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

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

Abstract

Inflammatory bowel disease (IBD), which includes Crohn's disease (CD) and ulcerative colitis (UC), is a multifactorial 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 contrast, 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
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 (NO2), 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 nitrosamines 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].
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].
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 osteoclasts 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-1α/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-10Rxα 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].
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 transcription 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/TH17 cells 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 microflora 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 fistulizing 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].
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 propria 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.

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