From Cytokines to Toll-Like Receptors and Beyond - Current Knowledge and Future Research Needs in Irritable Bowel Syndrome

Oscar Rodríguez-Fandíño, MSc1, Joselin Hernández-Ruiz, PhD2 and Max Schmulson, MD1*

1Laboratory of Liver, Pancreas and Motility (HIPAM), Department of Experimental Medicine, Facultad de Medicina-Universidad Nacional Autónoma de México (UNAM), México, 2Department of Clinical Research, Hospital General de México, México

The irritable bowel syndrome (IBS) is a complex disorder in which psychosocial, cultural and biological factors, interact. Recent knowledge in the pathophysiology of IBS, seem to combine issues such as a low grade inflammation or immune activation and dysbiosis that can trigger or exacerbate IBS. On the other hand, stress mediated through the hypothalamic-pituitary-adrenal axis can produce motility abnormalities that can modify the microbiota as well, with the subsequent immune activation in the mucosa and stimulation of nerve terminals, generating symptoms of IBS. Also, we speculate that, stress, dysbiosis or an underlying genetic predisposition, may increase the epithelial permeability leading to a contact between pathogens-associated molecular patterns and toll-like receptors in the deeper layers of the gut, developing a host immunity response and IBS generation. We believe that the role of toll-like receptors in IBS and elucidating the communication processes between the immune and the nervous system, warrant future research.

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Key Words
Immunity, Mucosal; Irritable bowel syndrome; Stress, psychological; Toll-like receptors

Introduction

Traditionally, irritable bowel syndrome (IBS) has been considered a functional gastrointestinal disorder (FGID), characterized by pain or discomfort associated with changes in bowel habit, such as diarrhea, constipation or alternating/mixture. As with other FGIDs, in IBS there is no evidence of organic, biochemical or structural abnormalities; however, recent data are shaping new concepts. Currently, IBS is diagnosed based on clinical symptoms by the Rome criteria, in the absence of alarm signs; but patients usually undergo exhaustive diagnostic evaluations looking for organic gastrointestinal pathologies before the diagnosis is accepted by both patients and physicians. Despite this, IBS is the most common FGID and may affect up to 20% of the adult population in industrialized and non industrialized countries. For example, in non industrialized countries, a 16% prevalence of IBS has been reported in the community, and up to 35% among volunteers. Furthermore, it is the first reason for consultation to gastroenterologists.
In recent years, new concepts have changed the main interest in IBS, energizing a growing group of gastroenterologists and scientists around the world. On the one hand, data from several groups have begun to blur the division between functional and organic disorders. For example, the recognition of inflammation in IBS, albeit its low grade, the participation of this inflammatory response on gut motility and sensitivity and finally the fact that IBS could represent an abnormal mucosal response to an altered intestinal microbiota, echoing current theories on inflammatory bowel disease (IBD), could answer the long time considered convergence between IBD, celiac disease and IBS. Additionally, there is evidence implicating gastrointestinal infections in the pathogenesis of IBS, the so called post-infectious IBS (PI-IBS). With this information, a rapidly growing body of evidence has supported a low grade inflammation and an altered fecal microbiota condition related to the pathogenesis of IBS. On the other hand, some antibiotics and probiotics have proven to reduce symptoms, leading to a new understanding of IBS.

Issues of Interest in the Pathophysiology of IBS

1. Genetic factors

During the last decade, studies on familial aggregation and on twins have supported the involvement of hereditary factors in the pathogenesis of IBS. However, the lack of consistent findings that support a specific genetic mechanism in IBS based on symptom phenotypes, has lead to a different approach: the examination candidate genes associations with FGIDs and with the underlying abnormalities in gastrointestinal and colonic physiology. Examples of this approach include the association of IBS with IL-10, serotonin transporter, alpha-2 adrenergic receptors and G protein functions. Gonsalkorale et al and van der Veek et al reported an association of IL-10 low producer polymorphism with the presence of IBS in different populations. The above findings have suggested a genetic predisposition for a reduced anti-inflammatory response, mediated by a lower production of this regulatory cytokine. Additionally, this pro-inflammatory condition could be synergistically stimulated, if patients have a higher production of tumor necrosis factor-alpha (TNF-α), as has been related by high producer polymorphisms in patients compared to healthy controls.

Recently, as a consequence of a gastroenteritis outbreak follow-up, Villani et al evaluated the genetic risk factors in patients with PI-IBS, reporting statistically significant relationships with several genes. The most striking features were the relationships between the immunity related toll-like receptor 9 (TLR9), IL-6 and adhesion E-cadherin 1 polymorphism genes, as risk factors.

2. Stress

Since the stress concept was borrowed from physics, Hans Selye identified the gut, along with the endocrine and immune systems, as the main targets altered by chemical and physical challenges. Recently, there have been several studies relating stressful events (psychological stressors) with the development of FGIDs such as IBS or symptom exacerbation, which intend to elucidate the stress related mechanisms that influence the pathophysiology of this disorder.

The release of corticotrophin-releasing factor (CRF) from the hypothalamus, in response to diverse physical and psychological stressors, plays a major role in orchestrating the behavioral, neuroendocrine and autonomic responses to stress. The hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system are the 2 core branches of this stress response system. Abnormalities in the stress response in the form of changes in adrenocorticotropin hormone, cortisol and catecholamine levels, have been reported in IBS. In fact, the importance of CRF has led research to develop CRF antagonists as possible treatments for IBS.

Additionally, alterations in the HPA axis have received much attention due to its possible implication in a non-standard immune activation in the gut wall, and in symptom exacerbation in different IBS subgroups. This non-standard immune activation is represented by an abnormal mucosal response to an altered microbiota including an increase infiltration of mononuclear, mast and enteroendocrine cells in the colonic mucosa, but without a frank histological inflammation by light microscopy and absence of clinical and laboratory markers of inflammation.

Also, in order to solve the therapeutic needs related to these factors, several groups have turned their scope to exploring the efficacy of probiotics, mainly due to the potential effects that can be developed by gut derived flora, in symptom improvement (pain and discomfort) and low grade inflammation in the intestinal mucosa, probably through several mechanisms and interactions, including possible modifications of the HPA axis balance.
3. IBS and the involved immunological response

Several groups have been reporting evidences that suggest the development of low grade inflammation in the gut mucosa (Table 1), mainly in patients with PI-IBS. Almost 20 years ago, Salzmann et al suggested an increased infiltration of mononuclear cells in the lamina propria of patients with IBS. On the other hand, the majority of individuals that suffer an acute gastroenteritis recover completely after the infectious agent is cleared, but a significant group develops FGIDs, such as IBS, functional dyspepsia or both. It is remarkable that non-gastrointestinal infections have also been reported as risk factors for the development of IBS.

1) Low grade inflammation-immune activation

The concept of low grade inflammation has been coined by several groups, and as previously mentioned, it is described as an increased infiltration in the colonic mucosa of T lymphocytes, mast cells and enteroendocrine cells (Fig. 1). However, the term low immune activation is probably more appropriate. The higher number of mast cells present in colonic biopsies, which has been reported as a key immunological trait of IBS patients, is an important issue. In fact, a higher number of mast cells in proximity to the colon sensory nerves have been correlated with the severity and frequency of abdominal pain, bloating, and probably with rectal hypersensitivity. It is noteworthy that Cremon et al reported higher numbers of T and mast cells in about 50% of the IBS patients. Their study compared the percentage of colonic mucosa infiltrated by immune cells in patients with IBS and those with truly inflammatory conditions, such as IBD (eg, microscopic colitis, inactive and active ulcerative colitis) and healthy controls, finding intermediate infiltration levels in IBS patients compared to controls, but lower levels when compared to patients with IBD. The authors concluded that inflammation could be a common pathophysiological factor triggering each one of these disorders, where IBS and IBD might represent the 2 ends of a wide spectrum of chronic inflammatory conditions.

Table 1. Immunological Abnormalities and Microbiota-Gut Interactions in Irritable Bowel Syndrome

| Study | Findings | Relevance in IBS |
|-------|----------|-----------------|
| Chadwick et al,11 2002 | Increased numbers of intraepithelial CD3+ and CD25+ lymphocytes | Low grade inflammation |
| Gwee et al,12 2003 | Increased production of pro-inflammatory cytokines | Increased production of pro-inflammatory cytokines |
| Barbara et al,13 2004 | Activated mast cells in proximity of colonic nerve endings | Correlated with the severity and frequency of abdominal pain/discomfort |
| O’Mahony et al,14 2005 | Altered IL-10/IL-12 ratio, indicative of pro-inflammatory state, normalized with B infantis | Correlated with improvement in the patients symptom scores |
| Kassinen et al,15 2007 | A significantly altered fecal microbiota in IBS | The microbial composition varies between IBS subtypes and controls |
| Liebregts et al,16 2007 | Higher TNF-α, IL-1β, IL-6 at baseline, and IL-6 levels after LPS induction | Pro-inflammatory cytokines differences according to bowel habit predominance |
| Cremon et al,17 2009 | IBS-D presented higher levels of cytokines than IBS-C | Inflammatory conditions as a continuum: Healthy-IBS-IBD |
| Piche et al,18 2009 | Greater number of mast cells in women than men with IBS | Immune activation is gender dependent |
| Öhman et al,19,20 2009 | Increased permeability and tight-junction protein expression | Increased permeability in the gut |
| Villani et al,21 2010 | Genetic predisposition to abnormalities in immunity and permeability | Genetic predisposition to abnormalities in immunity and permeability |

IBS, irritable bowel syndrome; LPS, lipopolysaccharide; IBS-D, diarrhea predominant-IBS; IBS-C, constipation predominant-IBS; IBD, inflammatory bowel disease; TLR9, toll-like receptor 9; CDH1, E-cadherin 1.
Figure 1. Possible immunological responses in the gut mucosa related to developing bowel disorders. In healthy controls there is a tolerogenic reaction towards commensal microorganisms. However, it is possible that bacterial populations present in some patients or a genetic predisposition, may shift the response towards an immune activation in irritable bowel syndrome (IBS) or to a full-blown inflammatory response in inflammatory bowel disease (IBD).

2) Mast cells

Mast cells are distributed along the digestive tract and in all layers of the intestinal wall, and might be in a permanent state of alert or activation due to the continuous presence of potential antigens within the gastrointestinal lumen. Furthermore, mast cell functions are diverse. Traditionally, they have been considered as the key effectors cells of allergic reactions. These cells could also be of major importance in recruiting and activating eosinophils, B and T cells in activated state (presence of CD4+ and CD25+ markers). On the other hand, mast cells have been involved in the communication with intrinsic and extrinsic nerves to modulate sensory and motility functioning. It is important to note that the resulting reactions from the mediators released by the above mentioned cells include responses in the enteric nervous system, visceral hyperalgesia and an increased mucosal permeability, as well as the generation of symptoms, such as changes in bowel habit and abdominal pain.

3) Enteroendocrine and other immune cells

Other immune cell types have also been observed to be increased in patients with IBS. That is the case for enteroendocrine cells, which have been related to PI-IBS. In this subgroup of patients, enteroendocrine cells have been reported to be present in higher numbers than in any other IBS subgroup. Additionally, neutrophils have been reported to be augmented in the gut mucosa of patients and in animal models, but unlike the above immune cells (mast cells and enteroendocrine cells) these observations seem to be scarce and possibly this cellular population is not an important mediator of the low immune activation present in IBS. In addition, the presence of neutrophils would imply a full-blown inflammatory process (Fig. 1).

4) Lymphocytes

Chadwick et al, reported increased numbers of T cells expressing CD25+ in the lamina propria of the colon that could be related with the low immune activation in the bowel. Recently, it has been hypothesized that CD4+ CD25+ T regulatory (Treg) cells are reduced in numbers and in their functional capabilities, producing an inadequate suppression of intestinal inflammatory activity. However, research data from Holmén et al did not find a significant difference in the mRNA expression of the FoxP3 - a specific marker of Treg cells -, between IBS patients and healthy controls, or in the suppressive activity of the peripheral CD4+ CD25+ cells from patients and controls. Despite the above research, the study was not able to rule out the possibility that the colonic Treg cells are dysfunctional in vivo or that alteration of other mucosal immunological cells with suppressive activity could be associated with the low grade inflammation that is found in the gut of patients with IBS.

Recently, Öhman et al reported several alterations in peripheral blood T cells, such as the presence of CD69+ lymphocytes (early activation) and the integrin alpha4beta7, receptor (intestinal homing) (Fig. 2). The same group also described a state of B cell activation, finding increased CD80 and CD86 expression, at least in a subpopulation of IBS patients. In addition, the slight but significant difference in the over expression of IgG and alpha4beta7, together with the increased expression of CD80 and CD86, suggests that IBS patients also have increased B cell activation in the extra-intestinal lymph nodes. In order to explain this cellular infiltrate, Öhman and Simrén suggested 2 possible explanations: (1) It could be due to an unregulated mild inflammation or (2) It can reflect a potent inflammatory suppressive process led by activated T lymphocytes. However, as of today, the profile of cytokines released by mast cells, enteroendocrine cells, T and B lymphocytes and Treg cells in IBS, as well as the level of activation of the infiltrated lymphocytes in the gut mucosa of these patients, is unknown.

5) IBS and microscopic colitis

The finding of increased numbers of T cells in the lamina propria of the colon in a subgroup of IBS patients, may need to
be differentiated from the presence of microscopic colitis. Microscopic colitis is a syndrome of chronic watery diarrhea with a chronic inflammatory cell infiltrate in the colonic mucosa, a normal colonic appearance in the colonoscopy and no laboratory abnormalities, therefore, the clinical presentation may be indistinguishable from diarrhea predominant-IBS (IBS-D). In fact, Limsui et al. have reported that 41% to 56% of patients with microscopic colitis, met symptom criteria for IBS. Also, Chey et al. found that the overall prevalence of microscopic colitis in suspected IBS patients is 1.5% and 2.3% in those older than 45 years. On the other hand, classic microscopic colitis encompasses 2 subgroups, collagenous colitis and lymphocytic colitis. The first one has an abnormally thickened subepithelial collagen band and the second one, has increased intraepithelial lymphocytes (IELs). Therefore, lymphocytic colitis may be similar with IBS respect to low grade inflammation, however, in microscopic colitis the IELs are increased from 5 per 100 surface epithelial cells in normal subjects, to 20 or more per 100 epithelial cells. Hence, colonoscopy with multiple biopsies is crucial to rule out microscopic colitis and to differentiate it from IBS. Furthermore, very recent data presented in abstract form have confirm that there is no difference between IBS and controls in the number of IELs from the cecum, transverse and sigmoid colon, and both groups have less than 10 IELs×100 enterocytes.

Figure 2. Integrative model of irritable bowel syndrome (IBS) pathophysiology. IBS can develop from centrally dominant factors such as stress or luminal factors like dysbiosis triggering an altered immune response. Stress alters gastrointestinal motility mediated through the hypothalamic-pituitary-adrenal (HPA) axis, and these motility abnormalities can modify the microbiota with the subsequent immune activation in the mucosa and stimulation of nerve terminals, generating symptoms of IBS. On the other hand, dysbiosis related to gastrointestinal infections, small intestine bacterial overgrowth or antibiotics may increase the epithelial permeability leading to contact between pathogens-associated molecular patterns (PAMPs) and toll-like receptors (TLRs) in the deeper layers of the gut with the subsequent host immunity response and IBS generation or symptom exacerbation. CRF, corticotrophin-releasing factor.
confirming the last statement.

4. Immune response molecules

The gastrointestinal tract, as well as other organs lined with mucosal tissue, is the first line of defense against luminal antigens and environmental pathogens. The epithelial cells in the mucosa form a layer that avoids the paracellular transit of substances with high molecular weight, such as pathogens or antigens. Also these cells produce large amounts of mucus and secrete anti-microbial peptides which limit bacterial populations. Under physiological conditions this epithelial barrier is not completely impermeable to all bacteria, developing a tolerogenic immune response. On the other hand, bacteria or other antigens pass through the paracellular space, leading to a local and transitory pro-inflammatory cytokine production that can slightly inhibit invasion by microorganisms (Fig. 2). This response occurs rapidly and does not trigger a measurable systemic pro-inflammatory state.

1) Cytokines

Research on the immunological response in IBS has been led by the analysis of differences in cytokines expression. Cytokines are intercellular signaling proteins, mainly produced by immune cells (mast cells, macrophages, glial cells and lymphocytes in proximity with afferent primary non-adrenergic non-cholinergic nerves in the gut mucosa). Their main function is to regulate and coordinate the immunological response, but they could also play an important role in the development of motor dysfunction, visceral pain and anxiety or depression, mediated by the CNS.

Gwee et al opened a research path, when finding higher levels of IL-1β mRNA expression in IBS patients both shortly after a gastrointestinal infection and three months after the infection was cleared. Later, O'Mahony et al reported an abnormal ratio of immunoregulatory/pro-inflammatory IL-10/IL-12 cytokines in non-stimulated peripheral blood mononuclear cells from patients with IBS, indicating an inflammatory state in these patients. In addition, the authors related the normalization of this cytokine ratio and the significant symptomatic relief to the use of a probiotic, the *Bifidobacterium infantis* 35624. Recently, Liebregts et al reported a markedly higher basal and lipopolysaccharide stimulated levels of pro-inflammatory cytokines (IL-1β, TNF-α and IL-6) released from peripheral blood mononuclear cells in patients with IBS. Moreover, the authors suggested that pro-inflammatory cytokines can partly explain symptom manifestations. More recently, O'Mahony et al evidenced a higher IFN-γ and TNF-α production from whole blood stimulated with lipopolysaccharide in an animal model relevant for IBS (rat maternal separation) compared to non-stressed rats. Also, Clarke et al using the same rat model, showed correlations between the IL-6 and the polyunsaturated fatty acid profile (negative correlations with omega-3 total levels, and positive correlations with the omega-6/omega-3 ratio), suggesting a phenotype that is primed for immune activation. Also, several other pro-inflammatory (IL-2, IL-12, IL-17 and IL-18) and anti-inflammatory (IL-4 and IL-13) cytokines have been shown to be present in a lesser extent in IBS.

2) Role of cytokines

The specific roles played by cytokines in IBS patients are not fully established. Microarray studies have supported the hypothesis of an immune activation, as they have shown a reduced expression of immunoregulatory cytokines together with an over expression of the pro-inflammatory ones. However, the cytokines are able to start several processes in different cellular types. For example, in vitro studies have demonstrated neuronal activation, probably mediated by glial cells, based on the fact these cells have a high density of receptors for cytokines and chemokines. The above suggests neuronal excitation mediated by intermediate cellular populations (glial cells). When stimulated by cytokines, the glial and endothelial cells locally release nitric oxide, arachidonic acid, prostaglandins, oxygen reactive species and excitatory aminoacids (mainly glutamate), which could modify the neuronal activity. Also, glial cells can produce chemokines which in turn can induce attraction of immunological cells, amplifying the inflammatory signals.

3) Chemokines

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5. Toll-like receptors

The innate immune system is the first line of defense and is promptly activated against pathogens or tissue damage, after the recognition of several pathogens-associated molecular patterns (PAMPs) or danger associated molecular patterns (DAMPs).
The most studied receptors for PAMPs and DAMPs are the TLRs, which have a significantly specific response in the innate immunity. Phylegenetic analysis classifies the TLRs in three main branches, which seem to correspond with the recognized ligands: (1) TLRs 1, 2, 4, 6 and 10 are involved with lipids recognition, (2) TLRs 5 and 11 recognize proteins and (3) TLRs 3, 7, 8 and 9 are sensitive to nucleic acids. TLRs display differential expression levels through the gut mucosal layers, with a spatial and cellular specific allocation probably related to their function.

It is remarkable how changes in the integrity of the epithelial barrier or permeability dysfunction lead to contact between PAMPs and TLRs in the deeper layers of the gut and the subsequent host immunity response (Fig. 2). However, despite the fact that there is not yet a study that has related mucosal permeability with TLRs in IBS patients, research has suggested that permeability abnormalities are related to IBS generation and symptom exacerbation, especially in the PI-IBS and IBS-D subgroups. In fact, ex vivo assays have shown an augmented permeability in all IBS subgroups based on bowel habit predominance, although the mechanisms involved in these changes are unknown. Recent reports have suggested alterations in the tight junction proteins, and their physiological processes are the possible underlying mechanisms of these changes.

Despite the fact that the TLRs are deeply related with some gastrointestinal chronic inflammatory disorders such as ulcerative colitis or Crohn’s disease, available data regarding their participation in the pathophysiology of IBS is quite limited. Recently, Brint et al reported a 5-fold up-regulation of TLR4 in women with IBS compared to healthy controls, while reporting a reduced expression of TLR7 and TLR8 in the same patients.

On the other hand, McKernan et al based on a rat model for IBS, reported many differences in the TLRs expression in chronically stressed rats compared to controls. The authors used 2 different models (maternal separation and stress susceptible Wistar-Kyoto rats), showing a significantly higher expression of the TLR3, 4 and 5 on both, while the Wistar-Kyoto which are genetically susceptible to stress, also had a significant over expression in the TLRs 7, 8 and 9 compared to the Sprague-Dawley which are not genetically susceptible to stress. The reasons involved in these differences are not clear; however, alterations in the gut microbiota could be related.

More recently, Villani et al evidenced the complex genetic scenario present in the gut mucosa of IBS patients. In this key study, the presence of genetic variants involved with TLR9, IL-6 and intestinal permeability E-cadherin, were found to be independent risk factors for developing IBS following an episode of acute gastroenteritis.

6. Microbiota interactions

The role of TLRs in the pathophysiology of IBS must be linked to the extremely taxonomically complex and ecologically dynamic community inside the gut, which comprises around 1,000 different species, the microbiota and the fine immune response, developing a tolerogenic reaction towards these commensal microorganisms in addition to the strongly combative response against the pathogenic agents. An abnormal or unbalanced response is currently considered a relevant issue in explaining the mechanisms underlying IBD. Although the relationship between the gut mucosal response and microbiota has not been completely elucidated in IBS, microbiota alterations due to the use of systemic antibiotics, small intestine bacterial overgrowth (SIBO) or acute gastroenteritis, are extensively considered risk factors for the development of IBS. In contrast, the use of some probiotics has proven to be effective in improving symptoms of IBS.

Despite the complexity of the gut flora ecosystem, some commensal microorganisms have been significantly related to IBS, including increased Clostridium perfringens (or more diverse Clostridium spp. in general) or decreased Bifidobacteria. Similarly, Codling et al reported higher gut microbial instability and diversity in controls compared to patients. In addition to the related immune mechanisms, very recently Tana et al also introduced the concept of the association of organic acids produced by bacterial fermentation derived from higher levels of Veillonella and Lactobacillus spp. with IBS. In summary, the available data has suggested the presence of a dysbiotic microbiota, especially in patients with IBS-D, even though the overall structure is similar between patients and healthy controls.

Finally, Collins et al have proposed that contrary to what occurs in normal subjects following a gastrointestinal infection or after the use of antibiotics, where the changes of microbiota are temporary and completely restored, in patients with IBS a vicious cycle is developed, where intestinal physiological changes, could lead to modifications of the bacterial diversity and richness. In addition, modifications in the microbiota, may trigger changes in the motor function and gut sensitivity, and perhaps produce a positive feedback sustaining the motility abnormalities and visceral hypersensitivity (Fig. 2).
Conclusion

In recent years there has been an increase in the knowledge of the pathophysiological mechanisms related to IBS. However, the evidence obtained from other chronic pain syndromes, where biological, psychological and cultural factors interact in a multifactorial fashion similar to what occurs in IBS, suggests that the data from research studies must be used with caution.98 Several elements are involved in this complex disorder, including an unbalanced immune response, dysbiosis or abnormalities in the intestinal permeability, all of them interacting to develop IBS. We must highlight that the mechanisms underlying the microbiota and host interaction warrant further research to elucidate the communication processes between the immune and the nervous system. We believe that the TLRs and their subsequent cellular activation and production of messenger molecules in IBS need to be investigated. In addition, the single contribution of each TLR and the interaction between 2 or more of these receptors to induce cellular activation and the mechanisms by which this event can affect the pathophysiology of IBS, also deserves future research.

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