of patients with CD tend to have a remitting and risk of CD relapsing course one-fifth remain active and about 13% enter long-term remission [80].

Meanwhile, for UC at the time of diagnosis, one-third of patients tend to have colonic involvement distal to rectosigmoid junction, one-third up to splenic flexure, while the remaining third have pancolitis, that is, contiguous involvement extending proximal to the splenic flexure [2]. The disease behavior is variable; 50% of UC patients with proctitis/proctosigmoiditis progress to extensive disease at 25 years [81]. While in regard to disease activity, based on data from prebiologic era, 57% of patients with UC tend to have a remitting and relapsing course, one quarter go into long-term remission, and about one-fifth remain active [39, 82].

6. IBD and morbidity

The key factors driving morbidity overlap between the two IBD subgroups—CD and UC. The predominant causes of morbidity in patients with CD are need for surgery, malnutrition followed by disease exacerbations and cancer [2, 4]. While among patients with UC, the major

burden of morbidity is due to the development of cancer followed by requirement for surgery and disease exacerbations [2, 4]. Overall, surgery remains the most common cause of morbidity in CD and a significant cause of morbidity in UC. Recently, the cumulative risk of IBD, particularly patients with CD requiring surgery has significantly decreased with rates of surgery being approximately 10–14% and 18–35% after 1 and 5 years, respectively [83–88]. This is attributed to adoption of more aggressive medical therapy in recent times [1, 2, 83, 84, 89, 90]. Based on age, location and behavior of CD, the greatest need for surgery is with ileocecal location and stricturing or penetrating/fistulizing disease phenotype [2, 86, 87]. Similarly, in UC, the likelihood of need for colectomy has decreased recently with estimated rates of 6 and 10% after 1 and 5 years, respectively [83, 91–93]. The highest probability of colectomy is in those with relatively recent diagnosis and severe disease especially pancolitis [2].

An interesting association has been observed between appendectomy and IBD [1]. While appendectomy is found to protect against future occurrence of UC, it may lead to an increased incidence of CD [94–96].

With regard to cancer as one of the drivers of morbidity, the overall risk of colorectal cancer is significantly higher in patients with IBD compared to the general population. The primary factors influencing this risk include persistent active inflammation, immunosuppression, long-standing disease, extensive disease, young age at diagnosis, family history of colorectal cancer and coexisting primary sclerosing cholangitis [2, 97]. Overall, patients with IBD have heightened risk of extraintestinal cancers such as lymphoproliferative and skin cancers [2, 98–100].

7. IBD and mortality

Whether or not having IBD confers a higher mortality remains debated. Population-based studies from 1980s to 1990s suggested a moderate increase in mortality rate in CD [101, 102]. However, recent European studies have failed to replicate these findings and indicate a comparable mortality rate in CD to the general population [103–105]. Major causes of mortality in CD include direct, such as surgical complications and malnourishment, and indirect related to smoking [101, 106–107].

Similarly, there is lack of definitive evidence to support higher mortality rate in patients with UC [105, 107–110]. However, unlike CD, most deaths in UC are due to colorectal cancer than from surgical or other complications [106, 109].

8. Conclusion

In conclusion, IBD is a condition with a unique etiopathogenesis and significant epidemiologic burden. To the present day, epidemiological studies continue to expand our understanding of the distribution, determinants and mechanisms of IBD. This has enabled us to recognize safer and more effective approaches to management and therapeutics outside of mere immunosuppression for IBD with emphasis on prevention, preemption and immunomodulation.

Author details

Sumant S. Arora[1*] and Talha A. Malik[2]

*Address all correspondence to: sumantarora@uabmc.edu

1 Department of Medicine, University of Alabama at Birmingham, Montgomery Residency Program, Montgomery, Alabama, USA

2 Department of Medicine-Gastroenterology and Department of Epidemiology, University of Alabama at Birmingham, Birmingham, Alabama, USA

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Chapter 2

Inflammatory Bowel Disease: The Association of Inflammatory Cytokine Gene Polymorphisms

Abdulrahman Al-Robayan, Misbahul Arfin, Ebtissam Saleh Al-Meghaiseeb, Reem Al-Amro and Abdulrahman K Al-Asmari

Additional information is available at the end of the chapter

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Abstract

The frequencies of alleles and genotypes of *TNF-α*, *TNF-β*, and *IL-10* genes were examined in Saudi subjects including IBD patients (UC and CD) and matched controls. Venous blood samples were collected from IBD patients and healthy control subjects, and genomic DNA was extracted using commercially available kit (Qiagen, CA, USA). In order to detect TNF-α (-308G/A), TNF-β (+252A/G), IL-10 (-1082G/A), (-819C/T), and (-592C/A) polymorphisms, the *TNF-α*, *TNF-β*, and *IL-10* genes were amplified using an amplification refractory mutation systems PCR methodology. Analysis of data showed that the frequencies of alleles and genotype of TNF-α (-308G/A), TNF-β (+252A/G), and IL-10 (-1082G/A), (-819C/T), and (-592C/A) polymorphisms differ between IBD patients and control subjects. Our study clearly indicated that the TNF-α (-308G/A), TNF-β (+252A/G), and IL-10 (-1082 G/A) polymorphisms are associated significantly with the risk of IBD susceptibility while other two, IL-10-819C/T and IL-10-592C/A, polymorphisms are not associated with IBD in Saudi population. However, well-designed epidemiological as well as genetic association studies with large sample size among different ethnicities should be performed in order to have better understanding of this relationship.

Keywords: tumor necrosis factor, interleukin-10, polymorphism, inflammatory bowel disease, Saudis, Crohn's disease, ulcerative colitis

1. Introduction

The inflammatory bowel diseases (IBDs), encompassing Crohn's disease (CD, OMIM 266600) and ulcerative colitis (UC, OMIM 191390), are chronic inflammatory disorders of the gastrointestinal tract. The incidence and prevalence of IBD have been increasing with time in

different regions around the world, indicating its emergence as a global disease [1–5]. Available literature indicates that IBD is a complex and multifactorial disease though the exact etiology is still not clear. However, it has been suggested that immune dysregulation caused by genetic and/or environmental factors plays an important role in the etiology of IBD [6–8]. IBD appears to be caused by overly aggressive T-cell responses directed against environmental factors and/or a subset of commensal bacteria/pathogens that inhabit the distal ileum and colon of genetically susceptible hosts. Patients with long-lasting IBD, both UC and CD, have been at increased risk of developing colorectal cancer, and CD patients are at increased risk of small intestine cancer [9].

The incidence of IBD is higher in North American and European populations compared with those in Asian and African, reflecting the role of both environmental and genetic factors. The rising prevalence of various autoimmune and inflammatory conditions in developed countries has been attributed to hygiene hypothesis, and they are thought to result from the lack of early exposure to select microbial agents due to stringent sanitation conditions [10]. The changes in dietary and intestinal microbial milieu have been suggested to play a key pathogenic role in the etiology of IBD, though the exact environmental factors responsible for changing IBD prevalence are not clearly defined [11]. Intriguingly, the characteristics of Western and Asian IBD patients differ in epidemiology, phenotype, and genetic susceptibility [12–15] highlighting ethnic variations. Various epidemiological and population-based studies have indicated that genetic factors contribute to the pathogenesis of IBD [16–18].

According to Jump and Levine [19], cytokines act as key signal in the intestinal immune system and participate in the disruption of the physiological inflammation of the gut. They are produced mainly by immune cells as small peptide proteins and facilitate communication between cells, by stimulating the proliferation of antigen-specific effector cells, and mediate the local and systemic inflammation in an autocrine, paracrine, and endocrine pathways [20]. A critical role is played by innate immune system in IBD pathology, and several cytokines secreted by activated dendritic cells (DC) and macrophages actively regulate the inflammatory response in IBD.

The production of cytokines can be affected by genetic polymorphisms within the coding and promoter regions of cytokine genes [21, 22]. Therefore, a genetic predisposition for the high or low production of a particular cytokine may affect disease susceptibility and clinical outcome [23, 24].

The IBD is believed to be caused by immunogenic responses against environmental factors and/or microbes inhabiting distal ileum and colon of genetically susceptible hosts. Inflammatory response in IBD is an important feature and proinflammatory cytokine; tumor necrosis factor-alpha (TNF) has been indicated to play a key role in the initiation and propagation of IBD. Increased expressions of TNF-α have been reported in peripheral phagocytes and intestinal tissues of IBD patients. High levels of TNF-α have also been documented in the serum of IBD patients [25–27]. Moreover, monoclonal antibodies against TNF-α have been effectively used to decrease inflammation in IBD [28]. Variations in levels/expression of TNF due to its genetic polymorphism have been linked with pathogenic role of this cytokine in various autoimmune and inflammatory diseases and thus have been regarded to be an appropriate target for management of diseases by interfering with the inflammatory responses.

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In view of the important immunoregulatory roles of TNF-α and TNF-β, they are considered as subject of interest for studies in IBD. TNF-α is produced mainly by monocytes and activated macrophages while TNF-β is produced mainly by activated T cells. Both *TNF-α* (OMIM 191160) and *TNF-β* (MIM153440) genes are located on chromosome 6 within the MHC III region and show close linkage to the HLA class I (*HLA-B*) and class II (*HLA-DR*) genes. It has been shown by various studies on monozygotic twins and first-degree relatives that 60% of variation in the production capacity of TNF-α is genetically determined [29]. A number of polymorphisms within the promoter region of *TNF-α* and the intron 1 polymorphism of *TNF-β,* in particular have been associated with variations in the serum levels of TNF-α [30, 31] One of the best described single nucleotide polymorphisms (SNPs) is located at nucleotide position -308 within the *TNF-α* promoter region (rs1800629) and affects a consensus sequence for a binding site of transcription factor AP-2 [32]. TNF-α (-308) promoter polymorphism leads to a less common allele-A (allele 2), which has been associated with increased TNF-α production in vitro [33, 34] and higher rate of TNF-α transcription than wild-type GG genotype [35, 36]. This polymorphism has been linked to increased susceptibility to several chronic metabolic degenerative, inflammatory and autoimmune diseases [37–41].

Of interest, G/A polymorphism at nucleotide position -308 within the human TNF-α promoter region is associated with elevated TNF levels, disease susceptibility, and poor prognosis in several diseases [42–45]. Adenine at position -308 makes the TNF-α promoter a much more powerful transcription activator than guanine [42].

TNF-β +252A/G (rs909253) polymorphism affects a phorbol ester-responsive element. The presence of G at +252 position refers to the less frequent mutant allele known as TNF-β * 1 (allele-1), which is associated with higher TNF-α and TNF-β production [42, 46].

TNF-β resembles to TNF-α in terms of several biological activities including apoptosis and gives rise to a similar proinflammatory response and has been shown to play a critical role in pathogenesis of many diseases. TNF-β has also been shown to contribute to the susceptibility of several inflammatory/autoimmune diseases. Association of TNF-β +252 A/G polymorphism has been reported with various autoimmune disorders including Gravis' disease [47] idiopathic membranous glomerulonephritis, IgA nephropathy, insulin-dependent diabetes mellitus [48], myasthenia gravis [49], asthma diathesis [50], SLE with nephritis [51], systemic sclerosis [52], plaque psoriasis [53], rheumatoid arthritis [54], and type 1 diabetes [55]. Recently, TNF-β +252 A/G polymorphism is reported to be associated with both susceptibility to and mortality from sepsis [56].

A few studies have been undertaken to determine the association of TNF-α polymorphisms and IBD in different parts of the world [57–59]. The results of these studies on association of TNF-α polymorphism with IBD are not consistent, and variations have been reported [60]. These variations might be due to genetic differences in populations or systemic variations in the ancestry of IBD patients and control subjects involved in the studies [27]. Moreover, differences have been found in the characteristics, epidemiology, phenotype, and genetic susceptibility to IBD in Western and Asian populations [15, 16]. Therefore, studies involving these unique features in different ethnic populations will help not only identifying the pathophysiology but also understanding the etiology of IBD.

No research has been done on the association between TNF-β polymorphism and IBD. TNF-α and TNF-β are closely related cytokines, and both are involved in the expression of TNF-α and in a suggested mechanism for autoimmune/inflammatory diseases; therefore, the joint analysis of polymorphisms in *TNF-α* and *TNF-β* genes will provide further insight into the pathogenesis of IBD and help in developing effective therapeutic agents. Saudi population is ideal for such genetic association studies because of the fact that it is a closed and isolated society with quite high rate of consanguinity. So, we studied and evaluated the possible association of alleles and genotypes of TNF-α (-308G/A) and TNF-β (+252A/G) polymorphisms with the susceptibility risk to IBD in this population.

On the other hand, interleukin-10 (IL-10) is an anti-inflammatory cytokine and can inhibit the synthesis of proinflammatory cytokines, such as interferon-γ, IL-2, IL-3, and tumor necrosis factor-α (TNF-α), produced by macrophages and regulatory T cells [61]. IL-10 is responsible for various functions. It shifts the Th1/Th2 balance by downregulating the Th1 responses and by suppression of proinflammatory cytokines [62]. Several studies have shown that serum IL-10 levels are significantly lower in IBD patients than in normal controls, suggesting that altered IL-10 levels may be involved in the pathogenesis of IBD and may be an IBD biomarker. IL-10 is capable of depressing the activated immune system. It has been reported that IL-10 knockout mice develop colitis when they are kept in unsterile environment [63], and the inflammation is reduced after administration of IL-10 in vivo and in vitro models [64]. Moreover, the production of IL-10 has been found to be impaired in severe cases of IBD [65, 66].

IL-10 suppresses CD4+ T helper, Th1, clones (which secrete IL-2, interferon-γ, and TNF-α) and promotes the immunomodulatory T helper, Th2, clones (which secrete IL-4, IL-10, and IL-13). The secretion of cytokines is responsible for regulating the balance between Th1 and Th2 cells which is critical for immunoregulation. In case of reduced capacity of T cells to produce IL-10 in response to a stimulus, Th1 responses continue with the breakdown of peripheral tolerance and are potential to develop autoimmunity [67].

IL-10 is a multifunctional cytokine mainly produced by immune cells, such as T cells, monocytes, appropriately stimulated macrophages, some subsets of dendritic cells (DCs), and B cells [68]. Non-immune cell sources of IL-10 also exist, including keratinocytes, epithelial cells, and some tumor cells [69, 70]. The human *IL-10* gene is located on chromosome 1q32.1 and contains five exons. Recently, IL-10 has been identified as an important player in the development of immunological and inflammatory responses involving in the pathogenesis of various diseases including IBD [71–73].

Several single nucleotide polymorphisms (SNPs) have been reported in the proximal and distal regions of the *IL-10* gene, out of which three promoter polymorphisms (rs18000896-1082A/G, rs1800871-819T/C, and rs1800872-592A/C) are involved in IL-10 transcription rate and directly affect its production level and expression [74–77]. The -1082G, -819C, and -592C (GCC) alleles have been associated with elevated levels of IL-10 production [78], while ACC and ATA haplotypes show intermediate and low *IL-10* gene transcription, respectively [79]. These IL-10 gene polymorphisms are reported to be associated with susceptibility/development to various inflammatory disorders [40, 80–82]. However, data are limited and inconsistent and therefore do not allow drawing unequivocal conclusions.

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Studies on the *IL-10* promoter polymorphisms and IBD susceptibility have also been inconsistent [71, 83–89]. Some studies have found an associations between *IL-10* polymorphism and IBD [71, 86, 87, 90], whereas other studies were unable to find any association between IBD and the *IL-10* promoter polymorphisms [83, 84, 88, 91]. In this study, we evaluated the association of five polymorphism in *IL-10, TNF-α,* and *TNF-β* genes with susceptibility risk of IBD in Saudi patients.

2. Methods

2.1. Subjects

Study groups consisted of 379 Saudi subjects including 179 IBD patients and 200 age- and sex-matched healthy controls visiting Gastroenterology Clinic of Prince Sultan Military Medical City (PSMMC), Riyadh. IBD patients included 20 cases of familial forms and 159 cases of sporadic forms. Of these patients, 95 were diagnosed to suffer with CD (57 men, 38 women) aged 17–65 years (mean age 32 years), while 84 patients with UC (34 men, 50 women) aged 22–68 years (mean age 34 years). Control group consisted of 120 men and 80 women matched for age and ethnicity (Saudi). Control subjects were screened for any history of IBD, diabetes, rheumatoid arthritis, systemic lupus erythematosus, or other autoimmune/inflammatory diseases and excluded if found positive. The diagnoses of CD and UC were based on conventional endoscopic, radiological, and histological criteria [92]. Demographic and clinical data were collected and used for exclusion and inclusion as described elsewhere [93]. This study was approved by the research and ethical committee of PSMMC, and written informed consent was obtained from all subjects to participate in this study.

2.2. Polymerase chain reaction (PCR) amplification

Venus blood (3 ml) was collected from all the participants, and genomic DNA was extracted using a commercially available kit (Qiagen, CA, USA). To detect polymorphisms at position -308 and intron 1 +252 of the TNF-α and TNF-β genes, respectively, and at position -592, -819 and -1082 of IL-10 gene, the amplification of TNF-α, TNF-β, and IL-10 genes was performed using an amplification refractory mutation systems PCR methodology described elsewhere [39, 93]. PCR amplification was carried out in PuReTaq Ready-to-Go PCR Beads (GE Healthcare, Buckinghamshire, UK) as described earlier [82]. The allele and genotype frequencies of all 5 polymorphisms were evaluated in IBD patients and controls. Hardy-Weinberg equilibrium was determined using Hardy-Weinberg Equilibrium Calculator for 2 Alleles. (http//www.had2know.com/academics/hardy-weinberg-equilibrium calculator-2alleles.html)

2.3. Statistical analysis

The difference between the frequency distribution of various alleles and genotypes in patients and controls was analyzed by Fisher's exact test using the CalcFisher software (http://www.jstatsoft.org/v08/i21/paper), and the *P*-values ≤0.05 were considered as significant. The odd ratio interpreted as *relative risk* (RR) indicated the strength of the association of disease with

respect to a particular allele/genotype and was calculated according to the method of Woolf as outlined by Schallreuter et al. [94]. The RR was calculated using the following formula only for those alleles and genotype, which were increased or decreased in IBD patients as compared to normal Saudis.

$$RR = \frac{a \times d}{b \times c} \tag{1}$$

where "a" indicates number of patients expressing the allele or genotype, "b" number of patients without allele or genotype expression, "c" number of controls expressing the allele or genotype, and "d" number of controls without allele or genotype expression.

The etiologic fraction (EF) is the hypothetical genetic component of the disease. Values >0.00–0.99 are significant. EF is calculated for positive associations where value of RR is >1 using the following formula [95]:

$$EF = \frac{(RR - 1)f}{RR}, \text{where } f = \frac{a}{a + c} \tag{2}$$

Preventive fraction (PF) shows the hypothetical protective effect of one allele/genotype for a disease. PF is calculated for negative associations where RR is <1 using following formula [95]. Values >0.00–0.99 indicate the protective effect of an allele/genotype against the manifestation of disease.

$$PF = \frac{(1 - RR)f}{RR(1 - f) + f}, \text{where } f = \frac{a}{a + c} \tag{3}$$

3. Results

The representative gel pictures of amplification of different genotypes for TNF-α (-308G/A) and TNF-β (+252A/G) are shown in **Figures 1** and **2**.

Allelic frequencies and genotype distributions of TNF-α (-308G/A) and TNF-β (+252A/G) polymorphisms were different in patients and controls. The allele frequencies of both patients and

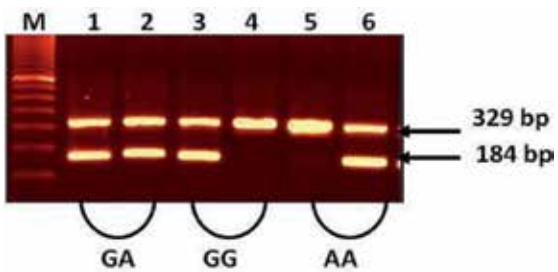

Figure 1. Shows the amplification of TNF-α (-308G/A) alleles (G and A). Lane M: 100-bp DNA marker, lanes 1 and 3: amplification of allele G, lanes 2 and 6: amplification of allele A, 184-bp band for target DNA, 329-bp band for internal control.

controls were in Hardy-Weinberg equilibrium. The frequencies of genotype GA and allele A were significantly higher, while those of genotypes (GG and AA) and allele A of TNF-α (-308G/A) were lower in IBD patients as compared to controls (**Table 1**). Allele A and genotype GA were susceptible to the IBD ($P < 0.001$), while allele G and genotype GG were protective against IBD ($P < 0.001$) in Saudi patients.

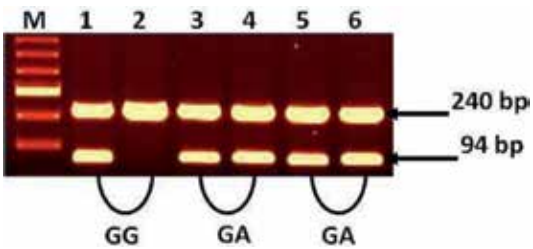

Figure 2. Shows the amplification of TNF-β (+252A/G) alleles (A and G). Lane M: 100-bp DNA marker, lanes 1, 3, and 5: amplification of allele G, lanes 4 and 6: amplification of allele A, 94-bp band for target DNA, 240-bp band for internal control.

Genotype/allele	IBD (n = 179)		Control (n = 200)		*P*-value	RR	EF*/PF
	n	%	n	%			
GG	5	2.79	110	55	0.00001♣	0.235	0.123
GA	173	96.65	76	38	0.0001♣	47.044	0.080*
AA	1	0.56	14	7	0.0009♣	0.075	0.449
G-allele	183	51.12	296	74	0.0001♣	0.367	0.397
A-allele	175	48.88	104	26	0.0001♣	2.722	0.396*

EF = Etiologic fraction, PF = preventive fraction.
♣Statistically significant.
*data for EF

Table 1. Genotype and allele frequencies of TNF-α (-308G/A) polymorphism in IBD patients and matched controls.

Because of the fact that two forms of IBD are characterized by different clinical pictures, it is reasonable to perform genetic association studies on homogenous group of patients; therefore, the genotyping results were stratified into UC and CD. However, similar association with TNF-α (-308G/A) polymorphism was noticed in the two groups. The genotype GA and allele A were significantly associated with CD and UC susceptibility in our population (**Table 2** and **Figure 1**). Allele A and genotype GA were susceptible to the UC and CD (*P*s < 0.01), while allele G and genotype GG were protective (*P*s < 0.01) in Saudi patients with UC and CD.

The association of TNF-α (-308G/A) polymorphism with UC, CD, or IBD in various ethnic populations worldwide has been summarized in **Table 3**. The association is not consistent, and

Genotype/allele	CD (95) n (%)	UC (84) n (%)	Control (200) n (%)
GG	3 (3.16)*	2 (2.38)*	110 (55)
GA	91 (95.79)*	82 (97.62)*	76 (38)
AA	1 (1.05)*	0 (0.0)*	14 (7)
G-allele	97 (51.05)*	86 (51.19)*	296 (74)
A-allele	93 (48.95)*	82 (48.81)*	104 (26)

*P value <0.05 compared to the frequency in controls.

Table 2. Genotype and allele frequencies of TNF-α (-308G/A) polymorphism in UC and CD patients.

ethnic variations are evident in the type or/and degree of association of TNF-α (-308G/A) polymorphism and IBD, UC, or CD susceptibility/severity or response to therapy.

On the other hand, studies on TNF-β gene polymorphism showed that the frequency of GG at position +252 of intron 1 was significantly higher in IBD as compared to controls, while the frequency of GA genotype was also higher in patient group but the difference was not statistically significant. The difference in the distribution of allele A and allele G was also not statistically significant in IBD and control groups albeit the frequency of mutant allele G is higher in IBD patients (**Table 4**).

The stratification of TNF-β gene polymorphism results for IBD patients into UC and CD showed that the distribution of genotypes GG and GA was different in UC as compared to controls indicating that the genotype GG is susceptible and GA protective only for UC but not for CD as almost similar distribution of genotypes and allele frequencies of TNF-β -intron 1 +252 polymorphism was found among the CD and controls (**Table 5** and **Figure 2**).

The frequency distribution of alleles and genotypes of both TNF-α and-β polymorphisms is not affected by gender or type of IBD (familial or sporadic) (**Tables 6** and **7**).

The representative gel pictures of amplification of different genotypes for IL-10 G (-1082)A, IL-10 C (-819)T, and IL-10 C (-592)A are shown in **Figures 3–5**.

The results of three promoter polymorphism of IL-10 gene are summarized in **Tables 8–12**. The genotype GG of IL10 (-1082) was significantly higher (P = 0.02) in IBD patients (15.08%) than control group (7.50%). Contrarily, the genotype AA was found to be significantly lower (P = 0.02) in IBD patients (9.50%) as compared to controls (17.50%). On the other hand, the heterozygous GA genotype was almost same in patients and controls (P = 0.99) (**Table 8**).

Upon stratification of genotyping results into CD and UC, we noticed that frequency of genotypes and alleles of IL-10 G (-1082)A differed significantly between CD patients and controls. Frequencies of genotype GG and allele G were higher in CD patients while those of genotype AA and allele A lower in CD patients as compared to controls. On the other hand, no significant different was found in the frequencies of alleles and genotypes between UC and controls (**Table 9**).

The frequency of -819 CC genotype was 33.52% in the IBD patients compared to 41.50% in controls, while CT was 38.12% in IBD patients as compared to 48.50% in controls. The frequency of homozygous TT genotype was similar in both IBD and control samples (10.61 vs. 10.00%). The frequencies of all genotypes of IL-10 (819C/T) polymorphism did not differ

Ethnicity/population	Type of association with IBD	Reference
American	UC susceptibility	[96]
*Asian	Associated with UC	[59]
*Asian	Associated with UC and CD	[58]
*Asian	Associated with UC susceptibility	[97]
Belgian	No association with CD treatment	[98]
Belgian	Associated with CD behavior	[99]
Brazilian	Associated with severity of CD	[60]
Canadian	No association with CD	[27]
Canadian	No association with IBD	[100]
*Caucasians	Better response to TNF blockers	[101]
Czech	Associated with IBD	[102]
Dutch	No association with IBD	[103]
English	IBD susceptibility	[104]
*European	No association with UC	[97]
*European	Associated with UC and CD	[58]
German	No association with IBD	[105]
Han Chinese	Association with UC susceptibility	[106]
Han Chinese	Association with UC susceptibility	[57]
Hungarian	IBD susceptibility	[107]
Indian	No association with IBD	[108]
Iranian	No association with IBD	[109]
Iranian	No association with IBD	[110]
Irish	No association with IBD	[83]
Israeli	No association with granulomas in CD	[111]
Italian	Associated with therapy	[112]
Japanese	UC susceptibility	[113]
Korean	Association with CD susceptibility	[114]
Korean	Association with CD susceptibility	[115]
Mexican-Mestizo	UC susceptibility	[116]
Portuguese	Pathological profiles of CD	[117]
Russian	UC susceptibility	[118]
Saudis	IBD susceptibility	[93]
Spanish	No association with CD	[114]
Spanish	No association with UC	[119]
Turkish	No association with IBD susceptibility	[120]
Turkish	Association with UC susceptibility	[121]

*meta–analysis

Table 3. Association of TNF-a 308G/A polymorphism in UC, CD, or IBD in various ethnic populations worldwide.

Genotype/allele	IBD (n = 179)		Control (n = 200)		*P*-value	RR	EF*/PF
	n	%	n	%			
GG	39	21.79	28	14	0.05♣	1.711	0.241*
GA	120	67.04	148	74	0.14	0.714	0.151
AA	20	11.17	24	12	0.87	0.922	0.037
G-allele	198	55.31	204	51	0.24	1.189	0.078*
A-allele	160	44.69	196	49	0.24	0.841	0.078

EF = Etiologic fraction, PF = preventive fraction.
♣Statistically significant.
*data for EF

Table 4. Genotype and allele frequencies of TNF-β (+252A/G) polymorphism in IBD patients and matched controls.

Genotype/allele	CD (n = 95) n (%)	UC (n = 84) n (%)	Control (n = 200) n (%)
GG	16 (16.84)	23 (27.38)*	28 (14)
GA	69 (72.63)	51 (60.72)*	148 (74)
AA	10 (10.53)	10 (11.90)	24 (12)
G-allele	101 (53.16)	97 (57.74)	204 (51)
A-allele	89 (46.84)	71 (42.26)	196 (49)

**P* value <0.05 compared to the frequency in controls.

Table 5. Genotype and allele frequencies of TNF-β (+252A/G) polymorphism in CD and UC patients.

Genotype/allele	Male (n = 89)		Female (n = 90)		*P*-value
	n	%	n	%	
GG	23	25.84	16	17.78	0.209
GA	57	64.05	63	70.00	0.429
AA	9	10.11	11	12.22	0.813
G-allele	103	57.87	95	52.78	0.340
A-allele	75	42.13	85	47.22	0.340

N = number of subjects.

Table 6. Genotype and allele frequencies of TNF-β (+252A/G) polymorphism in IBD male and female patients.

Genotype/allele	Familial (n = 22)		Sporadic (n = 157)		*P*-value
	n	%	n	%	
GG	4	18.18	35	22.29	0.780
GA	15	68.18	105	66.88	1.000
AA	3	13.64	17	10.83	0.717
G-allele	23	52.27	175	55.73	0.746
A-allele	21	47.73	139	44.27	0.746

Table 7. Genotypes and alleles of TNF-β (+252A/G) polymorphism in familial and sporadic IBD patients.

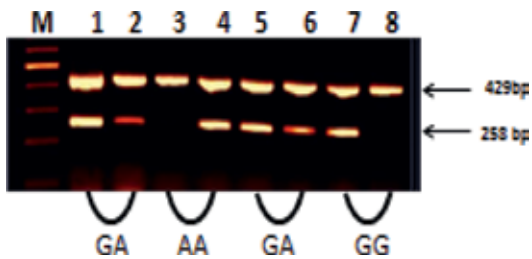

Figure 3. Shows the amplification of IL-10-1082G/A genotypes (GG, GA, and AA). Lane M: 100-bp DNA marker, lanes 1, 5, and 7: amplification of allele G, lanes 2,4, and 6: amplification of allele A, 258-bp band for target DNA, 429-bp band for internal control.

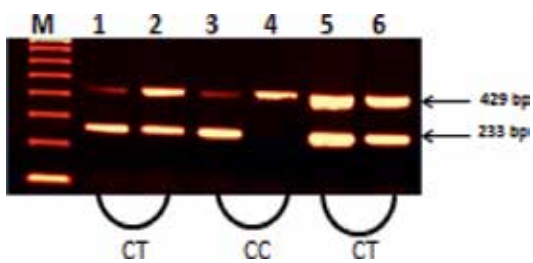

Figure 4. Shows the amplification of IL-10-819C/T genotypes (CT and CC). Lane M: 100-bp DNA marker, lanes 1, 3, and 5: amplification of allele C, lanes 2 and 6: amplification of allele T, 233-bp band for target DNA, 429-bp band for internal control.

significantly in patients and control groups. The allelic frequencies were also not different in patient and control groups (**Table 10**).

Upon stratification of subjects in to CD and UC, no significant difference was found in distribution of alleles and genotypes between patients and controls (**Table 11**).

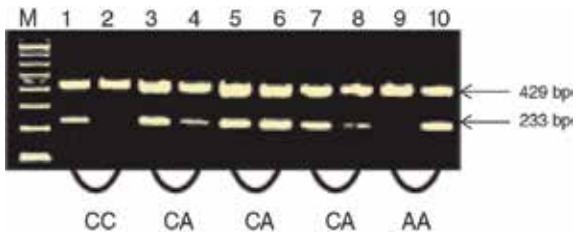

Figure 5. Shows the amplification of IL-10-592C/A genotypes (CC, CA, and AA). Lane M: 100-bp DNA marker, lanes 1, 3, 5, and 7: amplification of allele C, lanes 4, 6, 8, and 10: amplification of allele A, 233-bp band for target DNA, 429-bp band for internal control.

Genotype/allele	IBD (n = 179)		Control (n = 200)		*P*-value	RR	EF*/PF
	n	%	n	%			
GG	27	15.08	15	7.50	0.02♣	2.19	0.347*
GA	135	75.42	150	75.00	0.99	1.02	0.009*
AA	17	9.50	35	17.50	0.02♣	0.49	0.253
G-allele	189	52.79	180	45.00	0.03♣	1.36	0.135*
A-allele	169	47.21	220	55.00	0.03♣	0.73	0.138

EF = Etiologic fraction, PF = Preventive fraction.
♣Statistically significant.
*data for EF.

Table 8. Genotype and allele frequencies of (-1082G/A) IL-10 variants in IBD and matched controls.

Genotype/allele	CD (95) n (%)	UC (84) n (%)	Control (200) n (%)
GG	17 (17.90)*	10 (11.90)	15 (7.5)
GA	73 (76.84)	62 (73.81)	150 (75.0)
AA	5 (5.26)*	12 (14.29)	35 (17.50)
G-allele	107 (56.32)*	82 (48.81)	180 (45.0)
A-allele	83 (43.68)*	86 (51.19)	220 (55.0)

**P* value <0.05 compared to the frequency in controls.

Table 9. Genotype and allele frequencies of (-1082G/A) IL-10 variants polymorphism in UC and CD patients.

Similarly, the frequencies of alleles and genotypes of IL-10(-592C/A) polymorphism were not significantly different in IBD patient and controls (**Table 12**).

Upon stratification of subjects into CD and UC, no significant difference was found in distribution of IL-10(-592C/A) alleles and genotypes between patients and controls (**Table 13**)

Genotype/allele	IBD (n = 179)		Control (n = 200)		*P*-value	RR	EF*/PF
	n	%	n	%			
CC	60	33.52	83	41.50	0.11	0.91	0.039
CT	100	55.87	97	48.50	0.18	1.34	0.128*
TT	19	10.61	20	10.00	0.86	1.06	0.027*
C-allele	220	61.45	263	65.75	0.22	0.83	0.085
T-allele	138	38.55	137	34.25	0.22	1.20	0.084*

*data for EF

Table 10. Genotype and allele frequencies of (819C/T) IL-10 variants in IBD and matched controls.

Genotype/Allele	CD (95) n (%)	UC (84) n (%)	Control (200) n (%)
CC	31 (32.63)	29 (34.52)	83 (41.50)
CT	53 (55.79)	47 (55.95)	97 (48.50)
TT	11 (11.58)	8 (9.53)	20 (10.00)
C-allele	115 (60.53)	105 (62.5)	263 (65.75)
T-allele	75 (39.47)	63 (37.5)	137 (34.25)

Table 11. Genotype and allele frequencies of (819C/T) IL-10 variants polymorphism in UC and CD patients.

Genotype/allele	IBD (n = 179)		Control (n = 200)		*P*-value	RR	EF*/PF
	n	%	n	%			
CC	60	33.52	83	41.50	0.11	0.91	0.039
CA	100	55.87	97	48.50	0.18	1.34	0.128*
AA	19	10.61	20	10.00	0.86	1.06	0.027*
C-allele	220	61.45	263	65.75	0.22	0.83	0.085
A-allele	138	38.55	137	34.25	0.22	1.20	0.084*

*data for EF

Table 12. Genotype and allele frequencies of (-592C/A) IL-10 variants in IBD and matched controls.

The association of IL-10 promoter polymorphism with UC, CD, or IBD in various ethnic population worldwide has been summarized in **Table 14**. The association is not consistent, and ethnic variations are evident in the type polymorphism and IBD, UC, or CD susceptibility.

Genotype/allele	CD (95) n (%)	UC (84) n (%)	Control (200) n (%)
CC	31 (32.63)	29 (34.52)	83 (41.50)
CA	53 (55.79)	47 (55.95)	97 (48.50)
AA	11 (11.58)	8 (9.53)	20 (10.00)
C-allele	115 (60.53)	105 (62.5)	263 (65.75)
T-allele	75 (39.47)	63 (37.5)	137 (34.25)

Table 13. Genotype and allele frequencies of (592C/A) IL-10 variants polymorphism in UC and CD patients.

Ethnicity/population	IL-10 polymorphism	Type of association with IBD	Reference
Australian	1082 G/A, 592 C/A	CD susceptibility	[84]
Canadian	1082 G/A, 819 C/T, 592 C/A	No association with IBD	[100]
Canadian	819 C/T	Associated with CD	[87]
*Caucasian	1082 G/A, 819 C/T	Associated with IBD susceptibility	[89]
*Caucasian	592 C/A	No association with IBD	[89]
Caucasian	1082 G/A	Associated with IBD	[38]
*Mixed	819 C/T, 519 C/A	Associated with UC	[88]
*Mixed	1082 G/A	No association with CD or UC	[88]
Mixed	1082 G/A, 819 C/T, 592 C/A	Associated with CD phenotype	[73]
*Mixed	1082 G/A	Associated with CD, No association with UC	[122]
New Zealand population	1082 G/A	Associated with CD,	[72]
Indian	1082 G/A, 819 C/T, 592 C/A	No association with IBD	[123]
Mexican	1082 G/A, 592 C/A	Associated with IBD susceptibility	[90]
Danish	1082 G/A, 819 C/T, 592 C/A	No association with IBD	[124]
Tunisian	Promoter polymorphism	Associated with CD	[125]
Turkish	1082 G/A	No association with IBD	[120]
Spanish	1082 G/A, 819 C/T, 592 C/A	No association with UC or CD	[126]
Spanish	1082 G/A	Associated with CD	[71]
Hungarian	1082 G/A	No association with CD	[127]
Korean	Promoter polymorphism	No association with IBD	[114]
German	1082 G/A, 592 C/A	No association with IBD	[91]
Italian	1082 G/A	Associated with UC	[86]
Italian	819 C/T	No association with UC	[86]

*Meta-analysis.

Table 14. Association of IL-10 promoter polymorphisms in UC, CD, or IBD in various ethnic populations worldwide.

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4. Discussion

TNF-α being a key cytokine in the inflammatory response of IBD plays an important role in the digestive and systemic manifestations of the disease. Available literature on the TNF-α (-308G/A) polymorphism shows its importance in the pathogenesis of CD and UC [58–60]. From the outgoing results, it is clear that allele A and genotype GA of TNF-α (-308G/A) polymorphism are associated with IBD susceptibility in Saudi population. Our results are in accordance with the earlier reports from other populations. This polymorphism has been shown to affect the UC and CD susceptibility in Asians and Europeans. The allele A of TNF-α (-308G/A) is associated with UC susceptibility in Japanese and Han Chinese patients [57, 106, 113]. The genotype GA is a risk factor for UC in Asians, whereas homozygous genotype AA is risk for both UC and CD in European patients [58]. A meta-analysis besides supporting the association of TNF-α (308G/A) polymorphism with IBD in Asians suggested that genetic polymorphisms vary in Asians from Caucasians [59].

On the other hand, some reports support the association of TNF-α (-308G/A) polymorphism with the severity of IBD. TNF-α (-308G/A) polymorphism is reported to be significantly associated with the severity of CD and/or UC in Irish [83], Czech [102], Italian [112], Caucasian patients from New Zealand [128], and Brazilian patients [60]. However, it is not clear whether it is directly involved in pathophysiology of IBD or serve merely as markers in Linkage disequilibrium with susceptibility genes [83].

Moreover, the carriers of allele A are at greater risk of pancolitis and more likely to require bowel resection in UC and CD [102, 112]. The CD patients with allele A were reported to be more resistant to steroids compared with non-carriers. The CRP levels in UC and CD patients carrying allele A were found to be higher and reported to modify the disease phenotype, influence its activity, and lead to a more intense inflammatory response [112].

The increased inflammation, higher levels of C-reactive protein (CRP), TNF-α, and interleukin-1ß have been associated with the A-containing genotypes of TNF-α (-308G/A) polymorphism in the active phase of IBD [98, 99, 107, 129]. The higher frequency of allele A of TNF-α (308G/A) was found in anti-neutrophil cytoplasmic antibodies (ANCA)-positive than ANCA-negative IBD patients, which may have influences on the susceptibility IBD or the behavior of IBD [114].

On the other hand, homozygous genotype AA of TNF-α (-308) has been associated with susceptibility to CD in Portuguese patients, and it has been suggested that TNF-α (-308G/A) polymorphism is responsible for displaying distinct clinicopathological profiles in Portuguese CD patients [117].

However, contrary reports are also available in the literature. The lower frequency of allele A of TNF-α (-308G/A) has been reported in North European Caucasian and Korean patients with CD or UC as compared to healthy controls [103]. Although the frequency of allele G was reported to be slightly higher in Iranian Azeri Turkish IBD patients, it did not reach statistical significance [110]. Further, no association of TNF-α(-308G/A) polymorphism with IBD susceptibility was found in Australian [82], Brazilian [60, 130], Canadian [100], Chinese [106, 131],

Czech [132], French [133], Indian [108], Korean [115], Newfoundland [27], Spanish [126], and Turkish [120] populations. The reason for these differences in the TNF-α genetic associations with IBD etiology might be the variations in sample size, genotyping methods, and/or ethnicity itself as frequencies of alleles and genotypes of TNF-α (-308G/A) also vary in different ethnic healthy populations worldwide [39] (**Table 3**).

Our genotyping results for TNF-β (+252A/G) polymorphism showed that genotype GG was significantly associated with IBD susceptibility. Our results also indicated that genotype GA was slightly lower in IBD patient than the controls, but the difference did not reach statistical significance. However, when the results were stratified into CD and UC, it became evident that this polymorphism was associated only with UC but not with CD in Saudi population (**Table 4**, **Figure 2**). In contrast, some earlier reports suggested that the TNF-β (+252) polymorphism is not associated with CD or UC in Chinese, French, Korean, and Spanish patients [57, 106, 115, 119, 133]. It is possible that the TNF-β (+252A/G) polymorphism may be indirectly associated with IBD as it has been suggested to influence the expression/production of TNF-α [42].

Muro et al. [134] reported that the inflammatory response in IBD is effected by the changes in TNF-α and TNF-β levels and IBD patients are commonly treated with TNF-α inhibitors. Moreover, TNF-α gene polymorphisms are reported to affect the gene expression level of TNF-α, and a particular TNF-α genotype may influence the response of IBD patients treated with TNF-α inhibitors as mutated allele A of TNF-α(-308) and allele G of TNF-β(+252) polymorphisms have been associated with greater TNF-α transcription [35, 36, 42, 135].

Our study on Saudi IBD patients suggested a significant association between allele and genotype frequency of TNF-α (-308G/A) and TNF-β (+252A/G) polymorphisms and IBD susceptibility in Saudi population. It is evident from outgoing discussion that ethnicity plays a very important role in genetic association of TNF-α and TNF-β polymorphism with IBD. It is also inferred that the both the polymorphism may have synergistic effect on the susceptibility and may work in tandem to influence the etiology of IBD in Saudi population. The outcome of present study will not only help in the prognosis of IBD in Saudi population but also provide guideline for the treatment with anti-TNF therapy as individuals with different genotypes of TNF-α (-308G/A) respond differently to anti-TNF-α treatment [136, 137]. However, further studies are required involving other ethnic populations to strengthen these findings.

The genotyping results for IL-10-1082 G/A polymorphism indicated that genotype -1082GG and allele G are susceptible to IBD (RR = 2.19, EF = 0.347, RR = 1.36, EF = 0.135, respectively), while genotype AA and allele A are resistant to IBD (RR = 0.49, PF = 0.0.253, RR = 0.73, PF = 0.138, respectively). Upon stratification of genotyping results into CD and UC, we noticed that genotype GG and allele G of -1082 G/A polymorphism were associated with CD susceptibility, while genotype AA and allele A might be protective for CD. On the other hand, no significant association was found either with alleles or genotypes of -1082 G/A polymorphism and UC in Saudi patients. These results are in accordance with the various reports, which also indicted an association of IL-10-1082G/A polymorphism susceptibility to IBD [89, 122]. A meta-analysis including 18 case-control studies provided evidence for the association between IL-10-1082G/A polymorphism and susceptibility of CD [122]. Another met-analysis including 15 studies

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demonstrated clear association between the IL-10-1082G/A polymorphisms and the risk of IBD [89]. The allele G of -1082G/A polymorphism has been associated with the IBD, and higher serum levels of IL-10 concentration have been reported in IBD patients than in the controls [72, 90]. Earlier studies with CD patients also indicated that IL-10-1082 G/A polymorphisms contribute to susceptibility to CD [71] and -1082G allele was significantly increased in patients with CD than controls [73] while A allele of the IL-10-1082G/A was associated with decreased IL-10 production in CD patients and controls [78].

Contrary to these, some studies reported that there are no significant differences in the allele and genotype frequencies of the IL-10-1082G/A polymorphism between IBD patients and controls in various populations [88, 91, 120]. Klein et al. [91] reported that IL-10-1082G/A polymorphism is not demonstrably involved in the predisposition of IBD in German cohort. Similarly, no association between Turkish IBD patients and IL-10-1082G/A was found [120]. A meta-analysis by Zou et al. [88] observed that IL-10-1082G/A polymorphism is not associated with IBD. These data provide evidence that the effect of IL-10 gene polymorphisms on cytokine production differs in CD, UC patients, and controls in various populations.

Further, our results indicated that IL-10-1082G/A polymorphism is not associated with UC susceptibility in Saudi patients. These are in accordance with earlier reports indicating no association of IL-10-1082G/A polymorphisms with susceptibility of UC [88, 122]. Mendoza et al. [138] reported that IL-1082G allele is not associated with the phenotype of UC patients in Madrid's Spanish population.

On the other hand, IL-10-1082 G/A polymorphism has been reported to influence susceptibility to UC [38, 86, 126]. A gender effect has been reported, with women of AG/AA genotypes of IL-10-1082 G/A, having a higher risk of developing UC at a younger age and is related to the lower IL-10 production associated with the -1082A allele and to the IL10 downregulating effect of estrogens [86]. A mild influence of -1082 G allele in UC appearance has also been reported by Castro-Santos et al. [126]. In a stratified analysis, a highly significant association between the -1082 AA genotype and the steroid dependency was observed in IBD, and it was suggested that carriage of the -1082 AA genotype (low producer) is a relevant risk factor for developing steroid-dependent IBD. Tagore et al. [38] suggested that individuals genetically predisposed to produce less IL-10 are at a higher risk of developing IBD, and the frequency of the high IL-10 producer allele (-1082 G) is decreased in the whole IBD group and in the UC patients compared with normal.

The two other polymorphisms of IL-10 gene (IL-10-819C/T and IL-10-592C/A) are not associated with the susceptibility of IBD as the frequency distribution of genotypes and alleles of these two polymorphisms did not differ significantly between controls and IBD patient groups. The stratification of our results in to CD and UC patients also indicated that IL-10-819C/T and IL-10-592C/A polymorphisms are associated with neither with CD nor UC susceptibility in our patients. Similarly, Castro-Santos et al. [126] did not find any association between IL-10 (-812 C/T and -592 C/A) polymorphisms and UC or CD susceptibility. A recent meta-analysis demonstrated no significant association between the -592C/A polymorphism and IBD, CD, or UC, but a clear association with IL-10-819C/T polymorphism [89], while several other report showed that these polymorphisms are associated with IBD risk [72, 87, 88, 90, 139].

These differences in the association of IL-10-819C/T and IL-10-592C/A polymorphisms with CD or UC can be attributed to ethnic variations.

The exact mechanism by which IL-10 affects the susceptibility/pathogenesis of IBD is far from clear. It participates in the regulation of the immune response at several levels [69]. IL-10 regulates the inflammatory response, by inhibiting proinflammatory Th1 cytokines production [140].

IL-10 cytokine downregulates the expression of major histocompatibility complex (MHC) of class I and II molecules [141, 142]. It also has potent stimulatory effects on B lymphocytes, resulting in increased production of immunoglobulin and DNA replication [141]. The immune-stimulating effects of IL-10 have also been reported. IL-10 is shown to induce activated B cells to secrete large amounts of IgG, IgA, and IgM and in combination with IL-4 which results in the secretion of four immunoglobulin isotypes. The increased levels of IL-10 play a role in the amplification of humoral responses in some diseases [141].

Sanchez-Munoz et al. [143] suggested that the intestinal inflammation in IBD is controlled by a complex interplay of innate and adaptive immune mechanisms. Cytokines determine T-cell differentiation of Th1, Th2, T regulatory, and Th17 cells in IBD, and cytokines levels regulate the development, recurrence, and exacerbation of the inflammatory process in IBD. The dysregulation of T cells, or an over-production of effector T cells, results in the development and exacerbation of IBD [144]. Thus, the antigen-presenting cells (APCs), Th1, Th2, T regulatory cells, and Th17 and their cytokine products play an important role in the etiology of IBD [8]. These cellular interactions are modulated by pro- or anti-inflammatory cytokines (such as TNF-α, INF-γ, IL-1, IL-6, IL-4, IL-5, IL10, TGF-β, IL-13, IL-12, IL-18, IL-23) [145]. Although many common responses in IBD are mediated by cytokines, how cytokines determine the nature of the immune response in IBD may be quite different among different IBD forms [146].

A highly significant increase in IL-10 mRNA levels in T lymphocytes and in IL-10-positive cells in the colons of UC patients has been reported by Melgar et al. [147]. Moreover, IL-10 production by regulatory T cells has also been implicated as important factor in IBD [148]. Another regulatory B cells subtype called Bregs may also take part in UC etiology by producing IL-10 [149]. The significance of IL-10 produced by B cells has been indicated in IBD patients and animal models also [150, 151]. The Bregs can be responsible for the suppression and/or recovery from acquired immune-mediated inflammations by IL-10 and TGF-β1 in IBD [143, 149]. However, the exact mechanism is still far from clear and needs to be investigated.

5. Conclusion

Our study dealing with the five polymorphisms of proinflammatory and anti-inflammatory cytokine genes in Saudi IBD patients clearly indicates that the TNF-α (-308G/A), TNF-β (+252A/G), and IL-10 (-1082 G/A) polymorphisms are associated significantly with the risk of IBD susceptibility while other two, IL-10-819C/T and IL-10-592C/A, polymorphisms are not associated with IBD in Saudi population. However, due to several limitations in the present

study, it is suggested that well-designed epidemiological as well as genetic association studies with large sample size among different ethnicities should be performed in order to have better understanding of this relationship.

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Author details

Abdulrahman Al-Robayan[1], Misbahul Arfin[2], Ebtissam Saleh Al-Meghaiseeb[1], Reem Al-Amro[1] and Abdulrahman K Al-Asmari[2]*

*Address all correspondence to: abdulrahman.alasmari@gmail.com

1 Department of Gastroenterology, Prince Sultan Military Medical City, Riyadh, Saudi Arabia

2 Research Centre, Prince Sultan Military Medical City, Riyadh, Saudi Arabia

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Chapter 3

Genetic and Serological Markers in Identifying Unclassified Colitis

Valerie A. Fenech, John Schembri, Pierre Ellul, Godfrey Grech and Neville Azzopardi

Additional information is available at the end of the chapter

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Abstract

In 5–15% of the patients with inflammatory bowel disease (IBD) limited to the colon, it is difficult to distinguish histologically between ulcerative and Crohn's colitis. This is described as unclassified colitis. Distinguishing between the two is important in terms of prognosis, since patients with Crohn's disease (CD) have a higher risk of strictures and fistulae, which may predict a more severe disease course, as well as an increased risk for surgery. In addition, colectomy may be curative in ulcerative colitis patients not responding to medical therapy, while Crohn's patients undergoing colectomy can have relapses in other areas of the bowel and, therefore, need to be followed-up. In inflammatory bowel disease, intestinal inflammation is believed to occur secondary to an altered immune response in a genetically susceptible host. Genetic and serological markers (antibodies) may have a role in identifying unclassified colitis. Anti-*Saccharomyces cerevisiae* antibody (ASCA) and anti-neutrophil cytoplasmic antibodies (pANCA) have the highest sensitivity in distinguishing ulcerative from Crohn's colitis. Nucleotide oligomerization domain 2 (NOD2) and autophagy-related 16-like 1 (ATG16L1) polymorphisms are strongly associated with Crohn's disease, while epithelial barrier genes are significantly associated with ulcerative colitis. This chapter describes which gene polymorphisms and serological markers may be used to distinguish between ulcerative colitis and Crohn's disease in patients with histologically unclassified colitis.

Keywords: Crohn's disease, ulcerative colitis, unclassified colitis, serological markers, gene polymorphisms

1. Introduction

While inflammatory bowel disease (IBD) is broadly divided into ulcerative colitis (UC) and Crohn's disease (CD), there is significant overlap in clinical presentation, endoscopic appearance, and histological findings between these two disorders. In about 5–15% of the patients with inflammation limited to the colon, it is difficult to distinguish between UC and Crohn's colitis [1]. These cases of overlap, previously referred to as indeterminate colitis, are now called *unclassified* IBD [2].

Distinguishing between ulcerative and Crohn's colitis is important in terms of management and prognosis, since patients with CD have a higher risk of stricture and fistula formation that may predict a more severe disease course, as well as an increased risk of surgical intervention [3]. Surgery (colectomy) may be curative in UC patients not responding to medical therapy, while CD patients undergoing surgical resection of the colon can develop delayed inflammation in other areas of the bowel, therefore, needing closer follow-up.

The exact etiology of IBD is unknown and is probably multifactorial. One of the proposed mechanisms that leads to intestinal inflammation in IBD refers to an aberrant immune response in a genetically susceptible host [2]. This mechanism involves a complex interaction between environmental and microbial factors at the intestinal epithelium of patients with susceptibility genes that might lead to an altered innate and adaptive immune response. Intestinal homeostasis relies on the interactions between environmental factors, the epithelium, and the host immune system [4]. Breakdown of any of these components will disrupt the mucosal immune tolerance and promote inflammation.

The *immune defense mechanism* includes a huge armamentarium of complex signaling pathways which are involved in microbial recognition and antimicrobial function. These include pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), antimicrobial peptides produced by Paneth cells, mucus production from goblet cells, and secretory immunoglobulin A [4]. Genome-wide association studies (GWAS) have identified susceptibility genes that affect the intracellular processing of bacterial components, such as autophagy. Some of these are shared in common in CD and UC; however, other genes are unique to either of these diseases, helping us distinguish between the two. Nucleotide oligomerization domain 2 (Nod2) polymorphisms are one of the most studied genetic variants in CD. Nod2 plays an important role in immune defense and tolerance in that it is expressed in different cells of the immune system. For example, Nod2 stimulation in dendrtitic cells activates the nuclear factor-kB (NF-kB) pathway. Nod2 in T cells plays a role in T cell function including cytokine production [5]. Polymorphisms in the genes responsible for autophagy, mainly Nod2 and ATG16L1 genes, inhibit the recruitment of autophagy proteins that are responsible for phagocytosis of the pathogen [6].

Defects in the tight junctions of the intestinal epithelial cells and changes in the paracellular permeability will inhibit the epithelium from acting as an effective *mucosal barrier* against luminal pathogens and contribute to inflammation. Defects which have been described in IBD include T junction abnormalities, possibly mediated by tumor necrosis factor-alpha

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(TNF-alpha), alterations in the composition of the mucous layer secreted by goblet cells, and decreased defensin production by Paneth cells, especially in ileal CD [7].

Research has also focused on the identification of *environmental factors* as drivers of dysregulated immunity [8]. Alterations in the gut microbiota, leading to an imbalance of the pathogenic and nonpathogenic bacteria play another important role in immune tolerance. For example, a decrease in the diversity of *Firmicutes* is found in patients with IBD. It is still controversial whether it is a cause or a consequence of inflammation. There have been attempts to use gut microbiota as biomarkers; however, no microbial constituents were found to be specific to CD or UC [9].

Over the past decade, IBD research has focused on the role of genetic and serological markers in the phenotypic presentation of UC and CD. However, since UC and CD have different genetic and serological markers, such markers may also be used in identifying unclassified colitis. This review looks at the evidence behind these biomarkers and their role in different IBD subtypes.

2. Serological markers in differentiating CD from UC

There is no single marker that will determine the subtype of IBD; however, the combination of different serological markers may increase our ability in distinguishing between these subtypes. In patients whom it is difficult to differentiate between UC and CD using the traditional clinical, endoscopic, and histological criteria, serological markers are becoming increasingly helpful [10]. This area of research is rapidly expanding as new antibodies to different microbial antigens and autoantibodies are being discovered [2]. Antibodies to microbial agents include the anti-glycan antibodies, while antibodies to self-antigens include anti-neutrophil cytoplasmic antibodies (pANCA) and antibodies against exocrine pancreas (PAB).

Research has produced significant data on anti-*Saccharomyces cerevisiae* antibody (ASCA) and pANCA. These are the best available biomarkers in distinguishing between ulcerative and Crohn's colitis (ASCA has been linked to CD, while pANCA is associated with UC)[2]. ASCA positivity is found in 29–71% of the CD patients, while only 0–29% of the UC patients are ASCA positive. A positive ASCA result in isolation has a sensitivity ranging from 37 to 72% and a specificity from 82 to 100% for the diagnosis of CD. Combining a positive ASCA and a negative pANCA profile increases specificity to 92–99% [2].

However, more recently new serum biomarkers are being discovered. These biomarkers classify the type of colitis and also play an important role in predicting disease course, risk of complications, and response to treatment. Anti-glycan antibodies are strongly associated with CD, and their presence is also linked to a more severe disease type and risk of disease progression as well as an increased risk for IBD-related surgery [10]. The anti-glycan carbohydrate antibodies include anti-chitobioside IgA (ACCA), anti-laminaribioside IgG antibodies (ALCA), antimannobioside IgG antibodies (AMCA), and more recently, anti-laminarin (anti-L) and anti-chitin (anti-C).

The overall sensitivity of the newer anti-glycan antibodies in CD is low [1]; however, combining different serum biomarkers increases the predictive value for differentiating CD from UC. Being ASCA positive and pANCA negative increases the specificity and positive predictive value for diagnosis of CD compared to ASCA positivity alone [11]. In a study published in 2009 by Seow et al. evaluating more than 800 patients with IBD, it was found that 73% of the CD patients were positive for 1 or more anti-glycan antibodies [12]. In addition, all anti-glycan antibodies were specific for CD and more prevalent in CD rather than in UC.

These findings were confirmed in 2010 by Rieder et al. who also showed a higher prevalence of serum antibodies in CD than in UC [13], gASCA, or the combination of gASCA/pANCA were found to be most accurate for the diagnosis of CD, but using a combination of antibodies improved the differentiation between CD and UC [13].

Other antibodies that target microbial antigens include anti-outer membrane porin C (anti-OmpC), anti-Cbir1 flagellin, and anti-I2 antibody [2]. CD patients have an excessive secretion of IgA antibodies against OmpC, an outer membrane porin found in *Escherichia coli* [2]. CD patients are also more likely to have antibody expression against I2 which is produced by the microorganism *Pseudomonas fluorescens* [2]. However, adding anti-I2 and anti-OmpC to ASCA and pANCA only marginally improved the predictive capacity of distinguishing CD from UC [14].

Flagellin is an antigen present on most motile bacteria in the gut and is highly antigenic [2]. Flagellin CBir1 has been identified as a colitogenic antigen. Anti-CBir1 is more commonly found in CD rather than in UC patients (50–56% prevalence in CD versus less than 6% in UC) [2].

Antibodies targeting the exocrine pancreas (PAB) are also highly specific for CD. The autoantigen of PAB in CD is the membrane glycoprotein (GP2) on pancreatic acinar cells [2].

A number of antibodies have been identified in IBD, pANCA being most typically associated with UC. Despite this association, the use of antibodies has been limited in clinical use in UC. The combination of ASCA–/pANCA+ results in better diagnostic accuracy for differentiating CD from UC than either test alone. Additionally, CD patients who are pANCA positive may have colonic disease resembling UC-like disease. After resection, UC patients remain pANCA positive unlike CD patients in whom ASCA titres return back to normal [2].

The pANCA staining pattern is not specific to UC and is found in a number of autoimmune diseases and in up to 2.5% of healthy controls. Loss of antigenic response after DNase digestion of neutrophils, however, seems to be a dominant characteristic of UC-specific pANCA and is termed DNase-sensitive pANCA [15].

Serum biomarkers may, therefore, be used to help distinguish IBD from other diseases of the gut to identify the type of IBD in those patients who are difficult to classify using the classical clinical, endoscopic, histological, and radiological criteria, and to predict disease severity. The latter is related to both the number of positive serological antibodies present and their levels. There is no role in repeated testing for these biomarkers to assess disease activity [2]. Nor is it useful to predict response to treatment in CD.

In IBD patients with unclassified colitis, about half of these patients will eventually manifest as CD or UC. Usually, these are the patients who have a positive biomarker (ASCA or pANCA). The other half will remain with a diagnosis of unclassified colitis. Most of these patients are negative for both ASCA and pANCA [16]. This is where newer biomarkers might help to further classify the subtype of IBD.

2.1. Prediction of disease stratification and severity in CD

Both anti-laminarin (anti-L) and anti-chitin (anti-C) are associated with more aggressive CD phenotypes. In particular, anti-C has been associated with penetrating and perianal disease [12]. Anti-L appears to be associated with more steroid-dependency but is also useful in improving the differentiation between ulcerative and Crohn's colitis when used with ASCA and ANCA antibody status [17].

Besides differentiating between ulcerative and Crohn's colitis, serological biomarkers may also help in predicting the clinical course and behavior of the disease. Elevated titres of antimicrobial antibodies makes it more likely for a patient with CD to develop more severe and complicated disease, with an increased risk of requiring IBD-related surgery. The chance of having more severe disease increases with the number of serum biomarkers present and also with increasing titres of these antibodies [2]. Seow et al. have shown that an increasing number of positive antibodies is associated with early CD onset, fistulating and perianal disease, and increased risk for surgery [12]. These findings were confirmed by Rieder et al. who showed that a higher number of anti-glycan antibodies predicts a faster progression toward more severe disease [18]. Anti-GM-CSF antibody also correlates with disease activity and an increased risk of relapses [10].

Serological markers such as anti-GP2 and anti-CBir1 may contribute to better stratification of pouchitis in patients with UC undergoing ileal pouch-anal anastomosis (IPAA). A retrospective study published in 2012 by Coukos et al. analyzed ASCA IgG and anti-CBir1 antibodies in patients with UC who underwent IPAA. Both ASCA IgG and anti-CBir1 titres were significantly associated with postoperative IPAA complications. A positive anti-CBir1 test was found to be associated with CD of the pouch and/or fistula formation ($p < 0.001$) [19]. Identifying patients with these positive serological markers can help the clinician predict the risk of pouchitis in patients undergoing IPAA, and therefore, choosing more aggressive treatment to prevent pouch failure [19]. A similar association between GP2 antibodies and increased risk of pouchitis has also been demonstrated, especially when the inflammation exhibits CD-like complications. Elevated anti-GP2 was also associated with more frequent bowel movements per day and presence of at least one anti-glycan antibody. Therefore, the presence of these CD-specific pancreatic auto-antibodies (PAB) could be used as a predictor of pouchitis [20].

Notwithstanding their role in distinguishing IBD subtypes and predicting IBD phenotypes, serum biomarkers are frequently not useful in diagnosing clinical remission. Their levels remain elevated even in patients who are in endoscopic and histological remission. For example, the level of CBir1 antibody in the serum does not correlate with disease severity and stable CBir1 expression has been found in the serum of CD patients during both active disease and also when in endoscopically proven remission [21].

Serum biomarkers are not specific to CD but may also be found to a lesser extent in patients with other disorders of the gastrointestinal tract or in healthy individuals. For example, ASCA and PAB, which are highly specific antibodies in CD, can also be found in patients with coeliac disease, especially prior to commencing a gluten-free diet. Therefore, these serum antibodies are not to be used on their own to diagnose IBD, but are meant to be an extra tool in the accurate diagnosis and management of IBD [2].

3. Genetic markers

3.1. Genetic markers in Crohn's disease

Gut inflammation in IBD is believed to occur secondary to an interaction between the altered immune system of a genetically susceptible individual and environmental factors such as antimicrobial agents. IBD is regarded as a polygenic disorder with multiple susceptibility loci contributing to the overall risk of developing the disease [22]. There are frequently different genetic loci, which are involved in the pathogenesis of UC and CD, and this may be used in distinguishing between Crohn's and ulcerative colitis.

It may occasionally be difficult to differentiate functional from organic bowel diseases based on the clinical presentation and even more difficult to distinguish CD from UC. In a study published in 2008, von Stein et al. tried to identify genes from mucosal biopsy specimens which could help discriminate between functional disease and IBD and also genes which could distinguish between CD and UC [23]. The group identified seven IBD-specific genes that could help determine IBD type in patients with colitis. These genes were solute carrier (SLC)6A14, SLC26A2, small protein associated with PDZ domain-containing protein 1 (SPAP), regenerating protein IV (RegIV), Vanin-1, matrix metalloproteinase 7 (MMP-7), and growth-related oncogene alpha (GRO-alpha). These genes have been shown to play a role in IBD, be it in inflammation, tissue injury, or carcinogenesis. For example, GRO-alpha is a chemokine that recruits and activates neutrophils at the site of inflammation [24]. Using these seven biomarkers, one could correctly classify UC or CD patients in more than 92% of cases [25].

Wu et al. also tried to identify genes that are associated with CD, UC, and non-IBD colitis [26]. Genes differentially expressed in the CD patients were related to IFN gamma-inducible TH1 processes (IFITM1, IFITM3, STAT1, and STAT3) and antigen presentation (TAP1, PSME2, and PSMB8).

The role of several genes involved in epithelial defense has been studied as a possible contributing factor to the development of CD [27]. Some of the genes studied confirm a link between defects in the immune response and the role of intracellular bacteria in patients with CD.

3.1.1. Bacterial recognition

Nucleotide-binding oligomerization domain containing 2 (NOD2) gene is found on chromosome 16 and is one of the earlier genes to be linked to CD. NOD2 variants that alter the structure of the leucine-rich repeat domain of the protein can overactivate nuclear factor

NF-kB in monocytes, thereby altering the response of the immune system to microbial pathogens [28]. Polymorphisms in the NOD2 gene have been associated with more complicated disease in CD [29].

NOD2 gene polymorphisms may also have implications on drug treatment. Gutierrez et al. studied phagocytic and bactericidal activities in neutrophils of patients with CD. Patients with a NOD2-variant had less phagocytic and bactericidal activities and increased TNFα levels in response to the presence of bacterial DNA [30]. This might have an effect on the management of these patients as they may require more aggressive treatment, with an increased requirement for anti-TNFα agents.

3.1.2. Autophagy

Hampe et al. were among the first to show a potential role of autophagy in the pathogenesis of CD. The group studied the autophagy-related 16-like 1 (ATG16L1) gene, which encodes a protein that processes intracellular bacteria. A variant of this gene was found in CD but not in UC patients [31]. It supports the role of bacteria in the pathogenesis of CD as the processing of intracellular bacteria would be altered in patients having a variant of the gene.

Another gene involved in autophagy of intracellular pathogens is the immunity-related GTPase family M (IRGM) gene. IRGM is a CD susceptibility gene [32] that does not increase the risk of developing UC [33].

The protein tyrosine phosphatase nonreceptor type 2 (PTPN2) gene is also involved in the autophagy pathway and PTPN2 polymorphisms have also been associated with IBD. The PTPN2 polymorphism rs2542151 appears to be associated with both CD and UC, while polymorphisms rs1893217 and rs7234029 are associated with CD only [34].

3.1.3. Prostaglandin system

Variants of the prostaglandin receptor 4 (PTGER4) have been implicated to have a role in CD. In a German cohort of CD patients, Prager et al. found that the variant rs7720838 increased susceptibility to CD but not to UC. Patients with this gene variant were more likely to develop stricturing disease with the risk of stricture formation increasing further if the patient also had NOD2 mutations [35]

3.1.4. TH-17/IL-23 cytokine responses

Interleukin (IL)-23R mutations and their role in CD have been studied extensively. The IL-23 cascade contributes to the differentiation of the T helper (Th) 17 cells, which play an important role in immunoregulation in the gut [25]. Dysregulated Th17 differentiation may occur in CD patients [36, 37].

The rs11209026 SNP in IL23R was found to have a protective effect for IBD. Both CD and UC were associated with IL23R in a study conducted in over 500 Dutch patients with IBD [38]. In another study involving Korean patients with CD, two other polymorphisms (rs1004819 and rs1495465) were significantly associated with CD, and when the genotype was correlated to

the disease phenotype, it was found that it was associated with both stricturing and penetrating disease [39].

3.2. Genetic markers in UC

CD and UC may share some gene susceptibility loci but significantly differ at others. While genetically determined defects in the handling of intracellular bacteria (NOD2 and the autophagy genes ATG16L1 and IRGM) are specific to CD, multiple components in the Th17 pathway (IL23R, IL12B, JAK2, STAT3) are associated with both CD and UC [40, 41].

New potential pathogenic pathways for both CD and UC have been demonstrated, initially by genome-wide assocation studies (GWAS) and more recently via imputation and meta-analyses to combine the power of multiple individual GWAS. Meta-analyses of GWAS have shown that CD and UC share the majority of the 163 known genetic risk factors for IBD, although to varying extents, for example, IL23 pathway is more commonly linked to CD rather than to UC despite being strongly associated with both [42].

The major histocompatibility complex (MHC) seems to be one of the notable exceptions to this. In a large cohort of patients with IBD (18,405 Crohn's disease patients, 14,308 ulcerative colitis patients, and 34,241 controls), it was shown that the MHC region is a significant contributor to disease risk in IBD. However, while the majority of non-MHC susceptibility loci are shared between both UC and CD, most associated HLA alleles have a predominant role in either one or the other, with very few conferring shared IBD risk. Furthermore, whereas both class I and class II HLA variants contribute to disease risk in CD, class II variation seems to have a more important role in UC [43].

Altered epithelial barrier function may play a role in the development of UC but not in CD. A number of epithelial barrier genes are in fact specifically associated with ulcerative colitis (OCTN2, ECM1, CDH1, HNF4A, LAMB1, and GNA12). The role of epithelial barrier genes HNF4A, E-cadherin, and laminin was also first discovered by GWAS of a relatively small sample of 2361 white patients of European descent as part of the Wellcome Trust Case Control Consortium 2 [44]. The exact mechanisms by which such defects play a bigger role in the pathogenesis of UC than in CD are still poorly understood.

4. PSC-IBD phenotype

Primary sclerosing cholangitis (PSC) has long been associated with IBD, especially with UC. Up to 5% of patients with UC have PSC, while up to 3.6% of patients with CD have PSC, mostly those patients with extensive disease [45].

However, the phenotype of IBD in the context of a patient with both IBD and PSC is different from the IBD phenotype of a patient without PSC. De Vries et al. have recently published a systematical review of the literature to identify the distinctive features of IBD in patients with concomitant PSC [46]. The characteristics and clinical course of IBD in patients with PSC are different, making it distinct from the conventional IBD phenotypes.

The prevalence of IBD in PSC is high, ranging from 46.5 [47] to 98.7% [48]. More than 75% of these patients have UC, followed by CD and unclassified IBD.

Although the disease course of IBD in PSC is found to be quiescent, pancolitis is observed more frequently, with rates varying from 35 to 95% [49, 50]. Another two characteristics which are more commonly reported in IBD patients with PSC than in conventional IBD are backwash ileitis and rectal sparing [51]. Although the disease activity of Crohn's is similar in patients with and without PSC, the reported rates of complications like stricturing and penetrating disease are lower in patients with concomitant PSC [52]. On the other hand, patients with IBD and PSC who undergo proctocolectomy with ileal pouch—anal anastomosis (IPAA) have a higher risk of developing pouchitis than IBD patients without PSC [46].

The risk of dysplasia or development of colorectal carcinoma is increased in PSC-IBD. For this reason, guidelines on colorectal cancer surveillance classify patients with IBD-PSC in the high risk category, recommending increased frequency of surveillance colonoscopies. The cumulative 10-year risk varies between studies, but is significantly higher than in IBD patients without PSC. In addition to this, the predominant site of dysplasia or malignancy is different in IBD patients with PSC. It tends to occur in the proximal colon, as opposed to conventional IBD, where right-sided localization is less common [46].

PSC-IBD patients have been observed to develop dysplasia or colorectal carcinoma earlier than IBD patients without PSC, where the mean interval between the diagnosis of IBD and the development of dysplasia or colorectal carcinoma is longer [53].

Patients who undergo orthotopic liver transplantation for PSC still have high rates of IBD exacerbation despite being on immunosuppressant medication. Up to 51.5% of these patients experience an exacerbation [54].

The etiology of PSC is not clear. It has been shown in genetic studies that it shares risk loci with IBD. It is genetically more similar to UC, which might explain why UC is the predominant IBD phenotype associated with PSC. However, there are also several genetic loci, which are associated with PSC but not with IBD [46]. These differences, together with the characteristic features described above, suggest that PSC-IBD is a phenotype, which is distinct from UC or CD.

5. Conclusion

Serological and genetic markers may play an important role in identifying IBD type in patients with unclassified colitis. **Figure 1** summarizes some of the genetic loci and serological markers which can help us classify the type of colitis, and the degree of overlap which exists between CD and UC. These markers may have an important role in patients with unclassified colitis and may be used in these patients to create a risk score for IBD type. Thus, a patient with unclassified colitis who is ASCA+/pANCA− and carries the NOD2 and ATG16L1 polymorphisms has a significantly higher chance of having Crohn's colitis while patients who are ASCA −/pANCA+ and have polymorphisms in epithelial barrier genes are more likely to have UC.

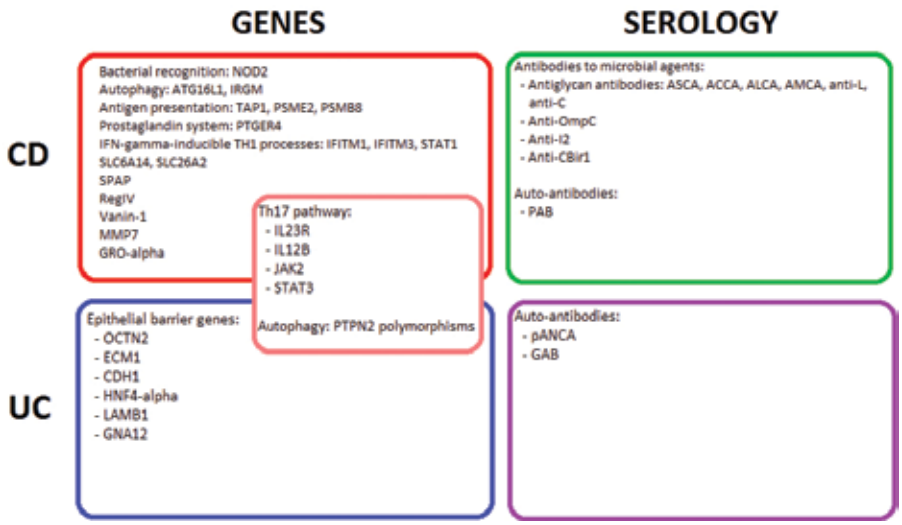

Figure 1. Genetic loci and serological markers, which may be used to distinguish between Crohn's disease (CD) and ulcerative colitis (UC) in unclassified colitis. (NOD2, nucleotide-binding oligomerization domain-containing protein 2; ATG16L1, autophagy-related 16-like 1; IRGM, immunity-related GTPase family, M; PTGER4, prostaglandin receptor 4; IFN, interferon; TH1, type 1 helper T-cell; IFITM, interferon inducible transmembrane protein; STAT, signal transducer and activator of transcription; SLC, solute carrier; SPAP, small protein associated with PDZ domain-containing protein-1; RegIV, regenerating protein IV; MMP, matrix metalloproteinase; GRO, growth-related oncogene; TH17, T helper 17 cells; IL23R, interleukin 23 receptor gene; JAK2, Janus kinase 2; PTPN2, protein tyrosine phosphatase, nonreceptor type 2; OCT, organic cation transporter; ECM1, extracellular matrix protein 1; CDH1, E-cadherin; HNF, hepatocyte nuclear factor; LAMB1, laminin β1; GNA12, guanine nucleotide binding protein, alpha 12; ASCA, anti-*Saccharomyces cerevisiae* antibodies; ACCA, antichitobioside carbohydrate antibodies; ALCA, antilaminaribioside carbohydrate antibodies; AMCA, anti-mannobioside carbohydrate antibodes; Anti-L, anti-laminarin antibodies; Anti-C, anti-chitin antibodies; Anti-OmpC, antibody to outer membrane porin C; Anti-I2, antibody to *Pseudomonas fluorescens*-associated sequence I2; Anti-Cbir1, antibody to bacterial flagellin; PAB, antibodies against exocrine pancreas; pANCA, anti-neutrophil cytoplasmic antibodies; GAB, antibodies to goblet cells.)

While there is significant evidence to link these markers to specific disease types, more evidence is needed. Since different gene polymorphisms are commoner in different geographical areas, population-based studies are needed to identify genes in specific populations. We also feel confident that with more research, other different disease-specific antibodies will be discovered. Long-term follow-up of patients with unclassified colitis may also help better characterize this disease and help us create a score based on serum biomarkers, which can predict the disease type in this group of patients.

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Author details

Valerie A. Fenech[1], John Schembri[1], Pierre Ellul[1], Godfrey Grech[2] and Neville Azzopardi[1*]

*Address all correspondence to: neville.azzopardi@gov.mt

1 Gastroenterology Department, Mater dei Hospital, Msida, Malta

2 Pathology Department, Laboratory of Molecular Pathology, University of Malta, Msida, Malta

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Section 2

The Role of Cytokines in Inflammatory Bowel Disease

Chapter 4

Cytokines in Inflamed Mucosa of IBD Patients

Tsvetelina Velikova, Dobroslav Kyurkchiev,
Ekaterina Ivanova-Todorova, Zoya Spassova,
Spaska Stanilova and Iskra Altankova

Additional information is available at the end of the chapter

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Abstract

Cells of the innate and the adaptive immune system have been identified as the key players in inflammatory bowel disease (IBD) pathogenesis, and the cytokines are central components of the inflammatory pathways that take place in the gut mucosa during the active and chronic phases of IBD. The effector cell response is largely determined by the type of cytokines that predominate in the intestinal mucosa. Here we describe the main cytokine players in intestinal inflammation during IBD—related to innate immune responses (tumor necrosis factor α—TNFα), TNF-like cytokine 1A, IL-8), and related to adaptive immune responses—Th1 (IL-1β, IL-18, IFNγ, IL-12), Th2 (IL-4, IL-5, IL-13, IL-11, IL-33), Th17 (IL-17A, IL-17F, IL-21, IL-22, IL-25, IL-27), cytokines required for Th17 development (IL-6, TGFβ, IL-23), anti-inflammatory cytokine IL-10 and Tregs along with IL-2. Recently described innate lymphoid cells (ILCs) could also be potential sources of IFN-γ, TNF, IL-5, IL-13, IL-17, and IL-22. The effects of cytokines in the gut are described in conjunction with the clinical implication and available biologic therapy. The data in the literature and our own results make us believe that in order to achieve immune homeostasis in the gut, pro-inflammatory and anti-inflammatory responses that define the mucosal cell immunophenotype should achieve balance.

Keywords: IBD, cytokines, mucosal inflammation, Th17, Tregs

1. Introduction

Both ulcerative colitis (UC) and Crohn's disease (CD), usually referred to as inflammatory bowel disease (IBD), are examples of complex disorders, which include inflammatory and

autoimmune features with prominent intestinal immune dysregulation. Cells of the innate and the adaptive immune system have been identified as the key players of IBD. Cytokines are central components of the inflammatory pathways that take place during the active and chronic phases of IBD. However, a clear picture of these processes is still missing. Since the inflammation is located in the intestinal mucosa, the latter is the main source of biomarkers in IBD allowing various immunological pathways to be explored in the gut. Thus, the determination of cytokine expression profile could help to elucidate the local immune responses during intestinal inflammation. Expression of IBD-related proteins such as cytokines, chemokines, adhesion molecules, and their corresponding cellular and soluble receptors has revealed their significant role in the pro- and anti-inflammatory processes in the inflamed gut mucosa. Indeed, the implication of some cytokines in the immunopathogenesis of IBD is investigated intensively and proved in experimental models of intestinal inflammation. Lack of enough investigation in humans, however, predetermines the need for further studies since it is proved that the common clinical phenotype of colitis may result from largely diverse genetic or immunological backgrounds.

2. Intestinal inflammation and cytokines

Since the pathogenesis of IBD is related to both dysregulated innate and adaptive immune pathways, which contribute to the aberrant intestinal inflammatory response in genetically susceptible individuals, the main focus of research attempts is directed to the initiation, perpetuation, and cessation of gut inflammation associated with IBD [1].

Cytokines are abundantly produced by the cells of the gut-associated immune system maintaining lymphocyte homeostasis under both steady-state and inflammatory conditions. These small, cell-signaling protein molecules act in a paracrine, autocrine, or endocrine manner, coordinate the communication between immune and non-immune cells of the intestinal compartment, and modify acute and chronic inflammatory responses at both local and systemic levels [2]. Moreover, elevation of pro-inflammatory cytokines is considered to be associated with the severity of gut inflammation [3]. Therefore, it is no surprise that cytokines have been a major therapeutic management of IBD [4].

It is believed that dysregulated immune mechanisms are related to T cells in the gut in IBD pathogenesis. Unregulated T lymphocytes activities can lead to autoimmunity, especially during inflammation when they can cause excessive tissue damage [5]. The ability of CD4+ T helper (Th) cells to alter the magnitude and outcome of the intestinal tissue-damaging inflammatory responses is mostly dependent on the production of distinct profiles of cytokines. Traditionally, the lesions in CD patients have been associated with a predominant activation of Th1 cells and production of large quantities of IFNγ under the stimulus of IL-12 through STAT4 signaling. By contrast, the lesions in UC patients were believed to be driven by Th2 cytokines, such as IL-4 and IL-13, through STAT6 activation. In the mouse model of IBD, CD3+ (T cell) depletion results in dramatic reduction of the gross pathology, neutrophil influx, and expression of pro-inflammatory cytokines and chemokines [6]. The cytokine

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expression pattern that strictly follows the polarization model of Th1 versus Th2, however, does not appear to be fully applicable in IBD. Nearly 20 years ago, Mosmann and Coffman concluded their paradigm with the prediction: "… further divisions of helper T cells may have to be recognized before a complete picture of helper T cell function can be obtained" [7, 8]. Indeed, several recent studies had led to the identification of more complex networks of cytokine interaction in IBD tissue, thus shedding light on the role of a distinct subset of T cells in the pathogenesis of IBD—Th17 cells. On the other hand, another T cell subpopulation, namely T regulatory cells (Tregs), is implicated in gut homeostasis and tolerance induction, and it is believed that Th17 and Tregs are in a mutually polarizing relationship [9]. An overview of the main cells and cytokines involved in intestinal inflammation is presented in **Figure 1**.

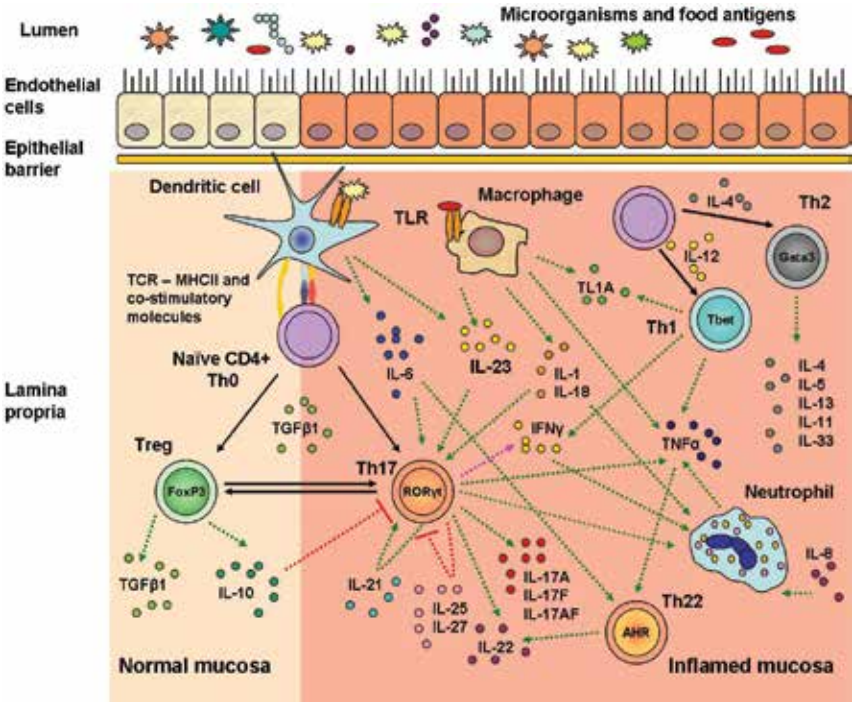

Figure 1. T-helper cells and cytokines interactions in normal and inflamed mucosa of IBD patients. The fate of naïve T cell depends on the interactions with the antigen-presenting cells (i.e. dendritic cells, macrophages) and the secreted cytokines. In normal mucosa, the abundant TGFβ1 directs naïve Th0 cells to Treg differentiation which secrete IL-10 and TGFβ1. "Danger" signals through TLR activation (on antigen-presenting cells), followed by secretion of IL-6, IL-23, IL-1, etc., with the simultaneous presence of TGFβ1, all favor the development of Th17 cells. The latter secrete many cytokines, for example, IL-21 acts as an autocrine positive regulator but IL-25 and IL-27 inhibit Th17 cells in autocrine manner. Th17 cells could also secrete IL-17 cytokines, TNFα, and in special circumstances—IFNγ; thus, Th17 cells play an intermediate role between innate and adaptive immune response, especially during inflammation in the intestinal mucosa. The balance between Th17 cells and Tregs is desired to maintain the immune homeostasis in the gut. However, Tregs and Th17 cells can convert into each other demonstrating same plasticity, depending on the cytokine milieu. Nevertheless, there are other players in the inflamed mucosa such as Th1, Th2, and Th22 cells. Legend: black arrow—cell differentiation; green arrow—secretion; pink arrow—possible secretion; red arrow—inhibition; TCR—T cell receptor; TLR—Toll-like receptor; MHCII—major histocompatibility complex—Class II; TL1A—TNFα-like 1 A.

Thus, the effector response is largely determined by the combination of cytokines that predominate in the intestinal mucosa, and it defines the mucosal T cell immunophenotype in each case [2].

2.1. Innate immune response and related cytokines

Dendritic cells (DCs), macrophages, epithelial cells, and myofibroblasts are able to recognize pathogen-associated molecular patterns (PAMPs) through their pattern-recognition receptors including Toll-like and NOD-like receptors. This recognition results in nuclear factor (NF)-kB activation with gene transcription and production of pro-inflammatory cytokines, such as IL-1 and TNFα, ensuring an effective innate response against microbial antigens. That also triggers antigen presentation, maturation, and up-regulation of co-stimulatory molecules which lead to efficient adaptive immunity involving T cell activation [10]. There is evidence for down-regulated protein level of TLR-3 in IBD, whereas TLR-2 and TLR-4 are up-regulated in intestinal mucosa of active IBD [11]. A specific mutation in NOD2 gene induces loss of NF-kB function during TLR-2 activation with a subsequent increased risk of infection with commensal bacteria and increased susceptibility to the ileal form of CD [12]. Recent studies suggest that increased mucosal permeability in the intestinal mucosa during IBD flare allows infiltration of a large number of granulocytes into the colonic mucosa. These leukocytes are activated, have a prolonged survival time, and release various pro-inflammatory cytokines (e.g. IL-1β, IL-6, TNFα, IL-18), which exacerbate and maintain the inflammation in the gut [13].

2.1.1. TNFα

TNFα links the innate and the adaptive immune responses and has crucial importance in the pathogenesis of IBD by inducing the differentiation of stromal cells into myofibroblasts and promoting their production of matrix metalloproteinases. The latter induce enterocyte apoptosis and digestion of gut basement membrane [10]. TNFα also exerts its pro-inflammatory effect through cytokines such as INFγ, IL-1β, and IL-6 [12].

TNFα is a well-established inflammatory mediator in CD whereas contradictory reports exist in UC [12]. There is a lack of studies on the mucosal expression of TNFα and the prediction of the clinical course, and only a few reports announced the predictive value of mucosal TNFα concentrations and the response to therapy in IBD patients. In fact, increased levels of TNFα and IL-15 have been previously reported in intestinal biopsies from IBD patients in remission without biopsy alterations [14]. Interestingly, the presence of TNFα in non-affected areas of IBD mucosa may not be sufficient to trigger mechanisms of mucosal damage. In preliminary reports, normalizing of mucosal TNFα seemed to predict a longstanding remission after stopping of anti-TNFα therapy in UC [12].

Certain TNFα polymorphisms (i.e. TNFα-308 A allele) are associated with increased serum levels of TNFα and therefore with higher susceptibility of IBD [15].

2.1.2. TNF-like cytokine 1A

TNF-like cytokine 1A (TL1A) is a novel member of TNF superfamily of proteins, produced by endothelial cells, macrophages, lamina propria T cells and plasma cells, monocytes, and monocyte-derived DCs [16]. Association with its functional receptor provides co-stimulatory signals for activation of T lymphocytes, leading to cell proliferation, cytokine secretion, and amplification of pro-inflammatory pathways, as well as induction of apoptosis in target cells [2]. Several studies have clearly demonstrated that TL1A and its receptor are up-regulated at mucosal protein and mRNA levels in IBD patients. TL1A is localized in the lamina propria and shows preferential expression on plasma cells and mucosal DCs. Of great importance is the fact that TL1A was shown to increase IL-13 secretion by natural killer T (NKT) cells, which are considered to be central to the mucosal injury that takes place in UC pathogenesis. Furthermore, TL1A induces IFNγ secretion in synergy with stimulation via TCR or IL-12/IL-18 [2]. TL1A expression is induced by TNFα and IL-1α as well and since the latter are abundantly expressed in the inflamed mucosa of UC patients, they may provide a strong stimulus for enhanced TL1A expression. On the other hand, several microorganisms were shown directly to stimulate TL1A secretion by DCs via TLR-signaling (TLR-4), LPS-induced and NFkB-dependent pathway [16]. Moreover, there is an inhibitory component of the TL1A receptor which could augment pro-inflammatory pathways at the intestinal mucosa by rendering activated lymphocytes resistant to apoptosis. Thus, increased expression of this inhibitory TL1A receptor may offer a survival advantage to effector lymphocytes, preventing their elimination and perpetuating tissue injury [2].

2.1.3. IL-8

IL-8, as a member of the CXC chemokines family, is not only a strong chemoattractant for neutrophils, monocytes, etc. but also triggers the secretion of superoxide anions and lysosomal enzymes in neutrophils, thus contributing to the tissue damage during inflammation. IL-8 mRNA expression in the inflamed mucosa is shown to be significantly higher than the level in non-inflamed mucosa of IBD patients or in the normal mucosa of non-IBD patients [13].

2.2. Th1 profile-related cytokines

Th1 cells are an essential part of the adaptive immune response, mainly against intracellular microorganisms and protozoa. The master transcription factors for Th1 definition are STAT4 and T-bet. Th1 cells in gut mucosa which are induced by increased levels of IL-12 and IL-18 are thought to cause intestinal inflammation in CD patients via production of high amounts of IFNγ. The latter induces enterocyte apoptosis and triggers the release of TNFα by activated mucosal macrophages. Th1 cells by themselves appear as an important source of TNFα [10].

2.2.1. IFNγ

IFNγ is a mediator of intestinal inflammation in CD, but contradictory reports exist for UC. However, increased levels of IFNγ have been observed in the inflamed mucosa from UC

patients too. IFNγ levels also correlated with the clinical activity but not with the endoscopic score in UC, whereas no correlation to the clinical activity was observed in CD patients [12].

2.2.2. IL-12

The role of IL-12 in intestinal inflammation will be discussed later along with IL-23.

2.2.3. IL-1

IL-1 exists in two forms, IL-1α and IL-1β, encoded by different genes but exhibit almost identical functions [16]. The major sources of IL-1 are activated myeloid cells and its production can be induced by bacterial lipopolysaccharide, TNFα, IFNα, IFN-β, IFN-γ, as well as IL-1. IL-1 was found to promote Th17 development in the presence of IL-6 and TGFβ, and also to potentiate their actions in humans but not in mice. Moreover, it has been reported that IL-1 can increase their effect on Th17 definition. However, the mechanism through which IL-1 influences Th17 differentiation is not fully determined yet [17]. Some suggestions include that IL-1β or IL-1α cooperates with IL-23 to enhance IL-17 production independent of TCR stimulation. Additionally, IL-1 may suppress the inhibitory effect which IL-2 exerts on Th17 cell production through induction of IL-1R, IL-23R, and transcription factor RORγt [16].

IL-1β was shown to be increased in CD and UC patients, whereas the IL-1-receptor/IL-1β ratio was negatively associated with the IBD activity. When comparing the IBD patients with controls, a significant variation in genotype frequency of the IL-1β promoter polymorphism was found. Higher levels of the pro-inflammatory cytokine IL-1β would be expected to increase the likelihood of developing IBD since higher levels of such cytokines occur in this disease [15].

2.2.4. IL-18

IL-18 is another member of the IL-1 pro-inflammatory cytokine family. IL-18 is an epithelial-derived cytokine that has been proposed to promote barrier function in the intestine, but its effects on intestinal T cells are poorly understood. Although IL-18 is mainly responsible for inducing IFNγ production and Th1 differentiation, this cytokine might be involved in Th17 cell definition as well. Antigen-presenting cells express IL-18R on their surface and its binding with the cytokine is required for generation of Th17 cells through an IL-23-dependent mechanism. Moreover, IL-18 synergizes with IL-23 in the induction of Th17 cell [16] However, there are more reliable proofs about the involvement of IL-18R in Th17 cell definition, but not for IL-18 itself. Probably this action might be fulfilled through binding of an unknown alternative ligand, distinct from IL-18, to the receptor [17]. In contrast, Maloy et al. [18] demonstrated that during steady state, intestinal epithelial cells constitutively secrete IL-18, which acts directly on IL-18R1-expressing CD4+ T cells to limit colonic Th17 cell differentiation. In addition, they found that IL-18R1 signaling was critical for Tregs-mediated control of intestinal inflammation, though IL-18R1 is not required for Tregs development [18]. Thus, since IL-18 may regulate differentially homeostatic and inflammatory subsets of T cells, this finding has potential for treatment of IBD and other chronic inflammatory disorders.

IL-18 was found elevated in the inflamed colonic mucosa of UC and CD patients and polymorphisms in the IL-18R1-IL-18RAP locus are associated with IBD susceptibility [18]. Moreover, the local expression of IL-18 has been shown to be associated with the grade of inflammation [19].

2.3. Th2 profile-related cytokines

Th2 cells, another important part of the adaptive immune system, are mainly involved in the effector responses against extracellular parasites, including helminths, as well as in allergy pathogenesis. They are defined by the transcription factors STAT6 and GATA3 [7]. The importance of Th2 response in IBD is still under debate. In UC, the inflammatory response is less skewed along specific pathways, even though there is enhanced production of IL-4, IL-5, and IL-13, cytokines made by Th2 cells, unlike CD where Th1 activation has been mainly employed in pathogenesis [20].

2.3.1. IL-13

IL-13 exerts the potential to increase intestinal permeability and induce both enterocyte differentiation and apoptosis. IL-13 is released mainly by Th2 cells but another source of that cytokine is NKT cells. NKT cells express surface CD161 but not invariant T cell receptor, which is a well-established characteristic of this population. They produce IL-13 in response to stimulation of antigen-presenting cells expressing surface CD1d. Most probably, these atypical NKT cells are stimulated to produce IL-13 in the colonic mucosa by flora-derived microbial products [2]. This was observed in patients with UC, but not in CD patients. Further studies revealed that CD161-expressing NKT cells showed IL-13-dependent cytotoxic activity against colon epithelial cells [2]. Moreover, IL-13 independently exerts harmful effects on epithelial barrier function, such as derangement of tight junction integrity, decreased restitution velocity, etc. [2]. Therefore, blockade of IL-13 downstream signaling may be an effective anti-inflammatory approach in UC which requires further investigations.

2.3.2. IL-11

IL-11 is a member of the IL-6 cytokine family and exerts pleiotropic effects on various cell types as it acts synergistically with other cytokines such as IL-3 and IL-4, thus it has been implicated in Th2-mediated sensitization and inflammation. IL-11 also prevents cell death and inhibits inflammation at sites of tissue injury. IL-11 mediates anti-inflammatory effects by down-regulation of LPS-induced NFkB activation, thus preventing transcription of inflammatory genes [12]. This may be implemented in IBD therapy, but still needs additional verification.

2.3.3. IL-33

IL-33 is the latest identified member of the IL-1 family of cytokines. mRNA and protein expression of IL-33 was detected in normal colonic cells both at the surface epithelium and in crypts, as well as in inflamed bowel onto lamina propria mononuclear cells (CD11b+ monocytes/macrophages and CD19+ B cells), endothelial cells, and subepithelial myofibroblasts.

During active intestinal inflammation, IL-33 actively participates in the epithelial-immune cell crosstalk that takes place in IBD mucosa. IL-33 expression is augmented under stimulation with IL-1β and TNFα, two cytokines that are enriched at the inflamed mucosa and are of pathogenic relevance in UC, as well as after TLR-3 and TGFβ signals [2].

Regarding mucosal expression, up-regulation of IL-33 appears to be specific for UC, as it was not observed in CD colonic inflammation [2]. Moreover, IL-33-expressing myofibroblasts were absent in fissuring areas in patients with colonic CD. Therefore, these observations may provide information of distinctive pathway between the two forms of IBD [2].

IL-33 was shown also to induce particularly the expression of Th2 effector molecules IL-5 and IL-13. Given the central role of IL-13 in UC, IL-33 may be involved in UC pathogenesis through the induction of IL-13 secretion. It has been proposed that IL-33 may function as "alarmin" for the gut-associated immune system activating toward intestinal inflammation or perpetuating the ongoing one [2].

2.4. Prerequisite cytokines for Th17 development

To emphasize the importance of Th17 in intestinal inflammation, here we start with the description of the prerequisite cytokines for the development of Th17 cells from naïve T cells.

2.4.1. TGFβ1

Transforming growth factor β (TGFβ) is a potent cytokine with multi-faceted regulatory and inhibitory activities and has two forms—TGFβ1 and TGFβ2. TGFβ1 is a pleiotropic cytokine best known for its potential to induce peripheral tolerance in the absence of IL-6 [12]. One of the mechanisms by which TGFβ1 is able to maintain tolerance is to support the survival of naturally occurring FoxP3+ Tregs (nTregs) in thymus. In addition, along with IL-2 and retinoic acid, TGFβ1 promotes the differentiation of induced Tregs (iTregs). Another mechanism of TGFβ-induced tolerance is to suppress the innate immune cells such as DCs and NK cells [5].

TGFβ1 also regulates the development of resident macrophages in the normal intestine, which possess some unusual features such as constitutive production of IL-10 and TNFα, refractory to TLR stimulation, high expression of MHCII and CXCR1, and avid phagocytic activity. Thus, this is another mechanism through which TGFβ1 favours local homeostasis [21].

TGFβ1 plays an important role under inflammatory conditions. In the presence of IL-6, TGFβ1 drives the differentiation of Th17 cells which promotes further inflammation and augmentation of ongoing autoimmune conditions. In addition, TGFβ1 in combination with IL-4 promotes the differentiation of IL-9-producing and IL-10-producing T cells, which surprisingly lack suppressive function and also promote tissue inflammation [12]. Increased protein levels of TGFβ1 are found in the mucosa of both CD and UC patients, whose levels correlated with the severity of disease in CD but not in UC patients [5, 12]. We also found significantly higher gene and protein levels of TGFß1 in the inflamed mucosa of CD patients alone [22]. This is not surprising since the tissue remodeling properties of TGFβ1 are well-established. Interestingly, TGFβ1 orchestrates the differentiation of both Tregs and Th17 cells

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in a concentration-dependent manner—low doses induce Th17 cell differentiation while higher doses inhibit Th17 cell development and promote Tregs [5, 11].

2.4.2. IL-6

IL-6 is a pleiotropic cytokine with regulatory effects on inflammation development. In addition to its stimulatory effects (i.e. induction of acute phase proteins), IL-6 also has inhibitory functions (i.e. cessation of the antiviral antibody response after certain immunizations). Recent studies have demonstrated that IL-6 has a crucial role in the regulation of the balance between Th17 cells and Tregs [23]. IL-6 activates a receptor complex consisting of IL-6R and the signal transducing subunit gp130 which activates downstream STAT1 and STAT3. STAT3 regulates IL-6-induced expression of RORγt and RORα, the crucial transcription factors for Th17 cells. In contrast to STAT3 activation, STAT1 inhibits the development of Th17 cells. Although IL-6 activates both STAT1 and STAT3, it has been demonstrated that in Th17 cell activation, they play two different roles—STAT3 maintains while STAT1 suppresses it [23]. Furthermore, STAT family members activated by various cytokines provide both positive and negative regulation for Th17 development (i.e. IL-27 inhibits Th17 differentiation through STAT1) [23]. TGFβ1 can induce gene activation of both FoxP3 and RORγt, but FoxP3 is able to associate with RORγt, thus inhibiting its transcriptional activation. Nevertheless, in the presence of IL-6 this inhibition is abrogated, so IL-6 could act as a potent promoter of Th17 instead of Tregs differentiation. All facts taken together, IL-6 appears as the main partner of TGFβ in priming naïve T cells to IL-17 production, playing a pivotal role in Th17 polarization and initiation of inflammatory immune response. Currently, it is also accepted that IL-6 is able to induce expression of IL-23R in T cells, making them responsive to IL-23 which sustains the Th17 phenotype [17].

Increased levels of IL-6 and its soluble receptor are up-regulated in active CD patients, and mucosal IL-6 levels were correlated with the degree of clinical activity in CD and UC [12]. In consent with these findings, in a group of 37 IBD patients, we also found both mRNA transcripts of TGFβ1 and IL-6 up-regulated in patients' mucosa compared to the mucosa of non-IBD persons, along with increased IL-17 mRNA in inflamed tissue [22, 24].

Several polymorphisms regarding the IL-6 gene are described to be also associated with susceptibility to IBD development, such as IL-6 174 [15].

Although anti-IL-6 antibodies therapy has become a novel therapeutic strategy for some inflammatory and autoimmune disease, including CD, IL-6 inhibitory treatment acts primarily on initial CD4+ T cells response including Th17 differentiation, rather than on the effector phase [23]. However, it still remains controversial whether this antibody can inhibit Th17 differentiation in a manner that is clinically meaningful.

2.4.3. IL-23

IL-12 and IL-23 share the common p40 subunit, but whereas IL-12 drives the classical Th1 response characterized by IFNγ production, IL-23 maintains an IL-17-secreting T cell population. Th1 responses may develop normally in the absence of IL-23, but in IBD patients, their manifestations require the presence of IL-23. The systemic inflammatory response and the

elevated concentrations of pro-inflammatory cytokines in the serum are driven by IL-12 while the local intestinal inflammation and production of IL-17 in the intestinal mucosa are controlled by IL-23 [11, 12, 25].

IL-23 is crucial in orchestrating the crosstalk between innate and adaptive immunity with a key role in driving early responses to microbes. In a recent study, Kamada et al. showed that IL-23 is secreted preferentially by a subset of sentinel mucosal cells expressing both macrophage (i.e. CD14, CD33, CD68) and DC markers (i.e. CD205, CD209) [26]. These cells are present in a large number in CD-involved tissue and produce IL-12 and IL-23 in response to environmental danger signals [8, 26]. The presence of pathogens or pathogen-related products (such as lipopolysaccharide) can strongly influence the production of IL-12 and/or IL-23 depending on the microbial agent. This happens within a few hours after exposure and these early events in pathogen encounter are likely to shape subsequent responses toward IL-12 or IL-23 expression [8]. It was shown that some of the pathogenic functions of IL-23 in the gut are mediated by atypical T cell populations, such as γδT cells, invariant NK cells, and innate lymphoid cells, inducing them to secrete Th17-related cytokines and contributing to intestinal inflammation [10]. IL-23 might be also closely associated with the neutrophil influx [12].

The precise function of IL-23 in Th17 regulation is still not entirely clear, although there are a lot of speculations. IL-23 failed to induce the differentiation of naïve T cells into Th17 cells due to lack of IL-23R on naïve T cells [16]. It was subsequently demonstrated that IL-23R is not expressed on naïve T cells. Instead, IL-23 acts as a survival signal for Th17 cells by the mechanism probably similar to TNFα [23, 27].

The synthesis of the common p40 subunit for both IL-12 and IL-23, and the functional heterodimeric IL-23 is enhanced in the gut of CD patients [11]. Along with other authors' findings, we detected up-regulated mRNA levels of IL-23 in inflamed mucosa, as well as significantly increased serum level of IL-23 among IBD patients in comparison with non-IBD persons [24], and we suggest that anti-IL-23 therapy could be beneficial for IBD patients.

Identification of multiple single nucleotide polymorphisms (SNPs) in the IL-23 receptor gene that has been associated with both UC and CD suggested that the IL-23 axis might play a central role in chronic inflammation. IL-23R SNPs that influence IBD susceptibility have provided a new picture of the way the local immune response can promote intestinal tissue damage [11]. Small differences in cytokine levels as a result of gene polymorphisms may have an important effect on the inflammatory response and thus, influence the pathophysiology of IBD [15]. Interestingly, one of these polymorphisms, Arg381Gln, confers protection against developing CD [20]. Nonetheless, the mechanism through which these SNPs confer either risk or protection from IBD remains unknown [15].

2.5. Th17 cells and produced cytokines

The discovery of an IL-23-dependent T cell population that produces IL-17 but not IFNγ or IL-4 suggested there is an additional Th cell subset. Th17 cells have derived their name from their ability to produce IL-17, also termed IL-17A. Th17 cells also produce other cytokines including IL-17F, IL-21, IL-22, TNFα, and IL-6 [17, 23]. However, analysis at the single cell level

has revealed that not all Th17 cells secrete the whole spectrum of cytokines, probably reflecting the heterogeneity of this cell's subset [25]. The IL-17 cytokine family also includes IL-17B, IL-17C, IL-17D (IL-27), IL-17E (IL-25), and IL-17A/F (**Figure 1**). The cytokines IL-27 and IL-25 have lowest protein homology to IL-17A. They are not produced by Th17 cells but act as negative regulators on the Th17 subset development. IL-27 is structurally related to IL-6 and is able to attenuate chronic inflammation by promoting IL-10 production [17]. In line with this, IL-27 and IFNγ are responsible for the inhibition of Th17 development in a STAT1-dependent manner [23], as described above. Another negative regulator of Th17 cells is IL-25, identified as a genetic homologue of IL-17, produced by Th2 and mast cells. IL-25 is involved in the expression of the Th2 cytokines IL-5 and IL-13, thus, favors Th2 responses. IL-25 deficiency is involved in pathologic inflammation, associated with increased expression of IL-17 and IL-23 [17].

CCR6, presented not only on Th17 cells, but also on Tregs, B cells, neutrophils and immature DC, plays a critical role in the migration of these cells to the sites of inflammation. TGFβ1 was shown to be the main factor for induction of CCR6 mRNA expression in Th17 cells and DCs [19]. IL-17-producing Th memory cells selectively express both CCR6 and CCR4, unlike Th cells producing IFNγ or both IFNγ and IL-17 which express CCR6 and CXCR3 [16]. Indeed, CCR4 is important for homing to the gut, where most RORγt+IL-17+ T cells are found [16].

The relationship among Th1, Th2, and Th17 cells is complex and still not clear. Th1- and Th2-related cytokines inhibit Th17 cell differentiation while IL-17 is not able to suppress Th1 or Th2 cells, or does it weakly. The suppression of IFNγ and IL-4 or their absence represents a way by which TGFβ1 could promote Th17 cell development. TGFβ1-driven Th17 cell differentiation can also occur in the absence of IFNγ and IL-4 [11]. In parallel with these findings, it was reported that IL-17-producing cells could be generated independent of the specific cytokines and transcription factors required for Th1 and Th2 differentiation [17]. Moreover, Th17 cells could develop from naïve T cells only in the combined presence of IL-6 and TGFβ1 [12, 20]. Thus, TGFβ induction of Th17 cells and also of Tregs, which are usually contradictory acting, is dependent on the presence of IL-6. This explains the apparent discrepancy of TGFβ1 involvement in both anti- and pro-inflammatory events in the intestine mucosa [17].

2.5.1. IL-17

IL-17 is an effector cytokine in gut immunity, which may have either pro-inflammatory or tissue-protective effects in the mucosa depending on the experimental or clinical model used. On one hand, IL-17 contributes to the mucosal barrier function by several mechanisms which, upon activation, result in a mucosal immune response toward pathogens [6]. IL-17 also promotes tight junction formation and increases trans-epithelial resistance in polarized intestinal epithelial cells by stimulating the production of antimicrobial peptides such as lipocalin-2, β-defensins, and calprotectin. This suggests that the latter are involved in the maintenance of immunological homeostasis and/or in the control of specific inflammatory pathways [19]. Thus, the Th-17-related cytokines mediate protective effects in host gut against various bacteria and fungi, particularly at mucosal surfaces [10, 11]. Interestingly, pathogens that have evolved to take advantage of various aspects of the mucosal response gain an edge

over the resident commensal bacteria and colonize the gut with priority. Despite that Th17 responses appear to be detrimental by promoting pathogen colonization of the mucosa, in the end, they result in decrease in bacterial dissemination from the mucosa that protects the host by inducing slight inflammation [6]. In line with this, it was shown that Th17 cells are constitutively present in the human and mouse intestinal mucosa and that Th phenotype is driven by the commensal bacteria in the gut. Additionally, stimulation of DCs with TLR ligands (e.g. fungal Dectin-1) induces synthesis of IL-6, TNFα, and IL-23 that promotes the differentiation of Th17 cells [11]. From this point of view, blocking Th-17 cytokines could have more deleterious than beneficial effects for the host [25].

On the other hand, IL-17 might mediate tissue inflammation by triggering several inflammatory pathways and by inducing various pro-inflammatory cytokines (e.g. IL-1, IL-6, TNFα, G-CSF, GM-CSF), chemokines (e.g. IL-18, CXCL-1, CXCL8, MIP-1), and enzymes (COX-2, matrix metalloproteinases). Both IL-17 and IL-22 stimulate granulopoiesis by inducing expression of the granulocyte colony stimulating factor (G-CSF) and IL-17A which rapidly recruits neutrophils to the inflammatory site. This mechanism has important evolutionary significance [25]. The neutrophil response gains time for the induction of the following antimicrobial Th1-IFNγ response which takes several days to develop. Once the appropriate immune effector functions occur, the IL-12/IFNγ axis becomes the dominant pathway in host defence. This is important for initial control of the infection, but if the IL-23/IL-17 immune pathway becomes dysregulated, there is a danger of autoimmune pathology development, such as IBD. These observations, including the fact that T-bet is expressed at lower level in Th17 cells, led McKenzie et al. to favour the hypothesis of a common lineage precursor of Th1 and Th17 cells [8]. Furthermore, the tissue localization and timing of IL-12 versus IL-23 responses explain the idea that IL-12/IFNγ axis is involved in systemic inflammatory conditions (such as lupus), whereas the IL-23/IL-17 axis appears to regulate tissue-specific disorders (such as IBD) [8].

Another layer of complexity to the mucosal existence of Th17 cells is other cell types, which can secrete IL-17-related cytokines: $\gamma\delta$T cells (secreting IL-17 in response to IL-23), NK, NKT cells (able to produce IL-17 and IL-22), and DCs (can secrete IL-22 in response to bacterial infection). Paneth cells, which are common in the ileum, also secrete IL-17A [6, 19]. As all these cells express the IL-23 receptor, the secretion of IL-23 by DCs comprises a trigger which potentiates early T cell activation and adaptive immunity development [6]. Thus, it appears that early activation of both adaptive and atypical innate-like T cells can lead to the expression of IL-17 and IL-22. However, dysregulated production of IL-17, IL-22, and TNFα in local tissue can result in chronic immune-mediated tissue destruction [8].

Studies in murine models of IBD strongly suggest that Th17 cells and their related cytokines contribute to tissue-damaging immune responses in the gut [25]. Up-regulation of Th17-related cytokines, however, does not represent a specific hallmark of IBD in humans, as increased levels of IL-17A and other Th17-related-markers have been seen in patients with rheumatoid arthritis, multiple sclerosis, psoriasis, etc. [11]. Immunohistochemistry studies have shown that in active UC, the IL-17-expressing cells were located mainly within the lamina propria, while in active CD, these cells were scattered throughout the submucosa and muscularis propria of the gut. Corresponding with this, it was shown that RNA transcripts for IL-17A and IL-17F

were up-regulated in the inflamed mucosa of UC and CD patients [3, 11, 22, 28]. Both IL-17 and IL-23 are correlated to the severity of UC [12]. More recently, Annunziato et al. demonstrated that the number of IL-17-producing T cells is higher in CD than in normal gut mucosa, and some of these cells also produce IFNγ [29].

Th17 cells have shown possession of functional plasticity. Some of the IL-17A-producing cells simultaneously express IFNγ (**Figure 1**). Majority of IL-17/ IFNγ-producing cells express CD161, a well-known marker of NKT cells, also identified recently on IL-17-producing memory T cells [11]. Th17 cells can be converted into Th1 cells if they receive appropriate stimuli, such as IL-12 which enhances the expression of Th1-related markers (i.e. T-bet and IFNγ) and down-regulates RORγt and IL-17. Additionally, recent studies have shown that treatment of intestinal lymphocytes with IL-23 can facilitate the production of either IL-17A or IFNγ in UC or CD, respectively [11].

This is in consent with the demonstration that some of the pathogenic effects of IL-23 in the gut are linked to the ability of this cytokine to turn on IFNγ production. Switching from IL-17A to IFNγ production occurs if Th17 cells are activated by a lack of TGFβ1 [25]. Th17 cells and their possible conversion to Treg direction is going to be described later.

This very complex and non-equivocal relationship of both pro-inflammatory and tissue-protective effects of IL-17 in the gut may explain the unsuccessful anti-IL-17 therapy in CD patients [10].

2.5.2. IL-21

IL-21, an IL-2-related cytokine produced by Th17 cells in response to IL-6, increases the expansion of this cell subtype by a positive autoregulatory feedback loop. IL-21, which is up-regulated in inflamed IBD mucosa, induces Th1 and Th17 immune responses in the mucosa [10], but a mixture of both Th1 and Th17 cytokines is needed to promote full pathology in the gut. In this context, a promising inducer could be IL-21, whose activity seems to be necessary for expanding both Th1 and Th17 cell responses in the intestine. [25]. As we have already noticed, IL-21 is overproduced in the gut mucosa of IBD patients, but the vast majority of IL-21-producing CD4+ T cells co-express IFNγ but not IL-17A. This fact suggests that Th1 but not Th17 cells are the major sources of IL-21 in the human gut [11]. There is evidence that IL-21 also enhances the expression of Th1-related transcriptional factors and IFNγ production in NK cells [11].

IL-21, like IL-17, stimulates gut fibroblasts to produce tissue-degrading matrix-metalloproteinases and enhances the secretion of chemoattractants (i.e. MIP-3α) by epithelial cells [10, 11]. IL-21, like IL-6, could also initiate Th17 differentiation together with TGFβ1 [23], even in the absence of IL-6 [16, 17]. IL-21 enhances the expression of IL-23R in Th17 cells, through a process that is dependent on STAT3 and RORγt, making these cells responsive to IL-23. IL-21 as well exerts additional biological functions that could contribute to its pro-inflammatory effect in the gut like inhibition of the peripheral differentiation of Tregs and making CD4+ T cells resistant to Treg-mediated immune suppression [11].

2.5.3. IL-22

IL-22 is a member of the IL-10 cytokine family and a Th17-related cytokine but it appears to be differentially regulated. IL-22 provides signals through a heterodimer comprising IL-22R and IL-10Rβ. The IL-22 receptor is highly expressed in tissues such as epithelial cells of the gastrointestinal tract. Via STAT3 signaling pathway, the activation of proliferative and/or anti-apoptotic programs starts, and this allows maintenance of epithelial barriers of the gut [5]. Most of the Th17 cytokines are highly dependent on the transcription factor RORγt for their expression, unlike IL-22 whose expression is dependent on the transcription factor aryl hydrocarbon receptor [5]. Th22 cells are another Th subpopulation characterized by the expression of this transcription factor and secretion of mainly IL-22 [5].

IL-22 has a dual functional nature in modulating the responses during tissue remodeling. IL-22 promotes induction of acute inflammatory proteins, mucins, and antimicrobial peptides (i.e. β-defensins), which are important for tissue integrity during inflammation. This mechanism ensures proper organ function and escape of potentially harmful effects by restricting the passage of luminal commensal flora and food antigens to the lamina propria [5, 25, 30]. It is important to point out that this process depends on the inflammatory context (the overall cytokine milieu and the tissues involved). Thus, IL-22 is important for control of pathogenic bacteria that need to translocate through host epithelial barriers to disseminate, especially in the gastrointestinal tract [5]. IL-22 also enhances intestinal barrier integrity by stimulating epithelial cell growth, goblet cell restitution, and mucus production, thus contributing to the healing of damaged tissue.

On the other hand, IL-22 can cause further inflammation by stimulating colonic fibroblasts to secrete inflammatory cytokines (e.g. TNFα, IL-8, IL-11, and leukaemia inhibitory factor), IL-6, chemokines, and matrix metalloproteinases [11]. It is not surprising that IL-22 is highly expressed during chronic inflammation [5] in mucosal samples of patients with active CD, because of the known dysbacteriosis and expected pathological microbial agents, and to a lesser degree in patients with UC, where autoimmune phenomena are more common.

IL-22 is also expressed by innate immune cells such as CD11c+ and NK cells located in the colon. The latter cells do not secrete IFNγ and are not highly cytotoxic [30]. IL-23, a traditional activator of NK cells, induces IL-22 expression in NK cells. Unlike TGFβ and IL-10 that directly modulate the immune response, IL-22 does not have direct effects on immune cells since these cells lack the expression of IL-22R [30]. This way, TGFβ1 and IL-10 are involved in maintaining immune homeostasis under steady-state conditions instead.

IL-22 is an ideal therapeutic candidate since it specifically modulates tissue remodeling and does not have direct effects on the immune response. Treatment with recombinant cytokine or gene therapy delivery of IL-22 may alleviate tissue destruction during inflammation owing to its selective modulation of tissue responses [5].

2.6. Role of FoxP3+ Tregs and related cytokines in gut inflammation

The main function of Tregs is to modulate the adaptive immune responses, and forkhead/winged helix transcription factor forkhead box P3 (FoxP3) is the master transcription factor

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for Tregs [23]. Two main subpopulations of Tregs have been best described: naïve (nTregs) and inducible Tregs (iTregs). The latter is believed to be derived by peripheral transformation of naïve T cells stimulated by IL-19, vitamin D3, antigens, and TGFβ1. So far, Treg function in IBD is not completely characterized [12].

Tregs are crucially involved in the maintenance of gut mucosal homeostasis by suppressing abnormal immune responses against the commensal flora or dietary antigens. They exert their function by producing the anti-inflammatory cytokines IL-10 and TGFβ, thus preventing both the activation and the effector function of T cells. Additionally, the regulatory activities of the immune response through mediators such as IL-10 and TGFβ still need to be profiled, especially those that might take place in the unaffected areas of IBD patients [14]. A certain number of Th17 and CD4+CD25+FoxP3+ Tregs cell is presented in the intestine even in the healthy state, partly due to the presence of enteric bacteria which favor the production of both Th17 and Tregs. DCs in the intestine or mesenteric lymph nodes also actively promote the production of both cell types. However, there are points of divergence, for example, the retinoic acid produced by DCs in the intestine induces only Tregs. In spite of the essential function of IL-2 as a growth factor of effector T cells, including Tregs, IL-2 has an inhibitory effect on Th17 cell production. Furthermore, IL-2 deficiency leads to systemic autoimmune disease, partly because of its involvement in the differentiation and survival of Tregs [16]. Recent studies have revealed that IL-2 deficiency promotes differentiation of Th17 cell subset in a STAT5-dependent mechanism. At present, the recognized precise mechanism is exerted by suppression of IL-17 expression by directly binding to the IL-17 gene promoter of STAT5 [16].

The importance of Tregs in maintaining immune homeostasis was once again emphasized with the X-linked IPEX syndrome (immune dysregulation, polyendocrinopathy, enteropathy), caused by mutation of FoxP3. IPEX patients quite often complain of gastrointestinal symptoms, suggesting that Tregs dysfunction may be involved in human IBD too [31].

A significant increase in production of Tregs in active-phase IBD mucosal lesions, as well as decreased numbers of Tregs in peripheral blood of IBD patients was described [9]. However, in active IBD a reduced number of peripheral Tregs have been reported to be reverted by anti-TNF treatment [12]. Indeed, Tregs are increased in the intestinal mucosa of IBD patients in comparison with the mucosa of healthy volunteers [22, 24, 27, 32]. Tregs isolated from inflamed tissue display no obvious defect in their suppressive function, at least in vitro [9]. However, Monteleone et al. found that Tregs obtained from the active-phase IBD mucosal lesions possess an ability to suppress T cell activation [11, 25]. Since Th17 cells appear to be resistant to the Tregs-mediated immunosuppression, it is likely that during chronic inflammatory process, such as in IBD, Tregs may be dysfunctional and might augment rather than suppress Th17-mediated immune responses [11]. At first, this phenomenon was explained as a feedback loop associated with an increase in the Treg cell attracted by IL-2 which is produced locally at sites of inflammation. On the other hand, however, up-regulated Th17 cells in response to increased production of pro-inflammatory cytokines were postulated [27]. Th17 cells, but not Tregs, are induced in the presence of pro-inflammatory cytokines, in addition to TGFβ1. Thus, Treg dysfunction may not be intrinsic but rather due to extrinsic milieu of activated cells that are resistant to suppression, and pro-inflammatory settings in the affected IBD mucosa [9, 33].

Plasticity of Tregs and Th17 is further demonstrated by the possibility of conversion between both subsets [27, 33]. Hu et al. have reported that Tregs express membrane-bound TGFβ and in the presence of IL-6, they convert to Th17 cells [34]. This could be an important warning regarding cell therapy with Tregs to treat chronic immune disease, including IBD, because the "homeostatic" Tregs may convert to pathogenic Th17 cells during inflammation where IL-6 is abundant [27]. Numerous studies have shown that in inflammatory cytokine environment, Tregs can lose FoxP3 expression and acquire expression of other transcription factors that define another lineage of CD4+ T cells as well as effector function. As we have already mentioned, exposure of Tregs to IL-6 results in a partial conversion to Th17 cells. Interestingly, although most IL-17-producing cells lost FoxP3 expression, some cells express both FoxP3 and IL-17. It is unclear, however, whether the resultant cells are suppressive [9]. So, once again it must be mentioned that the Th17/Tregs balance appears to play a very crucial role in IBD development [27].

2.6.1. IL-10

IL-10 is secreted by many types of immune cells including Th2, Tregs, Tr1 (IL-10-producing FoxP3-CD4+ T cells), Th3 (TGFβ and IL-10-producing CD4+ T cells induced in oral tolerance), NKT cells, B cells, macrophages, and DCs [5]. IL-10 binds to its heterodimeric receptor, composed of unique for IL-10 subunit (IL-10Rα) and shared with IL-22 subunit (IL-10Rβ). Although not completely sufficient, STAT3 is required for the inhibitory functions of IL-10. Importantly, STAT3 induces the expression of transcription factors that regulate various cytokine signaling pathways including IL-6. IL-10 down-regulates IL-12 production and expression of co-stimulatory molecules in macrophages and DCs, thereby reducing the Th1 response generation [5].

IL-10 is a key regulator of the immune system by limiting the inflammatory responses that could otherwise cause tissue damage. IL-10 is essential for homeostasis of the immune system, especially in the gastrointestinal tract where the tolerance is most needed. Evidence for that is the highly-susceptible-to-colitis IL-10-deficient mice which develop aberrant immune responses to commensal bacteria. This colitis is more severe when combined with a deficiency in TGFβ signaling [5].

Small intestine and colonic lamina propria showed the highest frequency of IL-10-expressing cells. Recent findings show that macrophages in the lamina propria preferentially induce IL-10-producing cells while DCs promote the generation of Th17 cells. On one hand, blocking IL-10 during infection can result in more severe pathology or even fatality of the host, but on the other hand, high production of IL-10 is associated with sustained chronic infections and its blockade promotes pathogen clearance. Thus, once again, the milieu of the intestines favors the generation of IL-10-producing T cells leading to tolerance against commensal bacteria, whereas the expression of IL-10 in peripheral tissues under infectious conditions leads to suppression of the immune response [5]. In line with this, when IL-10 was previously found to be abundantly expressed by macrophages in areas of dense inflammatory infiltrate, it had been directly related to the attenuation of the mucosal inflammation [14]. Knowing nowadays

about the dual role of IL-10, it is not unexpected that IL-10 is presented at a higher level in the inflamed mucosa of IBD patients [13]. These findings were confirmed by us as well [24].

Some IL-10 gene polymorphisms have been associated with susceptibility to IBD (i.e. IL-10—1082) and more significantly with UC alone. Whether the polymorphisms are directly involved in regulating cytokine production, and consequently disease pathophysiology of IBD, or serve merely as markers that are in linkage disequilibrium with susceptibility genes, is still unclear [15].

The involvement of IL-10 in the regulation of the pathogenic function of Th17 cells has been definitively demonstrated in experiments where non-pathogenic Th17 subtype expressing IL-10 is generated by IL-6 and TGFβ1, even though in the absence of IL-23. These cells also prevent the induction of the disease in an IL-10-dependent manner [35]. Even though IL-10 effectively treats colitis in mouse models and suppresses inflammatory cytokine production in vitro in intestinal cells of patients with IBD, clinical trials using recombinant IL-10 to treat IBD in humans have been largely disappointing, irrespective of the acceptable side-effect profile of the therapy [36].

2.7. Role of innate lymphoid cells in gut inflammation

Innate lymphoid cells (ILCs) are recently described cells that have been involved in both maintenance and loss of gut homeostasis. ILCs are phenotypically and functionally distinct subsets of cells that inhabit the intestinal mucosa. However, they produce cytokines associated with effector T-cell responses early in inflammatory lesions of patients with IBD [37]. The novel family of cells comprises three subsets: ILC1, ILC2, and ILC3 [38]. ILC1 express the transcription factor T-bet resembling Th1 cells with production of IFN-γ and TNF; thus, they contribute to host resistance to intestinal pathogens. ILC2 produce Th2 cytokines, such as IL-5 and IL-13, and they are dependent on the transcription factor GATA-3. ILC3 which express the transcription factor RORγt produce IL-17A and IL-22 mirroring Th17 cells [37]. ILC3 is involved in gut homeostasis by secreting IL-22 and promoting IL-10 and antimicrobial peptide production. Epithelial stress-induced ligands and inflammatory conditions may switch ILC3 to ILC1 secreting TNF and IFN-γ under the influence of IL-12. The pro-inflammatory cytokines of ILC1 and ILC3 lead mainly to epithelial apoptosis and neutrophil recruitment. ILC2 are able to contribute to IBD complications by producing the fibrogenic cytokine IL-13 [37].

Since ILCs might be substantial drivers of mucosal inflammation, targeting ILC subsets may be a new exciting treatment option for IBD patients.

3. Conclusion

From a clinical perspective, IBD is a chronic persistent disease characterized by repeated relapses and remissions. One explanation could be that memory Th cells created during the disease development persist in the body, including during remission, in a manner that is dependent on the various cytokine presentations. Effector cytokines in the mucosa may induce

inflammation at the time of the initial episode and during relapses. However, the ambiguity and contradictory actions of given cytokines confound the understanding of their interactions in dynamics of the immune response, and that leads to lack of synonymous conclusions about them. There is still strong need for further investigation, particularly in the gut mucosa, to fully comprehend their roles in the complex dynamic network of the immune mediators.

Th17 cells have been shown to play a central role in murine and human IBD. Inhibition of the Th17 pathway may be a promising treatment for IBD, with respect to the role of other subsets of Th1 and Th2 cells. The data in the literature and our own experience make us believe that in order to achieve immune homeostasis in the gut, pro-inflammatory and anti-inflammatory responses that define the mucosal cell immunophenotype, should achieve balance. Thus, following the clinical periods of remissions and relapses, it is important to observe their immunological equivalents in the gut and possibly in whole blood, namely regulatory and pro-inflammatory cytokines secreted by different types of immunocompetent cells.

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Author details

Tsvetelina Velikova[1*], Dobroslav Kyurkchiev[1], Ekaterina Ivanova-Todorova[1], Zoya Spassova[2], Spaska Stanilova[3] and Iskra Altankova[4]

*Address all correspondence to: ts_velikova@abv.bg

1 Department of Clinical Laboratory and Clinical Immunology, Medical University of Sofia, University Hospital St. Ivan Rilski, Sofia, Bulgaria

2 Clinic of Gastroenterology, University Hospital St. Ivan Rilski, Medical University of Sofia, Sofia, Bulgaria

3 Department of Molecular Biology, Immunology and Medical Genetics, Medical Faculty of Trakia University, Stara Zagora, Bulgaria

4 Clinical Immunology, University Hospital Lozenets, Sofia University, Sofia, Bulgaria

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Chapter 5

Interleukin 23 in IBD Pathogenesis

Ahmet Eken and Mohamed Oukka

Additional information is available at the end of the chapter

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Abstract

Interleukin-23 **(**IL-23) is a cytokine that belongs to the IL-12 cytokine family that is produced mainly by antigen-presenting cells. IL-23 receptor is expressed by various innate and adaptive immune cells, including group 3 innate lymphoid cells (ILC3), neutrophils, γδ T cells, Th17 and natural killer T (NKT) cells. IL-23 regulates various functions of the responding cells critical for host protective responses but is also implicated in many chronic inflammatory diseases including inflammatory bowel diseases (IBD). IL-23 receptor signaling components and downstream effector cytokines IL-17A/F, interferon-gamma (IFN-γ), IL-22, granulocyte macrophage colony–stimulating factor (GMCSF) have been shown to impact IBD-like disease development in various animal models; therapeutic approaches targeting the IL-23 pathway in IBD are in clinical trials. In this chapter, we attempt to review the literature on IL-23–mediated IBD pathogenesis. We did this by gathering the current information about the individual IL-23–producing and IL-23–responsive cells as to how they contribute to IBD pathology through various inflammatory mediators.

Keywords: IL-23, p19, Th17, ILC3

1. Introduction

1.1. IL-23 cytokine

Interleukin-23 (IL-23) is a heterodimeric cytokine that belongs to the IL-12 family cytokines and shares both ligand and receptor subunits with IL-12. IL-23 heterodimer is made up of p19 (IL-23A) and the shared beta chain, p40 (IL12β) subunit which also dimerizes with IL-12p35 and makes up IL-12 cytokine. Due to the shared use of p40, studies performed via the manipulation of p40 prior to the discovery of IL-23 suggested causality between many chronic inflammatory conditions and the IL-12/Th1 axis. With the genetic and immunologic

studies that targeted individual subunits of IL-12 and IL-23 in mice, a critical causal role for IL-23 in inflammatory bowel disease (IBD) pathogenesis has been established.

1.2. General features of IL-23 protein structure

Human p19 is a four-α-helix protein with 70% similarity with its mouse ortholog. It is encoded by its gene located on chromosome 12q13.2 which is composed of four exons and three introns. p19 protein contains five cysteine residues and several O-glycosylation but no N-glycosylation sites. Human p40 gene, however, is located on chromosome 11q1.3. It is made up of eight exons and seven introns. p40 has homology with soluble class I cytokine receptor chains such as IL-6Rα, and it is composed of three domains (D1-3). p40 is N-glycosylated and can form homodimers. p19 protein by itself does not have any known biological role. Both p40 and p19 has to be produced within the same cell for the generation of biologically active IL-23 heterodimers [1]. The heterodimeric interaction between the p19 and p40 subunits is stabilized by a disulfide bond between p19 residue Cys54 and p40 Cys177 [2].

1.3. Cellular sources of IL-23

IL-23 is expressed and secreted by professional antigen-presenting cells (APCs), chiefly dendritic cells, macrophages and monocytes. Epithelial cells were also shown to contribute to IL-23 production. These include keratinocytes [3], intestinal epithelial cells [4] and glomerular podocytes (epithelial cells in the Bowman's capsule especially during nephrotoxic serum (NTS) nephritis (NTN)) [5]. Furthermore, human fibroblast-like synoviocytes (*ex vivo* and *in vivo*) and human colon subepithelial myofibroblasts were shown to produce IL-23p19 upon IL-1β and TNF-α all of which suggest that non-hematopoietic sources may also contribute to IL-23 production to some extent, given the right stimulation [6, 7].

Different subsets of DCs exist, defined by their developmental origin, tissue location and surface markers [8, 9]. Stimulation with select ligands induces IL-23 production by $CD11c^+$ conventional DCs, pDCs or *ex vivo*-generated BMDC (mice) to varying degrees. The exact source of IL-23 *in vivo* among DC subsets during steady state, infection or chronic inflammation has been queried in various reports and, it appears, may be context dependent. Conventional DCs (cDCs) rely on transcription factor Zbtb46 and include $CD8^+$, $CD4^+$, $CD4^-CD8^-$ subsets in the lymphoid organs, and Langerhans cells in the skin, and interstitial single positive $CD103^+$ or $CD11b^+$ DCs in the connective tissues; $CD11b^+CD103^+$, $CD11b^-CD103^+$, $CD11b^+CD103^-$ as well as DN DC subsets are present in the gut [10]. $CD11b^+$ $CD103^+$ DCs were shown to be dependent on Notch2 and IRF4, and during *Citrobacter rodentium* infection, this subset was reported to be the primary source of IL-23 [10–12]. $CD11b^-$ fraction of $CD103^+$ DCs, which relies on Batf3 was shown to be dispensable for *C. rodentium* immunity [12]. Others also reported $CD11b^+CD103^+$ population as the main IL-23 source upon exposure to TLR5 ligand flagellin [13]. In the lung, $CD11b^+$ but not $CD103^+$ DCs were reported to be the major IL-23 source [10].

Siddiqui et al. showed that, in the context of intestinal inflammation (during T-cell transfer colitis and anti-CD40-induced colitis), E-$cadherin^+$ $CD11b^+$ DCs increase, and these cells are potent IL-23 producers. Despite the expression of CD103 by E-$cadherin^+$ DCs in the steady

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state, during inflammation, the investigators reported loss of CD103 expression, and inflammatory E-cadherin^{+}CD11b^{+} DCs were proposed to develop from Gr-1^{+} monocyte precursors [14].

Besides the reports implicating CD103^{+} DCs' role in IL-23 production, another study by Longman et al., however, reported that CX3CR1^{+} phagocytes not CD103^{+} DCs are the main IL-23 producer in mice [15]. Also in humans, they showed that CX3CR1^{+}CD14^{+} monocyte/macrophages, rather than CD103^{+} DCs, produced more IL-23 upon various Toll-like receptors (TLR) ligands. These are in line with previous reports which showed elevated macrophages (CD14^{+} CD68^{+} also CD205^{+}) with increased IL-23 production in the intestines of IBD patients [16]. Similarly, in *Helicobacter hepaticus*/anti-IL-10R model of murine colitis, CD103^{+} DCs were shown to be dispensable and produced low amounts of IL-23; the major source of IL-23 was MHCII^{+} Ly6C^{+} monocytes, CXCR1High F4/80^{+} macrophages and CX3CR1intLy6C^{low} macrophages/DC population [17]. Thus, in summary, the results regarding the source of IL-23 are divergent; information regarding IL-23 production by different APC subsets in the human intestine is incomplete (**Figure 1**).

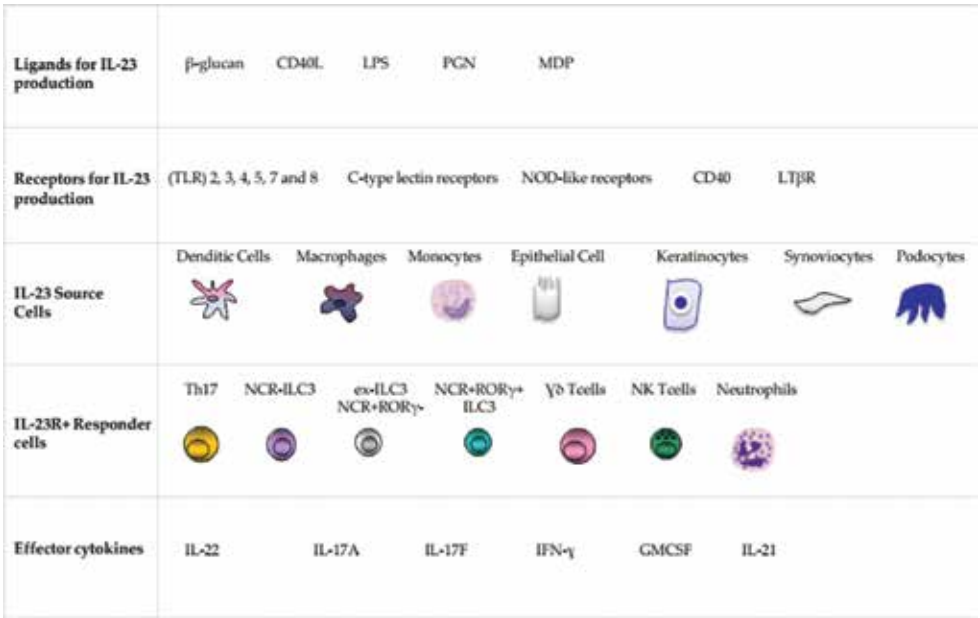

Figure 1. IL-23 inducers, producers and responders.

1.4. IL-23 stimulatory ligands

Microbiota (and pathogen-associated molecular patterns [PAMPs]) play an essential role in IL-23 production. As such, while IL-23 is constitutively expressed in the terminal ileum of SPF-housed mice, its expression is drastically reduced in the germ-free animals [18]. Pattern recognition receptors (PRR) link extracellular signals to p19 and p40 production [19,

20]. Stimulation of C-type lectin receptors, select Toll-like receptors (TLR), and CD40 by their corresponding ligands leads to IL-23 production [21]. β-glucan stimulation of APCs through C-type lectin receptor dectin-1 activates p19, p40 and p35 production (both IL-12 and IL-23) [22]. The ligand used here is curdlan, which is a pure β-glucan. β-glucan when combined with R848 (TLR7/8 ligand) or Pam2C (TLR2/6 ligand) also further increases IL-23 production [23]. TLR2 stimulation with peptidoglycan (PGN) alone, a gram-positive bacterial cell wall component, also induces preferential IL-23 production over IL-12 by DCs. LPS, a TLR4 ligand, can also induce IL-23p19 production, though not as potent as TLR2 ligand PGN [24]. The involvement of TLR4 in IL-23 production was shown using WT and LPS-deficient bacterial strains [25]. Bacterial nucleotide oligomerization domain 2 (NOD2)-ligand muramyl dipetide (MDP) can synergize with TLR2, TLR3, TLR4 ligands (PGN, dsRNA, LPS, respectively) and induces IL-23 production [26]. MDP can also synergize with TLR7/8 ligand R848 to promote IL-23 production [23]. TLR5 ligand flagellin also promotes IL-23 production [13]. It must be noted that DC type used in the abovementioned studies (BMDC, moDC or $CD11c^+$) is important and may result in differential degrees of IL-23 expression in response to abovementioned ligands.

CD40L stimulation of intestinal DCs preferentially stimulates IL-23 production and this induction is much higher compared with moDCs or splenic DCs [27]. Thus, not only microbial signals, but also those coming from T cells can regulate IL-23 production.

Prostaglandin E2 (PGE2), by engaging the G-protein coupled receptors E prostanoid 2 and EP4, also stimulates IL-23 production [28]. Similarly, extracellular nucleotides can signal through purinergic P2Y receptor for IL-23 production [29].

Non-hematopoietic cells also can express IL-23p19. IL-1β and TNF-α stimulation induces IL-23p19 production by synoviocytes [6]. LPS stimulation of TLR4 or ligation of agonistic antibody to LTβR in the colon epithelial cell line stimulates IL-23 production [4]. Similarly, colon epithelial cells, *in situ*, were shown to produce IL-23 in an LTβR-dependent fashion.

1.5. IL-23 gene expression

IL-23 expression is induced through various MAPKs including p38, JNK and ERK [30], as well as NFκB. The p40 gene expression is regulated at the transcriptional level by binding of NFκB, CCAAT/enhancer-binding protein (C/EBP), ets-2, PU.1, IRF1, IRF2, IRF5, IRF8 and activator protein 1 (AP-1) to the promoter region of p40 [31–34] upon stimulation with various ligands. The murine and human p19 promoter was also shown to contain three NFκB binding sites [30]. Two of these binding sites have been shown to be involved in TLR-mediated activation of p19 transcription. Smad3, AP-1 and activating transcription factor-2 (ATF-2) transcription factors were also shown to bind p19 promoter and positively regulate IL-23p19 expression. There are two binding sites for 2 interferon regulatory factor (IRF) genes, IRF3 and IRF7 in both human and murine p19 promoters. IRF3 was reported to be a positive regulator of p19 expression, and thus, its absence was shown to lead to the downregulation of p19 [35].

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1.6. IL-23 responsive cells

IL-23 receptor is expressed by both innate and adaptive immune cells. Group 3 ILCs (ILC3), dendritic cells, macrophages/monocytes, γδ T cells, and more recently, neutrophils were among the innate cells that were shown to respond to IL-23. Among IL-23R$^+$ adaptive immune cells are Th17, Th22 and some iNKT cells [36].

1.6.1. Th17 cells

The most studied IL-23 responsive cells are Th17 cells. IL-23 is needed for the maintenance/maturation and expansion of Th17 cells in humans and mice and is dispensable for their initial differentiation from naïve CD4$^+$ T cells. The maintenance of Th17 identity relies on IL-23-mediated induction of *Rorc, Il23r* and *Il17* expression [19]. Th17 cells also require IL-23 to fully acquire a pathogenic character [37]. In fact, several laboratories showed that Th17 cells are very weak inducers of EAE, a mouse model of human MS, unless they are generated in the presence of IL-23 or they express IL-23R. IL-23 exposure programs Th17 cells transcriptionally to have a unique effector cytokine profile compared to nonpathogenic Th17 cells which are not exposed to IL-23. Unlike nonpathogenic Th17 cells, which express only IL-17, IL-23-activated pathogenic Th17 cells express IFN-γ and GMCSF in addition to the IL-17. Various lines of evidence suggest that Th17 cells, and hence IL-23R signaling, is critical for the development of chronic inflammatory conditions such as Crohn's disease, ulcerative colitis, psoriasis, rheumatoid arthritis (RA) and systemic lupus erythematous (SLE) in addition to MS.

1.6.2. Group 3 ILCs

Rorγt$^+$ Group 3 innate lymphoid cells (ILC3s) are a heterogeneous population of cells which have an irreplaceable function in protective immunity against extracellular pathogens in the gastrointestinal mucosa. ILC3s have also been recently implicated in the pathogenesis of inflammatory bowel diseases (IBD) [38–40]. ILC3s express IL-23R and depend on IL-23 for their production of various effector cytokines including IL-22, IFN-γ and IL-17, which take part in the abovementioned processes.

1.6.3. γδ T cells

A fraction of γδ T cells ubiquitously express IL-23R and produce IL-22, IL-21 and IL-17 upon IL-23 stimulation [41]. Skin and mucosal surfaces, particularly intestinal intraepithelial compartment, contain more γδ T cells than other microenvironments. These cells are involved in protective immunity against various pathogens. Studies in mouse models of various chronic inflammatory diseases revealed that γδ T cells may take part in the pathogenesis of IBD, psoriasis, MS and rheumatoid arthritis (RA) via their effector cytokines [41].

1.6.4. Antigen-presenting cells

Data regarding expression of IL-23R by APCs are scarce but do exist. Rorγt was reported to be expressed by CD45$^+$CD11b$^+$ cells, but these cells were found to be CD11c$^-$Gr1$^-$ initially. However, this study focused more on LTi cells not APCs. Sakhina Begum-Haque reported the

presence of Rorγt$^+$ DCs in the context of EAE in the CNS [42]. More recently, Karthaus et al. profiled nuclear receptor expression in murine DCs from various tissues and reported expression of RORγ in pLN and SPLN-resident DCs, and some expression was even observed in BMDC [43]. MLN-resident DCs expressed much higher RORγ message. Conventional DCs expressed more mRNA message than pDCs. Protein levels, however, were not quantified in this work. Short Rorc isoform Rorγt was also not examined. In line with these studies, we reported GFP$^+$ CD11b$^+$ myeloid cells in our IL-23R GFP reporter mice [36]. A better characterization at the protein and functional level of IL-23R and Rorγt is needed to decipher the role of IL-23R in APC function and chronic inflammation.

1.6.5. Neutrophils

Neutrophils in both humans and mice were shown to express IL-23R, Rorγt, IL-17A and IL-22 and respond to IL-23. Due to their prompt recruitment to the sites of infection and abundance, they can limit the infections [44] and the damage associated with chronic inflammation [45].

1.6.6. NKT and other cells

A fraction of NK1.1$^-$ invariant Natural killer T cells (iNKT) express Rorγt, IL-23R and, in response to IL-23, produce IL-17 and IL-22. Rorγt$^+$ iNKT cells are present in the peripheral lymph nodes. IL-23R signaling appears to be important for maintaining the number of such iNKT cells in humans [46]. How IBD pathology is regulated via IL-23-dependent response of iNKT is unclear [47, 48].

A group of CD3$^+$ CD4$^-$CD8$^-$ Rag-dependent T cells were also reported to express IL-23R [49]. Such cells have been shown to increase in number in an IL-23-dependent manner during systemic lupus erythematosus and ankylosing spondylitis murine models.

1.7. IL-23 receptor signaling and down-stream inflammatory mediators

IL-23 signals through its heterodimeric receptor (IL-23R) that is composed of two subunits: IL-12Rβ1, which is shared by IL-12 receptor complex, and IL-23R, which is the unique subunit. The p19 subunit of IL-23 heterodimer interacts with IL-23R, whereas the p40 subunit interacts with IL-12Rβ1 chain. In both humans and mice, IL-23R locus is positioned proximal to IL-12Rβ2 on chromosomes 1 and 6, respectively, and thus is believed to evolve through a gene duplication process [50]. IL-23R is conserved among amniotes and the unique IL-23R subunit protein is made up of 629 and 659 amino acids in humans and mice, respectively. Mouse and human IL-23R has 84% similarity. IL-23R sequence is also highly similar to IL-12Rβ2 and gp130.

IL-23 receptor signals through JAK kinases and STAT transcription factors. IL-23 binding of IL-23R activates of Jak2 and Tyk2, which then phosphorylate the receptor, creating docking sites for the recruitment of STAT proteins. STAT1, 3, 4, 5 are subsequently phosphorylated by activated Jak2 and Tyk2 kinases. The major transcription factor activated by IL-23 stimulation is STAT3. Pathways activated upon IL-23 binding to its receptor include the P38 MAPK pathway, PI3K-Akt and NFκ-B pathway [51–53]. IL-23 signaling activates transcription of

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various effector cytokine genes including IL-17A, IL-17F, IL-22 and IFN-γ whose roles in IBD will be reviewed in the sections below.

1.8. IL-23 receptor signaling is involved in IBD in murine models and human studies

Various lines of evidence from murine studies built a pathogenic role for IL-23 signaling in IBD pathogenesis. Using *p19*$^{-/-}$, *p35*$^{-/-}$ and *p40*$^{-/-}$ mice and neutralizing antibodies against p19, p35 and p40, IL-23p19, but not IL-12p35, was demonstrated to be necessary for the development of spontaneous colitis in IL-10$^{-/-}$ mice [54]. Similarly, innate colitis induced by *H. hepaticus* in both Rag$^{-/-}$ or Rag sufficient hosts [55, 56] as well as adaptive T-cell colitis induced in Rag$^{-/-}$ mice via transfer of CD45RB^{high} naïve T cells [54–56] or CD45RB^{low} IL-10$^{-/-}$ memory T cells [54] were all dependent on IL-23p19 but not p35. Moreover, p19 KO mice were shown to be resistant to development of chemically induced colitis via DSS treatment [57]; conversely, IL-23p19 overexpression in mice resulted in enteropathy [58]. Similar to its ligand, IL-23 receptor is required for the development of adoptive naïve CD4^{+} T-cell–induced colitis [59], chemically induced DSS-driven colitis in the presence of adaptive immune cells [57] and innate cell-driven colitis induced via anti-CD40 treatment [60] in mice.

Data obtained from the studies with human IBD patients regarding the ligand as well as IL-23 receptor strongly suggest a role for this pathway in IBD development. In this regard, IL-23 was found to be elevated in the intestinal tissue of IBD patients [16]. Similarly, IL-23R mRNA was upregulated in individual lymphocytes (NK^{+}, CD4^{+}, CD8^{+} cells) obtained from both lamina propria and peripheral blood of CD and UC patients [61, 62]. Sophisticated genome wide association studies revealed IL-23R and downstream signaling molecules JAK2, TYK2, STAT3 variants as risk or resistance factors for CD and UC [63, 64]. Some of the identified variants have been studied. rs11209026 (or R381Q) SNP was discovered as a protective variant for CD [63] and UC [65] in Jewish and non-Jewish cohorts which was later shown to be a loss of function mutation in IL-23R [66, 67]. Arg-381 is located in the cytoplasmic domain of IL-23R protein and is well conserved among species, whereas Gln-381 allele is less frequent [63]. A later study demonstrated that CD8^{+} and memory CD4^{+} T cells purified from Gln-381 IL23R allele carriers produced less IL-17 and IL-22 in response to IL-23 stimulation, and that R381Q carriers contain fewer circulating Th17 and Tc17 cells compared to healthy Arg-381 carriers [68]. Peripheral blood mononuclear cells (PBMC) from individuals with R381Q variant also produce less IL-17 in response to the *Borrelia burgdorferi*, a potent inducer of Th17 responses [69]. Moreover, R381Q IL-23R transfected cell lines displayed reduced STAT3 phosphorylation compared with control IL-23R. These reports collectively provide a mechanistic explanation for the resistance to CD and UC of R381Q SNP allele. Other protective variants against Crohn's include p.Arg86Gln, p.Gly149Arg and p.Val362Ile [70]. The last two also protect from UC. Mechanistically how they affect IL-23R signaling remains unknown. p.Gly149Arg affects a highly conserved extracellular domain of IL-23R, whereas p.Arg86Gln and p.Val362Ile are variants in the poorly conserved domains [71]. These SNPs are believed to reduce IL-23R activity, but experimentally this has yet to be shown.

Risk variants of IL-23R for CD were also described [63]. They are thought to be gain of function mutations. rs10889677 is one such variant with a transversion in the 3′ UTR of IL-23R where

an A in the wild-type allele is mutated to C. This mutation was shown to abolish a regulatory pathway directed by miRNA Let-7e and Let-7f, which consequently resulted in elevated IL-23R mRNA and protein production in human PBMC and $CD4^+$ T cells [72].

Other SNPs in SAT3 (rs381676, rs744166 and rs11871801), in JAK2 (rs10758669), and in TYK2 have been described. However, mechanisms of action of these variants with regard to their impact on IL-23R signaling requires further study [73, 74].

2. IL-23-producing cells in IBD pathogenesis

Antigen-presenting cells are the primary source of IL-23. Thus, mutations in any of the genes responsible in the IL-23 production pathway by APCs would potentially have consequences for IBD pathogenesis. Indeed, examples of such defects in the literature exist. An example of a link between PRR and dysregulated IL-23 production was observed in individuals with NOD2 variant 1007fsinsC [75]. As described above, NOD2 is a cytosolic PRR that detects bacterial cell wall component, and three variants of this receptor were found in 40% of CD patients in western countries [76]. Recently, NOD2 was shown to crosstalk with TLR2 pathway to regulate IL-23 expression by dendritic cells via mobilizing miRNAs. miR-29 expression was shown to be augmented by this crosstalk, which directly targets IL-12p40 mRNA and indirectly IL-23p19. DCs with homozygous or heterozygote NOD2 variant 1007fsinsC from CD patients thus have defective miR-29, and consequently augmented IL-23 expression [75].

Susceptibility and protective variants of CARD9 have been discovered [77, 78]. CARD9 works downstream of β-glucan receptor dectin-1 and regulates IL-23 production. It is, however, unclear whether these variants are loss or gain of function mutations, but it is likely that these variants may lead to dysregulated IL-23 production by APC.

In some mouse IBD models, APCs were manipulated to determine their impact on pathogenesis. In DSS-induced chemical colitis, depletion of $CD11c^+$ DCs via DT injections in CD11c DTR mice during disease confers protection, whereas depletion before disease exacerbates the pathology [79–81]. Direct depletion of DCs during T-cell–induced colitis has not been performed although IL-23 production has been traced back to these cells in various IL-23-dependent colitis models and IL-23 neutralization or genetic deletion systems have been utilized along with targeting of various costimulatory molecules expressed by DCs. Nevertheless, transfer of E-$cadherin^+$ BMDCs which express IL-23 exacerbates T-cell–induced colitis [14].

Deletion of monocytes via CCR2 gene targeting or anti-CCR2 antibodies also confers protection from DSS-induced colitis [82, 83].

The importance of APC-derived IL-23 in murine colitis models based on infection has been documented by selective targeting of DCs, macrophages and monocytes [15, 17, 84], all pointing to the significance of APC-derived IL-23.

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3. IL-23 responsive cells in IBD pathogenesis

In this section, "how IL-23 responsive cells drive or prevent IBD pathology" will be discussed. Although IL-23R-expressing cells are manifold, effector cytokines produced upon IL-23 signaling are similar or identical by these innate and adaptive immune cells. The current literature regarding how each IL-23-dependent cytokine coming exclusively from a defined IL-23R$^+$ cell impact IBD pathology will be reviewed. A summary of the IL-23R positive cells are shown in **Figure 2**.

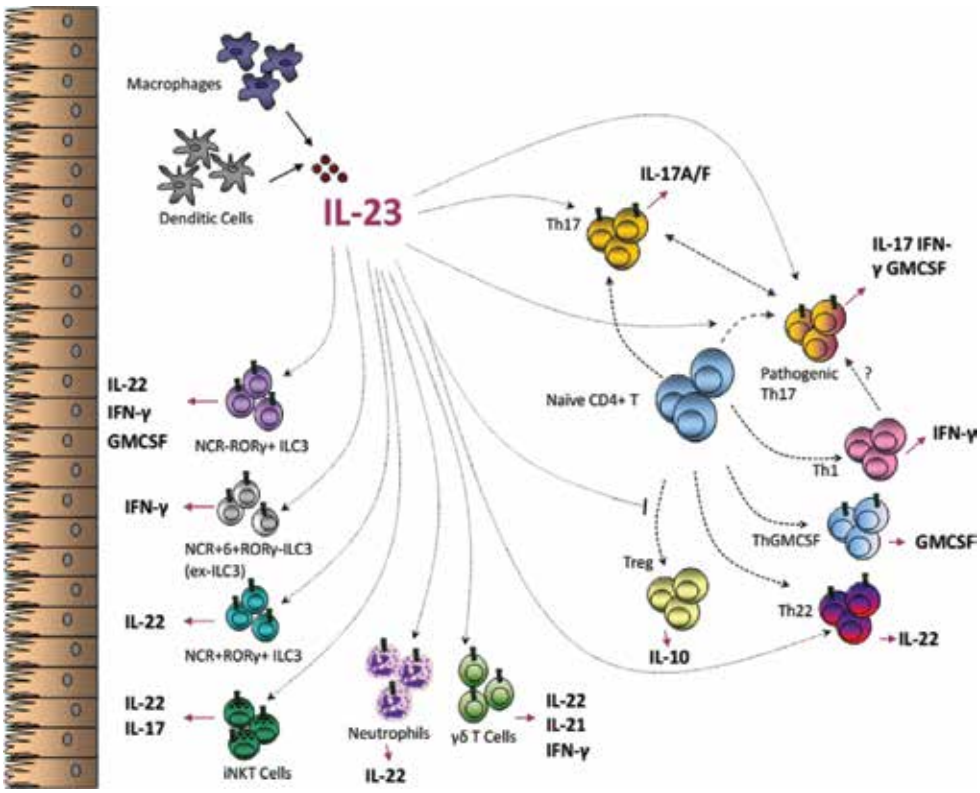

Figure 2. Summary of action of IL-23 on its target cells. IL-23 acts on both innate and adaptive immune cells to promote inflammation during IBD.

3.1. Th17 cells in IBD

3.1.1. IL-17A and F

IL-17 A and F are closely related (50% homology) Th17 signature cytokines that are produced in an IL-23-dependent manner. IL-17A and IL-17F are made of homodimers, however, IL-17A/F heterodimers also form. All of the combinations are recognized by the same heterodimeric

receptor composed of IL-17RA and IL-17RC subunits [19]. Both cytokines were expressed at high levels in the intestines of CD patients [62, 85–87]. Because these cytokines stimulate production of various inflammatory mediators by epithelial or endothelial cells that recruit neutrophils, monocytes and dendritic cells their involvement as pathogenic molecules were studied in IBD context [88, 89]. IL-17A neutralization in DSS model resulted in exacerbation of colitis with higher $CD4^+$ T-cell infiltrates and $CD11b^+$ granulocyte-monocyte infiltrates [90]. IL-17KO mice recapitulated this phenotype [91]. Similar to the chemically induced colitis, adaptive colitis induced by naïve $CD4^+$ T cells also developed more aggressively when *il17*$^{-/-}$ or *il17r*$^{-/-}$ T cells transferred as compared with WT T cells [92]. This protective role of IL-17 in murine models has been confirmed in Crohn's disease patients. Monoclonal anti-IL-17A secukinumab treatment exacerbated the disease, and adverse effects (high incidences of fungal infection) have been reported [93]. Recently, it was shown that IL-17 is critical for epithelial homeostasis and that the absence of IL-17A during colitis induced by DSS treatment further decreases epithelial integrity and barrier function, resulting in bacterial translocation across the intestinal epithelial barrier [94]. The same phenomenon of IL-17-dependent regulation of epithelial barrier function was reported by Maxwell et al. in a colitis model which was induced in *Abcb1a*-deficient mice upon *Helicobacter bilis* infection [95] which may provide a mechanistic explanation to protective role of IL-17A/F.

In some models of murine IBD, IL-17A and F demonstrated a pathogenic character. In this regard, IL-17FKO mice developed milder DSS-induced colitis compared with WT mice [91]. IL-17A neutralization also improved colitis in a T-cell transfer model in which colitis was induced by IL-17F$^{-/-}$ $CD4^+CD25^-$ T cells (Naïve + memory) [96]. Furthermore, Yen et al. had demonstrated a pathogenic role for IL-17A in $CD4^+$ T-cell transfer colitis induced by IL-10$^{-/-}$ T cells [54]. Lastly, deletion of IL-17RA, receptor for both IL17 and F (but also for IL-17C), or blocking of signaling via IL-17RA IgG in WT mice conferred protection from in TNBS-induced colitis [97].

3.1.2. IFN-γ

IFN-γ is a Th1 cytokine. However, Th17 cells and ILC3s also produce it when stimulated with IL-23. IL-17A expression by T cells is not required for naïve T-cell–driven colitis; in fact, its neutralization in IBD patients does not ameliorate the disease as described above. However, Th17 cells are needed for the pathogenesis of IBD in several murine models as RORγT [87], STAT3 [98], IL-23 [99], and IL-23R [59] manipulation by genetic and biochemical means alters the disease course. These data point to the involvement of other Th17-derived cytokines in colitogenesis. In fact, in naïve T-cell transfer-induced adaptive cell–driven coltitis, anti-CD40 or *Hepaticus*-induced innate colitis models, IL-23-dependent IFN-γ produced by either Th17 or innate ILC3 cells was shown to play a pathogenic role [38, 100]. IFN-γ in this context was shown to regulate myeloid inflammatory cell recruitment (neutrophils, monocytes and eosinophils) to the tissue [60, 101, 102]. In both $CD4^+$ T-cell transfer and *H. hepaticus*-driven murine adaptive models of IBD, Th17 cells that produce IFN-γ^+IL-17A$^+$ together have been described. These double producer cells were eliminated when donor T cells lack IL-23R in the T-cell–transfer colitis model, and colitis scores are improved [103]. IFN-γ^+IL-17A$^+$ double

producers increase in colitic mouse intestine. Independently-performed fate map studies have shown that IFN-γ^+IL-17A$^+$ double producers can further turn RORγT expression off and gradually turn into "alternative" Th1 cells through a process promoted by IL-23 and IL-12 [59, 104, 105]. It is unclear as yet what the relative contribution of Th17/Th1 double producers or alternative Th1 cells or the conventional Th1 cells to IBD pathogenesis are and if it applies to humans.

Besides acting as an inflammatory cytokine, IFN-γ was also shown to regulate IEC survival proliferation through Wnt inhibitor Dkk, which ultimately negatively impacts intestinal epithelial barrier function [106, 107]. Whether epithelial integrity is regulated by IFN-γ of Th17, or ILC3 origin is also unclear.

3.1.3. GMCSF

Granulocyte macrophage colony-stimulating factor (GMCSF) is a hematopoietic growth factor produced by various immune cells such as activated T and B cells, monocytes/macrophages, neutrophils, eosinophils and ILC3s, as well as other sources such as endothelial cells, fibroblasts epithelial cells, mesothelial cells, chondrocytes, Paneth cells and tumor cells [108]. It was shown that GMCSF is produced by Th17 cells in a Rorγt and IL-23-dependent fashion [109, 110]. More importantly, independent studies revealed that GMCSF is required for classical EAE development in mice, especially by activating microglia or mobilizing inflammatory myeloid lineages to the inflammation site [109, 110]. Studies from Fiona Powrie's lab more recently showed that IL-23-dependent production of GMCSF also contributes to the pathogenesis of colitis in naïve CD4$^+$ T-cell transfer- and *Hepaticus*-induced models of murine IBD [111, 112]. In both models, GMCSF was shown to promote eosinophil recruitment and activation in the colon which was needed for pathogenesis. On the other hand, work including with human cells proposed that a distinct lineage of Th cells is programmed to produce only GMCSF and that they constitute the major fraction GMCSF$^+$ Th cells (Though GMCSF$^+$IFN-γ^+ or GMCSF$^+$IL-17A$^+$ cells are also reported) [113]. STAT5 and IL-7 or IL-2 may be important in differentiation or activation of GMCSF$^+$ cells [114, 115]. It is noteworthy that STAT5$^{-/-}$ CD4 T cells are still able to induce colitis [114]. Thus, though GMCSF may have a role in IBD development, how much of that comes through IL-23-dependent pathway or from Th17 cells is unclear and more cell-specific deletion of GMCSF is needed to address this question.

3.1.4. IL-22

IL-22 is an alpha-helical cytokine which belongs to IL-10 family cytokine. Th17, Th22 and γδ T cells [19], neutrophils [45] and ILC3s produce IL-22 cytokine in response to IL-23 stimulation [116]. IL-22 signals through a heterodimeric cytokine composed of the specific IL-22R1 and IL-10Rβ subunits. Although IL-22 is mostly produced by cells of the hematopoietic lineage, IL-22 receptor is expressed by the non-hematopoietic compartment which includes epithelial cells in the skin, lung and intestines, liver and kidney [117]. IL-22 is needed in the mucosal surfaces for the containment of microbial flora at an arms distance of epithelia. IL-22 stimulates production of various antimicrobial proteins and peptides (Reg3β and γ, β-defensins, S100A7-9 etc.) as well chemokines and cytokines (CXCL1$^-$, 5, 9, IL-6, G-CSF) [116]. Thus, it is

also crucial for host defense against various pathogens including *C. rodentium* [118]. IL-22 promotes mucus production by goblet cells; acts as a growth factor and stimulates epithelial regeneration [118, 119].

IL-22 levels are elevated in the mucosal tissue of both UC and CD patients [120]. As with other Th17- specific cytokines, both protective and colitogenic roles for IL-22 have been described in murine IBD models. Sugimoto et al. were the first to demonstrate that IL-22 could improve murine IBD pathology. They observed a reduction in IL-22 levels after the disease onset in their spontaneous colitis murine model compared with control animals which developed disease due to a T-cell receptor defect ($Tcra^{-/-}$mouse) and went after IL-22 [121]. Using this model and DSS-induced colitis, these investigators showed that IL-22 overexpression improved colitis, and its neutralization via IL-22BP (the soluble receptor) or antibody, delayed recovery from colitis. Although the exact source of IL-22 in this work remained less defined due to the possible innate sources (γδ T cells, ILC3, neutrophils), during naïve $CD4^+$ T-cell transfer-induced colitis, IL-22 coming from exclusively Th17 cells were shown to be protective. This was shown by the transfer of IL-$22^{-/-}$ naïve $CD4^+$ T-cell transfer into $Rag^{-/-}$ mice which developed exacerbated colitis compared to that of WT T cells [122]. ILC3 also contribute to intestinal IL-22 production, as such IL-$23R^{-/-}$ $Rag1^{-/-}$ mice develop exacerbated colitis upon naïve $CD4^+$ T-cell transfer compared with control $Rag1^{-/-}$ hosts. IL-23R deficient Rag1KO mice had far less IL-22 in their intestines than control Rag1KO mice even after naïve T-cell transfer, showing that indeed ILC3 contribution to IL-22 is significant [60]. So, regardless of the cellular source, reduction in IL-22 levels impacted IBD development/recovery in naïve T-cell–induced colitis. IL-22-mediated protection from colon inflammation was demonstrated by targeting molecules responsible for the induction of IL-22 in different contexts. When AhR signaling was activated via its ligand Ficz, which increased IL-22 production, less colitis developed in TNBS, DSS and $CD4^+$ naïve T-cell–induced mice model; Ficz-dependent protection was reversed by neutralization of IL-22 [123, 124]. In all these models, IL-22 was believed to promote epithelial barrier regeneration. Conversely in its absence, epithelial barrier was breached and could not be repaired [122]. IL-22 receptor signaling activates STAT3; research shows that deletion of STAT3 in IL-22 responsive epithelial cells impairs IL-22-mediated intestinal epithelial repair which was demonstrated in a DSS-induced model [125]. This study revealed an important role for IL-22-induced mucin in IL-22-mediated protection from colitis.

IL-22 was also shown to drive colitis in noninfectious and infection-induced T-cell–dependent colitis models [126, 127]. Kamanaka et al. developed a T-cell–dependent colitis model by adoptive transfer of IL-10 unresponsive IL-$10dn^-$ $CD45RB^{low}$ $CD25^-$ $CD4^+$ memory T cells into $Rag^{-/-}$ hosts. The colitis developed in this model was IL-22-dependent (exclusively of TH17/Th22 origin), as such IL-$22^{-/-}$ $CD45RB^{low}$ $CD25^-$ $CD4^+$ memory T cells did not induce colitis compared with IL-22. It is noteworthy that colitis in this model, unlike the naïve T-cell transfer model, does not cause ulcers, but rather is characterized by mucosal thickening and hyperplasia consistent with proliferative potential of IL-22 [127]. *Toxoplasma gondii* infection-induced colitis in B6 mice has also been shown to be IL-22 driven (through its effects on MMP-2), thus IL-22 deletion ameliorated colitis in this context [128].

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3.2. ILC3, γδ T and NKT cells in IL-23R-mediated pathology

3.2.1. Group3 ILCs

Group 3 ILCs (ILC3) are Rorγ$^+$ innate cells that respond to IL-23 and are enriched in mucosal surfaces [129]. Although very rare in the circulation, in the intestinal lamina propria, Rorγ$^+$ ILC3s are enriched and constitute up to 8% of lymphocytes and ~70% of ILCs in the murine intestinal LP [39]. ILC3s include fetal LTi cells and adult ILC3s [130]. Various adult ILC3s were described in humans and in mice based on the expression of natural cytotoxicity receptors and cytokine production. These include (1) IL-22 producing NCR$^+$ ILC3 [131] which are also called ILC22, NK22, NKR-LTi or NCR22 [132]; (2) NCR$^-$IL-17A$^+$IFN-γ$^+$ double producing ILC3 [38] and (3) NCR$^-$IL-17A$^+$ ILC3s [133, 134] in mice [135]. Fetal and adult ILC3 were shown to differ in their CCR6 expression. Fetal LTi cells express higher CCR6, whereas adult ILC3s appear to be CCR6 low and accumulate after birth in a microbiota-dependent fashion. CCR6$^-$ adult ILC3s were also reported to rely on AhR and ligands acquired through diet [135]. All the ILC3 cells depend on Rorγt for their development and express IL-23R and produce Th17 cytokines in response to the IL-23, although the combination of cytokines differ with the microbial signal and ILC3 type.

Regardless of our incomplete understanding of their ontogeny, ILC3s have been shown to take part in the pathogenesis of IBD-like diseases in many murine models in the past 5 years. More importantly, not only Th17 cells but also CD3$^-$ ILC3s were reportedly elevated in the intestinal tissue of both UC and CD patients; they also contributed to elevated IL-22, IL-17A/F and IL-26 levels in tissue of IBD patients [136].

3.2.1.1. ILC3-derived IL-17 and IFN-γ

Although IL-17A neutralization trials failed at achieving a clinical benefit to IBD patients, IL-17A, particularly of ILC3 origin, has been shown to promote IBD-like pathogenesis in murine models. This was shown to be the case in *H. hepaticus*-induced colitis in Rag$^{-/-}$ mice, shown by Buonocore et al. in their landmark paper [38, 55, 56]. In this innate model, IL-17A$^+$ IFN-γ$^+$ ILC3 numbers elevated and neutralization of either cytokine alone or together ameliorated colitis. Additionally, deletion of ILC3 by crossing Rag$^{-/-}$ mice to Rorc$^{-/-}$ animals or ILC3 depletion via anti-Thy1 antibodies make them resistant to colitis induced by *H. hepaticus*. Pathogenicity of IL-17A was also reported in another innate colitis model, the *Tbx21*$^{-/-}$*Rag2*$^{-/-}$ (TRUC) mice. TRUC mice develop spontaneous colitis in a microbe-dependent fashion (which has recently been shown to be *H. Typhlonius*-dependent) [137]. Interestingly, colitis in this model is TNF-α-dependent until the age of 12 weeks after which blockade of TNF-α is ineffective. Through neutralization of IL-23 or IL-17A or blockade of IL-7R signaling, it was shown that ILC3s have been shown to drive colitis in this model via IL-17A. Both TNF-α and IL-6 appear to enhance the disease by enhancing IL-23 production or its signaling [133, 134].

In both *H. Hepaticus*-induced colitis and anti-CD40-induced colitis models (both of which are IL-23 mediated) ILC3-derived IFN-γ drives pathogenesis, and as such, IFN-γ neutralization in these models ameliorated colitis [27, 38, 100]. ILC3s indeed produce IFN-γ when stimulated with IL-23 [60]. Similar to Th17 cells, ILC3 cells have been reported to be plastic cells. Vonar-

bourg et al.'s studies revealed that that RORγT^{+}NKp46^{-} ILC3s (NCR^{-}ILC3), upon exposure to IL-12 and IL-15, upregulate NK cell marker NKp46 giving rise to NCR^{+}ILC3 *in vivo*. These cells subsequently downregulate RORγT and assume a Th1 or NK such as phenotype and called RORγt-NKR LTi. (currently considered as ILC1) [100]. These ex-ILC3s were shown to produce IFN-γ and were argued to be the major source of IFN-γ and the driver of colitis in the anti-CD40-induced colitis model. The plasticity of ILC3s has also been described in humans [138, 139]. In the presence of IL-12 and IL-2, human CD3^{-}CD127^{+}c-kit^{+} NKp44^{+} ILC3s downregulate Roγt and IL-23R; upregulate T-bet and then produce IFN-γ. These ex-ILC3s are categorized as non-NK ILC1 [138]. More recently, the ILC3-to ILC1 conversion has been shown to be a reversible process regulated by different subsets of antigen-presenting cell, presumably depending on the microbes or other external signals [139]. Elevated percentages of ILC1 have been reported in Crohn's disease-inflamed intestine [140], as well as in humanized mice treated with DSS [138]. However, causality with the disease, or whether their contribution is significant for pathogenesis in humans is also unclear given the scarcity of their number [136].

3.2.1.2. ILC3-derived IL-22 in IBD

ILC3s also produce IL-22 in response to the IL-23. Both pathogenic [60, 102] and protective [57, 141, 142] roles have been described for IL-22 that is coming from exclusively ILC3s. Deletion of ILC3s by crossing *Rag*$^{-/-}$ mice to *Rorc*$^{-/-}$ renders double KO mice more susceptible to DSS-induced colitis and also delays the recovery [57, 141]. Similarly, IL-22-deficient B6 mice or IL-23R$^{-/-}$ *Rag*$^{-/-}$ mice develop more severe intestinal damage in response to the DSS challenge which are reversible by recombinant IL-22-Fc injections [57]. IL-22 is needed for the healing of epithelia upon DSS-induced damage. However, too much of it in certain context may also promote colitis characterized by hyperplasia and mucosal thickening and myeloid inflammatory cell recruitment [119, 125]. We and others have shown this pathogenic effect of IL-22 using the innate cell-mediated colitis model induced by anti-CD40 injections. Neutralization of IL-22 in *Rag*$^{-/-}$ mice ameliorated colitis, conversely, restoring IL-22 expression in *IL-23R*$^{-/-}$*Rag*$^{-/-}$animals (which are protected from colitis), brought colitis back [60, 102]. How IL-22 mediates colitis is not entirely clear, but our data suggest that IL-22 may modulate IL-10, IFN-γ levels and neutrophil recruitment [60].

Protective effects of IL-22 may also be due to impact on microbial flora. Recent studies suggested that IL-22 may contribute to protection from IBD by restricting growth of certain genera of bacteria in the steady state [143]. A study by Zenewicz et al. revealed that intestine of *Il-22*$^{-/-}$ mice differs in representation of 14 different genera compared with WT mice and is more susceptible to DSS-induced colitis. More importantly, this susceptibility is transmissible to WT mice through co-housing of WT with *il22* KO mice, which points to functions of IL-22 independent of epithelial regeneration [143]. Supporting this view, another study using *AhR*$^{-/-}$*Rorc*$^{-/+}$ mice demonstrated that reduced IL-22 levels in the murine intestine allows overgrowth of SFB, which consequently promotes Th17 differentiation [144]. Thus *AhR*$^{-/-}$*Rorc*$^{-/+}$ mice develop spontaneous colitis owing to hyper-Th17 responses.

3.2.1.3. ILC3-derived GMCSF in IBD

Both human and mouse ILC3s form IBD patient intestine and murine intestine, respectively, were shown to produce GMCSF in an IL-23-dependent manner [101, 102]. Similar to adaptive cell-induced colitis models, during anti-CD40-induced colitis ILC3 contributed to GMCSF substantially, and its blockade via neutralizing antibodies blocked colitogenesis [101, 102]. GMCSF-dependent recruitment of myeloid effector cells (eosinophils-monocytes) may be the underlining mechanism for the pathogenic effects as described in adaptive cell-induced colitis models [111, 112]. GMCSF, however, was also shown to impact ILC3 motility out of crypto-patches, which may additionally contribute to its pathogenic role during innate cell-induced colitis [101].

3.2.2. γδ T cells

γδ T cells are nonconventional T cells with innate features and comprise 1–5% of lymphocytes in mice and human blood. Their numbers go up to 50% of lymphocytes in skin and mucosal tissues [145]. γδ T cells express Rorγt and IL-23R and are another source of IL-17 and IL-22, which can be produced both in IL-23-dependent and independent manner. In peripheral blood as well the intestines [146–148] of active IBD patients, elevated percentage and absolute number of γδ T cells were reported. Both tissue protective and pro-inflammatory roles in murine IBD models have been described for γδ T cells. In this regard, *Tcrδ*$^{-/-}$ mice developed more severe DSS-induced colitis accompanied by reduced regeneration and epithelial tissue repair [149, 150]. Depletion of γδ T cells also exacerbated TNBS-induced colitis in rats [151]. A recent study showed that this protective effect (of γδ T cells) was mediated through IL-22 and further enhanced by retinoic acid (RA) which induced RA receptor binding to IL-22 promoter [152]. More recently, γδ T cells were shown to be the major IL-17A source during acute DSS-induced colitis. In this model, IL-17 production was reported to be mostly IL-23 independent and regulated epithelial permeability through instructing localization of occluding, a tight junction protein [94].

Studies in some murine IBD models implicated γδ T cells as the contributor to pathology. Colitis in *Tcrα*$^{-/-}$ mice, which resembles to UC and spontaneously develops in a microbiota-dependent fashion, improved up on genetic deletion of γδ T cells [147]. In also a T-cell transfer model, γδ T cells enhanced colitis [153]. *Tcrβδ*$^{-/-}$ mice developed less colitis compared with *Tcrδ*$^{+/+}$ mice upon naïve CD4^{+} T-cell transfer. Cotransfer of IL-17^{+} CCR6^{+} γδ T cells but not CCR6^{-} IFN-γ^{+} γδ T cells with naïve T cells restored colitis in this model through potentiating Th17 and Th1 cells [153]. In another murine spontaneous colitis model which develops due to CD4^{+} T-cell-–specific deletion of phosphoinositide-dependent protein kinase 1 (Pdk1), γδ T cells were shown to be required for colitogenesis [154].

3.2.3. NKT cells

Type I NKT (iNKT) cells are characterized by their invariant T-cell receptor α-chain which is detectable by α-galactosylceramide loaded CD1d tetramers [155]. A population of NK1.1^{-} iNKT cells were shown to express RORγT and IL-23R [47, 48] and produce IL-17. These

RORγT + iNKT cells, when costimulated with IL-23 and IL1β, induce production of large amounts of IL-22 and IL-17 [47, 48]. Some studies documented a reduction in type I iNKT cells in the blood and intestinal tissue of CD and UC patients [156] (see the review for detailed role of iNKT in IBD [155]). Because iNKT cells produce IL-4, IL-13 and can promote Th2-responses, they have been experimentally shown to play a protective role in various murine IBD models including DSS [157, 158], TNBS [159], naïve $CD4^+$ T-cell transfer [160] and *T. gondii* induced [161, 162] models of colitis. However, exactly how IL-23–dependent production of IL-22 or IL-17 by iNKT cells confers protection or impacts IBD pathogenesis has not been fully elucidated.

3.2.4. Neutrophils

Some fractions of murine neutrophils (~20%) express Rorγt and IL-23R; even higher factions of human neutrophils (75%) have been shown to respond to IL-23 and produce IL-17A and IL-22 [163, 164]. A recent study demonstrated that during DSS-induced colitis, neutrophils significantly contribute IL-22 production; as such IL-22 WT neutrophil transfer improves colitis [45]. Neutrophils are recruited to intestine during T-cell transfer colitis and *Hepaticus*-induced colitis as well. Their neutralization in one study did not suggest any pathogenic role in these models [111]. It is unclear how neutrophils would impact intestinal pathology in other innate and T-cell–dependent colitis models.

4. Therapeutic approaches targeting IL-23 signaling in IBD

With the motivation from studies described above and the commonality of IL-23 signaling across a number of autoimmune/chronic inflammatory conditions, several companies have targeted IL-23 signaling pathway components with various means for therapeutic intervention in multiple inflammatory diseases including IBD. Most of these antagonists are monoclonal human or humanized antibodies that target specific (p19) or common (p40) subunits of IL-23 (**Table 1**). Others target downstream effector cytokines or cytokine receptors induced by IL-23 signaling such as IL-17A, IL-17F, IL-22 or IL-17RA. Few of those attempt to block IL-23 signaling and Th17 arm by inhibiting the transcription factors regulating IL-23 or IL-23R production via blockade with apilimod and Rorc inhibitors, respectively. Some of therapeutics are currently in use for conditions other than IBD; others are in the development–discovery stage, and some have been discontinued due to lack of efficacy or adverse effects (review by [165, 166]. **Table 1** gives a summary of the therapies directly targeting IL-23.

Ustekinumab is the only FDA-approved IL-23/IL-12 blocker that is currently used for treatment of psoriasis and psoriatic arthritis. It is a neutralizing fully human monoclonal antibody against the common p40 subunit. Several clinical trials are assessing its effectiveness against a list of autoimmune conditions. Ustekinumab phase III trials for Crohn's disease and ankylosing spondylitis showed promising results [167–169]. Ustekinumab is also being tested for atopic dermatitis and rheumatoid arthritis. Multiple Sclerosis patients, however, did not benefit from Ustekinumab for unknown reasons [170]. Briakinumab is also a p40-specific monoclonal

antibody developed by Abbott, but due to cardiac problems associated with its use, it did not make to the market [171]. Since Ustekinumab blocks both IL-12 and IL-23, the Th17 and Th1 arm of the helper T cells are affected together. IL-23p19-specific monoclonal antibodies which will exclusively target Th17 arm and spare Th1 lineage may be more beneficial for the long-term use. Although both Th1 and Th17 cells are implicated in many autoimmune conditions (Psoriasis, IBD, MS), the Th1 arm of the helper cells are crucial for immunity against intracellular pathogens and tumors, and thus selective targeting of Th17 may help to reduce the risk of certain infections or developing tumors during long-term use of immunosuppression.

Drug	Target	Company	Status	Disease
Ustekinumab	p40 (IL-12p40; IL-23p40) mAb human	Centocor Ortho Biotech and Janssen Research	Approved Approved Phase III Phase I Phase II Phase II Phase II Phase II Discontinued	Plaque psoriasis Psoriatic arthritis Crohn's disease CVID-dependent enteropathy Ankylosing spondylitis; Sarcoidosis atopic dermatitis; rheumatoid arthritis Multiple sclerosis
Briakinumab	p40 (IL-12p40; IL-23p40) mAb human	Abbott	Discontinued Discontinued Discontinued	Psoriasis Crohn's disease Multiple sclerosis
Guselkumab	IL-23 p19 antagonist mAb human	Janssen Research	Phase III	Psoriasis
BI 655066	IL-23 p19 antagonist mAb humanized	BoehringerIngelheim	Phase II	Crohn's disease; psoriasis
Tildrakizumab	IL-23 p19 antagonist mAb human	Schering-Plough/Merck	Phase III	Psoriasis
MP-196	IL-23p19 antagonist mAb	Effimune	–	Autoimmune disease
FM-303	IL-23p19 antagonist mAb	Femta Pharmaceuticals	Discovery	Inflammatory bowel disease
AMG 139	IL-23p19 mAb human	Amgen, AstraZeneca	PhaseII	Crohn's disease; psoriasis
IL-23 Adnectin	IL-23R	Bristol-Myers Squibb	Discovery	Immune disorder
Anti-IL-23 immunotherapy	IL-23R	Peptinov SAS	Discovery	Inflammatory disease
LY3074828	IL-23 p19 antagonist mAb humanized	Eli Lilly	Phase I	Psoriasis
Apilimod (STA-5326)	Blocks NFKB translocation, IL-12, IL-23 production	Synta Pharmaceuticals	Discontinued	Psoriasis; rheumatoid arthritis; common immunodeficiency

Adapted and modified from Tang and Iwakura [165] and Patel and Kuchroo [166].

Table 1. Identified interleukin-23 receptor (IL-23R) antagonists.

Guselkumab, Tildrakizumab, BI655066, AMG 139, MP-196 are monoclonal anti-p19 neutralizing antibodies that are now actively being tested by different companies for psoriasis at different phases (ClinicalTrials.gov). BI 655066 is additionally being tested on CD patients in a phase II trial. With positive results from psoriasis cases, the remaining p19 blockers are very likely to be extended to trials with Crohn's disease patients soon.

In addition to the IL-23 itself, several other downstream effector cytokines of IL-23R signaling pathway are being targeted with monoclonal antibodies to treat autoimmune diseases. Monoclonal antibodies against IL-17A, IL-17F, IL-17RA proved to be very effective treating psoriasis in various trials [166, 172]. However, secukinumab (anti-IL-17A) trial did not benefit CD patients [93], thus due to lack of any improvement with IL-17A neutralization brodaluzumab (IL-17RA antibody) development and trials were terminated [173]. As described in previous sections, recent studies suggest an important role to IL-17A intestinal barrier function which may be essential for the containment of microbiota. Thus, its removal may exacerbate the condition in IBD [94, 95].

IL-22 is another IL-23 regulated cytokine which went through clinical trials (ClinicalTrials.gov). Fezakinumab (ILV-094) is a monoclonal human IL-22 antibody and has been tested in psoriasis and RA with no results being revealed. Due to its involvement in various IBD models, IL-22 antibodies are also a likely candidate to through clinical trials in CD patients.

There are Rorc inhibitors that are being tested in healthy volunteers (VTP-43472 and JTE-151). They inhibit both Rorγ and Rorγt *ex vivo* and *in vivo* results are not yet available [166].

IL-23 receptor signals through JAK2/TYK2 kinases. Several JAK2 inhibitors are in clinical trials for treatment of cancer and autoimmune disease. Ruxolitinib is a JAK1/JAK2 inhibitor approved by FDA for myelofibrosis and is now being tested in RA and psoriasis patients. Baricitinib is another Jak1/Jak2 inhibitor in Phase II clinical trials in RA patients. Lastly, lestaurtinib is a JAK2 inhibitor and is in Phase II trials on psoriasis patients. These molecules (ClinicalTrials.gov) are eventually likely to be tested on IBD patients.

Author details

Ahmet Eken[1,2] and Mohamed Oukka[3,4*]

*Address all correspondence to: moukka@u.washington.edu

1 Medical Biology, Faculty of Medicine, Erciyes University, Kayseri, Turkey

2 Betül-Ziya Eren Genome and Stem Cell Research Center, Kayseri, Turkey

3 Center for Immunity and Immunotherapies, Seattle Children's Research Institute, Seattle, WA, USA

4 Department of Immunology, University of Washington, Seattle, WA, USA

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Chapter 6

Cytokines and Nitric Oxide in Immunopathogenesis of IBD and Potential Therapeutic Approaches

Imene Soufli, Ryma Toumi, Hayet Rafa and
Chafia Touil-Boukoffa

Additional information is available at the end of the chapter

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Abstract

Inflammatory bowel disease (IBD), which includes Crohn's disease (CD) and ulcerative colitis (UC), is a multi-factorial condition characterized by a chronic inflammation of the gastrointestinal tract. In IBD, the balance between pro/anti-inflammatory cytokines and immuno-regulatory cytokines is disturbed. An over-production of pro-inflammatory cytokines and nitric oxide characterizes the pathogenesis of IBD. In Crohn's disease the major cytokines are generated by Th1- and Th17-polarized T cells. In contract, UC is viewed more as an atypical Th2-type immune response characterized by the generation of high amount of IL-5, IL-4 and IL-13. Both Th1 and Th17 cytokines are involved in the up-regulation of iNOS expression in IBD and the production of high level of nitric oxide (NO). The latter, as an effect, causes tissue damages through the generation of reactive nitric oxygen species (RNOS). A better understanding of the pathogenesis of IBD has led to the development of new therapeutic strategies based on targeting cytokines and their receptors as well as NO modulation. Manipulation the microbiota with probiotics and helminthes may have potential use as anti-inflammatory agents in IBD by inducing anti-inflammatory cytokine pattern.

Keywords: cytokines, inflammatory bowel diseases, nitric oxide (NO), nitric oxide synthases (NOS), anti-cytokine therapy

1. Introduction

Inflammatory bowel disease (IBD), represented mainly by ulcerative colitis (UC) and Crohn's disease (CD), is a multifactorial condition characterized by a chronic inflammation of the gastrointestinal tract. It is widely accepted that IBD results from an uncontrolled mucosal immune response to intestinal microflora in genetically susceptible hosts [1, 2]. The inflamed intestine of patients with IBD is massively infiltrated by inflammatory cells that release a large amount of proinflammatory mediators such as cytokines and nitric oxide (NO) [3].

NO is a free radical which has several physiological and pathological functions. It is generated from the oxidation of the amino acid L-arginine by a family of enzymes called nitric oxide synthases (NOS). Three distinct isoforms of NOS are known: two isoforms constitutively expressed in neuronal (nNOS) and endothelial (eNOS) tissues and an inducible isoform (iNOS) expressed mainly in immune cells such as macrophages [4, 5]. The constitutively expressed isoforms release low levels of NO that exert physiological functions, whereas iNOS releases a high output of NO production under immunogenic and inflammatory stimuli [6, 7].

Cytokines are small soluble peptides which are produced by diverse immune and nonimmune cells. They exert their biological functions through specific receptors activating the JAK-STAT signaling pathway that control gene expression of target cells [8]. In IBD, the balance between pro-/anti-inflammatory cytokines and immunoregulatory cytokines is disturbed leading to distinguish a different T cell profile in CD and UC. Classically, Crohn's disease is described as TH1-type immune response characterized by the secretion of IFN-γ, IL-12, and TNF-α. In contrast, ulcerative colitis is viewed more as an atypical TH2-type immune response which generates high amount of IL-5, IL-4, and IL-13 [9, 10]. In addition, several studies have shown the involvement of TH17-type cytokines (IL-17, IL-23, IL-22, IL-6) in the pathogenesis process of both Crohn's and ulcerative colitis [11, 12]. Interestingly, both TH1 and TH17 cytokines are involved in the upregulation of iNOS expression in IBD. Indeed, a positive correlation between nitric oxide production and increased proinflammatory cytokines (TNF-α, IL-6, IL-17 IL-12, and IFN-γ) were observed in plasma of IBD patients [12, 13].

The considerable research conducted over the last year to better understand the pathogenesis of IBD has led to the development of new therapeutic strategies based on targeting cytokines, their receptors, as well as NO modulation. Unfortunately, some of those strategies showed limited efficacy. Hence, better understanding of the mechanisms underlying the inflammation and the immune response in IBD may give arise to new alternative complementary therapeutic strategies. Moreover, the assessment of NO production in IBD might be a useful inflammatory marker to predict the stage of the disease [14].

This chapter will address the cytokine involvement and their relationship with nitric oxide in IBD immunopathogenesis as well as potential therapeutic targets that may arise.

2. Nitric oxide and IBD

Nitric oxide is a lipophilic-free radical, which plays a key role in regulating homeostasis of many biological systems [15]. It is synthesized by a family of enzymes called NOS which catalyze the oxidation of the terminal nitrogen of the amino acid L-arginine and produce L-citrulline and NO [5, 7]. Three NOS isoforms have been identified and characterized in humans and in mice; their nomenclature respects the chronological order in which they were purified: The neuronal form (nNOS or NOS1), the inducible form (iNOS or NOS2), and the endothelial form (eNOS or NOS3). nNOS and eNOS are termed constitutive NOS (cNOS) as they are calcium-dependent, and are respectively expressed constitutively in neuronal and endothelial tissue [4, 5, 7]. The effects of NO differ on its rate, duration, and place of

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production and the nature of the target molecules [16]. Under physiological conditions, cNOS generates low levels of NO which have direct regulatory effects such as neurotransmission and regulation of blood vessel [17, 18]. On the other hand, iNOS generates high levels of NO which mediates antimicrobial and antitumor activities [16, 19, 20]. This isoform was first isolated in murine macrophages then it was found in several other cells type Including epithelial cells, hepatocytes, endothelial cells, and fibroblast. It is expressed after induction by immunologic and inflammatory stimuli [6, 16, 19, 20]. However, when NO is produced in excess, it becomes noxious. It causes deleterious effect indirectly through the creation of reactive nitric oxygen species (RNOS) such as peroxynitrite anion (OONO−), the nitroxyl anion (NO−), and dioxide nitrogen (NO_2), responsible for the oxidative stress [7, 21, 22]. Peroxynitrite, a molecule with high oxidative potential, can trigger cytotoxic processes such as lipid peroxidation and DNA damage leading to tissue damage and inflammation [22]. NO has been implicated as a pathogenic mediator in a variety of conditions, such as inflammatory bowel disease [23, 24] **Figure 1**.

The deleterious role of NO in IBD has been proposed after clinical studies that reported the presence of a high level of nitrite/nitrate in plasma, urine, and the lumen of the colon [14, 25, 26]. Moreover, a correlation between overexpression of iNOS and increased concentration of NO and the severity of diseases was shown [26]. In fact, an increased level of NO was found in serum, stool, and urine of patients with active phase of UC and CD compared to inactive phase [14, 24–26]. Although our study showed a significantly higher serum level of NO in CD patients compared to UC patients, data from previous studies reported no significant difference between these two categories of disease, whereas a higher systemic level of NO in UC compared to CD was reported [12–14, 24, 26].

As mentioned above, NO exerts its deleterious effects by combining with superoxide anion to form peroxynitrite. Thus, experimental model of colonic inflammation could be induced by intracolonic administration of peroxynitrite [27, 28]. Besides, high nitric oxide generation can be accompanied by the production of carcinogenic nitrosamnies from neutrophils in inflamed colonic mucosa. These nitrosamines may contribute to the increased risk of malignancy in IBD [28].

Moreover, recent studies carried out on patients with very onset inflammatory bowel diseases (VEOIBD) reported a genetic association with NOS2 single nucleotide polymorphisms (SNPs) and VEOIBD. Younger pediatric IBD patients develop a different disease phenotype compared to adults onset IBD, often characterized by a severe pancolitis and high expression of iNOS. The therapeutic inhibition of iNOS expression in VEOIBD could then be beneficial [29].

While several studies conducted on animal models report the deleterious effect of NO, some recent studies have shown that NO may also exert protective effect against colitis [29–32]. Indeed, because of its strong bactericidal and cytostatic properties, high NO generation by iNOS may represent a protective mechanism [28]. Recent study conducted on DSS-induced colitis model has shown that nitrite administration exerts both preventive and therapeutic effects in colonic inflammation [30]. More recently, iNOS deficiency was shown to aggravate inflammation in animal model of colitis through enhancing TH17 differentiation [31].

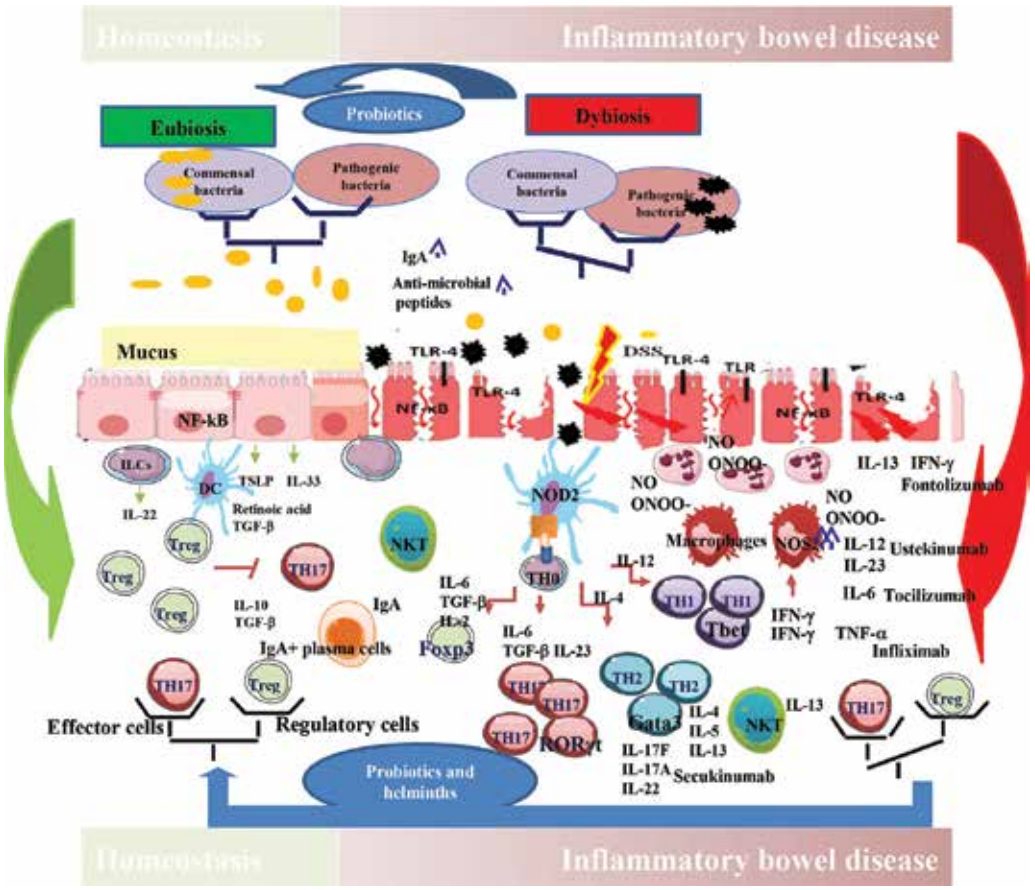

Figure 1. Involvement of cytokines and nitric oxide in IBD and the potential therapeutic targets. IBD is characterized by a defective regulatory and anti-inflammatory immune responses mediated by cytokines such as interleukine-10 and transforming growth factor (TGF)-β produced by regulatory T cells (Treg) and the over-production of interleukin (IL)-12, IL-6 and IL-23 and tumor necrosis factor (TNF)-α by dendritic cells (DC) and macrophages. Th1-polarized cells secrete interferon-γ, which induces the high production of nitric oxide (NO) by macrophages. Th2-polarized cells and natural killer T (NKT) cells induce an immune response mediated by IL-5 and IL-13. Th17-polarized T-cell generation is induced by transforming growth factor (TGF)-β, IL-6 and IL-23; they secrete IL-17A, IL-17F and IL-22. Biological therapies target several molecular pathways by blocking cytokine activity and restoring the microbiota through the use of probiotics and helminths. TLR, Toll-like receptor; NOD, nucleotide oligomerization domain; NF-κB,: nuclear factor kappa B; TSLP, thymic stromal lymphopoietin; DSS, dextran sulfate sodium; ROR, retinoid-related orphan receptor; ILCs, innate lymphoid cells; Foxp, Forhead box protein P3.

3. Cytokine regulation of nitric oxide in IBD

The inflamed tissue of patients with active IBD is characterized by a massive infiltration of immune cells that release several proinflammatory mediators and produce high *de novo* levels of NO. The expression of iNOS is highly regulated at both transcriptional and post-transcriptional level by several proinflammatory cytokines and immunogenic stimuli such as LPS [6, 7].

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In both patients and animal models of IBD, a positive correlation between the overproduction of proinflammatory cytokines, such as IL-1β, TNF-α, and IFN-γ, and an overexpression of iNOS was found. Its expression was mainly detected in lamina propria mononuclear cells and colon epithelial cells of inflamed mucosa [6, 12, 13, 25, 32–34]. Studies conducted on a DSS-induced experimental model of colitis in BALB/c mice showed that neutralization of endogenous TNF-α and/or IFN-γ ameliorated the chronic colitis and concomitantly decreased NO generation [32]. These data support the fact that IFN-γ and TNF-α are both involved in the exacerbation of DSS-induced colitis and may exert their detrimental role in the colonic mucosa partly through the induction of high output of NO [32]. These cytokines had an additive effect on the severity of histological damages and NO colonic levels. However, it seems that IFN-γ is the most potent inducer of iNOS in macrophages and epithelial cells than TNF-α since its neutralization was more effective in attenuating the experimental colitis [32].

Moreover, our studies reported an upregulation of iNOS expression in inflamed colonic mucosa which correlates with high systemic levels of NO, IFN-γ, and IL-12. These observations suggest that IFN-γ and IL-12 may play a pivotal role in IBD pathogenesis through NO pathway [12]. Human PBMC from IBD patients were shown to produce elevated level of NO compared to controls. Proinflammatory cytokines such as IFN-γ, IL-6, TNF-α, and IL-1β stimulate NO production *in vitro* in PBMC from patients with CD and UC suggesting that human PBMC may constitute another cellular source of NO in IBD [12, 13]. Interestingly, this study reported a positive correlation between TH17 cytokines including IL-6, IL-23, IL-17A, and NO production in plasma of patients with IBD [12]. Moreover, the mucosal alterations strongly correlated with high iNOS and pSTAT3 expression in colonic mucosa of patients with active IBD. These observations suggest that IL-17 may be a potent inducer of iNOS expression in inflamed mucosa of IBD patients leading to the exacerbation of the tissue damages. The mechanism by which IL-17 induces NO production is likely dependent on nuclear factor kappa B (NF-kB) expression. In fact, *in vitro* studies using osteoclastes cells showed that IL-17 induced high expression of mRNA of the NF-kB isoform RelA et p50 [35].

On the other hand, the negative regulation of iNOS could be achieved by TH2 derived cytokines such as IL-13 and IL-4. The inhibitory effect of these cytokines on iNOS protein and mRNA expression has been demonstrated in the HT-29 epithelial cell line induced by IL-1a/TNF-α/IFN-γ. Moreover, at low levels and in the presence of TNF-α, these cytokines exert inhibitory effect on iNOS expression and activation. Although a high level of these cytokines could inhibit iNOS mRNA induction in absence of TNF-α [36, 37]. The mechanism under the inhibitory effect of IL-13 on iNOS expression in epithelial cells is dependent on the activation of PtdIns 3-kinase pathway [37].

Furthermore, it has been shown that the immunosuppressive cytokine IL-10 inhibit iNOS expression depending on the cell type. Indeed, unlike IL-13, IL-10 had no effect on iNOS expression in colonic epithelial cells but was able to inhibit NO production in mouse-activated macrophages [6, 36]. Recently, it has been demonstrated that the inhibition of NO and ROS in mouse carrying a selective deletion of IL-10Rα in macrophages had less severe colitis than wild-type mice. These data suggest that the protective effect of IL-10 is mainly mediated through the downregulation of NO and ROS production by macrophages [38].

These observations and others suggest that cytokines modulate the iNOS expression and activity in colonic epithelium in human and experimental IBD, and might play homeostatic or inflammatory role in gut inflammation through iNOS modulation.

Several studies have shown that NO can in turn modulate the immune response by suppressing IL-12 production from dendritic cells and macrophages. In that manner NO may control the generation of TH1-type response [39]. More recently, a study reported that expression of iNOS in macrophages and dendritic cells can modulate inflammatory cytokine expression including TNF-α, IL-6, IL-12p70, and IL-23. Growing evidence supports this notion and suggests that NO may control T helper cell differentiation [31, 40]. Indeed, studies conducted in experimental model of colitis showed that iNOS deficiency aggravates inflammation and increased the percentage of TH17 cells. While an NO donor molecule suppressed IL-17 production in T cell-deficient NOS cultures and reduced the percentage of IL-17 producing CD4+ T cells. In fact, NO has been found to regulate IL-17 expression at the transcriptional level through the nitration of tyrosine residues in RORγt inhibiting therefore its binding to the promoter region of IL-17 gene [31].

4. Cytokines implication in IBD

The dysfunction of mucosal immune responses in IBD is characterized by abnormalities of both innate and adaptive immune systems. The final common pathway of this deregulated immune activation is an abundant infiltration of immune cells in the intestinal mucosa [11, 41–43]. These cells were found to release excessive proinflammatory mediators that amplify inflammatory cascade through the activation of *mitogen-activated protein kinases* (MAPK) and *nuclear factor kappa B*. Several studies have reported evidences about the contribution of cytokines, adhesion molecules, reactive oxygen metabolites (ROMs), and nitric oxide in triggering mucosal inflammation and injury in IBDs [8, 9, 23, 24, 43–45]. In IBD, the balance between proinflammatory cytokines (TNF-α, IL-1β, IL-8, and IL-17), antiinflammatory cytokines (IL-4 and IL-13), and immunoregulatory cytokines (IL-10 and TGF-β) is disrupted [45]. According to the cytokine environment found in IBDs patients, Crohn's disease and ulcerative colitis were conventionally associated to a different CD4+ helper T cells profile based on the paradigm TH2/TH1. Thus, Crohn's disease was described as TH1-type immune response promoted by the transcription factors STAT-4 and T-bet and characterized by the secretion of IFN-γ, IL-12, and TNF-α [9, 46]. Indeed, the studies conducted by our and other teams showed high levels of IL-12 and IFN-γ in CD patients with active disease [13]. IL-12 produced by macrophages/monocytes system and dendritic cells plays a pivotal role in enhancing natural killer (NK) cell-mediated cytotoxicity. Moreover, it is admitted that both IL-12 and IL-18 induce high level of IFN-γ production leading to the reinforcement of TH1 immune response [13, 47, 48]. In addition, TNF-α plays a pivotal role in the pathogenesis of IBD. It induces expression of adhesion molecules, increases the local release of nitric oxide, and enhances the production of metalloproteinases leading to the loss of epithelial integrity [49, 50]. In contrast, ulcerative colitis was viewed as a TH2-type immune response promoted by the expression of the transcription factors STAT-6 and GATA-3 and the secretion of IL-5, IL-4, and IL-13 [41].

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Furthermore, Fuss et al. demonstrated that UC patients, unlike CD patients, have atypical natural killer T cells. These cells produce high IL-13 levels and have cytotoxic activity toward epithelial cells [51].

Currently, the aforementioned classical concept of the pathogenesis of IBDs is reconsidered with the strong involvement of TH 17 cells. This subset of CD4+ T helper is promoted by the activation of the transcriptions factors STAT-3 and ROR-γt and is characterized by the production of IL-17A, IL-17F, IL-22, IL-21, IL-6, and IL-26 and the chemokine CCL20 [52, 53]. Several evidences support the implication of the TH17 cells in the intestinal mucosa protection against invading pathogens such as Candida and Salmonella, through chemotaxis of neutrophils and stimulation of antimicrobial peptides production by epithelial cells [54]. However, both in CD and UC high level of TH17 cytokines signature was demonstrated in the serum and inflamed mucosa. Increased IL-17A production can drive and aggravate the chronic inflammatory response [13, 55, 56]. More recently, another subset of TH17, TH1/TH17cells producing both IFN-γ and IL-17 has been identified in ileal form of active Crohn's disease and experimental models of colitis [57–59]. In addition, it has been reported that TH17 induce the production of high level of TNF-α, IL-1β, chemokines (IL-8), and matrix metalloproteinases such as MMP-9. Moreover, the expression of the cytokine IL-23 and CCL20, a chemoattractant for TH17 expressing CCR6, was highly upregulated in Crohn's disease lesions. IL-23 is a crucial effector necessary for the stabilization and expansion of TH17 cells. It enhances the expression of the master transcription factor (RORγt) following IL-6 and TGF-β stimulation. Moreover, it plays an important role in the development and propagation of the inflammatory response in the gut by inhibiting the expression of the transcription factor Foxp3 and the development of Treg cells [11, 52, 53, 58–60].

The TH17/Treg balance plays an essential role in maintaining intestinal homeostasis. The immunoregulatory cytokine TGF-β orchestrates the differentiation of TH17 and Treg cells in a dose-dependent manner. In the presence of high level of IL-6 and inflammatory mediators, TGF-β promotes the differentiation of TH17 cells. Conversely, high level of TGF-β and low level of IL-6 and inflammatory mediators promote the development of Foxp3+Treg-induced cells (iTreg) [61, 62]. Regarding the proinflammatory role of IL-6, elevated levels of this cytokine and its soluble receptor sIL-6R were found in colonic mucosa and sera of patients with inflammatory bowel disease. Compelling evidence in human and in animal models showed that IL-6 plays an important role in maintaining a chronic response by promoting the accumulation of T cells resistant to apoptosis. Besides, IL-6 induces the production of IFN-γ, TNF-α, and IL-1β and increases the expression of adhesion proteins such as ICAM-1 protein which participates in the migration and activation of inflammatory cells to the intestine [63, 64].

It is well established that ongoing inflammation in Crohn's disease and ulcerative colitis is mediated by uncontrolled T cell response. Altered Treg regulatory mechanisms have been documented in IBD. However, it is still not clear whether this defect is due to a numerical lack of Treg or to a defective TGF-β and IL-10 immunoregulatory activity [65, 66]. Interestingly, it has been shown in inflamed colon of CD patients a common CD4+T cell population, which coexpresses both Foxp3 and RORγt. This resident Treg cells showed plasticity toward TH17 in

inflammatory environment. Treg/TH17 balance is tightly regulated by intestinal factors such as endogenous mircroflora as well as the presence of retinoic acid. Indeed, it has been reported that the vitamin A metabolite, retinoic acid promotes Treg differentiation while inhibiting the formation of TH17 cells [55, 67, 68]. Thus, these data support the involvement of altered intestinal microenvironment in the development of IBD and rupture of gut homeostasis.

Other studies conducted on IBD experimental models reported the implication of other cytokines with immunomodulatory role such as IL-25, TSLP, and IL-22, opening therefore the way to new therapeutic strategies in IBD [69–71].

5. Therapeutic implications

Inflammatory bowel diseases are chronic conditions with no treatment to achieve a complete healing. As the exact etiopathology of these conditions is still not known, the conventional treatment (salazosulfamide, glucocorticoids, and immunosuppressive agents) remains symptomatic. It aims to attenuate inflammation and enable patients entering long-lasting remission.

It is well established that cytokines are key mediators in the pathogenesis of IBD. Thereby, their targeting represents a rational and promising therapeutic approach. Blocking proinflammatory cytokines such as TNF-α has led the revolution of biological therapies in several immune diseases including IBD. Chimeric (infliximab), humanized (certolizumab pegol), and fully human monoclonal anti-TNF-α antibodies (adalimumab) have been approved for the treatment of active refractory and fisulizing forms of Crohn's disease [72–74]. Even if the anti-TNF-α is the leader of biological therapies, many side effects have been assigned to its use such as infections and lymphoma risks [75]. Moreover, some patients were refractory or intolerant to anti-TNF-α therapy. Over the last years extensive therapeutic approaches have targeted other cytokines as well as their receptors and signaling pathways in treatment of IBD such as IFN-γ, IL-17A, IL-23/IL-12p40, and Jak1/3 signaling pathway. As described above, the axis IL-12/IFN-γ plays a key role in the pathogenesis of human IBD and experimental colitis. These findings lead to target IFN-γ or IL-12 for the therapy of IBD. Indeed, the monoclonal antibody ustekinumab, targeting the common p40 subunit of IL-12/IL-23, appears to be efficient in inducing clinical remission in moderate-to-severe Crohn's disease patient's nonresponding to anti-TNF-α therapy [76, 77]. However, the blockade of IFN-γ with specific monoclonal antibody, fontolizumab, had no clinical beneficial effect in patients with active CD [78]. Moreover, targeting TH17 cytokines in colitis with the anti-IL-17A antibody, secukinumab, showed disappointing results [79]. That result may be related to the cytokine pattern that can change depending on the location of the inflammatory injuries, the stage of the disease, and the T cell plasticity observed in inflamed mucosa of CD patients. In this context, studies conducted on CD patients and in experimental model of colitis showed a pronounced TH1–TH17 response as the disease becomes chronic. These results can be also explained by the plasticity between TH1/TH17 and TH17/Treg [80, 81].

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On the other hand, the use of immunoregulatory cytokines such as IL-10 and TGF-β has been extensively studied in order to restore the defective regulatory response in IBD [65]. Administration of recombinant human IL-10 has not been beneficial to patients with active UC and CD. However, the inhibition of Smad7 with a specific antisense oligonucleotide restores TGF-β1 signaling and showed safe and beneficial effects in a phase 1 study in active CD [82].

Another approach to downregulate T cell activation and resistance against apoptosis in IBD consists of neutralizing IL-6 receptor. Therapeutic benefit of blockade of IL-6R with a humanized anti-IL6R antibody, tocilizumab, was shown in established experimental colitis and in patients with Crohn's disease [83].

Based on experimental model of colitis, IL-13 is associated with the onset of inflammation in ulcerative colitis. Thus, targeting IL-13 or the factors that regulate its production might be a potential therapy in UC. Notably, treatment of patients with IFN-β exerted beneficial effect through the reduction of IL-13 production by the lamina propira T cells [84]. However, in a recent study, it has been shown that the efficiency of IFN-β treatment depends on TH17 cytokine profile of patients. Thereby, patients with low level of IL-17A showed positive clinical response to IFN-β than patients with high IL-17A levels [85].

Consistent with these data, an alternative therapeutic approach that aims to block intracellular signaling pathways of several cytokines has been explored. In particular, the JAK/STAT pathway which is responsible for signal transduction of various cytokine receptors involved in both the innate and adaptive immune response. Indeed, small molecule inhibitor of Janus kinases (JAKs) specific for JAK1 and JAK3, namely, tofacitinib, inhibit the signaling of several cytokines such as IL 2, IL 4, IL 7, IL 9, IL 15, and IL 21. It has been shown that tofacitinib can suppress T cell differentiation and activation conferring beneficial effects in IBD, particularly in ulcerative colitis [86–88].

Furthermore, given the complexity of cytokines network in IBD, it has been suggested that simultaneous neutralization of two cytokines using bispecific dual variable domain antibodies could yield promising result in treating IBD [89].

The limited efficacy that has shown certain cytokine-based therapy led to the search for alternative therapeutic pathways that regulate cytokines balance in IBD. There is growing body of evidence that suggest that probiotics and heminths may have potential use as antiinflammatory agents in IBD by inducing antiinflammatory cytokines pattern. The results of our studies and others demonstrated that some probiotics such as *Bifidobacterium infantis* and *Bifidobacterium longum* downregulate proinflammatory cytokines IFN-γ, IL-12, TNF-α, and IL-8 production and stimulate immunoregulatory cytokine IL-10 production [33, 90–92]. Moreover, it has been reported that *B. infantis* feeding in DSS-induced colitis model downregulate IL-17A expression and induce IL-10 production restoring thereby the TH17/Tregs balance [93, 94]. Human clinical trials showed encouraging evidence on the efficacy of the probiotics preparation VSL#3 and the probiotic *Escherichia coli* Nissle 1917 to maintain remission in ulcerative colitis. Unfortunately, very few studies reported the beneficial use of probiotics in CD [95, 96]. Furthermore, other studies have oriented the use of probiotics

lactobacillus to deliver cytokines such as IL-10 or anti-TNF-α locally to potentiate their action while limiting their side effects [97, 98]. Concerning the use of helminths in shaping the immune responses in IBD, there is overwhelming data showing their immunoregulatory effects. Indeed, immunity to helminth is TH2-type response dependent on the secretion of antiinflammatory cytokines (IL-4, IL-5, IL-13, and IL-9) and the induction of Tregs. Experimental studies demonstrated that helminthes infection attenuate damaging TH1-/TH17-driven inflammatory responses through the induction of regulatory responses [99–101] **Figure 1**.

Author details

Imene Soufli, Ryma Toumi, Hayet Rafa and Chafia Touil-Boukoffa*

*Address all correspondence to: touilboukoffa@yahoo.fr

Department of Cellular and Molecular Biology, Team "Cytokines et NOSynthases", University of Sciences and Technology Houari Boumediene, Algiers, Algeria

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Section 3

Mechanism Driving Inflammatory Bowel Disease

Chapter 7

MicroRNA in Inflammatory Bowel Disease

Kurt Fisher and Jingmei Lin

Additional information is available at the end of the chapter

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Abstract

Idiopathic inflammatory bowel disease (IBD) is a complex set of disorders that predominantly includes ulcerative colitis and Crohn's disease. The pathogenesis of IBD is multifactorial including genetic, infectious, and immunologic factors. MicroRNAs belong to a class of noncoding small RNAs that posttranscriptionally regulate gene expression, and they are an emerging class of genetic modifiers of IBD. Here, we focus on the use of unique microRNA expression patterns as biomarkers to classify and prognosticate disease severity in both mucosal tissue and serum from patients with either ulcerative colitis or Crohn's disease. Furthermore, we discuss specific microRNAs with respect to their roles in IBD pathogenesis and fibrosis. We also discuss the role of microRNAs in IBD-associated carcinogenesis, including their role as biomarkers, tumor suppressors, and oncogenes. Finally, we discuss the emerging therapeutic applications of microRNA manipulation to lessen the effect of IBD and its sequelae. Recent discoveries of the diverse roles of microRNAs in IBD pathogenesis have the potential to provide new targeted therapeutics for personalized medicine.

Keywords: inflammatory bowel disease, ulcerative colitis, Crohn's disease, microRNA, pathogenesis

1. Introduction

Idiopathic inflammatory bowel disease (IBD) is a group of chronic and recurrent inflammatory disorders that primarily involve the gastrointestinal tract. It predominantly includes ulcerative colitis (UC) and Crohn's disease (CD). The pathogenesis of IBD is multifactorial and not completely understood, but genetic, epigenetic, infectious, physiological, and immunological factors may all play important roles in the genesis and progression of the diseases [1–3]. A large number of genes have been linked to IBD susceptibility, pathogenesis, and carcinogenesis, and recent work has suggested that microRNAs can undertake the same roles.

MicroRNAs are encoded within the genomes of a wide variety of eukaryotes, and more than 2500 human mature microRNAs have been curated in miRBase database since their discovery in 1993 [4, 5]. MicroRNAs are evolutionarily conserved, single-stranded noncoding RNA molecules of 19–24 nucleotides, which represent a class of regulatory RNAs suppressing gene expression at a posttranscriptional level. MicroRNAs concurrently modulate the expression levels of dozens or more messenger RNA (mRNA) targets and any given mRNA sequence may be targeted by several different microRNAs creating intricate regulatory networks to fine-tune a cell's function [6–8]. At the time of publication, microRNAs are predicted to directly regulate the expression of at least 30% of the entire mammalian genome [9]. MicroRNAs have been found to be involved in the normal functioning of multiple pathophysiological networks and in the pathogenesis of a broad spectrum of human diseases, ranging from neoplastic to inflammatory conditions [10–15].

In this chapter, we focus on the role of microRNAs in IBD as recent publications have indicated that microRNAs play critical roles in the pathogenesis of IBD and IBD-associated carcinogenesis and may serve as critical future targets for personalized medicine.

2. MicroRNAs show a diverse array of aberrant expression in IBD

Extensive literature has shown that microRNAs undergo dysregulation in both tissue and peripheral blood of patients with IBD with a goal of discovering biomarkers and crucial initiators of pathogenesis.

2.1. MicroRNAs are differentially expressed in the mucosal tissue in UC

A search for biomarkers in UC in both non-affected and actively inflamed mucosa has identified a large number of microRNAs that show aberrant expression (**Table 1**) [16–28]. When compared to controls, a large number of microRNAs have been found to be upregulated including, but not limited to, miR-let-7e*, miR-let-7f, miR-7, miR-7i, miR-16, miR-20b, miR-21, miR-23a, miR-24, miR-29a, miR-29b, miR-31, miR-98, miR-125b-1*, miR-126, miR-126*, miR-127-3p, miR-135b, miR-142-3p, miR-146a, miR-150, miR-155, miR-195, miR-196a, miR-206, miR-223, miR-324-3p, miR-375, miR-422b, miR-548a-3p, miR-650, miR-663, and miR-4284. The decreased microRNA profiles include miR-143, miR-145, miR-188-5p, miR-192, miR-194, miR-194b, miR-196b, miR-200b, miR-215, miR-216b, miR-320a, miR-346, miR-375, miR-489, miR-548e, miR-559, and miR-630. Seven microRNA candidates have been identified by at least two independent groups, miR-21 [16–18, 26, 27], miR-29a [16, 19], miR-31 [19, 24], miR-146a [23, 24, 27], miR-155 [17, 23], miR-192 [16, 27], and miR-375 [16, 27], making them the most promising candidates for biomarkers of UC.

However, there are many variables that differ between studies, including anatomical tissue location, degree of inflammation, prior medication, and platforms used to measure microRNAs that all may contribute to the high discordance between studies.

Status	Tissue type	Control	Aberrant microRNA expression	Reference
Active UC	Sigmoid, *n* = 15	Healthy	Decreased: miR-192 and 375 Increased: miR-let-7f, 16, 21, 23a, 24, 29a, 126, 195, and 422b	Wu [16]
	Sigmoid, *n* = 12	Healthy	Increased: miR-21 and 155	Takagi [17]
	Sigmoid, *n* = 12	Healthy	Increased: miR-21 and 126	Feng [18]
	Colon, nonspecific, *n* = 10	Healthy	Decreased: miR-188-5p, 215, 320a, and 346 Increased: miR-7, 31, 135b, and 223	Fasseu [19]
	Colon, nonspecific, *n* = 5	Healthy	Increased: miR-150	Bian [20]
	Colon, nonspecific, *n* = 8	Healthy	Decreased: miR-143 and 145	Pekow [21]
	Colon, nonspecific, *n* = 20	Healthy	Increased: miR-let-7e, 20b, and 98*	Coskun [22]
	Colon, *n* = 10	Healthy	Increased: miR-146a and 155	Beres [23]
Active or inactive UC	Colon, distal-most, *n* = 10	Healthy	Decreased: miR-194b, 216b, 548e, and 559 Increased: miR-31, 146a, 206, and 663	Lin [24]
	Sigmoid, *n* = 26	Healthy	Increased: miR-4284	Koukos [25]
Inactive UC	Sigmoid, *n* = 15	Healthy	Increased: miR-16, 23a, 24, 29a, 375, and 422b	Wu [16]
	Colon, nonspecific, *n* = 8	Healthy	Decreased: miR-188-5p, 215, 320a, and 346 Increased: miR-29a, 29b, 126*, 127-3p, 196a, and 324-3p	Fasseu [19]
	Colon, nonspecific, *n* = 19	Healthy	Increased: miR-20b and 125b-1*	Coskun [22]
Unknown	Colon, nonspecific, *n* = 15	Healthy	Increased: miR-21	Yang [26]
	Colon, nonspecific, *n* = 12	Healthy	Increased: miR-7i, 21, 142-3p, and 146a Decreased: miR-192, 194, 200b, and 375	Zahm [27]
Active UC	Colon, nonspecific, *n* = 20	Inactive UC	Increased: miR-98	Coskun [22]
	Colon, left or sigmoid, *n* = 9	Inactive UC	Decreased: miR-196b, 489, and 630 Increased: miR-548a-3p and 650	Iborra [28]

Table 1. Aberrant microRNA expression in human colonic tissue in ulcerative colitis (UC).

2.2. MicroRNAs are differentially expressed in the mucosal tissue in CD

A search for biomarkers in CD has identified a large number of microRNAs that show aberrant expression (**Table 2**) [19, 23, 24, 28–32]. When compared to controls, miR-9, miR-9*, miR-16, miR-21, miR-22, miR-23b, miR-26a, miR-29b, miR-29c, miR-30a, miR-30b, miR-30c, miR-31, miR-34c-5p, miR-106a, miR-122, miR-126, miR-126*, miR-127-3p, miR-130a, miR-133b, miR-141, miR-146a, miR-146b-5p, miR-150, miR-155, miR-181c, miR-191, miR-196, miR-196a, miR-206, miR-223, miR-324-3p, miR-328, miR-375, miR-422a, miR-594, miR-663, and miR-885-5p have been found significantly upregulated [19, 24, 29, 31, 32]. The downregulated microRNA profiles include miR-let-7b, miR-7, miR-18a*, miR-19b, miR140-3p, miR-194b, miR-216b, miR-548e, miR-559, miR-629, and miR-629* [24, 30, 33].

Status	Tissue type	Control	Aberrant microRNA expression	Reference
Active CD	Sigmoid, *n* = 5	Healthy	Decreased: miR-19b and 629 Increased: miR-23b, 106a, and 191	Wu [29]
	Terminal ileum, *n* = 6	Healthy	Increased: miR-16, 21, 223, and 594	Wu [29]
	Colon, nonspecific, *n* = 16	Healthy	Increased: miR-9, 21, 22, 26a, 29b, 29c, 30b, 31, 34c-5p, 106a, 126, 126*, 127-3p, 130a, 133b, 146a, 146b-5p, 150, 155, 181c, 196a, 324-3p, and 375	Fasseu [19]
	Colon, nonspecific, *n* = 8	Healthy	Decreased: miR-7	Nguyen [30]
	Colon, nonspecific, *n* = 120	Healthy	Increased: miR-196	Brest [31]
	Colon, nonspecific, *n* = 15	Healthy	Increased: miR-31 and 141	Huang [32]
	Colon, nonspecific, *n* = 10	Healthy	Increased: miR-146a and 155	Beres [23]
Active and inactive CD	Colon, distal-most, *n* = 9	Healthy	Decreased: miR-194b, 216b, 548e, and 559 Increased: miR-31, 146a, 206, and 663	Lin [24]
Inactive CD	Colon, nonspecific, *n* = 8	Healthy	Increased: miR-9*, 21, 22, 26a, 29b, 29c, 30a*, 30b, 30c, 31, 34c-5p, 106a, 126*, 127-3p, 133b, 146a, 146b-5p, 150, 155, 196a, 223, and 324-3p	Fasseu [19]
Unknown	Colon, nonspecific, *n* = 10	Healthy	Increased: miR-122	Beres [23]
	Colon, nonspecific, *n* = 7	Healthy	Increased: miR-21 and 375	Zahm [27]
Active CD	Colon, left or sigmoid, *n* = 9	Inactive CD	Decreased: miR-let-7b,18a*, 140-3p, and 629* Increased: miR-328, 422a, and 885-5p	Iborra [28]

Table 2. Aberrant microRNA expression in human colonic tissue in Crohn's disease (CD).

At least two independent groups have found similar dysregulation of miR-21 [19, 27, 29], miR-31 [19, 24, 32], miR-106a [19, 29], miR-146a [19, 23, 24], miR-155 [19, 23], miR-223 [19, 29], and miR-375 [19, 27].

2.3. MicroRNAs are differentially expressed in blood samples in UC

Similar to the findings in tissue, microRNAs are also dysregulated in the peripheral blood of patients with UC (**Table 3**) [26, 28, 34–39]. When compared to controls, miR-let-7d, miR-let-7e, miR-let-7g, miR-let-7i*, miR-plus-E1271, miR-15b, miR-16, miR-17-5p, miR-19a, miR-20b*, miR-21, miR-22, miR-22-3p, miR-23a-3p, miR-24, miR-27a*, miR-28-3p, miR-28-5p, miR-29a, miR-30e, miR-30e-5p, miR-31, miR-92a-1*, miR-93, miR-103, miR-103-2, miR-103-2*, miR-128, miR-138, miR-140-3p, miR-142-5p, miR-143*, miR-146a-3p, miR-148b-3p, miR-150*, miR-151-5p, miR-155, miR-181b, miR-188-5p, miR-191-5p, miR-196b, miR-199a-3p, miR-199a-5p, miR-221, miR-223, miR-223a-3p, miR-302-3p, miR-320e, miR-330-3p, miR-340*, miR-345, miR-362-3p, miR-362-5p, miR-374b, miR-378, miR-378*, miR-422a, miR-423-5p, miR-500, miR-501-5p, miR-532-3p, miR-532-5p, miR-550*, miR-595, miR-598, miR-720, miR-760, miR-769-3p, miR-769-5p, miR-874, miR-941, miR-1246, miR-1271, miR-1274b,

miR-1296, and miR-4516 are upregulated in peripheral blood samples of patients with UC [26, 28, 34, 35, 38, 39]. When compared to controls, miR-150, miR-188-5p, miR-505*, miR-612, and miR-1827 have been shown to be downregulated [28, 34, 38].

Status	Tissue type	Control	Aberrant microRNA expression	Reference
Active UC	Peripheral blood, *n* = 13	Healthy	Decreased: miR-505* Increased: miR-plus-E1271, 28-5p, 103-2*, 151-5p, 199a-5p, 340*, 362-3p, and 532-3p	Wu [34]
	Peripheral blood, *n* = 88	Healthy	Increased: miR-16, 21, 28-5p, 151-5p, 155, and 199a-5p	Paraskevi [35]
	Peripheral blood, *n* = 62	Healthy	Increased: miR-595 and 1246	Krissansen [36]
Active and inactive UC	Peripheral blood, *n* = 18	Healthy	Decreased: miR-150 Increased: miR-let-7d, let-7e, let-7g, 15b, 19a, 24, 27a, 28-3p, 29a, 30e, 93, 103, 128, 142-5p, 196b, 199a-3p, 221, 223, 345, 374b, 423-5P, 532-5p, 598, and 760	Iborra [28]
	Peripheral blood, *n* = 46	Healthy	Decreased: miR-188-5p, 612, and 1827 Increased: miR-17-5p, 22-3p, 23a-3p, 30e-5p, 148b-3p, 191-5p, 223a-3p, 302-3p, and 320e	Polytarchou [37]
Active UC	Peripheral blood, *n* = 24	Inactive UC	Increased: miR-23a-3p, 148b-3p, 320e, and 4516	Polytarchou [37]
Inactive UC	Peripheral blood, *n* = 13	Healthy	Decreased: miR-505* Increased: miR-103-2, 362-3p, and 532-3p	Zahm [38]
	Peripheral blood, *n* = 10	Healthy	Decreased: miR-505* Increased: miR-103-2*, 362-3p, and 532-3p	Wu [34]
Unknown	Peripheral blood, *n* = 20	Healthy	Increased: miR-let-7i*, 20b*, 22, 27a*, 31, 92a-1*, 138, 140-3p, 143*, 146a-3p, 150*,181b, 188-5p, 330-3p, 362-5p, 345, 378, 378*, 422a, 500, 501-5p, 532-5p, 550*, 720, 769-3p, 769-5p, 874, 941, 1271, 1274b, and 1296	Duttagupta [39]
	Peripheral blood, *n* = 15	Healthy	Increased: miR-21	Yang [26]

Table 3. Aberrant microRNA expression in human peripheral blood in ulcerative colitis (UC).

Nine microRNAs have been found by at least two independent groups, miR-21 [26, 35], miR-28-5p [34, 35], miR-151-5p [34, 35], miR-199a-5p [34, 35], miR-345 [28, 39], miR-362-3p [34, 38], miR-505* [34, 38], miR-532-3p [34, 38], and miR-532-5p [28, 39].

2.4. MicroRNAs are differentially expressed in blood samples in CD

Similar to the findings in tissue, microRNAs are also dysregulated in the peripheral blood of patients with CD (**Table 4**) [28, 34–36, 38]. When compared to controls, a large number of microRNAs have been found to be upregulated in the peripheral blood including miR-plus-E1271, miR-let-7b, miR-16, miR-20a, miR-21, miR-23a, miR-27a*, miR-29a, miR-30e, miR-93, miR-106a, miR-107, miR-126, miR-140, miR-140-3p, miR-140-5p, miR-188-5p, miR-191,

miR-192, miR-195, miR-199a-5p, miR-200c, miR-340*, miR-362-3p, miR-484, miR-532-3p, miR-595, miR-877, and miR-1246. The significantly decreased microRNAs consist of miR-plus-F1065, miR-18a, miR-128, miR-140-5p, miR-145, miR-149*, and miR-877.

Status	Tissue type	Control	Aberrant microRNA expression	Reference
Active CD	Peripheral blood, *n* = 14	Healthy	Decreased: miR-plus-F1065 and 149* Increased: miR-plus-E1271, 199a-5p, 340*, 362-3p, and 532-3p	Wu [34]
	Peripheral blood, *n* = 46	Healthy	Increased: miR-let-7b,16, 20a, 21, 30e, 93, 106a, 140, 192, 195, and 484	Zahm [38]
	Peripheral blood, *n* = 128	Healthy	Increased: miR-16, 23a, 29a, 106a, 107, 126, 191, 199a-5p, 200c, 362-3p, and 532-3p	Paraskevi [35]
	Peripheral blood, *n* = 57	Healthy	Increased: miR-595 and 1246	Krissansen [36]
Active and inactive CD	Peripheral blood, *n* = 18	Healthy	Decreased: miR-877 Increased: miR-16, 27a*, 140-3p, 140-5p, and 195	Iborra [28]
Inactive CD	Peripheral blood, *n* = 5	Healthy	Decreased: miR-149* Increased: miR-340*	Wu [34]
Active CD	Peripheral blood, *n* = 9	Inactive CD	Decreased: miR-18a, 128, 140-5p, and 145 Increased: miR-188-5p and 877	Iborra [28]

Table 4. Aberrant microRNA expression in human peripheral blood in Crohn's disease (CD).

Six microRNAs have been found by at least two independent groups including miR-16 [28, 35, 38], miR-106a [35, 38], miR-195 [28, 38], miR-199a-5p [34, 35], miR-362-3p [34, 35], and miR-532-3p [34, 35].

2.5. Differential expression of microRNA that distinguishes UC from CD in indeterminate IBD

Although UC and CD have many overlapping features and symptomatology, they have distinct clinical, radiographic, endoscopic, surgical, and histologic findings. While most patients can be definitively classified as either UC or CD, 5–10% of IBD patients have equivocal features and are best classified as indeterminate colitis [40, 41]. The ability to determine which patients with indeterminate colitis act like CD would allow for better clinical and surgical management because patients with CD are much more likely to fail a pouch procedure.

Several microRNAs have been found to be differentially expressed between UC and CD, although the results have been inconsistent between most studies (**Table 5**) [19, 27, 34, 42].

In one study, a panel of five microRNAs (miR-19b, miR-23b, miR-106a, miR-191, and miR-629) was evaluated in 16 patients with a clinical diagnosis of indeterminate colitis. They found that 15 of 16 patients demonstrated UC-like expression patterns and concluded that microRNA

expression patterns in indeterminate colitis are far more similar to those of UC than CD [42]. These microRNA expression findings are similar to the data from long-term clinical follow-ups where most indeterminate colitis patients act much more like UC patients, rather than CD patients. The possibility to test microRNA profiles prior to surgery, with the hope of improving pouch outcome, is promising.

Status	Tissue type	Control	Aberrant microRNA expression	Reference
Inactive UC	Colon, nonspecific, $n = 8$	Inactive CD	Decreased: miR-100a-3p, 100b-5p, 150, 196b, 223, and 320a	Fasseu [19]
Active and inactive UC	Colon, distal-most, $n = 12$	Active and inactive CD	Increased: miR-19b, 23b, 106a, 191, and 629	Lin [42]
Unknown UC	Colon, nonspecific, $n = 12$	Unknown CD	Increased: miR-24	Zahm [27]
Active UC	Peripheral blood, $n = 13$	Active CD	Increased: miR-plus-E1035, plus-F1159, and 3180-3p,	Wu [34]

Table 5. Differential microRNA expression between ulcerative colitis (UC) and Crohn's disease (CD).

3. MicroRNA as a potential driver of pathogenesis and disease severity

Despite the vast numbers of microRNAs identified as deregulated in IBD, very few microRNAs are replicated in multiple studies. Here, we focus on the microRNAs with the best evidence as the driver of pathogenesis.

3.1. MiR-21 has an essential role in IBD development and disease severity

MiR-21 has been consistently identified as being upregulated in active UC and CD, suggesting a central role in the pathogenesis of IBD [16–18, 26, 43]. One study showed that the deletion of critical DNA methyltransferases (DNMT1 and DNMT3b) caused the dysregulation of approximately 10% of all microRNAs, highlighting the epigenetic regulation of microRNAs by DNA methylation [44]. The genome-wide association studies (GWASs) using Illumina CpG methylation assays have shown that the miR-21 locus was hypomethylated, and subsequently overexpressed, in samples of peripheral blood in patients with active CD [43]. A miR-21 knockout mouse model has been developed to assess the importance of miR-21 in IBD pathogenesis. Similar to human IBD, mice treated with dextran sodium sulfate (DSS) developed a chronic colitis causing significant morbidity and mortality with elevated tumor necrosis factor alpha (TNF-α) levels and miR-21 [45]. However, mice with genetic deletion of miR-21 were resistant to DSS-induced colitis. These findings strongly support the role of miR-21 in IBD pathogenesis, but further work is needed to clarify the mechanism of action.

The pathogenic effects of miR-21 in IBD are thought to be mediated through at least three separate mechanisms. First, miR-21 is thought to cause increased intestinal permeability in response to epithelial damage, a factor thought to initiate IBD. Despite no difference at baseline, treatment with DSS caused increased intestinal permeability in wild-type mice compared to miR-21 knockout mice [45]. Second, miR-21 is proapoptotic as the DSS-treated miR-21 knockout mice had less intestinal epithelial cell apoptosis than controls [45]. Several studies have indicated a role for miR-21 in the protection of free radical-induced apoptosis that linked the pro-survival phenotype to the inhibition of phosphatase and tensin homology (PTEN) with subsequent elevation of PI3K-Akt-mTOR activity [46–49]. Additionally, miR-21 has been shown to prevent renal tubular apoptosis by directly reducing levels of Rab11 protein [50]. The prevention of epithelial cell apoptosis may help maintain the epithelial cell barrier and limit intestinal permeability. Third, miR-21 has been associated with fibrosis in multiple disease models and has an emerging role in irreversible fibrosis of IBD. Multiple models of cellular injury have shown to be dependent on increased levels of TNF-α and subsequent induction of miR-21 [51, 52]. Increased serum levels of miR-21 were seen in human diseases with significant fibrosis, suggesting a role for miR-21 as a biomarker for disease activity [52, 53]. Elevated miR-21 expression is maintained throughout the development of dysplasia and carcinogenesis, but more controlled studies are needed to define its role in fibrosis [54].

3.2. MicroRNAs are associated with fibrosis and strictures in CD

Transmural inflammation is a hallmark of CD leading to irreversible fibrosis and stricture formation that marks disease severity. Several studies have attempted to identify the role of microRNAs in CD-related fibrosis. MicroRNA profiling of the serum from patients with CD with and without strictures has implicated miR-19a-3p and miR-19b-3p as potential pathogenic markers [55]. The authors found that low levels of both miR-19a-3p and miR-19b-3p were strongly correlated with stricturing CD and were independent of other potentially confounding variables such as site, gender, age, disease duration, and activity [55]. The studies in liver fibrosis have implemented miR-19b as a negative regulator of the pro-fibrotic tumor growth factor-β (TGF)-β-signaling pathway, but these experiments have not been confirmed in the setting of CD-associated fibrosis [56].

Several other microRNAs have been associated with TGF-β-signaling pathway and fibrosis in CD. TGF-β signaling has been shown to promote epithelial-mesenchymal transition, which leads to fibrosis in certain contexts. In patients with stricturing CD, the level of miR-29b was assessed in the mucosa overlying a stricture by comparing mucosa-overlying areas not affected by fibrosis [57]. Furthermore, the overexpression of miR-29b in fibroblast caused a decrease in TGF-β-mediated collagen deposition [57].

The complex interactions in the microenvironment between the mucosa and underlying stroma in CD are highlighted by studies of miR-200b. In IBD, the ability of the mucosa to withstand damage and remain intact is a crucial mechanism to limit disease severity. Researchers found that the miR-200b level was decreased in the mucosa of UC and CD, which correlated with the extent of damage incurred by the epithelium [58, 59]. MiR-200b has been shown to inhibit TGF-β-mediated epithelial-mesenchymal transition through the targeting of

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ZEB1 and SMAD2 expression [59]. Additionally, the overexpression of miR-200b promoted the growth of epithelial cells by stimulating cyclin D1 production [59]. These studies support that miR-200b can protect against damage from CD in both epithelium and underlying stroma.

These recent observations are laying the foundation for how microRNAs are intricately involved in the formation of fibrosis and strictures in idiopathic IBD.

4. The role of microRNAs in IBD-associated carcinogenesis

Molecular mechanisms of IBD-associated carcinogenesis are poorly understood and are an exciting area of research within the field [60, 61]. Multiple epidemiologic and basic science studies have shown that the risk of IBD-associated colon cancer increases as the extent of the disease, severity of inflammation, and duration increase [62–64]. Colonoscopies with multiple spatially distinct biopsies are used to assess for IBD-associated dysplasia. Although histologic examination can reproducibly identify dysplasia, IBD-associated dysplasia is difficult to be distinguished from sporadic adenoma based on histologic appearance alone, but certain features and patient characteristics can be helpful in suggesting IBD-associated carcinoma [65].

Molecular alterations have been shown to lead to dysplasia and carcinogenesis and the abnormalities have been demonstrated in normal-appearing nondysplastic mucosa from UC patients who had a remote dysplastic lesion [66–71]. Using microRNA expression microarrays from tissue with IBD-associated dysplasia, the dysplastic epithelium revealed that 22 microRNAs (miR-31, miR-31*, miR-96, miR-135b, miR-141, miR-183, miR-192, miR-192*, miR-194, miR-194*, miR-200a, miR-200a*, miR-200b, miR-200b*, miR-200c, miR-203, miR-215, miR-224, miR-375, miR-424*, miR-429, and miR-552) were upregulated. Ten microRNAs (miR-122, miR-139-5p, miR-142-3p, miR-146b-5p, miR-155, miR-223, miR-490-2p, miR-501-5p, miR-892b, and miR-1288) have been found to be downregulated [72].

Studies of microRNAs may elucidate distinct pathways to identify IBD-associated dysplasia and subsequent carcinogenesis from sporadic mutations. As such, developing reliable molecular markers to distinguish sporadic adenoma from IBD-associated carcinogenesis will aid in the surgical management of IBD patients who are under consideration for a total colectomy.

4.1. MiR-31 as an emerging biomarker of IBD-associated dysplasia

An assessment of the different anatomic locations in normal colon showed an equally low baseline expression of miR-31 [72]. MiR-31 is upregulated in both UC and CD, but not in other inflammatory conditions that have no association with dysplasia or malignancy [73]. Subsequently, the level of miR-31 was found to be 11-fold higher in IBD-associated dysplasia and carcinoma compared to that of IBD tissue without dysplasia, although no difference was seen in miR-31 expression level between IBD-associated dysplasia and IBD-associated carcinomas [72]. However, miR-31 was also found to be upregulated in sporadic colorectal adenocarcinomas [74–76].

The role of miR-31 in IBD-associated dysplasia or malignancy has only recently been examined and it is too early to determine if it is a useful marker of IBD-associated dysplasia or a generalized biomarker for carcinoma.

4.2. MiR-214 as an emerging oncogenic driver of IBD-associated carcinogenesis

Recent evidence suggests that microRNAs are not just biomarkers, as they can be crucial for carcinogenesis. A high-throughput genomic screen of microRNAs has identified miR-214 as a positive regulator of NF-kB activity [77]. Elevated levels of miR-214 were found in human tissue from patients with active UC or CD [77]. Two groups have previously shown that miR-214 promotes the growth of malignant osteosarcomas by directly reducing levels of the tumor suppressor, phosphatase and tensin homology [78, 79]. Using in vitro and in vivo models, it was displayed that chronic disease activity initiated interleukin-6 causing STAT3-mediated transcription of miR-214 with subsequent reduction of PTEN, PDZ, and LIM domain 2 (PDLIM2) causing enhanced activity of oncogenic NF-kB [77]. Furthermore, an inhibitor of miR-214 was able to inhibit DSS-induced carcinogenesis [77]. These data support not only the role of miR-214 as an oncogene but also provide insight into the molecular mechanisms of its carcinogenesis.

4.3. MiR-124a as a tumor suppressor epigenetically inactivated in IBD-associated carcinogenesis

Chronic inflammation has been shown to cause hypermethylation of CpG islands within the promoter of genes causing decreased gene expression [80]. Epigenetic silencing of well-characterized tumor suppressor proteins, E-cadherin, p14, and hMLH1, has been documented in UC-associated carcinogenesis [81–84].

The role of miR-124a as a tumor suppressor has been shown by demonstrating the direct suppression of oncogenic cyclin-dependent kinase 6 (CDK6) in acute lymphoblastic leukemia, medulloblastoma, and hepatocellular carcinoma [85–87]. The evaluation of patients with UC showed a strong correlation between increased miR-124a-3 hypermethylation and increased CDK6 expression [88]. The levels of methylation were highest in patients who had pancolitis and long-standing disease, and the individual with the highest value was later found to have high-grade dysplasia [88]. These studies are supported by the usage of high-throughput genetic screens that identified miR-124a as a negative regulator of oncogenic STAT3 in human colonic epithelial cells [89]. The authors demonstrated that miR-124a was downregulated through hypermethylation in pediatric patients with UC and strongly correlated with elevated levels of STAT3 and increased disease severity [89].

In combination, these studies demonstrate that the long-standing inflammation of IBD has the power to epigenetically downregulate important microRNA tumor suppressors that alters the downstream targets of those events. These findings might open up novel pathways that can be assessed for prognosis and potential personalized medicine.

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5. MicroRNA as potential therapeutic targets for IBD

The unique ability of microRNAs to posttranscriptionally regulate gene expression and affect multiple biological signaling pathways can lead to the development of antisense oligonucleotides as potential novel therapeutics in IBD. Multiple studies have shown that antisense oligonucleotides complementary to microRNAs can target specific microRNAs, abolishing their function in both in vitro cell models and in vivo animal models. Preclinical cell- and animal-based models have demonstrated that altering microRNA levels can modify the expression of either tumor suppressors or oncogenes as such to effect cancer growth [90–93]. Additionally, drug-induced microRNA changes have been shown to contribute to the therapeutic effect in in vitro models [94, 95].

With specific regards to the field of IBD, potential therapeutic targets consist of three general strategies: (1) to provide antisense oligonucleotides that inactivate microRNAs that are proinflammatory; (2) to replace the expression of tumor suppressor microRNAs; and (3) to provide antisense oligonucleotides that inactivate oncogenic microRNAs.

At the time of publication, no therapeutic manipulation of microRNAs in IBD has been published in either cell lines or animal models. However, a recent study has shown that the inhibition of miR-21, a central driver of IBD pathogenesis, slows the proliferation of a nasopharyngeal carcinoma cell line [96].

Although the delivery of microRNA-targeted therapeutics is technically challenging, recent advances in delivery methods suggest that we may soon see an explosion of microRNA-targeted therapies being assessed in vivo for the first time [97–99].

6. Conclusions

In summary, the rapidly expanding knowledge of microRNAs in the pathogenesis and carcinogenesis associated with IBD has created an emerging interest in their potential role for personalized therapies.

Author details

Kurt Fisher and Jingmei Lin*

*Address all correspondence to: jinglin@iupui.edu

Department of Pathology and Laboratory Medicine, Indiana University School of Medicine, Indianapolis, IN, USA

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Chapter 8

Intestinal Fibroblast/Myofibroblast TRP Channels in Inflammatory Bowel Disease

Lin Hai Kurahara, Keizo Hiraishi, Kunihiko Aoyagi,
Yaopeng Hu, Miho Sumiyoshi and Ryuji Inoue

Additional information is available at the end of the chapter

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Abstract

Inflammatory bowel disease (IBD) is characterized by the repeated cycles of inflammation and healing of the gut, which ultimately progress into intestinal fibrosis. Colonic fibroblast/myofibroblast's functions such as transformation, proliferation, invasion, migration, stress fiber formation, collagen synthesis, and cytokine/chemokine secretion are well estimated. However, the detailed mechanism can rarely be found so far. Thus, we focused on transient receptor potential (TRP) protein super family activated by various physical/chemical stimulations based on the above-described recognitions and also conducted the following examinations for the potential roles in Ca^{2+} signal transduction in fibroblast/myofibroblasts cells, which play an important role in intestinal inflammation and tissue remodeling. This chapter not only facilitates the understanding about the new role of intestinal fibroblast/myofibroblasts TRP channel for regulating inflammation, fibrotic processes but also suggests a novel molecular target of IBD treatment in future.

Keywords: inflammatory bowel disease (IBD), myofibroblast, TRP channels

1. Introduction

The prevalence of inflammatory bowel disease (IBD), a group of idiopathic disorders such as Crohn's disease (CD) and ulcerative colitis (UC) that cause chronic inflammation or ulcers in large- and small-intestinal mucosa, has been rapidly increasing since the Second World War. Because IBD follows a course of repeated severe diarrhea and constipation from a young age, it deteriorates an individual's quality of life for a long period of time as a refractory disorder.

Currently, most IBD treatments are limited to symptomatic relief. With increasing incidence, there is an escalating need to clarify a cause and establish definitive treatments [1, 2].

Located at the interface between the epithelium and lamina propria in most mucosal tissues, intestinal fibroblast/myofibroblast cells have ultrastructural features reminiscent of both smooth muscle cells and fibroblasts. Accumulating evidence suggests that myofibroblasts play crucial roles in intestinal homeostasis, inflammation, and neoplasia. In addition, these cells are known to play an essential role in modulating wound healing and fibrosis processes at the time of tissue damage or inflammation [3–5]. For instance, during skin-wound healing, fibroblast cells differentiate into myofibroblasts that secrete cytokines and growth factors to reduce wound size by contracting granulation tissue. Similarly, fibroblast-derived hepatic stellate cells (also known as Ito cells) located in the sinusoidal space of the liver support sinusoidal structure. Fibroblasts with similar transformation ability are also distributed in renal tubular epithelia, where they can be transformed in response to tissue damage, inflammatory substances, or growth factors to promote collagen production and stress fiber formation for tissue fibrosis [6–8]. Furthermore, fibroblast/myofibroblast cells produce cytokines, chemokines, growth factors, and inflammatory mediators involved in immune and inflammatory responses. The activation of myofibroblasts can induce excessive fibrosis, causing pathological tissue modifications (remodeling) such as wound closure, keloid formations, hepatic fibrosis (cirrhosis), and digestive tract obstructions [9]. However, mechanisms underlying myofibroblast transformation and cytokine secretion remain almost completely unknown, despite their importance in inflammatory tissue modifications.

Fibroblasts/myofibroblasts play important roles during the processes of intestinal inflammation and tissue remodeling [10, 11]; however, detailed mechanisms have rarely been identified. Based on previously described recognitions, we therefore focused on the transient receptor potential (TRP) superfamily as a new Ca^{2+} channel gene group activated by various physical and chemical stimuli. Mammalian TRP proteins form a non-selective cation channel superfamily that includes approximately 30 isoforms categorized into six subfamilies [12], including TRPC (canonical or classical: TRPC1–7), TRPV (vanilloid: TRPV1–6), TRPM (melastatin: TRPM1–8), TRPP (polycystin: TRPP1–4), TRPML (mucolipin: TRPML1–3), and TRPA (ankyrin: TRPA1). Implicated in a variety of cellular functions, TRP proteins form large non-voltage-gated cation channels constitutively activated by various physicochemical stimuli. Known activators for TRP channels include chemical stimuli (such as receptor stimulation, change in pH, and spicy or cooling agents), as well as temperature changes and various forms of mechanical stimuli including osmotic stress, membrane stretching, and shear forces. Proposed mechanisms are primarily associated with lipid bilayer mechanics, specialized force-transducing structures, biochemical reactions, membrane trafficking, and transcriptional regulation. TRP channels are assumed to form a tetrameric structure with four homologous subunits consisting of a six transmembrane segments, S1–S6, which are flanked by N- and C-terminal cytosolic regions. Although the six-time membrane-spanning configuration and a short helical pore loop between S5 and S6 segments are the hallmarks of voltage-gated cation channels, in TRP channels, periodically arranged, positively charged amino acid residues in the S4, which are essential for voltage-sensing, are missing [13]. Further, many additional protein-to-protein

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interaction domains and phosphorylation motifs exist within the N- and C-terminals of TRP channels. It is believed that, within specific membrane domains (e.g., caveola), a variety of signaling complexes are formed through these interaction sites, wherefrom diverse intracellular signal transductions are initiated. Owing to ubiquitous expression over the whole body including the central/peripheral nerve, cardiovascular, respiratory, digestive, renal urogenital, and erythroid/immune systems, TRP channels are thought to contribute to diverse biological functions, which are not restricted to innocuous and noxious multimodal sensory transduction (heat, cold, touch, proprioception, pain, taste, etc.) but also involve cardiac function, gut motility, psychomotor activity, and cell survival, proliferation, and death. In addition, several specific mutations have been identified in the *trp* genes for some hereditary disorders [12, 14–17].

The expression of TRP proteins in the alimentary tract is not confined to sensory neurons. The repertoire includes the other major classes of cells constituting the tract such as epithelial, endothelial, and smooth muscle cells and has recently been extended to fibroblasts/myofibroblasts [13], which belong to a special category of cells tightly associated with colonal/intestinal remodeling with the ability to transform and replicate to produce various cytokines under inflammatory circumstances. For instance, calcitonin gene-related peptide and substance P are known to be released by increased intracellular Ca^{2+} concentration through TRPV1 channel activation in sensory neurons [18, 19]. It has been proposed that excessive expression of this channel may be causally related with the occurrence and/or progression of IBD [20, 21]. Moreover, a nonselective cation channel TRPC4, which can be activated by muscarinic G-protein-coupled receptor stimulation, may be important for the excitatory control of intestinal smooth muscle cells [22–24]. Subsequent reports have implicated Ca^{2+} influx through TRPC4 channels in the initiation of spontaneous excitations in interstitial cells of Cajal, which regulate the gut automaticity [25]. More recently, we explored the potential roles of TRP channels in myofibroblastic Ca^{2+} signaling during intestinal inflammation and fibrosis. By using myofibroblast cell lines (CCD-18Co and InMyoFib) established from human colon epithelial and murine neonatal intestinal tissues, respectively, we could gain some key insights into the mechanisms underlying intestinal inflammatory and fibrotic remodeling processes [26].

In this chapter, we first describe the expression and function of TRPC channels in fibroblasts/myofibroblasts and then briefly discuss their potential roles in gastrointestinal disorders. Since the tumor-transforming factor (tumor necrosis factor (TNF))-α has been shown to affect the expression level of TRPC1 protein and its associated Ca^{2+}-transporting activity, the first part will be dedicated mainly to elucidating how TNF-α stimulates cyclooxygenase-2 (COX-2)-dependent prostaglandin E2 (PGE2) production through the activation of TRPC1 channels and enhances Ca^{2+} dynamics in CCD-18Co myofibroblasts. We next clarify the impact of PGE2 production on myofibroblastic function, with particular interest in Ca^{2+}-dependent regulation of transcription factors, that is, the nuclear factor of activated T-cell (NFAT) and the nuclear factor kB (NF-kB). The results suggest that negative feedback regulation of PGE2 production in intestinal myofibroblasts through TRPC1-associated Ca^{2+} influx may be of significant clinical importance to protect the gut from exacerbation of inflammatory process and, thus, progression of IBD [27].

In the second part, we describe the functional implications of transforming growth factor β1 (TGF-β1)-induced TRP channel activation in InMyoFib cells. Our studies so far suggest that TRP channels effectively regulate the expression of fibrosis-associated molecules and TGF-β signaling in InMyoFib cells. Consistent with this, expressions of TRP channels and fibrosis-associated factors were found to be increased in the stenotic but not in non-stenotic regions of biopsy samples from CD patients' intestines, implying a therapeutic potential of targeting the channels [28]. From these advances, we further anticipate gaining a good clue to elucidating the complex interplay among commensal microbiota, intestinal cells, and the immune system of the gut, and how such interactions, with genetic susceptibility and modification by environmental factors, contribute to the pathogenesis of IBD.

2. Roles of TRP proteins for the occurrence/progression of inflammatory bowel disease

Consultation with the literature indicates that there is close correlation between IBD initiation/progression and autoimmune abnormalities, which is characterized by aberrances in inflammatory responses of intestinal bacteria within the digestive tract. CD14-positive macrophages are markedly increased in the intestinal tract with CD pathology, where inflammatory cytokines including interleukin-6 and interleukin-23 (IL-6/IL-23) and TNF-α are excessively produced. The production of these cytokines, which can in turn activate adaptive immune reactions along with the production of IL-12 and IL-23, occurs at lower levels in the normal intestinal tract. However, suppressed immune responses of intestinal bacteria are inducible with higher production of IL-10, an anti-inflammatory cytokine involved in immune tolerance [29]. However, when chronic intestinal inflammation occurs, TNF-α or IL-6 can be excessively produced, initiating an excessive inflammatory response. Originally, adaptive immune responses were considered to play the dominant role in the pathogenesis of IBD; however, novel immunological and genetic studies have demonstrated that innate immune responses are of comparable significance in inducing gut inflammation. Recent progress in understanding IBD pathogenesis sheds light on related disease mechanisms, including innate and adaptive immunities, and interactions between genetic influences and microbial or environmental factors [2].

TNF-α is central to inflammatory processes and acts as an endogenous tumor promoter [30]. Therapeutic antibodies against TNF-α exert dramatic ameliorating effects on inflammatory bowel syndrome; myofibroblasts have been found to play a key role in this disorder [31]. TNF-α activates PGE2 production in myofibroblasts, fulfilling both protective and destructive roles in the gut. Although genetic deletion of the PGE2 receptor EP4 is detrimental to the gut, high concentrations of PGE2 analogs have also been shown to worsen clinical colitis (eventually leading to tumorigenesis), likely through the induction of pro-inflammatory reactions [32–34]. The formation of PGE2 in myofibroblasts is primarily catalyzed by COX-2, which is expressed at low levels in unstimulated conditions before being rapidly induced in response to inflammatory cytokines, growth factors, and tumor promoters [35].

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The myofibroblast cell line CCD-18Co expresses both COX forms and secretes PGE2, a feature that is significantly enhanced by TNF-α or IL-1β [30]. Evidence suggests that COX-2 expression and PGE2 production in myofibroblasts are controlled by intracellular Ca^{2+} concentration [36, 37]. However, the exact sources of Ca^{2+}, which contribute to this process, remain entirely unclear. In general, there are two distinct sources of Ca^{2+} for elevating intracellular Ca^{2+} levels: Ca^{2+} influx across the plasma membrane and Ca^{2+} release from the endoplasmic reticulum (ER). Ca^{2+} influx can occur through voltage-gated Ca^{2+} channels, receptor-operated Ca^{2+}-permeable channels (ROCs), and store-operated Ca^{2+} channels (SOCs). Recent studies have demonstrated that the canonical members of the TRP superfamily of proteins (TRPC) may contribute to SOC and ROC. The TRPC family consists of seven distinct isoforms designated as TRPC1-TRPC7 [12, 14, 38, 39]. Presently, TRPC1 is regarded as one of the most plausible candidate molecules for SOC in many cell types [38, 39] and plays a critical role in intestinal epithelial restitution [40]. In some cell types, TRPC1 dynamically assembles with both stromal-interacting molecule 1 (STIM1) and Orai1 to generate a greater complexity in store-dependent Ca^{2+} influx mechanisms [41], although whether TRPC1 serves as a pore-forming SOC subunit still remains unclear.

In CCD-18Co cells, treatment with TNF-α greatly enhanced both Ca^{2+} influx induced by store depletion and cell-surface expression of TRPC1 protein and induced a cationic conductance. Selective inhibition of TRPC1 expression occurs by small interfering RNA or functionally effective TRPC1 antibody targeting the near-pore region of TRPC1 antagonized enhancement of store-dependent Ca^{2+} influx by TNF-α, whereas TNF-α potentiated the induction of PGE2 production. Overexpression of TRPC1 in CCD-18Co produced opposite consequences [27]. We further elucidated that NF-kB and NFAT serve as important positive and negative transcriptional regulators, respectively, of TNF-α-induced COX-2-dependent PGE2 production in colonic myofibroblasts, at the downstream of TRPC1-associated Ca^{2+} influx [27]. NFAT and NF-kB are widely distributed Ca^{2+}-dependent transcription factors capable of regulating a multitude of physiological and pathophysiological processes [42–44]. NFAT is activated through dephosphorylation by calcineurin, which is activated upon binding of Ca^{2+}/calmodulin. NFAT is reported to regulate COX-2 expression in colon carcinoma cells [45], and its activation can occur through Ca^{2+} influx associated with TRPC1-, TRPC3-, or TRPC6-associated SOC or ROC activities [46, 47]. The NF-kB transcription factor family plays a key role in several cellular functions (inflammation, apoptosis, cell survival, proliferation, angiogenesis, and innate and acquired immunity) as well as in regulating the expression of more than 500 different genes involved in inflammatory and immune responses [48, 49]. The anti-inflammatory natural compound curcumin acts as a principal mechanism to suppress the NF-kB-mediated signaling, thereby modulating immune responses [50–52].

The fact that high doses of exogenous PGE2 analogs exacerbate clinical colitis in the TNBS model might be relevant to the use of misoprostol to prevent ulcers in patients who take anti-arthritis medication. The side effects listed for misoprostol include a variety of gastrointestinal tract problems, and these deleterious actions of PGE2 are likely associated with the stimulation of the release of interleukin-23 from activated dendritic cells, which in turn facilitate the differentiation of helper T lymphocytes to the pro-inflammatory phenotype Th17. These

opposing actions of PGE2 may imply that the extent of its production is crucial to determine the fate of intestinal mucosa, that is, the maintenance of integrity or disintegration. In this regard, the negative feedback regulation of PGE2 production in intestinal myofibroblasts through TRPC1-associated Ca^{2+} influx may be of significant clinical importance to protect the gut from exacerbation of inflammatory process and thus the progression of inflammatory bowel syndrome.

3. Intestinal fibroblast/myofibroblast TRP channel and fibrosis

Repeated cycles of inflammation and healing of the gut ultimately progress into intestinal fibrosis (**Figure 1**). Innate immune-signaling pathways are also important drivers of myofibroblast transdifferentiation, as they cause cellular activation and fibrosis. Numerous mediators, including PDGF, EGF, IGF-1 and -2, CTGF, IL-1, IL-13, stem cell factor, endothelins, angiotensin II, TGF-α, TGF-β, bFGF, and peroxisome proliferator activator receptor-γ, promote myofibroblast proliferation and extracellular matrix (ECM) production. These activated myofibroblasts are central to fibrogenesis [53, 54].

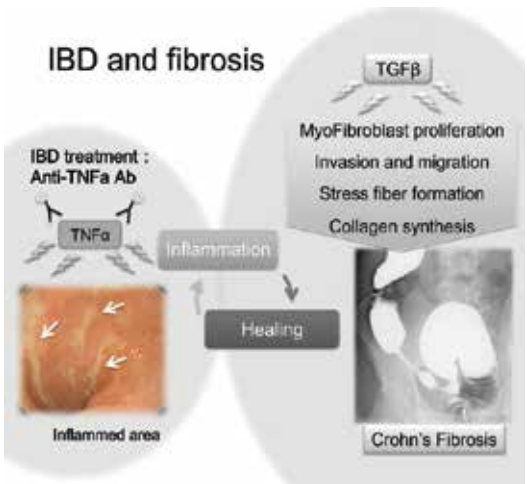

Figure 1. Inflammatory bowel disease and fibrosis. Repeated cycles of inflammation and healing of the gut ultimately progress into intestinal fibrosis. Endoscopic view of the inflamed area and a lower gastrointestinal series from a CD patient with fibrosis are shown. Colonoscopy and biopsy sampling showed a fibrotic lesion responsible for a colon stenosis.

TGF-β is principal to the development of fibrotic stenosis in CD and in numerous cell types. TGF-β secretion augments myofibroblast transformation. Canonical TGF-β signaling commences with its binding to a TGF-β type 2 receptor, which subsequently heterodimerizes with a TGF-β type 1 receptor to form an active TGF-βR1 receptor complex. Activated TGF-β type 1 receptor complex phosphorylates proteins against decapentaplegic homologs 2 and 3 (SMAD-2 and SMAD-3); activation of these transcription factors promotes collagen synthesis

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[55]. TGF-β can also signal through noncanonical pathways involving extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinase, and p38-mitogen-activated protein kinase (p38-MAPK). Both canonical and noncanonical TGF-β-signaling pathways are implicated in myofibroblast cytokine production and fibrosis in the gut [5, 53]. TGF-β levels are elevated in the inflamed intestines of CD and ulcerative colitis patients, and abnormal TGF-β signaling impairs intestinal immune tolerance and tissue repair [56]. In addition, TGF-β receptor-triggered-signaling cascades can be enhanced by calcineurin inhibitors cyclosporin A and FK506 [57, 58]. However, neutralizing TGF-β1 *in vivo* as an anti-fibrotic approach in CD may be highly problematic, as this may actually lead to disease exacerbation, despite the potent anti-inflammatory and immunoregulatory properties of this cytokine. In addition to TGF-β1, emerging evidence has shown that IL-13 and IL-17 are involved in intestinal fibrosis. IL-13 signaling via IL-13 receptor type 2 (IL-13R2) and subsequent TGF-β1 production comprises the main fibrotic pathway in a model of chronic colitis [59]. IL-17A expression was found to be increased in the inflamed areas of patients with inflammatory bowel disease [60].

In response to tissue injury and profibrotic mediators including TGF-β and PDGF, fibroblasts differentiate into myofibroblasts, and the activation and/or recruitment of fibroblasts with resistance to apoptosis result in fibrogenesis and subsequent fibrosis [61, 62]. It has been estimated that about 45% of human deaths are associated with fibroproliferative disorders including fibrosis [63]. Recently, anti-TNF-α antibodies were successfully introduced as anti-inflammatory IBD therapies. However, for patients with fibrotic stenosis, there are only surgical treatments such as balloon dilation [64]. Approximately one-third of CD patients have severe intestinal strictures and obstructions (caused by excessive fibrosis) that are eventually fatal. In addition, treating CD patients with anti-TNF agents increases the risk of developing recurrent intestinal stenosis and sub-obstructive symptoms [65], necessitating repeated surgery [66]. In fact, many IBD patients are still suffering from re-stenosis of surgically treated regions, which greatly impairs the quality of life and can risk the lives of patients. Thus, there is an urgent need to establish alternative anti-fibrotic strategies to treat CD patients and other individuals suffering from intestinal fibrotic complications beyond currently available anti-inflammatory therapies. Unfortunately, little is currently known about intestinal wound-healing processes and pathogenic mechanisms by which chronic intestinal inflammation causes detrimental fibrosis, although a complex scenario involving numerous humoral factors has been suggested in experimental models [6–8].

Fibroblasts (vimentin+, α-SMA−), located in the submucosal area of normal tissues, are central in maintaining structural formation, healing, and regeneration. Increased resident fibroblast populations are pivotal to fibrosis development. Fibroblasts isolated from IBD mucosa proliferate faster than normal, and this increase occurs after exposure to growth factors and pro-inflammatory cytokines, and after direct cell-to-cell contact with inflammatory cells. Fibroblast-to-myofibroblast (vimentin+, α-SMA+) transformation plays a critical role in wound healing and tissue remodeling after injury [8, 67]. Myofibroblasts synthesize ECM components and generate high contractile forces for wound retraction or tissue remodeling in developmental processes. However, persistent myofibroblast activity can underlie hypertrophic scarring, loss of tissue compliance, and even rampant fibrosis that is the basis for fibrotic

disorders of the heart, skin, lung, kidney, skeletal muscle, and liver [6, 68, 69]. The myofibroblast is considered a hybrid cell type with both smooth muscle and fibroblast properties [8]. A defining feature of myofibroblast differentiation is the formation of α-SMA stress fibers that provides a structural network for generating contractile forces [70]. Furthermore, intestinal stricture formation in CD is driven by the local excessive production of TGF-β [5, 71]. It is well known that fibrosis is associated with excessive accumulation of ECM components, such as collagens, matrix metalloproteinases (MMPs), and tissue inhibitors of metalloproteinases (TIMPs) [63, 72, 73]. In addition, other ECM proteins, such as fibronectins, elastins, and fibrillins, are upregulated during the development of fibrosis. This is due mainly to increased synthesis and decreased degradation of ECM components. Notably, during this process, MMPs that degrade the ECM are upregulated, whereas TIMPs are downregulated [74].

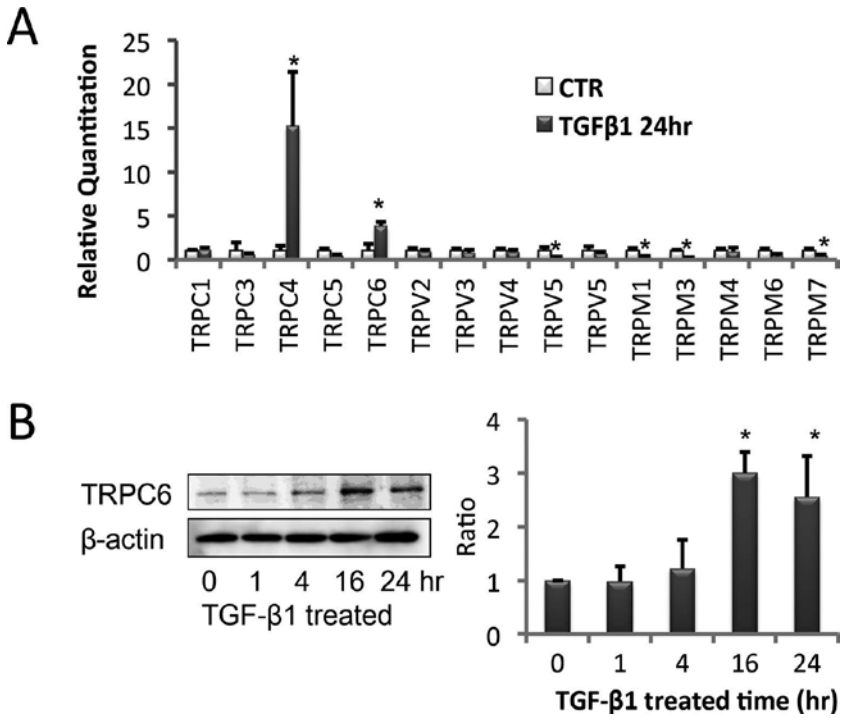

Figure 2. (A) *TRP* isoforms' mRNA in InMyoFibs. Results of real-time PCR analysis of the mRNA expression levels of *TRPC1*, *-C3*, *-C4*, *-C5*, *-C6*, *-V2*, *-V3*, *-V4*, *-V5*, *-V6*, *-M1*, *-M3*, *-M4*, *-M6*, and *-M7* after treatment with TGF-β1 (5 ng/mL, 24 h) are shown. (B) Immunoblot data of time-dependent changes in TRPC6 protein expression (left panel). Data were normalized to an internal control (β-actin) and are an average of four independent experiments (right panel). *$P < 0.05$ compared with untreated cells ($n = 4$). This figure was modified from a figure in Ref. [28].

TRP channels are cellular sensors for a wide variety of physical and chemical stimuli [75–77]. For example, they are involved in the sensation of touch, smell, taste, temperature, and pain [75, 78–80]. Recent studies have revealed that TRP channels also play essential roles in cell signaling and responses to innocuous or harmful environmental changes [15, 16, 81]. In addition, the activation of TRP channels changes the membrane potential, passes important

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signaling ions across the cell membrane, changes enzymatic activity, and initiates endocytosis or exocytosis [12, 75, 82]. Ca^{2+} is an essential signaling molecule implicated in various long-term cellular consequences, such as differentiation, gene expression, and cell proliferation, growth, and death, and it plays a significant role in regulating fibroblast functions [83–85]. TRPC channels are non-voltage-gated nonselective Ca^{2+}-permeable channels. Enhanced Ca^{2+} influx has been implicated in both differentiation and cytoskeletal rearrangements of various cell types. Accumulating evidence suggests that fibrosis-associated events in myofibroblasts are controlled by intracellular Ca^{2+} concentration, which is mediated by some members of the TRP channel superfamily [14, 86–88]. For example, TRPC1-mediated Ca^{2+} influx is essential for intestinal homeostasis/inflammation and progesterone-induced endometrial decidualization [27, 89]. Ca^{2+} signaling through TRPM7 channels likely plays a key role in TGF-β1-elicited fibrogenesis in human atrial fibroblasts [88]. Similarly, TRPC6/calcineurin-mediated signaling is essential for dermal and cardiac myofibroblast transformation, which occurs through complex interwoven pathways involving TGF-β, p38 mitogen-activated protein kinase, and serum response factor [70]. The formation of cell-to-cell contact is governed by Ca^{2+} signaling through TRPC4, which co-immunoprecipitates with junction proteins β-catenin and cadherin in vascular endothelial cells [90]. However, whether TRP channels play a role in intestinal fibrosis is not clearly understood.

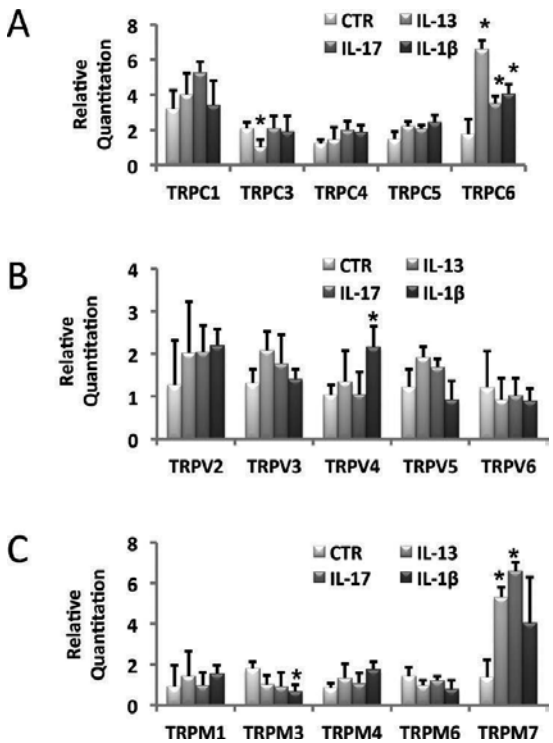

Figure 3. Real-time PCR analysis of *TRPC1*, *-C3*, *-C4*, *-C5*, *-C6*, *-V2*, *-V3*, *-V4*, *-V5*, *-V6*, *-M1*, *-M3*, *-M4*, *-M6*, and *-M7* after a 24-h treatment with IL-13 (10 ng/mL), IL-17 (10 ng/mL), and IL-1β (10 ng/mL). This figure was modified from a supplementary figure in Ref. [28].

In intestinal myofibroblasts, not only TGF-β1 but also IL-13 and IL-17 significantly upregulated TRPC6 expression (**Figures 2** and **3**). Myofibroblast TRPC6 is a key factor to modulate fibrosis through TGF signaling, and thus targeting TRPC6 may be a useful therapeutic regimen for CD patients with intestinal fibrosis [28]. The results showed that while increased TRPC6 activity promoted the TGF-β1-mediated expression of α-SMA and N-cadherin and strengthened interactions between the three molecules, it also negatively regulated collagen synthesis and secretion of anti-fibrotic factors, such as IL-10 and IL-11 (ERK and p38-MAPK dependent) [91–93]. Upregulated TRPC6 expression is essential for the formation of α-SMA stress fibers and N-cadherin-mediated adherens junctions, which, respectively, enable myofibroblasts to gain contractility and reinforce mutual intercellular connections [6, 94, 95]. Interestingly, adherens junctions appear in fibrotic tissues but are absent in normal tissues where fibroblasts do not develop the stress fibers [10]. These findings are consistent in part with a previous study that TRPC6-mediated Ca^{2+} influx was obligatory for myofibroblast differentiation in dermal and cardiac wound healing, although greater complexity appears to exist in the relationship between TRPC6-mediated signaling and intestinal fibrosis.

Furthermore, in our biopsy study, we examined samples from CD patients for the expression of TRPC4, TRPC6, α-SMA, N-cadherin, cytokines, and ECM, and found that these molecules were all increased in TGF-β1-treated InMyoFibs. The mRNA levels of *TRPC6*, *ACTA2*, *CDH2*, *IL-10*, *IL-11*, and *COL1A1* were significantly higher in stenotic areas than in non-stenotic mucosal areas of CD patients, whereas that of *TRPC4* was not significantly changed in 12 paired biopsy samples obtained from six patients (**Figure 4**). Stenotic lesions can be either inflammatory, fibrogenic, or neoplastic, or possess all of these characteristics. This means that therapeutic strategies distinguishing between these processes would yield improved outcomes compared with the currently available approaches. In this regard, more direct evidence that TRPC6 vitally contributes to the progression of excessive fibrosis in both an experimental model and in human tissues should help to elucidate the mechanism underlying the fibrotic process. This may be relevant not only to intestinal fibrosis but also to other fibrotic lesions of the skin, lung, and liver, where these channels are expressed at significant levels.

In addition to aforementioned mechanisms, the imbalance between MMP and TIMP, which maintain the state of remodeling and restitution, can accelerate structural changes of the bowel wall [1]. Microarray experiments showed that InMyoFib cells primarily express *MMP-1*, *MMP-2*, *TIMP-1,* and *TIMP-2*. When we next measured transcript expression of these molecules in stenotic areas from biopsy samples and TGF-β1-treated cells, we found that their mRNA levels were significantly unregulated; however, *TRPC6* siRNA pretreatment did not affect expression in TGF-β1-treated cells.

The studies with intestinal fibroblast/myofibroblast propose a new proof of concept that TRPC6 may act as an anti-fibrotic mediator. The upregulation of this channel appears to inhibit the signaling cascades associated with intestinal fibrosis including SMAD-2 phosphorylation and myocardin expression, which in turn modulate collagen synthesis, actin fiber formation, and expression of N-cadherin. Further evidence from biopsy samples suggests that the same mechanism may also operate in stenotic lesions of IBD. These results not only facilitate our

understanding about this new role for TRPC6 in regulating fibrotic processes but also provide a novel molecular target for anti-fibrotic therapies to treat IBD in the future.

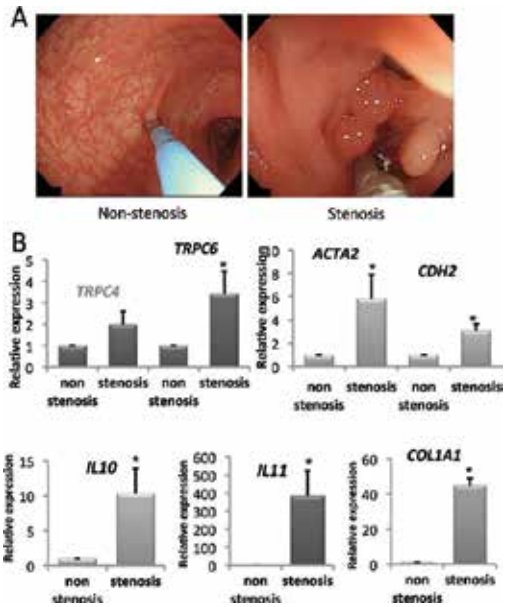

Figure 4. (A) Fibrosis in the colon: a clinical problem. Ulcerations and tissue damage are caused by chronic inflammation. This is followed by bowel wall fibrosis, leading to pseudopolyps or strictures reducing the colon. (B) Crohn's disease (CD) patient biopsies from non-stenotic and stenotic intestinal areas. The mRNA levels of *TRPC4*, *TRPC6*, *ACTA2* (α-SMA), *CDH2* (N-cadherin), *IL-10*, *IL-11*, and *COL1A1* in biopsies were examined by real-time RT-PCR in non-stenotic and stenotic-inflamed mucosal tissues of CD patients. $^*P < 0.05$ versus non-stenotic samples (12 paired biopsy samples obtained from six patients). **Figure 4B** was modified from a figure in Ref. [28].

4. Summary

Several studies including our study have underscored the importance of intestinal fibroblast/myofibroblast cells in IBD pathophysiology and epithelial barrier integrity, and accumulating evidence from preclinical and clinical studies has started to note an important contribution of TRP channels to many gastrointestinal remodeling processes. In this chapter, we summarized recent advances in this field, with particular emphasis on TNF-α-activated TRPC1 and TGF-β-activated TRPC6 expression and function in primary-cultured fibroblasts/myofibroblasts in the gastrointestinal tract, in conjunction with limited but interesting results from biopsy samples from CD patients. A noteworthy possibility from it is that the functionality of TRP channels may have unexpectedly tight correlation with inflammation- and fibrosis-associated processes in myofibroblasts *in vitro* and *in vivo*. Further investigation will be warranted to substantiate our yet-premature knowledge about this newly emerging field, which would hopefully lead to the exploitation of an unprecedentedly unique treatment for highly intractable inflammatory/fibrotic disorders with greatly compromised quality of life, such as IBD.

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Author details

Lin Hai Kurahara[1*], Keizo Hiraishi[1], Kunihiko Aoyagi[2], Yaopeng Hu[1], Miho Sumiyoshi[1] and Ryuji Inoue[1]

*Address all correspondence to: hailin@fukuoka-u.ac.jp

1 Department of Physiology, Fukuoka University School of Medicine, Fukuoka University, Fukuoka, Japan

2 Department of Gastroenterology, Fukuoka University School of Medicine, Fukuoka University, Fukuoka, Japan

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Chapter 9

Microbial Neuro-Immune Interactions and the Pathophysiology of IBD

Mònica Aguilera and Silvia Melgar

Additional information is available at the end of the chapter

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Abstract

Inflammatory bowel disease (IBD), encompassing Crohn's disease (CD) and ulcerative colitis (UC), is a group of debilitating disorders affecting patient's quality of life and with unknown aetiology. The collected evidence indicates that individuals can develop IBD as a result of genetic susceptibility, a dysregulated immune response and the influence of certain environmental factors. Common symptomatology includes abdominal pain, fever and bowel diarrhoea with blood and/or mucus excretion. The location and extent of disease differ between UC and CD, affecting the mucosal layer in the colon in UC patients, whereas in CD patients, a transmural inflammation is found anywhere in the gastrointestinal tract. Factors associated with IBD pathophysiology include alterations in immune responses, characterized by an atypically T helper (Th)-2 profile in UC, and a Th1/Th17 profile in CD, modifications in epithelial barrier function and alterations in the commensal microbiota composition with blooming of specific pathobionts, for example, adherent-invasive *Escherichia coli* (AIEC), and with diet. Recent research has uncovered that inflammation, *per se*, can activate the enteric nervous system inducing neurogenic inflammation and increasing visceral sensitivity, leading to pain. Similarly, alterations in the commensal microbiota composition/ligands have also led to modifications in intestinal nociceptive markers and in visceral pain. In this chapter, we aim to review the mechanisms implicated in microbial neuro-immune axis and its potential contribution to IBD pathophysiology and symptomatology. We focus on the findings identified in animal models and in IBD patients and on the prospective translation of targeting the microbial neuro-immune axis as future therapeutic treatment for intestinal inflammatory conditions.

Keywords: IBD, microbiota, intestinal neuro-immune interaction, visceral pain, microbiota–gut–brain axis

1. Introduction IBD

Inflammatory bowel disease (IBD) is a group of diseases comprising mainly two entities, ulcerative colitis (UC) and Crohn's disease (CD), of unknown aetiology. Ulcerative colitis was described in the late nineteenth century by Wilks and Moxon [1] and CD was described by Crohn et al. in the early 1930s as terminal ileitis [2]. Since the beginning of the twenty-first century, the incidence of IBD is increasing worldwide, especially in Westernized areas such as the United States, Europe, Australia and New Zealand as well as in South America, Asia and the Middle East and in specific populations, for example, paediatric-onset IBD. The prevalence in the Western World is currently up to 0.5% of the population [3].

IBD affects the patients' quality of life and is characterized by unpredictable flares of remission and relapses with symptoms of bloody diarrhoea, abdominal pain and rectal bleeding. The onset of IBD is at a young age ranging initially from 20 to 39 years and with a second onset in patients over 60 years of age [4]. IBD affects both males and females, with a higher prevalence of CD in females and no major differences in UC patients [5]. The inflammation in UC is localized to the colonic superficial mucosa while the inflammation in CD is transmural and can be found anywhere along the gastrointestinal (GI) tract, although the inflammation is predominantly located to the ileo-caecal area and the proximal colon [6, 7]. Ulcerative colitis is characterized by the formation of crypt abscesses, formed by extravasation of neutrophils through the intestinal epithelium while CD is characterized by the presence of skip lesions, granulomas, fibrosis and strictures. Extra-intestinal features in CD can result in major complications, for example, fibrotic strictures, and a subsequently need for surgery [8, 9]. To date, there is no cure for IBD, with most treatments primarily aiming to suppress disease severity and to keep the patient in remission by using biologics, anti-suppressants and steroids.

The cause of IBD is unknown but the collected evidence suggest that IBD can be manifested in genetically susceptible individuals who mount inappropriate local immune responses against microbial antigens after exposure to environmental factors [7, 10].

To date, genomewide association studies (GWAS) have identified at least 163 susceptible genes for IBD, with loci associated to bacterial recognition (NOD2) and autophagy (ATG16L1, IRGM) conferring a higher risk for CD. In contrast, genes involved in mucosal barrier function (e.g. HNF4a, CDH1, LAMB1, ECM1), IL-10 signalling and HLA haplotype DRB1*0103 have been associated with UC. Interestingly, genes linked with adaptive immune responses such as IL-23R, IL-12B and STAT3 confer a higher risk for both CD and UC. Despite the large number of loci identified, only approximately 20–25% of patients are linked to at least one of these loci suggesting that there are most likely other factors potentiating its development [11–13].

Alterations in barrier function, dysregulation in tight junction proteins and increased bacterial uptake has been reported in experimental models of colitis and in patients with UC and CD supporting the GWAS identified genes on barrier function [14, 15]. Others have also suggested that Peyer's patches are the sites of initial lesions in CD with M cells playing an important role in sampling microbes from the gut lumen and presenting to immune cells to mount inflam-

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matory responses [16, 17]. Furthermore, unaffected relatives of CD patients have shown increased intestinal permeability [14, 15].

2. Intestinal immune mechanisms and IBD

The main function of the intestinal immune system is to protect the host from harmful signals, for example, pathogens, by mounting specific responses as well as to keep a tolerance against A myriad of food and microbial antigens. A robust immune response against invading pathogens is critical for their clearance but an excessive or uncontrolled inflammation can result in chronic inflammation and lead to the development of inflammatory conditions such as IBD (**Figure 1**). The collected evidence to date suggest that the aberrant immune response in IBD patients is attributed to the dysregulated adaptive and innate

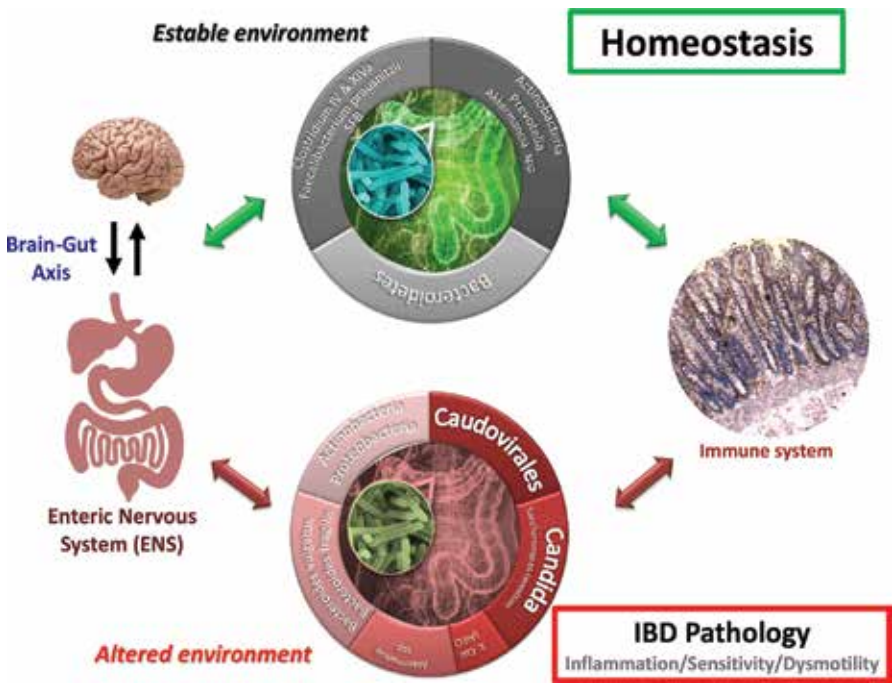

Figure 1. The gastrointestinal (GI) tract harbours up to 10^{14} bacteria, 10 times more than the number of cells of the human body. These bacteria include up to 1000 bacterial strains but are covered in few phyla. The most important ones in mammals are the Firmicutes (including Clostridium and Lactobacilli) and Bacteroidetes. Traditionally, it has been described that GI function is controlled by the intestinal immune system. Recent research has also highlighted that the enteric nervous system (ENS) and the gut commensal microbiota system play a crucial and an active role in influencing gut homeostasis. The ENS, mainly represented by the myenteric and the submucous plexus, Also known as the second brain due to it can work alone. The gut is connected to the CNS by the brain-gut axis, which maintains a bidirectional communication. When these three systems are balanced, there is a physiological homeostasis. An imbalance in any and/or all of these three systems can lead to the development of functional GI disorders and chronic inflammatory GI disorders such as IBD.

immune responses [7, 10]. The innate immune response is the first line of defence against harmful agents. Pattern recognition receptors (PRRs) detect microbial 'pathogen-associated molecular patterns' (PAMPs) or host-derived 'damage-associated molecular patterns' (DAMPs) inducing innate immune responses. Among PRRs, the intestinal Toll-like receptors (TLRs) are critical both in keeping intestinal homeostasis and in mounting innate immune responses. In humans, a total of 10 TLRs have been described, with the majority of them, except TLR3, signalling via the adaptor protein MyD88. Activation of TLRs via MyD88 induces several pathways including the transcription factor nuclear factor-kappa light-chain enhancer of activated B cells (NF-κB), mitogen-activated protein kinase (MAPK) and AP1, while the MyD88-independent pathway activates the interferon regulatory factor 3/7 (IRF-3/-7) signalling pathway [18]. In recent years, it has become evident that bacteria can penetrate/translocate through the intestinal barrier of IBD patients thereby inducing TLR-induced responses both by mucosal non-immune cells (e.g. epithelial cells) and innate immune cells (e.g. macrophages, dendritic cells). TLRs are expressed by

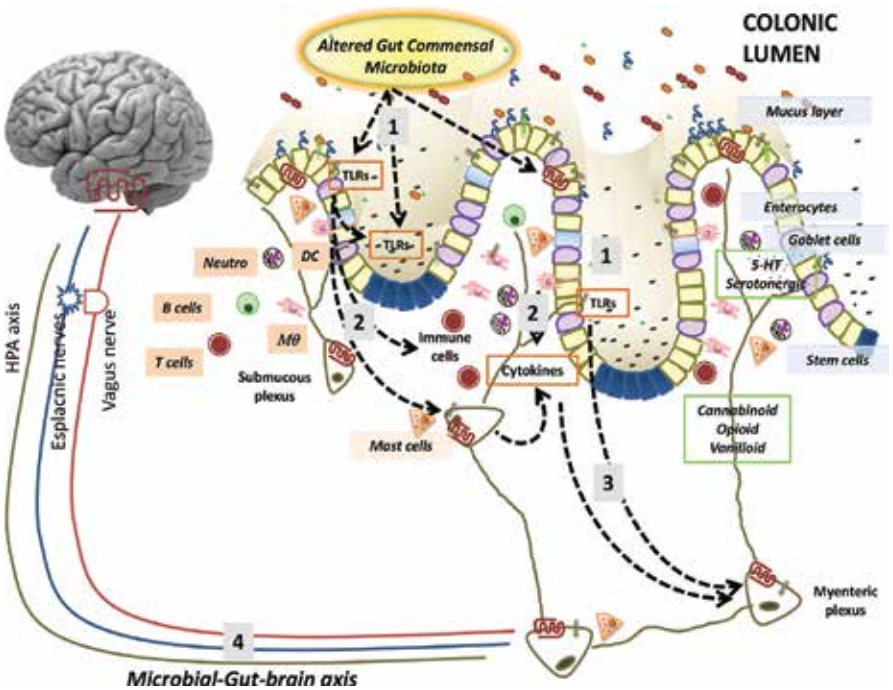

Figure 2. Representative schema of some of the putative mechanisms involved in IBD pathophysiology associated with microbial neuro-immune changes. The intestinal microbiota and microbial-derived products interact with the host bacterial recognition systems (such as TLRs) (1) generating a signalling cascade (2) that will lead to a local immune activation including mast cells, macrophages, T cells, neutrophils, dendritic cells and neuroendocrine systems (such as enterochromaffin cells) that seems to persist even when the overt inflammation is resolved. This persistent activation has the potential to influence sensory neural mechanisms within the gut depending upon the ENS (3) and the extrinsic innervation. In addition, the bidirectional communication between the gut and the CNS (4) is also altered that can offer an explanation for the altered perception of sensory signals and therefore altered manifestation of pain in patients suffering from IBD. Neutro—neutrophils; MΘ—macrophages; DC—dendritic cells; TLRs—Toll-like receptors.

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both epithelial and immune cells, and alterations in TLRs expression have been reported in both UC and CD tissue including increase expression of TLR4, TLR2 and TLR5 [18]. Activation of TLRs, for example, TLR4, leads to the activation of NF-κB pathway, which is responsible for the transcription of various pro-inflammatory cytokines and chemokines associated with IBD pathology (**Figure 2**). Other PRRs involved in CD pathology include NOD2, which is a cytosolic receptor belonging to the nucleotide-binding domain and leucine-rich repeat containing family (NLRs). NOD2 recognizes muramyl dipeptide (MDP), present in both Gram-positive and Gram-negative bacteria and activates the NF-κB pathway. NOD2 has also been identified as a susceptible gene for CD with 3 SNPs linked to ileal CD, suggesting that a defect in recognition and clearance of bacteria might be associated with CD development. However, the specific inflammatory mechanisms associated with NOD2 mutations are still largely unknown [18].

The intestine of IBD patients presents a chronic inflammation that differs in terms of immune cell subsets and cytokine profile. The colons of UC patients are heavily infiltrated with neutrophils, T and B cells with high levels of several pro-inflammatory cytokines including IL-1β, IL-6 and TNF-α and an atypical T helper (Th2) profile (IL-5, IL-10 and IL-13) [7, 10, 19]. Other chemokines such as IL-8 and GRO-α are highly increased in UC mucosa, with IL-8 levels correlating with the degree of inflammation and disease activity [20, 21]. Although neutrophils are indispensable for eliminating pathogens, their excessive presence in the tissue and their resistance to apoptosis [22] can lead to extensive tissue damage in UC, which can be caused by the persistent release of cytokines (IL-17, IL-6), reactive oxygen species (ROS) and proteases, all of which highly associated with patients with active UC [7, 19, 23]. The intestinal wall of CD patients is highly infiltrated by macrophages and T cells. It is acknowledged that CD is primarily mediated by Th17/Th1 cells as well high levels of innate pro-inflammatory cytokines including IL-1β, IL-6 and TNF-α [6, 10]. Further, the elevated levels of circulating and tissue B cells as well as their activity have also been reported in IBD patients [24, 25].

3. Intestinal neural pathways and visceral pain in IBD

A particular characteristic of the GI tract is the presence of an intrinsic nervous system, the enteric nervous system (ENS) also known as the second brain. Within the intestine, the ENS presents a clear distribution in two neuronal plexuses localized within the submucosa (submucosal or Meissner's plexus) and between the circular and longitudinal smooth muscle layers (myenteric or Auerbach's plexus). The ENS can maintain GI functions alone by the network around the gut wall formed by both plexi. It is composed by around 10^8 neurons consisting of intrinsic primary afferents, interneurons and motor neurons [26–28]. Enteric neurons are supported by glial cells (counterparts of the central nervous system (CNS) astrocytes), which can communicate with the mucosal immune system and the intestinal epithelium by producing different mediators including cytokines. The ENS controls intestinal motility, secretion and absorption, mucosal growth, local blood flow, the immune and barrier function and also carries nociceptive (painful) stimuli to the CNS [29–31] (**Figure 2**).

The gut receives also extrinsic innervation from the autonomic nervous system (ANS) and the spinal afferent nerve fibres that coordinate its activity. The ANS is composed of the sympathetic nervous system (SNS) and the parasympathetic nervous system (PSNS). The extrinsic innervation consists of vagal and spinal sensory nerves, vagal and sacral parasympathetic motor neurons, and sympathetic neurons from prevertebral ganglia, and it plays a key role in maintaining the bidirectional communication with the CNS as well as it is the anatomical basis of the gut–brain–gut axis [32–34].

The vagus nerve (cranial nerve X interfacing with the PSNS) has a motor and a sensorial division and three different endings in the gut: the intraganglionic laminar endings within the myenteric plexus, the intramuscular arrays within the smooth muscle layers and the mucosal fibres within the mucosa [32]. The SNS suppresses GI functions under vagus nerve's activation, cell bodies arise from the paravertebral sympathetic chain ganglia, adjacent to the spinal column and innervating the GI vasculature, as well as the prevertebral (celiac and superior/inferior mesenteric) ganglia, which controls motility and secretomotor neurons. Axons extend to the gut by the mesenteric nerves but also by the vagus nerve, cranially, which also contacts with the ENS [32, 35]. The spinal innervation of the gut comes directly from the dorsal root ganglia (DRG) of the spinal cord, and it is less extensive when compared to the ANS. They extend to the gut by the splanchnic (cranially) and the parasympathetic pelvic nerves (distally). The colon, which harbours large amounts of bacteria, has specific DRG in their innervation [32].

4. Visceral hypersensitivity

Nociception is the neural processes of encoding and processing noxious stimuli that can be accompanied, or not, with pain [26, 36]. Visceral pain originates from the internal organs and is initiated by nociceptors, which can detect mechanical, thermal or chemical changes above a basal threshold [37]. Perception of visceral pain relies mainly in spinal C and Aδ afferents fibres from DRG although vagal afferent stimulation can also mediate pain [38]. The strong compression, as well as chemical stimuli or irritation, of the colon generates afferent signals that can hypersensitize afferent nerves and become nociceptive [39–41].

Although most of the intestinal functions can be carried out by the ENS, extrinsic innervation is necessary to maintain a coordinated activity with the rest of the body and for sensory functions related to visceral pain perception within the gut. This is particularly important because visceral pain and/or altered visceral sensitivity (hypersensitivity) are frequent symptoms in several GI diseases including irritable bowel syndrome (IBS) and IBD. Visceral hypersensitivity generally originates from a local inflammation leading to an enhanced response to a painful stimulus (hyperalgesia) as a result of activation of the immune system, stressful conditions and the intestinal microbiota [42–44] (**Figures 1** and **2**). Alterations in sympathetic neural activity have specifically been implicated in IBD [25, 45]. A decrease in noradrenaline release from sympathetic varicosities in inflamed and uninflamed regions of the GI tract has consistently been reported in animal models of colitis,

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which appears to be due to the inhibition of N-type voltage-gated Ca^{2+} current in postganglionic sympathetic neurons [46]. However, specific alterations of sympathetic function and its role in IBD remain unclear [25]. In the last two decades, numerous morphological, pharmacological and molecular studies have characterized sensory-related systems within the gut, among them the serotonergic system, the endocannabinoid system, endogenous opiates and the vanilloid system have received particular attention due to their potential benefit as pharmacological targets for the treatment of visceral pain. A short description of each of these systems is outlined below.

4.1. The intestinal serotonergic system

The serotonergic system involves the neurotransmitter serotonin (5-hydroxytryptamine; 5-HT), which is mainly stored in mucosal enterochromaffin (EC) cells and in a lesser extent within the enteric neurons (up to 95% of body 5-HT is present in the gut). Tryptophan hydroxylase (TPH) is the limiting enzyme mediating 5-HT synthesis. Two TPH isoforms exist, namely TPH1, mainly expressed in EC cells, and TPH2, expressed in central and enteric neurons. TPH expression/activity is regarded as a reliable indicator of 5-HT availability, whereby high expression levels are indicative of a high rate of serotonin production and release [47–49]. Within the GI tract, 5-HT participates in motor, sensory and secretory functions modifying gut motility/sensation in several ways [50]. For example, 5-HT present within the enteric nerves and acting on 5-HT3 receptors of the vagal afferent nerve fibres can stimulate intestinal secretion and motor reflexes. 5-HT can also act on the receptors 5-HT3, 5-HT4 and 5-HT1P present on enteric neurons, thereby contributing to peristalsis and stimulating intestinal transit [51]. Expression of 5-HT7 receptor has been found on intestinal immune cells and demonstrated a key role in development of experimental colitis [52]. Intestinal inflammation is accompanied by alterations in enteroendocrine cells, among which EC is the most abundant. These cells are distributed throughout the GI tract, with many of them concentrated in the small intestine and rectum and in between epithelial cells, where they act as sensors of the intraluminal milieu. 5-HT release from EC cells is mediated by luminal or neuronal stimuli including mucosal stroking and endogenous chemical stimuli such as adenosine. Changes in the content, release and reuptake of 5-HT as well as increase numbers of EC cells have been reported 8 in both inflamed and non-inflamed gut of IBD patients and in experimental models of IBD [53, 54]. Some studies have also shown that changes in the microbial composition or stressful conditions can induce 5-HT release from EC cells, leading to the initiation of intestinal inflammation and the generation of abnormal sensory-related responses (i.e. altered viscerosensitivity) [48, 55–57]. The sodium-dependent serotonin transporter (SERT), a member of the Na^+/Cl^- neurotransmitter transporter family, is expressed by epithelial cells and neurons in the gut [47] and is involved in the reuptake of 5-HT. SERT expression is reduced in the inflamed and in the healing colonic mucosa of UC patients, thereby increasing 5-HT levels [25, 58]. Furthermore, deletion of SERT increases the severity of 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis in mice [59] and mice treated with the SERT inhibitor paroxetine presented alterations in GI motility and sensitivity [60]. Interestingly, the regulatory cytokine transforming growth factor-beta 1 (TGF-β1) was recently shown to stimulate SERT function suggesting a novel neuro-immune therapeutic strategy to treat GI disorders [61]. These

findings implicate that 5-HT signalling and its SERT-mediated termination can contribute to the symptoms associated with IBD pathophysiology and suggest that drugs targeting this pathway may benefit patients suffering from IBD and other inflammation-related gut disorders [25, 62].

4.2. The intestinal opioid system

The endogenous opioid system is composed by three G protein-coupled receptors: μ, δ and k opioid receptors. Within the GI tract, intestinal opioids, ligands and receptors are found in myenteric and submucosal neurons and in epithelial, endocrine and immune cells (including myeloid and $CD4^+$ T and $CD8^+$ T cells). Opioids have a well-characterized analgesic activity in visceral sensitivity [63, 64], which is mainly linked to the activation of μ and, to a lesser extent, k receptors. The expression of δ opioid receptor together with μ receptor is increased after administration of the inflammatory irritant mustard oil, thereby evoking allodynia and visceral hyperalgesia [25, 65]. μ-Opioid receptors (MOR) are overexpressed in active IBD mucosa, most likely as a compensatory analgesic mechanism generated in states of potentially increased sensitivity. MOR are also significantly enhanced by pro-inflammatory cytokines and repressed by NF-κB inhibitors in myeloid and lymphocytic cell lines [66]. Increased numbers of β-endorphin immunoreactive $CD4^+$ T cells and $CD11b^+$ macrophages are found in murine colonic lamina propria in chronic dextran sodium sulphate (DSS)-induced colitis, where the release of endogenous opioids decreases nociceptive signalling through the activation of μ-opioid receptors [67]. Therefore, it is speculated that the anti-nociceptive actions of peripheral opioids in colitis may indirectly result from a reduction of the neurogenic 'pro-nociceptive' components of inflammation, by decreasing CGRP and Substance P (SP) release that could counteract the pro-nociceptive effects of inflammatory mediators such as TNF-α during inflammation [68]. Recent studies have suggested that probiotics and microbial-related products can also module the intestinal expression of MOR [66, 69–72].

4.3. The intestinal endocannabinoid system

The endocannabinoid (CB) system comprises of two main receptors, CB1 and CB2, their endogenous ligands and their metabolizing enzymes, Mainly the fatty acid amide hydrolase, FAAH. Because of their chemical characteristics, endocannabinoid ligands are difficult to determine; therefore, the expression of CB1, CB2 and the enzyme FAAH, are used to assess endocannabinoid functionality. Within the GI tract, the endocannabinoid system controls intestinal motility, nociception and intestinal inflammation. CB1 and CB2 receptors are expressed on intestinal ganglionic neural cells within the ENS, in epithelial cells and immune cells in the gut [73–76]. The CB1 receptor is predominantly found in neural and epithelial cells, whereas the CB2 receptors are predominantly expressed in immune cells [77]. Upon activation, both receptors mediate analgesic effects and appear to have anti-inflammatory properties [75, 77–80]. Probiotics, bacterial products and stressful stimuli have been postulated to influence the endocannabinoid system [70, 81–83]. In IBD, an increased in CB1 expression has been identified in inflamed mucosa, while a reduction in the endocannabinoid agonist anandamide and no increase in CB2 expression were found.

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Ex vivo cultures of IBD biopsies and immune cells with the non-hydrolysable AEA analogue methanandamide (MAEA) resulted in a reduction in IFN-γ and TNF-α secretion [84]. In animal models of colitis, the CB2 agonist JWH-133 attenuates colitis in IL-$10^{-/-}$ mice and in DSS-induced colitis by decreasing the number of mucosal immune cells (including $CD4^{+}$ T cells, neutrophils, Mast cells and natural killer cells) [85]. Recent studies in humans and animals have identified a new strategy for the endocannabinoid system, whereby targeting of the enzyme FAAH can prove to be a better approach due to the potentially less side effects when compared to the currently available CB compounds [86–88]. Overall, the preclinical findings indicate that manipulating the endocannabinoid system can have beneficial effects in IBD patients, and therefore, the use of *Cannabis sativa* has also been studied, although further research is necessary in this context [89, 90].

4.4. The intestinal vanilloid system

The vanilloid system consists of one of six subfamilies of the transient receptor potential (TRP) channel family, with six types of transient receptor potential vanilloid (TRPV1-6) [91]. These receptors are calcium permeable, non-selective cation channels involved in thermo- and chemo-sensitive transduction [92]. In the intestine, TRPV1, 3 and 4 have been linked to viscerosensitivity and are characterized as pro-algesic receptors [79, 92–94]. TRPV are mainly expressed in intestinal afferent nerves, although they can also be found in EC cells as well as epithelial and immune cells [95–97]. In agreement with their pro-algesic effects, TRPV are upregulated in states of intestinal inflammation and visceral hypersensitivity; for example, TRPV1 is highly increased in immunoreactive nerves in IBD tissue and in quiescent IBD with IBS-like symptoms such as pain [98–102]. TRPV1 deletion prevented the development of post-inflammatory visceral hypersensitivity and pain-associated behaviours, while SP can sensitize TRPV1 function leading to a pro-algesic state [101, 103]. TRPV1 has been linked to the crosstalk between the microbiota and the neuro-immune response in the gut, because TRPV1 and CGRP can modulate cytokine response to lipopolysaccharide (LPS) independently of the adaptive immune response. It has been proposed that TLR4 can activate TRPV1 via intracellular signalling thereby inducing the subsequent release of anti-inflammatory CGRP to maintain mucosal homeostasis [104]. In addition, blocking of TRPV4 has also been shown to alleviate colitis and pain associated with the intestinal inflammation induced by TNBS in mice [105]. Similarly, intrathecal injection of antisense oligonucleotides to TRPA1, another member of the transient receptor potential channel family, decrease its expression and attenuates visceral hyperalgesia in TNBS-induced colitis [25, 65].

4.5. Neurotransmitters, neuropeptides and neurotrophins

More than two dozens of putative neurotransmitters have been described to date, with neurons usually expressing a combination thereof. Most of these mediators have been implicated in the neuro-immune communication associated with gut homeostasis and in the pathophysiology of intestinal inflammation but their specific functions are still to be established [106]. A short description of the most relevant mediators is outlined under this section.

Substance P (SP), an 11-amino acid peptide secreted by nerves and immune cells (including monocytes, macrophages, eosinophils and lymphocytes) belongs to the tachykinins family and acts by binding to the neurokinin-1 (NK-1) receptor. It functions in smooth muscle contraction, vasodilation and epithelial ion transport. It is a mediator of neurogenic inflammation due to stimulation of cytokine release from immune cells (e.g. macrophages, mast cells) and endothelium causing tissue damage and neurodegeneration [25, 107]. High expression of SP and NK-1 receptor was reported in the myenteric plexus and inflamed mucosa of patients with IBD. This is associated with a shift from mainly cholinergic innervation to a more extensive SP innervation, which correlates with the severity of UC and may be part of the neuronal basis for the observed altered motility disturbance seen in these patients [106, 108–110]. Antagonists of NK-1 receptors have been shown to ameliorate inflammation and protect from T-cell-induced colitis. Based on these findings, tachykinin antagonists have been proposed as potential anti-inflammatory treatment for IBD [25, 108, 111, 112].

Vasoactive intestinal polypeptide (VIP), a 28-amino acid peptide belonging to the pituitary adenylate cyclase-activating polypeptide (PACAP)/glucagon superfamily, is highly expressed in the myenteric plexus of the colon. VIP inhibits the peristaltic reflex in the circular muscle layer, controls intestinal blood flow and modulates the immune system by binding to both G protein-coupled VIP receptors 1 and 2. VIP is released from nerve terminals that contain nitric oxide synthase (NOS). These two peptides are thought to be the primary intestinal components of non-adrenergic, non-cholinergic nerve transmission. VIP expression is increased in colonic neurons of CD patients but not in UC patients [25, 113–115]. Treatment with VIP in murine TNBS-induced colitis reduces colitis severity and Th1-cell response [116, 117]. In addition, glucagon-like peptide 2 (GLP-2), a regulator of absorption with anti-inflammatory properties, decreases mucosal inflammation in TNBS-induced colitis in rats by activating VIP neurons of the submucosal plexus [118]. Neurotrophins are a family of proteins regulating neuronal activity in the CNS and PNS, belonging to a class of growth factors and playing a major role in visceral hypersensitivity in the inflamed gut. This is, partly linked to the effects of peripheral neurotrophic factors (NTFs) on local afferent neurons. Among these, nerve growth factor (NGF) is primarily involved in the regulation of growth, maintenance, proliferation and survival of certain target neurons and in innate and adaptive immune responses; brain-derived neurotrophic factor (BDNF) links the commensal microbiota and the CNS [119, 120]; and the family of glial cell line-derived NTFs (including GDNF, artemin and neurturin) are implicated in sensorial alterations observed in inflammatory and functional GI disorders [112].

5. Intestinal neuro-immune interactions

Intestinal inflammation, even if mild, causes significant alterations in neurally controlled gut functions including pain and altered motility. These symptoms are caused, in part, by persistent hyperexcitability of enteric neurons that can occur even after the resolution of colitis. Among cells generating inflammatory signals within the gut mucosa and affecting neural signalling in the ENS, mast cells and enterochromaffin cells seem to play a big role. Both of

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them are increased in the colonic mucosa of IBD patients [25]. The ENS and the mucosal immune system have the ability to regulate each other functions. In the intestinal wall, nerve cells are localized in close proximity to immune cells and they share several chemical mediators. The collected evidence point towards a major role of inflammatory signals affecting the enteric neurons and most likely generating IBD-associated symptoms [25, 121, 122] (**Figure 2**).

Inflammation-related alterations in the ENS are divided into those that alter the structural morphology of neurons and glial cells of the ENS and those that modify enteric neurotransmitters [25, 122–124]. During intestinal inflammation, morphological and functional alterations, including remodelling of visceral afferents, are also observed outside the primary region affected by the insult [112]. ENS structural changes are more marked in CD than in UC patients and are often associated with the extent of inflammatory infiltrate. In fact, it has been suggested that severe and extensive necrosis of gut axons may be a distinct feature in CD [25]. In support of this notion is the ablation of myenteric neurons, accompanied by a high neutrophil infiltration and an excessive production of the Th1 cytokines IFN-γ, TNF-α and IL-12 present in models of colitis. Interestingly, neuronal loss persisted for up to 56 days, that is when the inflammation had resolved in these models [125–128]. Others proposed mechanisms implicated in neuron loss, arisen from animal models and IBD patients, Which involve an increase in immune cell infiltrates, including eosinophils, lymphocytes, plasma cells and mast cells, in myenteric ganglia [25, 123, 126, 128, 129] as well as the activation of apoptotic pathways [130]. Furthermore, enteric glial cell ablation induces a significant decrease in the number of myenteric neurons, which appear to be associated with the loss of NOS-containing neurons in the myenteric plexus, likely underlying the alterations observed in smooth muscle relaxation and intestinal transit time [25, 131]. It is also believed that the reduced availability of neuroprotective factors due to neuronal cell loss may increase the susceptibility of enteric neurons to insults such as oxidative stress, which can have an important role in IBD pathophysiology. Overall, the collected data indicate that the loss of nerve cells is dependent on the time needed to develop inflammation, the type of inflammatory cells and the mediators profile required for nerve–immune interactions [107].

Immune cells found in the intestine, including dendritic and mast cells, lymphocytes and macrophages, express receptors for small molecule neurotransmitters and neuropeptides and produce cytokines targeting the enteric neurons [106]. Neuro-immune regulation includes degranulation of mast cells and influx of neutrophils due to neuronal activation. Neuropeptides released by enteric nerves including SP and VP can stimulate lymphocytes to induce their differentiation and alter immunoglobulin production. Signalling between immune cells and enteric neurons can also evoke alterations in gut function. Hyperexcitability of intrinsic primary afferent neurons may be secondary to activation of cyclooxygenase (COX)-2 and production of prostaglandins (PGE_2) from inflamed colon [25, 132]. Intestinal kinases have also been involved in intestinal inflammation. Protein kinase A activity in nerve terminals increases in previously inflamed colon and facilitates a fast synaptic transmission and the release-ready pool of synaptic vesicles [25, 133, 134]. There is also evidences that pro-inflammatory cytokines such as IL-1β and TNF-α exhibit pro-secretory effects in the human distal colon. Both IL-1β

and IL-6 are reported to increase excitability in submucous and myenteric neurons and to mediate effects on cholinergic and non-cholinergic transmission [135–137].

Mast cells are a major player in the innate immune response. Apart from their prominent role in immunoglobulin E (IgE)-dependent hypersensitivity, mast cells can release and modulate the release of several mediators including cytokines, growth factors, chemokines as well as histamine, proteases, and probably serotonin 22 receptors that regulate multiple important biological processes including neural actions in the human ENS [137]. Neuropeptides released from enteric and visceral afferent nerves regulate human intestinal mast cell mediator's release. In healthy individuals, mast cells are generally located in the lamina propria, in fewer amounts in the submucosa and sporadically found in the muscle layers or in the serosa. An estimated 70% of intestinal mucosal mast cells are in direct contact with nerves, and another 20% are within a 2-μm distance. Mast cells respond to neurotransmitters and nerves and can thereby regulate their activation threshold [137, 138], submucous neurons would respond with a transient excitation mediated primarily by 5-HT3 receptors [139]. Cytokines and chemokines can have different effect on mast cell functions. For example, the chemokine, macrophage inflammatory protein-1α (MIP-1α) is required for optimal mast cell degranulation in mice [140]. In contrast, the regulatory cytokine TGF-β1 can dose dependently inhibits stem cell factor-dependent growth of human intestinal mast cells by both enhancing apoptosis and decreasing proliferation [141] as well as it can influence mediator secretion by reducing histamine, cysteinyl leukotrienes and TNF-α release while prostaglandin D2 (PGD2) generation and COX1 and 2 expressions are upregulated. Mucosal mast cells can also respond to other mediators including adenosine triphosphate (ATP), somatostatin, calcitonin gene-related peptide (CGRP) and SP. Colorectal biopsies from patients with active CD or UC incubated with SP induce mast cell degranulation and histamine release [30, 142].

Histamine, proteases and TNF-α are stored as granules in mast cells and can be released within seconds. Other mediators such as lipid mediators and most cytokines are synthesized once the mast cells are activated. The most important mast cell mediator identified so far is histamine. Histamine influences fluid and ion transport, which is partly nerve mediated and directly excites submucous extrinsic sensory neurons [137, 142, 143]. There are four histamine receptors (H1, H2, H3 and H4), which are found as receptor clusters on submucous neurons, with the most frequent clusters being H1/H3 (29%), H2 (27%) and H1/H2/H3 (20%), respectively. The implication of histamine on sensory neurons comes from studies in rodents [142]. Rat dorsal root ganglion cells with projections to the viscera increased Ca^{2+} responses to a TRPV4 agonist and enhanced TRPV4 expression, when adding histamine or serotonin [144]. The pathophysiological relevance of histamine in both allergic and non-allergic conditions including IBD and IBS is established [141, 145]. In IBD, it has been reported that histamine secretion is increased in the jejunum of active CD and in urine of UC patients [146], although in a recent study, no differences in serum levels of histamine were identified [147].

Proteases, in particular the serine protease tryptase, are prominent mediators released from mast cells. Tryptase is present in almost all human mast cells, comprising up to 25% of their total proteins [148]. Proteases signal to nerves is mediated through protease-activated recep-

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tors (PARs), with four cloned PAR receptors identified in humans. PAR1, PAR3 and PAR4 are predominantly activated by thrombin, and PAR2 is activated by trypsin and mast cell tryptase [137]. In patients with UC, tryptase induces the release of inflammatory cytokines and chemokines, some of which may exert their effects through nerve pathways as outlined above [149]. Supernatants from stool of IBS and UC patients contain increased protease levels and when supernatants from UC patients were injected to mice, it promoted hypoalgesia, which was dependent on cathepsin-G-PAR4 activation [150]. PAR2 activation in mice increases intestinal permeability, which is mediated by SP and capsaicin-sensitive spinal afferent nerves while in rats PAR2 evoked visceral hypersensitivity [151]. Interestingly, PAR positive cells were increased in mucosa of UC patients and preferentially co-localized with tryptase$^+$ cells suggesting that mast cells activation via PAR2 might be involved in the pathogenesis of UC [149]. Similarly, mucosal biopsy supernatants from UC patients can activate mouse DRG neurons innervating the colon, via TNF-α regulation [137, 152].

6. Microbial alterations in IBD

The microbial community of the GI tract is composed by bacteria, virus, fungi, protozoa and yeasts. Gut colonization starts at birth and, when completed, it harbours about 100 trillion microbial commensals and symbionts belonging approximately to 5000 distinct species divided in the phyla Firmicutes, Bacteriodetes, Proteobacteria, Verrucomicrobia, Actinobacteria, Fusobacteria and Cyanobacteria [153–155]. The intestinal microbiota is not homogenously distributed along the GI tract. For example, *Proteobacteria* spp. (mainly Enterobacteria) and Lactobacillales preferentially populate the small intestine while Bacteroidetes and Clostridia populate the large intestine. The density of bacterial cells in the gut increases caudally with the maximal counts (10^{11}–10^{12} cells/g of content in both human and rodents) localized in the ceco-colonic region [156–159]. Intestinal bacteria can be transient i.e. bacteria introduced during adult life; they do not permanently colonize the gut and can have positive (probiotics) or negative (pathogens) effects on the host, or be innocuous, or permanent. The latter ones are long-term colonists of the gut, the true commensals, and they can have immunostimulatory effects (so called authobionts), or they can confer detrimental effects under certain specific conditions (so called pathobionts) [160].

Overall, the commensal microbiota serves the host with protection against pathogens, metabolizing complex lipids and polysaccharides and neutralizing drugs and carcinogens; but it can also modulate intestinal motility, influence the maturation of the intestinal immune system and modulate visceral perception [33, 161]. Changes in the normal composition of the microbiota, termed generally in the literature as dysbiosis, have been associated with chronic inflammatory and functional GI disorders such as IBD and IBS [154, 162] (**Figure 1**). Dysbiosis can occur in parallel to intestinal pathogenesis and can be either a consequence or a cause of the disease [163]. In fact, the causal effects of the microbiota in IBD are still a matter of discussion, with some authors considering that dysbiotic state a consequence and/or a perpetuating factor, rather than a cause of the disease [164, 165].

Many pathogenic organisms have been investigated as causing agents of IBD, including *Mycobacterium avium* subsp paratuberculosis *Helicobacter* spp, non-jejuni/coli *campylobacter* and *Escherichia coli* as well as viruses including Epstein–Barr virus, cytomegalovirus, paramyxoviruses and others [166, 167]. However, to date any pathogenic organism has proven to be a causative agent or even correlate to IBD severity. Recently, the focus has shifted with the conception that the gut commensal microbiota as a whole and/or in relationship to the host can influence disease outcome. This shift has arisen from reports showing that distal ileum and colon (containing the highest microbiota loads) are most susceptible to inflammation and that germ-free animals do not develop inflammation. Similarly, antibiotics and certain probiotics have shown therapeutic efficacy in certain IBD cohorts. An altered bacterial composition (dysbiosis) is associated with IBD patients, characterized by a reduction in bacterial diversity, especially the alpha diversity, which denotes the numbers of bacterial species and their abundance [168, 169].

Pathobionts have been identified and linked to intestinal pathology. For example, *Bacteroides vulgatus* can induce colitis in HLA/B27-β2m rats, but not in IL-$10^{-/-}$ mice and it can even prevent colitis in IL-$2^{-/-}$ mice [170, 171]. In stool samples and mucosal specimens from IBD patients, an increased abundance of Enterobacteriaceae (belonging to Proteobacteria), especially *E. Coli*, is repeatedly observed. Among this, the adherent-invasive *E. coli* (AIEC), which selectively colonizes the ileum of up to 40% of CD patients, has been suggested to be a strain-specific microbial factor in the pathogenesis of CD (**Figure 1**). The definition of AIEC was based on the ability of the AIEC-LF82 strain to adhere and invade epithelial cells and to persist within macrophages without induction of cell death and by inducing the secretion of pro-inflammatory cytokines such as TNF-α [169, 172–175].

In terms of commensals, a reduction in Firmicutes and a spatial reorganization of the Bacteroidetes has been described in patients with IBD [176–178]. For example, *Bacteroides fragilis* is responsible for a greater proportion of the bacterial mass in these patients. Some specific strains of Bacteroidetes and their polysaccharide A have been linked to harbour immunomodulatory potential, as shown by their protective effect on intestinal inflammation by suppressing IL-17 production and enhancing the production of IL-10 by intestinal $CD4^{+}Foxp3^{+}$ T regulatory cells [156, 179–181]. A higher abundance of *Actinobacteria* and a loss of *Prevotella* spp are identified in CD patients. A loss of the commensal *Faecalibacterium prausnitzii* (belonging to Clostridia) abundance has been described in IBD [177, 182]. *F. prausnitzii* was shown to have beneficial immune-regulatory effects on the host, with the A2-165 strain ameliorating inflammation in experimental models. *F. prausnitzii* has also been linked with a new subset of $CD4^{+}CD8\alpha\alpha^{+}$ T cells with regulatory/suppressive functions, a cell type that is less abundant in IBD patients. In addition to the anti-inflammatory properties, *F. prausnitzii* is an important supplier of butyrate to the colonic epithelium and it is found adherent to the gut mucosa where oxygen diffuses from epithelial cells thereby improving barrier function [183]. The loss of *F. prausnitzii* is speculated to be an indicator for increased IBD risk [184–187]. *Clostridium* spp. constitutes one of the largest families of the commensal microbiota and, probably due to *C. difficile* infections, it has traditionally been regarded as a pathogenic bacteria. However, recent data suggest that

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some members of the Clostridia group, , *Clostridium IV* and *Clostridium XIVa*, might have an anti-inflammatory potential in immune responses [180, 188] (**Figure 1**). Moreover, the Clostridia-related group of segmented filamentous bacteria (SFB) has been associated with both intestinal inflammation and immune regulation [180], but their role in human IBD pathogenesis is uncovered. Other commensal strains such as Lactobacilli and Bifidobacteria strains are typically considered to confer health benefits to the host and are frequently used as probiotics [189]. Interestingly, *L. acidophilus* seems to modulate sensory mechanisms leading to visceral analgesia [70] while Bifidobacteria can act as immunostimulants [190]. Probiotic treatment in IBD patients has, to date, not being as successful as in, for example, patients with pouchitis when compared to current treatments in UC patients. In CD patients, probiotic treatment appears to be even less beneficial [191–193]. Verrucomicrobia are a mucus-degrading group of bacteria that seems to affect intestinal barrier function through the degradation of the epithelial mucus layer [194] and some Verrucobacteria spp such as *Akkermancia muciniphila* alleviate experimental colitis and can also mediate intestinal immune tolerance [195, 196]. A reduction in *Akkermansia* spp has been identified in IBD patients [197] (**Figure 1**).

Recent research has identified diet as a major factor influencing commensal microbiota composition. Dietary fibres are often associated with reducing the risk of IBD as well as alterations in bacterial carbohydrate metabolism [177]. Fibres are metabolized to short-chain fatty acids (SCFA) by commensal microbiota in the distal GI tract. SCFA can influence the growth of pathogens, increase intestinal barrier function, influence visceral sensitivity and serve as energy source for colonocytes, and they can facilitate the generation and differentiation of intestinal regulatory T cells [198, 199]. Patients with CD and UC are associated with impairment in SCFA production [185], which is linked to a reduction in butyrate-producing bacteria, including *Roseburia inulinivorans, Ruminococcus torques, C. lavalense, B. uniformis* and *F. prausnitzii* as well as a reduction in butyrate levels. Less butyrate is linked to changes in visceral hypersensitivity [169, 200]. In contrast to dietary fibres, Westernized high-fat diet, full of refined carbohydrates, is strongly associated with the development of colitis in different IBD animal models, contrary to a diet highly based on fruits, vegetables and polyunsaturated fatty acid-3, which has a protective effect against disease progression in these models. Recent data have also revealed that specific changes in dietary intake, for example, feeding of milk-fat diet, can modify the composition of the gut microbiota, resulting in the emergence of pathobionts (*Bilophila wadsworthia)*. The correlations of these 'Westernized' diets and blooming of pathobionts in human IBD onset, development and/or relapse are Still to be further investigated [201–203].

The composition of the gut microbiota has recently been linked to the uptake and signalling effects of bile acids. Some members of the *Eubacterium* and *Clostridium* XIVa clusters possess the ability to 7α-dehydroxylate which are involved in secondary bile acid production. In fact, alteration in bile acid profiles may have the potential to protect against pathogens (such as *C. difficile*) [204] or pathobionts (such as *B. wadsworthia*). The latter one exacerbates colitis in IL-10$^{-/-}$ mice and is known to respond to alterations in bile acid profiles [201, 205].

Apart from bacteria, there are also alterations in the commensal fungi composition as well as the virome. Fungal microbiota is skewed in IBD; for example, CD patients show reduced fungal diversity together with an increased *Candida* taxa [206] and an increased Basidiomycota/Ascomycota ratio, and a decreased proportion of *Saccharomyces cerevisiae* has also been reported. Overall, the data indicate that the IBD gut environment might favour fungi at the expenses of bacteria [207]. An increase abundance of *Caudovirales* bacteriophages has also been reported in IBD patients. Some authors are suggesting that viral dysbiosis *per se* contributes to IBD pathology and changes in the bacterial ecosystem due to their predator–prey relationship [207, 208] (**Figure 1**).

7. Microbiota–gut–brain axis and IBD

There is a bidirectional signalling pathway between the GI and the brain, mainly through the vagus nerve, in which the commensal microbiota have an active role, denoted as the 'microbiota-gut-brain axis'. This axis is vital for maintaining homeostasis and it may be also involved in the aetiology of intestinal dysfunctions/disorders (**Figures 1** and **2**). There are evidences of the ability of the gut microbiota to communicate with the brain and thus modulate behaviour and pain and also transfer and eliminate micro-organisms for selecting the commensal profile. The proposal of a 'microbiota-gut-brain' implies that through a dynamic alignment, the microbiota inhabiting the intestinal lumen will affect the host's superior functions by changing CNS activity and vice versa, that is the brain activity and will also impact on microbiota development and composition. Apart from cognitive and vegetative functions, the 'microbiota-gut-brain axis' has been studied in visceral pain [209–212]. Although it has been traditionally studied in the context of IBS pathology, some of those findings can be translated to IBD, since IBD shares some overlapping mechanisms with IBS [161, 213, 214]. This includes the dysfunction of the brain-gut axis, the implication of TNFSF gene, the abnormal microbial composition and altered host functions, the low-grade inflammation and the presence of IBS symptoms in patients with IBD in remission [215]. Overall, there is evidence that host–microbe alterations might be not only divergent regarding the abundance of microbial community members but also in their metabolic activity.

The intestinal TLRs are critical for bacterial recognition and initiation of innate immune responses. In particular, TLR2, 4 and 7 have been directly implicated in the modulation of nociceptive markers and visceral hypersensitivity and pain [72, 104, 210, 216–221]. It has also been proposed that a neurochemical 'delivery system' exists whereby gut bacteria can send messages to the brain. This delivery system links the commensal gut microbiota to a number of neurotransmitters including GABA, serotonin, noradrenaline, dopamine, acetylcholine and melatonin, all of which are crucial for brain-regulated functions including visceral pain, brain development, anxiety or behaviour [33, 222, 223].

Some of the mechanisms described in the microbiota-gut-brain axis imply the activation of TLRs. Among them, TLR2, expressed in enteric neurons, glia and smooth muscle cells of the intestinal wall appear to regulate intestinal inflammation by controlling ENS structure and

neurochemical coding, along with intestinal neuromuscular function. Colitis in $Tlr2^{-/-}$ mice is more severe compared to wild-type mice that is associated with altered ENS architecture and neurochemical profile, intestinal dysmotility, abnormal mucosal secretion, reduced levels of GDNF and impaired signalling via Ret-GFR-α1. Treatment with GDNF to $Tlr2^{-/-}$ mice led to improved colitis [219].

TLR4, increased in IBD patients, has also been associated with severe colitis with impaired epithelial barrier, altered expression of anti-microbial peptide genes and altered epithelial cell differentiation [221]. A putative LPS–TLR4–TRPV1 axis has been described, directly implicating microbiota in changes of the nervous system by means of the innate immune system, that is the TLRs. In line with this notion, the local stimulation of TLR4 but also TLR7, both expressed in epithelial, immune and neural cells, can induce an immune activation that leads to changes in different nociceptive markers, implicating mainly the cannabinoid and the vanilloid system, without having an overt inflammatory response [216, 217]. These findings address some of the putative mechanisms associated with microbial neuro-immune responses, which can contribute to IBD pathophysiology (**Figure 2**).

8. Conclusions and perspectives

The intestinal immune system has as its main function to protect the host against invading pathogens as well as to tolerate the myriad of our commensal micro-organisms. If this crosstalk is altered due to genetic predisposition and/or environmental factors, the steady state will be broken and it will result in the development of chronic inflammation such as IBD. Recent research has also identified a third player, the nervous system consisting of both the ENS and the CNS, which can directly regulate the intestinal immune system (**Figure 1**). In this chapter, It is summarized the findings linking the intestinal neuronal pathways with the intestinal immune system and the microbiota in IBD patients. In several cases, the degree of inflammation appears to determine the alteration in neuronal pathways, for example, serotonin, the endocannabinoid system, the loss of neural axons, or the increase in EC and lía cell numbers [25, 26, 84, 106, 224–226]. However, it is worthy to note that an altered neuronal signalling can persist long after inflammation is apparently resolved in patients with inactive disease and in animal models after disease is resolved [227].

In conclusion, further studies addressing the triad gut microbiota nerves will be a major challenge in the future. Fundamental understanding of neuronal pathways in inflammatory conditions such as IBD is crucial for the discovery of future target strategies. These will in particular target the regulation of functional bowel symptoms such as abdominal pain, visceral sensitivity, which are prevalent in IBD patients with quiescent disease and are regulated by several of the outlined pathways. To date, the evidence on the gut–brain–microbiota axis in human IBD is scarce but future research will aim to delineate this axis in depth, with the goal to evolve our understanding on GI function, to elucidate the complex interaction of this axis with systemic organs and to cover new potential treatments.

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Author details

Mònica Aguilera and Silvia Melgar*

*Address all correspondence to: s.melgar@ucc.ie

APC Microbiome Institute, University College Cork, National University of Ireland, Cork, Ireland

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Section 4

Novel Therapeutic Perspecitves for Inflammatory Bowel Disease

Chapter 10

Oral Tacrolimus in Patients with Ulcerative Colitis

Takuya Inoue, Kazuki Kakimoto and
Kazuhide Higuchi

Additional information is available at the end of the chapter

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Abstract

Tacrolimus is a macrolide immunosuppressant that is structurally similar to rapamycin and has been found to have potent immunosuppressive properties, showing 10- to 100-fold higher potency for inhibiting lymphocyte activation than cyclosporine A (CsA). Because less variability in absorption and serum levels is observed among patients treated with tacrolimus compared with those who receive oral CsA, tacrolimus has been suggested to be more easily and safely administered to patients with refractory ulcerative colitis (UC) than CsA. However, because oral tacrolimus has a slower onset of action than intravenous CsA and food intake is known to reduce tacrolimus serum trough levels due to its low absorption rate, the proper method for administration of oral tacrolimus has not been determined. Moreover, the long-term effects of oral tacrolimus also remain unclear. In this chapter, key issues regarding the use of oral tacrolimus in patients with UC are reviewed.

Keywords: ulcerative colitis, refractory disease, accelerated step-up, cyclosporine A, top-down therapy

1. Introduction

Tacrolimus, also known as the macrolide immunosuppressant FK506 (formerly FR900506), is a powerful and selective anti-T-lymphocyte agent discovered in 1984. The first letter "T" represents Tsukuba, Japan, where tacrolimus was first identified. Because tacrolimus is a macrolide, the letters "ACROL" are included after the initial T. Finally, the letters "IMUS" represent the immunosuppressive effects of the drug [1]. Tacrolimus was isolated from the fermentation broth of the fungus *Streptomyces tsukubaensis*, which was isolated from a soil sample in Tsukuba, Ibaraki Prefecture, Japan [2].

The molecular structure of tacrolimus is completely different from that of cyclosporine A (CsA). However, their immunosuppressive properties are remarkably similar [2–5]. Initial reports showed that tacrolimus acts as an immunosuppressant by inhibiting interleukin 2 (IL-2) production and blocking the response of mixed lymphocyte culture at concentrations 100 times lower than that of CsA [5]. Early multicenter trials were conducted to evaluate the safety and efficacy of tacrolimus in patients who underwent liver transplantation; these trials showed that the effects of tacrolimus were equivalent to those of CsA-based immunosuppressive regimens [1, 6, 7]. Thus, clinically, tacrolimus was initially developed as a drug for the prevention and/or treatment of graft rejection in organ transplantation patients [8, 9].

Regarding the inflammatory bowel disease (IBD), tacrolimus has been used to treat fistulizing Crohn's disease (CD) and refractory ulcerative colitis (UC) [10]. Because topical administration of tacrolimus can result in high concentrations in the tissue and can effectively regulate the local immune response, topical tacrolimus has also been used to treat refractory distal colitis and extraintestinal UC, such as pyoderma gangrenosum [11–13]. Moreover, tacrolimus has a rapid onset of action and is highly effective in patients with refractory UC; therefore, tacrolimus is approved as an alternative treatment option for refractory UC under the national health insurance system in Japan [14]. The physicochemical properties of tacrolimus result in large variations in oral absorption and metabolism for clearance from the body [9]. Moreover, the therapeutic window of tacrolimus is narrow. Thus, therapeutic drug monitoring is necessary.

2. Clinical pharmacokinetics

Tacrolimus is highly lipophilic and is excreted from the body after undergoing extensive metabolism [9]. Food intake is known to reduce serum level of tacrolimus resulting from its low absorption rate [14]. After absorption, tacrolimus is metabolized by the liver and small intestinal microsomes containing cytochrome P-450 3A4 and 3A5, which are responsible for the biotransformation of tacrolimus [9]. After being metabolized, tacrolimus is mainly excreted in the feces and bile as conjugates. Using ^{14}C-labeled tacrolimus, Iwasaki et al. reported that urinary excretion accounts for less than 3% of the total dose administered and that less than 0.5% of the unaltered drug is detectable in feces and urine in health human subjects [15]. Because ketoconazole, fluconazole, erythromycin, diltiazem, cimetidine, methylprednisolone, and CsA are also metabolized by cytochrome P-450, tacrolimus should be used with caution in patients receiving these drugs [16]. On the other hand, coadministration of rifampicin significantly increases tacrolimus clearance; indeed, rifampicin treatment causes decreased levels of tacrolimus in the blood [17]. Because genetic polymorphisms are known to exist in cytochrome P-450 3A4 and 3A5, the determination of cytochrome P-450 3A genotypes in patients with refractory UC may provide useful information for selecting the optimal dosage of tacrolimus [9]. In patients with UC, Hirai et al. analyzed the association of cytochrome P-450 3A5 genetic polymorphisms with tacrolimus pharmacokinetics and efficacy in Japanese patients with UC. Their results showed that the trough level of tacrolimus is significantly higher in cytochrome P-450 3A5-nonexpressing (*3*3) patients than in cytochrome P-450 3A5-expressing (*1*3 and *1*1) patients and that the short-term remission rate is significantly

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different among these patient groups [18]. Additionally, lack of food intake and silencing of cytochrome P-450 3A5 are associated with achievement of optimal trough levels on multivariate analysis. The proton pump inhibitor lansoprazole is also metabolized by cytochrome P-450 3A4 and elevates the blood concentration of tacrolimus. Therefore, when lansoprazole is coadministered with tacrolimus, repeated therapeutic drug monitoring is needed to prevent the incidence of adverse events [19]. Because tacrolimus is also a substrate of P-glycoprotein, polymorphisms in P-glycoprotein may also determine tacrolimus response in patients with UC [20]. Elevated intestinal P-glycoprotein decreases tacrolimus absorption, thereby leading to decreased blood concentrations and decreased efficacy in patients treated with tacrolimus [21].

3. Immunosuppressive effects

Calcineurin inhibitors, such as CsA and tacrolimus, block the production of IL-2 and the activation of T lymphocytes [22]. CsA and tacrolimus belong to the family of immunophilin-binding drugs, and the drug-immunophilin complex inhibits calcineurin, preventing dephosphorylation of nuclear factor of activated T cells (NFAT) and resulting in decreased expression of cytokines, such as IL-2 [5, 8, 23, 24] (**Figure 1**). Although their immunosuppressive properties are similar, tacrolimus has been found to show 10- to 100-fold more potent inhibition of lymphocyte activation than CsA [5]. Additionally, tacrolimus acts on other pathways, including blockade of cytokine receptor expression and cytokine effects on target cells [25]. Interestingly, CsA, but not tacrolimus, suppresses nitric oxidase production, contributing to the side effects of hypertension and nephrotoxicity and enabling long-term use of CsA [26]. On the other hand, tacrolimus is known to be associated with many adverse effects, including hypertension and renal dysfunction [27].

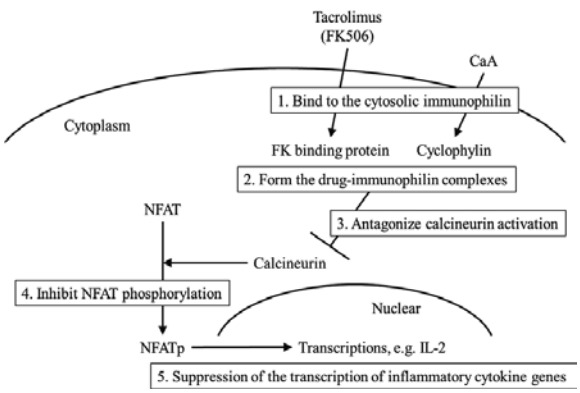

Figure 1. Calcineurin inhibitors in T cells. CsA and tacrolimus bind to immunophilin. The drug-immunophilin complexes inhibit calcineurin, preventing dephosphorylation of NFAT and resulting in decrease expression of cytokines, such as IL-2.

4. Tacrolimus in the treatment of refractory UC

UC is an idiopathic IBD characterized by a chronic relapsing/intermittent clinical course. Aminosalicylates are typically used as first-line treatment for patients with UC, while steroids are usually considered a second-line treatment and are used to induce remission when remission cannot be achieved with aminosalicylates [28]. Because steroids have a rapid onset of action and are highly effective, they are reserved for treatment in patients with severe UC who fail to respond to primary therapy. However, these agents are associated with considerable systemic adverse effects [29]. Nevertheless, approximately 20 % of patients with UC have chronically active disease that requires several courses of steroids [30]. As a result, many patients with refractory UC (e.g., steroid-refractory or steroid-dependent UC) experience severe complications associated with steroid treatment before stable remission can be achieved, and many of these patients ultimately require colectomy [30, 31].

Oral tacrolimus began to be used as an alternative treatment option for refractory UC in July 2009 under the national health insurance system in Japan [14]. Because tacrolimus does not depend on mucosal integrity for absorption, less variability in absorption and serum level is observed among patients treated with tacrolimus compared with those who received oral CsA [32]. Thus, oral tacrolimus has been suggested to be more easily and safely administered to patients with refractory UC than CsA. A study published by Ogata et al. was the first randomized controlled trial to demonstrate the efficacy of oral tacrolimus in refractory UC [33]. Importantly, they also confirmed that tacrolimus showed efficacy in a trough concentration-dependent manner. In their study, patients with refractory UC were randomly assigned to a high trough concentration (10–15 ng/mL) group, low trough concentration (5–10 ng/mL) group, or placebo group. A total of 68.4% of patients in the high trough concentration group improved within 2 weeks after administration of tacrolimus, whereas only 38.1% of patients in the low trough concentration group experienced disease improvement. To date, several uncontrolled and placebo-controlled studies have demonstrated that tacrolimus can induce remission in both adults and children, and these reports suggested that tacrolimus had a dramatic concentration-dependent effect, with the optimal target range appearing to be 10–15 ng/mL with a relatively short period of efficacy [34–38].

Nonetheless, we have still occasionally experienced patients who did not demonstrate improvement even though the appropriate trough level was achieved with oral tacrolimus using standard dosing (an initial dose of 0.025 mg/kg daily is approved under the national health insurance in Japan). We previously examined the short-term efficacy of tacrolimus in refractory UC and found that the clinical response rate at 4 weeks after the initiation of tacrolimus treatment correlated with the mean trough level at 8–21 days after treatment and that the primitive trough level was increased within 5 days after administration, which was important for obtaining the appropriate trough level at 8 days after tacrolimus administration [39]. Even when the starting dose of tacrolimus was set to 0.1 mg/kg/day to obtain early achievement of the appropriate trough level, more than 7 days was required to achieve the target tacrolimus blood concentration because food intake is known to reduce serum levels of tacrolimus by slowing the absorption rate [40]. Therefore, we conducted a prospective,

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multicenter, observational study to evaluate the efficacy and safety of rapid induction therapy with oral tacrolimus, starting at 0.1 mg/kg/day without a meal, in patients with steroid-refractory UC [41]. The dose was adjusted to maintain trough levels of 10–15 ng/mL for the first 2 weeks. Beginning at 2 weeks after the initiation of tacrolimus therapy, the tacrolimus trough concentration was gradually maintained at a lower level of 5–10 ng/mL. From this analysis, 0.15–0.16 mg/kg/day oral tacrolimus was needed to achieve the appropriate trough level; the mean trough level reached a peak on day 2, and 93.5% of patients could maintain high trough levels for the first 7 days of treatment. After 2 weeks, 73.1% of patients with refractory UC experienced clinical responses, and 75.4% of patients achieved clinical remission at 4 weeks after tacrolimus initiation.

Regarding the long-term efficacy in patients with refractory UC, Yamamoto et al. investigated the efficacy of tacrolimus as maintenance therapy for patients with refractory UC and reported that the cumulative colectomy-free survival rate was 62% at 65 months. They also reported that the colectomy-free survival rate was significantly higher in patients who responded to tacrolimus within 30 days than in those who did not and suggested that tacrolimus should be administered to achieve a low trough level (5–10 ng/mL) as maintenance therapy in patients with UC [42].

Currently, antitumor necrosis factor alpha (anti-TNFα) antibodies, such as infliximab and adalimumab, are also used as a treatment option for patients with refractory UC [43, 44]. Because there is no need to adjust the drug concentration when treating patients with these biologics and because they can be used for both induction and maintenance of remission in UC, such treatments have been widely used in the case of refractory UC. However, to date, there are very few reports comparing the efficacy of tacrolimus and anti-TNFα antibodies for refractory UC. Recently, Yamamoto et al. retrospectively compared the short-term safety and efficacy of tacrolimus versus anti-TNFα antibodies (infliximab or adalimumab) for moderate-to-severe active UC and reported that the response rate was higher in patients treated with tacrolimus, although no significant difference was observed [45].

Patients with refractory UC who failed second-line therapies with cyclosporine, tacrolimus, or infliximab have limited medical options to achieve remission and avoid colectomy. To date, several studies have evaluated the efficacy of infliximab as rescue therapy in patients who were refractory to tacrolimus and reported that the short-term response rates ranged from 25.0 to 75.0% [46–49]. Administration of infliximab in patients with refractory UC who did not respond to tacrolimus may be useful for induction remission and could help to avoid the need for colectomy. However, it is still unclear which sequential therapeutic strategies (tacrolimus switching to infliximab or infliximab switching to tacrolimus) should be used.

5. Top-down or accelerated step-up therapy with oral tacrolimus

In patients with CD, top-down therapy involves early introduction of biologics and/or immunomodulators in patients with newly diagnosed disease. With regard to UC, top-down therapy may not be suitable for all patients. Recently, studies have examined the use of

"accelerated step-up therapy," which involves a "step-up" aggressive treatment algorithm that preserves the concept of matching severity to treatment potency yet recognizes the potential benefits of the earlier use of biologics and/or immunomodulators [50]. For many patients with UC, such an accelerated step-up approach may be the best strategy [51]. Because differences in the onset of action of various agents are thought to influence the achievement and maintenance of disease remission, early intervention with tacrolimus may improve the long-term prognosis of patients with UC, similar to the effects of infliximab in patients with CD. Therefore, we evaluated the efficacy of oral tacrolimus in patients with moderate-to-severe UC not receiving concomitant steroid therapy. The results showed that early intervention with tacrolimus was highly effective at inducing remission (72.7% at 4 weeks and 90.0% at 12 weeks) and maintenance remission (72.5% at a mean follow-up of 10.4 months) [52]. Although additional studies are required to establish the efficacy and safety of oral tacrolimus therapy in patients with UC, oral tacrolimus may represent a top-down or accelerated step-up treatment option for patients with moderate-to-severe UC (**Figure 2**).

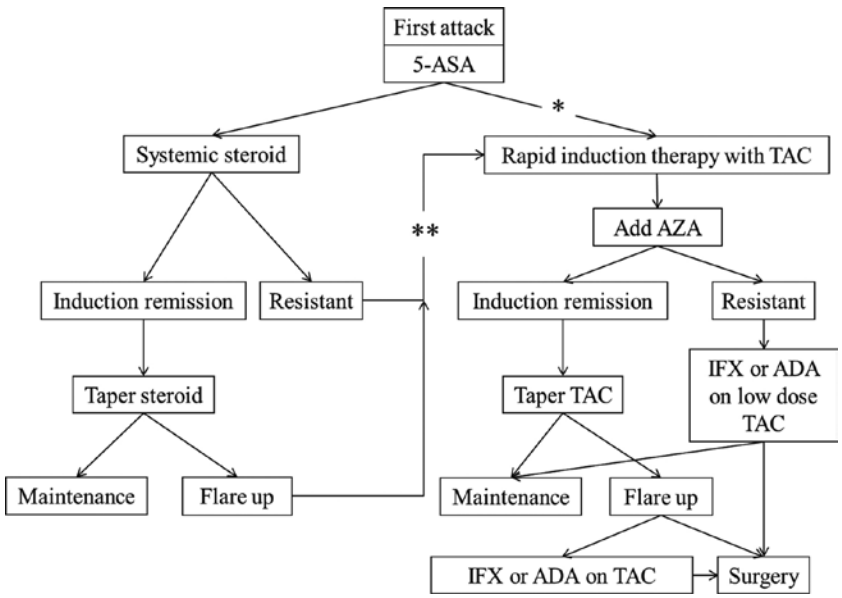

Figure 2. Treatment options for patients with moderate/severe UC. Because prompt intervention with potent medical therapy is crucial in the management of severe UC, top-down therapy with tacrolimus may be useful for avoiding steroid dependency and improving prognosis (*). Recognizing the potential benefits of the earlier use of immunomodulators/biologics and accelerating the introduction of these drugs are defined as "accelerated step-up therapy," thereby avoiding therapies that have minimal efficacy (**).

6. Impact of tacrolimus on cytomegalovirus (CMV) colitis

CMV infection has been reported to be a cause of refractory UC. Because the specific endoscopic features of refractory UC associated with CMV infection have not been clearly descri-

bed, diagnosing CMV infection at an early stage is difficult [53]. Although quantitative real-time polymerase chain reaction (qPCR) for detecting CMV infection in colonic mucosa has been shown to exhibit high sensitivity, the appropriate therapeutic approach for patients with UC having CMV-DNA-positive colonic mucosa remains unclear. In a randomized trial comparing tacrolimus and CsA for prevention of liver allograft rejection, the incidence of CMV infection was found to be significantly lower in patients receiving tacrolimus (15.7 and 25.0% for tacrolimus and CsA, respectively) [7]. Alessiani et al. also reported that tacrolimus treatment in liver transplant recipients resulted in a significantly lower incidence of symptomatic CMV infection compared with that observed after CsA treatment [54]. Shiraki et al. showed the suppressive effects of tacrolimus on CMV replication in vitro [55]. In contrast to tacrolimus, CsA has been reported to enhance the replication of CMV [55]. We used qPCR to identify patients with UC having CMV infection and assessed the outcomes of patients with CMV infections [56]. Our results showed that all CMV-DNA-positive patients who were treated with oral tacrolimus without ganciclovir showed clinical responses and decreased numbers of CMV-DNA copies (**Figure 3**). Because the use of steroids is known to increase the risk of CMV infection, which is associated with disease exacerbation and refractoriness, oral tacrolimus should be used prior to steroid therapy in patients with severe or refractory UC [57].

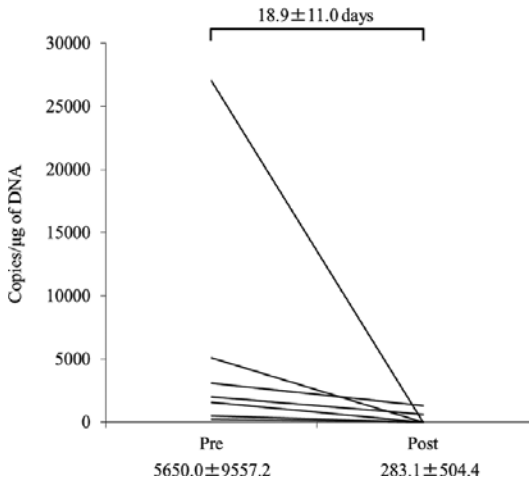

Figure 3. All CMV-DNA-positive patients who were treated with oral tacrolimus showed clinical responses and decreased numbers of CMV-DNA copies without concomitant ganciclovir [56].

7. Adverse effects

Similar to CsA, tacrolimus is known to be associated with many adverse effects, such as infections, renal dysfunction, hypertension, hyperglycemia, and neurological toxicity. However, these effects are generally mild and reversible. Although nephrotoxicity may be a limiting factor for the long-term use of tacrolimus, mean serum creatinine was not significantly elevated following short-term use of tacrolimus. With respect to blood glucose levels, Benson et al.

reported that 62.5% of patients with UC had elevated glucose levels; most of these patients were on corticosteroid therapy at the time [35]. Interestingly, because tacrolimus treatment has strong effects on steroid sparing, the mean fasting blood glucose level was reported to be significantly decreased after the initiation of tacrolimus treatment in patients with refractory UC [41]. During tacrolimus treatment, many patients develop hypomagnesemia (33.3–87.5 %) [33, 35, 41]. Additionally, other adverse effects, such as tremor, nausea, and headache, are often experienced. However, patients rarely have to discontinue tacrolimus therapy due to these adverse effects. Thus, induction therapy with tacrolimus is safe and well tolerated in patients with UC.

8. Conclusions

Tacrolimus is a potent immunosuppressive agent that is useful for inducing remission of refractory UC. Although the long-term efficacy and safety of tacrolimus in patients with UC have not been clearly elucidated, induction therapy with tacrolimus is safe and well tolerated in patients with refractory UC. Because of the requirement for tacrolimus dose adjustment and large variations in the oral absorption and metabolism of tacrolimus, physicians have to know the pharmacokinetics of tacrolimus to obtain maximum efficacy. Rapid induction therapy with oral tacrolimus in the early phase of treatment may provide excellent clinical outcomes and avoid the need for surgery for refractory UC. Therefore, oral tacrolimus may represent a top-down or accelerated step-up treatment option in cases of severe/extensive UC. However, thus far, tacrolimus is used primarily in Japan, and this drug is still not available in some other countries for the treatment of patients with UC because there are only two randomized controlled trials [33, 58] and a small number of studies confirming the efficacy and safety of tacrolimus. Further controlled studies with large numbers of patients are needed.

Author details

Takuya Inoue*, Kazuki Kakimoto and Kazuhide Higuchi

*Address all correspondence to: ureuretakuwan@yahoo.co.jp

Osaka Medical College, Takatsuki City, Osaka, Japan

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Chapter 11

Probiotics and Crohn's Disease

Karen Kroeker and Cathy Lu

Additional information is available at the end of the chapter

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Abstract

Crohn's disease (CD) is a chronic inflammatory condition that can affect any part of the gastrointestinal tract. The human gut microbiome is altered in patients with Crohn's disease. This knowledge has led to research directed at altering the microbiome for therapeutic potential. Probiotics are an attractive therapy, both from a researcher's perspective and also from the patients' perspective. In this chapter, we will review the current clinical evidence for the use of probiotics in the treatment of Crohn's disease. These studies are divided into three categories: induction of remission, maintenance of medically induced remission, and maintenance of surgically induced remission. Unfortunately, there is insufficient evidence to support the use of probiotics in the management of Crohn's disease at this time.

Keywords: Crohn's disease, remission, post-operative recurrence, CDAI

1. Introduction

Crohn's disease (CD) is one of the inflammatory bowel diseases (IBD) that can affect any part of the intestinal tract, from the gums to bum. This disease was first described in 1932 as regional ileitis; at that time, treatment was palliative [1]. It was known even then that this disease could cause perforation and fistulas. Crohn's disease is characterized by transmural inflammation, ulceration—from superficial aphthous ulcers to those that are deep and cause penetration, with skip lesions, and granulomas on pathological specimens. The pathogenesis of Crohn's disease is multifactorial—genetic susceptibility, altered host immune response, interplay with the environment, and altered gut microbiome.

The mainstay of treatment for Crohn's disease is medical with surgical intervention reserved for managing strictures and fistulas and for medically refractory disease. While there is no cure

for Crohn's disease, therapies are used to induce remission and maintain remission. When surgery is used to induce remission, strategies to prevent post-operative recurrence are important. Standard therapies for Crohn's disease focus on altering the immune system with corticosteroids, immunosuppressants, and biologic therapies that are directed at altering the immune system. Knowledge of the role of the enteric bacteria in the pathogenesis of Crohn's disease has led to interest in using probiotics for the treatment of this disease.

2. The altered microbiome in Crohn's disease

The microbiome of patients with Crohn's disease is known to be different than healthy controls. This difference is frequently called dysbiosis. The faecal microbiota in patients with CD has less complexity compared to the healthy controls [2]. Further, the temporal stability of dominant species of bacteria is lower in patients with CD compared to the controls [3]. Biopsy specimens of patients with IBD showed an abundance of *Enterobacteriaceae* compared to the controls [4]. Interestingly, in another study, biopsies from affected and unaffected areas of tissue of patients with IBD show significant differences in diversity [5]. It is uncertain whether the changes in the microbiota in IBD contribute to the disease development or the reverse is true. The Genetics, Environmental, Microbial (GEM) Project is looking for insight into this question by recruiting healthy first-degree siblings and offspring of patients with CD (www.gemproject.ca). Alterations of the microbiome may prove to be an effective approach for the treatment of IBD, especially if these changes in microbiome precede the onset of the disease.

Altering the microbiome as a way to treat active Crohn's disease (induce remission) or maintain remission induced by surgery or medications is being explored. Current methods to alter the microbiome include diet, antibiotics, probiotics, and more recently faecal microbial transplantation.

The use of enteral nutrition (EN) to induce remission in children with Crohn's disease has long been described [6]. In the recent ECCO/ESPGHAN guidelines, exclusive enteral nutrition is recommended as first-line therapy to induce remission in children with active luminal CD [7]. Recently, a systematic review of EN to maintain remission has also shown that EN is associated with a lower risk of relapse compared to a regular diet (34% vs. 64%, $p < 0.01$) [8]. Dietary therapy has rapid effects on microbiota composition and reduces inflammation [9].

Antibiotic exposure is known to be associated with dysbiosis, and this dysbiosis has been shown to be decreased with reduced intestinal inflammation in CD [9]. There are several studies looking at the antibiotics for the treatment of luminal Crohn's disease with some evidence to support the use of ciprofloxacin and metronidazole in treating luminal disease [10]. In surgically induced remission, antibiotics, in particular metronidazole and ornidazole, can reduce recurrence rates at 1 year [10].

Finally, probiotics are being used to attempt to alter the microbiome in patients with IBD. To date, the studies looking at probiotics to treat Crohn's disease have shown a rather modest

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benefit [11]. Nevertheless, patients and physicians alike remain interested in the potential of probiotics for use in the management of IBD. In a focus group study of patients with IBD and IBS conducted at the Cleveland Clinic, patients viewed probiotics favourably and understood them as a natural, low-risk option [12]. In addition to this, they had many unanswered questions about the use of probiotics. This further supports the need for health care providers to know and understand the evidence for the use of probiotics in the treatment of Crohn's disease.

3. Probiotic therapy in Crohn's disease

Medical treatment of Crohn's disease is often classified into the following categories: (1) induction of remission, (2) maintenance of medically induced remission, and (3) maintenance of surgically induced remission. The results of the available randomized and open-label clinical trials examining the effectiveness of probiotics will be presented for each of these three categories. In Crohn's disease, traditionally, clinical indices have been used to assess clinical efficacy for the treatment of Crohn's disease, with an emphasis on improving patient's symptoms and quality of life. The Crohn's disease activity index (CDAI) is most commonly used with values <150 being associated with remission and scores >450 indicating severe disease [13]. More recently, mucosal healing has emerged as an important and objective treatment endpoint in evaluating the efficacy for the treatments of Crohn's disease [14]. The majority of the studies of probiotics in Crohn's disease have used clinical endpoints, with the exception of the post-operative recurrence studies [15].

3.1. Induction of remission

The data to support the use of synbiotics or probiotics to treat active Crohn's disease are limited. In an open-label trial, Fujimori et al. examined the effect of synbiotic therapy (*Bifidobacterium breve, Lactobacillus casei, Bifidobacterium longum, and psyllium)* in 10 CD patients with active disease, the CDAI significantly improved (255-136, $P = 0.009$) with only two of the six responders successfully discontinuing steroid therapy in 13 months [15]. In a randomized controlled study of a different synbiotic (*Bifidobacterium longum* and Synergy 1 [inulin and oligofructose]), the CDAI of 35 patients with active CD also significantly improved at 6 months in the treatment group (219 ± 78 vs. 147 ± 74, $p = 0.02$) but not in the placebo group (249 ± 78 vs. 233 ± 155, $p = 0.81$) [16]. A criticism of this study is that baseline CDAI of the treatment group was lower than the placebo group, even though this difference was not statistically different ($p = 0.35$). Schultz et al. treated 11 CD patients with antibiotics and a tapering course of steroids. At 2 weeks, antibiotics were discontinued and the subjects were randomized to receive either *Lactobacillus GG* or placebo but found no difference in remission rates between the groups (80% vs. 83%) [17]. Twenty-five patients with mild/moderately active CD taking 5-acetylsalicylic acid (5-ASA) were treated in an open-label study with *Lactobacillus salivarius* for 6 weeks which resulted in significant improvement in clinical disease activity (217 vs. 150, $p < 0.05$) [18]. In a small open-label study of four paediatric CD patients using *Lactobacillus* GG for

6 months, Gupta et al. [19] showed a significant improvement in paediatric CDAI scores ($p < 0.05$) and 3/4 were able to taper their steroids.

In a recent meta-analyses that included 12 randomized trials studying remission induction in active IBD, subgroup analyses for CD showed no significant benefit with probiotics for inducing remission or response in active disease ($p = 0.35$, RR = 0.89) [20]. Overall, based on current evidence, probiotics cannot be recommended for use to induce remission in patients with active Crohn's disease.

3.2. Maintenance of medically induced remission

To date, the only study that demonstrated a statistically significant prolongation of medically induced remission in CD was that of Guslandi et al. [21], who compared *Saccharomyces boulardii* plus mesalamine versus mesalamine alone for 6 months. In this study, only 6.25% patients treated with probiotic plus mesalamine had a clinical relapse compared to 37.5% treated with mesalamine alone ($p = 0.04$). Prior to this, Malchow [22] completed a randomized, double-blind, placebo-controlled study of *Escherichia coli* Nissle 1917 in a group of 28 patients with active colonic CD with corticosteroid-induced remission. In this study of the patients that were able to successfully wean from steroids, 30% of the probiotic group relapsed compared to 70% of the controls; this difference was not statistically different.

Currently, in regards to *Lactobacillus*, there continues to only be one randomized, placebo-controlled trial in the adult population to evaluate if *Lactobacillus* GG is effective in inducing or maintaining medically induced remission [17]. All patients received a 2-week course of ciprofloxacin and metronidazole, along with a 12-week tapering course of corticosteroids starting at 60 mg. Eleven patients with moderate to active CD were initially enrolled to receive probiotic, LGG (2×10^9CFU/day), or placebo at week 2 of the study for six months. The primary endpoint was sustained remission defined as the absence of relapse at the 6-month follow-up visit. Relapse was defined as an increase in CDAI of >100 points. This study did not identify a benefit of *Lactobacillus* GG in maintaining remission in CD. However, a limitation of this study was inadequate power as the sample size was only 11 patients with only 5/11 patients completing the study. Of the five patients who remained in the study, two patients in each of the placebo and the probiotic groups had sustained remission. Systematic reviews and meta-analyses have also identified no benefits of *Lactobacillus* as a single probiotic agent in maintaining remission or preventing clinical or endoscopic relapses [20, 23, 24].

Bousvaros et al. [25] conducted a study in which 75 paediatric CD patients in remission were randomly assigned to receive either *Lactobacillus rhamnosus* strain GG (LGG) or placebo for 2 years. There were no significant differences between the groups with respect to the median time to relapse (9.8 vs. 11.0 months, $p = 0.24$ for the LGG and placebo groups, respectively) or the number of patients who relapsed ($p = 0.18$).

Most recently, Bourreille et al. [26] have conducted the only randomized-controlled trial (FLORABEST) in 165 patients with corticosteroid- or aminosalicylate-induced remission; patients were randomized to *Saccharomyces boulardii* or placebo for 1 year. The rate of relapse

was similar between the groups (47.5% were in the *S. boulardii* group vs. 53.2% were in the placebo group, $p > 0.05$) with no difference in the median time to relapse.

In a recent meta-analysis from 2014, subgroup analyses assessing seven studies recruiting CD patients revealed no significant difference in maintaining clinical remission with probiotics and placebo. The strains assessed included *E. coli* Nissle and *Bifidobacterium longum* [20]. Study limitations include the lack of consistency with probiotic, dose, concurrent IBD medications and the absence of endoscopic assessment of remission. Thus, there remains inconclusive evidence to support the use of probiotics to maintain remission in Crohn's disease and well-designed studies are required.

3.3. Maintenance of surgically induced remission

Recurrence of Crohn's disease post-resection continues to be an ongoing challenge in its management. The Rutgeerts score is a widely accepted scoring system for assessment of endoscopic recurrence post-ileocolonic resection. A number of studies have looked at different probiotics to prevent disease recurrence in CD patients with surgically induced remission.

Campieri et al. [27] reported in an abstract, a study of 40 patients treated with either rifaximin for 3 months followed by VSL#3 for 9 months versus mesalamine for 12 months, endoscopic recurrence rates at 1 year (80% for the probiotic group vs. 60% mesalamine group, no statistics reported). In another study of VSL#3, this combination product significantly reduced CD post-operative recurrence when the probiotic was administered immediately after surgery but not when administered some months after surgery [28]. In this multicenter study, 120 patients were randomly assigned to receive VSL#3 or placebo for 90 days, after 90 days of randomized treatment, all patients demonstrating either no or mild endoscopic recurrence were given VSL#3 for the remainder of this 365-day study. Colonoscopy was performed at days 90 and 365 to assess for endoscopic recurrence. At day 90, rates of severe endoscopic recurrence were similar (9.3% for the VSL#3 vs. 15.7 for placebo, $p = 0.19$). Endoscopic assessment at 365 days showed a trend toward less severe endoscopic recurrence if treated with VSL#3 for the year than those treated later (10% vs. 26.7%, $p = 0.09$).

In a randomized, double-blind trial by Prantera et al. [29], 40 patients received either *Lactobacillus GG* versus placebo following surgical resection for 1 year, there were no significant differences in clinical recurrence (16.6% vs. 10.5%, $p = 0.948$), or endoscopic recurrence (60% vs. 35.2%, $p = 0.297$) between the two groups. In 2006, Marteau et al. [30] conducted a larger trial ($n = 98$) over 6 months to investigate the efficacy of a single probiotic strain (*Lactobacillus johnsonii* LA1) to prolong the time to relapse in CD patients. The per protocol analysis confirmed that there was no significant difference between the two cohorts regarding endoscopic recurrence of disease at 6 months (64% vs. 49%, $p = 0.15$). Similarly, Van Gossum et al. [31] examined the efficacy of this same probiotic *Lactobacillus johnsonii* LA1 in a multicenter randomized controlled trial to prolong the time to relapse following elective ileocecal resection. Subjects were randomized to probiotic or placebo for 12 weeks at which time endoscopic recurrence was assessed; the proportion of patients with severe recurrence was similar (21% vs. 15%, $p = 0.33$).

In 2007, Chermesh et al. [32], conducted a small trial of Synbiotic 2000 (a commercial mixture containing four probiotics and four prebiotics) versus placebo. A total of 30 subjects were randomized 2:1 to probiotic: placebo. During the 2-year study, 21 subjects dropped out leaving only nine patients for analysis. No significant difference was found.

In summary, the evidence to support the use of probiotics to prevent recurrence in surgically induced remission is lacking.

4. Conclusion

The role of the microbiome as part of the pathogenesis of Crohn's disease has provided the impetus for much of the research at ways to influence the microbiome in patients with Crohn's disease. Probiotics, along with antibiotics, diet, and faecal microbial transplant, are being studied as options to treat this chronic inflammatory disease. Probiotics are appealing to patients likely due to them being perceived as natural, low-risk therapies for the treatment of IBD, in contrast to standard therapy which focuses on modulating the immune system. To date, the evidence to support the use of probiotics to induce and maintain remission in Crohn's disease is disappointing. Problems with probiotic research include the lack of knowledge about which probiotic to choose and at what dose. For probiotics to have a role in the management of Crohn's disease, more research is needed to align the pathogenic mechanism of the disease with the actions of the probiotics.

Author details

Karen Kroeker* and Cathy Lu

*Address all correspondence to: kkroeker@ualberta.ca

University of Alberta, Edmonton, AB, Canada

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Chapter 12

Autologous and Allogeneic Stem Cell Transplantation for Treatment of Crohn's Fistulae

Fernando de la Portilla, Ana M. García-Cabrera, Rosa M. Jiménez-Rodríguez, Maria L. Reyes and Damian García-Olmo

Additional information is available at the end of the chapter

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Abstract

Up to 20% of patients with Crohn's disease (CD) may have perianal fistula disease. Classically, surgery has played an important role; in recent years, medical treatment has taken a leading role. Immunosuppressants and biological trea tments have proven beneficial in many patients, but still, the percentage of patients who do not respond remains significant. In this scenario, cell therapy is envisaged as an effective alternative to surgery. The promising preclinical and clinical data that we review below suggest that cell therapy could represent a major advance in the clinical management of this difficult problem.

Keywords: stem cells, allogenic, autologous, transplantation, Crohn, fistulas

1. Introduction

Up to 20% of patients with Crohn's disease (CD) may have perianal fistula disease, which is frequently associated with perianal collections [1–3]. Classically, surgery has played an important role, by the placement of drains or setons creation of ostomies, and in severe cases, even proctectomy [4]. However, in recent years, medical treatment with or without the temporary placement of drains has taken a leading role. Immunosuppressants such as azathioprine, 6-mercaptopurine, methotrexate and cyclosporine have proven beneficial in many patients. In more complicated cases where these drugs are ineffective, biological treatments based on monoclonal antibodies have been shown to have some success for the

induction and maintenance of remission of perianal fistula disease and associated proctitis [5–11]. Still, the percentage of patients who do not respond or do so only partially remains significant. Furthermore, the existence of serious complications associated with treatment should not be overlooked [9, 12, 13].

It is as a result of these inadequacies in current treatment strategies that cell therapy has arisen as a complementary option [14]. The promising results published in recent years, both with autologous and in allogeneic cells, highlight a need for greater understanding of the basic principles of this new route and for clarification of the current state of the topic.

2. Basic concepts of cell therapy

Stem cells have both the capacity for self-renewal or self-replication and for production of daughter cells that proceed along specific developmental pathways that will eventually lead to differentiation into specialised cell types [15].

Embryonic stem cells are obtained from the inner cell mass of the embryo at the blastocyst stage. They are able to generate cell lines derived from any of the three embryonic germ layers (ectoderm, mesoderm and endoderm), giving them great therapeutic potential. In mature adult tissues, we find adult multipotent stem cells, which are generally only able to renew and regenerate tissues from the embryonic layer of which they come. However, based on the so-called phenomenon of cellular plasticity, in some instances, they can differentiate into cell populations different to those of their embryonic origin, providing many therapeutic options [16].

Finally, we have the so-called induced pluripotent stem cells (iPS), which are somatic cells that have been subjected to a process of nuclear reprogramming by ectopic expression of specific transcription factors. These acquire molecular and functional characteristics of pluripotency that make them akin to embryonic stem cells. They also display similar characteristics to these in terms of morphology, proliferation, gene expression, epigenetic status of pluripotent genes and their ability to differentiate *in vivo* and *in vitro* [17].

Although embryonic stem cells and iPS have great potential for cell-based therapies, there are several limitations to their use, including regulatory, ethical and genetic engineering considerations. As a result, there are currently no clinical trials evaluating their use [18].

On the other hand, adult stem cells can be obtained using much simpler methods and have no restrictions or ethical considerations. Furthermore, because of their autologous origin, they are not immunoreactive. Early studies using adult stem cells have focused on mesenchymal stem cells (MSCs). These can be found in the stroma of virtually every organ, for example, in subcutaneous adipose tissue and bone marrow. Being fibroblastoid cells, they are the precursors of all types of non-haematopoietic connective tissues (bone, fat, cartilage, etc.). MSCs are generally obtained by selection through adherence to tissue culture plastic, as they are able to adhere and grow in conditions where other cell types do not usually proliferate [19]. They are required to meet minimal criteria defined by the International Society for Cellular Therapy,

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namely, more than 95% of cells must express CD105, CD73 and CD90, as measured by flow cytometry; and <2% must be positive for CD45, CD34, CD14, CD11b, CD79a or CD19 and human leukocyte antigen (HLA) Class II. Moreover, they should be able to differentiate into osteoblasts, chondroblasts and adipocytes under standard *in vitro* differentiation conditions [20].

MSCs have a high capacity for proliferation and differentiation. Furthermore, under certain experimental conditions, they have displayed the ability to differentiate into non-connective cell lineages, such as neuronal and endothelial. Finally, as a particularly interesting property for the use at hand, they are capable, both *in vitro* and *in vivo*, of inhibiting immune response. This ability to immunoregulate includes inhibition of the activation of T, B and NKcells, the maturation of dendritic cells, as well as protecting against inflammatory and/or autoimmune pathologies, including transplant rejection [21].

3. Mesenchymal stem cells as therapies

Early studies with adult stem cells focused on MSCs isolated from bone marrow stroma, which have demonstrated adipogenic, osteogenic, chondrogenic, myogenic and neurogenic potential *in vitro*. However, obtaining stem cells from this source is painful for the patient and only provides a small number of cells [22]. Recently, methods of harvesting adult stem cells from adipose tissue by simple liposuction have been developed. Adipose tissue is rich in such cells, and their preparation is easier than that from bone marrow. Although there is some debate about whether stem cells originate in the fat tissue itself, or if perhaps they are mesenchymal or even peripheral blood stem cells passing through the fat, it is clear that adipose tissue represents a valuable source of potentially useful stem cells. These adipose-derived stem cells (ASCs) have been shown to have an inherent ability to self-renew, proliferate and differentiate into mature tissues, depending on the microenvironment that surrounds them. Such characteristics, intrinsic to all stem cells, make them highly attractive for use in cell therapy and regenerative medicine [23].

Interest in multipotent ASCs is increasing, owing to the ability to harvest large quantities of tissue under local anaesthesia via the liposuction process. Indeed, from just 1 g of adipose tissue, 5×10^3 stem cells can be obtained, which is much greater than the amount that can be acquired from bone marrow. Furthermore, compared to bone marrow MSCs, in the early stages, ASCs express CD34 to a greater extent (100–500 times higher) [24].

The terms adipose tissue-derived stromal cell (ADSC), adipose stromal–vascular cell fraction (SVF) and adipose-derived regenerative cells (ADRC) all correspond to cells obtained immediately after digestion of adipose tissue by collagenase. On the other hand, the terms processed lipoaspirate cells (PLA) and plastic-adherent adipose-derived stem cells (ASCS) describe those that are obtained after culturing those produced by the digestion process. As a unifying term, we refer to these cell types as adipose-derived stem cells (ASC), in accordance with the International Fat Applied Technology Society Consensus [25].

4. Utilisation of MSCs in the treatment of perianal fistula disease

The precise mechanism of the therapeutic action of MSCs is not fully understood, but is likely to reflect their inherent characteristics, in particular their differentiation potential [26, 27]. MSCs have the ability to migrate to the site of a lesion or inflammatory process, stimulate the proliferation and differentiation of resident stem cells through the secretion of growth factors, remodel the matrix and exert an immunomodulatory and anti-inflammatory effect. Together, these properties aid help the healing of tissues [28–31]. It has also been demonstrated that MSCs can induce an increase in epithelialisation and angiogenesis through a process of differentiation and paracrine interaction with skin cells [32–34].

Today, we know that Crohn's disease delays T-cell apoptosis [35, 36], and a mechanism of action of ASCs when injected into the inflammation site in the fistula tract has been postulated. Initially, the cells recognise proinflammatory cytokines such as IFN-γ, followed by activation of the indoleamine 2,3-dioxygenase (IDO) enzyme, which is ultimately responsible for creating a microenvironment—lymphocyte freezing by inhibition of phosphorylation. This results in a reduction in the release of proinflammatory mediators (TNF-α, IL-6, etc.) and an increase in that of anti-inflammatory species such as IL-10 [37].

5. Treatment protocol for anal fistulae

The protocol for stem cell treatment of anal fistulae inevitably starts with the harvesting of the MSCs, either from the patient's bone marrow or their fat (autologous), or from a healthy donor (allogeneic). Bone marrow cells are harvested by aspiration, and then, the MSCs are expanded *ex vivo* for subsequent use in the fistula tract [38, 39]. Although there are various protocols for expansion and differentiation of cells obtained from adipose tissue (with a consequent variation in results), ASCs are normally used after digestion with collagenase under constant stirring. The obtained solution is then centrifuged at low speed, and the resultant is filtered through a nylon mesh of 40–200 μm. The new solution is then centrifuged again, and the cells are re-suspended in fresh expansion medium. It is important to stress that this procedure must be carried out in extremely sterile conditions [40].

As for the route of administration, there is a single study in which allogeneic bone marrow MSCs were given intravenously, with the closure of fistulas being a secondary objective of the study [41]; all other published studies have employed the intralesional route [38, 39, 42–50].

Before intralesional injection of the isolated MSCs, the lesion site must be prepared with similarly intensive curettage, avoiding the use of cytolytic substances (hydrogen peroxide). The inner fistula orifice can then be sealed with an absorbable suture. At this point, half of the cell preparation is administered to the tissue around the inner hole, making small submucosal wheals. The other half is applied along the walls of the fistula tract, if possible along its whole length, while taking care not to go deeper than a few millimetres, again in small wheals (**Figure 1**). Several studies have investigated the use of fibrin glue as an adjuvant or scaffold, in order to enhance the attachment of cells in the fistula tract [43, 45–47]. The dose of cells

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required for optimum results remains to be determined; in published studies, this ranges from 3.5×10^6 to 40×10^6 cells [39–50].

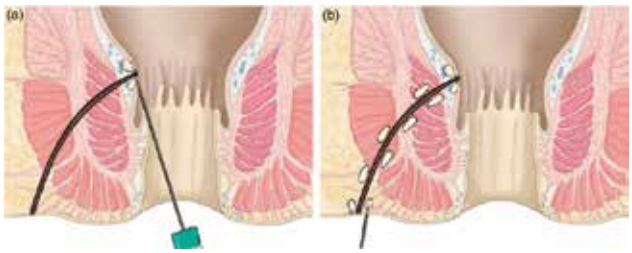

Figure 1. Implant points. (a) Wheal in the internal fistula orifice; (b) injection in the fistula tract at a depth of no more than 2 mm (courtesy of Tigenix).

Most studies have used ASCs, but there are also some that have evaluated the use of bone marrow cells. As for the cell source, the advantages of an allogeneic source (from healthy donors) are innumerable in comparison with those of an autologous source, especially in terms of greater accessibility, easy expandability and good stability. Their use is possible because of their low immunogenicity and limited persistence, which reduce the chances of provoking an adverse effect in the host [51].

6. Safety and efficacy of MSCs in the treatment of anal fistulae

The first experience with stem cells in the treatment of anal fistulae was reported by García-Olmo et al. [52]. Several studies have since been published, the majority of which are from Spanish groups. The MSCs used have mainly originated from adipose tissue, with only two studies using bone marrow MSCs. In these latter cases, both allogeneic and autologous cells have been used. In all studies, administration was intralesional, with fibrin glue often used [38, 39].

Today, any questions as to the feasibility and safety of such treatment seem to have been resolved, at least within the range of doses used. A retrospective study evaluating whether MSC treatment has any influence on fertility, course of pregnancy, birthweight or physical status was recently published [53]. Five patients with fistula associated with Crohn's disease treated with ASCs, and who indicated their intention to have children after completion of treatment, were tracked. Fertility and pregnancy course were not found to be affected by this therapy. Furthermore, no treatment-related malformations in newborns were observed. Therefore, it was concluded that in the patients analysed in the study, local injection of ASCs was not associated with adverse effects on the ability to conceive, pregnancy course or the newborn's condition.

In the published literature, there are differences in cure rate depending on the follow-up, but in general, it is estimated to be between 50 and 70% (**Table 1**).

Authors, year	Study design	Source of cells	Results
Garcia-Olmo et al., 2005 (Spain) [42]	Phase I clinical study ($n = 4$)	ASCs (autologous)	Complete closure: 50% of patients; 75% fistulas
Garcia-Olmo et al., 2009 (Spain) [42]	Open-label, multicenter, phase II study ($n = 14$)	ASCs (autologous); fibrin glue	Fistula healing: 71 vs 14%
Ciccocioppo et al., 2011 (Italy) [38]	Prospective study ($n = 10$)	MSCs (autologous)	Reduction in CDAI, PDAI and pain/discharge PDAI scores
Guadalajara et al., 2012 (Spain) [43]	Retrospective follow-up of Garcia-Olmophase II study ($n = 5$)	ASCs (autologous); fibrin glue	58% sustained fistula closure at end of follow-up by mean 3 years No safety problem
Cho et al., 2013 (Korea) [47]	Open-label, multicentre, dose escalationphase I study ($n = 10$)	ASCs (autologous); fibrin glue	Healing in 50% in the group with 2×10^7 cells
Lee et al., 2013 (Korea) [45]	Open-label, multicentre, phase II study ($n = 42$; 33 completed follow-up)	ASCs (autologous); fibrin glue	Fistula closure in 79%, recidive 11%
de la Portilla et al., 2013 (Spain) [48]	Open-label pilot study ($n = 24$)	ASCs (allogeneic)	Complete closure: 56.3% at 24 weeks
Ciccocioppo et al., 2015 (Italy) [44]	5-year follow-up of 2011 study ($n = 10$)	MSCs (autologous)	37% fistula relapse-free 4 years later
Cho et al., 2013 (Korea) [46]	Retrospective, 1-year follow-up from 2013 study	ASCs (autologous); fibrin glue	Complete closure maintained in 75% at 2 years ITT analysis; 80% PP analysis
Garcia-Olmo et al., 2015 (Spain) [49]	Retrospective, open-label ($n = 3$ with CD)	ASCs (allogeneic andautologous)	Healing in 2/3 CD fistula patients
Molendijk et al., 2015 (The Netherlands) [39]	Double-blind, placebo-controlledphase II study ($n = 21$)	MSCs (allogeneic)	Healing up to 85%
Park et al., 2015 (Korea) [50]	Multicentre, open-label, dose escalation pilot study ($n = 6$)	ASCs (allogeneic); fibrin glue	Group 1 (1×10^7 cells/ml); healing 100% Group 2 (3×10^7 cells/ml); healing 100%

ASCs, adipose-derived stem cells; CD, Crohn's disease; CDAI, Crohn's disease activity index; ITT, intention to treat; IV, intravenous; MSCs, mesenchymal stem cells/mesenchymal stromal cells; PDAI, Pouchitis disease activity index; PP, per protocol; SC, stem cells.

Table 1. Published studies using MSCs to treat Crohn's disease patients with perianal fistulas.

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Ciccocioppo et al. evaluated the long-term safety and efficacy of the use of bone-marrow-derived MSCs. In their study, 8 patients were followed prospectively for 72 months. These patients were part of a phase I/II trial previously conducted, in which a cure rate of 70% per year was reported, with improvement observed in the remaining 30% [44]. Patients received serialised injections of MSCs (4 on average) at intervals of 4 weeks. Secondary endpoints were the time patients remained without fistula and the time they were free of medical or surgical treatment. The Chrohn's Disease Activity Index (CDAI) increased over the first 2 years, followed by a gradual decline in the third year, and stabilisation at the end of follow-up at figures similar to those of the first year. The probability of remaining without fistula was 88% for the first year, 50% at 2 years and 37% over the next 4 years. The probability of patients being free from surgery was 100% for the first year, 75% for years 2–4 and 63% at years 5 and 6. Finally, the probability of patients being free from medical treatment was 88% for the first year, 25% at years 2–4 and 25% at years 5 and 6. No adverse effects related to treatment in these follow-up periods were recorded. The authors conclude that the fact that the activity indices increase again in the second year might suggest that this therapy is not curative, but that it does improve the remission rate in patients with refractory disease. Moreover, almost all patients required the reintroduction of biological or immunosuppressive therapy after the second year [44].

We are currently awaiting the publication of the results of a phase III, randomised, placebo, double-blind, multicentre, and international clinical trial employing Cx601, a preparation of allogeneic ASCs. It has recently been reported that, after 24 weeks, Cx601 was statistically superior to placebo in achieving the combined response (clinical and imaging) of complex perianal fistulas in Crohn's disease patients whose response to previous treatment, including anti-TNFs, had been inadequate.

7. Future perspectives

There is no doubt that a new avenue has opened for the treatment of Crohn's disease patients suffering from fistulae refractory to conventional therapy. Since the first description of the treatment, interest in this therapy has grown, so that in addition to the 11 studies published to date, at the time we write this chapter, there are more than a dozen clinical trials in recruitment or in the results publication phase.

While the safety of ASC therapy seems to have been well established, the optimal dosage, route of administration (intravenous versus intralesional), administration technique (alone or together with fibrin glue), among other matters, are yet to be adequately determined. However, these should be investigated and resolved in the coming years.

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Author details

Fernando de la Portilla[1*], Ana M. García-Cabrera[1], Rosa M. Jiménez-Rodríguez[1], Maria L. Reyes[1] and Damian García-Olmo[2]

*Address all correspondence to: fportilla@us.es

1 Department of General and Digestive Surgery, Colorectal Surgery Unit, "Virgen del Rocío" University Hospital/IBiS/CSIC/University of Seville, Seville, Spain

2 Department of Surgery (Fundacion Jimenez Diaz), Universidad Autonoma de Madrid, Madrid, Spain

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The incidence of chronic inflammatory and autoimmune diseases is steadily increasing. Inflammatory bowel disease (IBD) is one example of a chronic inflammatory disease, which primarily affects the intestine but may also affect extraintestinal organs. The exact pathogenesis of IBD is currently unknown. However, it is clear that the pathogenesis is complex, involving barrier defects, changes in the intestinal microbiome, and chronic immune activation. This book aims to summarize basic aspects of these complex interactions between barrier function, microbiome, and the immune system. Of note, there is currently no cure for IBD. However, several therapies have evolved in the last years, which are overall able to—at least temporarily—suppress IBD. These therapies and the underlying mechanism are discussed.

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