

Update on biologic pathways in inflammatory bowel disease and their therapeutic relevance

Matthew J. Hamilton · Scott B. Snapper ·
Richard S. Blumberg

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Abstract Results of recent genetic and immunologic studies have brought to the forefront several biologic pathways that allow for a better understanding of the mechanisms of tissue homeostasis, on the one hand, and inflammatory bowel disease (IBD) on the other. The explosion of research activity as a result of these newly identified targets is bringing the pathogenesis of these complex disorders into focus as well as creating new therapeutic opportunities. The greatest advances with perhaps the largest impact on our understanding of the etiology of Crohn's disease are those related to bacterial sensing, such as through nucleotide-binding oligomerization domain-containing protein 2 (NOD2) and its relationships to autophagy and the unfolded protein response as a consequence of endoplasmic reticulum stress. Interestingly, it appears as though these pathways, which are rooted in microbial sensing and regulation, are interrelated. Genetic studies have also renewed interest in previously studied pathways in IBD, such as the formation and function of the inflammasome and its relationship to interleukin (IL) 1-beta signaling. With the recent success of therapeutic agents designed to block tumor necrosis factor, the IL-12/23 pathways, and lymphocyte homing, insights have been gained into the biologic relevance and impact of these various inflammatory pathways in IBD. In this review, the exciting recent advances in these biologic pathways of IBD are discussed, particularly in light of their therapeutic relevance.

Keywords Bacterial sensing · Intestinal homeostasis · Inflammatory bowel disease · Crohn's disease · Autophagy · Ulcerative colitis

Introduction

Many recent advances have been made in our understanding of the pathogenesis of inflammatory bowel disease (IBD). While the clinical phenotypes have been well characterized, the molecular basis behind this group of disorders is now coming into focus. In this review, we provide insights into some of the biologic pathways that have been the source of the most recent discoveries in IBD research and their relationship to the development of new therapeutic approaches to disease. Pathways for bacterial sensing and response, inflammasome regulation, inflammatory cytokines, and lymphocyte homing and recirculation will be discussed. In this article we do not extensively cover recent advances in genetics and the intestinal microbiota nor provide a comprehensive discussion of all of the immunologic advances which have been recently reviewed elsewhere [1–3].

Pathways of bacterial sensing and response

Genetic studies have brought nucleotide-binding oligomerization domain-containing protein 2 (NOD2), autophagy, and the endoplasmic reticulum (ER) stress responses to the forefront of IBD research. NOD2, which is expressed on macrophages, dendritic cells, and intestinal epithelial cells (IECs), is an intracellular sensor of bacterial peptidoglycan as well as of ligands from mycobacteria and viruses whose activation leads to NF κ B induction and the

M. J. Hamilton (✉) · S. B. Snapper · R. S. Blumberg
Division of Gastroenterology, Brigham and Women's Hospital,
Harvard Medical School, Boston, MA, USA
e-mail: mjhamilton@partners.org

S. B. Snapper
Division of Gastroenterology, Children's Hospital of Boston,
Harvard Medical School, Boston, MA, USA

release of inflammatory cytokines and chemokines. The common genetic variants of NOD2 found in patients with Crohn's disease (CD) constitute the greatest known susceptibility to CD and abrogate this signaling [4]. While a complete understanding of how NOD2 leads to disease pathogenesis is not fully understood, it is known that NOD2 may also modulate Toll-like receptor (TLR) signaling [5–7] and affect the expression of α -defensins in Paneth cells [8, 9]. This decreased expression of defensins renders the host susceptible to model pathogens, as has been shown for *Listeria monocytogenes* [8].

The discovery that functional polymorphisms associated with *ATG16L1* are also associated with CD [10] introduced the concept that autophagy also contributes to disease pathogenesis. This is a well-conserved regulatory process by which protein and organelle turnover occurs in cells by autodigestion through lysosomal degradation [11]. In periods such as starvation, oxidative stress, or infection, autophagy is increased. In studies of mice where the *ATG16L1* gene is absent, autophagy of cell contents is impaired by a failure to assemble the proper destructive machinery [12]. The defense mechanism against invading *Salmonella typhimurium* is also rendered inadequate by dysfunctional autophagosomes in cultured human epithelial cells expressing the common variant of *ATG16L1* found in CD [13]. Dysfunctional autophagy leads to phenotypic and transcriptional defects in Paneth cells, further suggesting that these secretory IECs may be particularly vulnerable to genetic mutations that affect these pathways and contribute to IBD.

Genetic models have led to a third novel pathway in IBD which is the unfolded protein response (UPR). The UPR is induced by ER stress and caused by the accumulation of misfolded or unfolded proteins within the ER [14]. The UPR is controlled by three distinct pathways, among which the inositol-requiring enzyme (IRE)-X-box binding protein 1 (XBP1) pathway has been the most studied in CD and ulcerative colitis (UC). Deletion of XBP1 in intestinal epithelial cells leads to spontaneous enteritis, sensitivity to colitis induction by dextran sodium sulfate (DSS), increased ER stress in the IEC compartment, apoptosis of Paneth cells and reduction in the number of goblet cells, and finally impaired defense to *Listeria monocytogenes* [15]. Analysis of single nucleotide polymorphisms (SNPs) in the *XBP1* locus has demonstrated an association between *XBP1* and both UC and CD. Deep sequencing of the *XBP1* gene in a large cohort of CD, UC and control patients have further shown that the association signal is likely derived from hypomorphic rare variants within the coding region of the gene [15]. This suggests that genetic risk factors within the proper environmental context may lead to spontaneous intestinal inflammation which emerges directly from abnormalities in IECs.

These pathways highlight the common importance for appropriate sensing and responding to commensal bacteria in the maintenance of homeostasis and the avoidance of unrestrained intestinal inflammation. Moreover, recent evidence further suggests that these aforementioned pathways may be convergent (Fig. 1). NOD2 and autophagy are linked through the recognition and clearance of invading intracellular bacterial pathogens. Epithelial cells that express the most common mutation found in *NOD2* in association with CD display a profound defect in the autophagic destruction of intracellular bacteria [16]. Activation of NOD2 caused by these invading microbes appears to direct ATG16L1 to the plasma membrane adjacent to the point of bacterial entry in order to coordinate the clearance of the organism. In dendritic cells harboring either *NOD2* or *ATG16L1* CD susceptibility mutations, intracellular bacterial clearance was found to be similarly hampered by defective autophagy, which ultimately affected antigen presentation to CD4⁺ T cells [17].

Autophagy is also linked to UPR and ER stress. ER stress induced in various cancer line cells results in the appearance of autophagosomes and autophagy machinery that are not observed when a c-Jun N-terminal kinase (JNK), downstream of the IRE1–XBP1 pathway, or IRE1 are absent [18, 19]. Evidence also exists to support the concept that impaired autophagy may lead to ER stress. This is the case in animal models of obesity that display decreased levels of autophagy proteins and henceforth have increased ER stress [20]. How precisely autophagy affects ER stress and vice versa in IECs needs to be established in intestinal model systems.

It must be pointed out that IECs are particularly susceptible to defects in autophagy and ER stress. While the exact reasons for this are unknown, it appears as though more complex requirements exist at this interface between the intestinal microbiota and the mucosal compartment. For example, IECs express an additional isoform of IRE1, called IRE1 β , that may act to further protect IECs from ER stress in the setting of inflammation [21]. One can speculate therefore that the IECs have evolved to protect the host from bacteria or perhaps allow the host to be capable of adapting to local changes in the luminal environment as they are required.

There is further evidence that the bacterial sensing and regulation pathways that affect CD are linked to a common biologic pathway. The animal studies described above that result in hypomorphic NOD2, ATG16L1, and XBP1 function impair IEC Paneth cell function, resulting in deficient α -defensin production and impaired anti-microbial responses. Also gleaned from these studies is the notion that IECs and macrophages with hypomorphic NOD2, ATG16L1, and XBP1 function exhibit an excessive inflammatory response [e.g., tumor necrosis factor-alpha

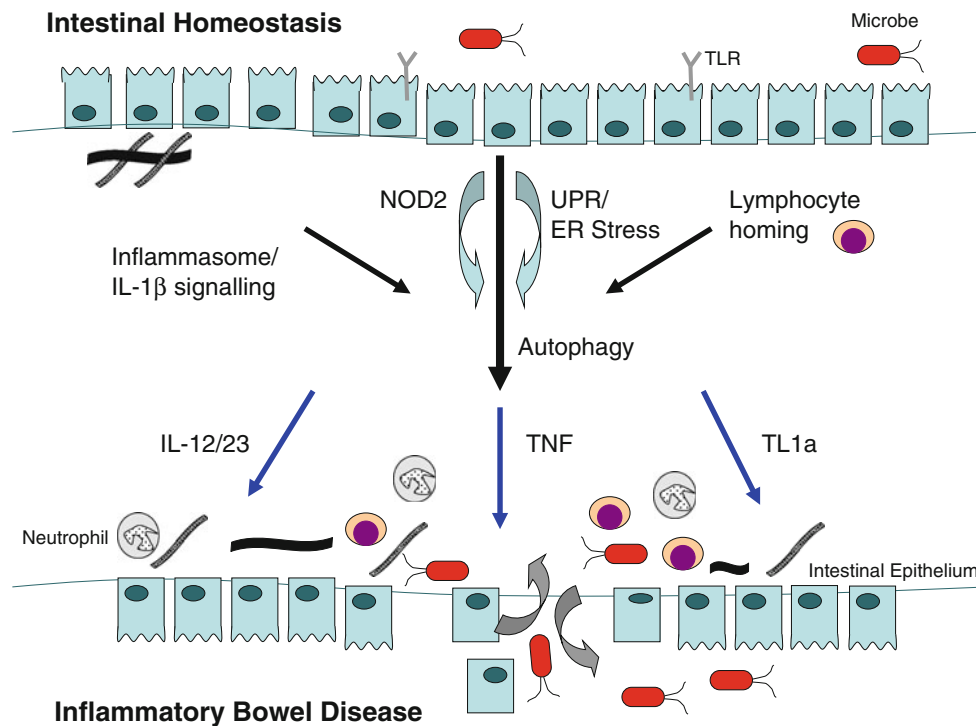


Fig. 1 Pathways that lead from intestinal homeostasis to inflammatory bowel disease. Homeostasis in the gut is altered by perturbations in the sensing of microbes by intestinal epithelia and subjacent immune cells by a variety of pattern recognition receptor systems, such as nucleotide-binding oligomerization domain-containing protein 2 (*NOD2*) and Toll-like receptors (*TLR*). This leads to cellular responses that place stress on immune and parenchymal cells, leading

(*TNF- α*) production] in response to microbial products. Similarly, myeloid cells expressing the CD variants for *NOD2* or lacking *ATG16L1* respond to bacterial stimuli with an increased inflammasome response. Therefore, the consequence of genetic abnormalities in these biologic pathways may lead to inappropriate stimulation of antimicrobial and inflammasome systems that in turn perpetuate a chronic inflammatory environment. These defects have significant consequences for the relationship between the host and commensal microbiota and likely explain many previous findings in this area. For example, CD patients have been shown to harbor increased bacteria within IECs [22], including adherent-invasive *Escherichia coli* [23, 24]. Interestingly, this bacterium was found to be increased in autophagosomes of *ATG16L1*-deficient cells [25]. Furthermore, alterations in these pathways may result in overall changes to the composition of the microbiota. With the development of improved molecular techniques, including 16 rRNA sequencing and metagenomic sequencing, it is now possible to characterize complex microbiomes [26]. Such studies have produced evidence indicating that the intestinal microbiomes of patients with IBD may be substantially altered. Subgroups of patients with IBD appear to contain fewer overall bacterial genes,

to adaptive unfolded protein responses (*UPR*) and autophagy. When maladapted, these pathways can lead to the inappropriate activation of inflammatory pathways (e.g. inflammasomes) and excessive release of inflammatory signals [e.g., interleukin (*IL*)-12/23, tumor necrosis factor (*TNF*), *IL-1 β* , and *TNF*-like factor *TL1A*]. *ER* Endoplasmic reticulum

particularly those in the “protective” *Firmicutes* and *Bacteroidetes* phyla [27]. Whether these changes in the microflora cause inflammation or are a consequence of inflammation has not yet been determined, and current knowledge may suggest that the etiology of inflammation lies somewhere in between.

Lymphocyte homing and recirculation

Recent studies from experimental animal models of IBD, genome-wide association studies (GWAS), and success in the treatment of patients with CD with therapeutic antibodies to $\alpha 4$ -integrins have brought forward the importance of homing pathways which coordinate the movement of lymphocytes into the site of intestinal inflammation. A meta-analysis of GWAS data in patients with CD [28] has identified an association between disease and *CCR6*. Chemokine receptor and ligand interactions are important for the process of migration of lymphocytes from the vasculature to the tissue microenvironment in inflammation. Chemokine receptor 6 (*CCR6*) is expressed on Th17 cells and interacts with its ligand *CCL20* on IECs. *CCL20* expression is increased in inflamed intestinal segments of

patients with IBD [29], and the Th17 cells in *Ccr6*^{-/-} mice exhibit abnormal migration patterns. These mice consequently develop less severe DSS-induced colitis [30]. However, it is important to note that these mice are also more susceptible to trinitrobenzene sulfonic acid (TBS)-induced colitis, suggesting different roles of this regulatory chemokine pathway in different models of intestinal inflammation. These seemingly disparate results in two models of experimental colitis also underscore the potential for expression of this chemokine receptor and ligand on different inflammatory cells in different tissue compartments which carry out different functions. In this regard, CCR6 is also expressed on Tregs [31]. Nonetheless, with the success of a blocking antibody directed against CCL20 [32] and with the results of a GWAS implicating the *CCR6* locus in CD, it is conceivable that pharmacologic blockade of this pathway may be a therapeutic possibility in select patients.

With this in mind, natalizumab is being increasingly used to treat patients with moderate to severe CD based on a large clinical trial that showed therapeutic responses in a select group of patients [33]. This drug is a fully humanized monoclonal antibody directed towards the $\alpha 4$ integrin subunit contained in the mucosal integrin $\alpha 4\beta 7$. Integrins are a group of heterogeneous transmembrane molecules expressed on multiple surfaces, including leukocytes where they serve as adhesion molecules for endothelium. They are composed of an alpha and beta subunit and the $\alpha 4\beta 7$ integrin homes leukocytes (except for neutrophils) to inflamed tissue compartments of the gut. The natalizumab pharmacologic approach may be problematic due to unintended blockade of integrin-driven leukocytes homing to tissues outside of the gut, such as the brain. Although clinical trials show this agent to be efficacious in the treatment of multiple sclerosis through the blockade of $\alpha 4\beta 1$ on brain-homing lymphocytes, this may result in progressive multifocal leukoencephalopathy, polyoma virus infection [34]. This infection occurs in 1:1000 patients receiving natalizumab, including those receiving this drug for CD. Therefore, clinical trials are underway to investigate gut-specific antibodies targeting components of this integrin pathway ($\alpha 4\beta 7$, MADCAM1, the endothelial ligand for $\alpha 4\beta 7$, and the $\beta 7$ subunit). It is also possible that blockade of lymphocyte homing may result in an undesired deficiency of regulatory cells, such as Tregs; therefore, more specific targets aimed at integrins expressed on pro-inflammatory immune cells are most desired.

While the process of homing lymphocytes to sites of inflammation has been extensively studied, a relatively new area of investigation in IBD is the process of resolving inflammation. It appears as though this is a coordinated and active process in order to prevent excessive and collateral damage, to provide defense against pathogens that may

take advantage of this environment, and to rid the tissue of unwanted inflammatory cells, necrotic cells, and other debris. The family of lipid-derived signaling molecules, including the lipoxins, resolvins, and protectins, play an increasingly recognized role in this process, and their functions have been recently reviewed [35].

In this light, it is important to highlight an interesting pathway associated with the resolution of intestinal inflammation that is linked to an environmental factor. Short chain fatty acids (SCFAs) are derived from the microbial fermentation of dietary fibers and bind to G-coupled receptor 43 (GPR43) that shows enhanced activity on certain immune cells, such as neutrophils, and has been linked to receptors associated with the innate immune response [36]. Interestingly, *Gpr43*^{-/-} mice exhibit a markedly delayed recovery to DSS-induced colitis due to a lack of response of their neutrophils to SCFAs, which normally induce the migration of neutrophils out of the inflamed tissues. This mechanism is highly similar to that of resolvins, which utilize an orthologous receptor, chem23, which is structurally similar to GPR43. When SCFAs are cultured with isolated human peripheral blood mononuclear cells, there is an increased production of anti-inflammatory prostaglandin-E2 and a decreased expression of lipopolysaccharide (LPS)-induced inflammatory cytokines [37]. From a clinical perspective, SCFAs in the form of *n*-butyrate have been found to be increased in the stools of patients with IBD, suggesting a possible inadequate response in the gut [38]. In another study, a 4-week treatment with germinated barley foodstuff was found to reduce clinical activity scores compared with placebo and to cause an increase of beneficial bacteria in the colon such as *Bifidobacterium* [39].

Inflammasome regulation

Inflammasomes are composed of an intracellular complex of proteins that assemble in myeloid cells and play an important role in the innate immune system through recognition of damage-associated molecular patterns. Activation of the inflammasome ultimately results in the formation of cytokines, such as interleukin (IL)-1 β , IL-18, and IL-33, to carry out various inflammatory and regulatory immune functions [40]. In general, these proteins are made as nonfunctional pro-proteins that must be cleaved by caspase-1 derived from the inflammasome to become active. In IBD, perturbation of the intestinal epithelial barrier might activate and potentiate the action of the inflammasomes that are poised to recognize pathogens and other “warning signals”. NOD-like receptor family pyrin containing 3 (NLRP3) encodes cryopyrin which activates caspase-1 to ultimately coordinate IL-1 β production.

Gain-of-function mutations in the nucleotide–oligomerization binding domain (NOD) component of NLRP3 results in the overproduction of IL-1 β and consequently rare fever-associated inflammatory diseases, including familial cold autoinflammatory syndrome, Muckle–Wells syndrome, and neonatal-onset multisystem inflammatory disease [41]. Interestingly, a recent candidate gene approach study identified *NLRP3* as a susceptibility factor for CD [42], resulting in yet more interest being directed towards the inflammome in IBD. In contrast to the three rare febrile diseases just mentioned, LPS-stimulated monocytes harboring CD susceptibility SNPs in *NLRP3* show a decreased production of IL-1 β . This is analogous to susceptibility mutations in NOD2 that result in a loss of function and decreased IL-1 β . A recent study examined the inflammasome function composed of another NOD-like receptor family pyrin member, NLRP6. In this study, *Nlrp6*^{-/-} mice harbored decreased levels of IL-18 in intestinal tissues and developed a dysbiosis with an accumulation of *Bacteroidetes* sp. that could be transmissible to wild-type mice and that conferred increased susceptibility to DSS-induced colitis [43].

These studies show how the loss of inflammasome function and, consequently, the production of cytokines, such as IL-1 β , under homeostatic conditions result in a disabled relationship between the epithelial barrier and the commensal microbiota. This accumulated data also raise the possibility that defective processing of IL-1 β contributes to the initiation of IBD. Alternatively, IBD is a chronic process, which could suggest that continued and inappropriate activation of caspase-1 and subsequent production of active IL-1 β could perpetuate IBD. There is evidence gleaned from the study of intestinal specimens of patients with IBD that indeed IL-1 β and IL-18 may be elevated in patients with certain forms of IBD [44–46]. Furthermore, animal studies of experimental colitis where IL-1 [47] or IL-18 [48] are blocked result in attenuated disease. Although it may be difficult to reconcile the different functions of the inflammasome pathway in IBD, several balancing factors are likely at play in disease pathogenesis during homeostasis versus chronic inflammation.

Inflammatory cytokine pathways

The success of antibodies directed towards TNF in the treatment of moderate to severe forms of CD and UC clearly identifies the centrality of this cytokine in IBD pathogenesis. Despite this knowledge, the mechanisms by which TNF may act in IBD have not been clearly determined, and genetic studies have not definitively implicated defects in TNF as conferring risk for these diseases. It may therefore be inferred that TNF has broad reaching roles in IBD pathogenesis or that it represents the final event in

converging pathways (Fig. 1). Nevertheless, it may be important to study mechanisms of TNF activity based on what is understood from clinical practice using anti-TNF agents and from the study of the TNFA ARE (AU-rich element in the 3' untranslated region that is important in TNF expression) mice that develop a spontaneous CD-like transmural ileo-colonic inflammation and ulceration with granulomas [49, 50]. For example, a TNF receptor, TNFR1, is expressed on the mesenchymal cells adjacent to IECs and may regulate mediators in parenchymal destruction, such as matrix metalloproteinases [50]. As such, this ability of the anti-TNF blockade to target innate pathways which might be shared between CD and UC may explain why TNF blockade as a therapy may succeed in not only CD but also in the more superficial inflammation of UC. Another possible activity of TNF that may establish shared mechanisms in both forms of IBD is its role in modifying epithelial apoptosis [51]. An examination of tissues in patients with CD suggests that apoptosis is increased in active disease and decreased with anti-TNF treatment. Reduced apoptosis by anti-TNF agents may at least also partially explain the clinical observation that these agents function to “heal the mucosa” [52].

Other TNF family members have been implicated in IBD, including TNF ligand-related molecule 1A (TL1A), which is encoded by *TNFSF15*. Interestingly, polymorphisms in this gene confer varying levels of risk in diverse ethnic populations, with Japanese and Korean populations being the most susceptible [53, 54]. TL1A is a TNF-like cytokine that is released by antigen-presenting cells and T cells and requires innate immune stimuli, such as TLR signaling. TL1A acts on T cells through the death receptor-3 (DR3) to ultimately help to induce proliferation of both Th1 and Th17 cells in an inflammatory milieu [55]. TL1A antibodies are effective in dampening colonic inflammation in DSS-treated mice and spontaneous intestinal inflammation in SAMP1/yit mice [56, 57]. Therefore, it is conceivable that blockade of TL1A may have therapeutic benefit in both forms of IBD in Asian populations.

The IL-12/IL-23 pathway is also of interest as it has been linked to CD and UC after the first GWAS detected polymorphisms in *IL23R* that conferred protection from disease [28]. Several drugs manufactured to block the components of this pathway are currently in clinical trials or in current practice so that an understanding of the biology in disease pathogenesis is important. IL-12 and IL-23 comprise subunit dimers-p35 for IL-12 and p19 for IL-23, as well as a p40 subunit that is common to both. These cytokines are produced by dendritic cells and function largely to induce and maintain Th1 (for IL-12) and Th17 (for IL-23) responses [58]. Blockade of IL-12 in TBS-induced colitis leads to amelioration of inflammation, thereby demonstrating the importance of this pathway in IBD [59]. The

role of IL-23 has been studied in a CD-like animal model of colitis (Rag2-deficient animals treated with anti-CD40 antibody) that display an exuberant production of Th1 cytokines, such as TNF and interferon alpha (INF α) as well as IL-12p40. In one study, blocking antibodies to the various subunits of IL-12 and IL-23 were used in a systematic manner in order to understand the roles of each component of the IL-12/23 pathway in intestinal inflammation [60]. A key finding to come out of all these studies was that the p19 subunit that is unique to IL-23 is necessary for colitis in this experimental model. Furthermore, Th17 expression was found to be markedly increased and dependent on IL-23. These results from animal studies indicate that inflammatory signals derived from innate antigen-presenting cells (e.g., dendritic cells) drive the chronic inflammation seen in IBD and that Th17 via IL-23 may be an important mediator in this process. The role of the Th17 pathway in IBD has been recently reviewed [55].

Based upon these studies and the efficacy of ustekinumab (humanized monoclonal antibody directed at the IL-12/IL-23-shared p40 subunit) in patients with psoriasis [61, 62], it has been predicted that this drug would have similar efficacy in patients with CD. Although remission rates in phase II studies did not demonstrate significant differences compared with placebo through blockade of IL-12p40 [63], there are several other drugs designed to block the components of the IL-12/IL-23 pathway currently in clinical development.

One novel inflammatory cytokine pathway that may play an important role in the pathogenesis of UC is the Th2 cytokine IL-13. A chronic model of oxalozone-induced colitis, which phenotypically resembles UC, showed that lamina propria natural killer T (NKT) cells that are restricted to CD1d produce increased IL-13 that is necessary for the development of inflammation and can attenuate the colitis if blocked [64]. In human studies, stimulated lamina propria T cells from patients with UC produce increased amounts of IL-13 compared to the T cells from patients with CD that produce small amounts [65]. This cytokine was subsequently found to augment the NKT cell-mediated cytotoxicity of HT-29 epithelial cells after LPS stimulation. Furthermore, IL-13 directly alters the electrical resistance of these cultured epithelial monolayers, in part due to increased apoptosis and changes in tight junction proteins, such as claudins [66]. Taken together, these data suggest that IL-13 derived from NKT cells works at the level of the superficial mucosal compartment to induce and/or potentiate chronic colitis observed in patients with UC [66]. In a recent proof-of-concept clinical study on the use of INF- β 1a therapy in patients with UC, a positive response to this agent correlated with decreased levels of IL-13 from lamina propria T cells compared to those observed in non-responders [67].

Conclusions

Our understanding of the pathogenesis of IBD has been significantly advanced by the discovery of genes and their immunologic pathways that confer risk in IBD. It has become clear that biologic pathways, such as those rooted in microbial sensing, are central to both the initiation and perpetuation of IBD. Many of these pathways are linked in a manner consistent with the complex interplay of often redundant systems that ultimately control homeostasis in the gut. In any pathway, perturbations at the level of the genes, environment (e.g., microbes), and/or altered immune responses disrupts homeostasis and ultimately determines the phenotype of the patient with IBD (Fig. 1). The most effective therapeutic option will target a molecule that represents the “final common pathway” for IBD, whatever that may be and which may vary among patients, highlighting the syndromic nature of these complex disorders.

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Conflict of interest None.

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