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Use of animal models in elucidating disease pathogenesis in IBD

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Abstract

Inflammatory bowel diseases (IBD) are a collection of diseases characterized by chronic gastrointestinal inflammation resulting from an exuberant immune response to commensal flora in genetically susceptible individuals. Rapid advances in the field of genomics have resulted in the identification of at least 163 loci that contribute susceptibility to both Crohn's disease (CD) and ulcerative colitis (UC). Similar to other complex diseases, however, the "curse of missing heritability" remains a significant concern in understanding the mechanisms underlying IBD. While genetic discoveries, to date, only account for 7–14 % of disease variance for IBD, studies have increasingly demonstrated a role for environmental factors in disease pathogenesis. Furthermore, the use of animal models of IBD has led to a greater understanding of disease pathogenesis implicating various aspects of the innate immune response including the bacterial, fungal, and viral microbiome and adaptive immune response such as the interleukin (IL)-23/IL-17 pathway.

Keywords

Crohn's disease; Ulcerative colitis; Microbiome; Innate immunity; Adaptive immunity

Introduction

Inflammatory bowel diseases (IBD) are a collection of diseases characterized by chronic gastrointestinal inflammation resulting from an exuberant immune response to commensal flora in genetically susceptible individuals. Rapid advances in the field of genomics have resulted in the identification of at least 163 loci that contribute susceptibility to both Crohn's disease (CD) and ulcerative colitis (UC) [1]. Similar to other complex diseases, however, the "curse of missing heritability" remains a significant concern in understanding the mechanisms underlying IBD. While genetic discoveries, to date, only account for 7–14 % of disease variance for IBD [1], studies have increasingly demonstrated a role for environmental factors in disease pathogenesis. Furthermore, the use of animal models of IBD has led to a greater understanding of disease pathogenesis implicating various aspects

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of the innate immune response including the bacterial, fungal, and viral microbiome and adaptive immune response such as the interleukin (IL)-23/IL-17 pathway.

Commensal bacteria

Commensal gut microbiota provides various benefits including modulation of gastrointestinal development, maintenance of immune homeostasis, and enhanced metabolic capabilities [2, 3]. It is predicted that the human gut contains up to 10^{14} bacteria, which contribute 100–150 times more genes than the human genome. Deep metagenomic sequencing helped to identify a "common core" microbiome, dominated by *Bacteroidetes* and *Firmicutes*, although it is well recognized that microbial composition is dependent on a host's physiological state [4–7].

Ribosomal RNA (rRNA) sequencing of surgical tissue samples obtained from CD, UC, and non-IBD subjects demonstrated the presence of four main bacterial phyla in the gut (*Actinobacteria*, *Proteobacteria*, *Bacteroidetes*, and *Firmicutes*), independent of disease status or gastrointestinal location [8]. While differing in the relative proportions of specific bacterial species, both small intestinal and colonic microbiota consisted predominantly of *Bacteroidetes* and *Firmicutes*. Differences in microbial composition are observed with disease status. Frank et al. [8] utilized hierarchal principal components analyses to separate study samples into two main groups and identified one major subset consisting of controls, CD, and UC samples and another smaller "IBD subset" which demonstrated a significant depletion of commensal bacteria including *Bacteroidetes* and *Firmicutes* and an enrichment in various *Actinobacteria* and *Proteobacteria*. This suggests that IBD and control specimens may have distinct microbial compositions, a finding also observed in IBD patients in clinical remission [9]. Quantitative PCR identified an overall tenfold decrease in total bacterial load in this IBD subset, which correlated with a higher occurrence of abscesses (for CD) and a younger age at time of surgery (for CD and UC) [8]. While other factors such as diet, disease history, and genetics were not examined, these observations are suggestive of a distinct microbial profiles associated with loss of commensal bacteria and disease severity.

In a comprehensive study, Gevers et al. [10] performed microbiome profiling of terminal ileum, rectal, and stool samples from 668 treatment naïve, new onset pediatric CD, and non-IBD controls. Concurrent with previous reports, decreased levels of *Bacteroidetes* and *Firmicutes Clostridiales* and increased levels of *Proteobacteria Enterobacteriaceae* were observed in CD. The authors identified an abundance of *Fusobacteriaceae*, *Proteobacteria Pasteurellaceae*, and *Neisseriaceae*, with strong association observed for the latter two *Proteobacteria* and earlier age (<10 years). These various bacteria can aggravate inflammation, invade intestinal epithelial cells, and adapt to survive under oxidative stress environments. Dysbiosis was predominantly observed in microbial profiles of mucosal tissue samples in comparison to fecal samples, stressing the importance of not only sample size but sample origin as well. CD-associated microbial functional changes included a loss of basic biosynthesis and increased oxidative stress and auxotrophy in mucosal tissue. Metabolic pathway analyses revealed CD-enrichment for bacterial species associated with glycan biosynthesis/metabolism, and CD-depletion of species associated with energy metabolism as well as amino acid, carbohydrate, and nucleotide metabolism. Utilizing these

microbial profiles and clinical variables, Gevers et al. [10] developed a model predicting disease severity over 6 months, with age of onset and *Enterobacteriaceae* levels negatively associated with future disease activity, and positive association observed with disease activity at time of diagnosis, and levels of *Fusobacterium* and *Proteobacteria Haemophilus*.

Other studies investigated whether specific IBD-associated genetic variants underlie observed changes in intestinal microbial composition. Genetic studies repeatedly identified both common and rare variants in *nod2* to be associated with CD susceptibility [1, 11–16]. Nucleotide-binding oligomerization domain-containing (NOD) receptors are intracellular pattern recognition receptors that recognize specific bacterial peptidoglycans and initiate an inflammatory response [17]. Additionally, NOD2 recruits autophagy protein ATG16L1 to the plasma membrane at the site of bacterial entry, forming a protein complex essential for autophagosome formation and bacterial clearance [18, 19]. NOD2-induced autophagy facilitates MHC class II processing and antigen presentation in dendritic cells [19]. Furthermore, NOD2 is established to be an important regulator of commensal gut microflora in mice and its expression is dependent on commensal microflora, suggesting that interplay between *nod2* and commensal bacteria may regulate intestinal homeostasis [20].

To investigate the role of *nod2* and microbiome, IBD patients and healthy controls with bacterial rRNA sequencing data were genotyped for the three common CD-associated NOD2 variants (R702W, G908R, and Leu1007fsInsC), as well as the T300A ATG16L1 variant. CD subjects demonstrated significant differences in the four bacterial phyla, with decreased frequencies of *Firmicutes Clostridium* (groups IV and XIVa) and increased frequencies of *Actinobacteria* and *Proteobacteria*, in comparison to controls [21]. Multivariate analysis identified ileal involvement, *atg16l1* genotype, *nod2* composite genotype, and *nod2*/age-at-surgery interaction to be associated with overall microbial composition changes. An allele dosage effect on intestinal microbiota of IBD patients was demonstrated for *atg16l1* and *nod2* [21], further indicating that specific disease-associated genetic variants may influence the microbiome and play an important role in IBD pathogenesis and/or disease heterogeneity. Follow-up studies with disease-unaffected ileal biopsies from CD, UC, and control patients demonstrated IBD phenotype, *nod2* genotype, and *Clostridium difficile* to be associated with shifts in overall bacterial composition [22]. Such alterations in microbial composition may, in part, help to explain increased susceptibility to *C. difficile* observed in IBD patients [23, 24].

Although it is well-established that IBD is associated with decreased *Bacteroidetes* and *Firmicutes Clostridia*, some reports demonstrated increased *Bacteroidetes* and *Firmicutes* load in non-inflamed ileal biopsies taken from CD patients homozygous for the NOD2 Leu1007fsInsC frameshift mutation, as well as in terminal ileum of *nod2*-deficient mice [25]. While the specific bacterial loads reported may vary due to the presence of genetic factors, disease natural history, biopsy location, sample type, environmental effects, or varying medical therapies, it is nevertheless well-recognized that NOD2 is an integral component of the host-microbiota interaction.

In 2012, Natividad et al. [26] demonstrated that changes in microbial composition could impact the phenotype of *nod* knockout mice, further supporting the link between NOD and

microbiota. In comparison to pathogen-free (SPF) *nod1*+/−*nod2*+/− controls, SPF *nod1*- and *nod2*-deficient mice exhibited more severe dextran sodium sulfate (DSS)-induced colitis and compromised intestinal barrier dysfunction, evidenced by increased colonic permeability and decreased colonic expression of E-cadherin and RegIIIγ. Colonization of *nod*-deficient mice with altered Schaedler Flora (ASF) or treatment with probiotic *Bifidobacterium breve* resulted in increased RegIIIγ expression and decreased susceptibility to DSS-induced colitis in the presence of commensal bacteria.

Fucosyltransferase2 (*fut2*) regulates the expression of ABO antigens in saliva and gastrointestinal mucosa and approximately 20 % of Caucasians are homozygous for *fut2* W134X loss-of-function allele and do not express ABO antigens (termed “non-secretors”) [27]. Although the specific polymorphisms vary, numerous genetic variants result in non-secretor phenotypes across several populations [28]. In recent years, polymorphisms in *fut2* are associated with CD susceptibility [29], and changes in microbial composition [30–32]. Rausch et al. [30] reported significant variation in microbial composition between individuals to be dependent on CD status, as well as the interaction between disease status and *fut2* genotype. Overall, a 15 % reduction in microbial genes is demonstrated in mucosal lavage samples from the cecum and sigmoid colon of healthy heterozygotes and homozygote non-secretors, in comparison to homozygote secretors [31]. Decreased short-chain fatty acid (SCFA) producing bacteria (such as *Roseburia* and *Faecalibacterium*) and increased *Bacteroidetes* are reported in non-secretors independent of disease status [30, 31]. Distinct bacterial taxa, such as *Bacteroidetes Alistipes* and unclassified *Firmicutes Lachnospiraceae*, are observed in CD non-secretors [30]. Non-secretor status is associated with reduced bifidobacterial diversity and abundance in an analysis of fecal microbiota of 71 healthy subjects [32].

Despite these principal studies with *nod2* and *fut2*, additional investigation is required to better comprehend the consequences of specific IBD-associated genetic variants on gut microbiota and disease pathogenesis. Studies are expanding to include characterization of microbial metabolic pathways and biological processes affected in IBD. In a detailed investigation of 204 IBD subjects and 27 healthy controls, Morgan et al. [33] provided an in-depth analysis of the interplay between disease status, environmental factors, medical therapies, and the microbiome. Several factors independently associated with changes in microbial composition included sample origin (stool vs. biopsy), age, smoking, and medication/antibiotic treatment, signifying that such factors must be taken into account in future analyses of the IBD microbiota. After adjusting for confounding covariates, various bacterial clades were found to be differentially abundant in IBD (various genus-level phylotypes of *Firmicutes Clostridia* and *Firmicutes Bacilli*, and *Proteobacteria Enterobacteriaceae*). Interestingly, ileal CD demonstrated a distinct microbial composition, including reduced *Firmicutes Ruminococcaceae* family, which is involved in carbohydrate metabolism; enrichment of genera *Escherichia*/*Shigella* of the *Enterobacteriaceae* family, which produce lipopolysaccharides known to activate Toll-like receptors that can initiate an inflammatory cascade; and reduced genus *Odoribacter* of the *Bacteroidetes* phylum, which is hypothesized to affect host inflammation. A critical contribution of this study was the unique microbial metabolic profiles identified in IBD, including reduced amino acid biosynthesis and SCFA production, and increased markers of oxidative stress, bacterial

secretion, and nutrient transport. Unique metabolic changes were observed with ileal CD, including an overrepresentation of genes involved in bacterial secretion, adherens, and invasion [33].

Utilizing lavage samples collected from non-involved intestinal regions from IBD and control subjects, Tong et al. [9] identified five microbial modules representing functional microbial communities (FMC) within the intestinal microbiome. These FMCs were present across all subjects, yet with varying abundance dependent on disease status, two of which were highly preserved across two independent datasets. One FMC, most strongly associated with CD, included pathogenic bacteria such as *Enterococcus* and *Escherichia*, and enrichment of Kyoto encyclopedia of genes and genomes (KEGG) pathways including bacterial invasion of epithelial cells and pathogenic *Escherichia coli* infection. A second FMC was negatively correlated with IBD status and included the anti-inflammatory commensal *Faecalibacterium* and other SCFA producing bacteria. Recently, metagenomic analyses identified changes in energy metabolism specifically associated with FUT2 secretor status in 39 healthy subjects. Reductions in amino acid biosynthesis/metabolism, and an enrichment of glycan biosynthesis/metabolism and enriched metabolism of lipids, carbohydrates, vitamins, and cofactors are noted in non-secretors and heterozygotes [31]. These observations were consistent across both human and mouse datasets and in agreement with other reports [33]. These metagenomic variations further correlated with changes in microbial metabolic activity and proteomic changes [31]. Thus, genetic variation in IBD susceptibility loci such as *fut2* can influence microbial composition as well as microbial function.

Such studies are maiden explorations into deciphering the interplay between host genetics and the intestinal microbiome in IBD. While a significant majority of these studies focused on gut bacterial communities, reports have also highlighted a growing role for virus and fungi as critical contributors to the commensal microflora in the gut.

Viruses and IBD

The identification of CD-associated polymorphisms in *atg16l1* and *irgm* implicates an important role for autophagy in the development of CD [34–37]. Mice with reduced (hypomorphic) protein expression of ATG16L1 (ATG16L1^{HM}) exhibit decreased autophagy with distinct Paneth cell abnormalities, including irregular packaging and secretion of granules containing antimicrobial peptides and lysozyme [38]. Similar Paneth cell abnormalities were observed in ileocolonic resections from patients with CD homozygous for the *atg16l1* risk allele (T300A) [38]. ATG16L1^{HM} mice raised in an enhanced barrier facility free of pathogens did not exhibit these distinct Paneth cell anomalies suggesting an environmental contribution and subsequent experiments implicated both commensal flora and murine norovirus (MNV-CR6) in driving the development of this phenotype [39]. These observations bear a striking resemblance to abnormalities observed in CD patients homozygous for *atg16l1* risk allele [38].

Uninfected ATG16L1^{HM} and wild-type mice exhibited comparable DSS-induced intestinal injury. However, MNV-CR6-infected, DSS-treated ATG16L1^{HM} mice further displayed intestinal fibrosis, inflammation, and ileal mucosal atrophy similar to CD. These anomalies

were mediated in part by TNFα, IFNγ, and the presence of commensal bacteria, as the presence of blocking antibodies or antibiotic treatment specifically alleviated DSS-treated, viral-induced intestinal pathologies observed in ATG16L1^{HM} mice [39]. Aberrant gene expression in Paneth cells of ATG16L1^{HM} mice has been previously reported [38]. A comparison of the response to infection between ATG16L1^{HM} and wild-type mice revealed a differential expression of genes with ligase activity and transporter function. Additional genes involved in intra-cellular protein trafficking, protein targeting and localization, and amino acid and carbohydrate metabolism also exhibited differential expression when taking both infection and ATG16L1^{HM} status into account [39]. Thus, Cadwell et al. highlighted a novel virus-susceptibility gene interaction, establishing that given the appropriate genetic make-up, a viral infection could be a critical deciding factor between inflammation and health. While other viruses, such as CMV, EBV, and Parvovirus B19 are implicated in IBD, similar virus-susceptibility-gene interactions are not demonstrated and conclusive evidence of causality is lacking [40–43]. These studies emphasize the importance of continued investigations into the role of the virome in IBD pathogenesis.

Fungi

The fungal component of the microbiota constitutes approximately 0.1 % of the total gut microbiome and is emerging as an important player in IBD pathogenesis [44, 45]. Many innate immune receptors are known to interact with fungal pathogens and antifungal immunity depends on a complex interplay between receptors, downstream signaling molecules, and cytokines [46]. DECTIN-1, a non-Toll-like receptor pattern recognition receptor for fungal wall β-glucan recognition [47, 48], and CARD9, a central component of the Dectin-1/Syk signaling pathway [49], have emerged as key components of the innate immune response to fungal pathogens. Family studies have further supported a role for DECTIN-1 and CARD9 in fungal immunity, having demonstrated loss-of-function mutations leading to DECTIN-1 and CARD9 deficiencies in patients with mucocutaneous fungal infections [50, 51]. Studies have begun to lay the framework for a role for the mycobiome in IBD pathogenesis. Increased mean fungal diversity and alterations in intestinal fungal composition are observed in CD patients in comparison to controls [45]. Additionally, association between anti-glycan antibodies to the cell wall mannan of *Saccharomyces cerevisiae* and CD, including disease behavior, location, and severity, are documented [52–54]. Moreover, genetic association with IBD susceptibility and single nucleotide variants in *card9* are established [1, 4, 55, 56].

A report in 2012 by Iliev et al. [57] established that commensal fungi are abundantly present in mouse intestinal mucosa, are recognized in the fecal material by DECTIN-1, and are able to produce a fungal antibody response following DSS-induced intestinal inflammation. High-throughput sequencing of fungal rRNA isolated from murine feces identified a rich fungal diversity with >100 fungal species representing greater than 50 genera and an equal number of novel unannotated fungi. Ten fungal species made up approximately 97 % of the identified fungal sequences, with 65 % of the sequences belonging to *Candida tropicalis* [57].

In comparison to wildtype, controls, *clec7a*$^{(-/-)}$ mice exhibiting DECTIN-1 deficiency demonstrated increased susceptibility to DSS-induced colitis, evidenced by increased weight loss, mucosal erosion, crypt destruction, and inflammatory cytokine production, and interestingly, an increased proportion of opportunistic pathogenic fungi, including *C. tropicalis*. Fungi were observed invading inflamed colonic tissues in these DSS-induced *clec7a*$^{(-/-)}$ mice, yet remained localized in the lumen of DSS-induced wild-type mice. Several lines of evidence suggested that these observations seemed to be fungal-specific: no differences were observed in the bacterial flora of DECTIN-1 deficient compared to wildtype; anti-fungal therapy improved colitis in *clec7a*$^{(-/-)}$ mice; mice deficient in DECTIN-1 supplemented with *C. tropicalis* demonstrated an aggravated disease phenotype compared to wildtype; and fecal microflora transplants did not transfer colitis susceptibility to wild-type mice nor alleviate disease phenotype in *clec7a*$^{(-/-)}$ mice [57].

GWAS and other IBD genetic studies have not previously reported an association with *clec7A*. Having uncovered a role for *clec7A*/DECTIN-1 in intestinal fungal immunity, Iliev et al. [57] further demonstrated a novel association with genetic variation in *clec7A* and severe medically-refractory UC (MR-UC) and an earlier progression to surgery [57]. Together with observations that DECTIN-1 deficient mice do not develop spontaneous colitis but rather demonstrate an exacerbation of symptoms upon DSS-induced intestinal inflammation, these genetic findings implicate *clec7A* as an IBD severity gene, potentially via its role in mediating host-fungal immune responses.

Studies such as the ones highlighted in this section lead one to conclude that multiple interactions with various susceptibility loci and a plethora of both commensal and environmental factors may underlie IBD heterogeneity and may advance our understandings of the mechanisms of disease pathogenesis.

IL-23/IL-17 pathway

IL-23 production occurs by macrophages and dendritic cells and this cytokine comprises two subunits, p19 (encoded by the gene *Il-23A*) and p40 (the subunit encoding IL-12B) [58]. There is increased expression of IL-23 by lamina propria (LP) macrophages isolated from patients with CD implicating IL-23 as an important cytokine in the development of colitis [59]. IL-23 promotes differentiation of naïve T cells into Th17 cells characterized by the transcription factor RORγt in an IL-6-and TGF-β-rich environment [60]. Th17 cells produce the cytokines IL-17A, IL-17F, IL-21, and IL-22. Genome-wide association scans (GWAS) identified several genes involved in Th17 differentiation, including *Il-23R*, *Il-12B*, *jak2*, *stat3*, *ccr6*, and *tnfsf15*, as susceptibility genes to the development of IBD [4, 55]. Furthermore, clinical trials using a monoclonal antibody to p40 (the shared subunit between IL-12B and IL-23) demonstrate higher response rates defined as a reduction in Crohn's disease activity index (CDAI) within the induction phase of treatment compared to placebo [61, 62]. These response rates were significantly lower compared to trials done in patients with psoriasis [63, 64], which may be attributable to pharmacokinetics and diversity in the biological alterations that underlie the pathogenesis of CD in those individuals. These data implicate the importance of IL-12 and IL-23 in disease pathogenesis.

Animal models played an important role in elucidating the role of IL-23 and IL-17 in promoting intestinal inflammation. Using two types of murine models, *Il-10*$^{(-/-)}$ mice and lymphocyte-deficient *rag* knockout mice, Yen et al. [65] sought to determine how IL-23 contributes to the intestinal inflammation seen in these animals. *Il-10*$^{(-/-)}$ mice spontaneously develop intestinal inflammation that resembles CD by 3 months of age. *Il-10*$^{(-/-)}$ mice backcrossed with mice lacking the p35 subunit of IL-12 (thus lacking the ability to produce IL-12) developed diarrhea and rectal prolapse indicating underlying colitis, which was confirmed histologically by 7 weeks of age. This finding suggested that in *Il-10*$^{(-/-)}$ mice, the development of underlying intestinal inflammation is not dependent on IL-12. Interestingly, *Il-10*$^{(-/-)}$ mice backcrossed with mice lacking p19 (the subunit comprising IL-23) did not develop any intestinal inflammation by 12 months of age implicating IL-23 as a critical cytokine in the development of chronic mucosal inflammation. CD4+ T cells from *Il-10*$^{(-/-)}$ × *p19*$^{(-/-)}$ mice exhibited an enhanced Th1 response compared to their *Il-10*$^{(-/-)}$ counterparts as they produced significantly more IFNγ suggesting that the manifestation of disease does not result from an impaired Th1 response, rather it requires the presence of IL-23. Stimulation of CD4+ CD45RB^{low} memory T cells from *Il-10*$^{(-/-)}$ mice with IL-23 resulted in enhanced *Il-17* gene and protein expression and blockade of IL-17 using a neutralizing antibody attenuated the histologic intestinal inflammation typically seen in *rag* knockout mice reconstituted with T cells from *Il-10*$^{(-/-)}$ mice. Thus, IL-23-induced intestinal inflammation appears to result in part by the induction of memory-activated T cells expressing IL-17 [65].

The importance of IL-23 and the IL-23R pathway in the development of colitis is supported by the work of Ahern et al. [66] who used T cells from *Il-23r*$^{(-/-)}$ mice to demonstrate that the expression of IL-23R on T cells is required for the development of intestinal and not systemic inflammation. Transfer of CD4+ CD45RB^{hi} naïve T cells isolated from wildtype mice into B and T cell deficient *rag1*$^{(-/-)}$ mice resulted in rapid expansion of these cells and the development of systemic inflammation and severe colitis. In contrast, transfer of CD4+ CD45RB^{hi} cells from *Il-23r*$^{(-/-)}$ mice resulted in minimal intestinal inflammation, which is thought to be related to a decrease in the number of CD4+ T cells within the colon that occurs as a result of a decreased ability to proliferate due to the lack of IL-23. There was no difference in the systemic inflammatory response between the wildtype and *Il-23r*$^{(-/-)}$ mice as measured by splenomegaly, splenic T cell accumulation, hepatic infiltrates, and systemic concentrations of pro-inflammatory cytokines, IL-6, TNFα, and IFNγ. Interestingly, the reduced intestinal inflammation in the *Il-23r*$^{(-/-)}$ mice was associated with a reduced expression of IL-17A, IL-21, and IL-22, all of which are Th17-related cytokines. In a T cell transfer model of colitis, cells isolated from the spleen, mesenteric lymph nodes (MLN), and colon revealed a significant decrease in the number of IL-17A^{+}IFNγ^{+} cells in mice receiving *Il-23r*$^{(-/-)}$ T cells. IL-17 and IFNγ double positive CD4+ T cells are a T cell subset thought to be pathogenic and causes severe colitis in experimental models [66–69]. This implicates IL-23 as an important cytokine for the differentiation of this effector cell population [66].

The family of IL-17 cytokines comprises IL-17A, IL-17B, IL-17C, IL-17D, IL-17E (also known as IL-25) and IL-17F, which act through the IL-17R to induce cellular responses [70]. IL-17 acts to induce other cytokines such as IL-6, chemokines, and prostaglandins by a

variety of cell types [70]. Numerous animal models have supported IL-17's role as a pro-inflammatory cytokine. In an acute trinitrobenzenesulfonic acid (TNBS) model of colitis, Zhang et al. [71] showed that IL-17 protein levels are increased in wild-type mice treated with TNBS. *Il-17R*$^{(-/-)}$ mice that were treated with TNBS developed only mild inflammation with less neutrophilic infiltration, and a reduction in the amount of weight loss compared to wildtype mice. Measurement of IL-6 protein levels revealed a reduction in the amount of IL-6 produced in *Il-17R*$^{(-/-)}$ mice substantiating the lack of IL-17 signaling. These data reflect the importance of IL-17 signaling in the development of acute colitis in mice treated with TNBS.

The mechanism by which IL-17 contributes to IBD disease pathogenesis is complex as data from animal models and human samples suggest both a pro-inflammatory [65, 72, 73] and protective role in IBD [74–76]. The induction of Th17 responses by commensal organisms such as segmented filamentous bacterium (SFB) can be protective against pathogenic invasion through its induction of IL-22 and IL-17A. SFB, however, does not induce the secretion of IFNγ by CD4+ T cells [77]. Furthermore, IL-17 producing Th17 cells express FOXP3 and are thought to arise from a lineage of regulatory T cells which may reflect the complexity of these cells in mucosal homeostasis [78]. IL-17 producing FOXP3+ CD4+ T cells were found to be elevated in inflamed areas of patients with CD compared to UC patients and control patients with colon cancer [79]. Interestingly, a population of FOXP3+IL-17 producing CD4+ T cells were found in inflamed areas of patients with CD but not in UC or normal controls. These FOXP3+IL-17 producing CD4+ T cells appear to be gut specific as they also express CCR6 and α4β7 integrin. These cells are similar to Th17 cells as they express the transcription factor RORγt and secrete IL-22 and IFNγ. Furthermore, they have characteristics of T regulatory cells, including expression of increased levels CD101 and low levels of CD127 and suppression of the proliferation of CD3/CD28-activated CD4+ T cells. These cells, however, unlike regulatory T cells, do not secrete IL-10. These findings implicate a plasticity that may make them pro-inflammatory in the appropriate environment [79].

The hallmark of the pathogenesis of CD involves the induction of Th1 and Th17 responses. Sakuraba et al. [80] evaluated the induction of the Th1 and Th17 responses in patients with CD using CD4+ T cells and dendritic cells (DCs) isolated from MLN in CD patients, UC patients, or normal controls. In order to determine effector cell polarization, these CD4+ T cells were initially stimulated with anti-CD3/CD28 antibodies after which they were stimulated with phorbol 12-myristate13-acetate (PMA) and ionomycin and IFNγ production was measured. IFNγ production was significantly elevated in patients with CD compared to those with UC or normal controls. Although there were low numbers of IL-17 producing cells in all three patient groups, they were significantly increased in CD patients compared to UC patients. In order to determine the mechanism of induction of the effector T cells responses, Sakuraba et al. isolated mature DCs, myeloid DCs, and plasmacytoid DCs and conducted mixed lymphocyte reactions. Mature and myeloid DCs were able to induce higher Th1 responses measured by IFNγ production in CD patients compared to UC patients and normal controls. In addition, DCs from MLN of CD patients produced higher amounts of IL-12p40 and IL-23p19 and lower amounts of IL-10 compared to patients with UC. These

data suggest that the induction of the Th1 and Th17 responses in MLN of patients with CD relates to the production of IL-12 and IL-23 and lack of IL-10 by myeloid DCs [80].

Understanding the development and the molecular signature of pathogenic Th17 cells is critical to understanding their contribution to disease pathogenesis in IBD and for successful development of therapeutic targets in the IL-17 pathway. Using a mouse model of experimental autoimmune encephalomyelitis (EAE), Lee et al. [81] were able to determine that the pathogenicity of Th17 cells was dependent on the induction of TGF-β3 and these TGF-β3-induced Th17 cells had a distinct molecular signature. Table 1 lists the characteristics of pathogenic versus nonpathogenic Th17 cells. In this model, they determined that although IL-6 and TGF-β1 were critical in the differentiation of Th17 cells from naïve T cells, IL-23 exposure for extended periods was required for the onset of severe disease. Using microarray technology, it was discovered that the addition of IL-23 to IL-6 and TGF-β1 in the differentiation of Th17 cells resulted in induction of TGF-β3. The induction of TGF-β3 was dependent on IL-23R signaling, as CD4+ T cells taken from *Il-23r*-deficient mice were unable to induce TGF-β3 expression under Th17 polarizing conditions. Interestingly, it is the IL-23R signaling and not specifically IL-23 that is needed for TGF-β3 expression as IL-6 can induce TGF-β3 when IL-23R signaling is intact. Furthermore, TGF-β3-induced Th17 cells expressed similar amounts of IL-17 to TGF-β1-induced Th17 cells; however, they expressed more IL-22 and IL-23R. When transferred into naïve recipient mice, TGF-β3-induced Th17 cells resulted in severe disease and increased mortality compared to mice receiving TGF-β1-induced Th17 cells suggesting that TGF-β3-induced Th17 cells are extremely pathogenic. It was determined that TGF-β3-induced Th17 cells were functionally distinct from TGF-β1-induced Th17 cells and that difference is largely determined by differences in downstream signaling through TGF-βRII. Characterization of the transcriptional signature was done via whole genome microarray and revealed higher expression of cytokines and chemokines such as IL-22, transcription factors such as STAT4, and effector molecules and lower expression of immunoregulatory molecules such as IL-10 in TGF-β3-induced Th17 cells. This contrasted transcripts in TGF-β1-induced Th17 cells, which had higher expression of immunoregulatory molecules such as IL-10 and molecules involved in IL-10 regulation. Lastly, this group determined that T-BET, a transcription factor normally characterized by Th1 development was required for the induction and maintenance of TGF-β3 which is critical for Th17 pathogenicity. Collectively, these data highlight a distinct subgroup of Th17 cells that may contribute to the pathogenesis of autoimmune diseases and provide a useful target, TGF-β3, for regulation of inflammation caused by these TGF-β3-induced, pathogenic Th17 cells [81].

The importance of IL-17 in the pathogenesis of CD is seen in both animal models of CD and in human disease. In a case control study using 763 patients with CD and 254 controls, novel associations in haplotypes of *Il-17A* and *Il-17RA* were associated with CD risk using Illumina technology [82]. Furthermore, the pro-inflammatory role of IL-17 is supported by findings of increased numbers of IL-17 positive cells in the lamina propria of CD patients with both active and inactive disease compared to controls. This correlated with higher expression of IL-17 as increased IL-17 messenger RNA (mRNA) transcripts were expressed in active and quiescent disease and fecal concentration of IL-17 was elevated in CD patients with active disease. IL-23, however, had increased mRNA expression only in active CD

patients [83]. IL-17F mRNA transcripts also appear to correlate with active CD compared to quiescent CD and do not appear to be increased in UC [84].

TNFSF15/TL1A and the IL-17 pathway

Tnfsf15 and its protein TL1A is implicated in the pathogenesis of many autoimmune inflammatory diseases [85] including IBD, and contributes susceptibility to IBD in multiple ethnic groups [85]. TL1A can be membrane bound or soluble in form and upon binding to its receptor, death domain receptor 3 (DR3) initiates a number of immune responses, including activation and proliferation of T cells resulting in the production of pro-inflammatory cytokines such as IFNγ. Induction of TL1A occurs through the innate immune system through activation of Fc gamma receptors, TLRs and NLRs [86–91].

In chronic DSS models, TL1A enhances Th1 and Th17 function through its upregulation of IFNγ and IL-17 production, under Th1/Th17 polarizing conditions [92]. Treatment with an anti-TL1A antibody decreases the amount of IFNγ and IL-17A produced and attenuates weight loss, colon shortening, and the histologic inflammation seen in this model of chronic colitis. These findings are corroborated in two other models of T cell-mediated chronic colitis [92, 93]. TL1A's effect on the production of Th1- and Th17-related cytokines is supported using T cells and macrophages isolated from the intestinal LP and peripheral blood (PB) of patients with CD. Stimulation of LP and PB CD4+ T cells with soluble TL1A induces IFNγ production and IL-17A production by LP and not PB CD4+ T cells and synergizes with IL-23 to enhance production of IFNγ and IL-17A [90]. These data suggest that TL1A plays an important role in the development of gut mucosal inflammation.

It appears that the role TL1A plays in promoting Th1 and Th17 responses is complex as there is evidence that TL1A acts as a differential regulator in generation of Th17 cells from naïve T cells. Naïve CD4+ T cells isolated from peripheral blood of healthy donors activated with anti-CD3/antiCD-28 antibody reduce the expansion of CD4+ IL-17 producing cells and IL-17A production when co-stimulated with TL1A [94]. Wild-type mice exhibited decreased numbers of Th17 cells in inguinal lymph nodes compared to DR3-deficient mice when stimulated with TL1A secondary to the lack of TL1A signaling in DR3-deficient mice. Unlike DR3-deficient mice, wild-type TL1A-stimulated splenic T cells inhibit the expansion of IL-17 producing CD4+ T cells [94]. Furthermore, TL1A's effect on de novo Th17 differentiation was investigated using naïve CD4+ T cells exposed to TGF-β and IL-6 given that TGF-β and IL-6 are required for differentiation of naïve T cells into Th17 cells [95–97]. The addition of TL1A to naïve CD4+ T cells exposed to IL-6 and TGF-β results in decreased numbers of Th17 cells [94]. This indicates that TL1A inhibits Th17 expansion and production of IL-17A. This may be possible if TL1A differentially affects naïve T cells and T cells already committed to a Th17 lineage. Our group and others have previously shown that TL1A enhances Th17 effector function through production of IL-17A by activated CD4+ T cells [90, 92]. Those activated T cells may already be committed to Th17 differentiation and thus TL1A stimulation resulted in production of Th17-dependent cytokines. TL1A stimulation of naïve T cells under Th17 polarizing conditions inhibits expansion of Th17 cells suggesting that TL1A does not promote de novo differentiation of

Th17 cells. Collectively, these findings highlight the complexity of the IL-17 pathway and the role that TL1A plays in the induction of Th17 responses.

Anti-IL-17 in the treatment of CD

A humanized monoclonal antibody against IL-17A (Ixekizumab) is effective in treating patients with chronic plaque psoriasis [98]. Since IL-17 is also implicated in IBD disease pathogenesis, a human monoclonal antibody against IL-17A (secukinumab) was evaluated in a double-blind, randomized, placebo controlled trial to evaluate its efficacy in active Crohn's disease. Fifty-nine patients with moderate to severe Crohn's disease were treated with two doses of 10 mg/kg IV secukinumab or placebo. The primary endpoint assessed was the probability of a ≥50 point reduction in CDAI compared to placebo at week 6. The results of the trial revealed that there was <0.1 % probability that secukinumab reduces the CDAI by ≥50 points when compared to placebo. In addition, 18 patients discontinued the drug with 10 people lacking a therapeutic benefit from the drug. Interestingly, post hoc exploratory pharmacogenetic analysis showed that a minority of patients who responded to IL-17 blockade carried a *tnfsf15* (rs4263839) single nucleotide polymorphism (SNP) [99]. At DDW in 2014, Wallace et al. [100] presented their investigation of the potential functional consequences of the *tnfsf15* (rs4263839) SNP on the differential effects of anti-IL-17 therapy in IBD. They first showed that the *tnfsf15* (rs4263839) variant is in perfect linkage disequilibrium with the *tnfsf15* SNP (rs6478109), which led to elevated TL1A production [101]. The adoptive T cell transfer model in mice was used to simulate the anti-IL-17A trial. Compared to wild-type, *rag1*$^{(-/-)}$ mice that received *Il-17a*$^{(-/-)}$ or TL1A-Tg (sustained TL1A expression), naïve T cells exhibited worsened colonic inflammation compared to *rag1*$^{(-/-)}$ mice that received wild-type naïve T cells. Similar to the human IL-17 trial, IL-17A deficiency under TL1A-driven conditions (TL1A-Tg/*Il-17a*$^{(-/-)}$) ameliorated colonic inflammation seen in both *Il-17a*$^{(-/-)}$ and TL1A-Tg mice. Mucosal *Il-17a*$^{(-/-)}$ and TL1A-Tg CD4+ T cells had a increased activated phenotype (CD69, CD44), which was reduced with TL1A-Tg/*Il-17a*$^{(-/-)}$. Compared to either *Il-17a*$^{(-/-)}$ or TL1A-Tg, TL1A-Tg/*Il-17a*$^{(-/-)}$ mice had reduced expression of IFNγ and IL-9 and increased expression of the regulatory cytokine IL-10. Consistently, in vitro-differentiated naïve *Il-17a*$^{(-/-)}$ and TL1A-Tg T cells expressed increased IFNγ and IL-9. In contrast, in vitro-differentiated naïve TL1A-Tg/*Il-17a*$^{(-/-)}$ T cells expressed lower IL-9 but higher IL-10 levels and had higher regulatory function. Our study [100] showed that IL-17 blockade induced mucosal inflammation by enhancing pro-inflammatory and reducing regulatory responses. However, under TL1A-driven conditions, inhibiting IL-17 may be beneficial by reducing pro-inflammatory responses and enhancing regulatory responses. In summary, IL-17 blockade induced mucosal inflammation by enhancing Th1, Th9 effector pathways, and reducing regulatory responses. Under TL1A-driven condition, inhibiting IL-17 may be beneficial by reducing Th1 and Th9 effector responses and enhancing regulatory responses. This may be but one of the mechanisms explaining why a subset of IBD patients with TL1A polymorphisms improved with IL-17 blockade, while most trial patients did not. This highlights the importance of understanding pathway-pathway interactions and patient genetics in designing clinical trials.

Conclusion

The use of animal models in studying the mechanism of disease pathogenesis for multiple autoimmune inflammatory diseases has provided the scientific community with invaluable information that ultimately has guided the development of various therapeutic drugs. These models highlighted the importance of the microbiome and revealed the complexity of the role of IL-17 in IBD disease pathogenesis. The knowledge gained from such animal models and advances in understanding genetic susceptibility to diseases will drive the era of personalized medicine in which clinical trials are designed using small groups that are homogenous from both a genetic and phenotypic perspective.

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Table 1

Characteristics of pathogenic versus nonpathogenic Th17 cells [81]

	Nonpathogenic Th17 cells	**Pathogenic Th17 cells**
Cytokine Millieu	IL-6, TGF-β1	IL-6, IL-23, TGF-β3
Transcriptional signature (increased expression)	CXCL3, IL-22, IL-3, CCL4, GZMB, LRMP, CCL5, CASP1, CSF2, CCL3, TBC21, ICOD, IL-7R, STAT4, LGALS3, LAG3	I6ST, IL1RN, IKZF3, MAF, AHR, IL-9, IL-10