UNDERSTANDING CROHN'S DISEASE: IMMUNITY, GENES AND MICROBES

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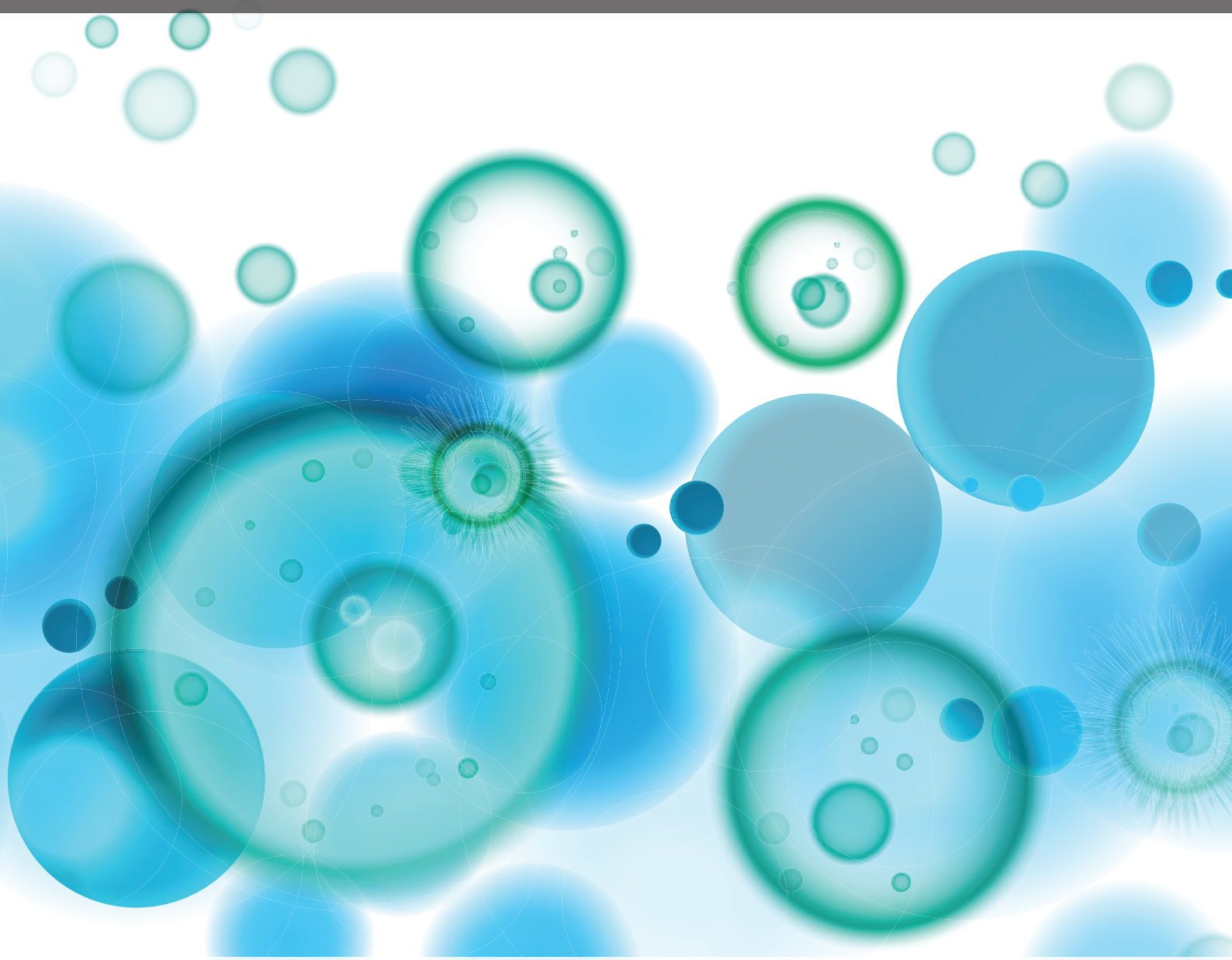

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UNDERSTANDING CROHN'S DISEASE: IMMUNITY, GENES AND MICROBES

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Crohn's disease (CD) is a chronic, relapsing, inflammatory bowel disease resulting in considerable morbidity and reduced quality of life. Although still under intense debate, CD seems to result from an enhanced and uncontrolled immune response to the gut microbiota. CD is thought to be multifactorial depending on genetic and environmental determinants. In recent years, nearly 100 single nucleotide polymorphisms (SNPs) were associated with increased risk of developing CD (some of the SNPs also associated with susceptibility to ulcerative colitis, another type of IBD). These SNPs are mostly located in genes involved in innate and adaptive immunity mechanisms, such as autophagy, expression of pattern-recognition receptors and citokine signaling. Epigenetics is also probably playing a role in CD susceptibility, as it is sensitive to environmental conditions and may mediate gene-environment interactions. Environmental factors possibly involved in CD development include diet, gut microbiota composition and infection with specific pathogens, of which the most consistently associated to CD are Mycobacterium avium subsp. paratuberculosis and adherent-invasive Escherichia coli.

This Topic aimed at bringing together contributions covering different genetic, epigenetic, immunological and microbial processes involved in the development of CD, helping to drive forward the understanding of CD immunopahtology.

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EDITORIAL
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Editorial: Understanding Crohn's Disease: Immunity, Genes and Microbes

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Keywords: inflammatory bowel diseases, Crohn's disease, genetic susceptibility, T cells, intestinal microbiota

Editorial on the Research Topic

Understanding Crohn's Disease: Immunity, Genes and Microbes

Crohn's disease (CD) is a chronic debilitating syndrome, associated with considerable morbidity and resulting in elevated public health costs every year. CD and ulcerative colitis (UC) are the two major forms of inflammatory bowel disease (IBD), which has long been characterized as an exacerbated inflammatory response to common antigenic stimuli in the gut due to immune dysregulation. Although both CD and UC share some common patterns, they are distinct diseases. In fact, while UC is characterized by a diffuse mucosal inflammation involving mainly the rectum and adjacent colonic tissue, CD is a transmural inflammatory disease that may involve any part of the gastrointestinal tract, from the mouth to the anus, although in most cases the terminal ileum is affected. Etiology of IBD, particularly CD, has been long debated and is likely to involve the contribution of multiple factors, making its study challenging. Among those contributing factors are genetic inheritance, epigenetic mechanisms, infection with particular pathobionts, and the gut microbiota in general. This topic aimed at bringing together contributions covering aspects related primarily to CD etiology and immunopathology, helping to drive forward a more comprehensive understanding of this challenging syndrome.

Genetic inheritance is an important predisposing factor for IBD. In a comprehensive review, Loddo and Romano point out the importance of genetic traits in susceptibility to IBD. The authors discuss the success of next-generation sequencing in the investigation of rare monogenic susceptibility variants, implicated in early-onset and very early-onset IBD. They also address the importance of epigenetic mechanisms, such as DNA methylation, in linking environmental stress and gene expression and discuss the use of microRNAs as biomarkers and therapeutical targets in IBD. The first genetic variant conferring susceptibility to ileal CD was located in the nucleotide oligomerization domain 2 (NOD2) gene (1), a gene implicated in recognition of bacterial muramyl dipeptide. Sidiq et al. highlight the role played by epithelial cell NOD2 expression in maintaining gut homeostasis and regulating ileal microbiota composition and address the consequences of a deficient NOD2 variant in gut dysregulation. As described in an original research article, Parkhouse and Monie tested whether loss-of-function NOD2 variants conferring susceptibility to CD (R702W, G908R, and L1007fsincC) exhibited deficient binding to receptor-interacting protein kinase 2, an adaptor protein implicated in NOD2 signaling. They found that impairment of NOD2 signaling shown by variants containing CD-susceptibility polymorphisms did not correlate with deficient RIP2 binding, concluding that the causes for impairment are multifactorial.

A set of articles included in this topic specifically relate to patterns of immune system function in CD. Di Giovangiulio et al. contributed with a comprehensive review on the neuromodulation

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of the gut immune system. Indeed, the cross talk between the nervous system and the gut immune system can shape the mucosal immune response to gut antigens and contribute to gut homeostasis. It is widely accepted that T cell function, particularly the interplay between regulatory and effector T cells, is of major importance in the maintenance of gut tolerance to intestinal antigens. Sarrabayrouse et al. discuss the important role played by a unique human subset of Treg cells, the IL-10-producers CD4CD8αα cells present in the intestinal mucosa. These cells are induced by clostridial bacteria to suppress T cell proliferation in a process independent on Foxp3 expression, since CD4CD8αα subset does not express this transcription factor. In a mini-review, Omenetti and Pizarro discuss the dynamic balance between intestinal Th17 and Treg cells, the plasticity allowing interdifferentiation and the participation of the gut microbiome in driving the differentiation toward each phenotype. Mucosal-associated invariant T cells (MAIT cells) are a non-conventional T cell subset possibly playing a role in CD (2). MAIT cells make up 10% of peripheral blood and intestinal lamina propria T cells and express Th17 cells markers. In an opinion article, Treiner elegantly discusses arguments in favor of the participation of MAIT cells in CD pathogenesis and presents two putative mechanisms by which activation of this T cell subset might occur in the gut.

In recent years, evidence is accumulating in favor of a central role of gut microbiota in CD pathogenesis. Three reviews included in this topic highlight the relevance of gut microbiota composition in health and disease. Haag and Siegmund discuss the interplay between intestinal microbiota and the innate immune system and the ways by which an altered microbiota may influence barrier integrity and activate innate immunity, leading to chronic inflammation. Buttó et al. also address current approaches available to investigate microbiota-associated mechanisms of disease, such as new animal models. Oberc and Coombes discuss the impact of antibiotic treatment and infectious gastroenteritis (two external risk factors for CD) on gut microbiota composition, leading to dysbiosis and consequently to disease onset. Sechi and Dow mini-review addresses the long debated participation of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in CD etiology, discussing MAP exposure, human genetic susceptibility to mycobacterial infection and MAP possible involvement in CD, and other human inflammatory diseases.

Finally, in line with increasing recognition of the role played by nutrition in disease prophylaxis and therapy, Ferguson discusses the beneficial effects of directed nutritional therapy for CD patients, according to the genetic background and presence of particular susceptibility polymorphisms. Although data on the benefits of particular nutrients already exist, systems biology approaches would allow to validate efficacy of particular diet constituents to CD patients. This would help to drive nutritional therapy into the primary therapy in CD, integrated with currently accepted approaches.

I hereby wish to manifest my appreciation to all the authors that participated in this research topic. Their articles significantly contributed to a more comprehensive view on CD immunopathology and associated factors, helping to improve current understanding of this debilitating disease.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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MINI REVIEW
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Inflammatory bowel disease: genetics, epigenetics, and pathogenesis

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Inflammatory bowel diseases (IBDs) are complex, multifactorial disorders characterized by chronic relapsing intestinal inflammation. Although etiology remains largely unknown, recent research has suggested that genetic factors, environment, microbiota, and immune response are involved in the pathogenesis. Epidemiological evidence for a genetic contribution is defined: 15% of patients with Crohn's Disease (CD) have an affected family member with IBD, and twin studies for CD have shown 50% concordance in monozygotic twins compared to <10% in dizygotics. The most recent and largest genetic association studies, which employed genome-wide association data for over 75,000 patients and controls, identified 163 susceptibility loci for IBD. More recently, a trans-ethnic analysis, including over 20,000 individuals, identified an additional 38 new IBD loci. Although most cases are correlated with polygenic contribution toward genetic susceptibility, there is a spectrum of rare genetic disorders that can contribute to early-onset IBD (before 5 years) or very early onset IBD (before 2 years). Genetic variants that cause these disorders have a wide effect on gene function. These variants are so rare in allele frequency that the genetic signals are not detected in genome-wide association studies of patients with IBD. With recent advances in sequencing techniques, ~50 genetic disorders have been identified and associated with IBD-like immunopathology. Monogenic defects have been found to alter intestinal immune homeostasis through many mechanisms. Candidate gene resequencing should be carried out in early-onset patients in clinical practice. The evidence that genetic factors contribute in small part to disease pathogenesis confirms the important role of microbial and environmental factors. Epigenetic factors can mediate interactions between environment and genome. Epigenetic mechanisms could affect development and progression of IBD. Epigenomics is an emerging field, and future studies could provide new insight into the pathogenesis of IBD.

Keywords: inflammatory bowel disease, genetics, epigenetics, IBD, Crohn's disease, early-onset IBD

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INTRODUCTION

Inflammatory Bowel Diseases (IBDs) are complex, multifactorial disorders characterized by chronic relapsing intestinal inflammation. The two major subtypes of IBD are Ulcerative Colitis (UC) and Crohn's Disease (CD).

Ulcerative Colitis and CD are important worldwide health problems, with an incidence in Europe of 12.7 and 24.3 per 100,000 person-years, respectively, and prevalence of 0.5 and 1.0%.

The incidence of IBDs is continually growing among children and adults, all over the world (1).

Although the exact etiology is still not completely known, recent studies have indicated that personal genetic susceptibility, environment, intestinal microbiota, and immune system are all involved in the pathogenesis of IBDs (**Figure 1**).

The heritability model of CD and UC is still unknown, but many genetic and environmental factors are likely to be involved. These conditions tend to occur in multiple familial cases, and having an affected relative is an important risk factor for the onset of IBD.

Population-based studies have provided compelling evidence that genetic factors contribute to the pathogenesis of IBD; an 8- to 10-fold greater risk of IBD among relatives of UC and CD probands has been demonstrated and, most importantly, that there is concordance between twins (2).

Twin studies have provided the best evidence for genetic predisposition to IBD, which is stronger for CD than UC. In particular, twin and family studies for IBD have shown that a child has a 26-fold increased risk for developing CD when another sibling already has it, and the risk is ninefold increased in the case of UC (3).

However, the fact that genetic factors account for only a part of general disease variance indicates that microbiota and environment may interact with genetic susceptibility in the pathogenesis of IBD.

The adaptive immune system has been classically considered to play the main role in the pathogenesis of IBD. Recent research in genetics and immunology has confirmed that the innate immune system maintains great importance in inducing gut inflammation.

New advances in understanding IBD pathogenesis explain important disease mechanisms, including not only the innate and adaptive immunity, but also interactions between genetic, microbial, and environmental factors (4).

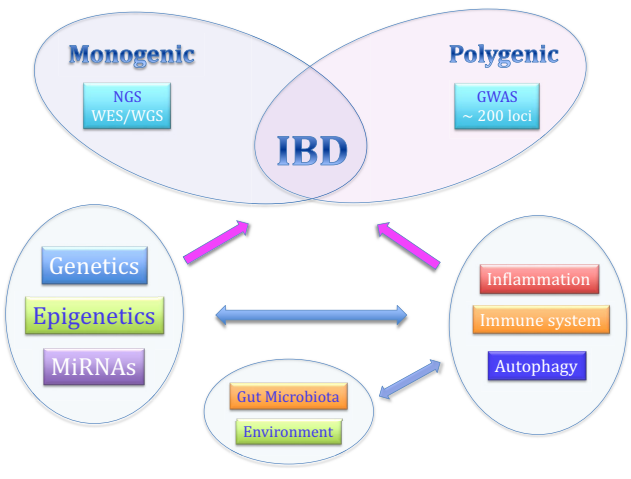

FIGURE 1 | The complex pathogenesis of inflammatory bowel diseases.

GENETICS AND PATHOGENESIS

Over the past few decades, there have been important advances in our understanding of genetic contributions to IBD. This is due to technological progress in genetic testing and DNA sequencing that has allowed many genome-wide association studies (GWAS), which have identified new single nucleotide polymorphisms (SNPs) (4, 5).

Nucleotide-binding oligomerization domain containing 2 (NOD*2*) was the first susceptibility gene for CD discovered in 2001. This gene codes for a protein that acts as an intracellular receptor for bacterial products in monocytes and transduces signals leading to NFkB activation. The activation of NOD2 with muramyl dipeptide induces autophagy in dendritic cells (DCs). DCs from CD patients with susceptibility variants in NOD2 gene are deficient in autophagy induction and also show reduced localization of bacteria in autophagolysosomes (6).

Genome-wide association studies have identified and confirmed many susceptibility loci for IBDs. The identification of susceptibility loci has enhanced understanding of causes of the disorder by providing important clues in crucial and disturbed pathways of the intestinal immune system (7).

Genetic analyses have reported two other autophagy-related genes, *IRGM* and *ATG16L1*, showing an important role for autophagy in immune responses in IBD. Genetic variants that have been found to confer an increased risk of CD indicate the importance of innate immunity, autophagy, and phagocytosis in its pathogenesis.

Other genes, like *IL23R* and *PTPN2*, are also associated with autoimmune disease, suggesting another aspect of Crohn's pathogenesis.

Recent progress in the genetics of IBD can explain the underlying pathogenesis of the disease.

Conventional IBD is a group of polygenic disorders in which hundred(s) of susceptibility loci contribute to the overall risk of disease.

The most recent and largest genetic association study, which employed genome-wide association data for over 75,000 adolescent and adult-onset IBD patients and controls, identified 163 susceptibility loci for IBD, encompassing ~300 potential candidate genes. Of these 163 loci, 110 conferred risk to both IBD subtypes, whereas 30 loci where unique to CD and 23 loci were unique to UC. More recently, a trans-ethnic analysis including over 20,000 individuals of European and non-European ancestry identified an additional 38 new IBD loci, highlighting shared genetic risk across populations and increasing the number of known IBD risk loci to 200 (8, 9).

The evidence of shared IBD risk loci across diverse populations suggests that combining genotype data from cohorts of different ancestry will enable the detection of additional IBD-associated loci.

However, all identified loci individually contribute only a small percentage of the expected heritability in IBD (3).

The increasing number of susceptibility gene loci described in IBD indicates that genetic components are important factors involved in the disease pathogenesis. Identified genetic factors

account for only a small proportion of the disease variance: 13.1% for CD and 8.2% for UC. Overall, explainable susceptibility loci and genetic risk factors discovered so far, account for only 20–25% of the heritability (genetic risk) (8, 10).

MONOGENIC FORMS AND VERY EARLY-ONSET IBD

Approximately 20–25% of patients with IBD are diagnosed before 16 years old. The onset of intestinal inflammation in children can affect development and growth. Age of onset can also provide information on type of IBD and associated genetic features.

Although most cases of IBD are correlated with a polygenic contribution toward genetic susceptibility, there is a spectrum of rare genetic disorders that produce IBD-like intestinal inflammation (11).

Early-onset inflammatory bowel diseases (EO-IBD) (before 5 years) and very-early-onset inflammatory bowel diseases (VEO-IBD) (before 2 years) are rare, particularly severe disease presentations. Few case series have reported on early-onset of IBDs, often described as CD or CD-like, but starting within the first year of life with typical characteristics of CD. The majority of VEO-IBD are caused by genetic defects (monogenic diseases).

The genetic variants that cause these disorders have a wide effect on gene function. These variants are so rare in allele frequency that the genetic signals are not detected in GWAS of patients with IBD. With recent advances in genetic mapping and sequencing techniques and increasing awareness of the importance of these rare disorders, ~50 genetic disorders have been identified and associated with IBD-like immunopathology.

Monogenic defects have been found to alter intestinal immune homeostasis via several mechanisms.

These include disruption of the epithelial barrier and the epithelial response, as well as reduced clearance of bacteria by neutrophil granulocytes and other phagocytes. Other single-gene defects induce hyperinflammation or autoinflammation or disrupted T- and B-cell selection and activation.

Hyperactivation of the immune response can result from defects in immune inhibitory mechanisms, such as defects in IL-10 signaling or dysfunctional regulatory T-cell activity (3).

The main of the IL-10 pathway within the colonic mucosa is confirmed by the occurrence of severe colitis during the first weeks of life in infants carrying mutations in *IL10*, *IL10RA*, or *IL10RB* genes. Immunosuppression has failed to correct the defect in this pathway, which seems to be important to control colon inflammation (12).

Loss-of-function defects in IL-10 and its receptor (encoded by *IL10RA* and *IL10RB* genes) cause VEO-IBD with perianal disease and folliculitis within the first months of life. All patients with loss-of-function mutations that prevent IL-10 signaling develop IBD-like immunopathology, indicating that these defects are a monogenic form of IBD with 100% penetrance (13, 14).

A functional IL-10 pathway is essential for immune homeostasis within the colon, and defects in this cytokine or one of the subunits of its receptors cause extensive inflammation of the colon and perianal region. Thus, on a clinical basis, we can suspect a defect in the IL-10 pathway in children, presenting deep ulcerations and granuloma (CD-like), especially in consanguineous families (12).

It is a challenge to diagnose the rare patients with monogenic IBD, but accurate genetic diagnosis is important for assessing prognosis, and proper treatment of patients.

This group of diseases has high morbidity, and subgroups have high mortality if untreated. Based on the causes, some require different treatment strategies from most cases of IBD. A genetic diagnosis should always be carried out due to the differences in prognosis and medical management (3).

IBD IN THE ERA OF NEXT-GENERATION SEQUENCING

Next-generation sequencing (NGS) is a new technology with the potential to identify every genetic variation throughout the human genome in a single experiment.

Next-generation sequencing technologies have revolutionized the field of medical genetic research and are currently being used to search for Mendelian disease genes, and applied for the diagnosis of patients with genetically heterogeneous disorders. This can be performed much faster and more cost efficiently than with traditional techniques.

It is often a less expensive option than traditional Sanger sequencing for diseases characterized by genetic heterogeneity. This is a novel approach to discover lower prevalence with higher effect size (15).

Because of the large amount of data that are being generated, bioinformatic analysis plays an important role in research and diagnostics (16, 17).

The two sequencing approaches for detecting variations in the genetic code are whole genome sequencing (WGS) and whole exome sequencing (WES).

Whole genome sequencing is the ultimate approach for detecting all genomic variations in a patient's genome. However, current NGS instruments are limited in terms of throughput and cost efficiency and this approach is often only used for large-scale research studies and gene discovery projects.

Whole exome sequencing is a more cost-efficient strategy for novel disease gene discovery and diagnostics in human genetics. The protein-coding regions constitute the exome, ~1% of the human genome or ~30 Mb, split across ~180,000 exons. Currently, the great majority of mutations responsible for Mendelian diseases in humans affects sequences within the coding regions of exons or are located within a few nucleotides of the exon boundaries. Exome sequencing basically refers to the enrichment of sequences corresponding to all (or nearly all) protein-coding exons followed by next-generation sequencing.

Exome sequencing was introduced in 2009. Since then, it has been used to discover several hundred novel disease genes and has begun to significantly improve diagnostics for patients with rare genetic diseases. It has rapidly become one of the main tools for studying the genetic causes of diseases and we have learned much from exome sequencing.

Whole exome sequencing covers only coding areas of the genome, and costs less than WGS, providing higher-depth coverage and therefore greater certainty regarding novel discoveries.

But only together with subsequent functional studies on identified proteins and pathways will novel technologies elucidate underlying pathogenic mechanisms.

Use of WES in clinical diagnostics has grown significantly since clinical laboratories started performing it. Many patients with rare recessive and dominant disorders, who had previously spent years on an uninformative diagnostic odyssey, have now had diagnoses made through WES (16).

Interestingly, results of diagnostic applications of NGS indicate that there is a much wider phenotypic spectrum associated with mutations in many genes than was suspected from initial clinical definition and Sanger sequencing.

The advent of NGS techniques has allowed new large-scale approaches with unexpected diagnostic power. In particular, WES is changing the diagnostic paradigm in medical genetics practices.

The traditional diagnostic approach with clinical evaluation and laboratory analysis provides a diagnosis in ~50% of patients. WES offers the possibility to have an answer in most of the remaining cases, providing diagnosis in another 25–30% of patients (18).

The WES approach has been successfully used to identify single variants in very early-onset IBD and has been quite successful in elucidating novel monogenic forms of IBD and new susceptibility genes. However, many polymorphisms that affect disease susceptibility are located in non-coding areas of the genome: for this reason, the ENCODE (Encyclopedia of DNA Elements) project has highlighted the importance of non-coding regions in disease risk, trying to identify all functional elements in the human genome sequence (19).

EPIGENETICS AND IBD

Inflammatory bowel diseases could be caused by interactions between the patient and the environment, in particular the genome, the immune system, the intestinal microbiota, and specific environmental factors such as the effects of breastfeeding, food, smoking, drugs, and so on. Epigenetics may be defined as mitotically heritable changes in gene expression without altering the DNA sequence.

Gene expression can be altered by changes to the structure and function of chromatin. Different cells in the body are characterized by different functions and different levels of gene expression despite each sharing the same genetic code. This variation in gene activity from cell to cell is achieved by mechanisms and processes that are collectively termed epigenetics. The main epigenetic mechanisms include DNA methylation, histone modification, RNA interference, and the positioning of nucleosomes. These epigenetic mechanisms, in particular DNA methylation, appear to be very important in the interaction between environment and genome. It is potentially reversible and heritable over rounds of cell division (20). Variation in DNA methylation is a well-recognized cause of human disease and is likely to play a pivotal role in the cause of complex disorders. Several well-known disorders of imprinting are known including Beckwith–Wiedemann syndrome, Temple syndrome, Wang–Ogata syndrome, Silver–Russell syndrome, Angelman syndrome, and Prader–Willi syndrome. Imprinted genes are thought to play an important role in fetal growth and their carefully regulated expression is important for normal cellular metabolism and human behavior.

The challenge is to identify consistent epigenetic alterations of etiological significance, given that epigenetic modification of DNA differs between tissues, occurs at different times of development within the same tissue and is sensitive to continual environmental factors.

Epigenetics has developed into one of the most promising concepts in all areas of biomedical research. Recent epigenetic studies have shown that interactions between genome and environment play an important role in the phenotypical expression of diseases, explaining also the differences in disease expression in monozygotic twins (1).

Much evidence supports the idea that IBD is caused by a complex interaction between genetic mutations of multiple genes and environmental factors. There is growing evidence that epigenetic factors can play an important role in the pathogenesis of IBD.

A number of potential clinical applications of epigenetics in diagnostics and therapeutics are receiving attention. The diagnostic applications of epigenetics include the use of biomarkers to confirm diagnosis, stratify disease course and response to chemotherapy, and predict development of cancer (11).

DNA methylation is the most studied epigenetic modification and during the last decade its correlation to IBD pathogenesis has been well established. Several reports have suggested that there are significant differential DNA methylation statuses between normal and inflamed tissues from CD and UC patients. Therefore, there are evidences that the hypermethylation of many gene promoters is associated with IBD patients.

Genes from different molecular pathways have been studied but till now there is no standardized database of methylated genes in IBD.

DNA methylation patterns have proven to be most useful in the sensitive detection of disease.

DNA-methylation-based technologies have a promising future in both clinical diagnostics and therapeutics. DNA methylation markers have been developed using targeted candidate gene approaches and have applications in diagnostics, but can also contribute to therapeutics as predictors of therapeutic response (20). Further studies of epigenetic factors associated with IBD could lead to new therapeutic strategies, whether they specifically target epigenetic mechanisms or affect the pathways they control.

DNA methylation should be studied in depth to understand the molecular pathways of IBD pathogenesis, and epigenetic studies of IBD discussed that may have a significant impact on the field of IBD research.

THE EMERGING ROLE OF MIRNA IN IBD

The field of microRNA (miRNA) research is expanding rapidly. MiRNAs are strongly implicated in the pathogenesis of many common diseases, including IBDs, playing an important role in the development, regulation and differentiation of the innate and adaptive immune system (21).

MicroRNAs are a class of endogenous small non-coding single-stranded RNA molecules, ~18–24 nt long, encoded in genomic DNA, which act as post-transcriptional regulators of gene expression. The biogenesis of miRNAs goes from transcription in the nucleus to generation of the mature miRNA in the cytoplasm.

MiRNA genes are located throughout the genome, either within intronic sequences of protein-coding genes, within intronic or exonic regions of non-coding RNAs, or set between independent transcription units (intergenic). Some miRNAs have their own promoters and are transcribed independently, some share promoters with host genes, while others are co-transcribed as a single primary miRNA transcript (21, 22).

It is estimated that miRNAs regulate more than 60% of protein-coding mRNAs and that more than one-third of human genes are targets for miRNA regulation.

In particular, each miRNA can target hundreds of mRNAs and each mRNA can be regulated by several miRNAs, resulting in mRNA destabilization and/or inhibition of translation.

They regulate important cellular functions such as differentiation, proliferation, signal transduction, and apoptosis and exhibit highly specific regulated patterns of gene expression.

Emerging evidence suggests the regulation of miRNAs expression through epigenetic mechanisms such as DNA methylation, histone modifications, and circular RNAs (circRNAs). DNA methylation, the addition of methyl groups at CpG islands by DNA methyltransferases, is associated with transcriptional repression. Similarly, acetylation or deacetylation of histones may alter transcriptional activity. This process adds complexity to our understanding about regulation of gene expression (21).

Although a large number of miRNAs have been identified, little is known about their function (23).

There is growing evidence that miRNAs play a role in the induction of cancer, inflammatory, and autoimmune diseases. In the intestinal tract, miRNAs are involved in tissue homeostasis, intestinal cell differentiation, and the maintenance of intestinal barrier function (24).

Nowadays, research is interested in the possibility to use miRNAs as biomarkers and therapeutic target in IBD. There are emerging data from human diseases studying miRNAs as novel biomarkers in diagnosing and predicting disease course and response to therapy.

Recently, several studies have shown a differential expression of miRNAs in tissue samples and blood from patients with IBDs compared with healthy controls, suggesting that miRNAs may be considered as novel biomarkers of these diseases (25).

Given that CD and UC differ in their clinical presentations, genetic associations, gene expression patterns, and immune responses, differing miRNA profiles are expected for these two conditions.

Studies to date have identified unique miRNA expression profile signatures in IBD and preliminary functional analyses associate these deregulated miRNAs to canonical pathways associated with IBD pathogenesis (26).

Crohn's Disease and Ulcerative Colitis patients have unique miRNA expression profiles in their target organs. While some differentially expressed miRNAs are common to other immune-related disorders, most are unique.

Moreover, it has been shown that CD and UC differ not only in their tissue miRNA profiles, but also in their peripheral blood miRNA profiles. Then there are unique miRNA tissue profiles and distinct miRNA profiles in peripheral blood.

While UC and CD represent distinct diseases with some overlap, identification of distinct miRNA expression profiles may provide an early method to determine a patient's disease course. After the functional consequences of alterations in miRNA expression are established, miRNA may also become the target of future treatments.

Further investigation about the roles of miRNAs in the human context will improve our knowledge of miRNAs in the pathogenesis and diagnosis of IBD and will be useful for the development of miRNA-based therapies (27).

CONCLUSION

The number of potential IBD susceptibility genes continues to increase. The increasing number of genetic loci associated with IBD requires other studies to understand how they involve immunity and inflammation in susceptible individuals.

The identification of genetic variants may define a specific disease phenotype to help follow clinical progression and eventually develop new targeted therapies.

However, the evidence that genetic factors contribute in small part to disease pathogenesis confirms the important role of microbial and environmental factors.

Epigenetic mechanisms may affect development and progression of IBD, mediating interactions between genome and environment. Epigenomics is an emerging field, and future studies could provide new insight into the pathogenesis of IBD.

The role of miRNA in IBD represents a new pathway for discovery of disease mechanisms, diagnostics, and therapeutics.

These new discoveries on the genetics of IBD imply that future research on interactions between genes and between genes and environment will be essential to better understand the pathogenesis of these diseases and more appropriate medical therapy.

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REVIEW
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Nod2: A Critical Regulator of Ileal Microbiota and Crohn's Disease

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The human intestinal tract harbors large bacterial community consisting of commensal, symbiotic, and pathogenic strains, which are constantly interacting with the intestinal immune system. This interaction elicits a non-pathological basal level of immune responses and contributes to shaping both the intestinal immune system and bacterial community. Recent studies on human microbiota are revealing the critical role of intestinal bacterial community in the pathogenesis of both systemic and intestinal diseases, including Crohn's disease (CD). NOD2 plays a key role in the regulation of microbiota in the small intestine. *NOD2* is highly expressed in ileal Paneth cells that provide critical mechanism for the regulation of ileal microbiota through the secretion of anti-bacterial compounds. Genome mapping of CD patients revealed that loss of function mutations in *NOD2* are associated with ileal CD. Genome-wide association studies further demonstrated that *NOD2* is one of the most critical genetic factor linked to ileal CD. The bacterial community in the ileum is indeed dysregulated in *Nod2*-deficient mice. *Nod2*-deficient ileal epithelia exhibit impaired ability of killing bacteria. Thus, altered interactions between ileal microbiota and mucosal immunity through *NOD2* mutations play significant roles in the disease susceptibility and pathogenesis in CD patients, thereby depicting NOD2 as a critical regulator of ileal microbiota and CD.

Keywords: NOD2, ileal Crohn's disease, Paneth cells, microbiota

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NOD2, A MEMBER OF THE NLR FAMILY, REGULATES INNATE AND ADAPTIVE IMMUNE RESPONSES

NLR [Nucleotide binding domain (NBD)-leucine rich repeats (LRR) containing or nucleotide-binding oligomerization domain (NOD)-like receptor] proteins play an important role in shaping the immune system. Humans have 22 NLR protein members and mice have 34 members whereby abundance may vary in relation to cell types (1–4). While certain NLR protein members are critical for the early embryogenesis (5, 6) or upregulation of major histocompatibility complex (MHC) molecules (4, 7–10), other group of NLR proteins have important roles in recognizing pathogen and damage-associated molecular patterns (PAMPs and DAMPs, respectively) in the cytoplasm and eliciting innate immune responses (1, 11–16). NLRs have a tripartite structure consisting of an amino-terminal effector domain, a central NBD, and a carboxy-terminal LRR (17). Most NLR proteins contain distinct protein–protein association domains, such as caspase recruitment domain (CARD), pyrin domain (PYD), or a baculovirus inhibitor repeat (BIR). These variations of the amino-terminal domain are used to categorize NLRs into major subfamilies, which include NLRCs (NLRs containing CARD), NLRPs (NLRs containing Pyrin), and others (1, 3, 11, 14, 18–20). The central NBD, found in all NLRs, is involved in oligomerization and activation (1, 3). The carboxy-terminal LRRs may be

used for the recognition of PAMPs and DAMPs by many NLRs, except NLRP4 and NAIP (Neuronal Inhibitor Apoptosis Protein) that do not use LRRs for ligand recognition (1, 3, 20–24).

NOD2 is a 110 kDa cytosolic protein (1040 amino acids) with two CARD domains, thereby is a part of the NLRC subfamily (13, 15). Known as a major genetic risk factor for Crohn's disease (CD), *NOD2* gene is located on human chromosome 16p21. Upstream of the transcription start site of *NOD2,* are two NF-κB-binding sites within the promoter at 26 and 301 bp as well as vitamin D receptor-binding sites within the *NOD2* gene (25–27). *NOD2* is highly expressed in myeloid cells, such as dendritic cells and macrophages, but expression is low in T cells (27–30). Furthermore, *NOD2* is highly expressed in Paneth cells in the ileum with a lesser degree in epithelial cells from the intestines, oral cavity, and lungs (28, 29, 31–33). *NOD2* expression is induced by bacterial components (e.g., LPS), short-chain fatty acids (e.g., butyrate), hormonal vitamin D (1,25-dihydroxyvitamin D_3), and pro-inflammatory cytokines (e.g., TNF-α) (25, 27, 28, 34–39). The expression of *NOD2* and downstream kinase, receptor-interacting serine/threonine-protein kinase 2 (*RIP2)* in the intestine is largely dependent on the presence of gut microbiota as the expression of these two genes is significantly low in germ-free mice and can be restored by colonization of single strains of bacteria or microflora from mice under SPF conditions (28, 31, 35).

It has been shown that NOD2 recognizes muramyl dipeptide (MDP), *N*-acetylmuramyl-L-alanyl-D-isoglutamine (40–42). LRRs at C-terminus are responsible for direct recognition of MDP, although affinity is not strong for *in vitro* association assays (13, 15). MDP is derived from peptidoglycan found mainly in Gram-positive bacteria and to a lesser extent in Gram-negative bacteria. MDP can be transported into the cytoplasm of mammalian cells from endo/phagosome through transporters, such as solute carrier family protein 5 (SLC15A4) (43–45). Both MDP recognition and NOD2 oligomerization are enhanced when ATP binds to the Walker A motif in the NBD, known to regulate the activity of most NLRs (13, 15).

NOD2 activation promotes oligomerization and recruitment of RIP2 by CARD-CARD homophilic interactions (**Figure 1**) (36, 46–48). E3 ligases [TNF receptor associated factor 6 (TRAF6), TNF receptor associated factor 2 (TRAF2), cellular inhibitor of apoptosis protein (cIAP2)] are recruited to this NOD2-RIP2 platform to ubiquitinate NEMO (NF-κB essential modulator) at amino acid R285 and R399 (42, 47, 49, 50). Whereas NOD2 is subsequently ubiquitinated and undergoes proteasomal degradation (51), TGFβ-activated kinase 1 (TAK1) and ubiquitinated NEMO form a complex within proximity to activate IκB kinase (IKK) that initiates the NF-κB signaling pathway (37, 46, 50). Once phosphorylated by IKK, IκB-α is targeted to proteasomal degradation that permits free NF-κB to translocate into the nucleus (35, 42, 46, 47, 50) (**Figure 1**).

In addition to NF-κB activation, the mitogen-activated protein kinases (MAPK), such as p38 and ERK, are activated in a TRAF6-dependent manner (42, 50) (**Figure 1**). Activation of these signaling cascades upon NOD2 stimulation induces the production of a variety of cytokines, chemokines, and antimicrobial peptides (TNF-α, IL-6, IFN-γ, IL-1β, IL-10, IL-8/CXCL8, α-defensin) depending on cell types (30, 52). NOD2 signal cascade is enhanced by toll-like receptor (TLR) agonists through NF-κB. Along with MDP, this relationship further highlights the impact of microbial components on the NOD2 pathway (1, 30, 53).

Through the innate immune system, NOD2 provides a defensive strategy to protect the hosts against bacterial infection. Various epithelial cells throughout the human body not only create a physical barrier but also produce antimicrobial peptides to regulate bacterial colonization (29, 31, 54). In addition to *NOD2* gene, mutations in *ATG16L1* (Autophagy-related 16 Like 1) gene are a risk factor for CD (55). ATG16L1 is a component of a large protein complex essential for autophagy, a mechanism that degrades intracellular components using the lysosome. As NOD2 is capable of recruiting ATG16L1 to the plasma membrane at the bacterial entry site, *NOD2* mutants failed to recruit ATG16L1, ultimately impairing autophagosomal encapsulation of invading bacteria in dendritic cells (56, 57). Therefore, both ATGL16 and NOD2 have interrelated roles for regulating the microbial invasion that has yet to be studied in Paneth cells (**Figure 1**). However, in Paneth cells, NOD2 recruitment onto dense vesicles helps coordinate cargo-sorting events that support host defenses (58). Therefore, NOD2 function and related responses regulate interactions between innate immunity and the intestinal microbiota.

As noted above, NOD2 and TLR signaling cooperate to enhance immune responses. This synergy of NOD2 and TLR activation affects the polarization of T cell adaptive immunity (Th1 vs. Th2 immune responses) (42, 52, 59, 60). While it is well documented that the NOD2 pathway cooperates with signals through NOD1 or various TLRs (e.g., TLR3, TLR4, TLR9) to enhance Th1 immune responses, Th2 responses are also induced through the cooperation of NOD2 with TLR2 agonists (35, 52, 61, 62). Regarding humoral immunity, NOD2 is capable of influencing antigen-specific immunoglobulin production based on studies using *Nod2*-deficient mice (42, 52). Thus, *NOD2* expression impacts immune responses as well as bacterial killing and colonization in the ileum (25, 31, 34, 35, 52, 61–63). In addition to bacterial components, dietary fiber as well as vitamin D-containing products also induces expression of *NOD2* to further increase the regulation of the microbiota (26, 28, 35).

ETIOLOGY AND PATHOPHYSIOLOGY OF CROHN'S DISEASE

Crohn's disease is a chronic, relapsing inflammatory disorder of the gastrointestinal tract, most commonly involving the ileum and colon. CD patients typically suffer from frequent and chronically relapsing flares with diarrhea, abdominal pain, rectal bleeding, and malnutrition. Histologically, CD is characterized by transmural infiltration of lymphocytes and macrophages with granuloma. Because of the clinical and histological features, most CD patients require lifelong treatment, such as medication and surgery. For efficient treatment of CD, it is important to identify patients who carry risk factors for complications of the disease and initiate appropriate therapy at early phase of the clinical course (64).

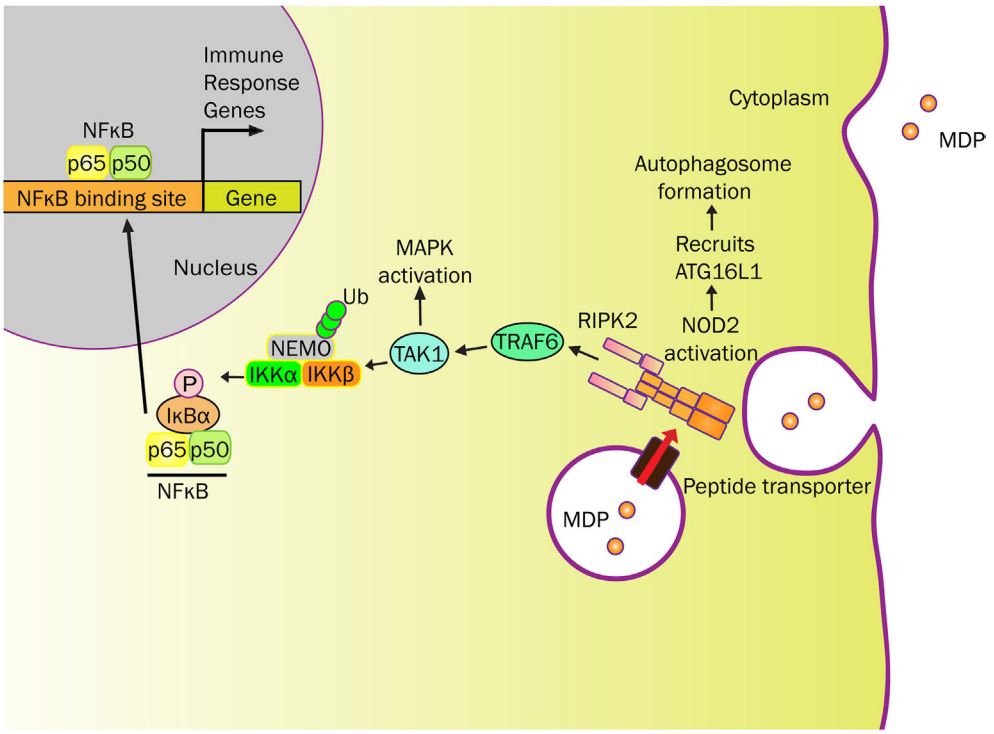

FIGURE 1 | NOD2 senses MDP and elicits immune responses. MDP-derived from bacterial cell wall can be transported into the cytosol by endo/phagosome through peptide transporters (e.g., SLC15A4). MDP activates NOD2 to initiate RIPK2 recruitment resulting in platform formation. This cytosolic NOD2-initiated structure localizes E3 ligases (e.g., TRAF6) to ubiquitinate NEMO, for which NEMO and TAK1 forms a complex near IKK. In addition to activating the mitogen-activated protein kinase (MAPK) pathway, TAK1 phosphorylates IKK to initiate the NF-κB signaling pathway. IκB-α is phosphorylated by IKK, which targets IκB-α to proteasome degradation. No longer restricted, free NF-κB translocates into the nucleus and activates various immune response genes. NOD2 also recruits ATG16L1 near the plasma membrane to promote autophagosome formation, known to degrade antigenic structures. The release of cytokines and chemokines, initiated by NOD2, recruits immune cells to influence Th1 and Th2 responses.

In the healthy human intestine, the intestinal epithelium provides an effective barrier against luminal bacteria with the help of epithelial tight junction and the mucus layer (**Figure 2**). However, in patients with inflammatory bowel diseases, this barrier function is often compromised due to genetic, environmental, microbial, and immunological factors (65, 66). Dysfunctional mucosal barrier allows the penetration of bacterial products, leading to direct interaction with immune cells (**Figure 2**). Innate immune cells, such as macrophages and dendritic cells produce cytokines, such as TNF-α, IL-1β, IL-6, IL-12, IL-23, and chemokines upon recognition of microbes and microbial products. Activated dendritic cells promote the differentiation of $CD4^+$ T cells into pro-inflammatory T cell subsets, such as Th1 or Th17 effector T cells, which secrete additional cytokines and chemokines (65, 66). Recently identified novel family of innate immune cells termed innate lymphoid cells (ILCs), in particular the type 1 ILC (ILC1) subset, produce high amount of pro-inflammatory cytokines, such as IFN-γ and TNF-α (67–69). High levels of chemokines produced by these innate and adaptive immune cells further recruit lymphocytes, creating a vicious positive feedback cycle of inflammation in CD.

NOD2: THE HIGHEST RISK FACTOR IN ILEAL CD

Recent Genome-wide association studies (GWAS) revealed 163 susceptibility loci for IBD (inflammatory bowel disease), 30 of them being specific to CD (70). Among them, *NOD2* was the first gene identified as a risk factor for ileal CD (71, 72), discovered by the genetic mapping study of the CD susceptibility locus (73). One of the cell types that express *NOD2* at a high level is the Paneth cell, most of which are located in the terminal ileum (32). Paneth cells play an important role in the innate regulation of gut microbiota by synthesizing and secreting antimicrobial peptides or proteins. Upon the stimulation with bacterial products, such as the NOD2 ligand, MDP, Paneth cells secrete antimicrobial peptides, including lysozyme, secretory phospholipase A2 (sPLA2), and human α-defensins 5 and 6 (HD5 and HD6) in the intestinal lumen (74). Therefore, *NOD2* mutations lead to dysregulation of host–microbe interactions, which increases the susceptibility to abnormal ileal inflammation. In addition to this microbial dysbiosis, other genetic or environmental factors trigger the development of ileal CD.

A meta-analysis showed the risk for CD development was increased to 17.1-fold in *NOD2* homozygotes or compound

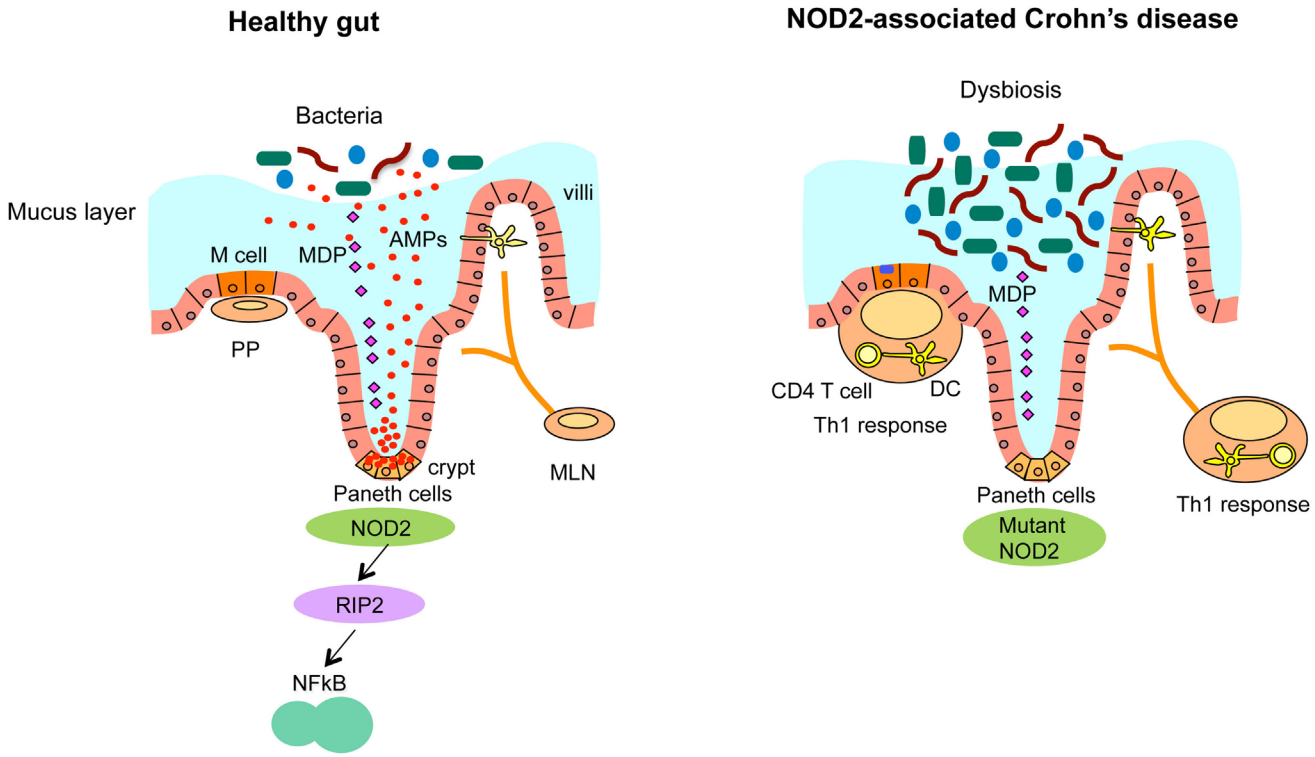

FIGURE 2 | NOD2-associated dysregulated microbiota leads to the susceptibility of CD. Ileum in healthy individuals (left panel). NOD2 senses microbiota-derived MDP and activates NF-κB through the downstream kinase receptor-interacting protein 2 (RIP2), which is critical for the bacterial killing activity of Paneth cells via the secretion of anti-bacterial compounds. Ileum with loss of *NOD2* function (right panel). In CD, that is associated with *NOD2* mutations, CD-associated *NOD2* mutants disturb many characteristics of gut immune homeostasis, including reduced MDP sensing, and impaired antimicrobial responses in Paneth cells, leading to defective bacterial clearance. This dysbiosis caused by impaired Paneth cell function is characterized by increased load of bacteria and abnormalities of Peyer's patches (PP) and mesenteric lymph nodes (MLN) that stimulate the mucosal immune system to induce Th1 immune response, leading to chronic inflammation.

heterozygotes, and 2.4-fold in simple *NOD2* heterozygotes (**Table 1**) (75). Three main variants or polymorphisms in *NOD2* gene (i) a frame shift mutation at position 1007 (1007fs); (ii) a glycine to arginine conversion at amino acid residue 908 (G908R); and (iii) an arginine to tryptophan conversion at amino acid residue 702 (R702W) were highly associated with susceptibility to CD (71, 72, 76). All three mutations are located within or close to the LRR domain that recognizes MDP. Indeed, patients with ileal CD showed reduced levels of HD5 and HD6 derived from Paneth cells (77, 78). Additionally, the production of α-defensins is particularly reduced in patients with non-functional *NOD2* mutations (79, 80). These findings showed that CD-related mutations in *NOD2* are loss of function and individuals carrying *NOD2* mutations are at high risk for ileal CD.

Furthermore, many studies have suggested that *NOD2* mutations increase the risk of complications in CD (**Table 1**). It was shown that any of the *NOD2* mutations are independent predictive factor for stricturing [odds ratio (OR) = 1.82], fistulizing (OR = 1.25), and need for surgery (OR = 2.96) (81). A meta-analysis reported that the risk for complicated disease, such as stricturing or fistulizing, was increased by 8% [relative risk (RR) = 1.08] in *NOD2* heterozygotes and 41% (RR = 1.41) in *NOD2* homozygotes or compound heterozygotes (82). Likewise, the risk of surgery was increased by 58% (RR = 1.58) with any of the *NOD2* mutations (82). In terms of each risk allele, p.1007fs mutation is the strongest disease predictive factor. p.1007fs was associated with stricturing (OR = 1.38) and the necessity of an operation (OR = 1.69) (83). The homozygous carriers of p.1007fs were significantly younger at the time of diagnosis (under the age of 26 years). Fourteen out of 19 homozygous carriers of p.1007fs (73.7%) required surgery and 11 out of 14 patients (78.6%) underwent the re-operation due to re-stenosis (84). The mutation of p.G980R is also associated with disease complications [RR = 1.33 (82), OR = 1.65 (83)]. Interestingly, multiple studies indicated that association of *NOD2* mutations with CD were found in European and Ashkenazi Jewish ancestry population, but not in CD patient cohort of Asian ancestry, such as Japanese, Chinese, Korean, or Indian, indicating the impact of ethnic genetic background on the CD susceptibility caused by *NOD2* mutations (85–88).

Taken together, *NOD2* mutation and the susceptibility of ileal CD are strongly related via altered interaction between ileal microbiota and mucosal immunity. Carrying the *NOD2*

TABLE 1 | *NOD2* mutations in CD patients.

Reference	*NOD2* mutation type	Risk for	Increase of risk
Economou et al. (75)	Single risk allele[a]	Development of CD	2.39-fold (OR = 2.39)
	Two or more risk alleles[a]	Development of CD	17.1-fold (OR = 17.1)
Cleynen et al. (81)	Any *Nod2* mutations	Ileal location	1.90-fold (OR = 1.90)
		Stricturing	1.82-fold (OR = 1.82)
		Fistulizing	1.25-fold (OR = 1.25)
		Need for surgery	2.96-fold (OR = 2.96)
Adler et al. (82)	Single risk allele[a]	Stricturing or fistulizing	8% (RR = 1.08)
	Two risk alleles[a]	Stricturing or fistulizing	41% (RR = 1.41)
	Any risk alleles[a]	Need for surgery	58% (RR = 1.58)
	G980R	Stricturing	33% (RR = 1.33)
Weersma et al. (83)	1007fs	Ileal location	1.83-fold (OR = 1.83)
		Stricturing	1.38-fold (OR = 1.38)
		Need for surgery	1.69-fold (OR = 1.69)
	G980R	Stricturing	1.65-fold (OR = 1.65)
Seiderer et al. (84)	1007fs	Early onset	73.7% (14/19 patients)
		Re-operation (re-stenosis)	78.6% (11/14 patients)

[a]*Risk allele: 1007fs, G908R or R702W.*
OR, odds ratio; RR, relative risk.

mutation is the strong risk factor for ileal CD with multiple complications.

ROLE OF MICROBIOTA IN ILEAL CD

The surfaces of the body, particularly the gastrointestinal tract, are exposed to a large number of diverse microbes that are collectively referred to as the microbiota. Composition of the microbiota can be significantly affected by the genetic background of hosts along with several other factors, such as diet, age, stress, and diseases (89). While commensal bacteria colonize all mucosal surfaces and the skin, the intestinal tract harbors the largest bacterial load (up to 10^{14} total bacteria) (90, 91). It is becoming increasingly clear that the close symbiotic relationship between host and an adaptable microbiota forms a vital part of the intestinal homeostasis (92, 93). The host microbiota plays important roles in several processes such short-chain fatty acid production (94), the development of intestinal epithelium (95, 96), protection against invading pathogens, and, importantly, the development of mature immune system (96). The intestinal microbiota provides both inflammatory and anti-inflammatory products, which modulate the immune responses (97, 98) and dysbiosis in the microbiota is considered pivotal to the origin of many diseases. It is well known that the disease outcome can be influenced by the host microbiota either due to the increased colonization of pathogenic strains or the introduction of new bacterial species. This is well-exemplified in IBD patients who typically exhibit imbalances in the microbiota composition. Moreover, reducing exposure to intestinal bacteria in these patients has been shown to frequently alleviate inflammation (99, 100).

ROLE OF NOD2 IN THE INTERACTION BETWEEN MICROBIOTA AND ILEAL HOST IMMUNITY

NOD2 plays an important role in mediating host–microbe interactions in the intestine. Interestingly, NOD2 has been shown to contribute to the homeostatic maintenance of the gut microbiota. Abnormal *NOD2* function associated with LRR polymorphisms leads to an inflammatory response mediated by innate immune functions (42, 71, 72, 101). NOD2 controls the expression and secretion of antimicrobial peptides thereby acting as an important regulator of the ileal commensal microbiota in mice (31). *Nod2*-deficient mice exhibit reduced bactericidal activity, higher loads of commensal bacteria and increased susceptibility to colonization by pathogenic bacteria (31, 102), and are, thus, prone to bacterial infections and intestinal inflammation (30, 42). NOD2 function, therefore, may be pivotal in understanding the balance between the host immune system and commensal bacteria.

NOD2-MEDIATED REGULATION OF ILEAL MICROBIOTA

NOD2 is critical for regulating the bacterial flora in the ileum through the secretion of anti-bacterial compounds (31, 42, 103–105) (**Figure 2**). Earlier studies in *Nod2*-deficient mice indicated the role of NOD2 in the homeostasis of gut epithelium, possibly linking it to the development of CD (42). Furthermore, increased *NOD2* expression is observed at the sites for α-defensin secretion, such as intestinal crypts in mice (42) and Paneth cells in human (32, 33). *Nod2*-deficient mice harbor larger loads of bacteria in the ileum due to impaired Paneth cell function, compared to littermate controls even in the same cage (31). *Nod2*-deficient mice are susceptible to Th1-dominant granulomatous inflammation in the ileum, which is induced by an opportunistic pathogen *Helicobacter hepaticus* and ileal dysbiosis (2, 30). Furthermore, *NOD2* expression in the intestine is regulated by signals from the microbiota, given that germ-free mice had lower *NOD2* expression that was reversible upon monocolonization with commensal bacteria (31). Zhang et al. found that NOD2-mediated lysozyme trafficking in Paneth cells is directed by commensal bacteria, which is important for regulating intestinal infection (58). Two different steps, selective lysozyme trafficking and final secretion (106) are separately regulated by commensals implicating a new mechanism by which symbiotic bacteria direct a lysozyme-trafficking event to promote host defense against pathogens.

Several studies have shown the role of NOD2 in host protection against pathogens in a number of bacterial infectious models including *Listeria monocytogenes* (42), *Staphylococcus aureus* (107), *Chlamydophila pneumoniae* (108), *Streptococcus pneumoniae* (109), and *Mycobacterium tuberculosis* (110). *Nod2*-deficient mice were susceptible to *L. monocytogenes* infection via oral but not systemic routes, suggesting that NOD2 might have a non-superfluous role in intestinal antimicrobial responses (11).

The discovery that *NOD2* mutations are strongly associated with CD highlighted the importance of NOD2 in the regulation

of antimicrobial responses. Loss of function mutations in *NOD2*, rendering it unresponsive to peptidoglycan component, MDP, have been implicated in the development of CD (41, 111, 112). Interestingly, patients with mutant variants of *NOD2* have altered microbiota in the ileum (32, 33, 113–117). There was a significant decrease in the relative frequencies of *Clostridia* and an increase in the *Actinobacteria* and *Proteobacteria* in CD patients compared to healthy controls (117).

Apart from having role in the bactericidal ability of Paneth cells, NOD2 also shapes the microbial community profiles via local production of reactive oxygen species (118–120). Adult *Nod2*-deficient mice display a substantially altered microbial community structure and a significantly elevated bacterial load in their feces and terminal ileum compared to their wild-type counterparts (114). Interestingly, these findings were also present in weaning-age mice, indicating a subtle influence of NOD2 on the early development and composition of the intestinal microbiota. Increased load of the *Bacteroidetes* and *Firmicutes* phyla as well as the genus *Bacteroides* was observed in the terminal ileum of CD patients with homozygosity in *NOD2* mutations (114). In addition, altered frequencies of *Faecalibacterium* and *Escherichia* have also been associated with *NOD2* risk alleles in CD patients. *Nod2*-deficient mice harbor increased bacterial load in the feces and ileum along with increased abundance of *Bacteroidetes* and *Firmicutes* in comparison to wild-type mice (31, 114, 121). These studies are supported by the observations of Petnicki-Ocwieja et al., who used the most strict experimental conditions taking into account the usage of littermates and bacterial quantification by qPCR, with the prominent difference observed only in ileum (31).

Several abnormalities in the small-intestinal epithelium of *Nod2*-deficient mice were investigated which included the inflammatory gene expression and goblet cell dysfunction (102). Ramanan et al. demonstrated that these abnormalities were associated with excessive interferon-γ production by intraepithelial lymphocytes (IELs) and Myd88-dependent signaling, and were dependent on the expansion of a common member of the intestinal microbiota, *Bacteroides vulgatus*. Although this study implies NOD2 prevents harmful immune responses by controlling the expansion of a pro-inflammatory member of the microbiota (102), the composition of the gut microbiota may vary among mice from different laboratories, which may lead to the variations in the experimental outcome (31, 114, 121).

NOD2 AND GUT IMMUNE HOMEOSTASIS

The constant exposure of the intestinal tissue to gut microorganisms maintains the mucosa in a state of physiological inflammation, which balances tolerogenic and pro-inflammatory type responses to maintain homeostasis. In line with the idea that NOD2 signaling might indirectly regulate T cell populations, results presented in a recent study indicated that there are dysfunctional and reduced numbers of IELs in *Nod2*-deficient mice (122). These effects of *NOD2* deficiency could hamper the integrity of intestinal epithelium and lead to an altered immune response to the resident microbiota. A recent study showed that there is an increased sensitivity of *Nod2* as well as *Rip2*-deficient mice to DSS-induced colitis and colonic adenocarcinoma as a result of dysbiosis, and that the effect can be transmitted to wild-type mice through the microbiota (123). However, such differences in the composition of gut microbiota as observed in naive mice deficient in NOD2 signaling components is debatable as many other studies could not arrive at the same conclusion (124–126), depicting that, in accordance with the study in TLR-deficient mice (127), changes in the relative abundance of certain bacterial groups in *Nod2*-deficient mice emulate caging conditions and familial transmission rather than their NOD2 genotype. In regard to this discrepancy, the question of whether *NOD2* deficiency can cause different composition of intestinal bacteria requires additional study. Likewise, different reports in human studies suggest a reduced abundance of certain bacteria, including *Faecalibacterium prausnitzii* and other butyrate-producing organisms (128) in patients with colitis, but whether these changes occur before the onset of inflammation is an important question in the field. Continuing studies, such as these will help to determine whether dysbiosis occurs before disease onset and whether particular microbial profiles are indeed early biomarkers of disease susceptibility.

Studies conducted in healthy SPF mice support the observation in which failure of NOD2 to induce secretion of antimicrobial compounds allows for increase of certain bacterial groups. Studies by Petnicki-Ocwieja et al. (31) reported increased loads of *Bacteroides* and *Firmicutes* groups in the ileum of *Nod2*-deficient mice compared to wild-type/heterozygous mice from the same litter. However, no such difference was observed in the feces of these mice. This observation was attributed to the decreased bactericidal activity of crypt-secreted factors, such as α-defensins (42). Similarly, increased abundance of *Bacteroides* was also reported in the ileum of *Rip2*-deficient mice compared to wild-type littermates (31). However, Rehman et al. (114) reported immense abundance of *Bacteroidetes* in the feces of adult *Nod2*-deficient mice, whereas greater load of *Firmicutes* was observed only in the terminal ileum of these mice. Mondot et al. (121) reported increased colonic abundance of the *Rikenellaceae*, *Bacteroidaceae* and *Prevotellaceae* families in *Nod2*-deficient mice compared to wild-type mice with no specific housing conditions. In conclusion, these studies indicate the increased bacterial load in the ileum of *Nod2*-deficient mice although the variations observed in these studies can be attributed to different housing conditions.

Microbial dysbiosis in CD is likely to be shaped by the chronic inflammatory state of the intestine. Abnormal microbial composition in association with the detrimental genetic background increases the predisposition of *Nod2*-deficient mice to inflammatory diseases. Several studies on the microbiota in *Nod2*-deficient mice reveal its altered diversity as well as richness (114, 121). These studies emphasize the importance of NOD2 in regulating the intestinal microbiota composition and the subsequent outcome of CD.

Previous studies indicate an important role of NOD2 in the development of GALT (Gut associated lymphoid tissues) during the bacterial colonization of gut (129). In *Nod2*-deficient mice, high proportion of $CD4^+$ T cells, increased inflammatory cytokine levels, and higher antigen and bacterial permeability

rates (124) were observed, indicating an increased stimulation of Peyer's patches (PP) by resident gut microflora. All these changes observed in these mice were suppressed after treatment with oral antibiotics. Therefore, apart from influencing the development of the GALT, NOD2 has a role in modulating the immune response by limiting the development of a Th1 immune response toward bacteria. The results described above support the observation that activation of NOD2 in dendritic cells controls their ability to induce a polarized Th1 response in $CD4^+$ T cells (130), thus elucidating the role of NOD2 in hematopoietic cells. However, this cannot explain why the phenotype of *NOD2* mutation is only associated with ileal lesion. Also, the Th1 immune response phenotype was not observed in other studies unless colonized with *Helicobacter* species (30).

OTHER POSSIBLE MECHANISMS CAUSED BY *NOD2* MUTATIONS

Amendola et al. reported that the absence of spontaneous colonic inflammation in *Nod2*-deficient mice can be attributed to altered gut permeability caused by the high mucosal cytokine production and increased activity of MLCK. MLCK, myosin light chain kinase, is a factor that likely causes the increased gut permeability in *Nod2*-deficient mice due to its effects on tight junctions (131). *NOD2* deficiency, therefore, causes the permeability changes that increase the exposure of dendritic cells to factors, such as TLR ligands that in turn influence Treg cell development and subsequent changes in the microbiota. These results are in agreement with the studies in IBD patients where increased gut permeability and an altered microbiota are associated with *NOD2* polymorphisms with no inflammation (116, 117, 132, 133). Recently, it was found that *NOD2* is expressed in Lgr5+ stem cells using culture of intestinal crypt organoids, indicating that NOD2 may contribute to epithelial homeostasis by regulating stem cell function in addition to bacterial killing activity of Paneth cells (134).

Autophagy has emerged as a pivotal component of the innate immune response to intracellular bacteria and is also involved in the delivery of microbial ligands to intracellular compartments containing TLRs to trigger an antimicrobial response. Three groups revealed that autophagy is activated by NOD2 to augment intracellular bacterial killing (56, 57, 135). The studies by Travassos et al. (57) demonstrated that bacterial sensing by NOD proteins is linked to the induction of autophagy, thus providing a functional association between *NOD2* and *ATG16L1*, two of the most important genes associated with CD. Cooney et al. demonstrated that bacterial killing and MHC class II-dependent antigen presentation in primary human dendritic cells are increased by MDP-activated autophagy and this process involves ATG16L1, NOD2, and RIP2, but is independent of NLRP3 (56). Dendritic cells from CD patients expressing CD-associated *NOD2* or *ATG16L1* risk variants have the defect in autophagy induction, bacterial trafficking, and antigen presentation. Hence, *NOD2* polymorphism along with the defective lysosomal degradation, and impaired induction of antigen-specific $CD4^+$ T cells would lead to bacterial persistence and thereby the inflammatory responses in CD (56). Furthermore, a recent study showed that ATG16L1- or NOD2-deficient DCs have impaired capability to induce Tregs upon stimulation with immunomodulatory molecules from the commensal *Bacteroides fragilis*, indicating the immunosuppressive role of NOD2 and ATG16L1 in intestinal dendritic cells (136). However, this suppressive function does not explain the critical role of NOD2 and ATG16L1 in Paneth cells and ileum specific inflammation (137).

Nod2 deficiency in mice, thus, results in increased intestinal bacterial load and greater susceptibility to pathogenic bacterial colonization (31), which could result from either Paneth cell dysfunction, defects in intestinal autophagic responses, or more likely, a combination of the two. Thus, in addition to Paneth cell defects, *ATG16L1* and *NOD2* risk variants affect the intestinal epithelial cell antimicrobial responses, probably changing the gut microbiome and enhancing CD susceptibility (135).

PERSPECTIVES

Although more studies are needed to fully understand the CD pathogenesis caused by *NOD2* mutations, recent studies have shown the important roles of NOD2 in the interactions between microbiota and the intestinal immune system. In the studies using *Nod2*-deficient mice, it was shown that NOD2 is critical for the function of bactericidal activity of ileal crypts and the regulation of ileal microbiota. Therefore, it is tempting to speculate that ileal CD caused by *NOD2* mutations is mainly due to the dysfunction of Paneth cells. Under physiological condition, microbiota in the ileum is tightly regulated by bacterial killing activity of Paneth cells in healthy individuals. This regulates both pathogenic and non-pathogenic bacteria as well as bacterial antigens that constitutively stimulate the intestinal immune system. An increased load of microbiota alone is likely not sufficient to induce CD pathogenesis, since inflammatory responses in mucosal immunity are tightly regulated by various mechanisms. However, in the presence of other genetic, environmental, and immunological factors, dysbiosis of bacteria may increase susceptibility to ileal inflammation by enhancing stimulation of the intestinal immune system beyond the controlled physiological threshold, leading to pathological changes and, finally, to chronic inflammation. Although the studies of Paneth cells and microbiota in *Nod2*-deficient mice and CD patients support this scenario, further research is required to fully elucidate the molecular mechanism of CD. Additional studies on CD-associated microbiota and animal models of ileal CD may provide a greater insight to develop better treatment and management methods of CD patients.

AUTHOR CONTRIBUTIONS

All authors listed have made substantial, direct, and intellectual contribution to the work, and approved it for publication.

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Dysfunctional Crohn's disease-associated NOD2 polymorphisms cannot be reliably predicted on the basis of RIPK2 binding or membrane association

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Polymorphisms in NOD2 represent the single greatest genetic risk factor for the development of Crohn's disease. Three different non-synonomous NOD2 polymorphisms – R702W, G908R, and L1007fsincC – account for roughly 80% of all NOD2-associated cases of Crohn's disease and are reported to result in a loss of receptor function in response to muramyl dipeptide (MDP) stimulation. Loss of NOD2 signaling can result from a failure to detect ligand; alterations in cellular localization; and changes in protein interactions, such as an inability to interact with the downstream adaptor protein RIPK2. Using an overexpression system, we analyzed ~50 NOD2 polymorphisms reportedly connected to Crohn's disease to determine if they also displayed loss of function and if this could be related to alterations in protein localization and/or association with RIPK2. Just under half the polymorphisms displayed a significant reduction in signaling capacity following ligand stimulation, with nine of them showing near complete ablation. Only two polymorphisms, R38M and R138Q, lost the ability to interact with RIPK2. However, both these polymorphisms still associated with cellular membranes. In contrast, L248R, W355stop, L550V, N825K, L1007fsinC, L1007P, and R1019stop still bound RIPK2, but showed impaired membrane association and were unable to signal in response to MDP. This highlights the complex contributions of NOD2 polymorphisms to Crohn's disease and reiterates the importance of both RIPK2 binding and membrane association in NOD2 signaling. Simply ascertaining whether or not NOD2 polymorphisms bind RIPK2 or associate with cellular membranes is not sufficient for determining their signaling competency.

Keywords: Crohn's disease, inflammation, innate immunity, NLR, NFκB, signal transduction, membrane localization, RIP2

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Abbreviations: CARD, caspase activation domain; LRR, leucine-rich repeat; MDP, muramyl dipeptide; NFκB, nuclear factor κB; NOD2, nucleotide oligomerization domain 2; RIPK2, receptor-interacting serine/threonine kinase 2; SNP, single nucleotide polymorphism.

INTRODUCTION

Nucleotide oligomerization domain 2 (NOD2) is a cytoplasmic pattern recognition receptor that has been connected with a variety of inflammatory disorders including Crohn's disease, Blau syndrome, asthma, sarcoidosis, and arthritis (1). NOD2 is organized into three primary functional domains: an N-terminal effector region consisting of two caspase activation domains (CARDs); a central nucleotide-binding domain; and a C-terminal series of leucine-rich repeats (LRRs). NOD2 is maintained in an inactive state in the cell by interactions with chaperones, such as HSP90 and SGT1 (2, 3). However, detection of muramyl dipeptide (MDP), a component of bacterial peptidoglycan, along with binding and hydrolysis of ATP initiates receptor activation. Formation of the active signaling complex, most likely occurs at cellular membranes and signal propagation requires interaction with specific adaptor proteins. This primarily involves CARD-mediated interaction with the adaptor kinase receptor-interacting protein kinase 2 (RIPK2), but other proteins, such as CARD9 and TRAF4, can also be involved. This stimulates a pro-inflammatory immune response via nuclear factor kappa B (NFκB) and mitogen-associated protein kinase (MAPK) pathways.

Crohn's disease is a chronic inflammatory bowel disorder in which intestinal immune responses, most commonly in the ileum and colon, become dysregulated. The etiology of Crohn's disease is complex and whilst the precise cause, or causes, is uncertain, it is clear that genetic factors are an important contributory component to disease progression. Of the more than 70 genes associated with Crohn's disease, polymorphisms in *NOD2* remain the single greatest genetic risk factor (4, 5). Three non-synonomous polymorphisms – R702W, G908R, and L1007fsincC – account for around 80% of all *NOD2*-polymorphism associated cases of Crohn's disease reported, with homozygotic mutation resulting in around a 10-fold greater risk than heterozygotic mutation. Somewhat counter-intuitively all three of the major *NOD2* polymorphisms associated with Crohn's disease result in a loss of receptor function and reduced inflammatory signaling (6, 7). The reduction in NOD2 function has, however, been proposed to contribute to disease via a number of different mechanisms that includes: disruption of the host microbiota, dysregulation of intestinal tolerance, and enhanced activation of other pro-inflammatory signaling pathways (8–11). The precise contribution of these mechanisms to disease pathogenesis, however, remains enigmatic.

Genetic studies have reported numerous other polymorphisms in *NOD2* that associate with Crohn's disease. We were interested in seeing whether these polymorphisms also displayed a loss of function in response to ligand stimulation, and in identifying whether or not dysfunction could be related to disruption of RIPK2 binding and/or membrane association. We generated over 50 NOD2 constructs containing these polymorphisms and assessed their response to ligand stimulation. Twenty-three variants showed a significant reduction in signaling capacity with nine of them (R38M, R138Q, L248R, W355stop, L550V, E825K, L1007fsinC, L1007P, and R1019stop) showing a near complete loss of signaling capacity. Whilst no single cause for the loss of function could be identified, both RIPK2 binding and membrane association were important factors. Overall our data is consistent with the view that Crohn's disease-associated NOD2 polymorphisms result in receptor dysfunction, but that the causes of this dysfunction are multi-factorial.

MATERIALS AND METHODS

Chemicals, Plasmids, Antibodies, and General Methods

Chemical reagents were obtained from Sigma-Aldrich, UK, unless otherwise specified. HEK293T and HeLa cells were maintained in DMEM supplemented with 10% fetal calf serum, 100 µg/ml penicillin/streptomycin and 2 mM L-glutamine at 37°C and 5% CO_2. All transfections were performed using jetPEI™(Polyplus-Transfection) as per the manufacturers' instructions. pCMV-FLAG-NOD2, encoding N-terminally FLAG-tagged full length NOD2; pCI-myc-RIPK2, encoding N-terminally myc-tagged full length RIPK2; and pEF6-V5-mCARD9, encoding N-terminally V5-tagged full length murine CARD9 were kind gifts from Professors Thomas Kufer (12), Kate Fitzgerald, and David Underhill, respectively. Crohn's disease-associated single nucleotide polymorphisms (SNPs) were identified using published literature (5, 6, 13–18) and the NCBI SNP database and generated using site directed mutagenesis. Mutant sequences were verified by DNA sequencing of the entire open reading frame. Plasmids encoding Firefly luciferase under the control of an NFκB (pluc) or IL-8 promoter (pluc-IL8) and Renilla luciferase controlled by a constitutive promoter (phrG) were kind gifts from Prof Clare Bryant. Antibodies used in this work were rabbit anti-FLAG (F7425, Sigma-Aldrich), mouse anti-FLAG M2 (F3165, Sigma-Aldrich), mouse anti-V5 (ab27671; Abcam), mouse anti-GAPDH (ab9485, Abcam), rabbit anti-Myc (ab9106, Abcam), goat anti-rabbit (ab6721, Abcam), goat anti-mouse (A4416, Sigma-Aldrich), Alexa-488 goat anti-mouse (A11001, Life Technologies), and Alexa-555 goat anti-rabbit (A21428, Life Technologies).

HEK293 Reporter Assays

HEK293T cells in 96-well plates were transfected with 2 ng pLuc or pLuc-IL-8, 1 ng phrG, 0.1 ng wild-type or mutant pCMV-FLAG-NOD2, and made up to 0.1 µg total DNA with empty plasmid. One hundred nanograms per milliliter MDP (Invivogen) was added concomitant with transfection. Cells were lysed 24 h post-transfection with 1× passive lysis buffer (Promega) and luminescence measured with a LUMIstar Luminometer (BMG Labtech). Each SNP was tested in triplicate in a minimum of three separate experiments. Reporter assays routinely show variations between experiments as a result of differences in transfection efficiency and cell passage number. In order to allow comparison between experiments, data was normalized to the signal obtained using wild-type NOD2 and expressed as a percentage of wild-type signaling activity.

Data was plotted and analyzed with GraphPad Prism 5. To correct for unequal variance, data was log transformed, then subjected to statistical analysis using a one-way analysis of variance

with a Bonferroni multiple comparison *post hoc* test. Data are expressed as mean + SEM and a p-value of <0.05 was taken as significant.

Immunoprecipitation

HEK293T cells seeded in 6-well plates (Costar) were transfected with 1 μg of the appropriate pCMV-FLAG-NOD2 construct and 0.5 μg pCI-myc-RIP2 and incubated overnight. Cells were lysed and incubated with Dynabeads (Life Technologies) labeled with mouse anti-FLAG (Sigma) as per the manufacturers' instructions. Samples were denatured by the addition of SDS-loading buffer (Invivogen) and heating for 5 min at 90°C prior to separation by SDS-PAGE using 4–20% Tris-glycine gels (NuSep) for 1 h at 200 V. Proteins were transferred to polyvinylidene fluoride membranes for detection by Western Blot using the appropriate antibodies.

Immunofluorescence

HeLa cells were seeded into 12-well plates containing a sterilized 19-mm diameter glass coverslip. Cells were transfected either with 1 μg of wild-type or mutant pCMV-FLAG-NOD2, or with 0.5 μg of each of the appropriate constructs for co-immunofluorescence studies. Cells were incubated overnight before washing (1× PBS) and fixing for 15 min (4% paraformaldehyde in PBS). Cells were washed again (1× PBS), permeabilized [0.4% Triton X-100 (VWR) in 1× PBS] for 10 min, blocked for 20 min (2.5% goat serum, 1% bovine serum albumin in 1× PBS), then incubated with appropriate primary and secondary antibodies. Cells were subsequently washed three times with 1× PBS, the second wash containing a 1:5000 dilution of 10 mg/ml Hoechst 33258 in PBS to stain the nucleus. Coverslips were mounted onto microscope slides (VWR) using Mowiol mounting solution containing 2.5% 1,4-diazabicyclo[2.2.2]octane to reduce fading. Cells were visualized using an AXIO Imager.M2 microscope (Carl Zeiss Ltd., Cambridge, UK) and images created using Image J.

Subcellular Fractionation

This was performed using a Subcellular Protein Fractionation Kit (Perbio Science UK) as per the manufacturers' instructions from HEK293T cells overexpressing the appropriate NOD2 mutant constructs and grown in 12-well plates. Cytosolic and membrane fractions were isolated and analyzed by SDS-PAGE and western blotting. The proportion of NOD2 associated with the membrane was calculated by densitometry using Image J (19).

Bioinformatics

Full length human NOD2 (NP_071445.1) was used to search the non-redundant protein database at NCBI. Recovered sequences with at least 90% coverage of human NOD2 were retained and manually curated to remove proteins that were described as predicted or hypothetical, or which were clear species duplications. In the latter circumstance, the protein with the highest scoring alignment for that species was retained. In total, NOD2 sequences from 30 different species were subsequently submitted for multiple sequence alignment using MUSCLE (20). Accession codes are detailed in Figure S2 in Supplementary Material. Homology models of the NOD2 CARDS were generated using the NOD1 CARD [PDB 2DBD] as a template.

RESULTS

Selection of NOD2 Single Nucleotide Polymorphisms for Study

At the outset of this study, 98 SNPs in NOD2 associated with either Blau Syndrome or Crohn's disease were identified using published data (5, 6, 13–18) and the NCBI database of NOD2 SNPs (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?locusId=64127). The Blau Syndrome-associated polymorphisms were studied, and have been reported, independently of those associated with Crohn's disease (21). Seventeen SNPs previously shown to have an NFκB activity comparable to wild-type NOD2 (6) were excluded from further study, as were three SNPs for which alternative polymorphisms existed at the residue of interest. Nine polymorphisms could not be successfully generated in the NOD2 expression vector. In total, 53 Crohn's disease-associated polymorphisms were taken forward for functional characterization in conjunction with wild-type NOD2 and the inactive Walker-B mutant D379A as control constructs.

Crohn's Disease-Associated NOD2 Polymorphisms can Result in a Defective Response to MDP Stimulation

To determine the effect of the NOD2 polymorphisms on receptor function, we assessed their ability to signal in response to MDP stimulation using NFκB and IL-8 reporter assays. Twenty-three of the polymorphisms, along with the Walker-B mutant D379A, showed a significant reduction in signaling (**Figure 1**). All polymorphisms that showed a significant reduction in signaling activity with the exception of A105T, D113N, D357A (IL-8 only), and P727C (NFκB only) were significantly impaired in both reporter systems. Nine of the polymorphisms (R38M, R138Q, L248R, W355stop, L550V, N825K, L1007fs, L1007P, and R1019stop) and the control D379A showed major functional impairment with signaling below 15% of wild-type NOD2 (**Figure 1**). None of the polymorphisms tested resulted in receptor hyperactivation in response to MDP (Figure S1 in Supplementary Material).

Amino Acid Conservation can be Indicative but does not Correlate with Functional Impact

Amino acids with important functional roles regularly show higher levels of evolutionary conservation. We updated our previous cross-species alignment of NOD2 (22) and compared the level of evolutionary conservation for each of our polymorphic NOD2 variants (**Figure 2**; Figures S1 and S2 in Supplementary Material). Consistent with a key functional role, nine of the polymorphisms that showed a significant reduction in signaling were completely conserved across all aligned species, and a further two (E825 and L1007) were conserved in 29/30 species (**Figure 2**). However, high levels of residue conservation could not be used as a direct predictor of functional impact as seven completely conserved residues showed no impact on signaling (**Figure 2A**). Interestingly, V162 is only conserved in five of the 30 species and despite all of the substitutions being conservative hydrophobic

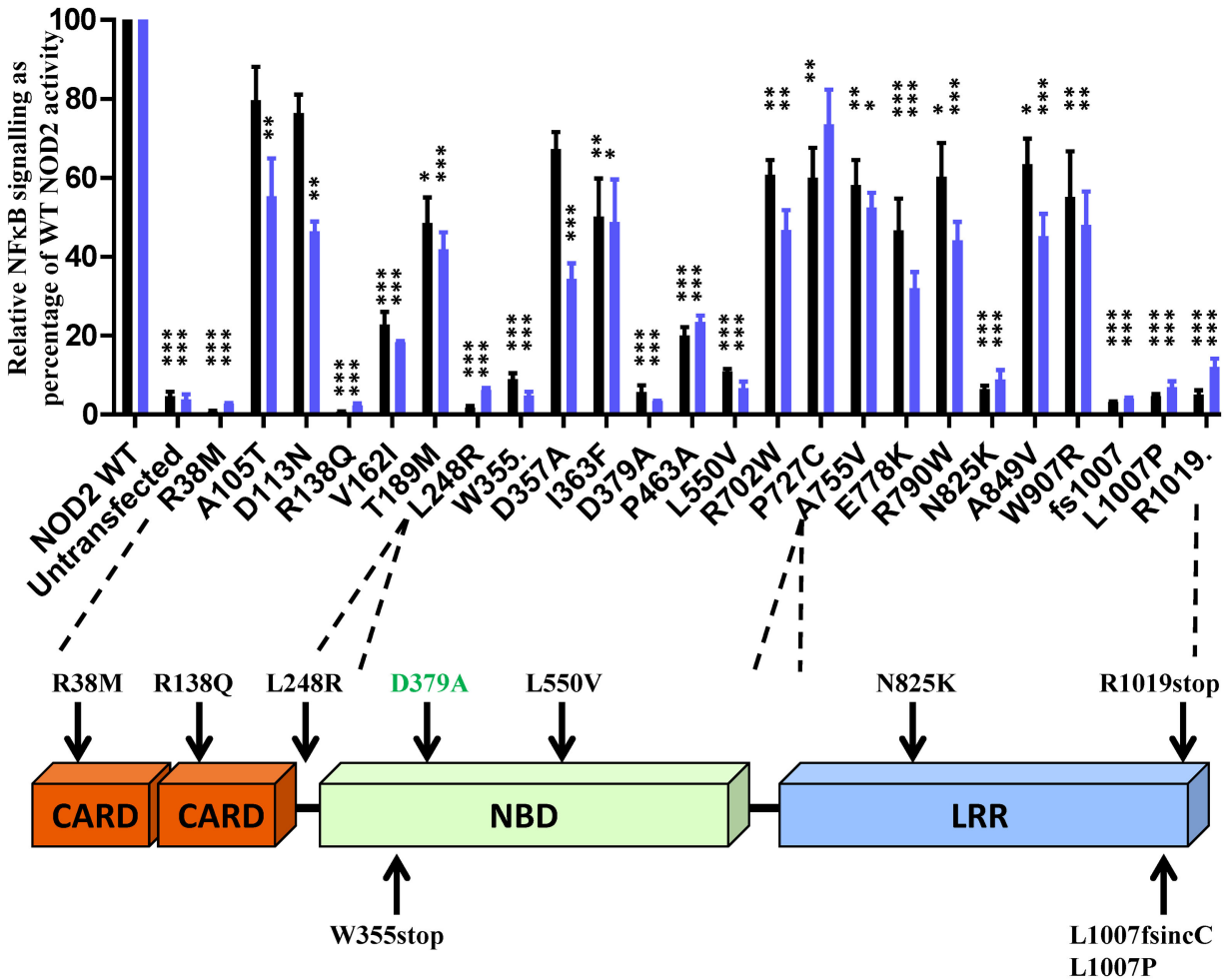

FIGURE 1 | Twenty-three NOD2 polymorphisms show a significant reduction in signaling following stimulation with MDP. The ability of NOD2 polymorphisms to signal via NFκB (black bars) or activate the IL-8 promoter (blue bars) was determined following stimulation with 100 ng/ml MDP. Signaling functionality is expressed as a percentage of wild-type receptor activity. All polymorphisms were tested in triplicate in a minimum of three separate experiments. Error bars show SEM. Data was log transformed and statistical significance determined using one-way ANOVA with Bonferroni post-test for multiple samples. Only polymorphisms showing a significant reduction in signaling are plotted (data for the other polymorphisms is provided in Figure S1 in Supplementary Material) – $^{*}p < 0.05$, $^{**}p = < 0.01$, $^{***}p = < 0.001$. A schematic of the NOD2 domain structure is provided with the location of polymorphisms showing <15% activity marked.

ones, the polymorphism V162I signals at <25% of the wild-type protein (**Figures 1** and **2B**). In fact 10 species, including members of the bovine, porcine, caprine, and canine families, all possess an isoleucine in this position and therefore may show impaired NOD2 functionality.

RIPK2 Binding is not Sufficient for NOD2 Signaling

Following activation of NOD2, signal propagation requires interaction with the adaptor protein RIPK2 in a CARD-dependent manner. To determine if NOD2 SNPs with a signaling defect were impaired in their ability to interact with RIPK2 we performed co-immunoprecipitations in HEK293T cells between FLAG-tagged NOD2 constructs and Myc-tagged RIPK2. With the exception of R38M and R138Q, all the tested NOD2 polymorphisms retained the ability to bind RIPK2 (**Figure 3A**; Figure S1 in Supplementary Material), thereby indicating that whilst the interaction with RIPK2 is necessary for signal transduction, it is not sufficient. This is consistent with recent observations between NOD1 and RIPK2 (23). The inability of R38M and R138Q to interact with RIPK2 was confirmed by immunofluorescence in HeLa cells (**Figure 3B**). Wild-type NOD2 showed membrane-based co-localization with RIPK2 following overexpression. However, whilst both R38M and R138Q still associated with the cell membrane they did not localize with RIPK2 (**Figure 3B**). In fact, in the presence of R38M and R138Q RIPK2 showed a punctate distribution in the cell interior (**Figure 3B**) that matched its distribution when transfected into HeLa cells in the absence of NOD2 (**Figure 3B**). Homology models of the NOD2 CARDs (**Figures 3C,D**) indicated that both R38 and R138 are predicted to be located in equivalent positions on the first helix of the first and second CARDs, respectively. This

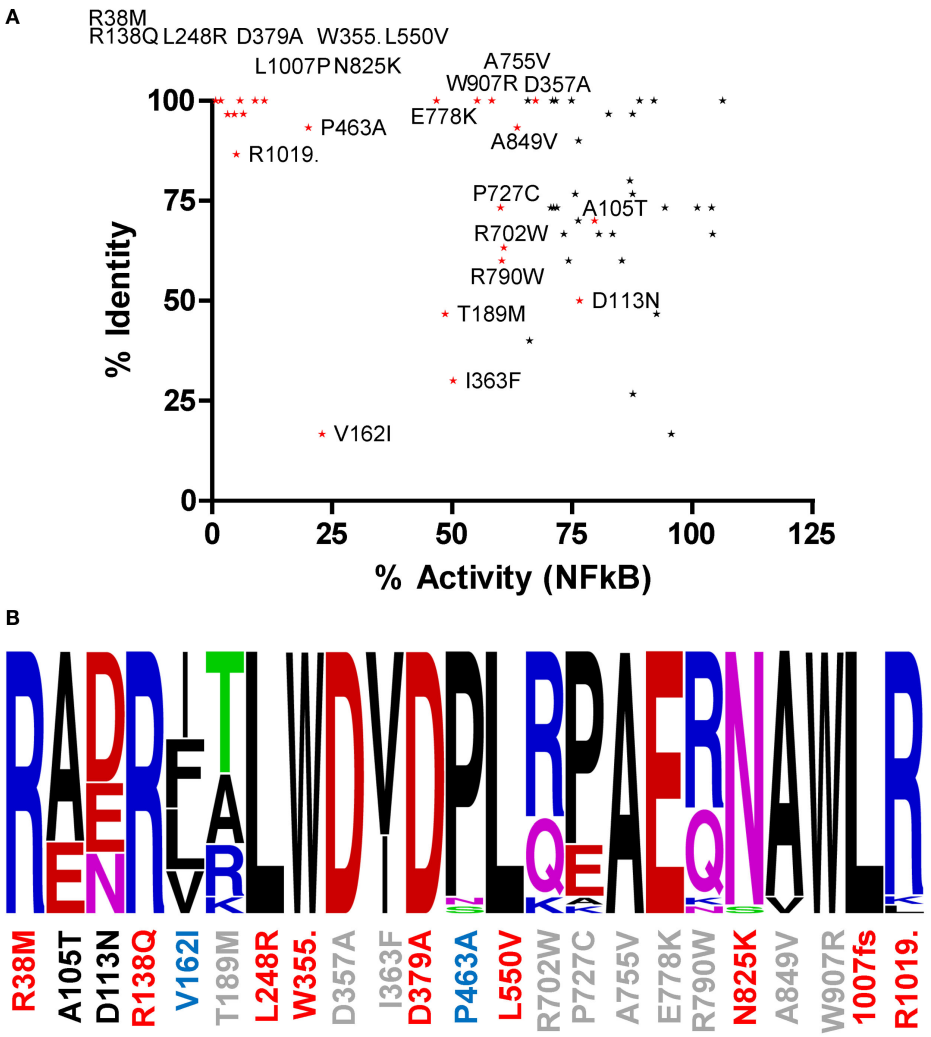

FIGURE 2 | The relationship between residue conservation and functional impact. (**A**) Scatter plot of percentage residue identity and percentage NFκB signaling activity for all polymorphisms studied. Polymorphisms that cause a significant reduction in signaling are colored red and labeled. The points for R38M and R138Q overlap on the graph. (**B**) WebLogo representation of residue conservation for those polymorphisms producing a significant reduction in signaling. Polymorphism position is denoted on the *x*-axis and colored by level of NFκB signaling compared to WT NOD2 – red <15%, blue 15–45%, gray 46–75%, and black >75%. Amino acid residues are colored as follows: basic residues – blue; acidic residues – red; hydrophobic residues – black; polar acidic residues – green; and polar basic residues – pink.

strongly suggests that this region on both CARDs is crucial for the interaction with RIPK2.

Association of NOD2 Polymorphisms with Membranes does not Directly Relate to Functionality

Our immunofluorescence of R38M and R138Q (**Figure 3B**) indicated that these polymorphisms were still recruited to the membrane despite being unable to signal. This suggested that whilst the association of NOD2 with cellular membranes is important for signal transduction, it does not in fact guarantee that signaling will occur. To study whether the other dysfunctional polymorphisms showed altered membrane association we performed immunofluorescence and subcellular fractionation on a collection of overexpressed polymorphisms displaying a range of signaling activity (**Figure 4**). The extent of membrane association was estimated by densitometry of the subcellular fractionation data (**Figure 4A**). As shown by the scatter plot in **Figure 4C**, no clear correlation between the degree of membrane association and the extent of receptor signaling could be drawn. However, polymorphisms associated with a significant reduction in signaling could be assigned into three main groups. Those with a membrane association and signaling activity below 15% (L248R, D379A, N825K, L1007P, L1007fs, and R1019stop); those with membrane association similar, or fractionally lower than wild-type and low signaling activity (R38M, R138Q, V162I, W355stop, P463A, and L550V); and those with slightly impaired signaling, but similar or slightly low membrane association. With the exception of I363F none of the polymorphisms with <20% membrane association signaled at levels above 15% of the wild-type receptor. Analysis of a wider range of functional and dysfunctional NOD2

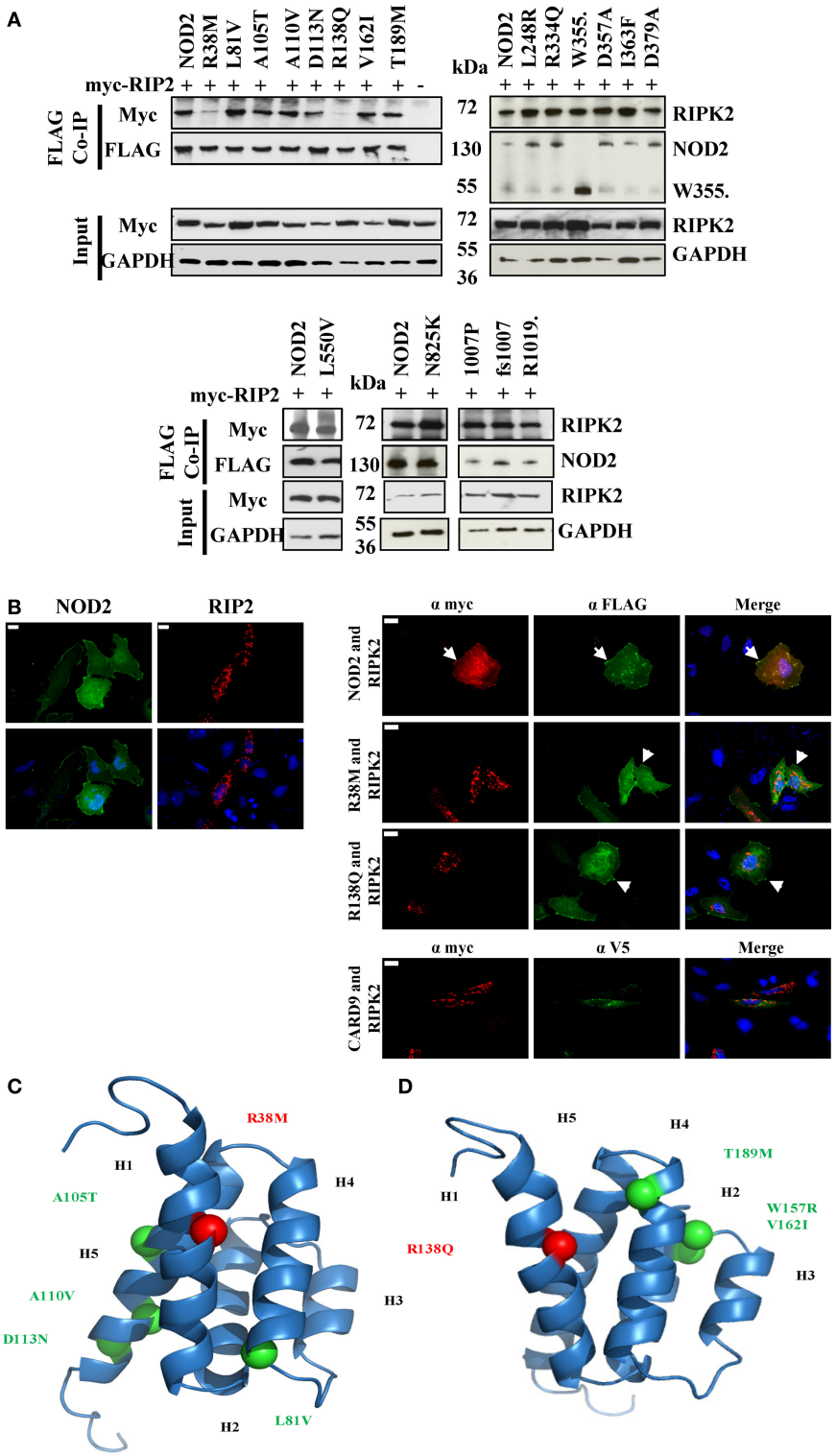

FIGURE 3 | Impact of NOD2 polymorphisms on the interaction with RIPK2. **(A)** HEK293T cells seeded in 6-well plates were transiently transfected with 0.5 µg pCI-myc-RIP2 and 1 µg pCMV-FLAG-NOD2 polymorphic constructs as labeled. After 24 h, cell lysates were immunoprecipitated using Dynabeads labeled with mouse anti-FLAG antibody. Proteins were separated by SDS-PAGE and proteins detected using the antibodies specified. **(B)** Immunofluorescence was performed in 12-well plates using HeLa cells seeded onto coverslips using 0.5 µg pCMV-FLAG-NOD2 or pCI-myc-RIP2 and the protein localization was visualized following antibody staining (left panel). Co-localization studies were performed by transfecting 0.5 µg of pCI-myc-RIP2 with 0.5 µg of each of pCMV-FLAG-NOD2/R38M/R138Q or pEF6-V5-CARD9 (right panel). Examples of membrane-associated fluorescence are indicated by arrows. The scale bar indicates 20 µm. Cartoon representation of homology models of the first **(C)** and second **(D)** CARDs of NOD2 showing the conserved location of R38 and R138 (red spheres) and the location of other CARD located polymorphisms (light green). Helices are annotated as H1–H6.

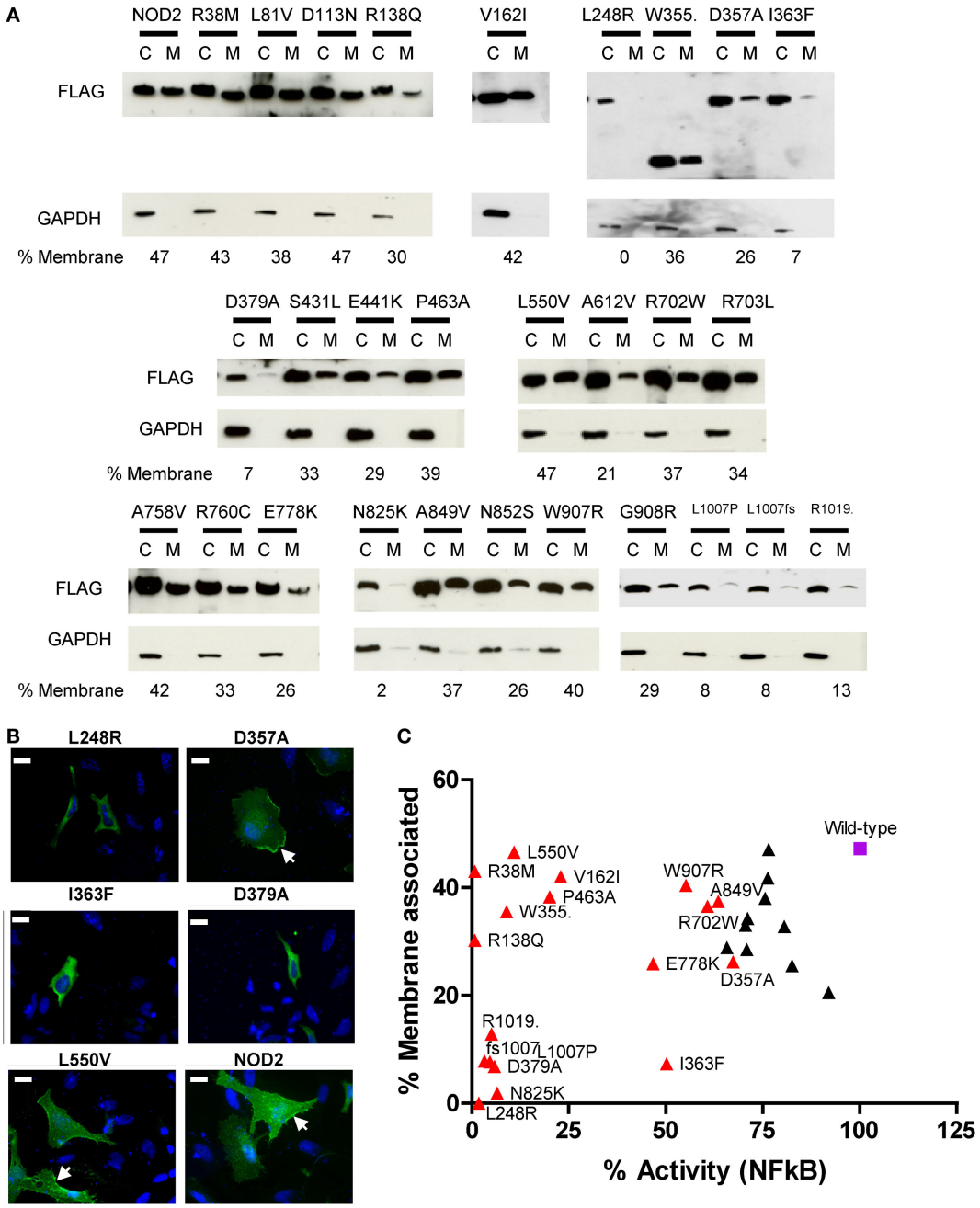

FIGURE 4 | Membrane association of NOD2 polymorphisms. (**A**) NOD2 polymorphisms were transiently transfected in HEK293 cells. After 24 h cells were harvested and the membrane and cytoplasmic fractions separated using the Pierce Subcellular Fractionation Kit. Proteins were resolved using SDS-PAGE and NOD2 constructs detected with anti-FLAG antibody. Detection of GAPDH confirmed the relative purities of the membrane and cytoplasmic fractions. The proportion of NOD2 associated with the membrane was estimated by densitometry using ImageJ. (**B**) Representative immunofluorescence images showing the cellular localization of overexpressed NOD2 constructs in HeLa cells. One microgram of the relevant DNA was transfected into HeLa cells seeded into 12-well plates containing a coverslip. After 24 h, cells were fixed using 4% PFA in PBS and permeabilized using 0.4% Triton X-100. Proteins were stained using mouse anti-FLAG primary antibody (Sigma) and Alexa Fluor® 555 goat anti-rabbit secondary antibody (Life Technologies). Cells were visulaised using an AXIO Imager.M2 microscope (Zeiss) and images created using Image J. Membrane localization is indicated by arrows and the scale bar equals 20 μm. (**C**) Scatter plot of percentage membrane association plotted against percentage NFκB signaling activity. Polymorphisms that showed a significant reduction in signaling are colored red and labeled. The wild-type receptor is represented as a purple square and labeled.

polymorphisms is needed before it can be determined whether this is a strict correlation. The association of R38M, R138Q, and W355stop at levels broadly similar to wild-type NOD2 suggest that whilst membrane association is likely to play an important role in NOD2 signaling, it is not sufficient for signal transduction to occur.

DISCUSSION

The etiology of Crohn's disease is complex and is influenced by combinations of genetic, lifestyle, and environmental factors. Polymorphisms in NOD2 present the strongest genetic susceptibility factor although over 70 separate genes have been reported to be associated with the risk of disease development and progression (4). Our understanding of how NOD2 SNPs predispose to Crohn's disease is improving and in general, they appear to result in defective NOD2 function (10). However, the extent and severity of this defect is variable and appears to show an element of cell-type and/or stimuli specificity. In this work, we have analyzed 53 Crohn's disease-associated SNPs for their effect on three key functional consequences of NOD2 stimulation, namely: basal and ligand-induced NFκB-driven signaling, interaction with RIPK2, and membrane localization. Due to the low population frequency of the SNPs studied, we employed an overexpression system, which despite their acknowledged shortcomings, still provide a valuable system for understanding the functional impact of mutations on protein function.

It is clear from our results, and in agreement with the work of other researchers investigating different NOD2 polymorphisms, that not all Crohn's disease-associated NOD2 polymorphisms result in the same degree of functional impact. Indeed, only 23 of the SNPs we tested showed significant functional impairment. This may reflect the presence of subtle functional impacts that are masked by protein overexpression; that the polymorphisms affect functions not tested here, such as signaling via CARD9; or that their reported association with Crohn's disease is artifactual. These are all elements that require further study.

Earlier studies have implicated R38, following mutation to alanine, as important for the interaction of NOD2 and RIPK2 (24). Our observations, in which R38M and R138Q were the only polymorphisms to lose the ability to efficiently interact with RIPK2, are consistent with this assertion and also suggest a crucial role in RIPK2 binding for R138Q. Homology models show that R38 and R138 occupy equivalent position in the first helix of the first and second CARDs, respectively (**Figures 3C,D**) in a region that forms part of a basic patch on the surface of each CARD. We observed dramatic reductions in signaling capacity for R38M and R138Q, with the latter observation consistent with earlier work (6). That other signaling defective NOD2 polymorphisms retain the ability to interact with RIPK2 is consistent with our recent observations between NOD1 and RIPK2 (23) and confirms that for both NOD1 and NOD2, the engagement of RIPK2 is necessary, but not sufficient for signal propagation.

It has been widely reported that membrane association is an integral requirement for NOD2 signaling (17, 25–27). Our observations in this work suggest that the connection between NOD2 signaling and membrane association requires further clarification. Five polymorphisms (L248R, N825K, L1007P, L1007fs, and R1019stop) plus the Walker-B mutant (D379A) showed minimal capacity to signal following ligand stimulation and had very low levels of membrane association. Whilst I363F despite showing <10% membrane association, still signaled at around half the level of wild-type protein. In contrast, other polymorphisms showed low (V162I and P463A) or very low (R38M, R138Q, W355stop, and L550V) levels of activity, but have levels of membrane association little different to wild-type NOD2. In the case of R38M and R138Q, the lack of signaling clearly relates to their failure to recruit RIPK2. However, V162I, W355stop, P463A, and L550V all associate with membrane and all engage RIPK2, but are still heavily impaired in their signaling capacity, suggesting that the reasons for NOD2 dysfunction in these cases are somewhat more complex and elusive.

Although residue conservation does not provide a clear and definitive correlation with the functional impact of the polymorphisms, it can help rationalize why certain residues do, or do not, have functional consequence when mutated. For example, amino acids that showed high levels of evolutionary variation, such as W157, R235, R708, and R760, generally showed limited impairment in signaling. Whereas, the functional impact of polymorphic changes at highly conserved residue locations, such as R38, R138, L248, and L1007, was much more dramatic. In some instances, the reason for the dramatic impact and hence evolutionary conservation are clear. For example, R38 and R138 are needed for interaction with RIPK2; N825 forms part of the LRR asparagine ladder and hence contributes to structural integrity; and L1007 and R1019 are in the C-terminus of the protein and important for membrane localization. On the other hand, why L248 and L550 are completely conserved and functionally crucial is less apparent. For L248, its importance may relate to its proximity to CARD9 (28) and TRAF4 (29) binding sites and the introduction of a large basic amino acid in the polymorphism may disrupt these interactions. Similarly, mutation of L550 could result in destabilization of the protein structure or interfere with the interaction with chaperone proteins and hence disrupt the transition from the inactive to active conformation of NOD2. Reasons for the lack of functional impact for polymorphisms of the other highly conserved residues are less clear, but in at least the case of N852S, this reflects a change of residue that is permitted in the structural context of that amino acid, i.e., serine can functionally substitute for asparagines in the LRR asparagine ladder (30).

Our work here provides one of the most detailed investigations into the functional impact of Crohn's diseases-associated polymorphisms. By assessing not just receptor signaling, but also the interaction with RIPK2, evolutionary conservation, and receptor localization, we have provided an important insight into the diverse range of functional impacts displayed by these polymorphisms. Importantly, the work highlights the benefit of studying the functional impact of polymorphic variation through highlighting the complex relationship between NOD2 and its association with RIPK2 and cellular membranes in relation to signal transduction.

AUTHOR CONTRIBUTIONS

RP performed and analyzed the data, drafted the manuscript, and approved the final version; TM conceived the study, analyzed the

data, wrote and revised the manuscript, and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2015.00521

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REVIEW
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The Neuromodulation of the Intestinal Immune System and Its Relevance in Inflammatory Bowel Disease

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One of the main tasks of the immune system is to discriminate and appropriately react to "danger" or "non-danger" signals. This is crucial in the gastrointestinal tract, where the immune system is confronted with a myriad of food antigens and symbiotic microflora that are in constant contact with the mucosa, in addition to any potential pathogens. This large number of antigens and commensal microflora, which are essential for providing vital nutrients, must be tolerated by the intestinal immune system to prevent aberrant inflammation. Hence, the balance between immune activation versus tolerance should be tightly regulated to maintain intestinal homeostasis and to prevent immune activation indiscriminately against all luminal antigens. Loss of this delicate equilibrium can lead to chronic activation of the intestinal immune response resulting in intestinal disorders, such as inflammatory bowel diseases (IBD). In order to maintain homeostasis, the immune system has evolved diverse regulatory strategies including additional non-immunological actors able to control the immune response. Accumulating evidence strongly indicates a bidirectional link between the two systems in which the brain modulates the immune response via the detection of circulating cytokines and via direct afferent input from sensory fibers and from enteric neurons. In the current review, we will highlight the most recent findings regarding the cross-talk between the nervous system and the mucosal immune system and will discuss the potential use of these neuronal circuits and neuromediators as novel therapeutic tools to reestablish immune tolerance and treat intestinal chronic inflammation.

Keywords: intestinal immune system, oral tolerance, sympathetic system, parasympathetic system, peptidergic pathway, neuropeptide, inflammatory bowel disease

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INTESTINAL IMMUNE HOMEOSTASIS AND ORAL TOLERANCE

A main function of the immune system is to distinguish between "danger" or "non-danger" signals and to respond appropriately. This is crucial in the gastrointestinal (GI) tract, where the immune system is constantly exposed to a multitude of food antigens and symbiotic microflora, which are essential for providing vital nutrients to the body. Therefore, the balance between immune activation versus tolerance should be tightly regulated to maintain intestinal homeostasis. In recent

years, it has become clear that the mucosal immune system has developed an ingenious mechanism, referred to as oral tolerance, to fulfill this task. In detail, *lamina propria* antigen-presenting cells (APCs), such as dendritic cells (DCs) and macrophages (MFs), are "educated" by intestinal bioactive factors, such as transforming growth factor-beta (TGF-β), retinoic acid, thymic stromal lymphopoietin, and mucins (1–5), to suppress inflammation and promote immunological tolerance via the induction and expansion of antigen specific anti-inflammatory regulatory T cells (Tregs) in the mesenteric lymph nodes (MLN).

To date, oral tolerance is widely accepted to represent the cornerstone of intestinal immune homeostasis (3, 6). In healthy individuals, intestinal immune tolerance against food and microbiota antigens is, therefore, crucial to prevent an immune reaction against harmless food (3). Nevertheless, in individuals with genetic or environmental predisposition (altered microbiota, viral or bacterial infection, chemical additives, or pollution) oral tolerance is broken resulting in immune activation against luminal antigens. Loss of this delicate equilibrium indiscriminately results in chronic and excessive immune activation indiscriminately against luminal antigens leading to invalidating intestinal disorders, such as inflammatory bowel disease (IBD). So far, there is no cure for IBD. The actual goal of IBD treatment is to reduce the inflammation that triggers symptoms and tissue alterations. In a group of cases, this may lead to long-term remission and reduced risks of complications but in a large number of patients disease relapses are common. Thus, the ultimate aim in IBD research is to explore novel therapeutic methods to reinstall intestinal immune tolerance.

Lately, experimental and clinical evidence suggests that an additional actor, i.e., the nervous system, may play a critical role in modulating the intestinal microenvironment, preserving immune homeostasis and tolerance. In the current review, we will highlight the most recent findings regarding the cross-talk between the nervous system and the mucosal immune system. Furthermore, we will discuss the potential employment of some of these neuronal circuits and neuromediators as novel therapeutic tools to reestablish immune tolerance and treat intestinal chronic inflammatory diseases, such as IBD.

THE NERVOUS SYSTEM AS MODULATOR OF IMMUNE RESPONSE

The cross-talk between the immune and nervous systems occurs through a complex set of neurotransmitters, cytokines and hormones and is undoubtedly playing a crucial role in the regulation of an immune response (7). The ground-breaking idea that neurotransmitters could serve as immune modulators emerged with the discovery that their release and diffusion from nervous tissue could lead to signaling through typical neurotransmitter receptors expressed on immune cells (8).

Inflammatory mediators released locally can activate sensory nerves and send signals to the nervous system. Through the induction of the so called "inflammatory reflex," efferent nerves also convey signals from the nervous system to the periphery where the release of neural mediators affects immune responses and inflammation (9). Consequently, the nervous system is able to rapidly sense and regulate inflammation in peripheral tissues as well as restore immune homeostasis via the release of mediators acting locally on immune cells. In several chronic inflammatory diseases such as rheumatoid arthritis (RA), systemic lupus erythematosus, and IBD, the tone of the sympathetic nervous system (SNS) is also increased (10). This suggests that an autonomic nervous system imbalance with a dominant activation of the SNS and inadequate parasympathetic tone may have a key role in the pathogenesis of various immune related disorders including IBD (11).

NEUROIMMUNE INTERACTION IN THE GUT WALL

The enteric nervous system (ENS), also known as the "Little brain of the gut," forms a complex and independent nervous system within the GI tract (12). The ENS, together with the assistance of the extrinsic innervation, enables us to perceive the inner world of our intestine and its contents, to regulate motility and to digest nutrients (12) (**Figures 1A,B**).

The ENS consists of a network of hundreds of millions of neurons and glial cells clustered in small ganglia connected by nerve bundles organized in two major layers embedded in the gut wall, the myenteric *plexus* (or *Auerbach's plexus*) and the submucosal *plexus* (or *Meissner's plexus*) (13, 14).

Functionally, the ENS resembles the central nervous system (CNS) as it uses similar sensory and motor neuronal fibers, information processing circuits "or interneurons" and releases a comparable set of neurotransmitters (**Figure 2**). The chemical neuromediators of the ENS were initially thought to be limited to neurotransmitters, such as acetylcholine (ACh) and serotonin, but, subsequently, purines, such as ATP, amino acids (γ-aminobutyric acid or glutamate), and peptides, such as vasoactive intestinal polypeptide (VIP) and neuropeptide Y (NPY), have been identified (15). More recently, nitric oxide (NO) has emerged as an important neurotransmitter in the ENS (16, 17). Overall, more than 20 candidate neurotransmitters have now been identified in ENS, and most neurons contain several of them (15). Growing evidence now supports the ground-breaking idea that these neurotransmitters can convey signals to immune cells and modulate their function (8). Indeed, many neurally derived molecules, such as ACh, serotonin and glutamate, have potent inhibitory effects on various immune cells including APCs or T cells (8, 18).

Besides the intrinsic innervation, the gut is extrinsically innervated by the autonomic nervous system composed of the sympathetic (SNS) and parasympathetic (PNS) system (**Figure 2**). Cell bodies of preganglionic sympathetic neurons are located in the CNS, between the first thoracic and third lumbar spinal cord segment, from where its axon connects to the postganglionic neurons where they send axons to the intestinal wall. On the contrary, parasympathetic preganglionic neurons are found directly in the brainstem were they form the vagus nerve (VN) that densely innervates the GI tract.

Sympathetic and parasympathetic fibers convey afferent information from the gut to the brain informing the CNS about the

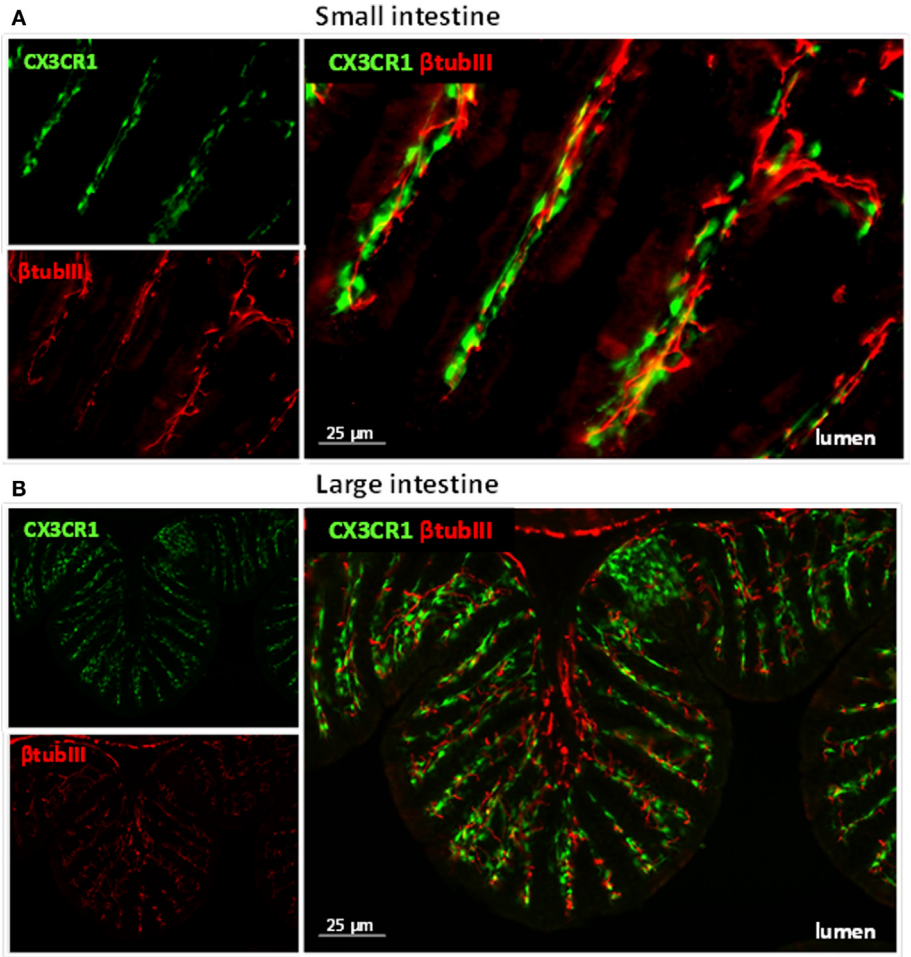

FIGURE 1 | Immune cells and neuronal fibers are in close proximity in the gastrointestinal tract. Immunofluorescent picture of the ileum **(A)** and proximal colon **(B)** from CX3CR1$^{eGFP/WT}$ mice showing in red neuronal fibers and in green CX3CR1^{+} macrophages. Neuronal fibers were visualized with a rabbit antitubulin III (red; Covance, 1:2000) followed by a donkey anti-rabbit Cy5 antibody (Jackson Immunoresearch). The green GFP signal (i.e., macrophages) highlights the CX3CR1^{+} macrophages.

intestinal microenvironment (19). We have recently identified a direct activation of neurons (c-fos expression) in the dorsal motor nucleus of the VN in a mouse model of intestinal inflammation (20). This evidence strongly supports the idea that inflammatory stimuli can activate the autonomic nervous system and induce the release of immune-modulatory mediators in the periphery by nerve endings.

PARASYMPATHETIC NERVOUS SYSTEM AS MODULATOR OF INTESTINAL IMMUNE HOMEOSTASIS

In line with the neuroimmune cross-talk hypothesis, the CNS, via the VN, is playing a crucial role in regulating immune response in the periphery through the cholinergic anti-inflammatory pathway (CAIP) (21). Tracey et al. originally reported that vagotomy significantly enhanced proinflammatory cytokine production and accelerated the development of septic shock, whereas electrical stimulation of the efferent VN prevented systemic inflammation and improved survival via the release of ACh in the spleen (22, 23). More recently, they also showed that in sepsis, the vagal anti-inflammatory reflex requires an intact splenic nerve (23) and alpha 7 nicotinic receptor (α7nAChR) expression on splenic MFs (24). Since the spleen is devoid of cholinergic innervation, this concept was lately challenged. However, this discrepancy was resolved by showing that upon vagus nerve stimulation (VNS), ACh released in the celiac mesenteric ganglia activates postsynaptic α7nAChR of the splenic nerve, leading to the release of norepinephrine in the spleen (25, 26). Surprisingly, norepinephrine induces the synthesis of ACh in a subpopulation of splenic memory T cell expressing choline acetyltransferase (ChAT), resulting into the anti-inflammatory effect of VNS (27). The discovery that activation of the VN has a potent anti-inflammatory effect implies that afferent vagal fibers will detect activation of the innate immune system in peripheral tissue and send an integrated efferent vagal input to that same site modulating the inflammatory response.

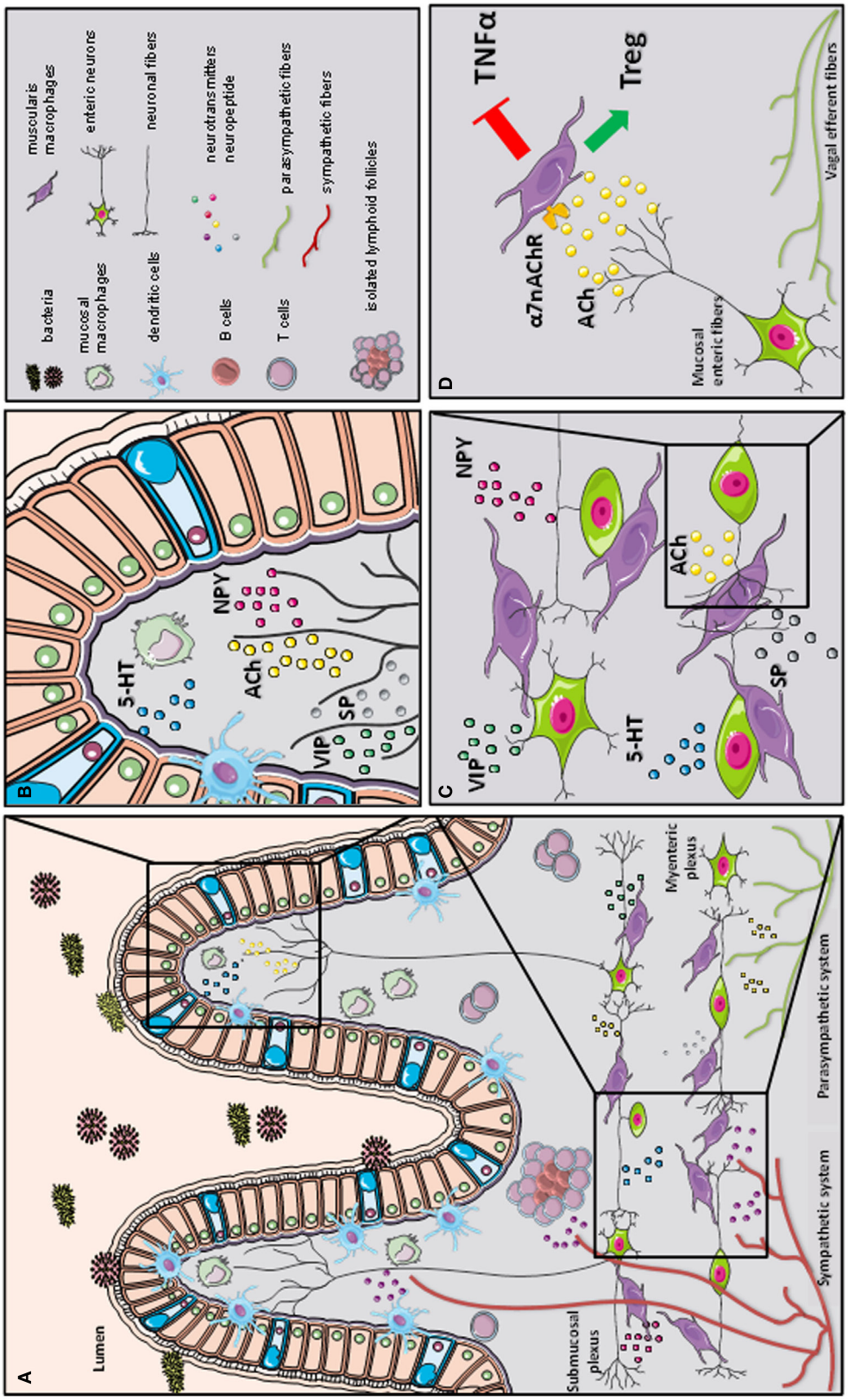

FIGURE 2 | A schematic representation of the cross-talk between the nervous and immune system in gastrointestinal tract.

(Continued)

FIGURE 2 | Continued
The gastrointestinal tract is highly innervated via the autonomic nervous system (sympathetic and parasympathetic system) and enteric nervous system (via the myenteric and submucosal *plexus*). While the parasympathetic fibers (vagus nerve) extensively innervate the gut wall up to the myenteric *plexus*, the sympathetic fibers directly contact immune cells, secondary lymphoid organs (such as isolated lymphoid follicles), and enteric fibers in the submucosal/mucosal compartment **(A)**. Innate and adaptive immune cells, such as dendritic cells (DCs), macrophages (MFs), and T cells, located in the mucosal villi are affected by the presence of several immune-modulating neurotransmitters released by the enteric neural fibers, such as substance P (SP), vasoactive intestinal peptide (VIP), serotonin (5-HT), and neuropeptide y (NPY) **(B)**. In the myenteric *plexus*, a dense network of enteric neurons is present in close proximity to resident macrophages. The release of acetylcholine (ACh) and multiple of neuropeptides (SP, VIP, 5-HT, and NPY) condition the resident macrophages residing in the myenteric *plexus* **(C)**. During inflammation, vagal efferent fibers directly activate cholinergic enteric neurons in the myenteric *plexus*. The release of ACh triggers α7 subunit of the nicotinic acetylcholine receptor (α7nAChR) expressed on resident macrophages. The activation of α7 nAChR decreases tumor necrosis factor alpha (TNFα) release and favors regulatory T cells (Treg) conversion (our observation) leading to the control of intestinal inflammation and restoration of intestinal immune homeostasis **(D)**.

Thus, the CAIP represents a new counter-regulatory mechanism to control the immune system and in contrast to circulating hormones or cytokines, provide a rapid local modulation of the immune response (27).

Early anatomical evidence highlights that efferent vagal nerve fibers extensively innervate the GI tract with a typical rostro-caudal gradient of vagal preganglionic innervation, with the highest density observed in the stomach followed by a subsequent decrease in the small bowel and colon (28). Therefore, it is not surprising that the vagal anti-inflammatory pathway plays a crucial role in the regulation of the intestinal immune response.

A few years later, we and others have extended the concept of the vagal anti-inflammatory pathway to the GI tract by showing the benefical effect of electrical VNS in a model of postoperative ileus (POI) (29) (**Figure 2**). POI is an immune-mediated condition evoked by surgical handling of the intestine in which the inflammation is primarily restricted to the intestinal muscular layer. In this model, electrical, nutritional and pharmacological activation of CAIP have been shown to prevent both surgery-induced inflammation and delayed GI transit (29, 30).

Similar to the spleen, we identified α7nAChR-expressing MFs residing in the *muscularis externa* as the final target of vagal anti-inflammatory pathway in the gut (**Figure 2D**) (31, 32). In addition, we have clearly verified that in POI, where inflammation only occurs in the *muscularis externa* the intestinal vagal anti-inflammatory effect is independent of the spleen and of ACh-producing T cells. As in the spleen, immune cells in the gut wall are indirectly modulated by the VN as vagal efferents solely synapse with cholinergic enteric neurons in the myenteric *plexus* (31). This implies that enteric neurons rather than vagal nerve endings interact with the intestinal immune system and release ACh.

Over the years, it is becoming increasingly clear that the microenvironment in the gut mucosa and submucosa determines the immune response to the initial exposure of luminal antigens. Given the potent anti-inflammatory effect of the cholinergic innervation, one might assume that the cholinergic tone in the submucosal compartment may have an important impact on mucosal immune homeostasis. The ENS forms a dense network of nerve fibers in close vicinity with intestinal immune cells, both in the submucosal (*lamina propria*) and *muscularis externa* compartment of the intestine (33). This could imply that vagal signals are amplified by the ENS inducing a substantial release of ACh in the intestinal microenvironment leading to modulation of the immune response. In line with this hypothesis, electrical and pharmacological activation of CAIP has been widely studied as a novel approach to treat IBD in several animal models. Reduced vagal cholinergic input induced by abdominal vagotomy results in an increased susceptibility to develop colitis with an elevated proinflammatory cytokines production following dextran sulfate sodium (DSS) administration (34). In line, nicotine or α7nAChR specific agonist treatments were protective in experimental colitis (34, 35). In addition, reduced mucosal levels of ACh in a murine model of depression were also associated with exacerbation of colitis (36, 37). However, the actual determinant of the increased susceptibility to colitis after vagotomy is still unknown. Interestingly, O'Mahony et al. recently showed that vagotomized mice have basal increase of activated nuclear factor (NF)-κB level in the gut and reduced splenic Tregs (38).

However, the crucial role of the α7nAChR revealed in models of sepsis and POI still remains ambiguous in colitis. α7nAChR$^{-/-}$ mice had a higher severity of acute DSS-induced colitis which was in line with choline-chloride (α7nAChR specific agonist) treatment able to decrease inflammatory parameters in a model of depression-induced colitis (37). On the other hand, Snoek et al. described that treatment with specific α7nAChR agonists (AR-R17779 and GSK1345038A) reduced inflammation, such as NF-κB activity and cytokines, but these treatments did not improve the clinical signs of colitis (39), indicating that during colitis the receptor involved in vagal anti-inflammatory effect is still uncertain.

In accordance with the ambiguous effect of α7nAChR in experimental models of colitis, nicotine, an ACh nicotinic receptor agonist, has an opposite effect in the two main IBD forms: ulcerative colitis (UC) and Crohn's disease (CD). Clinical observation indicates that UC patients experience more severe disease upon quitting smoking, while it improves again after returning to smoke (40, 41). In addition, a lower incidence of UC has been observed in smokers, while a negative effect of cigarette smoking has been observed in CD patients (41, 42). Smoking, namely, worsened symptoms compared to non-smokers in CD patients (43) due to increased influx of neutrophils into the intestinal mucosa (44, 45), suggesting that nicotine might affect colonic inflammation in IBD via CAIP. Galitovskiy et al. proposed a possible experimental explanation for the dichotomous effect of nicotine in colitis. In this study, the authors showed that nicotine attenuated oxazolone colitis, resembling UC (Th2-induced inflammation), increasing colonic regulatory T cells and reducing Th17 cells. On the contrary, nicotine exacerbated trinitrobenzene sulfonic acid (TNBS-) induce colitis, resembling CD (Th1-induced inflammation), by

increasing colitogenic Th17 cells (46). These findings suggest that nicotine might influence the inflammation according to the type of immune response partially explaining the controversial effect of smoking observed in UC and CD patients.

Various studies have tried to correlate autonomic dysfunction, such as alteration of the vagal tone, with clinical outcome in IBD patients (47, 48). Bonaz and colleagues reported negative correlation between low vagal tone and increase plasma levels of tumor necrosis factor alpha (TNF-α), suggesting that the CAIP may be altered in these patients. However, a clear correlation between IBD and vagal tone has still not been convincingly verified. Recently, Clarençon et al. described the first attempt of VNS in a patient with CD. The patient subjected to long-term low frequency VNS, showed significant improvement with reduction of both clinical disease activity index and endoscopic remission. This beneficial effect was correlated to an increased parasympathetic tone (49). Even though this report is interesting in this patient proposing a therapeutic role for the VNS in IBD, results should be taken with caution considering the size of the study and the fact that placebo effect could not be ruled out using this experimental approach.

Currently, electrical VNS is explored as therapeutic treatment in patients affected by chronic inflammation (50). Ongoing clinical trials are investigating the possible beneficial effect of VNS in patients with RA, POI, and CD (NCT01552941, NCT01569503, and NCT01572155). Additional preclinical and clinical data will hopefully clarify whether the CAIP will be an alternative therapeutic approach to treat intestinal inflammatory diseases.

SYMPATHETIC NERVOUS SYSTEM

Besides the PNS, the GI tract is extensively innervated by the sympathetic nervous system (SNS) mostly involved in the modulation of blood flow, secretion, and motility.

Sympathetic fibers mainly innervate the myenteric and submucosal *plexus* as well as the mucosal layer (51). Of note, on the contrary to the PNS, sympathetic fibers have been found in direct contact with immune cells residing in gut-associated lymphoid tissues including Peyer's patches and MLN (52, 53). Anatomical studies have undoubtedly shown large amounts of noradrenergic fibers both into the dome region of the follicles where fibers are in direct contact with lymphoid cells and in the *lamina propria* where fibers are mainly associated with blood vessels (52). Interestingly, various innate and adaptive immune cells express receptors for the typical sympathetic neurotransmitters, including noradrenaline (NA) and adrenaline (A), supporting the idea that also the SNS may regulate immune response and inflammation in peripheral tissues including the GI tract (25, 54, 55). Catecholamines bind a large family of adrenergic receptors. These are G-couple receptors composed of different subunits: three α1 (A, B, and D), three α2 (A–C), and three β (β1–β3) receptor subtypes. Interestingly, adrenergic receptors have different threshold of activation depending on the cathecholamine concentration: high concentrations activate β-adrenoreceptors, subsequently increasing cAMP levels, whereas low concentrations activate preferentially α-adrenoreceptors leading to decreased cAMP levels (56). This difference may explain the controversial results obtained in experimental and clinical studies investigating the pro- and anti-inflammatory role of sympathetic innervation during local tissue inflammation. It is important to mention that catecholamines can also be produced and released by various immune cells, such as T- and B-cells (57). Interestingly, production of catecholamines after immune cell activation has been proposed as an autocrine loop involved in the regulation of inflammation (58).

Together with anatomical proximity of noradrenergic fibers to various immune cells (59), also a direct functional interaction has been proven on DCs. Recent studies, demonstrated direct effects of NA on DC migration, antigen uptake, cytokine production and T cell polarization via the intracellular signaling pathways PI3K and ERK1/2 (60–63). Additionally, adrenergic fibers also affect T cell polarization repressing Th1 polarization, while favoring Th2 cell induction (64, 65), suggesting possible effect of NA in the skewing of T cell responses.

The reciprocal cross-talk between the sympathetic fibers and immune system has been extensively studied also in experimental models of intestinal inflammation.

In order to study the role of sympathetic immune-regulation during inflammation, chemical sympathectomy has been performed using 6-hydroxydopamine (6-OHDA) treatment resulting into depletion of NA in the peripheral nerve terminals. In rats, 6-OHDA treatment revealed an alteration in migration and accumulation of lymphocytes (B and T cells) in the gut-associated lymphoid organs during inflammation (66, 67). In more detail, sympathetic denervation decreased inflammation in acute DSS- and TNBS-induced colitis (68, 69). On the contrary, Straub and colleagues showed in two different chronic models of colitis (i.e., chronic DSS-induced colitis and IL10-deficient mice) that chemical sympathectomy significantly exacerbated disease, suggesting that catecholamines may play a favorable effect in the chronic phase of inflammation by promoting tissue repair (70). These controversial results might be explained by a dual role of these fibers in which the SNS confers proinflammatory effects at the beginning of colonic inflammation, while it exerts anti-inflammatory effects in the chronic phase of inflammation. Of note, similar results have been recently described in a murine model of rheumatoid arthritis, another chronic model of inflammation. Indeed, mice that underwent early sympathectomy showed improvement of arthritis scores while animals subjected to late sympathectomy had significantly increased arthritis scores compared with control mice (71).

In addition to sympathectomy, several studies have attempted to mimic the effect of the SNS during colitis by pharmacological activation of adrenergic receptors. Treatment with a β_3-AR agonist was shown to be beneficial as it reduced the severity of TNBS-induced colitis in rat (72). However, this anti-inflammatory effect of the agonist might be indirect, since it was associated with cholinergic-mediated contractions of the colon known to improve mucosal healing (73). However, in another study activation of β_3-ARs was also able to reduce colonic cytokines release, further supporting its anti-inflammatory effect in colitis (51). Additionally, during TNBS-induced colitis, mice treated with α2-adrenoceptor antagonist, RX821002, showed a reduced expression of colonic proinflammatory genes (TNF-α and IL-1β) (74). Overall, preclinical data clearly indicate the immunomodulatory

effect of the SNS in experimental models of colitis. However, the effect of the SNS is still controversial with studies reporting both pro and anti-inflammatory effects, depending on the preclinical model used and on the disease stage assessed (69).

As previously mentioned, accumulating evidence suggests that abnormalities in the neural autonomic profile may be an aggravating factors in the pathogenesis of IBD (50). Of note, enzymes involved in the synthesis of NA are reduced in patients affected by both UC and CD even though lower level of NA has been only detected in CD patients (75, 76). Furthermore, a reduction of sympathetic nerve fibers has been observed in biopsies from CD patients, while sympathetic fibers are increased in tissue of UC patients (77, 78).

The idea that SNS dysfunction may have an impact on IBD has been recently tested with promising results in a clinical trial in UC patients. In this study, treatment with the α_2-AR-agonist clonidine, induced normalization of the SNS activity and significantly improved disease severity in patients with active UC (79).

Although the involvement of the SNS in the inflammatory response has been extensively proven, further studies should be conducted to better understand how modulation of the SNS may enter in the therapeutic armamentarium of IBD.

THE PEPTIDERGIC PATHWAY: NEUROPEPTIDES

Neuropeptides are neuronal signaling peptides involved in a wide range of neuronal functions. Often neuropeptides are coreleased with neurotransmitters complicating studies evaluating their specific effects. Neuropeptides modulate neuronal communication by acting on cell surface receptors. Interestingly, various subtypes of neuropeptide receptors are also expressed on immune cells suggesting possible influence of these molecules on the immune system (**Table 1**). This idea is supported by clinical and experimental observations obtained in IBD patients and animal models of colitis that are discussed below.

VASOACTIVE INTESTINAL PEPTIDE

Vasoactive intestinal peptide is a 28-amino acid neuropeptide that is widely expressed in different tissues, including intestine, central and peripheral nervous system, pancreas, and lung (80). In the intestine, VIP is mainly produced by enteric neurons in the myenteric and submucosal *plexus* and regulates both intestinal motility and chloride secretion (81, 82, 84). In addition, VIP has emerged as a potent anti-inflammatory peptide affecting both innate and adaptive immune responses. Due to its anti-inflammatory properties, the therapeutic potential of VIP in the treatment of inflammatory disorders, such as IBD, has been extensively investigated. In the intestine, the receptors for VIP (VPAC1 and VPAC2), are mainly expressed by smooth muscle cells and immune cells including T cells, DCs, and MFs, suggesting a possible immune-modulatory effect of VIP (80, 83, 85). The *in vitro* anti-inflammatory properties of VIP on myeloid cells are well documented and highly relevant for IBD. Indeed, one of the proposed pathogenic drivers in IBD is the loss of immune tolerance against harmless antigens leading to chronic production of inflammatory mediators by MFs and enhanced Th1/Th17 polarization by inflammatory DCs (149, 150). Interestingly, VIP inhibits inflammatory MFs through inhibition of NF-κB activation leading to reduced production of proinflammatory cytokines (TNFα, IL-6, and IL12p40) and enhanced production of the tolerogenic cytokine IL-10 (151–155). Moreover, VIP treatment reduces expression of toll-like receptor 4 (TLR4) in MFs rendering them less responsive to lipopolysaccharide (LPS) (86, 87). In addition, DCs acquire an anti-inflammatory phenotype upon VIP treatment. VIP-induced activation of VPAC1 in bone marrow-derived DCs induces a tolerogenic phenotype with low levels of costimulatory molecules CD80, CD86, and CD40, and high levels of the anti-inflammatory cytokine IL-10 (88, 89). These VIP-conditioned DCs induce polarization of naïve $CD4^+$ T cells into $CTLA4^{pos}$ IL-10 secreting Tregs**.** In addition to its effects on innate immune cells, VIP also acts directly on T cells to promote Th2 differentiation through activation of VPAC2 (90, 91). Notably, during Th2 differentiation both VIP and VPAC2 are upregulated in T cells, suggesting that VIP-VPAC2 participates in a Th2 autoregulatory loop (92). It has indeed been demonstrated that VIP supports survival of Th2, but not Th1 cells (90). Taken together, by acting on both the innate and adaptive arm, VIP actively counteracts Th1 responses. These findings highlight VIP as an attractive therapeutic candidate for the treatment autoimmune disorders with a typical Th1 profile, including CD. The therapeutic potential of VIP in CD is indeed supported by the beneficial effects of VIP in TNBS-induced colitis, an experimental model of CD (156). Administration of VIP induces a remarkable amelioration of TNBS-induced colitis through activation of VPAC1. Reduced disease severity correlates with lower levels of proinflammatory chemokines and cytokines, inhibition of Th1 responses and induction of a Th2 immune response (156, 157). Although VIP showed very profound effects in this study, it should be noted that other studies could not confirm the beneficial effects of VIP in TNBS-induced colitis (158). Moreover, inhibition or genetic deletion of VIP does not exacerbate colitis, but rather induces resistance to both TNBS- and DSS-induced colitis (93, 159). These findings make the interpretation of the preclinical therapeutic evidence of VIP in colitis difficult and argue for further fundamental research to better understand the promiscuous role of VIP in intestinal physiology and pathology. Also from a clinical point of view, the role of VIP in IBD is far from clear. Although there are some reports showing hypertrophic VIPergic nerves and increased VIP levels in rectal biopsies of patients with IBD, other reports demonstrate no increase or even decreased levels of VIP (94–96). In summary, although VIP exerts potent anti-inflammatory effects *in vitro*, there is currently not enough preclinical and clinical evidence to support translation of VIP treatment in IBD to the clinic.

NEUROPEPTIDE Y

Neuropeptide Y is a 36-amino acid peptide and is considered as one of the most abundant peptides in the central and peripheral nervous system (97). NPY is highly conserved among species and in mammals its effects are mediated trough binding of six different G-coupled receptor subtypes (Y1, Y2, Y3, Y4, Y5, and Y6) (98,

TABLE 1 | Expression and effect of selected neurotransmitters during intestinal inflammation.

Neurotrasmitter	Main source	Receptors	Target cells	Experimental evidence	Clinical evidence
Catecholamines					
Noradrenaline–adrenaline	Sympathetic fibers, T and B cells (59)	β-adrenoreceptors (58) α-adrenoreceptors (58)	DCs, T cells (25, 56, 57)	Sympathetic denervation improves DSS, TNBS (78, 79) Sympathetic denervation worsens IL10$^{-/-}$ and chronic DSS (77, 80, 81) β_3-AR agonist improves TNBS (82) α2-Adrenoceptor antagonist improves TNBS (83)	Active UC patients typically have increased SNS activity (71–73), it is decreased in CD patients (74) α2-AR-agonist improve severity of UC in human (71)
Acetylcholine	Parasympathetic fibers Cholinergic enteric neurons T and B cells (23–27)	α7 nicotinic acetylcholine receptor (26)	Macrophages (22, 26, 31)	Vagotomy worsens DSS (34) Choline-chloride improves depression-induced colitis (37) Nicotine improves oxazolone colitis (46) Nicotine worsens TNBS (46) α7nAChR$^{-/-}$ mice had a higher severity of acute DSS-induced colitis (39)	VNS improves severity CD patient (49)
Neuropeptides					
Vasoactive intestinal peptide (VIP)	Enteric neurons (81)	VPAC1, VPAC2 (80)	Smooth muscle, T cells, DCs, macrophages (80–82, 84, 85)	Immunomodulatory effects (86–92) VIP treatment ameliorates TNBS colitis (93)	Limited clinical evidence: both ↑ and ↓ of VIP level observed in IBD (94–96)
Neuropeptide Y (NPY)	Central and peripheral nervous system (97) Immune cells (105)	Y1, Y2, Y3, Y4, Y5, and Y6 (GPCRs) (98, 99)	Innate and adaptive immune cells (monocytes, lymphocytes, and granulocytes) (100–103)	Proinflammatory effect: shown in mouse and rat models of DSS Induced colitis (106–108) and TNBS-induced colitis (109)	IBD patients: no change in NPY plasma levels (104)
Calcitonin gene-related peptide (CGRP)	α-CGRP: central and peripheral nervous system β-CGRP: gut, pituitary gland, and immune system (119–121)	Calcitonin receptor-like receptor (CRLR) (110)	TRPV1 and CGRP are colocalized on peripheral neurons and on immune cells, such as MFs and DCs (111–115)	Anti-inflammatory effect Shown in mouse and rat models of DSS-induced colitis (122) and TNBS-induced colitis (123)	UC and CD patients: ↓ CGRP^{+} cells in the intestinal *muscularis* layer (116–118)
Substance P (SP)	Central and peripheral nervous system (124–126) Innate immune cells: monocytes, MFs, eosinophils and lymphocytes (133–136)	Neurokinins-1 (NK-1), NK-2, and NK-3 (GPCRs) (127–130)	Enteric neurons, smooth muscle, endothelial cells, immune effectors, and mucosal epithelial cells (127–130)	Contradictory results Proinflammatory effect: shown in mouse and rat models of DSS colitis (137) and TNBS colitis (138) Anti-inflammatory effect: role in mucosal healing shown in mouse models of DSS and TNBS colitis (140, 141)	UC patients ↑ SP in rectum and colon (77, 131, 132) SP in severe inflammatory colon lesions (139) CD patients: decreased (131), unchanged (142), and increased (77) SP levels
Serotonin	Enterochromaffin cells, enteric neurons (143, 144)	5-HT receptors (143, 144)	Enteric neurons, immune cells (145, 146)	↓ Serotonin (genetic and pharmacological) ameliorates DSS and DNBS colitis (147); ↑ serotonin worsens colitis in IL10$^{-/-}$ mice (148)	No clinical evidence available

99). In the CNS, NPY is mainly present in the hypothalamus and is involved in modulating anxiety, appetite, blood pressure, and nociception (160–162). On the other hand in the periphery, it is mainly expressed in sympathetic nerves where it is colocalized and coreleased with NA. Within the gut, enteric neurons of the *myenteric plexus* and *submucosal plexus* are the major source of NPY (163). The biological effects of NPY on the GI system are of inhibitory nature and include effects on pancreatic and GI secretion, blood pressure, and GI motility as well as modulation of intestinal inflammation with direct interaction with the immune system (164). NPY can affect both the cells of the innate and adaptive immune system as it modulates neutrophil chemotaxis, granulocyte oxidative burst, and NO production, T helper cell differentiation, natural killer cell activity, suppression of lymphocyte proliferation and activation of APCs (165–168). Interestingly, next to nerve-derived NPY, immune cells themselves are able to express NPY enabling them to modulate the immune cell function in a paracrine or autocrine manner (105). The expression of several Y receptor subtypes have been reported on immune cells (100–103). As Y1 receptor is the most abundant receptor, it has been intensively investigated and appears to be present in each immune cell investigated so far (100). Although less information has been gathered about the expression of other Y receptors, human neutrophils express Y1, Y2, Y4, and Y5, with Y4 being the most abundant (169). Furthermore, Y1, Y2, and Y5 expression was also demonstrated in mouse MFs and rat granulocytes (103, 170). NPY can exert pro- or anti-inflammatory effects, depending on which receptor is activated and on

which immune cell. In particular, NPY levels were shown to be increased in both DSS-induced colitis (171) and TNBS-induced colitis (109). Further evidence for the role of NPY in promoting inflammation in the gut is provided by the fact that NPY knockout (KO) mice are resistant to the induction of DSS colitis (106, 107). Similar results were obtained by using an NPY antisense oligodeoxynucleotide in rats (171). Receptor Y1 seems to mediate the proinflammatory effect of NPY as KO or antagonism of the receptor results into a comparable attenuation of inflammation (108). In addition, receptor Y1-deficient mice have impaired APC function and consequently a decreased number of effector T cells, as well as a decreased MF production of TNF-α and IL-12, explaining the observed protective phenotype in experimental colitis (102). During gut inflammation, there is a considerable amount of cross-talk between NPY and TNF-α. This was shown by the decreased production of TNF-α by enteric neuronal cells from NPY-deficient mice. Conversely, block of TNF-α causes a reduction in colonic NPY expression (172). Additionally, NPY also enhances nNOS, which is associated with oxidative stress, in a murine model of DSS colitis (106). Although NPY plasma level is not altered in IBD patients (104), targeting NPY or its receptors might be an interesting therapeutic approach for treating IBD.

CALCITONIN GENE-RELATED PEPTIDE

Calcitonin gene-related peptide (CGRP) is a 37-amino acid neuropeptide that exists in two isoforms, α-CGRP and β-CGRP. These isoforms are encoded by two different, but closely related genes. α-CGRP is mainly produced in the central and peripheral nervous system, whereas β-CGRP is primarily produced in the gut, pituitary gland and by immune cells. The biological actions of both isoforms are largely overlapping (119–121). Although CGRP participates in development and maintenance of pain, it has been also described as a potent regulator of inflammatory responses (173). In addition in the GI tract, CGRP participates in the regulation of gastric acid secretion and intestinal motility (174, 175). Release of CGRP by nerve endings and immune cells is induced by activation of the transient receptor potential vanilloid 1 (TRPV1). TRPV1 and CGRP are colocalized on peripheral neurons as well as on immune cells, such as MFs and DCs (111–115). Whereas the anti-inflammatory effect of CGRP on LPS-induced inflammation is well described, its role in intestinal inflammation still needs to be fully elucidated. Up to now numerous studies have pharmacologically investigated the role of CGRP in experimental models of colitis. The effect of systemic administration of CGRP and its antagonist, hCGRP, has been tested in a rat TNBS model. Intravenous administration of CGRP protected the colonic mucosa against TNBS in both the early and late phases of acute colitis, while hCGRP exacerbated TNBS-induced inflammation (123). On the other hand, the contribution of TRPV1 in gut inflammation is still controversial, as it was shown that disease severity was reduced in TRPV1 KO mice in model of DSS colitis, suggesting that TRPV1 activation may enhances inflammation (176). On the contrary, acute stimulation of sensory neurons by capsaicin, a known TRPV1 agonist, ameliorated disease symptoms in TNB-colitis in rats (177). This could be due to the corelease of other neuropeptides from sensory $TRPV1^+$ fibers, such as substance P, which could promote a more proinflammatory milieu (122). Recently, CGRP expression has also been reported in TRPM8 expressing (temperature-sensitive TRP channels for cold sensation) mucosal fibers. In line, TRPM8-dependent CGRP release have been shown in the colon upon DSS exposure (122). In the same study, genetic deletion of TRPM8 increased the susceptibility of mice to acute colitis. This was correlated with an increase in CGRP levels in mucosal fibers suggesting that TRPM8-CGRP signaling may be involved in dampening intestinal inflammation (122).

Considering the possible participation of CGRP in the pathogenesis of IBD many studies have attempted to gather clinical data in both UC and CD patients. However, while early studies showed a decrease of CGRP positive cells in the intestinal *muscularis* layer of UC and CD patients (116–118), a more recent report did not confirmed lower CGRP level in tissue from patients during moderate and severe UC (178).

SUBSTANCE P

Substance P is a neuropeptide which is composed of 11-amino acids (179) and is widely expressed in the brain and periphery (124) including the GI tract where it is mainly expressed by neurons of the *myenteric* and *submucosal plexuses*, as well as intrinsic and extrinsic sensory neurons (125, 126). Interestingly, SP is also expressed by a variety of innate immune cells, such as monocytes (133), MFs (136), eosinophils (134), and lymphocytes (135). SP binds specific G protein-coupled receptors named neurokinins-1 (NK-1), NK-2, and NK-3. Within these three receptors, NK-1 has the highest affinity for SP and is abundantly expressed throughout the GI tract by different kind of cells: enteric neurons, smooth muscle, endothelial cells, immune effectors, and mucosal epithelial cells (127–130). Several studies have correlated SP-NK-1 signaling with intestinal inflammation. In a model of TNBS colitis, mice lacking neutral endopeptidase (NEP), an enzyme that degrades SP in the extracellular fluid, displayed exacerbated inflammation (138). Accordingly, the use of an NK-1 antagonist improved the severity of colitis in different experimental models (137, 138). Binding of SP to its receptors leads to the activation of mitogen-activated protein (MAP) kinase, protein kinase C (PKC), and NF-kB pathways leading to production of proinflammatory cytokines, such as IL-1b, IL-6, IL-8, and TNFα (180–183). Although these findings suggest a proinflammatory effect of SP in intestinal inflammation, studies have emerged that highlight a role for SP in mucosal healing and thus propose a beneficial effect of SP in colitis. Castagliuolo I et al. described the development of DSS and TNBS-induced colitis in NK-1 receptors deficient mice, and showed that there was an increase in severity of colitis as well as an increased mortality in these mice (140). The protective effect of NK-1 is associated with the transactivation of epidermal growth factor receptor (EGF-R), which in turn leads to cell proliferation in the colon (140, 141). Moreover, SP has been described to trigger cell proliferation in a multitude of cell types, such as T cells, smooth muscle cells (184). Although clinical evidence suggests a role for SP in the pathophysiology of IBD in patients, the exact role of SP in intestinal inflammation needs to be further elucidated. Several studies investigated levels of SP in the serum

and locally in the gut. Increased levels of SP have been observed in the rectum and colon of UC patients, and were correlated with disease activity (77, 131, 132). On the other hand, another study showed that SP containing nerves increased in hypervascularized lesions, while they decreased in severe inflammatory lesions in the colon of UC patients (139). Contradictory results have also emerged in studies investigating SP in CD patients. While some reported decreased levels of SP in the mucosa in CD patients (131), others showed that there is no difference (142) or even an increase in SP levels (77). These controversial results might be explained by the variation in methodology, tissues used and different stages of the disease (185–187). Additionally, changes in NK-1R expression were investigated in IBD patients. An increased number of NK-1R positive lymphoid cells was observed and additionally also increased NK-1R mRNA expression in inflamed mucosa was reported. Furthermore, an altered pattern of epithelial NK1-R expression was found in UC, while in CD an increased expression was reported in the *myenteric plexus* (125). Clearly, further studies are needed in order to clarify the interaction between SP and the immune system and its possible involvement in intestinal inflammation.

SEROTONIN

Although serotonin or 5-hydroxytryptamine (5-HT) is well known as a neurotransmitter of the CNS, the majority of serotonin in the human body is produced in the GI tract (143). Intestinal 5-HT is mainly produced by enterochromaffin cells (ECs) and enteric neurons of the myenteric plexus (144, 188). In both cells types, biosynthesis of 5-HT depends on the conversion of dietary L-tryptophan to 5-hydroxytryptophan by tryptophan hydroxylase (TPH). Synthesis of 5-HT in the intestine is performed by two different types of TPH, namely TPH1 and TPH2 (145). The bulk of 5-HT is produced by ECs and depends on TPH1. In contrast, enteric neurons only have a minor contribution to total 5-HT levels in the intestine and rely on TPH2 for the production of 5-HT. The distinct regulation of 5-HT synthesis in these two compartments also has important functional implications. Whereas myenteric production of 5-HT by TPH2 is essential for normal motility, mucosal 5-HT synthesis by TPH1 in ECs influences intestinal inflammation (145, 147). Indeed, depletion of mucosal 5-HT via genetic deletion ameliorates both DSS- and DNBS-induced colitis in mice. This effect is eliminated when TPH1 knockout mice are replenished with 5-HT (147). In line, mice that develop spontaneous colitis due to IL-10 deficiency have increased disease severity when the actions of 5-HT are amplified by genetic deletion of the serotonin transporter SERT (148). Enterocytes of SERT knockout mice are unable to remove 5-HT from the extracellular space leading to enhanced 5-HT effects. Taken together, these data clearly show that mucosal 5-HT has profound proinflammatory effects and can exacerbate development of colitis. Hence, selective inhibition of mucosal 5-HT levels might be an interesting therapeutic option for the treatment of IBD. In a recent study, the therapeutic potential of selective TPH1 inhibition has been validated. Indeed, oral administration of telotristat etiprate, a potent TPH1 inhibitor, effectively reduced disease severity and intestinal inflammation in mice with TNBS-induced colitis (146). Of note, telotristat etiprate did not cross the blood–brain barrier nor did it affect serotonin levels in myenteric neurons (189, 190). This is important to ensure the safety of the treatment and to avoid side effects due to depletion of 5-HT in the CNS and ENS. Another way to reduce the proinflammatory effects of 5-HT is by inhibition of 5-HT receptors that are selectively expressed on intestinal immune cells. Although the mechanisms by which 5-HT exerts its proinflammatory actions is not completely understood, it was recently shown that DCs isolated from TPH1 deficient mice produce less IL-12 compared to wild-type DCs (191). Treatment of these DCs with 5-HT led to restoration of IL-12 production, indicating that 5-HT can polarize DCs to become proinflammatory. Hence, selective pharmacological inhibition of 5-HT might be an interesting therapeutic approach since the proinflammatory actions of 5-HT on intestinal DCs could be reduced. Unfortunately, the 5-HT receptors that are commonly found on intestinal DCs are also involved in other processes in both CNS and ENS. Accordingly, inhibition of 5-HT receptor signaling has a relatively poor therapeutic potential given the severe side effects, including behavioral and motility problems. In summary, reduction of mucosal 5-HT through selective inhibition of TPH1 likely holds most promise for future treatment of inflammatory intestinal disorders. However, further research is needed to better define the cellular players involved in the proinflammatory actions of 5-HT.

CONCLUSION AND PERSPECTIVE

An intricate network of immune and non-immune cells and their mediators function in unison to protect us from toxic elements and infectious microbial diseases that are encountered in the intestinal lumen. This network operates efficiently by use of a single cell epithelium layer, fortified by adjacent cells and lymphoid tissues that protect its integrity. On occasion alterations of the steady state due to genetic background and/or environmental microbes result in inflammatory diseases or infections including development of IBD. Thus, intestinal immune homeostasis is finely regulated by several redundant strategies to counteract excessive and unnecessary immune responses. Lately, an intimate bidirectional interaction between the nervous and immune system in the gut has been increasingly demonstrated. This pathway is a hard-wired connection between the immune and nervous system closely interacting to regulate inflammation. Based on our and others' findings, we can now conclude that intestinal neuronal circuits modulate the immune response directly into the gut wall. Therefore, we hypothesize that there may be two stages of neural modulation in the gut: in case of subtle and localized intestinal inflammation, the local innervation involving the ENS will be activated, while in more systemic inflammatory responses, such as in severe colitis, the autonomic innervation will come into play and modulate the immune response even in distant organs, such as the spleen or the bone marrow.

In conclusion, studying the immune-modulatory properties of nervous systems as well as endogenous neuropeptides will be a fundamental challenge for the next years. Although further fundamental understanding of their role in specific immune disorders is required, it is evident that neuroimmunomodulatory

therapies hold great promise, as evidenced by the ongoing clinical trials evaluating the effect of CAIP in autoimmune disorders. Further understanding of the neuronal circuits and receptors involved will likely support the development and use of specific receptor agonists and antagonists in the treatment of different neuroimmune pathologies. These new insights will not only affect our understanding of GI function, but might help to elucidate the complex interactions in other organs and systems. Unraveling of the mechanisms by which the autonomic and intrinsic innervation may reinstall intestinal immune homeostasis will, therefore, have a major impact on the therapeutic approach of many so far untreatable disorders, such as IBD.

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Microbiota-specific CD4CD8αα Tregs: role in intestinal immune homeostasis and implications for IBD

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In studies in murine models, active suppression by IL-10-secreting Foxp3 regulatory T cells (Tregs) has emerged as an essential mechanism in colon homeostasis. However, the role of the equivalent subset in humans remains unclear, leading to suggestions that other subsets and/or mechanisms may substitute for Foxp3 Tregs in the maintenance of colon homeostasis. We recently described a new subset of CD4CD8αα T cells reactive to the gut bacterium *Faecalibacterium prausnitzii* and endowed with regulatory/suppressive functions. This subset is abundant in the healthy colonic mucosa, but less common in that of patients with inflammatory bowel disease (IBD). We discuss here the physiological significance and potential role of these Tregs in preventing inflammation of the gut mucosa and the potential applications of these discoveries for IBD management.

Keywords: Tregs, *Faecalibacterium prausnitzii*, IBD, microbiota, inflammation

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DIVERSITY OF PERIPHERALLY DERIVED Tregs (pTregs)

CD4 regulatory T cells (Tregs) inhibit inflammatory responses (1). They can be subdivided into natural Tregs, which differentiate in the thymus (tTreg) and peripherally derived Tregs (pTregs), which differentiate in secondary lymphoid organs or tissues (2). These populations differ in terms of their non-redundant roles: tTregs play an essential role in maintaining tolerance toward self-structures, whereas pTregs are involved in the responses to externally delivered antigens or commensal microbes. Furthermore, the tTreg population appears to be stable, whereas that of pTregs may be more labile (3). This functional dichotomy results from differences in differentiation due to exposure to different TCR ligands (self and non-self antigens, respectively) and specific factors (cytokines, route of exposure, and antigen-presenting cells) in contrasting settings (4). The two Treg subsets can also be distinguished on the basis of the presence or absence of constitutive expression of the Foxp3 transcription factor. Constitutive Foxp3 expression and more particularly, the demethylation of a specific region of the Foxp3 locus are characteristic features of tTregs (5). Three main subsets of CD4 pTregs have been described in mice: $Foxp3^{+}CD25^{+}$ lymphocytes (3, 6), which are particularly abundant in the colon lamina propria (LP) (7) and two $Foxp3^{-}$ subsets: the type 1 regulatory T (Tr1) cells and the T helper 3 (Th3) cells. The Tr1 subset secretes IL-10 and TGF-β in the absence of IL-4 and IL-17 (8–10) and is abundant in the small intestine (7). The Th3 subset may also secrete IL-10, but it differs from Tr1 in its expression of membrane-bound TGF-β (11, 12). The Tr1 Tregs are induced *in vitro* by IL-10 (8–10) and *in vivo* by TGF-β and IL-27 (9, 13) in the context of diverse immune responses (14) and upon chronic stimulation with antigens in the presence of IL-10 (10). The suppressive action of Tr1 Tregs is essentially IL-10-dependent, but it is also at least partly governed by TGF-β (8, 9). Moreover, the suppressive function of these cells may be

mediated by a cytotoxic mechanism dependent on granzyme B and perforin (15). The Th3 subset is induced in the gut mucosa by oral immunization (12, 13). Its suppressive effects are essentially mediated by TGF-β, but also partly by IL-10 (11, 16). Much remains unknown about the typical features of Tr1 and Th3 cells and their relative contributions to immune regulation in general and to gut homeostasis in particular. Recent studies have shown that the development of colonic Foxp3$^+$ Tregs in mice is induced by gut clostridial bacteria and their metabolites, and that these Tregs play a key role in the prevention of colitis (17, 18). In humans, however, the role of gut Foxp3$^+$ Tregs in irritable bowel disease (IBD) remains unclear (19, 20), leading to suggestions that these cells may be less crucial in humans than in mice for the maintenance of colon homeostasis (21, 22).

HUMAN COLON DP8α T CELLS ARE pTregs INDUCED BY CLOSTRIDIAL BACTERIA

We recently reported that the CD4CD8αα (DP8α) lymphocytes of the colon LP are Foxp3$^-$ IL-10-secreting Tregs highly skewed toward the recognition of *Faecalibacterium prausnitzii*, a gut bacterium belonging to cluster IV of the genus *Clostridum* (23). In the healthy colonic mucosa of colon cancer patients, these cells account for about 12% of the CD4 lymphocytes present. We have shown that about 2% of the CD4 PBLs have the same CD4CD8αα phenotype and that 15% of these cells, on average, also react with *F. prausnitzii* (23). Together with the role of clostridial antigens in the induction of mouse colonic Tregs (17, 24) and the demonstration that segmented filamentous bacteria (SFB) antigens induce Th17 lymphocytes in the small intestine (25), our data suggest that *F. prausnitzii* participates in the induction of human DP8α colonic Tregs through antigen presentation. Support for this hypothesis is provided by our recent observation that *F. prausnitzii* imprints a phenotypic tolerogenic profile including a failure to secrete IL-12 on LPS-matured human DCs *in vitro* (unpublished data). Interestingly, *F. prausnitzii* is the most abundant bacterium of the human intestinal microbiota in healthy adults (26, 27) and decreases in its abundance have been linked to dysbiosis in IBD (28–32). The unique anti-inflammatory potential of this bacterium has recently been demonstrated, both *in vitro* and *in vivo* (33, 34). We found that there were fewer DP8α Tregs in the inflamed colonic mucosa and blood of Crohn's disease patients and in the blood of ulcerative colitis (UC) patients than in healthy individuals (23). These results suggest that lower levels of *F. prausnitzii* are associated with lower levels of *F. prausnitzii*-specific Treg anti-inflammatory activity in IBD patients, and that this may contribute to the disease. As a corollary, this suggests that DP8α Tregs may play a role in colon homeostasis and IBD prevention. However, this hypothesis requires confirmation and a number of important questions about these cells remain to be answered, to define more precisely their contribution to IBD prevention.

DP8α Treg: A NEW pTreg SUBTYPE

We must first consider whether DP8α lymphocytes represent a new pTreg subtype. If Tr1 cells are defined as Foxp3$^-$ Tregs secreting IL-10, then DP8α T cells could be considered to be Tr1 cells. Gagliani et al. (35) have suggested that human and mouse Tr1 cells are defined by the coexpression of CD49b and LAG3. We have also reported the expression of LAG3 by colonic DP8α cells *ex vivo* (23), but we did not consider their expression of CD49b. Nevertheless, our data revealed significant differences between Tr1 cells and DP8α Tregs. For example, DP8α Tregs stably express the CD8αα homodimer, CD25 and the transcription factor GATA-3, but do not express PD1, considered to be a canonical marker of Tr1 cells (9, 35). Moreover, whereas suppression by Tr1 and Th3 Tregs is largely dependent on IL-10 or TGF-β secretion, respectively (7, 9), the inhibition of T-cell proliferation by DP8α Tregs *in vitro* was little affected by a blocking anti-IL-10 antibody and not at all affected by an anti-TGF-β receptor antibody (23). It is, therefore, possible to distinguish DP8α Tregs from the Tr1 and Th3 Treg subsets.

One surprising finding of our work is the lack of Foxp3 expression by DP8α Tregs. However, they otherwise strongly resemble mouse Foxp3 colonic Tregs in terms of their regulatory markers (CD25, CTLA-4, GITR, and LAG3), regulatory functions (inhibition of T-cell proliferation, inhibition of DC maturation, and IL-10 secretion) and induction by related clostridial species (23). In both mice and humans, Foxp3 expression is required to maintain the Treg cell program and suppressive functions of tTreg (36) by repressing the activation-dependent expression of a number of genes, as elegantly shown in a recent study (37). In mice, Foxp3 is also expressed by the pTregs induced by clostridial bacteria (17). We have reported that DP8α Tregs have highly stable regulatory properties (23). This implies a high degree of commitment of these cells to their Treg status, with the expression of a Foxp3-independent genetic program in these cells. We are currently trying to decipher the genetic basis of DP8αα Treg commitment by comparing the transcriptomic signatures of the three main subtypes of CD4 lymphocytes in the colon LP: DP8α Tregs, conventional CD4 (CD4$^+$CD25$^-$CD127^{High}), and Foxp3 Tregs (CD4$^+$CD25$^+$CD127^{low}), with and without activation.

COLONIC DP8α Tregs: FUNCTIONAL HOMOLOGS OF THE pTregs INDUCED BY CLOSTRIDIAL BACTERIA IN MICE?

One important question raised by our results is whether human DP8α Tregs are functional homologs of the mouse Foxp3 Tregs induced by clostridial species (17), as shown in **Figure 1**. Alternatively, clostridial bacteria might induce both Treg subsets, with these subset playing complementary roles in colon homeostasis. This second hypothesis is unlikely in mice, because most of the IL-10-secreting Tregs of the colon LP express Foxp3, so IL-10-secreting Foxp3-negative lymphocytes are missing from this compartment (7). CD4CD8αα IL-10-secreting T lymphocytes have been described in the mouse gut mucosa. However, these cells were located in the epithelium of the small intestine (38), a compartment clearly different from the colon in terms of the composition and function of its immune components (39). In addition, we found no reactivity to *F. prausnitzii* in freshly sorted human colonic Foxp3 Tregs, suggesting

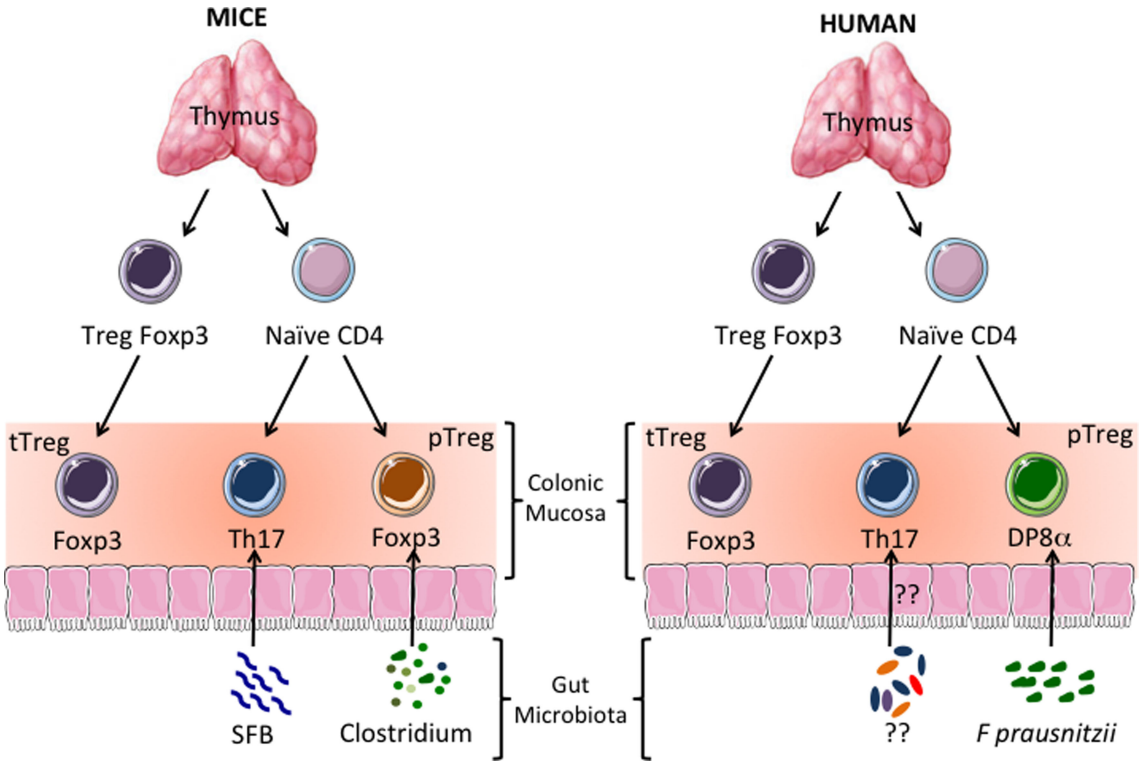

FIGURE 1 | Human CD4CD8αα (DP8α) regulatory T cells (Treg): functional homologs of the Tregs induced by clostridial bacteria in mice? In mice and humans, a subpopulation of $CD4^+$ thymocytes develops into Tregs expressing the transcription factor Foxp3. These thymus-derived Tregs (tTregs) migrate to all tissues, including the colonic mucosa, where they prevent autoimmune reactions. Thymus-derived naïve $CD4^+$ T cells also migrate to the colonic mucosa. There, depending on the type of antigen-dependent signals they receive, they develop into effector lymphocytes or peripherally induced Tregs (pTregs). It has been shown in mice that colonic Th17 and $Foxp3^+$ IL-10-secreting pTregs are induced by segmented filamentous bacteria (SFB) and clostridial bacteria, respectively. In the human colonic mucosa, IL-10-secreting DP8α Tregs induced by *Faecalibacterium prausnitzii* (*F. prausnitzii*), a gut bacterium belonging to the *Clostridium* cluster IV, seem to be the homologs of the mouse Foxp3 pTregs.

that this bacterium (or at least its antigens) is not involved in the induction of Foxp3 lymphocytes in the human colon LP (unpublished data). This may appear to conflict with the induction of Foxp3 Treg development by human clostridial bacteria in the colonic mucosa of GF mice (24), but the true meaning of this result remains unclear, because another study has shown that the human microbiota cannot restore normal mouse colon development upon transfer into GF animals (40). It, therefore, appears possible that during evolution, both humans and rodents have selected clostridial symbionts on the basis of their capacity to maintain colon homeostasis via Treg induction, but that these two groups diverged in terms of the molecular mechanisms involved in this process. Consistent with the hypothesis that DP8α Tregs may be functional homologs of mouse Foxp3 pTregs, the role of human Foxp3 Tregs in the prevention of colitis remain unclear (19). Moreover, the manifestations of enteropathy in IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) patients (who lack functional Foxp3 Treg), which are often considered to provide support for a role for $Foxp3^+$ Tregs in the prevention of IBD, clearly differ from those in IBD (41). This suggests that IPEX-associated colitis results from autoimmune attacks rather than from defects in tolerance to the microbiota and, thus, that mechanisms other than Foxp3 Treg-dependent suppression, possibly including suppression by DP8α Tregs, are involved in human colonic homeostasis (21, 22).

ARE ALL BLOOD DP8α LYMPHOCYTES REGULATORY T CELLS?

About 2% of CD4 PBLs have the same double-positive phenotype as DP8α LPLs, raising questions about their function. Most DP8α PBLs lacked regulatory markers *ex vivo*, but they acquired these markers and regulatory functions after a short period of *in vitro* activation or establishment in culture (which also requires TCR activation), whereas their CD4 homologs did not. Moreover, *ex vivo*, about 10% of DP8α PBLs expressed the gut homing receptor CCR9, and about the same proportion recognized *F. prausnitzii* (23). Therefore, most DP8α PBLs appear to be Tregs, although only a limited fraction of these cells react to *F. prausnitzii*. It is possible that some of these cells are pTregs induced by microbiota components present outside the gut, in the pulmonary mucosa, or the skin, for example. It is also possible that some of the circulating DP8α lymphocytes are not Tregs. Additional studies will be required to address these questions and to determine the specificity of the TCRs of regulatory DP8α PBLs that do not recognize *F. prausnitzii*.

DOES THE CD8αα MOLECULE PLAY A ROLE IN DP8α Treg FUNCTION?

CD8αα expression can be transiently induced on human $CD4^+$ T lymphocytes by activation in the presence of IL-4 (42). However, this molecule is expressed constitutively by the DP8α lymphocytes of the human colon LP and blood (23). This raises questions about the possible role of this molecule in DP8α Treg function. Like the CD8αβ coreceptor, CD8αα binds to MHC class-I molecules (43). In mice and humans, it also binds to the specific ligands thymus leukemia antigen (TL) (44) and gp180 (CEACAM5)/CD1d (45, 46), respectively. The human CD8αα ligand is expressed in the gut, by non-lymphoid cells, such as colonic epithelial cells and polynuclear neutrophils, two types of non-professional APCs that can activate MHC class II-restricted T lymphocytes. Previous studies have shown that the interaction between the CD8αα molecule and its ligands costimulates both the TCR activation induced by specific MHC/Ag complexes or by CD3 antibody, and T-cell function (44, 47). Moreover, CD8αα has been shown to play a role in the selection of high-affinity CD8αβ T cells (48). We have observed (unpublished data) that the triggering of CD8αα by an anti-CD8 antibody (OKT8) enhances the activation of DP8α Tregs induced by an anti-CD3 antibody (OKT3). This suggests that ligation of the CD8αα molecule of DP8α Tregs may increase their TCR-dependent activation by microbiota-derived antigens. It has recently been shown that TCR signaling is critical for the maintenance of the suppressive capacity of Foxp3 Tregs in mice, particularly in the colonic mucosa (49). It would be interesting to determine whether there is a similar dependence on TCR signaling in DP8α Tregs and whether the CD8αα receptor contributes to this process.

DP8α Tregs, BIOLOGICAL MARKERS AND THERAPEUTIC TARGETS IN IBD

Our observation that DP8α Treg levels are low in the colonic LP and blood of IBD patients, who frequently also have low levels of *F. prausnitzii* in their gut microbiota, provides evidence in support of a correlation between the levels of these bacteria and DP8α Treg levels in these patients (**Figure 2**). As recently suggested (50), such a correlation might result from a feedback loop between the selection, by follicular regulatory T cells (Tfr), of an adequate

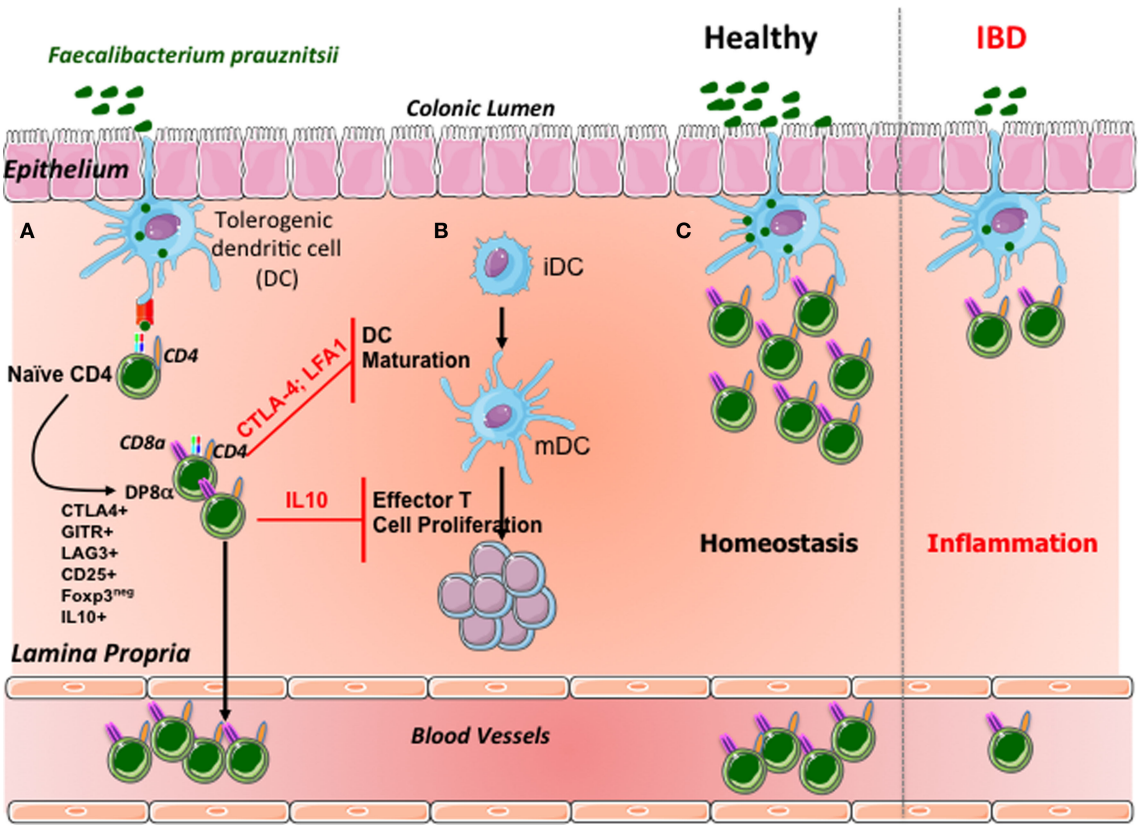

FIGURE 2 | Suggested model of DP8α Treg induction and homeostasis control in the human colonic mucosa. **(A)** In the colonic mucosa, *F. prausnitzii* antigens are presented by dendritic cells. Simultaneously, these bacteria may imprint a tolerogenic phenotype on dendritic cells. The recognition of *F. prausnitzii* antigens by the naïve CD4 T cells equipped with a specific TCR contributes, in this context, to the differentiation of these cells into Foxp3-lacking Tregs coexpressing CD4 and CD8α. DP8α T lymphocytes express most of the regulatory markers of Foxp3 Tregs and secrete IL-10. A fraction of these cells migrate into the blood. **(B)** Like Foxp3 Tregs, DP8α Tregs inhibit the maturation of dendritic cells in a CTLA-4- and LFA-1-dependent manner. Moreover, these cells decrease effector T-cell proliferation, via a mechanism involving interleukin 10. **(C)** Under normal circumstances, colonic *F. prausnitzii* expands the pool of DP8α Tregs in the colonic mucosa and in the blood; in IBD patients, the low levels of *F. prausnitzii* may compromise the expansion or survival of the DP8α Treg population, reducing the frequency of these cells in the mucosa and blood of patients.

IgA repertoire fostering microbiota diversity, particularly as concerns the abundance of clostridial bacteria, which in turn govern the development or survival of DP8α Tregs. In this context, the possible presence of DP8α Tfr should be investigated.

It is currently difficult to determine whether there is a strong correlation between the levels of DP8α Tregs and *F. prausnitzii* in the colonic mucosa, as no method for quantifying DP8α lymphocytes in biopsy specimens is available. There is an urgent need to develop such a method, based on CD4 and CD8α colabeling by immunohistochemistry, although this approach would not distinguish between CD4CD8αα and CD4CD8αβ lymphocytes, or, preferentially, quantitative RT-PCR, if a specific marker of colonic DP8α lymphocytes can be identified from the transcriptomic signature of these cells. Efforts are currently being made to identify such a marker.

Only about 15% of DP8α PBLs appear to be specific for *F. prausnitzii*, suggesting that the remaining circulating DP8α lymphocytes are not induced by the gut microbiota. Nevertheless, the total frequency of DP8α PBL and the frequency of these cells for *F. prausnitzii* are lower in the blood of IBD patients than in controls (23). The question as to whether the frequency of circulating DP8α lymphocytes and/or of DP8α lymphocytes reactive to *F. prausnitzii* can be viewed as a biological marker of IBD is an important issue as there are currently no specific biomarkers of this disease. It will be necessary to determine whether DP8α levels are correlated with disease type and activity and predict disease progression in a large cohort of IBD patients to answer this question.

If DP8α levels in the blood or the colonic mucosa are found to be predictive of disease progression, this would provide an objective means of assessing the contribution of these Tregs to the prevention of IBD. Such an advance would open up new possibilities for treating IBD by manipulating the frequency of *F. prausnitzii* in the gut microbiota or increasing the number of circulating DP8α Tregs through specific *in vivo* stimulation or induction, or adoptive transfers of these cells. We have found that DP8α Tregs proliferate well *in vitro*, whilst maintaining their regulatory phenotype and functions (23).

CONCLUDING REMARKS – FUTURE ORIENTATIONS

We have identified, for the first time in humans, a mechanism by which the gut microbiota can affect gut homeostasis: the induction of DP8α Tregs in a mucosa exposed to frequent stimulation with microbiota-derived immune stimuli, both PAMPs and microbe antigens. The precise physiological significance of DP8α Tregs remains to be determined, but the discovery of these cells has potentially wide-ranging implications for the management of IBD and, potentially, of other immune diseases involving the abnormal induction and/or function of microbiota-induced DP8α Tregs.

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The Treg/Th17 Axis: A Dynamic Balance Regulated by the Gut Microbiome

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T-helper 17 (Th17) and T-regulatory (Treg) cells are frequently found at barrier surfaces, particularly within the intestinal mucosa, where they function to protect the host from pathogenic microorganisms and to restrain excessive effector T-cell responses, respectively. Despite their differing functional properties, Th17 cells and Tregs share similar developmental requirements. In fact, the fate of antigen-naïve T-cells to either Th17 or Treg lineages is finely regulated by key mediators, including TGFβ, IL-6, and *all-trans* retinoic acid. Importantly, the intestinal microbiome also provides immunostimulatory signals, which can activate innate and downstream adaptive immune responses. Specific components of the gut microbiome have been implicated in the production of proinflammatory cytokines by innate immune cells, such as IL-6, IL-23, IL-1β, and the subsequent generation and expansion of Th17 cells. Similarly, commensal bacteria and their metabolites can also promote the generation of intestinal Tregs that can actively induce mucosal tolerance. As such, dysbiosis of the gut microbiome may not solely represent a consequence of gut inflammation, but rather shape the Treg/Th17 commitment and influence susceptibility to inflammatory bowel disease. In this review, we discuss Treg and Th17 cell plasticity, its dynamic regulation by the microbiome, and highlight its impact on intestinal homeostasis and disease.

Keywords: T-regulatory cells, T-helper 17 cells, intestinal homeostasis, gut microbiome, inflammatory bowel disease

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INTRODUCTION

The gastrointestinal tract represents the largest surface area of the human body that comes into direct contact with the external environment. Consequently, the gut mucosa is exposed to a massive amount and diverse range of foreign antigens. Host detection of pathogenic microbes by antigen-presenting cells (APCs) results in cytokine production, as well as recruitment and differentiation of T-helper (Th) cells. The nature of the offending organisms is crucial for the differentiation into

Abbreviations: AhR, aryl hydrocarbon receptor; AMPK, adenosine monophosphate-activated kinase; APC, antigen-presenting cell; ATP, adenosine triphosphate; BFT, *Bacteroides fragilis* toxin; DC, dendritic cell; GF, germ-free; GPR, G-protein receptor; HIF1α, hypoxia-inducible factor 1α; IBD, inflammatory bowel disease; IL-23R, IL-23 receptor; Ig, immunoglobulin; iTreg, induced Treg; LP, lamina propria; mTOR, mammalian target of rapamycin; nTreg, natural Treg; PSA, polysaccharide A; RA, retinoic acid; SCFA, short-chain fatty acid; SFB, segmented filamentous bacteria; SI, small intestine; TCR, T-cell receptor; Th, T-helper; Treg, T-regulatory cell; Tr1, T-regulatory type I cell.

Th cells, and once contained, effector responses are counterbalanced by Tregs that limit collateral damage. In peripheral organs, such as the gut, the balance between Treg/effector cells is normally achieved by *in situ* induction of these cells from naïve T-cells, recruitment of differentiated Treg/effector cells into the tissue, and reprogramming of already differentiated Treg/effector cells towards other lineages in peripheral tissues (1, 2).

Treg/Th17 AXIS IN HEALTH AND DISEASE

Treg and Th17 Cells: Similarities Beyond Functional Opposites

Th17 cells have only recently been identified as a unique $CD4^+$ T-helper subset, characterized by IL-17 production that promotes tissue inflammation (3, 4). Understanding their function during homeostatic and inflammatory conditions is continuously evolving; however, it is increasingly clear that Th17 cells are critical in protecting mucosal surfaces against microbial pathogens, including bacteria, fungi, and viruses (5, 6), particularly in the lamina propria (LP) of the small intestine (SI), where they are abundantly present (7). Notoriety of Th17 cells initially emerged with the report that IL-17-producing T-cells, driven by IL-23, were major contributors to autoimmune inflammation (8). Indeed, the initial discovery that IL-23, rather than IL-12, was required to develop disease in experimental models of inflammation (9, 10) led to the reevaluation of T-cells that drive IL-23-dependent inflammation.

Over the last two decades, Tregs have been identified as dedicated suppressors of diverse immune responses and inflammation, and central keepers of peripheral tolerance. Tregs are generated in both the thymus (natural Tregs and nTregs) and the periphery (iTregs). While iTregs resemble nTregs in phenotype and function, there are also differences, most prominently regarding their epigenetic and transcriptional status, as well as their inherent stability (11–13). Indeed, when naïve $CD4^+$ T-cells recognize antigen presented as self, in the absence of any inflammatory stimuli, tolerance is induced and these cells, at least partially, differentiate into Tregs. Accordingly, organs exposed to a wide repertoire of foreign antigens, such as the gut, may be dominated by Tregs arising from peripheral conversion, rather than thymic-cell differentiation. The peripheral antigenic landscape may also affect selective expansion of Treg T-cell receptor (TCR) clonotype (14) that is presumably dependent on a peripheral antigenic niche (15). According to this scenario, iTregs represent an essential, non-redundant regulatory subset that supplements nTregs, in part by expanding TCR diversity (16).

Although Tregs and Th17 cells fundamentally differ in function, they also display many common features. Both populations are abundantly found in the periphery, particularly in the intestine (7, 17), and are composed of heterogeneous subpopulations that are able to change effector or suppressor capabilities under different conditions (2). Moreover, shared mechanisms and key mediators (e.g., lineage-specific transcription factors, cytokines) regulate Th17 cells and Tregs, similar to other T-helper subsets. The pleiotropic cytokine, TGFβ, for example, is essential for differentiation of both cell types. TGFβ is non-redundantly required for the generation of Tregs (18) but dispensable for the development of Th17 cells (19). IL-1β can substitute TGFβ in IL-6-mediated generation of Th17 cells (20). Thus, in the absence of proinflammatory signals, such as IL-6 produced by microbial-activated dendritic cells (DCs) or IL-21 produced by IL-6-stimulated T-cells (21–23), priming of naïve $CD4^+$ T-cells by antigen in an environment rich in TGFβ promotes the development of iTregs (24, 25). Conversely, activation in an environment wherein both TGFβ and IL-6 are available promotes Th17 development, at least at mucosal sites (26).

Nonetheless, it is perplexing how the same cytokine can regulate differentiation of T-cells with opposing functions. The answer likely lies in TGFβ's concentration-dependent function. At low concentrations, TGFβ synergizes with IL-6 and IL-21 to promote IL-23 receptor (IL-23R) expression, favoring Th17 differentiation (21–23), whereas at high concentrations, TGFβ represses IL-23R and favors $Foxp3^+$ Tregs, which in turn inhibits RORγt function (27). Conversely, IL-21 and IL-23 can relieve Foxp3-mediated inhibition of RORγt, thereby promoting Th17 differentiation (27). Therefore, the decision of antigen-stimulated cells to differentiate into either Th17 or Tregs depends upon the cytokine-regulated balance of the two master regulators of these cells, RORγt and Foxp3, respectively. Several other mediators can also influence the balance between Th17 and Tregs. RA, a metabolite of vitamin A, preferentially induces Tregs over Th17 cells by enhancing TGFβ signaling while blocking IL-6R expression (28). Moreover, aryl hydrocarbon receptor (AhR), highly expressed on Th17 and Tregs, can promote the induction of both cell types by integrating environmental stimuli (29, 30). Environmental stimuli affecting gastrointestinal immunity via AhR can consist of both dietary- and bacteria-produced ligands, which can interact directly with AhR (31, 32). Interestingly, the loss of bacteria-producing AhR ligands may influence gut immunity and increase the risk of colitis (33).

Treg/Th17 Plasticity

Several studies have established that differentiation of $Foxp3^+$ Tregs is not static and that Tregs can differentiate into Th17 cells. This phenomenon was first reported in mice, wherein IL-6 was shown to convert $Foxp3^+$ cells to Th17 cells in the absence of TGFβ (34–36), which was confirmed in humans (37, 38). In contrast to "Th1-like" Tregs, IL-17-secreting Tregs are suppressive *in vitro* but lose this capacity upon stimulation with IL-1β and IL-6 (38). Accordingly, among $ROR\gamma t^+T\alpha\beta$ cells derived from different murine tissues, the presence of $Foxp3^+$cells that function as Tregs has been reported that coexist with IL-17-producing $ROR\gamma t^+T\alpha\beta$ cells (39). In this study, the ratio of $Foxp3^+$ to IL-17-producing $ROR\gamma t^+T\alpha\beta$ cells is skewed in favor of IL-10 production by Foxp3 and CCL20 and in favor of IL-17 by IL-6 and IL-23. It is unclear why only some $IL\text{-}17^+$ cells express Foxp3, and how this is biological relevant. It is possible that Foxp3 activation occurs during Th17 programming, or alternatively, that Foxp3 expression may signify a distinct differentiation pathway. A recent report showed that under arthritic conditions, $CD25^{lo}Foxp3^+CD4^+$ T-cells lose Foxp3 expression and undergo IL-6-dependent transdifferentiation into Th17 cells, which accumulate in inflamed joints. Once

adoptively transferred into mice, these cells are able to accelerate the onset, and increase severity, of arthritis and associate with loss of Foxp3 expression in the majority of transferred T-cells (40). Interestingly, IL-17-producing $Foxp3^+CD4^+$ lymphocytes are also observed in inflammatory bowel disease (IBD) patients (41). These cells share phenotypic characteristics with both Th17 and Tregs and show potent *in vitro* suppressor activity (42) and increased sensitivity to Th17-generating cytokines in IBD patients versus controls (41). Although Tregs are not sufficient at controlling inflammation in IBD, it is unclear whether or not they retain their suppressive function. Increasing, albeit confounding, evidence points to the different cell origins responsible for this discrepancy (43, 44), adding further complexity to the biological relevance of the functional and phenotypic overlap between Treg and Th17 cells observed in IBD (41, 42).

Whether Th17 cells represent a terminally differentiated lineage or a metastable state is still an area of debate. Multiple studies have identified a Th17 subset that coproduces IFNγ, such as in the inflamed intestine, where they display developmental plasticity (45, 46). Generally, Th17 cells can retain an $IL\text{-}17A^+$ phenotype, or lose IL-17 and acquire expression of IFNγ, in a process driven by IL-12 and IL-23 via a STAT4- and T-bet-dependent manner (47), thus giving rise to Th1-like cells. The latter Th17 subset does not possess colitogenic potential, whereas the former, derived from a Th17 precursor, can mediate experimental colitis via STAT-4 and T-bet, but not through IL-2 or IFNγ receptors (48). One reason that Th17 cells display considerable developmental plasticity may be that RORγt does not participate in stabilizing positive feedback toward transcription factor activation, thus rendering its expression sensitive to environmental signals (49).

Until recently, the conversion between Treg and Th17 was thought to be a one-way street, wherein Tregs can unidirectionally convert into Th17 cells (34). Although coexpression of Th17 and Treg signature genes has been reported in the same cells (38, 39), it is unclear whether Th17 cells can undergo a global reprogramming that drives conversion to another Th-type or that they simply display phenotypic plasticity. Gagliani et al. made the seminal discovery that under homeostatic conditions, intestinal Th17 cells can lose IL-17 expression and a fraction of these "exTh17" cells acquire regulatory features resembling $CD4^+Foxp3^-$ Type 1 Tregs (Tr1) (50). This conversion is determined by reprogramming of the Th17-relevant transcriptional profile, referred to as "transdifferentiation" (51, 52). The functional reprogramming experienced by exTh17 into Tr1 is irreversible; indeed, these cells display anti-inflammatory properties by preventing Th17-mediated colitis (50). While Th17 cells generated with TGFβ1/IL-6/IL-23 are able to promote colitis, exTh17 Tr1 cells generated under the same conditions fail to induce disease. In fact, although TGFβ1 is important for exTh17 Tr1 cell development, Th17 cells remain colitogenic, despite the presence of TGFβ1, as long as they do not convert into Tr1 cells (50). While the main cytokines orchestrating Treg/Th17 plasticity have been identified (**Figure 1**), the fine balance of environmental stimuli required for directing T-helper cells toward one lineage versus another is still known.

REGULATION OF THE Treg/Th17 AXIS BY THE GUT MICROBIOME

Metabolic Control of Th17 and Tregs

Environmental signals and microbiome sensors can profoundly affect T-cell differentiation and response to immune stimuli. Generally, activated effector T-cells are anabolic, primarily employing glucose as their carbon source and utilizing glycolysis for fast access to adenosine triphosphate (ATP). Memory and resting T-cells are instead catabolic with the ability to metabolize fatty and amino acids, in addition to glucose, and depend on oxidative phosphorylation to generate ATP (53). Two key mediators of the glycolytic and lipogenic pathways in T-cells are mammalian target of rapamycin (mTOR) and adenosine monophosphate-activated kinase (AMPK), which promote *de novo* fatty acid synthesis and fatty acid oxidation, consequently inducing either energy production or storage, respectively (54). Both mTOR and AMPK act as crucial cellular energy sensors and are regulated by the availability of nutrients (55, 56). Th17 cells depend on acetyl-CoA carboxylase 1-mediated *de novo* fatty acid synthesis and the underlying glycolytic-lipogenic metabolic pathway for their development, whereas Tregs rely on oxidative phosphorylation and consume their required fatty acids exogenously (57). Upregulation of the glycolic pathway in Th17 cells can also be activated by the transcription factor, hypoxia-inducible factor 1α (HIF1α) (58) that binds to the locus encoding RORγt and enhances its expression while inhibiting Foxp3. Together, this promotes T-cell differentiation toward Th17 and prevents Treg commitment under both normoxic and hypoxic conditions (59). Differently from $Foxp3^+$ Tregs, Tr1 metabolism is supported by glycolysis via HIF1α in early metabolic reprogramming and by AhR at later stages, which then promotes HIF1α degradation (60). Both hypoxia and extracellular ATP increased at inflammatory sites (61, 62), triggered AhR inactivation, and inhibited Tr1 differentiation (60). Therefore, metabolic factors present in the microenvironment have immune-modifying potential, which can skew the balance between inflammation and immune tolerance by biasing the decision of T-cell fate toward either Th17 or Treg lineages.

Regulation of Th17 and Treg by the Commensal Flora

The importance of the gut microbiome in regulating the Treg/Th17 axis became widely appreciated when different groups reported that germ-free (GF) mice demonstrate a decreased frequency of SI Th17 cells and colonic Tregs (63, 64). One of the most widely investigated commensal bacteria in the context of Th17 immunity is segmented filamentous bacteria (SFB), a *Clostridia*-related species (65) that displays features between an obligate and facultative symbiont (66), suggesting that these bacteria obtain nutritional requirements from their host (65). Interest peaked when SFB was reported to specifically induce Th17 cells in the SI (67, 68) and in extraintestinal sites during autoimmune inflammation (69, 70). SFB antigen is presented to $CD4^+$ T-cells by DCs in a major histocompatibility complex-dependent manner, which is required for the induction of SFB-specific intestinal

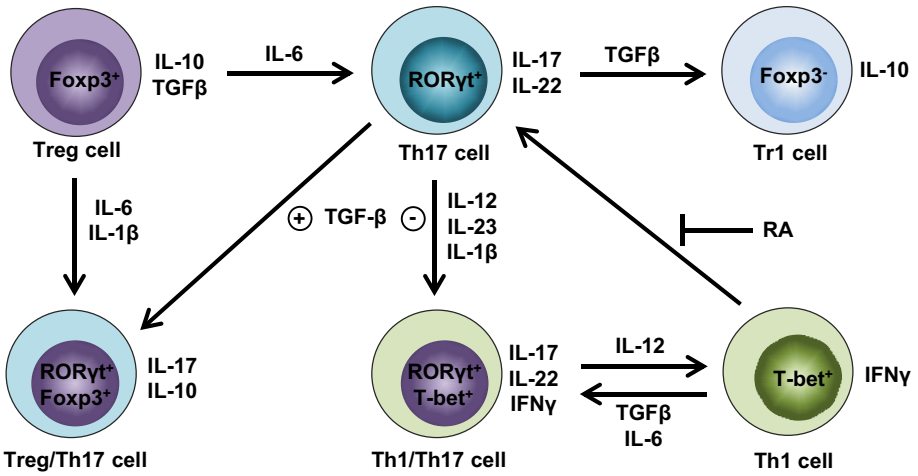

FIGURE 1 | Cytokine milieu orchestrates Treg and Th17 cell plasticity. Th17 cells lose stability in the absence of TGFβ and presence of IL-12, IL-23, and IL-1β, favoring IFNγ expression and differentiation into Th1/Th17 cells that produce both Th1 (e.g., IFNγ) and Th17 (e.g., IL-17, IL-22) cytokines. Further augmentation of IL-12 can fully convert Th1/Th17 cells into Th1 cells, whereas this process can be reverted by either TGFβ and IL-6 or in the absence of retinoic acid (RA) in favor of Th1/Th17 or Th17 cells, respectively. Alternatively, the abundance of TGFβ in the absence of IL-6 drives Th17 cells toward regulatory phenotypes, such as either RORγt$^+$Foxp3$^+$ Treg/Th17 cells or Foxp3$^-$ Tr1 cells. If proinflammatory cytokines are present, including either IL-6 or IL-1β and IL-6, Foxp3$^+$ Tregs have the ability to transdifferentiate into either Th17 or Treg/Th17 cells, respectively.

Th17 cells (71, 72). SFB colonization of GF mice activates a wide range of antimicrobial defenses, including immunoglobulin (Ig) A secretion and LP production of antimicrobial peptides and proinflammatory cytokines (63, 67, 68). SFB colonization is potentially beneficial since it attenuates bacteria-induced colitis (68), but it can also induce colitis in genetically susceptible mice (73), suggesting that while SFB can normally enhance immune control of infection, its presence can also result in inflammation. The abundance of SFB, together with gut barrier function, is regulated by the IL-23R/IL-22 pathway (74). When the intestinal barrier is disrupted, systemic dissemination of microbial products occurs, which invokes the IL-23 pathway and initiates barrier repair, as well as Th17 responses aimed to neutralize invading commensal microbes (74). Moreover, SFB-induced-IL-23 results in production of IL-22 by type 3 innate lymphoid cells, which is critical for the production of serum amyloid A proteins 1 and 2 by epithelial cells (75). This circuit promotes IL-17 expression in RORγt$^+$ T cells, especially in the terminal ileum, which is the site of SFB attachment to the epithelium, the essential condition for Th17 induction by SFB (75, 76). SFB-induced activation may also result in the generation of autoreactive Th17 cells in response to presentation of autoantigen in the setting of a breached intestinal barrier. SFB-mediated induction of Th17 immune responses can also occur indirectly via other cell types. Indeed, Treg-specific MyD88 deficiency is sufficient to impair intestinal IgA responses to SFB and results in the expansion of Th17 cells (77).

Another resident of the human gut microbiome influencing T-cell homeostasis is the symbiont, *Bacteroides fragilis*. *Bacteroides* species are normal constituents of the intestinal microbiome; however, under certain circumstances, these microbes can become pathogens. Polysaccharide A (PSA), the most abundant capsular polysaccharide expressed by *B. fragilis*, mediates conversion of CD4$^+$ T-cells into IL-10-producing Foxp3$^+$ Tregs via TLR2 and suppresses Th17 responses, thus facilitating colonization of *B. fragilis* (78). Consistently, PSA is able to both prevent and ameliorate experimental colitis (79), suggesting that *B. fragilis* facilitates Treg differentiation in the gut and induces mucosal tolerance. Nevertheless, strains of *B. fragilis* secreting the zinc metalloprotease, *B. fragilis* toxin (BFT), have been implicated in IBD (80, 81) and in IL-17-dependent inflammation-associated colon cancer (82). Indeed, BFT can alter the function of intestinal epithelial tight junctions, resulting in increased permeability and diarrhea (83).

Other bacterial strains, such as *Clostridia*, are able to induce Tregs within the gut. Most *Clostridia* maintain a commensal relationship with the host, with a few exceptions, including *Clostridia perfringens*, *Clostridia difficile*, and *Clostridia tetani*, which produce toxins and are pathogenic. Colonization of GF mice with a defined mixture of 46 *Clostridium* strains belonging to clusters XIVa and IV induces the differentiation of colonic Helios-negative Tregs in a MyD88-independent manner (64). Additionally, a mixture of 17 strains from *Clostridiales* clusters VI, XIVa, and XVIII isolated from human feces also exhibits Treg-inducing activity (84) and suggests that *Clostridium*-dependent induction of Tregs may contribute to the maintenance of intestinal immune homeostasis. Similarly, colonization of GF mice with altered Schaedler flora, a standardized cocktail of benign intestinal commensal microbiota, results in the *de novo* generation of Tregs and downregulation of Th1 and Th17 immunity (85). A summary of bacterial strains influencing Treg and Th17 intestinal immune responses are depicted in **Figure 2**.

The precise mechanism(s) underlying colonic Treg induction by the gut microbiome remains unclear, although several reports suggest that commensal, microbe-derived short-chain fatty acids

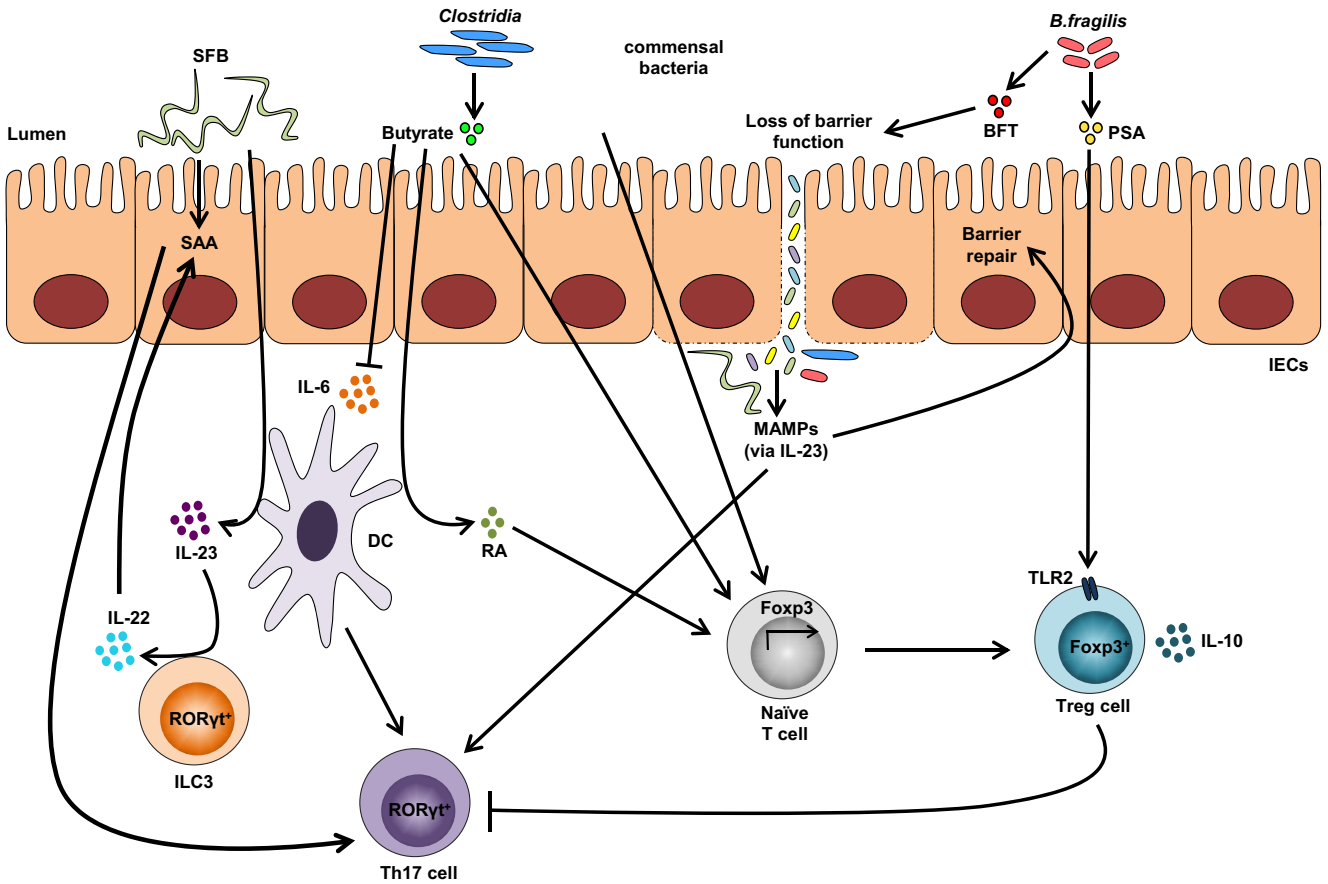

FIGURE 2 | Impact of the gut microbiota on Treg and Th17 immune responses. Colonization with segmented filamentous bacteria (SFB) occurs by intimate attachment to the intestinal epithelium and promotes the development of Th17 cells via intestinal epithelial cell (IEC)-derived cytokines, serum amyloid A (SAA), as well as antigen presentation by dendritic cells (DCs). Adhesion of SFB to IEC can potentially generate a circuit, wherein DC-derived IL-23 stimulates IL-22 production by type 3 innate lymphoid cells (ILC3), which in turn induces SAA from IEC and can lead to Th17 cell differentiation. Conversely, colonization of beneficial commensal bacteria induces *de novo* generation of Tregs and downregulates Th17 immune responses. Commensal bacteria, including most *Clostridia* species, produce short-chain fatty acids, i.e., butyrate, which participates in the *de novo* generation of Tregs by suppressing proinflammatory cytokines, by promoting RA production from DCs, and by inducing Foxp3 transcription. Among different *Bacteroides fragilis* strains, those expressing polysaccharide A (PSA) mediate the generation of Tregs via TLR2, while those secreting *B. fragilis* toxin (BFT) alter the function of IEC tight junctions. Upon disruption of barrier function, dissemination of microbial products, recognized by microbe-associated molecular patterns (MAMPs), occurs and activates the IL-23 pathway, resulting in subsequent barrier repair and stimulation of Th17 immune responses.

(SCFAs), particularly those from *Clostridiales*, may be involved (86–88). SCFAs, together with organic acids and alcohols, are metabolic end products generated in the lower gastrointestinal tract from fermentative growth of carbohydrates and proteins that cannot be degraded (89). Specifically, locally produced butyrate participates in colonic *de novo* Treg development, whereas oral administration of acetate and propionate contributes to Treg migration into the colon by upregulating G-protein receptor (GPR)15 (90). Indeed, *in vivo* administration of butyrate suppresses proinflammatory cytokines from macrophages and DCs, likely through inhibition of histone deacetylases (87, 91), and ameliorates experimental colitis (88). Butyrate participates in Treg differentiation by facilitating histone H3 acetylation in the promoter and conserved non-coding sequence regions 1 and 3 of the Foxp3-encoding locus (88) or by activating its receptor, GPR109a, that promotes RA production in DCs and leads to induction of Treg differentiation (92). Interestingly, T-cell regulation by SCFAs is dependent on the cytokine milieu and immunological context. Indeed, acetate promotes IL-10-producing T-cells during steady-state conditions and effector Th1 and Th17 cells during active immune responses (93). Other dietary-related factors, such as fat-enriched diets, have been implicated in gut microbial regulation of intestinal immunity. In fact, high-fat-diet-derived microbiota decreases Th17 cell frequency and the ability of intestinal APCs to generate Th17 cells *in vitro*, thus contributing to low-grade inflammation (94).

Dysbiosis Affecting the Th17/Treg Axis in IBD

Dysbiosis is considered an alteration of the resident commensal microenvironment compared to commensal communities found

in healthy individuals and has been reported in many diseases, including IBD (95). Dysbiosis can be classified into three different, non-mutually exclusive, categories: loss of beneficial microbial organisms, expansion of pathobionts, and loss of overall microbial diversity (96). Interestingly, reduced abundance of butyrate-producing bacteria, i.e., *Clostridiales* cluster IV and XIVa, has been found in IBD patient fecal samples (95), supporting the hypothesis that presence of beneficial bacteria inducing Treg differentiation is important to prevent IBD. Although there is no single organism capable of inducing IBD, a few pathogens have been implicated. Two colitogenic proteobacteria, *Proteus mirabilis* and *Klebsiella pneumonia*, have been identified in ulcerative colitis-like T-bet$^{-/-}$Rag2$^{-/-}$ mice that spontaneously develop dysbiosis and colitis (97, 98). However, maternally transmitted endogenous microbes are also required to maximize inflammation in these mice (98), suggesting that microbe interaction may determine whether a pathobiont will display a pathogenic profile. Similarly, IBD patients display an increased number of *Actinobacteria* and *Proteobacteria* (95). Intestinal T-cell homeostasis appears to be dependent not only on the type of bacteria present but also on overall microbial diversity. Indeed, the transfer of over 30 different human *Clostridia* strains into GF mice induced a threefold expansion of Tregs compared to uncolonized controls, whereas transfer of a single strain from the same *Clostridia* collection induced a more modest Treg response (64, 84), suggesting that greater microbial diversity maximizes host immune responses and its reduction may contribute to inflammatory processes, such as in IBD (99).

CONCLUSION

Although progress has been made in clarifying the role of the microbiome in Treg and Th17 mucosal immunity, its impact on disease, such as IBD, remains controversial. A better understanding of the mechanisms regulating these processes may aid in the development of therapeutic agents aimed to maintain appropriate Treg/Th17 balance and restore homeostatic function during disease states.

AUTHOR CONTRIBUTIONS

SO and TP conceptualized review. SO provided an initial draft of the review, while TP performed the final edits.

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Mucosal-associated invariant T cells in inflammatory bowel diseases: bystanders, defenders, or offenders?

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The quest for new therapeutics and better follow-up of patients with inflammatory bowel diseases (IBD) requires the clearest possible picture of the immunological mechanisms underlying these complex pathologies. We identified recently a potential new player in this destructive game, a non-conventional T cell subset called Mucosal-Associated Invariant T (MAIT) cells. These cells were initially identified on the basis of their use of a semi-invariant TCR, made of the invariant Vα7.2–Jα33 TCRα chain (now TCRAV1S2–AJ33) paired to a limited number of different TCRβ chains (1). Human MAIT cells are mostly CD8+ T cells with an effector/memory phenotype and expression of various chemokine receptors involved in extra-lymphoid migration. They also express most markers associated with IL-17 producing T cells, such as RORγt, high CD161, IL-23R, and CD26. They make up to 10% of peripheral blood and intestinal lamina propria T cells, and are even more abundant in the liver (2). The most striking feature of MAIT cells is their recognition of highly conserved microbial-derived metabolites associated to a monomorphic MHC class-I like molecule, MR1 (MHC-related 1) (3). These ligands structurally belong to the pterin family and are derived from the riboflavin synthesis pathway. Recent experiments showed that virtually all MAIT cells are stained by fluorescent MR1 tetramers loaded with these specific metabolites. This pathway is absent in vertebrates, but many bacterial and fungal species produce riboflavin and therefore, MAIT cells-specific ligands. In this respect, these metabolites behave like microbial innate signals, and may alert MAIT cells that an invasive infection is ongoing. Upon activation, MAIT cells release TNFα, IFNγ and become cytotoxic; they also produce IL-17 in specific conditions. In fact, they represent the great majority of naturally occurring IL-17-producing CD8+ T cells in the human peripheral blood. These cells are very likely to perform important anti-microbial functions, as suggested in humans and mice models (4). However, numerous reports suggest that MAIT cells are recruited from the blood to inflamed tissues in chronic inflammatory diseases such as multiple sclerosis, psoriasis, and systemic lupus, among others. We recently showed that Crohn's disease (CD) patients display a decreased number of blood MAIT, balanced by their accumulation in the inflamed portions of the gut (5). As already stated, MAIT cells are equipped with chemokine receptors allowing migration toward tissues, in particular in conditions of inflammation. Therefore, it might be suggested that they are non-specifically attracted to sites of inflammation and are only bystanders in this process. However, we wish to discuss in this opinion article the arguments in favor of a relevant role for this T cell subset, at least in the context of CD.

We showed in our study that blood MAIT cells from CD patients showed an altered phenotype, increased *in vivo* proliferation, and, interestingly, a shift in cytokine production with decreased IFNγ and increased IL-17 production (5). While this description does not allow any formal conclusions about the direct involvement of MAIT cells in the pathophysiology of the disease, it has several important implications. Indeed, although blood MAIT cells may be non-specifically attracted to the inflamed gut by locally produced chemokines, it must be reminded that a significant number of them are found in the gut lamina propria in the healthy intestine. Therefore, it is more than likely that these intestinal cells are also activated in CD, and produce cytokines, which are highly relevant to the pathology, i.e., IFNγ, TNFα, and IL-17. Hence, it is difficult to suggest that these local cells (as well as newcomers from blood), strongly activated and producing inflammatory cytokines, have no consequences on the local inflammation. The question that needs to be addressed is the mechanisms by which MAIT cells may be activated; we propose two non-mutually exclusive hypotheses:

1. The microbial ligands recognized by the MAIT cells TCR (named RL antigens after their ribityl-lumazine composition) derive from a conserved pathway of riboflavin synthesis. This pathway is conserved among many bacterial species, and may explain MAIT cells activation and recruitment in response to infections by *Salmonella typhimurium*, *Mycobacterium tuberculosis*, *Shigella flexneri*, *Vibrio cholerae*, and others. However, these ligands are also synthetized by non-pathogenic bacteria, including several species found in the normal gut microbiota. In mice, peripheral maturation of MAIT cells is dependent upon the colonization of the intestine by the commensal flora. Therefore, there are strong interactions between MAIT cells and ligands derived from the commensal flora. Nevertheless, mechanisms must exist to ensure

that intestinal MAIT cells do not aggressively respond to gut bacteria in the steady-state. In IBD, the disruption in local immune homeostasis results in dysbiosis, increased permeability of the mucosa, and subsequent increased bacterial translocation. Therefore, it is likely that these events induce also an increased availability of antigens derived from the commensal flora and therefore, MAIT cells activation.

2. Human MAIT cells constitutively express receptors for IL-12, IL-23, and IL-18. It is now demonstrated that they can be activated in the absence of their cognate antigens by a combination of IL-12 + IL-18 (6). MAIT cells activation by cytokines result in exclusive IFNγ secretion, at least in the blood and liver (7). Furthermore, both cytokines play an important function in MAIT cells activation by bacteria, independently of their capacity to produce riboflavin, since blocking antibodies to IL-12 and IL-18 strongly inhibit activation. This pathway depends on activation of the inflammasome in monocytes, resulting in cytokine secretion and MAIT cells activation in co-culture experiments. IL-12 and IL-18 are both over-produced in the intestinal mucosa of CD patients. IL-23R, NLRP3, IL-18R1, and IL12B2 gene polymorphisms are significantly associated with CD, further suggesting a prominent role for these cytokines in the pathophysiology of the disease. Therefore, it is likely that this cytokine environment participates locally in MAIT cells activation, and may influence their survival and/or proliferation. Altogether, the cytokines produced in large quantities by intestinal antigen-presenting cells create an environment that favors MAIT cells activation, both in the presence and absence of their cognate antigens.

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The next important question is obviously the role that activated MAIT cells may play during CD. In most reports describing MAIT cells implications in autoimmune diseases, the authors suggest that these cells are pro-inflammatory and deleterious. This is inferred from the fact that *in vitro* mitogen-activated MAIT cells produce IFNγ, TNFα, and IL-17. Given the reported inflammatory role for IL-17 in several chronic inflammatory diseases, this cytokine secretion pattern is interpreted strictly as pro-inflammatory. However, it is important to pinpoint several facts. First, in most reports, MAIT cells activation *in vitro* by riboflavin-producing bacteria induce mostly IFNγ and TNFα secretion, with little or no IL-17. Accordingly, cytokines-induced MAIT cells activation results solely in IFNγ production. Second, very little is known about the regulation of cytokine secretion by MAIT cells *in vivo*. Of note, there is no published description of the functional response of mucosal MAIT cells to bacteria, their cognate antigens and/or cytokines. It is likely that the mucosal specific environment (cytokines, antigen-presenting cells, and other features) skews MAIT cells functions, as already described for conventional T cells. This question is of great importance in the context of CD, where the major inflammatory cytokine is suggested to be IFNγ, although IL-23 and IL-17 are produced in great quantities in the inflamed mucosa (8). Indeed, several studies reported a protective role for IL-17 in the intestinal mucosa (9, 10), and clinical trials with the anti-IL-17 antibody secukinumab demonstrated an absence of efficiency in CD (11), suggesting IL-17 is not the major cytokine in the inflammatory process (12). In our own study, we reported that blood MAIT cells from CD patients show a shift in cytokine secretion with lower IFNγ and higher IL-17. Therefore, it is possible that in CD, MAIT cells recruited from the circulation to the mucosa display enhanced protective IL-17 secretion in response to the local inflammation, as an endeavor to heal the aggressed mucosa. In other words, there is insufficient data available to demonstrate at this point that MAIT cells are necessarily pro-inflammatory, especially in the context of IBD.

CONCLUDING REMARKS

There is no demonstration to date that MAIT cells are directly involved in autoimmune/inflammatory diseases. However, there is sufficient data to foster more studies on this topic, especially in CD. The main problem is that there is no relevant animal model available, as mice display a defect in MAIT cells development. Therefore, it is important to pursue the analysis of human MAIT cells *in vitro* and *ex vivo*, in healthy donors and CD patients. There is a need to study these cells in the mucosa, where they probably behave differently than at the systemic level. It will be also useful to analyze the role that some important gene polymorphisms involved in CD may have on MAIT cells activation and functional response. Finally, longitudinal analysis of MAIT cells phenotype and functions in CD patients undergoing various targeted therapies will also shed light on their role in the disease, and, possibly, their potential use as biomarkers.

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Intestinal microbiota and the innate immune system – a crosstalk in Crohn's disease pathogenesis

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Crohn's disease (CD) is a chronic, relapsing inflammatory disorder that can occur anywhere along the gastrointestinal tract. The precise etiology of CD is still unclear but it is widely accepted that a complex series of interactions between susceptibility genes, the immune system and environmental factors are implicated in the onset and perpetuation of the disease. Increasing evidence from experimental and clinical studies implies the intestinal microbiota in disease pathogenesis, thereby supporting the hypothesis that chronic intestinal inflammation arises from an abnormal immune response against the microorganisms of the intestinal flora in genetically susceptible individuals. Given that CD patients display changes in their gut microbiota composition, collectively termed "dysbiosis," the question raises whether the altered microbiota composition is a cause of disease or rather a consequence of the inflammatory state of the intestinal environment. This review will focus on the crosstalk between the gut microbiota and the innate immune system during intestinal inflammation, thereby unraveling the role of the microbiota in CD pathogenesis.

Keywords: Crohn's disease, innate immune system, inflammation, microbiota, dysbiosis

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Introduction

Crohn's disease (CD) is a chronic, relapsing inflammatory disorder affecting the gastrointestinal tract and together with ulcerative colitis (UC) commonly included in the collective term inflammatory bowel diseases (IBD). Inflammation associated with CD is characterized by a discontinuous, transmural pattern, and can affect any part of the gastrointestinal tract (1). Although the precise etiology of CD remains unclear, several factors are believed to play a role in its development and progression. Given the results from genome-wide association studies (GWAS), it is undisputed that genetic susceptibility plays an important role in disease development (2, 3). Among immune-related conditions, CD is special in that the genetic contribution to disease is high with concordance rates up to 50% among monozygotic twins (4, 5). However, in countries that have adopted a "modernized" lifestyle the incidence rates of IBD have steeply increased in the last few decades. These epidemiological observations clarify that the host genotype alone accounts for a significant but limited proportion of CD risk and rather underline the multifactorial nature of the disease (6–10). The role of the intestinal microbiota in disease pathogenesis has become more and more appreciated. Evidence from genetic, immunological, and microbial studies implicates that chronic intestinal inflammation results from a dysregulated immune response toward components of the commensal intestinal microbiota in genetically susceptible individuals (11). This perception is supported by

the identification of several risk loci associated with CD, including genes involved in intracellular processing and killing of bacteria (e.g., NOD2, ATG16L1, IRGM) (12, 13).

Starting with the intestinal lumen, the first part of this review will focus on the intestinal microbiota composition and its changes during inflammation. In the following sections, we will then take a closer look at deeper layers of the intestinal tissue to dissect the interplay of the intestinal microbiota with cells of the host immune system.

Human Intestinal Microbiota

Intestinal Microbiota – Composition

The assembly of the human gut microbiota starts during birth with *Bifidobacterium* and *Lactobacillus* derived from the vaginal canal and breast milk displaying the microorganisms that initially colonize the human intestinal tract (14). During adolescence, phylogenetic diversity of the microbial community increases leading to a complex, diverse, and dynamic microbiota. The adult human gastrointestinal tract contains an abundant microflora, including bacteria, archaea, eukarya, and viruses (15). Recent development of culture-independent molecular profiling methods has greatly advanced our understanding of the microbiome (16). Nowadays, the composition of microbial communities is typically evaluated by targeting the bacterial 16S ribosomal RNA gene as a phylogenetic marker (15). These culture-independent molecular techniques indicate that the human microbiota contains about 200 strains of bacteria comprising over 100 different bacterial species, dominated by just a few phyla (16). Even though these techniques revealed a high level of variability between individuals at the bacterial species level, *Firmicutes* and *Bacteroidetes* represent the predominant phyla across all vertebrates, representing over 90% of all intestinal bacteria. It is assumed that the individual composition of the microbiota is relatively stable over time, a term called "resilience." Factors that promote microbial diversity in healthy adults include diet, environment, gender, and genetics among others (17, 18). Studies focusing on gut microbiota profiles of adult humans with varying degrees of genetic relatedness effectively demonstrated the impact of genetic and environmental factors on gut microbiota development. The intestinal microbiota composition of monozygotic twins indicated a high degree of similarity, but was yet distinct. Individuals who were living in the same environment and shared similar eating habits showed the least similarity, while siblings displayed an increased similarity in their species profile (1, 19). Recent studies in nucleotide-binding oligomerization domain 2 (*Nod2*)-deficient mice and humans carrying *NOD2* variants point to an essential role of *Nod2* for the development and composition of the host microbiota. *Nod2*-deficient mice displayed an increased load of commensal microbiota and an altered microbiota composition. Additional studies in weaning mice illustrated that NOD2 may affect the microbial community early in life. Furthermore, the substantial changes in the microbiota composition caused by *Nod2* deficiency was paralleled by an increased colitis severity following chemically induced injury. Subsequent co-housing and cross-feeding experiments revealed the transmissibility of the observed genotype-dependent disease risk (20). Taken together, these studies clearly indicated that the host genotype has a lasting effect on the intestinal microbiota composition and *vice versa* the intestinal microbiota is capable of determining the host phenotype.

Intestinal Microbiota – Shaping the Gastrointestinal Immune System

In health, the relationship between the host and the intestinal microbiota provides mutual benefits. On the one hand, the microbiota benefits from the nutrient-rich environment of the gut paving the way for the establishment of a relatively stable ecosystem. In turn, the intestinal microbiota enriches the host with vital functions that the host itself cannot perform. The intestinal microbiota is substantial for mucosal barrier function, affects the development of the mucosal immune system, and is essential for a number of physiological metabolic processes as described further on (21). The profound effects of the commensal microbiota on intestinal and immune cell development have best been highlighted by the engraving phenotype of germ-free (GF) mice. One of the first deficiencies observed in GF mice was a profound reduction of secretory immunoglobulin A (IgA) levels in the intestine. Subsequent mono-association of these mice with various bacteria was shown to lead to an increased IgA expression (22). In addition to numerous defects in antibody production, GF mice display various morphological tissue defects in their intestines [**Table 1**; (23)]. These developmental impairments are attenuated following the introduction of gut bacteria, once more illustrating the indispensable connection between the ultrastructural development of the intestine and the commensal microbiota. Next to defects in intestinal organ development, investigations on GF mice also revealed cellular defects in intestinal epithelial and lamina propria lymphocytes as well as in mesenteric lymph nodes (**Table 1**). Normal functioning of intestinal epithelial cells, including the expression of microbial recognition receptors,

TABLE 1 | Defects in the intestinal mucosal immune system in GF mice.

Defects in intestinal organ development in germ-free (GF) mice

	Site	Phenotype in GF mice
Small intestine	Peyer's patches	Fewer, less cellular
	Lamina propria	Thinner, less cellular
	Germinal centers	Fewer plasma cells
	Isolated lymphoid follicles	Smaller, less cellular
MLN	Germinal centers	Smaller, less cellular Fewer plasma cells

Cellular defects in germ-free (GF) mice

	Cell type	Phenotype in GF mice
IEL	$CD8^+$ T cells	Fewer, reduced cytotoxicity
LPL	$CD4^+$ T cells	Proportional decrease in number
	$CD4^+$ T cells	Decreased Th17 cells (small intestine)
	$CD4^+$ T cells	Increased Th17 cells (colon)
MLN	$CD4^+CD25^+$ T cells	Reduced expression of FoxP3
	$CD4^+CD25^+$ T cells	Reduced suppressive capacity

IEL, intestinal epithelial lymphocytes; LPL, lamina propria lymphocytes; MLN, mesenteric lymph nodes; GF, germ free [adapted from Ref. (23)].

defensins, and antimicrobial peptides (AMPs), was shown to be impaired in GF animals compared to their conventionally raised counterparts (23).

Detailed investigations on certain members of the intestinal microbiota served to unravel mechanisms by which commensal bacteria induce immune tolerance. For example, investigations on segmented filamentous bacteria (SFB) revealed that these Gram-positive bacteria are sufficient to promote T helper 17 cells (Th17) development in the small intestinal lamina propria (24). In addition, colonization of GF mice with SFB resulted in an increased production of serum amyloid A in the terminal ileum, which in turn enhanced IL-6 and IL-23 production by lamina propria dendritic cells (DCs), thereby stimulating a Th17 inducing environment (24). Th17 effector cytokines enhance epithelial cell tight junctions, induce mucin production, and have been associated with induction of AMPs. Colonization of GF mice with SFB resulted in the induction of multiple AMP genes, for example, *RegIIIγ* (24). Even though Th17 cells are crucial for protecting the host against pathogenic infection, it should be noted that these cells also display an inflammatory potential as observed in different murine models of autoimmune diseases (25, 26). Recently, certain strains within *Clostridia* clusters XIVa, IV, and XVIII were shown to induce regulatory T cell (Treg) responses in the colon (27). Another prominent human commensal, *Bacteroides fragilis*, was found to direct the development of $FoxP3^+$ Tregs via the immunomodulatory molecule polysaccharide A. Monocolonization of GF mice with *B. fragilis* was accompanied by an increased suppressive Treg capacity and the induction of an anti-inflammatory cytokine profile emerging from $FoxP3^+$ T cells in the gut (28).

Intestinal Microbiota – Metabolic Functions

Next to their ability to promote immune system development and maturation as outlined above, the intestinal microbiota enriches the host with metabolic functions by synthesizing vitamins and degrading complex indigestible dietary carbohydrates and proteins. Fermentation of dietary fiber leads to the production of short chain fatty acids (SCFAs), primarily acetate, propionate, and butyrate (21, 29). The functions of SCFAs in promoting colonic health range from displaying a unique energy source for colonocytes to mediating anti-inflammatory and anti-tumorigenic effects (30). Therefore, it is easily comprehensible that conditions coming along with reduced SCFA-levels, including diversion colitis, fiber-free diet, or GF conditions, present with metabolic starvation and consecutive colonic atrophy (31). Among SCFAs, butyrate has received most attention for its effects on colonic health. Butyrate displays an anti-inflammatory effect by decreasing the expression of pro-inflammatory cytokines via inhibition of NF-κB activation and was shown to exert the ability to influence gene expression in the colon through histone deacetylase inhibition (32, 33). Moreover, butyrate elicits biological effects by binding to G protein-coupled receptors, namely GPR109A and GPR43. Butyrate serves as an endogenous agonist for GPR109A, expressed in the colonic epithelium, adipose tissue, and on immune cells, while GRP43 is activated by all three SCFAs (34). The activation of GPR109A results in a decrease of intracellular cAMP-levels accompanied by controlling electrolyte and water absorption, thereby potentially affecting the incidence of diarrhea (35). Activation of GPR109A by butyrate was found to impose anti-inflammatory properties in colonic macrophages and DCs and enabled them to induce the differentiation of Tregs and IL-10 producing T cells. Furthermore, GPR109A signaling was shown to be decisive for butyrate-mediated induction of IL-18 in the colonic epithelium (36). Comparative analysis in GF mice and their conventionally colonized counterparts revealed markedly reduced SCFA concentrations in the intestines of GF mice. Application of SCFAs via drinking water restored colonic Treg homeostasis and function in GF mice, suggesting that their lack of SCFAs may account at least partially for their immune defects, especially their reduced colonic Treg numbers (37). In a murine model of colitis, dextran sulfate sodium (DSS)-induced inflammation in GF mice was ameliorated through additional application of acetate. Conventionally colonized mice lacking Gpr43 showed a markedly increased inflammatory response following DSS application as compared to wild-type (wt) mice. Subsequent acetate application via drinking water resulted in a decrease of colonic inflammation but only in wt mice. Additional acetate application in $Gpr43^{-/-}$ mice did not lead to alterations in the severity of inflammation indicating that the protective effect of acetate seen in wt mice occurred through binding to GPR43 (38). Most recently, Mackay and colleagues provided evidence that diet deficient or low in fiber content exacerbates colitis induced by DSS, whereas a high-fiber diet was shown to exert a protective effect. Moreover, it was demonstrated that fiber mediates its protective properties via activating the NLRP3 inflammasome (39).

Given the aforementioned tremendous metabolic functions of the intestinal microbiota, the question raises whether inflammation in peripheral tissues is also influenced by intestinal microbiota-derived metabolites. Recently, Marsland and colleagues found evidence that dietary fermentable fiber and SCFAs are capable of shaping the immunological environment not only in the murine intestine but also in the lung (40). Oral administration of a high-fiber diet was accompanied by increased circulating SCFA levels and concomitant protection against allergic inflammation in the lung following exposure to house mite extract (HM) through intranasal administration. By contrast, feeding mice with a low-fiber diet resulted in decreased SCFA levels and an increase of allergic airway disease in response to HM application. Accordingly, histological sections revealed enhanced eosinophilic and lymphocytic infiltrates in the airways of mice fed a low-fiber diet as well as enhanced concentrations of IL-4, IL-5, IL-13, and IL-17A in lung tissue homogenates. The detected differing outcome in response to HM exposure underlines the impact of SCFAs on shaping the immunological environment in the lung, thereby impinging on the severity of allergic inflammation.

Intestinal Microbiota in Disease – Changes in the Gut Microbial Ecosystem

Changes in the microbiota composition display a hallmark of CD, commonly described as dysbiosis. Individuals with IBD have been characterized by marked qualitative and quantitative changes in their microbiota composition (41). Several culture-dependent and -independent analyses focusing on the microbiota

profile of CD patients revealed less complex microbiota profiles and higher numbers of mucosa-associated bacteria compared to healthy individuals (41–45). Metagenomic-based studies have reported a reduction in members of the phyla Bacteroidetes and Firmicutes in patients suffering from CD or UC (41, 46, 47). Among members of the Firmicutes phyla, significant reductions in the butyrate-producing bacterium *Faecalibacterium prausnitzii* (*F. prausnitzii*) have been observed repeatedly in CD patients (7, 48). This bacterium displays anti-inflammatory properties, which have been intensively studied both *in vitro* and *in vivo*. Stimulation of peripheral blood mononuclear cells with *F. prausnitzii* induced very low levels of the pro-inflammatory cytokines IFNγ and IL-12 paralleled by high levels of IL-10 (48). These *in vitro* effects were confirmed *in vivo* by using a 2,4,6-trinitrobenzenesulphonic acid (TNBS)-induced model of colitis. Oral administration of *F. prausnitzii* markedly reduced the severity of TNBS colitis and tended to rectify the dysbiosis associated with colitis in this model. Follow-up studies after ileal resection provided evidence that a low proportion of *F. prausnitzii* on resected ileal mucosa of CD patients was associated with an increased risk of endoscopic recurrence after 6 months.

The changes in abundance and biodiversity of intestinal microbiota during inflammation are further characterized by an increase of members associated with the Proteobacteria and Actinobacteria phyla. CD patients were shown to harbor increased loads of *Enterobacteriaceae*, in particular *Escherichia coli* belonging to the taxonomic lineages B2 and D (49). Adherent-invasive *E. coli* (AIEC) pathovar has been commonly identified in the intestinal mucosa of patients with CD, particularly associated with ileal mucosal lesions (50, 51). Isolates of these certain strains of *E. coli* were shown to not only adhere to epithelial cells but also to invade and replicate intracellularly. Moreover, AIEC is able to survive and replicate within macrophages without triggering host cell death accompanied by the release of large amounts of tumor necrosis factor (TNF)-α (52). Compared to healthy controls, mucosal biopsy specimens from CD patients displayed a 10-fold higher presence of bacteria that penetrate the mucus layer. These results from fluorescent *in situ* hybridization analyses indicate that in CD the microbiota might have closer contact with the mucosa, a hypothesis that will be discussed in a later section. However, this finding could be explained by the increased numbers of mucolytic bacteria, such as *Ruminococcus gnavus* and *Ruminococcus torques*, observed in macroscopically and histologically unaffected intestinal epithelium of CD patients (53). In 2011, the so far largest cohort study provided a detailed description of the microbiome in CD patients (54). A lower occurrence of *F. prausnitzii*, *Bifidobacterium adolescentis*, *Dialister invisus*, and an uncharacterized species belonging to *Clostridium* cluster XIVa as well as a higher number of *R. gnavus* characterized the dysbiosis signature of CD patients (54). From a metabolic point of view, this comes along with an increased mucolytic and reduced butyrate-producing capacity. Moreover, this study not only confirmed previous findings but also propounded that these changes were markedly characteristic for the disease as this profile was not found in unaffected relatives of analyzed patients.

Intestinal Microbiota in the Pathogenesis of Crohn's Disease

Experimental and Clinical Evidence

Over the last decades, a consistent body of evidence has accumulated supporting the role of the intestinal microbiota in precipitating IBD. Studies in IL-2- and IL-10-deficient mice were one of the first highlighting the role of the intestinal microbiota in the induction and perpetuation of chronic inflammation. Spontaneous colitis observed in IL-2 deficient mice when raised under specific-pathogen-free (SPF) conditions was unverifiable in animals with the same genetic background bred under GF conditions. Similarly, IL-10-deficient animals developed an attenuated inflammation with regard to disease severity and expansion when kept in a facility with a defined microbial environment (55, 56). Subsequently, *Tbet*-deficient mice were shown to develop chronic colitis with a histopathological similarity to human UC. The study revealed also a correlation of the presence of *Proteus mirabilis* and *Klebsiella pneumoniae* and colitis in these animals. Furthermore, healthy wt mice developed chronic colitis upon co-housing with T-bet$^{-/-}$/Rag2$^{-/-}$ mice (TRUC) or following gavage of feces from TRUC mice (57, 58). These data indicate that loss of T-bet influences bacterial populations to become colitogenic and that colitis is transferable to genetically intact hosts. More recently, studies in TNF$^{\text{delta ARE}}$ mice provided clear experimental evidence for the causal role of bacterial dysbiosis in the development of chronic small intestinal inflammation. Deletion in the TNF adenosine–uracil (AU)-rich elements (ARE) leads to TNF-driven spontaneous small intestinal inflammation, characterized by both transmural manifestation and a predominant ileal involvement, thereby mimicking key features of human CD pathology (59). Haller and colleagues assessed the impact of intestinal bacteria in this model of ileitis by using different hygienic conditions (GF, SPF, and conventional housing) as well as antibiotics. CD-like ileitis development was completely absent in GF mice. Antibiotic treatment resulted in an amelioration of disease severity and a recurrence of inflammation was observed following the relapse of microbiota composition. Moreover, transfer of dysbiotic cecal microbial communities from SPF–TNF$^{\text{delta ARE}}$ mice into GF recipients resulted in the development of ileitis, mimicking inflammation severity of corresponding donors (60). These aforementioned studies just provide examples for the experimental evidence that has accumulated over the last years.

Event though CD can manifest anywhere along the human alimentary tract, it is mainly observed in areas containing the highest concentrations of bacteria (i.e., terminal ileum and colon). One of the first clinical references for the involvement of the intestinal microbiota in disease pathogenesis came from experiments showing that diversion of the fecal stream from an inflamed segment of the small intestine improved symptoms of CD patients. Furthermore, restoration of fecal stream and postoperative exposure of the neoterminal ileum to luminal contents induced inflammation, indicating that the microbiota acts as a trigger in postoperative recurrence of CD (61, 62). Numerous arguments in favor of the involvement of the human microbiota in disease pathogenesis have emerged over the last years (**Table 2**).

TABLE 2 | Clinical evidence – involvement of the human microbiota in Crohn's disease pathogenesis.

Arguments on behalf of the involvement of intestinal microbiota in CD	Reference
Feacal stream diversion improves symptoms of CD	(62)
Reinfusion of luminal contents results in recurrent disease	(61)
Antibiotic therapy is associated with clinical improvement	(63–65)
Mucosal barrier defects and increased translocation	(66, 67)
Higher loads of mucus-associated bacteria	(42)
Higher concentrations of mucolytic bacteria	(53)
Decrease in *Faecalibacterium prausnitzii*	(41, 46, 48)
Decreased concentrations of AMP	(68)
CD susceptibility genes: involvement in killing of intracellular bacteria and secretion of AMP	(69–75)
Siblings of CD patients exhibit mucosal dysbiosis	(76)

CD, Crohn's disease; AMP, antimicrobial peptides.

Although there has accumulated a large body of experimental and clinical evidence supporting the role of dysbiosis in the pathogenesis of CD, it is still up for discussion whether these alterations in the gut microbiota composition represent a cause or a consequence of chronic intestinal inflammation. Taking into account that the development of inflammation was shown to be dependent on the presence of a gut microbiota in different murine models and given the insufficient data to recommend probiotics for use in CD implies a pathogenetic role (55, 56, 77, 78). Investigations on fecal microbiota composition in first-degree relatives of patients with IBD have coined the term "predysbiosis." As already outlined above, several studies indicated a significant impact of the host genotype on the composition of the intestinal microbiota. Relatives of CD patients are at much higher risk of developing CD as compared with the general population. The risk of falling ill is highest in first-degree relatives, especially siblings, and also extends to more distant relatives (54, 79). The relative risk of developing CD was shown to be over 30-fold higher in siblings of patients with CD compared to that of the general population (76). Moreover, an over 50-fold increase in the incidence of IBD was reported within multiply affected families (80, 81). Unaffected relatives of CD patients were found to have a different composition of their intestinal microbiota compared to healthy controls without familial predisposition (54). This subclinical dysbiosis observed in asymptomatic relatives was different from the dysbiosis detected in CD patients and was characterized by lower numbers of both *Collinsella aerofaciens* and an unspecified member of the *E. coli – Shigella* group as well as higher numbers of the mucolytic bacterium *R. torques* compared with healthy subjects. *R. torques*, a non-butyrate-producing member of the *Clostridium* cluster XIVa is capable of degrading gastrointestinal mucin. In contrast to the dysbiosis detected in CD patients, the subclinical dysbiosis in their relatives was not characterized by a diminished butyrate-producing capacity but enhanced mucin degradation might be assumed in these individuals. Given the differences between the observed dysbiosis in CD patients and the subclinical dysbiosis in unaffected relatives, the results from this cohort study suggest that a "predysbiosis" may precede the clinical manifestation of CD. Moreover, the increased numbers in mucolytic bacteria let one suppose that an enhanced mucin degradation capacity of the intestinal microbiota represents an interim step, leading from normobiosis to the investigated dysbiosis among the patient cohort (54). In a recently published study, Hedin and colleagues focused on the mucosal microbiota in healthy siblings of CD patients and described a mucosal dysbiosis characterized by a reduced diversity of core microbiota and a lower abundance of *F. prausnitzii* in these at risk individuals. The lower abundance of *F. prausnitzii* is also one of the convincingly observed species-specific findings in CD patients' dysbiosis, therefore also suggesting the causative role rather than being a consequence of chronic inflammation (76). Further arguments in favor for an etiological contribution of dysbiosis in CD are provided by the transmissibility of inflammation to genetically susceptible hosts as demonstrated in numerous *in vivo* models. However, similar patterns of microbial changes in diverse hosts as well as the fact that inflammation *per se* is capable of inducing dysbiosis pleads for the theory of dysbiosis as a consequence of the inflammatory milieu. The fact remains that the altered microbial composition entails metabolic consequences and subsequent changes in the intestinal milieu deriving from the variety of essential functions provided by the intestinal microbiota and the crosstalk between the microbiota and the host immune system.

Host–Microbiota Crosstalk – Innate Mechanisms for Maintaining Intestinal Homeostasis

The innate immune system consists of the intestinal epithelium and cells of the innate immune system, such as neutrophils, DCs, monocytes/macrophages, and innate lymphoid cells (ILCs). The luminal surface of the gastrointestinal tract with approximately 300–400 m^2 represents the largest interface between the host and the environment. The diverse and abundant indigenous microflora exists in close proximity to the immune system of the intestinal mucosa and immune cells in the underlying tissue. Moreover, the gastrointestinal mucosa is continuously exposed to antigens derived from the food and has to deal with pathogenic microorganisms that can cause tissue damage. The intestinal immune system therefore possesses multiple layers of protection to respond against invading bacteria, thereby limiting their exposure to the systemic immune system while maintaining tolerance toward luminal bacterial antigens and food antigens. Given that a dysregulated immune response toward components of the intestinal microbiota is thought to be a fundamental pillar of chronic intestinal inflammation development, the complex interaction of the host with the plentiful intestinal microbiota has to be precisely regulated.

Barriers of Protection

The Intestinal Mucus Layer – A Protective Blanket

The intestinal epithelium lies at the interface between the intestinal lumen and the gastrointestinal-associated lymphoid tissue, thereby building the first barrier against excessive microbial translocation to the lamina propria (82). The intestinal epithelium itself is covered with a mucus layer constituting the first physical

barrier to luminal antigens. Mucus is secreted by goblet cells and typically contains several major components, which in the intestine comprise of MUC2 and MUC5AC. These densely glycosylated proteins are resistant to digestive enzymes and give the mucus its gel-like properties (7). The type of mucus organization in the small and large intestine are clearly different. The small intestinal mucus fills the luminal space between the villi and is not attached to the epithelial surface. Antimicrobial products such as AMPs derived from epithelial cells and Paneth cells as well as IgA interfuse with the mucus secreted from the crypts, thereby generating an antibacterial gradient that acts to keep luminal bacteria away from epithelial surfaces. Of note, the small intestinal mucus layer is penetrable to bacteria but still provides a diffusion barrier (83). The mammalian large intestinal mucus is organized in a two-layer system. The loose outer layer of the mucus is composed of mucin, diluted antimicrobials, and is the normal habitat for commensal bacteria, whereas the inner layer is firmly attached to the epithelial cells, rich in antimicrobials, and displays a low bacterial density (84). The physiological relevance and protective function of the mucus layer has best been highlighted in studies using MUC2$^{-/-}$ mice. Muc2-deficiency was shown to lead to colonic inflammation accompanied by the presence of bacteria in direct contact with the intestinal epithelium (85). Moreover, these animals displayed a much higher susceptibility to infection by pathogens (86). From studies in GF mice, it emerged that intestinal microbiota have a role in shaping the colonic mucus barrier (87). Mice housed under GF conditions have been characterized by an extremely thin adherent colonic mucus layer. Exposure to bacterial products, such as lipopolysaccharide or peptidoglycan, leads to the re-storage of the mucus thickness to levels observed in conventionally housed mice. A recently published work reinforced the assumption that intestinal bacteria affect the host mucus barrier properties. In this study, two colonies of C57BL/6 mice were housed and bred in different rooms but both under SPF conditions. Analysis of the microbiota composition by 16S rRNA gene sequencing revealed significant differences at multiple taxonomic levels between the two separately housed colonies. Interestingly, the two colonies not only differed in their microbiota composition but also have a different mucus phenotype that was shown to be specific for each colony. Whereas the thickness of the mucus layer was similar in both groups, a major difference regarding permeability was detected. One colony displayed a mucus layer impenetrable to bacteria, the other colony had an inner mucus layer that was penetrable to both bacteria and beads. The causal role of the intestinal microbiota in the detected mucus phenotypes was underpinned by subsequent experiments showing that the different mucus properties were transmissible by transfer of cecal microbiota to GF mice (88). Although genetic risk loci in *MUC1* and *MUC19* have been identified in IBD, there is only limited and widely varying data regarding changes of the mucus layer during CD (3). One study investigated the thickness and continuity of the mucus barrier in rectal biopsies from CD patients but only provided a small number of cases (89). In this study, no significant differences between CD patient specimens and controls were found. Another study reported a depletion of the mucus layer at inflamed sites of the gut of CD patients compared to healthy controls as well as compared to non-inflamed areas in the same patient (90). Of note, there were no significant differences regarding the thickness of the mucus layer between non-inflamed areas of CD patients and healthy controls. These results are in direct contrast to previously published data measuring the thickness of the colonic mucus in surgically resected specimens from CD patients. Pullan and colleagues reported increased values compared to healthy controls (91). As briefly outlined above, the colonic mucus is organized in two layers. Given the contrary results, one has to consider that in the study showing a thicker mucus layer in CD patients a PAS/AB staining technique was used. During PAS/AB staining variable amounts of the loose outer mucus layer might remain thus contributing to the larger thickness observed in CD specimens. The different results might also reflect different stages of disease and moreover, inflammation in CD is characterized by a discontinuous appearance therefore a general mucus barrier dysfunction is unlikely to occur.

Antimicrobial Peptides and Antibodies

A substantial mechanism for controlling the contact between intestinal epithelial cells and luminal antigens is the secretion of AMPs. One of the intensive studied proteins among AMPs is the antibacterial lectin *RegIII*γ produced by multiple epithelial lineages, including enterocytes and Paneth cells and capable of mediating direct killing of Gram-positive bacteria (92). Mouse *RegIII*γ and its human counterpart HIP/PAP are primarily expressed in the small intestine. Inflammatory conditions were shown to increase the expression of *RegIII*γ in the mouse intestine, likewise HIP/PAP expression is increased in the mucosa of IBD patients (93). Reconstitution of GF mice with an intestinal microbiota from conventionally raised mice resulted in a sharp increase in the abundance of *RegIII*γ transcripts in Paneth cells (92). This study also explored the mRNA expression of *RegIII*γ during weaning reflecting an early stage of intestinal development. *RegIII*γ mRNA levels showed a sharp upward movement that was missing in GF controls. To further unravel the host–microbial interactions underlying the regulation of *RegIII*γ, Cash and colleagues investigated the dynamic of this antibactericidal protein in mice lacking IgA. *Bacteroides thetaiotaomicron* and *Listeria innocua* – under state conditions compartmentalized in the intestinal lumen – showed an increased mucosal adherence in the absence of IgA and were found to substantially trigger a vast *RegIII*γ mRNA increase in GF mice lacking IgA. These results indicate that an increased contact between microbiota and the intestinal epithelium drives *RegIII*γ expression. The role of *RegIII*γ as a key element of the intestinal mucosal defense has further been validated in murine *RegIII*γ-deficiency studies that revealed an increased bacterial colonization of the intestinal epithelial surface (94).

Another major group of AMPs is represented by α-defensins, largely produced by small intestinal Paneth cells and β-defensins produced by most epithelial cells (95). Human defensin (HD)-5 and HD-6, both belonging to the group of α-defensins as well as the human β-defensin (HBD)-1, are expressed constitutively (16). HBD-2 and HBD-3 belong to the group of inducible defensins and are expressed in the large intestine. The induction was shown to be mediated by pro-inflammatory cytokines, such as IL-1β,

involving NF-κB pathways (96, 97). Induction was also observed following the recognition of intestinal bacteria by pattern recognition receptors (PRRs) (98). Decreased defensin levels can result in a weakening of the intestinal epithelial barrier and might be involved in the pathophysiology of chronic inflammation. Ileal CD is associated with reduced expression of Paneth cell-derived α-defensins and colonic CD was reported to be associated with reduced expression of β-defensins by enterocytes (68). Next to their antimicrobial property, α-defensins possess an additional homeostatic role in regulating and shaping the composition of the small intestinal microbiota. Transgenic expression of DEFA5 (α-defensin) comes along with a significant decrease of members of the phylum Firmicutes, paralleled by an increase in the percentage of Bacteroidetes. Moreover, transgenic expression of DEFA5 also causes a loss of SFB and concomitantly Th17 in the lamina propria (99).

A third immune mechanism serving to control the microbiota and reduce the contact between intestinal bacteria and epithelial cells is the secretion of IgA by lamina propria plasma cells (100). DCs within the Peyer's patches and subepithelial dome region continuously sample luminal bacteria. Bacteria- and antigen-loaded DCs interact with and prime T- and B-cells in the Peyer's patches or find their way to the gut-draining mesenteric lymph nodes via the afferent lymphatics. The interaction of antigen-bearing DCs with naïve B cells induces the activation and differentiation of naïve B cells to IgA producing plasma cells. These plasma cells leave the Peyer's patches or mesenteric lymph nodes, respectively, enter the systemic circulation via the efferent lymphatics and return to the intestinal lamina propria where they secrete IgA into the intestinal interstitium. The secreted IgA is specific for commensal microbiota, subsequently taken up by epithelial cells and delivered to their apical surface. When released into the intestinal lumen, IgA binds to commensal microbiota, thereby limiting their capability to penetrate the epithelial barrier (7, 101). Remarkably, live bacteria transported by DCs do not gain access to the systemic circulation at any time thus are not able to induce systemic immune responses. Recent studies provide evidence that goblet cells, supposedly purely secretory cells, also act as luminal sensors for the innate immune system. McDole and colleagues were able to show that in the absence of inflammation, small intestinal goblet cells act as passages delivering low molecular weight soluble antigens from luminal to $CD103^+$ DCs in the underlying lamina propria (102). The interconnection between goblet cells and DCs with tolerogenic potential illustrate that goblet cells contribute to the communication between luminal antigens and the innate immune system.

Taken together, these aforementioned innate barriers of protection are crucial for intestinal homeostasis. Moreover, a finely balanced interplay between the intestinal microbiota and cells of the host immune system is indispensable for ensuring the functionality of these barrier mechanisms.

Epithelial Barrier and the Crosstalk Underneath

In contact with the inner mucus layer, there is the intestinal epithelium consisting of four main types of epithelial cells, namely enterocytes, enteroendocrine cells, goblet cells, and Paneth cells. The intestinal epithelium contributes to absorption, digestion as well as secretion, and functions as a mucosal barrier (84). Mucosal barrier integrity is maintained by tight junctions, desmosomes, and adherence junctions. Impairment of the epithelial barrier is accompanied by increased intestinal permeability and bacterial translocation leading to persistent immune activation, a condition that has been observed in both CD and UC patients (103). In health, only small amounts of luminal antigens are allowed to pass across the epithelium. Murine models displaying an impaired barrier function were shown to develop intestinal inflammation. Junctional adhesion molecule-A (JAM-A) is a key structure of tight junctions and mandatory for controlling cell migration into the underlying tissues. Studying of CD tissue specimens for JAM-A expression revealed a loss of epithelial JAM-A expression (104). In line with these findings are the decreased levels of further tight junction proteins, such as claudins, observed in CD (66).

Lying between the luminal microbiota on the one side and immune cells in the lamina propria on the other, the intestinal epithelium functions to communicate with both. Sensing of microbial antigens by epithelial cells as well as by innate immune cells, such as macrophages and DCs, is crucial for maintaining intestinal homeostasis and is mediated by PRRs (95). These receptors include the family of toll-like receptors (TLRs) as well as intracytoplasmatic receptors, such as NOD-like receptors (NLRs). Polymorphisms in TLRs and NLRs have been implicated in increased susceptibility to IBD (3, 69). Whereas TLRs are capable of detecting a variety of bacterial components, such as lipopolysaccharide, lipoproteins, and CpG DNA, NLRs recognize peptidoglycan molecules on the bacterial cell wall (105). Activation of PRRs results in down-stream signaling cascades that are widely mediated by *Myd88*. *MyD88* represents a cytosolic adaptor protein, equipped with a toll/IL-1 receptor (TIR) domain, thereby able to directly bind TLRs (106). Once engaging TLRs, *MyD88* mediates MAP kinase activation, recruitment of a variety of signaling molecules and drives nuclear translocation of NF-κB and subsequent production of pro-inflammatory cytokines. Given the abundance of commensal antigens in the intestinal lumen, it is crucial that activation of PRRs not exclusively drives inflammatory responses. Excessive stimulation can lead to detrimental inflammation. Otherwise, repetitive stimulation of TLRs due to commensal bacterial exposure was shown to result in a down-regulation of the NF-κB pathway and stimulation of AMP production (1). Moreover, triggering of PRRs also promotes antigen presenting cell maturation and is involved in proliferation of Tregs (107). Already about one decade ago, Rakoff-Nahoum and colleagues provided experimental evidence that the recognition of the commensal microbiota by TLRs is crucial for maintenance of intestinal epithelial homeostasis and required for regeneration after mucosal injury (108). TLR expression and activation has to be tightly regulated and this regulation is exerted by a number of mechanisms, such as adjustment of their expression, their localization, and their positioning within the tissue. Most surface TLRs are principally found on the basolateral side of IEC and are only up-regulated during inflammation (109).

Punctilious sampling and processing of luminal antigens is essential for avoiding the development of intestinal inflammation

and establishing and sustaining a healthy relationship between the commensal microbiota and the host. DCs are an indispensable element in this crosstalk as they are continuously exposed to antigens derived from commensal microbiota, dietary products, and intestinal pathogens. The ductility of DCs is warranted by their ability to adapt to influences of the microenvironment. In this context, different DC populations with different functionality have been described. Gut-resident DCs display tolerogenic functions as they encounter and sense commensal bacteria and act, for example, by inducing a highly tolerogenic response through differentiation and expansion of Tregs (110, 111). The triangular crosstalk between epithelial cells, DCs, and the intestinal microbiota is orchestrated by PPR signaling. Using an *in vitro* model, intestinal epithelial cells were shown to release cytoprotective factors, such as thymic stromal lymphopoietin and transforming-growth factor-β upon activation of TLR-signaling, leading to the presence of a predominantly tolerogenic DC phenotype. These tolerogenic DCs were found to secrete IL-10, leading to an immune response dominated by Tregs (112).

Even though the intestinal epithelium with its numerous lines of protection provides a barrier separating luminal antigen from the underlying tissue, it is inevitable that antigens gain access to the lamina propria. As mentioned above, this might not only occur through injury or infection but also happens when commensals undergo epithelial cell transcytosis or translocation via M cells or DCs (113). In response to transforming-growth factor-β and IL-8, chemokines abundant in the extracellular matrix of the lamina propria, circulating blood monocytes gather to the non-inflamed lamina propria where they become resident intestinal macrophages. Located strategically in the subepithelial lamina propria, intestinal macrophages are part of the armament of innate defense mechanisms. The interaction between macrophages and microorganisms is mediated by PRRs, including TLRs and NLRs. In contrast to peripheral macrophages, intestinal macrophages were shown to perform their defense activities without releasing inflammatory cytokines while retaining their phagocytic and bacteriocidal activity. Consequently, intestinal macrophages do not provoke a pro-inflammatory cytokine milieu in the non-inflamed mucosa despite the close proximity to and interaction with immunostimulatory microorganisms. This so-called "inflammatory-anergy" was shown to be at least in part attributable to matrix-bound transforming-growth factor-β and Tregs causing a down-regulation of PRRs and related adapter proteins, resulting in NF-κB inactivation (113, 114). In the case of inflammation or intestinal infection, blood monocytes accumulate in the lamina propria and actively combat invading microorganisms through uptake and degradation, including the release of inflammatory mediators.

Over the last decade, an emerging family of innate immune cells, termed ILCs has been characterized. Since ILCs reside primarily at mucosal sites and are enriched at mammalian barrier surfaces, including the intestine, these cells are in close proximity to environmental antigens and commensal microbiota in particular. Commensal microbiota were found to provide signals for the development, differentiation, and function of ILCs and this crosstalk, the interplay between the microbiota and ILCs is indeed an area of intense research. ILCs have been shown to contribute to tissue repair and remodeling at barrier surfaces and were found to influence inflammatory conditions, thereby orchestrating host–commensal relationships (115). Being derived from an Id2-dependent lymphoid progenitor cell population, ILCs are of lymphoid origin but do not require antigen receptors generated by somatic recombination (116). Based on transcription factors required for their development, their cytokine production patterns and surface markers, the family of ILCs is categorized in three main branches. In brief, group 1 ILCs (ILC1) are Tbet- dependent and are composed of ILC1s and NK cells. ILC1s respond to IL-12 and IL-18 and produce IFN-γ. Group 2 or GATA3$^+$ ILCs secrete IL-5, IL-9, IL-13, and amphiregulin in response to IL-33 and IL-25. Finally, group 3 ILCs (ILC3) express RORγt and include lymphoid tissue-inducer (LTi) cells and natural cytotoxicity receptor (NCR) expressing ILCs. Group 3 ILCs respond to IL-1β and IL-23 and are the main producers of IL-17 and IL-22 (117). Given the spatial proximity of ILCs and commensal microbiota in the intestine, multiple groups have investigated the possible role for commensal microbiota in the development of ILCs. Studies using GF mice indicated that NK cells and GATA3$^+$ ILCs develop properly without receiving signals from commensal microbiota (118, 119). Regarding RORγt$^+$ ILCs, there have been controversial reports on the requirement of commensal microbiota for their development. It seems that the development of at least subsets of RORγt$^+$ ILCs occurs regardless of the presence of a commensal flora as evident by the presence of LTi cells as well as the generation of secondary lymphoid structures in the sterile environment before birth (115, 120, 121). However, the ripening of intestinal cryptopatches into isolated lymphoid follicles was found to be compromised in GF mice, suggesting at least a functional impairment in some populations of LTi-like RORγt$^+$ ILCs (122). Several groups reported normal development of RORγt$^+$ ILC subsets in both GF and antibiotic treated mice (115, 123, 124). By contrast, other studies proposed that commensal bacteria are capable of enhancing the development of NCR$^+$ RORγt$^+$ ILCs and IL-22 production. IL-22 produced by this population in turn contributed to epithelial homeostasis by regulating genes involved in tissue repair and antimicrobial defense mechanisms, such as *RegIIIβ* and *RegIIIγ* (125–127). Factors accounting for these differing results may relate to host genetics, a differential presence of other cell types and cytokines and not least to a varying exposure to non-live bacterial- or diet-derived signals. Although it seems that the development of most ILCs is not reliant on bacterial interaction, signals derived from the commensal microbiota directly affect the function of ILCs. The regulation of ILC function by commensal bacteria can happen indirectly or directly through engagement of PRRs on ILCs themselves. For instance, human NK cells were shown to express functional TLR2 and TLR9 (128, 129). Moreover, stimulation of human RORγt$^+$ ILCs with TLR2 agonists resulted in IL-2 production which in turn enhanced IL-22 expression (130). In addition, there is accumulating evidence that NCRs of NK cells and NCR$^+$ RORγt$^+$ ILCs act as direct sensors of components of the commensal bacteria (131–133). Next to direct signaling through TLRs and NCRs, different groups provided evidence that the aryl hydrocarbon receptor (AhR), expressed by RORγt$^+$ ILC3s and well known for its critical function in ILC

development and IL-22 production has also a role in the commensals regulatory function. More specifically, the AhR can be stimulated by ligands derived from the tryptophan metabolism of commensal microbiota, thereby having an intermediate position between direct and indirect regulation (134). In addition to direct regulation, commensal bacteria also take advantage of an indirect route of influencing the function of ILCs via triggering cytokine signals from epithelial cells or other innate immune cells. As one example, commensal bacteria captured by DCs lead to the induction of IL-12 that acts on ILC1s by stimulating their IFN-γ production that in turn accelerates macrophage phagocytosis (116). Next to the effects of the commensal microbiota on ILCs, there is evidence that ILCs are capable of influence the microbial community. This reciprocal interaction is, for example, simply attributed to signals derived from ILCs that act on the intestinal permeability and thus affect bacterial translocation.

Due to displaying a major source of IL-17 and IL-22, ILC3s are supposed to be critical for the promotion of inflammation and tissue repair in the intestine as well as for dealing with extracellular bacteria (135). Murine models provided evidence that NCR^+ ILC3s rapidly response to infection with the enteric pathogen *Citrobacter rodentium* by producing IL-22, which is essential for host protection. This ILC3 response is promoted by the occurrence of a positive feedback loop stimulating DCs to the production of both lymphotoxin-β and IL-23 (124, 136). IL-17 and IL-22 derived from ILC3s promotes neutrophil recruitment to the intestine and stimulates *inter alia* the production of RegIIIγ, RegIIIβ, as well as mucus production, thereby also pointing out the important role of ILC3s in tissue repair (135, 137). Moreover, ILC3s were shown to have the potential to regulate not only innate but also adaptive immune responses, a capacity that might be crucial especially with regard to chronic inflammatory disorders like IBD. Mortha and colleagues recently reported that ILC3s are the primary source of granulocyte-macrophage colony-stimulating factor (GM-CSF) in the intestine. GM-CSF production was depend on and regulated by the ability of macrophages to sense commensal microbiota and produce IL-1β. ILC3-derived GM-CSF influenced myeloid cell homeostasis and was essential for the generation of subsequent Treg responses toward food antigens and maintenance of oral tolerance (138). Furthermore, mouse and human ILC3s were also found to express MHCII, and directly interact with $CD4^+$ T cells. Most interestingly, murine studies revealed that depletion of ILC3-intrinsic MHCII leads to the development of spontaneous $CD4^+$ T cell-driven microbiota-dependent inflammation (139, 140).

Of note, this only provides a brief insight into the complex world of ILCs but states incontrovertibly that the emerging family of ILCs is essential in the context of the crosstalk between the microbiota and the innate immune system, thereby displaying a key determinant in regulating the host–commensal relationship.

Conclusion

The innate immune system provides multiple layers of protection to regulate and control interactions between the intestinal microbiota and the host. However, the functionality of these protective mechanisms depends on and is positively affected by finely balanced signals derived from the commensal microbiota, thereby ensuring their reliability and performance. In CD, these mechanisms of defense and tolerance are impaired at multiple levels. Intestinal dysbiosis and concomitant changes of the intestinal luminal milieu and environment weaken the intestinal epithelial barrier and entail an increased epithelial permeability. Epithelial barrier dysfunction is followed by an increase of translocation to the lamina propria, where defective handling of antigens might elicit a strong inflammatory response, maintained and enhanced by ineffective phagocytosis, and bacterial clearance as well as impairment of adaptive immune responses. Although it remains unclear, if dysbiosis precedes disease or results from active inflammation, the changes observed during active CD have a lasting and debilitating effect on the numerous host mechanisms that function to maintain homeostasis. Two recent randomized controlled trials provide additional insight by indicating that re-establishing of the intestinal microbiota composition by fecal microbiota transplantation (FMT) ameliorates active UC in a patient subgroup. The first trial conducted by Moayyedi et al. randomly assigned patients with active UC to receive either FMT via enema from healthy anonymous donors or placebo (water via enema) once weekly for a period of 6 weeks (141). The second study also investigated the therapeutic impact of FMT in patients with UC (142). Remarkably, in this study by Rossen et al., autologous fecal microbiota served as control and the administration route of FMT was via nasoduodenal tube. In brief summary and without discussing all study characteristics and statistical outcomes in detail, the decisive insight obtained from these studies is that responsiveness to FMT was accompanied with changes in the microbiota composition and especially with a significant increase of the patients microbial diversity. Moreover, patients with a recent diagnosis of UC seem to be more likely to respond to FMT. Given that seven of the nine responding patients in the study conducted by Moayyedi et al. received fecal material from the same single donor, this leads to the suggestion of a donor-dependent effect and underlines the relevance of the microbiota composition in intestinal homeostasis. Furthermore, it points out that intestinal dysbiosis – irrespective of being cause or consequence – comes along with changes in the intraluminal milieu and together with a genetic predisposition might be capable of triggering a vicious cycle resulting in an abnormal inflammatory response.

This increasing evidence on the interplay of the intestinal microbiome, barrier, and intraepithelial as well as mucosal immune cells displays a complex network that is tightly regulated. Nevertheless, the data discussed indicate several target structures that require in-depth exploration, such as targeting the luminal site via a defined change in the microbiota composition or via selected nutritional restriction that strengthen barrier function and exert anti-inflammatory effects on immune cells. FMT will have to be replaced by a defined administration of a bacterial mix. The definition of this "mix" or additional food compounds that influence the mucosal balance will be the center of research and might ultimately provide novel therapeutic targets not only for IBD.

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REVIEW
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Mechanisms of Microbe–Host Interaction in Crohn's Disease: Dysbiosis vs. Pathobiont Selection

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Crohn's disease (CD) is a systemic chronic inflammatory condition mainly characterized by discontinuous transmural pathology of the gastrointestinal tract and frequent extraintestinal manifestations with intermittent episodes of remission and relapse. Genome-wide association studies identified a number of risk loci that, catalyzed by environmental triggers, result in the loss of tolerance toward commensal bacteria based on dysregulated innate effector functions and antimicrobial defense, leading to exacerbated adaptive immune responses responsible for chronic immune-mediated tissue damage. In this review, we discuss the inter-related role of changes in the intestinal microbiota, epithelial barrier integrity, and immune cell functions on the pathogenesis of CD, describing the current approaches available to investigate the molecular mechanisms underlying the disease. Substantial effort has been dedicated to define disease-associated changes in the intestinal microbiota (dysbiosis) and to link pathobionts to the etiology of inflammatory bowel diseases. A cogent definition of dysbiosis is lacking, as well as an agreement of whether pathobionts or complex shifts in the microbiota trigger inflammation in the host. Among the rarely available animal models, SAMP/Yit and TNF^{deltaARE} mice are the best known displaying a transmural CD-like phenotype. New hypothesis-driven mouse models, e.g., epithelial-specific Caspase8$^{-/-}$, ATG16L1$^{-/-}$, and XBP1$^{-/-}$ mice, validate pathway-focused function of specific CD-associated risk genes highlighting the role of Paneth cells in antimicrobial defense. To study the causal role of bacteria in initiating inflammation in the host, the use of germ-free mouse models is indispensable. Unraveling the interactions of genes, immune cells and microbes constitute a criterion for the development of safe, reliable, and effective treatment options for CD.

Keywords: IEC, dysbiosis, TNF, Paneth cells, barrier dysfunction, microbiota, Crohn's disease, IBD

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INTRODUCTION

Crohn's disease (CD) is one of the two dominant phenotypes of inflammatory bowel diseases (IBD) characterized by chronic and relapsing inflammation of intestinal segments (1). There is overwhelming evidence corroborating the notion that the pathophysiology of CD is under the control of several contributing factors, including heritable traits, environmental cues, abnormalities in intestinal mucosal barrier integrity (2) and function (3), immune regulation (4, 5), and gut microbiota (6). Exaggerated immune responses are presumably directed against normal commensal enteric bacteria in genetically susceptible hosts. Host genetic susceptibility may be related to defective mucosal barrier

function and/or bacterial killing, leading to an overexposure to luminal antigens and to inadequate immunoregulation, resulting in abnormal responses and tissue damage (7–11).

GENETIC EVIDENCE FOR MICROBE–HOST INTERACTION IN CROHN'S DISEASE

Genome-wide association studies (GWAS) have postulated that the heritability of IBD may arise from polymorphisms in associated genes, each contributing in an additive fashion to the overall disease risk (12–15). The most recent GWAS meta-analysis has ascertained 163 susceptibility loci for IBD, revealing similar genetic predispositions in childhood and adult onsets (15). A number of these genetic risk factors are related to innate immunity, most specifically to microbial recognition and its subsequent elimination. The first susceptibility gene described and associated to increased risk of developing CD in Caucasian populations is the nucleotide-binding oligomerization domain 2 (NOD2, also called CARD15) gene. NOD2 gene encodes a cytoplasmic pathogen-recognition receptor (PRR) that recognizes muramyldipeptide (MDP), a component of both Gram-positive and Gram-negative cell walls, and therefore it is thought to play a role in the clearance of intracellular bacteria (16–20).

Additional CD-susceptibility genes that affect bacterial killing include autophagy-related protein 16-1 (ATG16L1) and immunity-related GTPase M (IRGM) (21–26). ATG16L1 gene encodes for an integral protein of the autophagy pathway. Autophagy is an important host defense mechanism for handling intracellular microorganisms and for the response to cellular stress, and it requires the generation of curved membranes to envelop cytoplasmic organelles and intracellular pathogens (27). A working hypothesis is that variants in components of the autophagy machinery may result in reduced pathogen clearance and intracellular homeostasis. IRGM gene encodes for one of the IRG proteins that orchestrate immunity toward intracellular pathogens including autophagy functions (28). While Irgm-deficient mice do not consistently exhibit spontaneous intestinal inflammation, they are much more susceptible to dextrate sodium sulfate (DSS) colitis compared to the WT counterparts, developing acute epithelial injury, loss of Paneth cell granules, altered antimicrobial peptide (AMP) production, and authophagy function of the epithelium (29).

Further CD-susceptibility loci related to aberrant microbial recognition and handling encompass toll-like receptor 4 (TLR4) (30), leucine-rich repeat kinase-2 (LRRK2) (31), and neutrophil cytosolic factor-4 (NCF4) (25). TLR4 is critical for host defense against Gram-negative bacteria since it recognizes lipopolysaccharide (LPS), and it is expressed on intestinal epithelial cells (IECs) and myeloid cells, such as macrophages (MΦs) and dendritic cells (DCs) (32). Two single nucleotide polymorphisms of human TLR4, D299G, and T399I lead to hyporesponsiveness to LPS by interfering with recruitment of TLR adapter molecules, such as myeloid differentiation primary response 88 (MYD88) and TIR domain-containing adaptor-inducing interferon β (TRIF) (33–35). LRRK2 gene is highly expressed on bone marrow-derived myeloid cells, but it is not expressed in IECs (36). Lrrk2-deficient mice do not develop spontaneous inflammation, but they are more susceptible to DSS experimental colitis compared to WT littermates, suggesting a role for innate immune cells in IBD progression (37). NCF4 encodes the p40-phox subunit of nicotinamide adenine dinucleotide phosphate oxidase that is crucial for reactive oxygen species (ROS) production by phagocytic cells in response to microbial infection. Genetic alterations in this CD-susceptibility gene result in abnormal neutrophil and MΦ recruitment, exacerbated cytokine secretion, impaired ROS production, and reduced bacterial clearance in CD patients (38, 39).

A proposed mechanism for the pathogenesis of CD is an exaggerated T (T_H1/T_H17) cell response toward luminal microbiota, which results in the breakdown of the mucosal tolerance to enteric bacteria (40). High levels of antibodies against these microbes correlate with disease progression, and T_H1 and T_H17 cells are massively recruited to mucosal sites and secrete cytokines, such as TNF-α, IFN-γ, IL-17, and IL-22, which participate to the inflammatory state in CD patients (41–45). GWAS indicated that IL-23 receptor gene and five additional genes involved in T_H17 differentiation (*IL12B*, *JAK2*, *STAT3*, *CCR6*, and *TNFSF15*) are associated with susceptibility to CD (40). Anti-IL-12/IL-23 antibody therapy, which targets both T_H1 and T_H17 cells, is effective in CD (42, 46). Finally, the exact specificity of these T cells, their pathways of activation, and the mechanisms of their dysfunction in CD are still poorly understood. These aspects have been elegantly discussed by others (47, 48) and will not be examined in the current review.

Compelling evidence regarding the marginal role of heritable traits in triggering the onset and causing flares in CD has been provided by GWAS with monozygotic twin cohorts. Monozygotic twins have an (almost) identical genetic makeup, and they also have presumably similar environmental exposure and dietary habits in early childhood. They also have a similar gut microbial composition when healthy (49). Despite familial aggregation being a risk factor for developing IBD (2, 50), germline variation in CD loci of monozygotic twins only accounts for ~25% of estimated heritability, suggesting that the contribution of genetic factors to the etiology of CD is modest (51, 52). Differential exposure to environmental factors (so-called exposome) might account for the low level of concordance in monozygotic twins. Another working hypothesis is that T and B cell receptors (TCRs and BCRs) are not identical in monozygotic twins and that specific receptor rearrangements predispose to an immune-related sensitivity or autoimmune disease (53). In addition, a potential reason for relatively low concordance rates in monozygotic twins may relate to epigenetic changes. The study by Satsangi's research team provides compelling evidence for epigenic modifications in several regions of the genome of pediatric and adult CD patients (54, 55).

SENSING OF MICROBES

Good fences make good neighbors
(Robert Frost, 1914) (56)

The mammalian intestine is inhabited by complex bacterial communities that contribute to the host metabolism and immunity. It remains under investigation how the host maintains homeostasis toward these bacterial populations, discriminating between commensal and pathogenic bacteria-derived signals. A growing body of evidence suggests that IECs contribute to mucosal homeostasis through the integration of microbial signals and interaction with immunocompetent cells (57). Current hypotheses suggest that the breakdown of the intestinal epithelial barrier function leads to increased translocation of food and bacterial antigens, to loss of immune tolerance to commensal bacteria, and to chronic intestinal inflammation, triggering the onset of CD. IECs contribute to tissue homeostasis by maintaining a rigorous spatial separation between microbiota and host, which limits immune activation and promotes induction of tolerance. Specialized IECs may sense the intestinal bacterial community through PRRs, such as TLRs and NOD receptor, and preserve mucosal homeostasis exploiting several strategies to restrict the colonization of resident and pathogenic bacteria and avoid immune overactivation. Processes engaged to this end encompass the secretion of an apical mucus layer by globlet cells (58), the secretion of AMPs by Paneth cells (59, 60), and the transport of secretory immunoglobulin across the epithelial barrier (61, 62). Mucus production has been shown to increase in germ-free (GF) mice exposed to TLR ligands, suggesting that bacteria-derived signals regulate goblet cell functions (58). Paneth cells are specialized cells in the crypts of the small intestine from which they exert a role in crypt homeostasis and maintenance of the intestinal stem-cell niche by secreting AMPs that keeps commensal and pathogenic bacteria at bay and regulate the composition of the gut microflora (63, 64). The expression of AMPs, such as RegIIIγ, is dependent on the recognition of bacterial molecules through Myd88-dependent TLR sensing. Evidence of this mechanism is provided by the proof that epithelial cell-specific Myd88-knockout mice and RegIIIγ-knockout mice loose the segregation between the microbiota and the intestinal surface, leading to a dramatic increase in mucosal-associated bacteria in the terminal ileum (65). Similar loss in spatial organization has been observed in biopsies from CD patients, characterized by lower AMP levels and consequently increased mucolytic bacteria and compromised mucus layer (66–68). Similarly, Muc2-deficient mice develop spontaneous colitis (69).

Small numbers of bacteria that breach epithelial barrier are killed by MΦs or can survive briefly in DCs promoting the induction of IgA by T-dependent and T-independent mechanisms (70). Hence, secretory IgA is selectively induced by DCs loaded with commensal bacteria resulting in local secretion of IgA that limits the penetration and overgrowth of commensal bacteria (71–73). The host may as well produce secretory IgA that is pathogen specific, but it is believed that indigenous bacteria are bound by low-affinity IgA, whereas enteric pathogens are highly coated with IgA (74). Flavell group recently demonstrated that GF mice colonized with highly IgA-coated taxa from IBD patients were much more susceptible to DSS colitis and displayed a bacteria-enriched inner mucus layer compared to GF mice humanized with low IgA-coated bacteria (75). These findings suggest that IgA coating is a defense mechanism that the host engages to mark and distinguish disease-driven bacteria from other members of the intestinal microbiota. The transport of IgA across the intestinal epithelium is modulated by the expression of the polymeric immunoglobulin receptor (pIgR) on the basolateral membrane of IECs. It has been reported that commensal bacteria may activate Myd88- and NF-κB-dependent signaling in IECs and consequently promote the expression of pIgR (62, 76). In addition, microbiota-specific T_H17 cells may induce epithelial pIgR expression, therefore promoting intestinal IgA secretion and contributing to intestinal homeostasis. This finding is corroborated by the evidence that IL-17 receptor-deficient mice display reduced IgA levels in the gut that can be restored by T_H17 cell transfer, and increased bacterial translocation and colitis scores upon DSS treatment (77). The serum of CD patients is enriched with high levels of IgA autoantibodies (78, 79). Furthermore, CD patients harbor a stronger humoral immune response against intestinal bacteria than healthy subjects, as shown by the detection of elevated IgG-coated bacteria in CD stool samples (80). This finding corroborates the concept of loss of mucosal tolerance for intestinal bacterial communities in CD patients.

Intestinal epithelial cells are currently emerging as key mediators of inflammatory and immune mechanisms in mucosal tissues. The transcription factor NF-κB has been shown to play a crucial role to control epithelial integrity and immune homeostasis toward gut bacteria in gnotobiotic mice and rats (81–83). Functional proof for the role of NF-kB signaling in the epithelium was provided by an elegant study from Nenci et al. (84). Mice-harboring IEC deletion of *Nemo*, upstream modulator of NF-κB, displayed destruction of intestinal barrier, dramatic translocation of bacteria from the lumen into the mucosa, and developed spontaneous colitis that was Myd88 and TNF receptor-1 dependent (84). Consistent with this protective role of inflammation-related signaling in the epithelium, the absence of TLR-related mechanism conferred increased susceptibility to DSS-induced colitis (85).

Nevertheless, the question remains how much TLR-related microbial sensing confers protection rather than tissue inflammation. It has been proposed that low expression of TLRs at the epithelial level, especially TLR2 and TLR4, is associated with mucosal homeostasis by maintaining a hyporesponsive state toward the presence of commensal bacteria (86–88). Notably, epithelial tolerance to microbial ligands seems to occur immediately after birth in order to promote a stable intestinal host–microbe homeostasis (89). Nevertheless, the high turnover rate of the epithelium may require additional immunosuppressive mediators of the mucosa such as transforming growth factor (TGF)-β and prostaglandin (PG) J2 in order to maintain epithelial cell homeostasis in response to the constant bacterial challenge (82, 83, 90). In the context of infectious or chronic inflammatory conditions, IFN-γ and TNF-α upregulate TLR expression in IECs (91). Consistently, TLR4 is significantly increased on the apical side of IECs throughout the lower gastrointestinal tract of CD patients (86). With respect to the maintenance of mucosal

immune homeostasis, it has also been postulated that the expression of individual TLR is limited to specific cell lineages (92), and it is spatially restricted on the apical or basolateral intestinal surface (93, 94). For instance, TLR1, TLR2, and TLR4 seem to be coexpressed on a subpopulation of human and mouse cells located in the intestinal crypt and belonging to the enteroendocrine lineage (92). Analysis of polarized human IECs *in vitro* indicated that TLR5 is expressed on the basolateral surface and from this location triggers the production of cytokines and chemokines in response to flagellin (93).

Macrophages and DCs sense microbial components of the intestinal lumen, providing a key link between the microbiota and epithelial barrier functions (95, 96). The primary role of MΦs is to phagocytose cellular debris and microbes and stimulate lymphocytes and other immune cells to respond to the antigen through the secretion of cytokines and chemokines. Depending on the requirements of the surrounding tissue environment and of the stimuli encountered, MΦs express different biological functions exhibiting a remarkable plasticity (97). Gastrointestinal mucosal MΦs play a crucial role in maintaining gut homeostasis (98). Considering the huge bacterial load present in the intestinal lumen, it is likely that commensals (and pathogens) breach the epithelium. Mucosal MΦs are strategically located adjacent or in proximity of the epithelia and along the lamina propria. Intestinal MΦs are characterized by low production of proinflammatory cytokines but with an intact phagocytic ability (99). Indeed, this MΦ population helps to maintain a low level of inflammation in the lamina propria (state of physiologic inflammation), in order to prevent inappropriate immune responses to microbes. This is possible because intestinal MΦs express low level of TLR2 and TLR4, the two main receptors involved in sensing bacterial cell wall components (100). However, even though they may express TLR1, TLR3, and TLR5–9 to different extents (101), mucosal MΦs do not release proinflammatory cytokines in response to TLR ligands. They express low levels of TRIF, Myd88, and TRAF6 proteins, leading to an inability to phosphorylate NF-κB p65 and MAPKs (98). Intestinal MΦs retain a highly phagocytic activity, but they do not present antigen in normal intestinal mucosa as they lack constitutive expression of the costimulatory molecules CD40, CD80, and CD86, displaying a tolerance-inducing phenotype (98). In contrast, intestinal MΦs from CD patients express high levels of costimulatory molecules (102–104), increased activation of NF-κB signaling and oxidative burst activity (105, 106), high expression of TLR2 and TLR4 (107–110), and secretion of cytokines, including TNF-α and IL-23 (111).

Dendritic cells located in the lamina propria can penetrate the epithelium without disrupting the barrier function and acquire antigens from food or directly sample gut-associated bacteria and take them to mesenteric lymph nodes, where they are presented to $CD4^+$ T cells. In contrast to DCs loaded with harmful bacteria that reach systemic secondary lymphoid structures (spleen and lymph nodes) and activate systemic immunity, DCs loaded with commensal bacteria migrate to the MLNs but remain confined in the mucosal lymphoid tissue (112). IECs release mediators (e.g., TGF-β, thymic stromal lymphopoietin, and PG E2) that maintain DCs in a quiescent state and promote the induction of regulatory T cells (113). This mechanism allows the host to develop systemic tolerance in response to commensal colonization which appears compromised in CD patients (114). During inflammation, IL-12 and IL-23 produced by DCs restrain regulatory T cells and promote T_H1 or T_H17 effector cells, respectively (42). The crucial role of IL-23 in promoting chronic mucosal inflammation has been emphasized by evidence suggesting that this cytokine, produced by innate immune cells, may inhibit $FoxP_3$ Treg cell function and consequently positively modulate T_H17 differentiation (115).

ILEITIS PHENOTYPE IN MOUSE MODELS WITH DEFICIENCY IN CROHN'S DISEASE-ASSOCIATED GENES

Genetic polymorphisms associated to CD may alter immune responses to commensal bacteria and mucosal barrier function impacting the microbiota composition in the gut. The possible mechanism for genetic regulation of enteric microbiota include altered Paneth cell function but also altered barrier functions and mucus production, defective secretion of IgA, and altered innate and adaptive immune responses.

Paneth cells are specialized IECs located adjacent to the stem cell zone in the base of the crypts in the small intestine. They are critically involved in host defense against enteric pathogens by secreting AMPs and TNF-α (116, 117). Mutations in genes associated with CD, usually highly expressed in Paneth cells, predispose to the development of ileal lesions in humans (118). In line with this, mouse model with deficiency in several CD-associated genes, including *Nod2*, *Atg16l1*, and *Xbp1*, displays Paneth cell defects and susceptibility to intestinal inflammation (119, 120).

Nucleotide-binding oligomerization domain 2 is a well-acknowledged susceptibility gene for CD (16, 18, 121). NOD2 is thought to play a relevant role in maintaining microbial tolerance at the intestinal barrier (16) and to activate innate and adaptive immunity (63). Three common mutations in the C-terminal leucine-rich repeat region of the NOD2 gene, namely G908R, R702W, and the frameshift deletion mutation L1007, and several other rare polymorphisms have been discovered and associated to increased risk of developing CD. How the NOD2 variants increase susceptibility to CD remains debated. For instance, the frameshift variant L1007 has been shown to decrease NF-κB activity in HEK293 cells stimulated with a number of common bacteria (17). This observation contradicts the evidence that CD clinical specimens are characterized by elevated NF-κB activity (122). One possible explanation is that the mutation in NOD2 leads to a defect in the innate immune response allowing intracellular bacteria to escape the first line of host defense, resulting in an enhanced adaptive response. In support of this hypothesis, elevated proinflammatory cytokine production was detected in splenocytes and blood mononuclear cells from Nod2-deficient mice challenged with TLR2 ligands (123, 124). Furthermore, $Nod2^{-/-}$ mice become more susceptible to colitis as a result of enhanced TLR2 responses characterized by increased production of IL-23 and IL-12 (124). However, the role of NOD2 as negative regulator of TLR2 is controversial since the same L1007 frameshift mutation has been shown to protect mice from systemic infection (and inflammation) by *Enterococcus faecalis*

that is a Gram-positive bacterium and therefore sensed mainly by TLR2 (125). Notably, Nod2$^{-/-}$ mice do not spontaneously develop an inflammatory phenotype (63) but exhibit a higher load of commensal bacteria (i.e., *Bacteroidaceae*) in the terminal ileum and Peyer's patches and a reduced ability to prevent pathogenic bacteria colonization (126–128), suggesting a genotype-driven selection of a pathobiont-enriched microbiota. Similarly, the NOD2 variant L1007 is associated with higher colonization of the intestinal mucosa by the *Bacteroidaceae* in humans (129). It is still not clear whether the observed dysbiosis is the cause or a consequence of the disease, but it is tempting to speculate that NOD2 variants are associated with changes in the composition and load of the commensal microbiota in the terminal ileum that may facilitate disease progression and pathology. In line with this speculation, WT mice develop colitis when recolonized with dysbiotic fecal microbiota from Nod2-deficient mice (130). In addition, crypts and Paneth cells from Nod2$^{-/-}$ mice have attenuated antibacterial activity and decreased expression of α-defensin and cryptidin leading to increased susceptibility to *Listeria monocytogenes* infection *in vivo* (63, 126). These findings are in agreement with the deficiency in α-defensin production observed in CD patients (3, 131, 132).

In the gut, bacterial growth and division contribute to the remodeling of components of the bacterial cell wall, i.e., pepdidoglycan (PGN), by bacterial autolysins, e.g., muramidases and amidases (133). During this process, soluble PGN fragments can be transferred to the bloodstream and initiate innate immune responses through PRRs, including NOD2 and TLR2 (134, 135). Specific classes of bacteria, such as Actinomyces and Mycobactera, use hydroxylases to covalently modify MDP-generating moieties that activate more potently the NOD2 pathway. This evidence raises the intriguing possibility that bacterial components may account for the differential activation of immune cells resulting in detrimental effects on a host immunity already compromised, such as in CD.

Nucleotide-binding oligomerization domain 2 has been reported to account for stem cell protection and to contribute to stem cells regeneration via responses triggered by MDP recognition and leading to the recruitment of NF-κB at the membrane surface of IECs (136–139). Conversely, NOD2 mutant 3020insC, which is associated with CD, shows an impaired ability to activate NF-κB following MDP stimulation *in vitro* (138, 140, 141). In the absence of bacterial invasion into the host cytosolic compartment, it has been postulated that MDP can cross plasma membrane and localize into the cytosol via the plasma membrane transporter, PepT1 (142, 143). During chronic inflammation, such as CD, PepT1 expression has been shown to be upregulated in the colon (144, 145). Nevertheless, a recent study argues against the indication of a role for PepT1 in the development of intestinal inflammation, showing that in animal models resembling Crohn-like ileitis (TNF$^{\text{deltaARE}}$) and colitis (IL-10$^{-/-}$, IL-10XTLR2$^{-/-}$, and Rag2$^{-/-}$) and in human intestinal tissues from IBD patients, severity of inflammation correlated with lowered PepT1 expression levels (146).

Nucleotide-binding oligomerization domain 2 is involved in the cellular protection mechanism called autophagy, by directly interacting with the ATG16L1. Since both of these genes are CD-susceptibility loci, it has been proposed that loss of autophaghy-related function is implicated in the pathogenesis of CD. DCs (147), MΦs (148), and epithelial cells (149) containing ATG16L1 and NOD2 variants show defects in antibacterial autophagy. In DCs, these defects are associated with an impaired ability to process pathogens (such as *Salmonella typhimurium*) and present exogenous antigens (such as MDP) to CD4^{+} T cells (147). Murine Atg16l1- and Nod2-knockout MΦs secrete aberrant IL-1β levels in response to LPS (150) and MDP (122), respectively. These findings highlight that CD-susceptibility genes require interaction with environmental (microbial) cues to manifest a disease phenotype. This is further supported by the observation that GF hypomorphic Atg16l1 mice are disease free, whereas colonized mice display aberrant Paneth cells morphology and antimicrobial protein expression, and they are highly susceptible to DSS-induced colitis (119, 150, 151). This phenotype has been recently associated to dysfunctional IECs due to mutations in the autophagy machinery rather than arising from defects in granule formation in Paneth cells (152). In fact, the author exploited IECs-specific Atg16l1-knockout mice to demonstrate that the deletion of the autophagy-related gene is responsible for reduced Paneth cell number, abnormal granule morphology, reduced expression of AMPs, and increased inflammation and systemic translocation of *S. typhimurium* compared with control mice (152). Similarly, CD patients carrying the ATG16L1 T300A mutation show an autophagy-associated defect in Paneth cells with granule abnormalities (153). Exploiting mice with epithelial cell-specific Myd88 deletion, Hooper group demonstrated that only invasive bacteria, such as *S. typhimurium* and *E. faecalis*, can activate autophagy in IECs in a Myd88-dependent fashion (154). In addition, the authors showed that autophagosome formation *in vivo* is TRIF independent, while in RAW MΦs autophagy was reported to be depended on this adaptor molecule (155). Nod2$^{-/-}$ mice do not harbor defects in autophagosome formation, rather lack of the intracellular PRR is associated to an increased number of bacteria within ileal IECs compared to WT mice, as shown by FISH analysis (154). This finding suggests that bacterial breach of the intestinal epithelium triggers autophagy in IECs in Nod2-independent manner. Mice with an IEC-specific deletion of the essential autophagy gene *Atg5* display decreased numbers of autophagosomes but no histological evidence of pathology and an increased number of intracellular *S. typhimurium* (154). These findings highlight the crucial role of IECs as a selective barrier that contributes to maintaining mucosal homeostasis by finely tuned communications with gut microbiota and the luminal environment, peripheral tissues, and the immune system (156).

Multiple cellular stress responses were observed in IBD, including endoplasmic reticulum (ER) and mitochondrial (mit) unfolded protein responses (UPRs). The accumulation of misfolded proteins in the ER and mit lumen of IECs activates several processes, including inflammation and UPR signaling pathways, and the integrated stress response (157, 158). TLR signaling and autophagy cooperate in bacterial sensing actively interacting with cellular stress responses, i.e., UPR. UPR signaling is mainly driven by inositol requiring, ER-to-nucleus signaling protein 1α–X-box-binding protein-1 (XBP1) pathway. XBP1 gene deletion may lead to increased ER stress as a consequence

of a defective UPR in secretory IECs (i.e., goblet and Paneth cells), thereby affecting their function (120). While Xbp1-deficient mice are protected from ileitis in GF housing, colonized Xbp1$^{-/-}$ mice develop spontaneous transmural inflammation, resembling Crohn's ileitis phenotype in humans (120, 159). In particular, mice with Xbp1-deficient IECs develop spontaneous enteritis and display ER stress with high level of the chaperone grp78 and C/EBP homologous protein (Chop), Paneth cell loss, and reduced globlet cell number and size, compromised response to pathogenic bacteria (i.e., *L. monocytogenes*), and they are more susceptible to DSS colitis (120). Overexpression of CHOP in IECs aggravates DSS-induced colitis and impairs mucosal wound healing (160). *In vitro*, XBP1 deficiency induced ER stress that led to a heightened JNK-dependent proinflammatory response of epithelial cells to flagellin and TNF-α (120). The emerging role of ER stress in the progression of CD is supported by the detection of high levels of ER stress markers (i.e., grp78) in ileal and colonic epithelia of CD patients (120, 161, 162). Generation of multiple cellular stress responses, and consequently inflammation, in the intestinal epithelium is multifactorial and includes genetic and environmental factors (163, 164). Paneth cells from patients with quiescent CD and healthy controls carrying the ATG16L1 T300A risk allele are characterized by increased ER stress (153). This finding indicates an interaction between ER stress mechanisms and autophagic pathways. For instance, CD patients may harbor a defect in barrier function which results in overactivation of Paneth cells by bacterial ligands, requiring a higher demand of secretory AMPs, potentially leading to ER stress (165). It is possible to speculate that progression of ER stress in CD patients may be facilitated by the production of ROS induced by the inflammatory cytokine TNF-α (166).

The evidence of barrier dysfunction in CD patients, associated with the inability to achieve mucosal healing or to seal off the damaged epithelium, confers a role of relevance to tight junction proteins as risk factors for disease development (167). Notably, aberrant (high) tight junction-dependent paracellular permeability (168, 169) and exacerbated TNF-α level has been observed in CD patients as well as in their healthy first-degree relatives (170–173). A proposed mechanism is that inflammatory cytokines, i.e., TNF-α, may activate myosin light chain kinase (MLCK) which induces tight junction disruption ultimately leading to cytoskeletal-related barrier defect (174–177). This phenomenon leads to the activation of systemic T cell-mediated immune responses and impaired barrier function (178). A major validation of this molecular mechanism comes from the observation that wild-type mice treated with MLCK inhibitor and mlck-knockout mice are protected from barrier dysfunction (174, 178). In contrast, transgenic mice expressing mlck constitutively (CA-MLCK Tg) displayed increased paracellular permeability within the small intestine and colon, without developing spontaneous disease (179). Intriguingly, transfer of colitogenic T cells into CA-MLCK Tg mice accelerated the onset and severity of colitis (179). This finding suggests that increased gut permeability is insufficient to trigger disease in the absence of other predisposing factors, which most likely include immune dysregulation and altered luminal microbiota.

WHAT IS THE CONTRIBUTION OF MICROBIOTA TO CD?

The microbial composition in the intestinal tract is considered another potential risk factor in individuals with CD (51, 180). There are several lines of evidence for microbial involvement in IBD. For instance, (i) inflammation occurs in regions with higher bacterial density, such as distal ileum and colon, (ii) GF animals do not develop ileitis (181) or colitis (182), (iii) antibiotics have shown some therapeutic efficacy in IBD patients (183), (iv) the severity of the disease correlates with the bacterial density in the intestinal mucosa, and (v) a large number of studies reported an altered bacterial composition in IBD patients compared to healthy individuals, so-called dysbiosis (184).

THE GASTRO INTESTINAL ECOSYSTEM – WHO IS THERE?

The mammalian gastrointestinal tract is habitat to taxonomically diverse microorganisms in very close proximity to the host. The totality of these microorganisms, so-called "microbiota," includes bacteria, viruses, archaea, and eukaryotes (yeasts, protozoa), and their genes represent the "intestinal microbiome." The human and murine intestines are dominated by the bacterial phyla *Bacteroidetes* and *Firmicutes* with a minor proportion represented by *Proteobacteria*, *Actinobacteria*, *Verrucomicrobia*, *Tenericutes*, and *Fusobacteria* (185, 186). At lower taxonomic levels, the diversity is very high, with ~100–200 bacterial species per individual (187). Notably, great interindividual differences were observed in the overall microbial community, highlighting the limitation in defining the absolute composition of a "healthy" microbiota (188–191). In the last decades, compelling evidence emerged pointing out the pivotal role of the intestinal ecosystem in defining the host immune homeostasis (e.g., by inducing pro- or anti-inflammatory responses) (192). Therefore, the study of shifts in the intestinal ecosystem is of fundamental importance to understand associations and causalities between gut microbes and immunity.

CHARACTERISTICS OF DYSBIOTIC ECOSYSTEMS

Under physiological conditions, the microbiota shows both plasticity and high resilience, i.e., upon short-term perturbations (e.g., change in dietary pattern), the microbial composition adapts to alterations in the intestinal milieu, though soon resembles a predisturbance state (189). The microbial ecosystem can also be changed without pathologic consequences for the host and stabilize within a new "alternative state" (193). Therefore, the microbiota is capable of adapting to exposomal factors including diet, smoking, antibiotics, or intrinsic factors like host genetics. Nevertheless, diet seems capable to overwrite the influence of genetic imprint (194–196). Rapid resilience to perturbations is a key requirement for the intestinal homeostasis and the host's health in order to maintain a health-associated composition of the ecosystem (eubiosis). However, this resilience is lost in some

pathologic conditions, like IBD. In coexistence with genetic susceptibility to inflammatory processes – like in IBD patients – the microbiota is causative for disease development and is therefore considered as "dysbiotic" (197).

For different pathologies, there is a strong evidence for the role of "dysbiosis," defined as an alteration in the ecosystem associated to pathology (180, 198–200). While for CD and UC, the connection between dysbiosis and pathology seems well established; also other pathologies like obesity, diabetes, cardiovascular disease, and even depression or multiple sclerosis have been recently connected to intestinal dysbiosis (198, 201–204)

However, dysbiosis displays several features, which will be in the present review categorized in (i) reduced bacterial diversity, (ii) expansion of pathobionts, (iii) changes in the microbial composition, i.e., increase or reduction in indicator species, and (iv) change in microbial functional capacity (205). Thereby, the appearance of these characteristics may occur solitarily, successively, or simultaneously.

The most widely discussed attribute of dysbiosis is *reduced bacterial diversity*. Based on the numbers of bacterial species and their abundance found within one sample, the alpha diversity can be calculated. Many studies associated lowered bacterial diversity to disease, with the rationale of loss in metabolic redundancy (180, 206–209). Several reports have addressed dysbiosis and decreased diversity in IBD patients' display compared to healthy individuals (51, 180, 200, 206, 210–214). Furthermore, the ability to outcompete pathogens by a low-diverse microbiota is diminished. In patients who underwent frequent antibiotic treatment, the deteriorated intestinal diversity was shown to increase the risk of infection by opportunistic pathogens, such as *Clostridium difficile* (215). In animal models, pathogens including *Salmonella*, *Citrobacter rodentium*, or enterohemorrhagic *Escherichia coli* fail to colonize in the presence of a diverse, undisturbed microbiot but elicit pathogenic traits if competing strains are missing (216–219). The mechanisms of competitive exclusion may correspond to rivalry for nutrients or virulence modulation of intruding strains (220).

Dysbiosis may also be simplified by linking it to *expansion of pathobionts*, i.e., single strains of the commensal microbiota that outgrow and cause detrimental effects in the host. The term pathobiont was defined by Chow and Mazmanian as "…symbiont that is able to promote pathology only when specific genetic or environmental conditions are altered in the host" (221). While pathobionts are found only in low abundance in a healthy microbial setting, they overgrow in dysbiosis and cause disease in the susceptible (e.g., immune compromised) host. Hereby, specificity in the combination of microbe and host susceptibility is required. In animal studies, it was shown that *Bacteroides vulgatus* induced colitis in HLA/B27-ß2m rats, but not in IL-$10^{-/-}$ mice and even prevented colitis in IL-$2^{-/-}$ mice (222, 223). Early studies regarded *Mycobacterium avium* subspecies *paratuberculosis* as the responsible pathobiont or even pathogen in IBD. However, this hypothesis could not be verified, as summarized by Packey and Sartor (224). In stool samples and mucosal specimens from IBD patients, an increased abundance of *Enterobacteriaceae* is repeatedly observed, and among these the *E. coli* strain LF82 is discussed to be a pathobiont (225–227). The group around Darfeuille-Michaud was the first to describe adherent-invasive *E. coli* (AIEC), which selectively colonize the ileum of CD patients, suggesting that dysbiosis in IBD may also relate to strain-specific virulence factors (6, 228–231). The authors showed that the AIEC strain LF82 is able to persist within MΦs and epithelial cells and selectively colonizes the ileum of CD patients.

Apart from single pathobiont alterations, dysbiosis is in most cases regarded as *shifts in the overall microbial composition*, i.e., simultaneous increased or decreased abundance of certain commensals. Due to the new sequencing techniques and improved databases, great progress has been made in characterizing the intestinal microbiome also in larger cohorts. However, most samples were taken from patients who already underwent some sort of treatment, which exerts changes in the ecosystem and thereby impede conclusive interpretation of findings (232–234). A study from Gevers et al. elegantly solved this issue by picturing the treatment naive microbiome in children recently diagnosed for CD, before shifting the intestinal ecology by different forms of pharmaceutical or nutritional intervention. They described an increase in *Enterobacteriaceae* preceding the onset of CD, an observation made by other studies as well (199, 234, 235). It is worth noting, however, that small inflammatory lesions may result in changes in microbiota composition prior to the diagnosis of disease. Thus, prospective cohorts would be of high value but demanding considering the low incidence of IBD. Among the multitude of studies performed to detect IBD-associated bacterial taxa, little congruence is found between different cohorts. By combining the sequencing data from several studies, Walters et al. showed shifts in the bacterial composition at different taxonomic levels, which were at least partly consistent for several studies and cohorts (227). They showed higher abundance of *Actinobacteria* and *Bacteroides* sp. and a loss of *Prevotella* sp. in CD patients compared to healthy controls. They also described at lower taxonomic level the loss of some *indicator species* in IBD, like *Faecalibacterium prausnitzii*. This health-associated species was found in significantly lower levels in the inflamed intestine compared to healthy specimens and exerts positive immune-regulatory effects on the host (236, 237). Therefore, loss of *F. prausnitzii* is speculated to be an indication for increased IBD risk (211, 236, 237). However, use of just a single indicator species as diagnostic tool may not be sufficient, as IBD-associated strains may be cohort or individual specific. Nevertheless, the novel approach of using the analysis of overall drifts in the intestinal, preferably stool microbiota as a diagnostic tool is a promising new tactic in IBD diagnosis. Walters et al. highlighted some universally valid disease-related shifts, which may be powerful enough to securely diagnose IBD, by combining the sequencing results from several studies (227). By using stool microbiota, this non-invasive approach is even more advantageous.

By great advances in 16S rRNA profiling methods, shifts in the prevailing bacterial members of the intestinal composition can be more easily assessed and discussed. However, most descriptions of intestinal dysbiotic communities fail to take fungal and viral contributions into consideration. Recently Chehoud et al. could show reduced fungal diversity in pediatric CD accompanied by increased *Candida* taxa (238). Norman et al. showed marked differences in the intestinal virome in CD and UC patients compared

to healthy controls (239). The main difference was an increase in *Caudovirales* bacteriophages which was not secondary to bacterial dysbiosis, but it is more likely to assume that viral dysbiosis contributes to pathology and changes in the bacterial ecosystem due to a "predator–prey" relationship (240–242). However, studies of the role of other microbial taxa in IBD pathogenesis are awaited and will be essential to unravel dysbiotic patterns.

In addition to bacterial changes in composition and diversity, changes in the *functional metabolic capacities* are characterizing a dysbiotic ecosystem on a functional level. As shown by the human microbiome project, the healthy intestinal ecosystem may be highly different in composition, while its metabolic activity is highly similar (190). An interesting study in IBD patients by Morgan et al. showed a small perturbation of the intestinal composition, though a quite distinct change in microbial function (235). In dysbiotic conditions, microbial pathways for oxidative stress tolerance, immune evasion, metabolite uptake, and carbohydrate as well as amino acid biosynthesis were upregulated. An increase in carbohydrate metabolism and especially changes in the metabolic capacity to utilize fucose were reported in dysbiotic settings in CD patients or animal models of inflammation by others as well (181, 243–245). Metaproteome analysis in a cohort of CD patients also proved a distinct protein signature associated to CD (246). By correlating these functional shifts to the bacterial ecosystem, *Bacteroides*-derived proteins related to survival in challenging environments (e.g., DnaKs and other chaperones) were found overrepresented. Dysbiosis may also be described by changes in the intestinal microbial function and consecutively produced metabolites (247, 248). This leads to the assumption that dysbiosis may be more precisely characterized by changes in the microbial function rather than composition.

Dietary fibers are often associated to be reducing the IBD risk, as fibers are metabolized to short-chain fatty acids (SCFA) by microbes in the distal gastrointestinal tract (249). Those SCFA were found to hamper the growth of pathogens, increase the intestinal barrier function, and serve as energy source for colonocytes (250–255). Furthermore, they also facilitate the generation and differentiation of regulatory T cells in the gut and thereby are important to maintain a homeostatic environment (252, 254, 256). Machiels et al. have reported that UC is associated with impairment in SCFA production (237). Though the authors observed a reduction in butyrate-producing *Roseburia hominis* and *F. prausnitzii*, as well as a reduction in butyrate levels, they could not draw a direct correlation between these findings. This provides evidence that the microbiota may not only be divergent regarding the abundance of community members but also in their metabolic activity.

Furthermore, the definition of dysbiosis should not be a mere one-sided microbial consideration but should take the host into account as well. Palm et al. showed that IBD patients display an *altered immune recognition of a dysbiotic microbiota* correlating with an increased and divergent IgA coating (75). By using an animal model of chemically induced colitis, they also transferred the disease susceptibility and thereby proved the true causal role of this IgA-coated ecosystem. Consequently, dysbiosis may also be defined as an "alteration of symbiosis" with pathophysiologic consequence (257).

WHAT CAUSES DYSBIOSIS?

The sum of environmental triggers, so-called exposome, including diet, early nutrition (breast or formula feeding), mode of delivery, hygienic milieu (contact to disinfectants or animals), stress, drugs, lifestyle, and geography (e.g., air pollution and location) may contribute to shape the intestinal ecosystem (**Figure 1**) (185, 208, 258–266). Diet has an enormous impact on the intestinal ecology. For instance, diet high in fat shifts the microbiota toward a more dysbiotic pattern associated with increased risk of intestinal inflammation. David et al. effectively showed the plasticity and the stability of the intestinal ecosystem upon short-term perturbations by feeding mice an exclusively animal- or plant-based diet (267). Animal-based diet promotes bile-tolerant microorganisms, such as *Alistipes* or *Bilophila* (194, 267). For instance, the increase in *Bilophila* is of interest considering that *Bilophila wadsworthia* was previously shown to exert pathobiont behavior in IL-10$^{-/-}$ (268). In another study, a western diet (rich in fat and sugar) induced dysbiosis and favored the increase in AIEC LF82 upon colonization in genetic susceptible CEABAC10 mice (269). Several other studies showed that dramatic shifts in the intestinal ecosystem are not induced by short-term changes in dietary habits or application of single nutrient but require long-term pressure on the ecosystem (189, 270).

Beside exposomal factors, also *intrinsic factors*, i.e., host genetic, shape the intestinal microbiota. Several genes associated with altered immune function, microbial recognition, or antimicrobial defense were shown to influence the intestinal ecosystem. In a meta-analysis, Knights et al. highlighted the significant association between the NOD2 risk allele and increased abundance of *Enterobacteriaceae* in IBD patients (271). This observation was backed up by cohousing experiments of NOD2-deficient mice which develop a genotype-induced dysbiosis. By transferring this dysbiotic microbiota to WT cage-mates, they became more susceptible to DSS colitis (130). In another study, Rausch et al. showed an association between the intestinal microbiota and the FUT2 (secretor) gene – a physiological trait that regulates gastrointestinal mucosal expression of blood group A and B antigens. Approximately 20% of humans lack the FUT2 gene, which was shown to be a risk factor for CD development (272). Within the group of CD patients, the microbiota from FUT2 carriers differed from non-secretors in composition, diversity, and functionality (245, 273).

In intestinal pathologies, such as IBD, dysbiosis is associated to inflammation. It has been proposed that the shift in bacterial composition is due to the inability of some bacterial taxa to adapt to the inflammatory milieu characterized by increased secretion of AMPs (274). The potential of AMPs to shape the intestinal ecology and their importance in IBD has been shown by others before (275). The inflammatory process may also induce stress or upregulation of virulence-associated genes in the microorganism, as shown in monoassociation studies with the gut commensal strains *E. faecalis* OG1RF (276) or *E. coli* NC101 (277, 278). For instance, metalloprotease gelatinase E (GelE) secreted by *E. faecalis* contributes to the development of chronic intestinal inflammation in mice that are susceptible to intestinal inflammation (IL-10$^{-/-}$ and TNF$^{\text{deltaARE}}$ mice) by

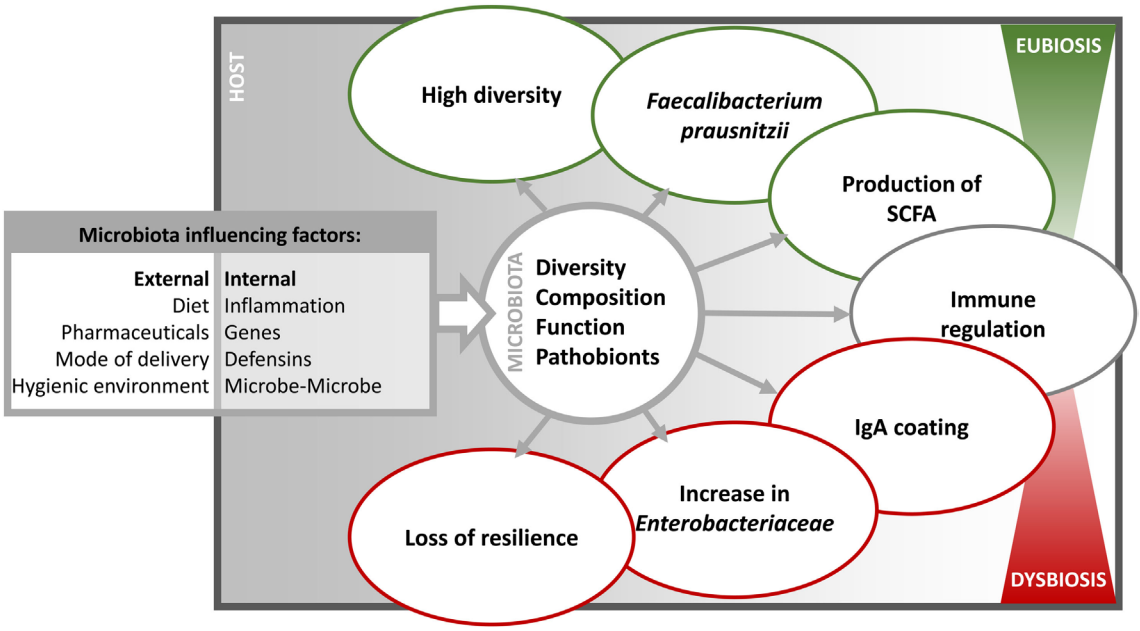

FIGURE 1 | The different influencing factors and characteristics of dysbiosis. The intestinal microbiota is influenced in diversity, composition, function, and prevalence of pathobionts by internal and external factors. Consequently, the microbiota varies in immune relevant characteristics and thereby shifting from a eubiotic to a dysbiotic state.

impairing epithelial barrier integrity (279). *E. faecalis* isogenic mutants lacking GelE, enterococcal polysaccharide antigen, or lipoproteins (Lgt) show significantly decreased inflammation in the distal colon of monoassociated IL-10$^{-/-}$ mice and impaired TLR2-mediated activation of DCs (276, 279). *E. coli* NC101 cells isolated from inflamed IL-10$^{-/-}$ mice displayed upregulated bacterial-stress response genes (e.g., heat shock proteins) compared to isolates from WT mice (277, 278). However, inflammation may also promote growth advantages and virulence of pathogens. For *S. typhimurium*, it was shown that the host produces tetrathionate under inflammatory circumstances, which consequently promotes the ethanolamine utilization of *S. typhimurium* and thereby its colonization fitness (280, 281). Also nitrate produced by the host during inflammatory processes is assumed to promote growth of *Enterobacteriaceae*. Consequently, the often discussed increase of *Enterobacteriaceae* in IBD may also regard as secondary to inflammatory processes rather than causative (282).

Apart from host-derived factors, also microbe-derived mechanisms exert impact on other commensals. The transfer of single strain or bacterial consortia to GF mice enables to study mechanisms of *microbe–microbe interaction*. Commensals are capable of inhibiting growth of pathogens by, e.g., competition for nutrients, quorum sensing-mediated colonization repression, as well as expression of virulence factors (220, 283–285). Metabolic interaction (food chain) of commensals and pathobionts are also shaping the intestinal ecosystem, as some organisms depend on the conversion of dietary components or induction of host-derived nutrients by other members of the microbiota (217, 286–288). Also quorum sensing-mediated repression of colonization is regarded as an important mechanism of shaping the intestinal ecosystem, and recent findings from Thompson et al. demonstrate the role of autoinducer-2 in reshaping antibiotic-induced dysbiosis in the gut (284, 289).

THE ROLE OF THE MICROBIOTA IN ANIMAL MODELS OF INTESTINAL INFLAMMATION

Most dysbiosis-influencing factors cannot be studied under highly controlled and standardized conditions in humans. To discover causative dysbiotic shifts in the microbiota prior to disease onset, large prospective cohort screenings would be necessary, which are hardly feasible due to low incidence rates. Therefore, animal models of intestinal inflammation are gaining more and more importance, as confounders like diet, genetic background, and hygienic environment can be continuously controlled. *Genetically driven animal models* developing inflammation without any chemical inductor (e.g., DSS or TNBS) are especially appropriate to study the interaction of the intestinal microbiota and pathology, as the chemical compound *per se* would influence the ecosystem. Ever since the availability of *GF and gnotobiotic animal models*, great progress has been made to portray microbe–host interaction. Hereby, many IBD mouse models were shown to be free of disease in GF conditions and thereby clearly prove the causality of microbial triggers in IBD development.

The IL-10$^{-/-}$ mouse – incapable of producing the anti-inflammatory cytokine IL-10 – is the best-studied model of colitis. In

GF housing, IL-10$^{-/-}$ mice do not develop inflammation, and interestingly the time point of microbial colonization seems to be of importance, as IL-10$^{-/-}$ mice colonized at adult age developed more severe inflammation (182). A more recent model of UC is the Rag2$^{-/-}$ × Tbx21$^{-/-}$ (TRUC) mouse model. Those mice develop UC-like colitis due to the absence of an adaptive immune system. TRUC mice were free of colitis in GF conditions as well (290). Another model protected from inflammation under GF conditions is the TCR alpha-knockout model. These mice are characterized by a defective immunity as well, as αß T cells are absent (291). Also severe combined immunodeficiency (SCID) mice, having an impaired B and T cell functions, are free of colitis in GF-housing conditions (292).

However, all the above-mentioned models display a colitis phenotype. To study mechanisms relevant in CD, which is characterized by ileal involvement, just few animal models are available. The SAMP1/YitFc mouse model is one of the rare models displaying a CD-like inflammation, with still uncertain mechanism of pathology induction (293, 294). Nevertheless, a recent study suggests that inflammation may be mediated by loss of CCL21 signaling and DC migration from the ileal lamina propria to mesenteric lymph nodes (293–297). Another model of CD-like ileitis is the TNF^{deltaARE} mouse model. These mice have increased TNF levels due to a deletion of the adenosine–uracil-rich element in the TNF transcript (298). The pathology in TNF^{deltaARE} mice with their transmural ileitis and the Th-1-driven immune response give a very good resemblance to the inflammation in CD patients. In line with this, TNF antibodies, which are a treatment option in CD patients, were shown to hamper inflammation in TNF^{deltaARE} mice (299). Just recently, the necessity of a microbial trigger for inflammation development was demonstrated, as TNF^{deltaARE} mice are free of inflammation in GF housing (181). In contrast to SAMP1/YitFc mice, the inflammation in TNF^{deltaARE} mice is not characterized by the formation of "cobblestone" structures – a thickening of the gut wall with protruding lesions found also in some patients with progressive IBD in humans (300, 301). These divergent three-dimensional structural inflammatory phenotypes again highlight the necessity and basic requirement of different mouse models to study the mechanisms of inflammation development and to be able to characterize the complexity of inflammation in IBD.

Mice with a conditional deletion of caspase 8 (a protease involved in apoptosis regulation) in the IECs (Casp8^{deltaIEC} mice) spontaneously develop chronic inflammation in the terminal ileum due to increased necroptosis (302). Notably, antibiotic-treated Casp8^{deltaIEC} mice were rescued from ileitis, indicating an important role for the intestinal microbiota in the development of the disease (303).

Finally, the development of animal models that mirror human genetic risk factors for IBD or other pathologies may be an important step toward unraveling mechanisms of pathology. The disease-free status of animal models in GF housing is exploited as valuable tool to investigate the development of dysbiosis, its influencing factors, and also mechanisms of microbe–host interaction in gnotobiotic setups.

DYSBIOSIS AND MICROBE–HOST INTERACTION IN GNOTOBIOTIC ANIMAL MODELS

Specific mechanisms of disease induction can be studied through the colonization of GF mice with single bacterial strain. Therefore, many colonization studies have been performed for models of intestinal inflammation as well as for other pathologies, including obesity, diabetes, and multiple sclerosis, as recently summarized (304). However, by colonizing immunocompromised GF animals with selected single strain, several obstacles are met. Not all microbes colonize equally, and the induction of inflammation depicts bacteria as well as model-specific traits.

In the IL-10$^{-/-}$ model, several monoassociations with candidate pathobionts failed to induce inflammation, such as *Helicobacter hepaticus* and *E. coli*, while other bacteria were effective in developing pathology, such as *E. faecalis* or *B. wadsworthia* (279, 305, 306). These findings suggest that an association with only one bacterial strain may not be sufficient to induce the necessary immune maturation required for the establishment of the inflammatory status. Atarashi et al. could show that one strain of *Clostridium* was not sufficient to induce regulatory immune functions in former GF animals, as this relies on concerted actions of different strains (307, 308). It is tempting to speculate that not all members of the microbiota are equally sufficient to induce pathology but rather requires the interaction with other commensals. An example of this hypothesis encompasses monoassociation of IL-10$^{-/-}$ mice with *H. hepaticus* or *Lactobacillus reuteri*, which as single strain did not induce inflammation, while the combination of the two bacteria induced colitis (306). Therefore, current studies tend to use complex microbial settings or defined microbial consortia to ensure sufficient immune maturation in GF animals. Garrett et al. showed that colitis in TRUC mice correlates with increased abundance in *Klebsiella pneumonia* and *Proteus mirabilis*. However, the combined colonization of TRUC or Rag2$^{-/-}$ recipient mice with both these two bacterial strains did not induce inflammation. Just a combined colonization of *K. pneumonia*, *P. mirabilis*, and a complex microbiota induced inflammation in recipient Rag2$^{-/-}$ mice (290). However, it has to be mentioned that WT cage-mates were also inflamed, suggesting a rather pathogenic than dysbiotic trait of the microbiota. Interestingly, Powell et al. identified *Helicobacter typhlonius* as a key driver of pathogenesis in TRUC mice (309). The transfer of a complex microbiota from inflamed TRUC mice induced colitis in gnotobiotic TRUC mice as well. However, when the microbiota from antibiotic-treated mice in remission was transferred, the recipients developed only attenuated inflammation (310). In the SCID mouse model, Stepankova et al. showed inflammation development by segmented filamentous bacteria (SFB) only in combination with complex specific pathogen-free (SPF) microbiota (292). SFB are known to be potent inducers of the host's immune response as summarized elsewhere (292, 311). This also points out the fact that monocolonizations may pose elegant ways to prove causality, whereas complex mechanisms of disease initiation may need more complex interactions. TNF^{deltaARE} mice colonized with the pathobiont *E. coli* LF82 did not develop ileitis, whereas the disease was induced upon colonization with

microbiota from inflamed donors (181). Those mice showed in SPF housing high variance in ileitis development due to spontaneous shifts in the intestinal microbiota, i.e., inflamed mice displayed microbial patterns distinct from non-inflamed mice. By transferring the microbiota from inflamed $TNF^{deltaARE}$ to GF mice, the $TNF^{deltaARE}$ recipients develop inflammation. The WT cagemates did not develop inflammation, showing that the microbiota was truly dysbiotic and not pathogenic, as susceptibility of the host is needed for inflammation development. By transferring the microbiota from non-inflamed $TNF^{deltaARE}$ mice, no inflammation was observed in $TNF^{deltaARE}$ or WT recipients. This clearly pinpoints that the concerted action of a complex microbiota with dysbiotic patterns and the genetic susceptibility are precondition for the CD-like pathology in this model.

The studies mentioned in this review clearly emphasize the role of dysbiosis in intestinal pathology, as well as its multifactorial etiology. Until now, no single microbial trait could be identified as a clear indicator of a dysbiotic state itself. It was rather shown that dysbiosis is characterized by multifaceted variations in community networks and displays specificity for the respective host and inflammatory condition. Several human studies reported dysbiosis before onset of disease (234, 312). In animal models of intestinal inflammation, the causal relationship of a dysbiotic ecosystem in inducing inflammation was effectively shown by transferring the microbiota directly or by cohousing animals and thereby inducing pathology in recipients (130, 181, 290, 310). In these animal models which have different mechanisms of pathology induction, a single microorganism is not capable to induce tissue inflammation but needs the concerted action of a complex microbiota. These observations are further supported by the fact that no single pathobiont has been found that is consistently present in different IBD cohorts. More and more studies show that dysbiosis is characterized by changes in the microbial composition and microbial function rather than mere reduction in diversity or expansion of pathobionts. This may also explain why by now, no single probiotic treatment was found able to restore dysbiosis by administration of just one single probiotic strain, suggesting that one single species may not be powerful enough revert complex dysbiotic shifts.

REBIOSIS AS CONCEPT FOR CLINICAL THERAPY

Even though a high number of studies have been performed on the benefits of probiotics in IBD, new meta-analyses show no overwhelming or placebo-superior effect (313, 314). Strain-specific mechanisms were investigated showing the potential of probiotics to enhance intestinal barrier integrity, to counteract proinflammatory cytokines, or to induce an anti-inflammatory immune response (315–317). Newer studies go from single probiotic strain or mixtures with low defined numbers to more complex setups. In *C. difficile*-induced infection, fecal microbiota transplantation (FMT) seems to be very promising and superior to antibiotic treatment regimens (318–322). Hereby, the complex fecal microbiota of a healthy donor (often a relative or spouse of the patient) is processed, standardized, and subsequently transferred to the patient by either nasogastric route or colonoscopy. Although the bacterial taxa associated with a successful FMT-donor microbiota have not been discovered yet, the subsequent increase in the intestinal microbial diversity of the recipient is in most cases associated with recovery (323). However, due to the novelty of this treatment approach, appropriate cohort numbers and well-controlled trials are to be provided in the future. The generation of biobanks for frozen donor microbiota (e.g., OpenBiome; Microbiome Health Research Institute Inc.) is of utmost importance. A future prospect is FMT capsules for therapeutic treatment of infectious diseases, such as *C. difficile* infection, which is currently under development (324). Despite the fact that FMT was highly effective in *C. difficile* infection, its therapeutic implementation in IBD is unclear. A recent study by Moayyedi et al. showed a superior placebo effect of FMT in UC patients, though this effect is donor and time dependent (325). An overall analysis of uncontrolled studies with small patient cohorts shows limited success for FMT as standard therapy in IBD, and the rationale of introducing new antigen pools in a milieu that has been overreacting to microbial stimuli is questionable (326, 327).

The causality of dysbiosis in IBD development has been reported, but clear compositional patterns and functionality are still to be unraveled. These aspects are of importance for basic understanding of which consortia may be effective for therapeutic potential. Finally, the intestinal bacterial community is under the influence of environmental-, host-, and microbial-derived factors, and therefore, microbial composition is not the only trait to be considered in order to choose the suitable donor, but also the recipient genetic background and the exposome should be taken into consideration.

CONCLUSION AND OUTLOOK

Despite a growing body of evidence suggesting a causative role of the intestinal microbiota in CD pathogenesis, the impact of bacteria on disease progression, the development of the various disease phenotypes, the risk to develop therapy refractoriness, or the risk to relapse is completely unclear. An increasing number of sequencing studies in human cohorts show associations of certain bacterial groups and CD development. However, due to the nature of the study or high interindividual variation, they often lack the possibility to depict mechanisms causal for disease initiation. Therefore, animal models of intestinal inflammation are important to show functionality and to depict characteristics of dysbiosis. The possibility to unravel certain mechanisms of interaction in animal models of inflammation is generating important insights. The role of antigenic surface compounds or microbial-derived metabolites is of great interest to elucidate mechanisms of microbiota-induced shifts in intestinal homeostasis. Even though new mouse models were recently generated which display more hypothesis-driven mechanisms of intestinal inflammation, it is evident the paucity of model organisms that accurately reproduce all aspects of multifactorial disease, such as CD.

Questions remain about the reliability of mouse models as tool to draw interpretations on human disease. In some cases, mouse research has led to major advantages in the ability to treat serious conditions. For instance, work on the acute promyelocytic leukemia mouse model resulted in successful treatment

for this type of cancer in human patients (328). Knocking out the leptin gene in mice manifested the role this hormone has in regulating appetite and, by extension, preventing obesity (329). In contrast, mice are not always reliable as models for human disease as shown in the case of specific IL-17-deficient mouse strains which generated conflicting results in preclinical models of IBD (330).

Similar questions arise from the use of humanized mice in order to unravel human molecular mechanisms or functions. The humanization process consists in repopulation of mice with human hematopoietic cells or in colonization of the gut of GF mice with human microbiota. The potential of these tools for the study of human immune function and causality of the complex host–microbiota interactions *in vivo* is apparent. Nevertheless, it should be taken into consideration whether the mouse system is able to support development of human immune cells and whether antigens from human origin enable murine immune system maturation. Additionally, it has been shown before that bacterial strains sharing similar genomic content exert differential ability to stimulate the host response (331, 332), suggesting that molecular and biochemical differences in cell envelope architecture may account for the variation in cytokine profiles. This evidence leads to the hypothesis that some bacterial strains may harbor host specificity, and therefore, isolates specific for the human setup might not be able to induce maturation of the mouse immune system, as previously suggested (333).

Finally, it should be noted that even though GWAS have linked genetic variations to several human conditions, they do not provide information on their functions. Animal models are clearly a fundamental tool to identify gene function, and how mutations in gene associated to the disease alter these functions.

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REVIEW
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Convergence of external Crohn's disease risk factors on intestinal bacteria

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Crohn's disease (CD) is an immune-mediated intestinal illness that significantly compromises health in many developed countries. Although definitive causes remain elusive, the required contribution of microbes in the progression of disease has become an accepted concept. Known CD risk factors, such as antibiotic use and acute infectious gastroenteritis, may impact the gut. This concept is now being explored with a view toward understanding the beneficial and unfavorable microbes that may be altered in numbers during such external insults. A comprehensive understanding of the microbial component to CD could be useful clinically as future therapies may focus on preventing risk exposures on susceptible individuals, eliminating harmful microbes, or restoring a protective gut microbiome. Here, we examine how acute infectious gastroenteritis and antibiotic exposure may impact the gut microbiota in the context of inflammation in CD.

Keywords: Crohn's disease, adherent-invasive *E. coli*, gastroenteritis, antibiotics, microbiota

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INTRODUCTION

Crohn's disease (CD) is part of the inflammatory bowel disease spectrum that can affect any portion of the gastrointestinal tract. CD is becoming increasingly common in many high-income nations, particularly among the pediatric population (1). Due to its high prevalence and chronic disease course, CD imposes significant directs costs on the health care system, along with a high attendant societal cost (2). Since the classification of CD in the 1950s, significant investments have been made in understanding the root causes of the disease, as well as in new treatments to induce remission that target mainly the aberrant inflammatory response underlying the disease course. Although genetics are known to play an important role in CD (3, 4), penetrance is low for any one genetic risk allele. Furthermore, the sharp rise in CD in many countries over the past 40 years indicates that genetics alone does not fully explain the etiology of disease, but rather that environmental exposures may be antecedent to disease initiation and progression in a genetically susceptible population (5). Understanding the nature and origins of such environmental risk factors is of paramount importance in curbing the rise of CD worldwide.

Clinical and animal studies have provided robust data indicating that intestinal microorganisms, both luminal and tissue-associated, play an important role in CD pathogenesis (5–9). For example, a pathogenic role for luminal bacteria is consistent with the beneficial effects of experimental fecal stream diversion following ileocolonic resection (10) and levels of epithelial-adherent bacteria have been found to correlate clinically with CD severity (11, 12). Environmental exposures, such as infectious agents or use of certain xenobiotics, can create intestinal vulnerabilities, rendering one more

susceptible to the functional and microbial changes associated with CD. In this review, we briefly summarize the microbial composition changes that have been described in CD patients, and discuss two CD risk factors – acute infectious gastroenteritis and exposure to antimicrobial agents – that are known to induce pervasive changes to the microbial composition in the gut. The potential for dysbiosis-inducing external insults such as these to select for the expansion of pathobiont-like bacteria that affect the inflammatory tone of the gut is discussed.

MICROBIAL DYSBIOSIS IN CROHN'S DISEASE

The advent of high-throughput sequencing has allowed researchers to investigate the gut microbiome in health and disease states, which was previously difficult to study due to the immense quantity and diversity of species present as well as the difficulty in getting broad representation of the intestinal community by culturing methods (13). Several high-throughput methods have been developed over the past decade that have allowed researchers to identify intestinal bacteria based on genetic signatures. One method involves using fluorescence *in situ* hybridization (FISH) probes designed to label certain groups of bacteria. These labeled bacteria can then be quantified using flow cytometry (FCM-FISH) (14). This method has the advantage of being relatively simple and inexpensive; however, the FCM-FISH method has relatively poor resolution and can only identify broad changes in bacterial groups. High-throughput sequencing has set a new benchmark, allowing researchers to analyze the population structure of complex microbial communities (15). In this regard, 16s rRNA is a useful genetic marker as it is universally present among all bacteria. It contains both variable regions useful for genomic classification and conserved regions that can be used to design universal primers. The choice of primers is extremely important, as even "universal" primers may poorly amplify some bacterial families. Although sequencing larger segments of the 16s rRNA region provides more resolution, most high-throughput sequencing technologies are limited to sequencing several hundred base pairs. For this reason, most studies analyze only one or several variable regions of the 16s rRNA gene. Nonetheless, even short reads of variable regions around 100 bases are usually sufficient to differentiate many microbes to the genus level, although using different variable regions can lead to different results (16). When comparing the results of 16s rRNA sequencing, it is important to consider differences in primer choice and the length of DNA sequenced from each fragment. Additionally, differences in data clean up as well as data analysis can lead to additional variability. For example, different analysis procedures between groups can result in considerable discrepancies in data generation and interpretation that should be taken into consideration when comparing observations from different publications. Nevertheless, some themes are emerging from the literature, which are discussed below.

The human microbiome is composed primarily of anaerobes within the bacterial phyla *Firmicutes* and *Bacteriodetes*. One of the first studies to investigate the CD microbiome examined fecal bacteria from six healthy individuals and six CD patients using both 16s rRNA sequencing and FCM-FISH (17). All CD patients were in remission, had no exposure to antibiotics for at least 3 months, and had previous ileal involvement in the disease course. In this study, CD patients had much lower microbial diversity than the control group, with 88 ribotypes represented in healthy subjects compared to only 54 in CD patients. This change was largely due to decreased diversity in the *Firmicutes* phylum, in particular, the *Clostridium leptum* group. *C. leptum* are known to produce butyrate, a short chain fatty acid (SCFA) and energy source for the intestinal epithelium. Butyrate has also been shown to down regulate pro-inflammatory mRNA within enterocytes and to acidify the intestinal lumen that is thought to inhibit certain pathogens such as *Salmonella* and *Escherichia coli* (18, 19). A similarly designed study using FCM-FISH analyzed fecal samples from 14 CD patients and 13 healthy controls that had not received sulfasalazine, antibiotics, or laxatives in the month prior to analysis (20). Using FISH with group-specific probes the study also found that *C. leptum* was significantly decreased in the CD group.

A larger study examined intestinal biopsy samples rather than fecal samples, from CD and UC patients and healthy controls ($n = 190$) using 16s rRNA sequencing (21). *Bacteroidetes* and *Lachnospiraceae* groups were greatly decreased in these subjects, whereas *Actinobacteria* and *Proteobacteria* groups were increased. Another study investigated both fecal and mucosal samples from healthy ($n = 27$), CD ($n = 121$), and UC patients ($n = 75$) utilizing 16s rRNA sequencing of V3–V5 regions (22). *Roseburia*, *Phascolarctobacterium*, and *Ruminococcaceae* were all reduced in CD patients. These microbial groups are also important producers of SCFA. In ileal CD patients, *Ruminococcaceae* and *Faecalibacterium* groups were particularly reduced while *Escherichia/Shigella* species were enriched, a finding that has been reproduced in several studies. Interestingly, the use of 5-ASA was strongly correlated with a decrease in *Escherichia/Shigella*. Various genera within *Clostriales* order were decreased in patients treated with antibiotics, possibly due to their sensitivity to ciprofloxacin/metronidazole often used in CD treatment. The genes involved in several major metabolic pathways have been well characterized in model bacteria. Since many of the genome of gut microbes have been sequenced, it is possible to predict if a microbe lacks a particular classical pathway based on an absence of conservation to known genes typically involved in that pathway. Overall, microbes from IBD patients had decreased pathways for amino acid synthesis coupled with increased amino acid uptake pathways. There were also increased levels of carbohydrate and lipid uptake and metabolism pathways, particularly in ileal CD. Many of these changes may aid in the metabolism of mucin, which is often overproduced during intestinal inflammation. The reduction in amino acid synthesis resembles phenotypes elicited by murine pathobionts known as segmented filamentous bacteria (23, 24). The microbiome showed increased glutathione uptake, which may be important for surviving oxidative stress from inflammation (25). Finally, the ileal CD metagenome was enriched in bacterial secretion systems, in particular, the type II secretion system that is often used in the export of toxins (26).

Another recent study analyzed the treatment-naïve microbiome of pediatric CD patients ($n = 447$) and healthy controls

(n = 221) using tissue biopsies and feces and sequencing the 16s rRNA V4 region (27). When comparing intestinal biopsy samples from healthy individuals to CD patients, the *Bacteroidales* and several species within the *C. leptum* groups had lower abundance in pediatric CD patients in accordance with previous studies. Several bacterial families that were found to be more abundant were *Enterobacteriaceae*, *Pasteurellaceae*, *Veillonellaceae*, *Neisseriaceae*, and *Fusobacteriaceae*. The use of antibiotics in patients already diagnosed with CD is controversial (28, 29). However, in this study, a subset of CD patients had been treated with antibiotics providing for a separate analysis. When this group was analyzed separately, it was found that antibiotic exposure amplified the microbial dysbiosis associated with CD. Importantly, in new onset patients, the microbial dysbiosis was poorly reflected in the stool compared to tissue biopsy samples, which might suggest that the bacterial community of pathogenic significance in the disease is tissue associated.

Overall, these studies consistently show that CD is associated with decreased abundance and diversity of the *Firmicutes* phylum with an increased abundance in the *Proteobacteria* phylum (**Figure 1**). Tissue samples appear to be more indicative of dysbiosis compared to fecal samples at least in early disease. Although *Firmicutes* appear decreased in overall abundance in CD, some specific members of the *Firmicutes* are conversely increased. These studies occasionally differ on the particular groups of bacteria that are elevated or reduced in CD patients. These differences may be due to differences in sample type (luminal vs. tissue) as well as differences in patient populations (patients with active disease vs. remission, treated patients vs. treatment naïve), or in method of analysis. How these shifts in microbial ecology contribute to the pathophysiology of CD remains an active area of research. A recent study using a murine model of CD demonstrated that bacteria gut dysbiosis played a causal role in intestinal inflammation (30). Several mechanisms may explain why dysbiosis is associated with CD. It is possible

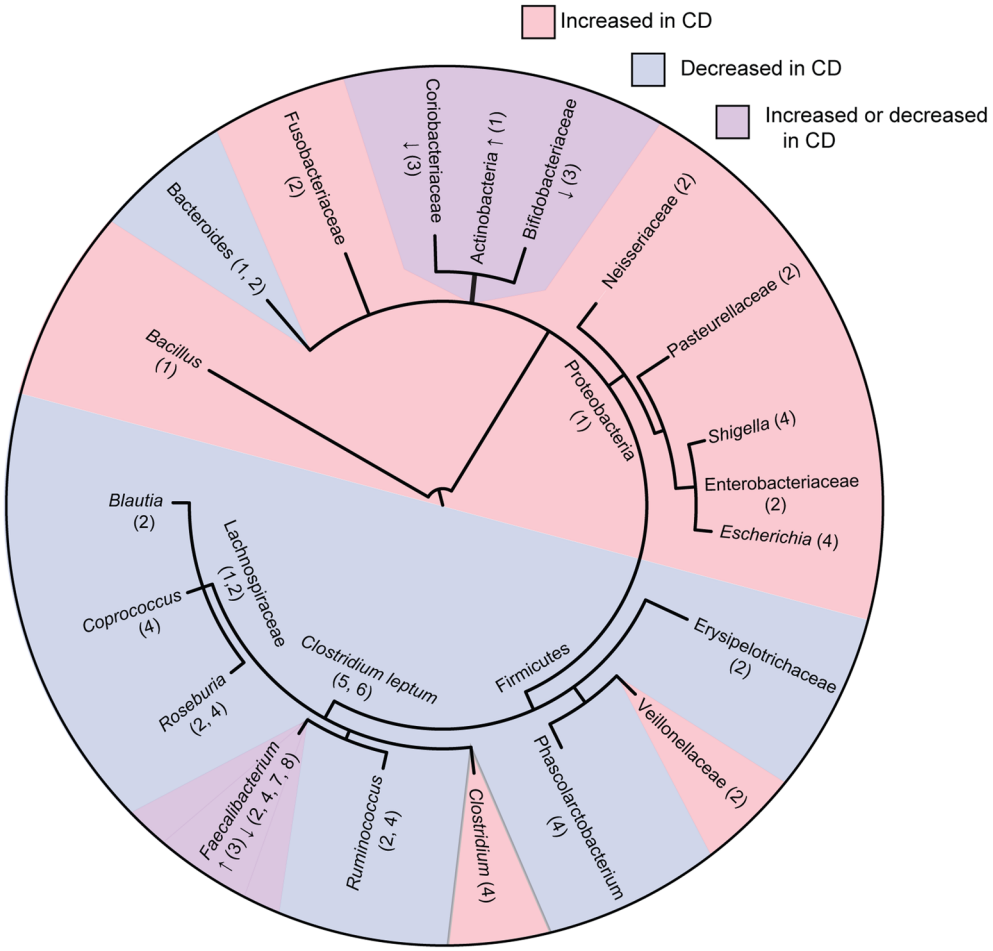

FIGURE 1 | Phylogenetic tree of bacterial groups associated with increased or decreased abundance in the gut microbiome of Crohn's disease patients. In cases were bacterial groups have been associated both increases and decreases, studies showing increased abundance are indicated with "↑" and studies showing decreased abundance are indicated with "↓." Numbers indicate references as follows; 1, Frank et al. (21); 2, Gevers et al. (27); 3, Hansen et al. (31); 4, Morgan et al. (22); 5, Sokol et al. (20); 6, Manichanh et al. (17); 7, Sokol et al. (32); 8, Sokol et al. (33). Tree topology was created using phyloT (http://phylot.biobyte.de/) and the figure was made using ITOL (http://itol.embl.de/).

that dysbiosis contributes to CD because the loss of SCFA producing bacteria has multiple impacts on the gut environment, including impaired survival of enterocytes, increased production of inflammatory cytokines, and decreased suppression of potentially pathogenic *Proteobacteria*. It is also possible that dysbiosis is caused by the intestinal inflammation in CD. Bacterial species from *Proteobacteria* are facultative anaerobes that tend to have a higher resistance to reactive-oxygen species produced during inflammation, possibly giving them a selective advantage over the predominantly obligate anaerobes from *Firmicutes* and *Bacteroidetes*. These mechanisms are not mutually exclusive, and it is likely that microbial dysbiosis both contributes to and results from the intestinal inflammation seen in CD.

One member of the *Firmicutes* that has received much attention in the context of CD is *Faecalibacterium prausnitzii*, a major member of the *C. leptum* group of *Firmicutes* that is decreased in CD patients (32). In particular, a higher abundance of *F. prausnitzii* was associated with a higher rate of remission following resection surgery (33). *In vitro* analysis showed that this bacterium was capable of inhibiting NFκB and the secretion of pro-inflammatory cytokines, as well as stimulating the release of anti-inflammatory cytokines, such as IL-10. *In vivo* studies using dinitrobenzene sulfonic acid (DNBS) (34) and trinitrobenzene-sulfonic acid (TNBS) (33)-induced colitis models showed that *F. prausnitzii* and its culture supernatant were capable of attenuating inflammation when introduced both orally and into the peritoneal cavity. An intriguing recent study has identified a protein secreted by *F. prausnitzii* that may explain its anti-inflammatory properties (35). However, the exact role of *F. prausnitzii* in the pathogenesis of CD has yet to be elucidated. Two studies of pediatric patients with newly diagnosed, untreated CD conflictingly showed increased (31) and decreased (27) levels of in *F. prausnitzii* compared to healthy controls. It is possible that the decreased levels of *F. prausnitzii* are a characteristic of adult CD but not pediatric CD. It is also possible that *F. prausnitzii* is lost due to medications used to treat CD such as antibiotics rather than having a direct link to disease expression. Additional research is needed to determine how levels of *F. prausnitzii* might dictate the fine balance between health and disease in the gut.

INFECTIOUS GASTROENTERITIS AS A RISK FACTOR FOR CD

Amply powered clinical studies show that acute infectious gastroenteritis caused by enteric pathogens (including *Salmonella* and pathogenic *E. coli*) increases the short- and long-term risk of developing CD (36–38) (**Table 1**). In one study, Garcia-Rodríguez et al. compared a cohort of individuals with acute gastroenteritis ($N = 43{,}013$) with matched controls without documented gastroenteritis ($N = 50{,}000$), excluding individuals with previous IBD diagnosis (36). Over a mean follow-up period of 3.5 years, there was an increased risk of developing IBD after a single episode of acute gastroenteritis (HR 2.4 [1.7–3.3]). The association was stronger with CD compared to ulcerative colitis (UC), particularly in the first year after gastroenteritis (HR 6.6 [1.9–22.4]). A second case–control study by Porter et al. compared individuals diagnosed with CD with healthy controls (37). The study examined previous diagnoses of infectious gastroenteritis in both groups. Patients with IBD were more likely to have had a previous diagnosis of infectious gastroenteritis (AOR 1.40 [1.19–1.66]). Once again, the risk was higher for CD (AOR 1.54 [1.17–2.04]) than for UC (AOR 1.36 [1.08–1.72]). Finally, a retrospective study compared cohorts of healthy individuals in Denmark to those diagnosed with acute gastroenteritis caused by either *Salmonella* or *Campylobacter*, tracking subsequent IBD diagnoses over the following 15 years (38). Overall, the gastroenteritis group had an increased risk of subsequent IBD diagnosis over the entire 15-year period (HR 2.9 [2.2–3.9]), with the greatest risk occurring in the first year following an episode of acute gastroenteritis.

Due to the observational nature of these studies, it is difficult to determine whether infection predisposes individuals to CD or if individuals with a susceptibility CD are more susceptible to infection. It is possible that low-grade inflammation in prodromal CD may lower the colonization resistance to enteric pathogens, a phenomenon has been demonstrated in murine models (39). Several studies have shown that infectious gastroenteritis is associated with microbial dysbiosis, albeit transiently (40, 41), that might underlie the microbial triggers of chronic disease. Though the mechanisms are currently unknown, the long-term risk of developing CD following acute infectious gastroenteritis might suggest that resident gut microbes could perpetuate inflammatory reactions in the post-infectious period. One central hypothesis is that exposure to infectious pathogens creates an environment favorable to colonization by other pro-inflammatory bacteria. These may be existing members of the microbiota typically restricted by microbial and host processes, or they may be acquired *de novo* through vulnerabilities created by the disruption of the resident microbiota during gastroenteritis. For example, human (42) and animal studies (43, 44) indicate that inflammation during gastroenteritis selectively disrupts the resident intestinal microbiota in favor of *Enterobacteriacea*, such as *E. coli*. Whether such disruptions have long-term consequences on the host in the post-infectious period has not been rigorously studied.

TABLE 1 | Epidemiological studies associating infectious gastroenteritis and Crohn's disease.

Study	Type	Location	Population size	Type of association	Risk [95% CI]
Garcia Rodriguez et al. (36)	Retrospective cohort	United Kingdom	IGE: 43,013 Cont: 50,000	Overall	HR 6.6 [1.9, 22.4]
Porter et al. (37)	Case–control	United States	CD: 1,037 Cont: 11,646	Overall	OR 1.54 [1.1, 2.04]
Gradel et al. (38)	Case–control	Denmark	IGE: 13,148 Cont: 26,216	Overall	HR 3.0 [1.7, 5.3]

IGE, infectious gastroenteritis; HR, hazard ratio; OR, odds ratio.

TABLE 2 | Epidemiological studies associating antibiotic treatment with subsequent CD diagnosis.

Study	Type	Location	Population size (CD:control or cohort size)	Type of association	Risk [95% CI]
Card et al. (49)	Adult Case–control	United Kingdom	587:1460	2–5 years before CD diagnosis	OR 1.32 [1.05, 1.65]
Margolis et al. (50)	Adult Retrospective cohort	United Kingdom	94,487	Overall	HR 1.62 [1.04, 2.53]
Shaw et al. (48)	Adult Case–control	Canada	1025:22,346	2–5 years before CD diagnosis	AOR 1.29 [1.18, 1.40]
Hildebrand et al. (53)	Pediatric Case–control	Sweden	1098:6550	Pneumonia <5 years old	OR 3.56 [1.79, 7.08]
Hviid et al. (51)	Pediatric Retrospective cohort	Denmark	577:627	Overall	RR 3.41 [1.45, 8.02]
Virta et al. (52)	Pediatric Case–control	Finland	233:2380	2 years before CD diagnosis	AOR 1.87 [1.37, 2.56]

OR, odds ratio; HR, hazard ratio; AOR, adjusted odds ratio; RR, relative risk.

ANTIBIOTICS AS A RISK FACTOR FOR CD

Antibiotics are commonly prescribed for a wide range of illness and are occasionally used in the treatment of CD (45). Antibiotics have long been known to have negative impacts on commensal microbes and are known to be an important risk factor for gastrointestinal illnesses, such as *Clostridium difficile* infection (46, 47). Several epidemiological studies have linked exposure to antibiotics with CD, which are summarized in **Table 2**. A case–control study in Canada compared antibiotic use in individuals with incident IBD diagnosis and non-IBD controls and found that patients diagnosed with IBD were more likely to have been prescribed antibiotics in the preceding 5 years before diagnosis (48). Of interest, there was a dose-dependent relationship between the number of antibiotics dispensed and the risk of subsequent IBD diagnosis. The risk of subsequent IBD diagnosis was highest 2 years after antibiotics and diminished over time. Nearly all antibiotics included in this study, with the exception of clindamycin, were associated with an increased risk of IBD diagnosis. Interestingly, metronidazole had the highest risk (AOR 2.86 [2.24–3.65]) followed by quinolones (AOR 1.45 [1.27–1.64]). Both of these antibiotics are also used as treatments for CD.

A case–control study using data from the UK examined patient medical history at least 5 years prior to diagnosis of CD (49). Again, patients who were later diagnosed with CD were more likely to have taken antibiotics (OR 1.32 [1.05–1.65]). When individual antibiotics were examined, only metronidazole and tinidazole (OR 1.71 [1.05–2.76]) and tetracylines (OR 1.33 [1.01–1.77]) were associated with subsequent CD. This study did not find a dose-dependent relationship between the numbers of antibiotics dispensed and overall CD risk.

A cohort study from the UK examined the association between oral tetracycline for the treatment acne in adults and adolescents and subsequent IBD diagnosis (50). Once again, there was an association between tetracycline use and IBD (HR 1.39 [1.02–1.90]) and a stronger association with CD in particular (HR 1.62 [1.04–2.53]). Furthermore, the risk of CD varied based on the type of antibiotic, with minocycline conferring the lowest risk (HR 1.28 [0.72–2.29]) followed by tetracycline (HR 1.61 [1.00–2.63]) and finally doxycycline (HR 2.25 [1.27–4.00]).

Several studies in pediatric patients have shown similar results. A study in Denmark followed all singleton children born between 1995 and 2003 (51) and tracked antibiotic prescriptions and IBD diagnoses. CD, but not UC, was associated with previous antibiotic prescriptions (RR 3.41 [1.45–8.0]). This relationship was dose dependent, with the highest risk being among children prescribed ≥7 antibiotics (RR 7.32 [2.14–24.9]). Interestingly, the risk of diagnosis was highest within 3 months of antibiotic exposure (RR 4.43 [1.88–10.44]). Penicillin V and extended spectrum penicillins conferred the highest risk (RR 2.92 [1.22–6.97] and RR 3.13 [1.33–7.40], respectively). Another study from Finland similarly found an association between antibiotic use 2 years preceding CD (AOR 1.87 [1.37–2.56]), but not UC, diagnosis (52). Cephalosporin antibiotics were associated with the highest risk. Finally, a Swedish study which used childhood infections as a proxy for antibiotic treatment found a position association between pneumonia, diagnosed at <5 years old, and subsequent CD (OR 3.56 [1.79–7.08]) (53).

These studies have provided evidence for a correlation between antibiotic use and subsequent diagnosis of CD; however, most of the studies do not agree on a specific class of antibiotic that confers the highest risk, which may relate to differences in patient populations or prescribing practices. Due to the observational nature of these studies, it is difficult to show causality between antibiotics and CD and it remains possible that the phenotype that makes one susceptible to CD may also make one susceptible to infections requiring antibiotics. Conversely, among patients already diagnosed CD, the use of antibiotics is associated with a reduced chance of flaring (54). Relatively short courses of antibiotics are known to lead to dramatic shifts and loss of diversity on the gut microbiome, which can persist for years (55). It is possible that antibiotic exposure may trigger an unfavorable microbial community structure that increases the potential for long-term dysbiosis and risk of CD in susceptible individuals. Microbiome comparison between CD patients with and without exposure to antibiotics supports this claim, showing that antibiotics magnify the dysbiosis signature associated with CD (27). Nonetheless,

robust experimental evidence is needed to clarify the impact of antibiotic use on host susceptibility to disease.

TISSUE-ASSOCIATED BACTERIA ARE INCREASED IN CROHN'S DISEASE

Inflammation in CD can involve both the small and large bowel and is accompanied by changes in the microbial composition and distribution at these sites (6, 13). Clinical observations are consistent in finding increased numbers of bacteria associated with the intestinal epithelium in Crohn's patients (5, 12, 22). The intestinal mucosa, made up of the epithelium and underlying lamina propria, is a site rich in immune cell populations that protect the integrity of the epithelial surface and direct the innate and adaptive immune responses. Since this site is consistently breached in CD, attention has focused on the relationship between the innate immune system and the microbiota (56), with a particular emphasis on the bacteria that penetrate into this normally aseptic site. Examination of the mucosa in ileal CD has been informative. It is here that the density of *E. coli*, enriched in virulence and secretion pathways, is elevated as determined by culture and molecular methods (11, 22, 57). Use of bowel-specific aminosalicylates is associated with normalization of this *E. coli* bloom in ileal disease (22, 58), suggesting that inflammation might somehow benefit certain bacteria that are associated with the mucosal surface. This finding is important clinically because the severity of ileal disease has been shown to correlate directly with the density of mucosa-associated *E. coli* (12). The implication of course is that reducing the burden of mucosa-associated bacteria would have favorable outcomes on the disease course, possibly opening up new therapeutic avenues beyond immunosuppression. Together, these data support the general view that mucosa-associated *E. coli* are of pathogenic significance in the disease and that their abundance can be manipulated using pharmacologic treatments directed at host inflammation. These results stimulate many important and interesting questions. For example, they imply that the selective environment favoring *E. coli* expansion at the mucosal surface is inflammatory in nature. Although the exact source of this selection is not yet known, fruitful lines of investigation can be envisaged to uncover it. Furthermore, does the inflamed mucosa render a host more susceptible to *de novo* colonization by bacteria with pathobiont-like features, or is this expansion seeded from a resident population of opportunistic bacteria that exploit this favorable niche? Lastly, the genetically encoded bacterial adaptations needed for mucosal expansion in the inflamed gut are not known, but uncovering them would offer valuable insight into the evolutionary process that selects for such pathobionts. The upshot of this information would be new useful genetic markers for detecting pathobionts in hosts and other potential environmental reservoirs, and in identifying new potential targets for antimicrobial drug discovery.

ADHERENT-INVASIVE *E. coli* IN CROHN'S DISEASE

Much experimental and observational data implicate infectious agents in the initiation and maintenance of chronic inflammation in the intestine (6). *E. coli* is a Gram-negative species in the intestine where it can have a positive effect on gut homeostasis. However, through acquisition of virulence factors, such as toxins, adhesins, and secretion systems, *E. coli* can develop pathogenic traits that participate in intestinal and extraintestinal disease processes (59). Work originating from the laboratory of Dr. Darfeuille-Michaud identified *E. coli* in ileal biopsies from patients with CD (57), which they called adherent-invasive *E. coli* (AIEC) to reflect their atypical ability to adhere to mucosal epithelial cells and invade and survive within human cells. These *E. coli* were found to lack known virulence factors typical of pathogenic *E. coli*, including type III secretion systems or phage-encoded toxins, suggesting that they were a newly described pathovar. A series of papers followed that detailed the intracellular lifestyle of AIEC and the inflammatory response by cultured cells to AIEC infection (60, 61). AIEC are capable of replicating within macrophages and causing these cells to release high amounts of TNFα *in vitro* (62). It also appears possible that AIEC exploit cell phenotypes associated with genetic CD risk factors, such as defects in autophagy (63).

Several studies have now confirmed that AIEC are enriched in humans with CD, where they are six-times more likely to be isolated from ileal and colonic samples compared to healthy controls and represent the dominant bacterial species present (57, 64, 65). A growing body of work has uncovered genetic and phenotypic diversity among AIEC isolated from adults (12, 65–67), children (68, 69), and companion animals (70) indicating that different host environments can select for the AIEC phenotype. Since AIEC can be isolated from seemingly healthy individuals (albeit much less frequently than in Crohn's), this genetic diversity suggests that interactions between AIEC and other CD risk factors might be needed to elicit disease in a subset of individuals.

In 2013, Small et al. established a novel model for chronic AIEC infection in conventional mice (8), that over time, develop transmural inflammation and fibrosis in the small and large intestine, the very hallmarks of CD. Importantly, this happens over timescales consistent with a progressive chronic disease without the need for foreign chemicals to induce inflammation. Using this model, it was shown that AIEC is resistant to innate antimicrobial defenses at the epithelial surface, including resistance to antimicrobial peptides released by Paneth cells and colonocytes (71). The molecular mechanism for this resistance involves, in part, the expression of a surface-localized protease that cleaves cationic host defense peptides to render them inactive. In addition to resistance to host-derived antimicrobial molecules, AIEC tend to be resistant to multiple xenobiotic antimicrobials, particularly in isolates obtained from patients with ileal disease (72). This likely reflects the selective environment in which AIEC evolve within hosts, as CD patients are often given antibiotics as part of their treatment regimen. As of yet, no studies have examined whether eradicating AIEC is a viable or efficacious strategy in the treatment of CD; however, animal models permitting chronic colonization and disease progression will help facilitate such studies in the future.

CONCLUSION

Intestinal bacteria play an important role in the pathogenesis of CD in a manner that is incompletely understood. External risk factors, such as acute infectious gastroenteritis and antibiotic exposure, are

moderately associated with CD, yet neither can be considered sufficient to elicit disease on their own. It is likely that a combination of genetic and environmental risk factors is necessary in the pathogenesis of CD; however, exactly how such a constellation of risk factors manifest in disease expression has been a challenging problem to address. The study of mucosal-associated *E. coli* with pathobiont-like properties has yielded promising new leads into the pathogenesis of CD but more work needs to be done to understand where such microbes come from, where they evolve their pathogenic features, and what the effect of their eradication might have on susceptible hosts. A mechanistic understanding of the pathogenic microbes in CD will offer more therapeutic targets and perhaps paradigm shifting approaches toward curative treatments for this disease.

AUTHOR CONTRIBUTIONS

AO and BC wrote the paper.

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Mycobacterium avium ss. *paratuberculosis* Zoonosis – The Hundred Year War – Beyond Crohn's Disease

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The factitive role of *Mycobacterium avium* ss. *paratuberculosis* (MAP) in Crohn's disease has been debated for more than a century. The controversy is due to the fact that Crohn's disease is so similar to a disease of MAP-infected ruminant animals, Johne's disease; and, though MAP can be readily detected in the infected ruminants, it is much more difficult to detect in humans. Molecular techniques that can detect MAP in pathologic Crohn's specimens as well as dedicated specialty labs successful in culturing MAP from Crohn's patients have provided strong argument for MAP's role in Crohn's disease. Perhaps more incriminating for MAP as a zoonotic agent is the increasing number of diseases with which MAP has been related: Blau syndrome, type 1 diabetes, Hashimoto thyroiditis, and multiple sclerosis. In this article, we debate about genetic susceptibility to mycobacterial infection and human exposure to MAP; moreover, it suggests that molecular mimicry between protein epitopes of MAP and human proteins is a likely bridge between infection and these autoimmune disorders.

Keywords: paratuberculosis, MAP, Crohn's, autoimmune, molecular mimicry, type 1 diabetes, autoimmune thyroiditis, multiple sclerosis

INTRODUCTION

In 1913, a concise description of what today is known as Crohn's disease was offered by Scottish surgeon Kennedy Dalziel (1). Twenty years earlier, in 1895, German veterinary Johne H. A. described the cause of an incurable profuse diarrhea in cattle. He noted acid-fast bacteria (most often indicating the organism that causes tuberculosis) that, when transferred to a guinea pig, did not cause tuberculosis (2). Johne first labeled the disease "pseudotuberculosis" and it eventually became known as paratuberculosis.

Infected cow's intestines had the same cobblestone aspect of Dalziel's patient and microscopically, the patient's and cattle's diseased intestines were so alike that Dalziel wrote that the tissue characteristics were:

> . . . so similar as to justify a proposition that the diseases may be the same (1).

He hypothesized that the disease in cattle and the disease in people shared the same cause. The disease in humans was later named after Dr. Crohn who described a series of patients in 1932 (3).

The heart of this 100-year controversy revolves around the fact that the usual diagnostic techniques to detect bacteria are commonly inefficacious to detect *Mycobacterium avium* ss. *paratuberculosis* (MAP) in humans. A short explanation is that it is just very difficult to grow MAP from humans; and, MAP exists with a modified cell wall – the component of the bacterium that takes up the characteristic acid stain. In this state, the bacterium is no longer "acid fast" and cannot be detected microscopically. Recent work has identified the capacity of MAP to undergo a morphologic change to become spore-like. The spore morphotype survives heat and other stressors and may lead to an increased persistence in hosts and the environment (4).

Understanding the difficulty in detection and appreciating the work of specialty labs that have shown MAP bacteremia in Crohn's disease patients, there has been a warming to the association of MAP in Crohn's (5).

MYCOBACTERIUM AVIUM ss. *PARATUBERCULOSIS*

Mycobacterium avium ss. *paratuberculosis* is an acid-fast staining small rod-shaped bacterium (6, 7). As with members of the Mycobacteriaceae genus, its cell wall structure rich in complex lipids is unique. The tough and peculiar cell wall of mycobacteria is, in large part, responsible for the persistence of these bacteria, both in the environment and inside the host. Paradoxically, the pathogenic potential of mycobacteria increases as their growth rate decrease. In fact, slow-growing mycobacteria are more pathogenic than fast growing mycobacteria. Except the uncultivable *Mycobacterium leprae* (the cause of leprosy in humans), MAP has the slowest growth rate among harmful mycobacteria. After inoculum of infected samples from infected animals and incubated under optimal conditions, MAP colonies usually appear not before 3 months or more (8).

MAP AND HUMAN EXPOSURE

Mycobacterium avium ss. *paratuberculosis* can be found in pasteurized milk (9, 10), milk powder for children (11), surface water (12–14), soil (12), cow manure that contaminates the soil and surface water, moreover cow manure is usually applied as fertilizer in different crops (15) and supply of drinking water (16) all

contributing to human exposure. Soil and plants in grazing areas retain MAP; its DNA can be detected in the upper greens of plants, their roots and in the soil below the roots to a depth of 80 cm (17, 18). MAP DNA was detected in over 80% of domestic water samples in Ohio (19). Chlorination and filtration may help to survive mycobacteria rather than eliminate these organisms by killing off their competitors (20). Moreover, mycobacteria organisms have been reported on tap water pipes (21) in biofilms (22) and plastic water bottles (23). One estimate is that mycobacteria could be present in drinking water in "massive numbers," on the amount of up to 700,000 or 7×10^5 organisms per liter of water (22). A recent study reported testing infant formula for MAP in 65 samples from 18 countries: >40% tested positive for viable MAP (24).

MAP AND HUMAN DISEASES

In addition to Crohn's, MAP has been associated with multiple diseases: sarcoidosis and Blau syndrome (25), type 1 diabetes (26–32), Hashimoto's thyroiditis (33–36), and multiple sclerosis (MS) (37–49). In autoimmune diabetes, thyroiditis, and MS, MAP is thought to induce pathology due to molecular mimicry between protein elements of itself and the targeted organ elements of the host, e.g., MAP 3865c and Znt8 in autoimmune (type 1) diabetes and thyroiditis (31, 35, 36). **Figure 1** shows how MAP may trigger autoimmune diseases.

If humans are so readily exposed to MAP, why is there not pervasive Crohn's disease and the other diseases mentioned in this article?

GENETICS

CARD15

A good example about the interaction between the genetic susceptibility and microbial infection can be found in Crohn's and Blau syndrome (50), both having polymorphisms within the CARD15 gene.

The gene was originally referred as the NOD2 gene and linkage studies have placed it on chromosome 16; now it is known as the CARD15 gene (51). The CARD15 gene is part of the ancestral innate immune system that recognizes bacteria peptidoglycan in particular mycobacterial glycolylated form of muramyl dipeptide MDP (52–54).

CARD15, BLAU SYNDROME, AND CROHN'S DISEASE

Insights into the consequence of genetic susceptibility to MAP infection may be observed in the rare inflammatory disease, Blau syndrome. This granulomatous inflammatory disorder is characterized by uveitis, arthritis, and dermatitis (50). Although rare, Blau syndrome has been of interest in recent medical literature because of the inherited or *de novo* mutation within the CARD15 gene, the same gene associated with Crohn's susceptibility (55, 56). However, Blau syndrome susceptibility component of the CARD15 gene is located at the nucleotide binding site domain (55, 56) whereas the Crohn's susceptibility can be found at the N-terminal leucine-rich repeat domain (57–59).

Blau syndrome shares the same clinical characteristics of juvenile sarcoidosis; in fact, new CARD15 mutations are consistently found in cases of sporadic juvenile sarcoidosis – Blau syndrome (60, 61). For these reasons – the clinical appearance of sarcoidosis and a shared genetic susceptibility with Crohn's – it was proposed that MAP could have a role in Blau syndrome. A series of Blau tissues comprised of skin, synovial samples as well as Blau graulomas of the liver and kidney were tested for the presence of MAP. Six tissues of five patients representing three different families were all found to have MAP present in the tissue granulomas (25).

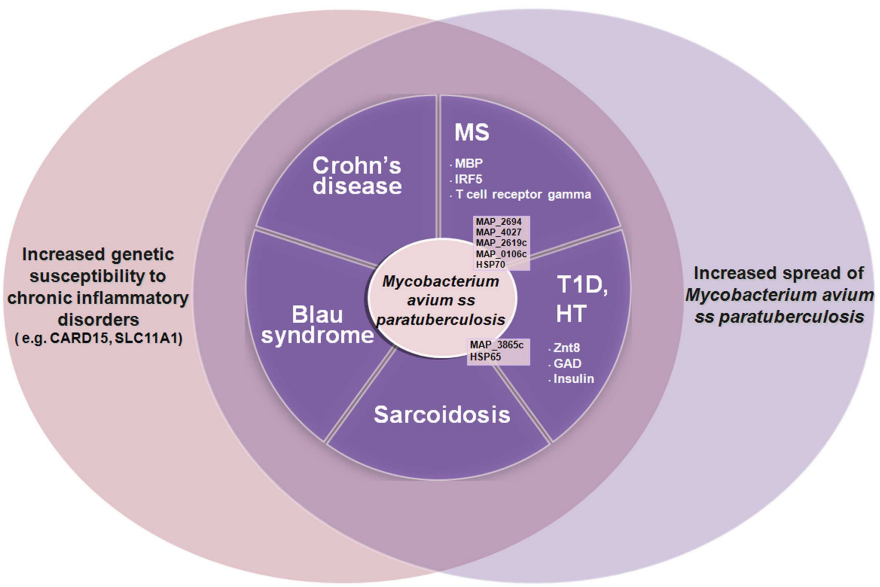

FIGURE 1 | The increased spread of *Mycobacterium avium* ss. *paratuberculosis* associated with genetic susceptibility to intracellular pathogens such as MAP (e.g., CARD15, SLC11A1) is leading to an increase of autoimmune diseases and inflammatory diseases such as type 1 diabetes (T1D), Hashimoto thyroiditis (HT), multiple sclerosis (MS), Crohn's disease, Blau syndrome, etc. Some of the MAP proteins involved are indicated (in black) with the human homologous target proteins (in white).

The proposed etiopathology is that following MAP exposure, an individual genetically susceptible with mutations within the nucleotide binding domain of CARD15 will exhibit Blau syndrome whereas if the mutations are within the leucine-rich-repeat domain of the same gene, they may exhibit Crohn's disease. Moreover, it has been reported that CARD15 defects of the leucine-rich-repeat domain, are associated in an aggressive phenotype of Crohn's disease (62). Recent work has reviewed the susceptibility genes associated with Crohn's (63).

SLC11A1

An additional gene linked with Crohn's susceptibility is the solute carrier 11a1 (SLC11A1) gene (64). SLC11A1 was previously identified as natural resistance-associated macrophage protein 1 (NRAMP1) (65). Polymorphisms within this gene and its promoter are recognized as having a role in the susceptibility of humans and animals to a number of infections, in particular mycobacterial infections, and it has been related to the susceptibility to autoimmune and inflammatory disease as well (64, 65). The SLC11A1 gene, located on chromosome 2q35, is around 14 kb in length. It encodes an integral membrane protein of 550 amino acids that is localized within the acidic endosomal and lysosomal compartment of resting macrophages (65).

The product of the SLC11A1 gene modulates the cellular environment in response to activation by intracellular pathogens by acidifying the phagosome thus killing the pathogen (66). As such, it plays a role in host innate immunity (67). Mutations of SLC11A1 may impair phagosome acidification yielding a permissive environment for the persistence of intracellular bacteria (68).

SLC11A1 IN INFECTIOUS AND AUTOIMMUNE DISEASE

Sarcoidosis, an other systemic disease associated with MAP, has been associated with polymorphisms of the SLC11A1 gene (69). Susceptibility to mycobacterial diseases, leprosy, and Buruli's ulcer were also associated with polymorphism of the SLC11A1 gene (70). Similar polymorphisms have been associated with Johne's disease (paratuberculosis) in cattle (71), goats (72), and sheep (73). A SLC11A1 defect mouse was created by researchers at the Belgium Pasteur Institute to develop a murine model for MAP infection (74).

Due to the capability of SLC11A1 to modulate innate immunity, it is not surprising that the relationship between polymorphisms in SLC11A1 and a number of mycobacterial as well as autoimmune diseases has been explored (75). In addition to leprosy (76) and tuberculosis (77), an association is found in rheumatoid arthritis (78), MS (39, 79), inflammatory bowel disease (80–82), and type 1 diabetes – all diseases associated with MAP (83, 84).

MOLECULAR MIMICRY

Molecular mimicry by a microorganisms has been hypothesized to initiate and exacerbate an autoimmune response through sequence or structural similarities with self-antigens (85, 86). Rheumatic fever is one of the best examples for molecular mimicry between group A streptococcus and host antigens leading to the glomerulonephritis and rheumatic heart disease (87, 88). The development of post-streptococcal sequelae is characterized by damage to the heart, joints, and the central nervous system (Sydenham's chorea). Damage of the heart is the most critical effect and is present in 30–45% of the cases – mostly causing damage to the heart valves.

MAP AND TYPE 1 DIABETES

Type 1 diabetes mellitus (T1DM) is an autoimmune disease manifest by progressive T cell-mediated autoimmune destruction of insulin-producing beta cells in the pancreatic islets of Langherans (89). Sechi in 2008 found the DNA of MAP in the blood of autoimmune (type 1) patients (32) but not non-autoimmune (type 2) diabetes (27, 28). Sechi also found an association of polymorphisms of the SLC11a1 gene and MAP in T1DM patients (59, 64, 82).

While it may be intuitive to envision an occult presence of MAP as an infective agent producing a granulomatous lesion of Crohn's or sarcoidosis (**Table 1A**); it may be more difficult to assign a role for MAP in T1DM. The link connecting MAP and T1DM is molecular mimicry: protein elements of the pathogen "look like" elements of the host's endocrine pancreas; and immune responses directed at the pathogen sometimes may attack the host (**Table 1B**). Childhood exposure to cows milk-based infant formula is a strong risk factor for juvenile autoimmune diabetes (30)

Table 1 | (A) Map-related granulomatous diseases. (B) Map-associated autoimmune diseases.

(A) MAP-RELATED GRANULOMATOUS DISEASES		
Disease	**Shared genetic susceptibility**	**Reference**
Crohn's	CARD15, SLC11A1	(8, 51, 52, 57, 59, 62, 64)
Sarcoidosis	SLC11A1	(54, 69)
Blau syndrome	CARD15	(52–56, 60)

These granulomatous diseases are ones where evidence of MAP can be found in the granuloma. CARD15, caspase recruitment domain gene 15; SLC11a1, solute carrier 11a1 gene.

(B) MAP-ASSOCIATED AUTOIMMUNE DISEASES		
Disease	**Mimicking elements**	**Reference**
Autoimmune diabetes	HSP65/GAD MAP3865c/ZnT8 – pancreatic	(31, 86–88, 90–94)
Autoimmune thyroiditis	MAP3865c/ZnT8 – thyroid	(35, 36)
Multiple sclerosis	HSP70, MAP_2694, MAP4027, MAP_2619c 352-61, MAP_0106c protein 121–132	(37–49)

These autoimmune diseases have autoantibodies. There are share molecular elements between MAP proteins and host organs. HSP65, heat shock protein 65; GAD, glutamic acid decarboxylase; MAP3865c, M. paratuberculosis protein 3865c; ZnT8, zinc transporter 8; HSP70, heat shock protein 70; MAP-0106c, M. paratuberculosis 0106c protein (aa. 121–132); MBP85-98, myelin basic protein (aa. 85–98).

and, as mentioned in the "exposure to MAP" section of this paper, viable MAP is found in infant formula (24).

The proposed links is the mimicry of mycobacterial protein MAP3865c and the human homolog Znt8 (31, 35) along with the heat shock protein of MAP (HSP65) and pancreatic glutamic acid decarboxylase (GAD) (30). Different islet autoantibodies (aAbs) may characterize the period preceding T1D clinical onset, aAbs against islets antigens such as insulin, glutamic acid decarboxylase (GAD65), insulinoma associated protein-2, and zinc transporter 8 (ZnT8) may be detectable for months up to years before disease onset. Sechi et al., for example, reported that anti-MAP and anti-ZnT8 antibodies (Abs) targeting homologous membrane-spanning sequences are cross-reactive and capable of eliciting strong immune responses in T1D adult patients (91). One of the sequences was also able to elicit a T cell response (95). An association between MAP and T1D in children was demonstrated by Cossu et al. (96), Additional evidence of the involvement of MAP in the early phases at T1D onset appear from two studies (91, 92) where an association between Abs positive for ZnT8 and MAP homolog epitopes in Sardinian and Italian children at T1D onset was demonstrated. Moreover, Sechi et al. (93) reported a similar high antibody response against insulin epitopes and its MAP homologous peptides in children; those both at risk for T1D and at T1D onset. A review on the topic was previously reported (94).

MAP AND AUTOIMMUNE THYROIDITIS

The most common autoimmune disease associated to T1D is autoimmune thyroid disease, its frequency is estimated at >90% among patients with T1D and autoimmune diseases (97). Different articles associate MAP to autoimmune (Hashimoto's) thyroiditis (HT) (33, 34). The same molecular mimicry principle is suggested as the link between MAP and Znt8, one of the organ-specific autoantigens of thyroiditis (33–36). Though ZnT8 is primarily expressed in pancreatic islet cells, it is also expressed in the follicular and para-follicular epithelial cells of the thyroid gland. In view of the evidence accounting for a cross-recognition of MAP3865c/ZnT8 homologs sequences in T1D subjects, and applying the theory which proposes MAP as an HT environmental trigger (acting trough a molecular mimicry mechanism) (35, 36), it is natural to consider MAP for a causal role in HT. Moreover, it has been reported that the occurrence of islet aAbs (especially Znt8) was associated with a positive titer of thyroid peroxidase antibodies (ATPO) in newly diagnosed adult-onset autoimmune diabetic patients (98).

HEAT SHOCK PROTEINS

Heat shock proteins (HSPs) are expressed at high level in response to environmental stress. They stabilize proteins and are involved in the folding of denatured proteins helping cells survive stressful conditions and promoting recovery (99). HSPs are synthesized to respond to the presence of invading pathogens. However, pathogens may also produce their HSPs. The increased expression of both self and infective stress proteins and the extensive sequence homology between microbial and human HSP (50–80% amino acid homology of mycobacterial HSP65 and human HSP60) have led to the concept that HSPs are involved in the etiology and pathogenesis of many immune-mediated disorders (100). Antibodies to mycobacterial HSPs have been found in various autoimmune diseases (101). Just to mention some, the mycobacterial 65 kDa HSP has been associated to rheumatoid arthritis (102–104), autoimmune hepatitis (105), primary biliary cirrhosis (106), and systemic sclerosis (107). HSP65 has been reported in different vasculitis-associated systemic autoimmune diseases such as Kawasaki disease (108), Behcet's disease (109) Takayasu's arteritis (110), moreover, Hsp70 has also been associated with MS (90).

MAP AND MULTIPLE SCLEROSIS

Sechi et al. have published studies implicating MAP in MS (37–39, 41–49). Molecular mimicry and SLC11A1 associations are central to this association as well (40, 41). MAP has been associated with Epstein–Barr virus (EBV – thought to be one of the triggers of MS) (44): peptides of each microorganism (MAP and EBV) cross react with anti-myelin basic protein (MBP) (43) and interferon regulatory factor 5 (IRF5) in MS patients (48). Interferon-beta therapy influence antibody response against MAP (49). An extensive review on the topic has been previously published (46).

THE FUTURE – MAP AND HUMAN DISEASE

The role of MAP in Crohn's disease has progressed from controversial to conspicuous to compelling. The century-old striking similarities existing between Johne's and Crohn's diseases on a tissue level are now validated at cellular and molecular levels (90). There is an increasing awareness and call for resolution (111, 112). Improved testing strategies for ruminant herds such as metabolomic profiling (113) will aid in the public health approach to animal disease and sources of human exposure. On a limited basis, Crohn's disease has been treated successfully with antibiotics (114, 115). As the MAP/Crohn's debate resolves and as more diseases are linked to MAP, there will likely be a major shift in the public health approach to MAP and human disease. Early indications of such a shift are two clinical trials employing anti-mycobaterial drugs: clarithromycin, rifabutin, and clofazimine. One is a 60-center trial in Crohn's disease (116) and another is the same treatment for MS (117). Positive outcomes from efforts like these – curing Crohn's disease and MS with anti-mycobacterial medication as well as prevent autoimmune diabetes and thyroiditis – will further solidify the role of MAP as a zoonotic agent in human disease and, perhaps after more than a century, will resolve this medical controversy.

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Nutritional modulation of gene expression: might this be of benefit to individuals with Crohn's disease?

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The incidence of inflammatory bowel diseases (IBD), including Crohn's disease (CD), is increasing worldwide, especially in young children and adolescents. Although hospitalized patients are usually provided with enteral or parenteral support, continuing care typically requires a trial-and-error approach to suppressing symptoms and maintaining disease remission. Current nutritional advice does not differ from general population guidelines. International collaborative studies have revealed 163 distinct genetic loci affecting susceptibility to IBD, in some of which host–microbe interactions can be seen to play an important role. The nature of these loci enables a rationale for predicting nutritional requirements that may not be evident through standard therapeutic approaches. Certain recognized nutrients, such as vitamin D and long-chain omega-3 polyunsaturated fatty acids, may be required at higher than anticipated levels. Various phytochemicals, not usually considered in the same class as classic nutrients, could play an important role. Prebiotics and probiotics may also be beneficial. Genomic approaches enable proof of principle of nutrient optimization rather than waiting for disease symptoms to appear and/or progress. We suggest a paradigm shift in diagnostic tools and nutritional therapy for CD, involving a systems biology approach for implementation.

Keywords: vitamin D, phytochemicals, probiotics, genetics, genomics, microbiota

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Introduction

Inflammatory bowel diseases (IBD) cover a spectrum of gastrointestinal disorders, including both Crohn's disease (CD) and ulcerative colitis (UC), differentiated by their location and behavior. While these diseases were seen as rare in the early twentieth century, they have become increasingly common, causing gut inflammation, ulceration, and other symptoms, in up to 1 in 250 people worldwide. For example, they had not been previously described among Bedouin Arabs in Southern Israel, but numbers are increasing now, almost certainly because of their increasingly urbanized lifestyle (1). A recent commentary described IBD in Asia as the "emergence of a Western disease" (2). While observed previously in Australasia, these diseases are becoming more common in young children and adolescents (3).

There are a number of accepted treatments for CD, aimed not only to reduce the inflammation but also to improve the long-term prognosis (4). The most desirable end point, achieved in only a relatively low proportion of cases at present, is long-term remission. One of the two approaches is used for CD treatment. "Step-up" starts with milder drugs first, while "top-down" gives people stronger

drugs earlier in the treatment process. Anti-inflammatory drugs are often the first step in the treatment. These include oral 5-aminosalicylates, such as sulfasalazine and mesalamine, or corticosteroids. However, all of these lead to undesirable side effects. Immune system suppressors also reduce inflammation by targeting the immune system. These drugs include azathioprine (Imuran), mercaptopurine (Purinethol), infliximab (Remicade), adalimumab (Humira), and certolizumab pegol (Cimzia). The latter three drugs are inhibitors of the immune system protein tumor necrosis factor-alpha (TNF-α). It is important to realize, however, that the inflammatory response is a necessary part of response to an infection or injury, and long-term suppression of this may be disadvantageous.

Not only is CD itself debilitating, it also carries with it an increased risk of colorectal cancer (CRC) (5). The nature of this cancer is fundamentally different from the classic disease, which arises from an adenomatous polyp (6). The type of CRC that develops from either form of IBD is almost inevitably lethal. However, like sporadic CRC, it arises through genomic instability.

Genomic instability refers to the accumulation of mutations during the life cycle of cells (7–9). These mutations can involve changes at the level of nucleotide base pairs, chromosome rearrangements, or aneuploidy. Genomic instability can initiate cancer, augment cancer progression, and influence the overall prognosis of the affected patient. It arises from many different pathways, including telomere damage, centrosome amplification, epigenetic modifications, and DNA damage from endogenous and exogenous sources. Protection against genomic instability, or at least reduction of the probability, is an important function of certain nutrients and phytochemicals (7).

Although nutrition is an important consideration in hospitalized patients, non-hospitalized patients are not currently being given nutritional guidelines outside those for the general population. We explore the possibility that thinking outside the current square in terms of nutrition might have significant beneficial effects beyond protection from malnutrition. That is, we suggest that nutrition could move from being an adjunct to therapy, to the point where it becomes primary therapy in its own right, integrated with currently accepted approaches (10). There is reason to suggest that this could have significant benefits in terms of delaying IBD progression and reducing the possibility of CRC formation. Such information would ideally be tailored according to individual patient genetics, and validated using systems biology approaches, as detailed in the following sections.

Diet in Non-Hospitalized CD Patients

Either prior to hospitalization or following discharge from hospital, dietary selection is determined by something of a random process (11). While the diet may be based on previous recollections of foods which affected symptoms, either in a positive or negative sense, it is often guided by advice from others with the disease. It has been clear for some time that certain foods may have adverse effects in most subjects, exacerbating disease symptoms by increasing inflammation and/or producing flatulence. In some groups, there has been endorsement of a "low FODMAP" diet, as this appears to reduce some of the symptoms in CD and other functional gastrointestinal disorders, especially irritable bowel syndrome (12, 13). FODMAP refers to the combination of saccharides and polyols and is an acronym of "Fermentable Oligo-, Di-, mono and polyols." Against the recommendation for a low-FODMAP diet, there is a concern that such a structured dietary regime will have adverse effects in reducing dietary diversity. Of particular concern is the recognition that this same group of excluded nutrients plays a major role in modulating the composition of the gut microbiome (14).

It has been recognized that there is a considerable danger of malnutrition exacerbating the already difficult symptoms of CD. Attention has been drawn to the possible need for higher than normal levels of vitamins such as vitamin D or lipids such as long-chain omega-3 polyunsaturated fatty acids (*n*-3 PUFAs) (15, 16). What has not always been recognized is the role of dietary components that are not currently included in essential lists. For example, prebiotics and probiotics may be important in regulating the gut microbiota, and their importance may be missed in some assessments (17–19). Phytochemicals are sometimes called "non-nutrients." Although these are not included in lists of essential nutrients, many of them play key roles in maintaining genomic stability and in modulating the composition of the gut microbiome (7, 20, 21). These could be key factors in helping to prevent the development of CRC in IBD.

Irrespective of nutrient composition, it has repeatedly been observed that foods considered to be beneficial for some individuals may actually cause adverse effects in others (11, 22). Individuals with the diseases differ in genetic, epigenetic, and phenotypic characteristics, so it is not surprising that a single approach is unlikely to be beneficial to all. However, studies showing the importance of genotype in the phenotypic characteristics of the diseases encourage the possibility of tailoring diets based on genetic and genomic information (23).

Genetic Basis of CD

Crohn's disease was one of the subjects of early reports on genome-wide association studies (GWAS). At that time, it appeared that there could be up to eight loci affecting disease susceptibility (24). Since that time, large international groupings have performed a meta-analysis of CD and UC GWAS scans, as part of activity of the International IBD Genetics Consortium (IIBDGC) (25). This was followed by extensive validation of significant findings, from data on a combined total of more than 75,000 cases and controls. These efforts initially revealed 163 distinct genetic loci affecting susceptibility to IBD overall (26), but this number has more recently been expanded to 201 (27). A number of these genes are involved in primary immune deficiencies, characterized by a dysfunctional immune system and increased susceptibility to serious infections. In particular, there is a considerable overlap between IBD loci and the immune-mediated disorders, ankylosing spondylitis and psoriasis (27). This study found considerable overlap between susceptibility loci for IBD and mycobacterial infection, which relationships were emphasized by further analyses using coexpression network analysis. Many of the identified loci contain genes involved in microbial handling,

and host–microbe interactions clearly play an important role in disease susceptibility (26).

In further analyses of these datasets, the IIBDGC detailed fine-mapping project, clarified the nature of these genes and their interactions, and suggested that phenotypically there should be three rather than just two main classes of IBD and that CD should be categorized into two distinct classes depending upon its location (27). Each of these classes of disease has characteristic genetic and phenotypic characteristics. The detailed nature of the genes and networks involved in disease susceptibility enables a rationale for predicting nutritional requirements that may not be evident through standard therapeutic approaches.

Possible Deviations from Conventional Nutrient Requirements in CD

There is a considerable scientific input into the setting of recommended daily nutrient intakes. While these may be good for the general population, they may often not reflect the actual requirements for CD patients. **Figure 1** illustrates the identification of specific dietary components or items that may be important for different groups of CD patients, according to their genotype and the nature of the gut microbiome. Genetic testing can indicate the primary single-nucleotide polymorphisms (SNPs) of importance at an individual level. If, for example, some of the interleukin (IL) genes are affected, these are primarily involved in inflammation (28, 29), although they may have a downstream effect on the microbiota. Thus, supplementation with long-chain *n*-3 PUFA or fish oils may be a nutritional approach to prioritize (30–32). Similarly, identification of NOD2 or ATG16L1 gene variants indicate that ability to respond appropriately to colonic bacteria is compromised (33, 34), and prebiotics and/or probiotics may provide an important starting point (14, 35–37). For all these individuals, especially those carrying a number of variant SNPs, it becomes important to protect against genomic instability since this will reflect a propensity for cancer development. In this respect, certain nutrients, especially vitamin D and phytochemicals, may play an important role (7).

While it is desirable to have studies in CD patients, in practice much of this work comes from mouse models, developed to reflect our understanding of the genetics of CD (41–44). Two of these models will be described here.

The IL-10 gene-deficient (*IL-10*$^{-/-}$) mouse model lacks a functional version of the anti-inflammatory cytokine, IL-10. *IL-10*$^{-/-}$ mice, inoculated with normal intestinal bacteria, have been used to investigate the role of various dietary components in intestinal inflammation, including mechanistic studies that consider transcriptomic, metabolomic, and proteomic effects

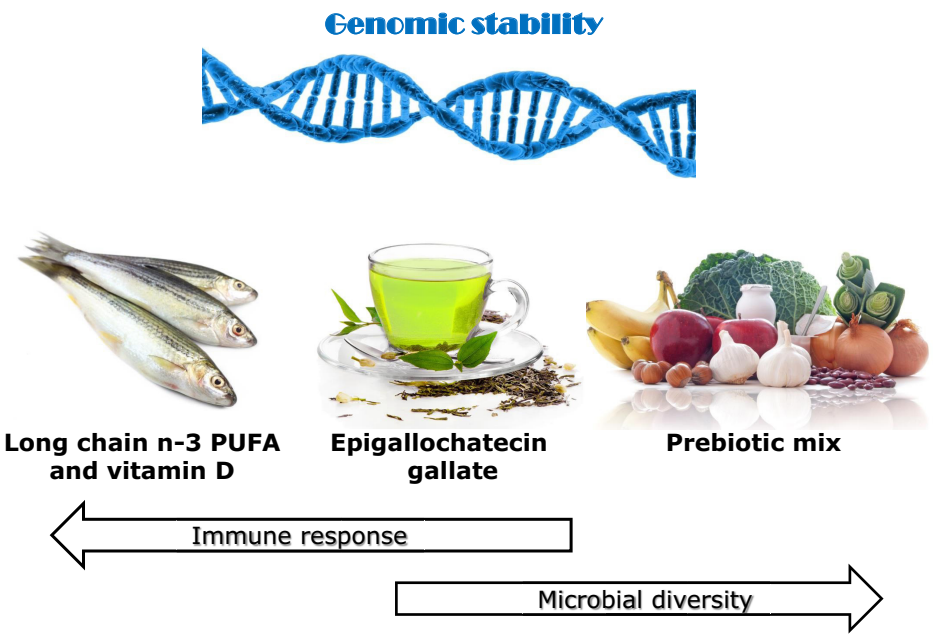


FIGURE 1 | Effects of various identified dietary components on immune response, microbial diversity, and genomic stability, with use justified by genetic testing. The use of the immunochip supplemented with fine mapping enables very detailed understanding of the nature of the genes involved in CD susceptibility of an individual. A number of the affected genes play key roles in inflammatory response. If the majority of the affected genes favor inflammation, then a primary recommendation may be to enhance the intake of long-chain *n*-3 PUFA, especially EPA and DHA. Both of these play distinctive and major controlling roles in inflammatory response (16). If genes affecting microbial response and signaling pathways predominate, then probiotics may be an especially important dietary component (35). In addition, various prebiotics such as the food combination illustrated here act to stimulate the growth of beneficial gut microbes (14, 37). Both vitamin D and various phytochemicals, including the active component in green tea, EGCG, are recognized as having roles in each of these processes and are likely to be beneficial to CD patients at higher than standard dietary recommendations (15, 38–40). Maintenance of genomic stability will play an important role in slowing progression of CD or the development of CRC (7, 21). Thus, diets containing a good balance of such components, possibly tailored according to genotype, may act to complement or slow the need for recognized therapeutics in CD. Images from Can stock photos.

(41, 45, 46). The multidrug-resistant (*mdr1a*$^{-/-}$) mouse model carries a deletion mutation enabling disease-causing microorganisms (bacteria, viruses, fungi, or parasites) to resist a range of important drugs (antibiotics, antifungal, antiviral, and antiparasitic drugs) targeted at eradicating the organism (47). *Mdr1a* knockout mice are susceptible to developing a severe spontaneous intestinal inflammation in pathogen-free animal facilities.

Micronutrients

Fenech has reviewed the role of various micronutrients in slowing the progress of genomic instability, a key component in the progression of digestive diseases and the initiation of cancer (8, 48, 49). He points to the importance of individualizing dietary components according to genotype and shows increased dietary intake of vitamin E, calcium, folate, retinol, and nicotinic acid being associated with less DNA damage and a need to define the optimal amount being especially important for riboflavin, pantothenic acid, and biotin. These three have been distinguished because of increased DNA damage being especially evident at higher doses. Fenech has described high-throughput nutrient arrays that enable defining, on an individual basis, the optimal combination of nutrients for DNA damage prevention, maintenance of telomere integrity (important in cancer risk), and cancer growth control (48).

We have more generally reviewed vitamin and mineral requirements to maintain genomic stability, especially in the context of the micronutrient genomics project (50). It is noteworthy that certain of these nutrients may be required in higher than usual amounts in CD, since they are utilized in the control of immune response and inflammation. Our own studies have particularly emphasized the importance of getting not only the correct form of selenium but also the appropriate level according to genotype (51, 52).

Vitamin D is an important vitamin that appears to be required at higher than anticipated levels in CD patients (15). This may partly be caused by genetic requirements, and it is of interest that a number of SNPs associated with vitamin D uptake and distribution actually appear on the immunochip, used in the important study of IBD risk genes by Jostins and coworkers (26). Again, there is a specific link with inflammatory processes and with control of microbiota in the identified genes. Higher plasma vitamin D levels have been associated with a reduced risk of *Clostridium difficile* (53), whereas reduced levels of circulating vitamin D enhance the risk of cancer and other inflammatory diseases (54, 55). We suggest that such an observation may add fuel to an argument that higher than current recommended daily intakes of vitamin D may be particularly appropriate to CD patients.

Dietary Lipids

Many naturally occurring agents directly bind with and activate peroxisome proliferator-activated receptor gamma (PPAR-γ or PPAR gamma), a type II nuclear receptor that in humans is encoded by the PPAR-γ gene. Agents binding this include various PUFAs including arachidonic acid and arachidonic acid metabolites. PPAR-g regulates fatty acid storage and glucose metabolism. The genes activated by PPAR-g stimulate lipid uptake by adipocytes and play an important role in regulating inflammation and cancer cell growth (46).

Peyrin-Biroulet and coworkers (56) demonstrated antimicrobial functions of the PPAR-γ gene products in maintaining epithelial expression of a type of colonic beta-defensin (mDefB10 in mice, DEFB1 in humans). In mutant mice carrying this mutation, these authors showed defective killing of a number of bacteria including *Candida albicans*, *Bacteroides fragilis*, *Enterococcus faecalis*, and *Escherichia coli*. It appears that colonic involvement in CD is linked to reduced expression of DEFB1, independent of inflammation. Thus, it has been suggested that PPAR-g-targeting by either drugs or nutrients could prevent colonic inflammation by restoring antimicrobial immunity in CD.

There have been variable results across various studies in relation to the association of common PPAR-g variants (C161T and Pro12Ala) with IBD. While Hume and coworkers reported no association in an Australian cohort (57), Shrestha et al. found suggestive relationships in Chinese but not Dutch patients (58). However, the Pro12Ala variants appeared to be protective against the development of CD in European Caucasians (57). Such studies have led support to the suggestion that there are significant ethnic differences in the phenotypic expression of this variant. There is reason to believe that diet, including lipids, may play a key role in this effect.

Conjugated linoleic acid has been shown to modulate immune responses in patients with mild to moderately active CD (59), and there is some support for this being through modulation of PPAR-g. In their Caucasian CD cohort, Ferreira and coworkers (60) found that a high intake of total, saturated, and monounsaturated fats and a higher ratio of *n*-6/*n*-3 PUFAs were associated with a more active phenotype. They studied the effects of four genetic polymorphisms, including the two PPAR-g SNPs 161C/T and Pro12Ala SNPs, in a case–control population. Although they reported no significant effects of these SNPs on disease susceptibility *per se*, they found that the presence of either of these SNPs led to a more detrimental effect of a high intake of total and trans fats.

The effects of omega-3 polyunsaturated fatty acids (*n*-3 PUFAs) are also modified by genotype, especially the nitric oxide synthase (NOS3) gene (61). These authors reported associations of plasma fatty acid composition and NOS3 SNP genotypes (rs11771443, rs1800783, rs1800779, rs1799983, rs3918227, and rs743507) in 450 individuals with the MetS from the LIPGENE dietary intervention cohort. They found that several markers of inflammation were significantly different between the genotype groups. There was a significant gene–nutrient interaction between the NOS3 rs1799983 SNP and plasma *n*-3 PUFA status on plasma triacylglycerol (TAG) concentrations. Minor allele carriers (AC + AA) were considerably more responsive to changes in plasma *n*-3 PUFA than major allele homozygotes. Such individuals are likely to benefit even more from long-chain *n*-3 PUFA consumption than the general population in order to reduce inflammation in CD (16).

Phytochemicals

Polyphenols are secondary metabolites of higher plants and one of the largest groups of natural products. Although not usually

considered in the same class as essential nutrients, various polyphenols could play an important role in CD susceptibility and progression. A range of polyphenols have been shown to modulate inflammation, especially in genetically deficient mouse models. For example, a green tea extract enriched in polyphenols was able to modulate colonic inflammation in mice lacking the multidrug resistance gene (*Mdr1a*$^{-/-}$). Mice were fed control or green tea-enriched diets for 21–24 weeks of age, after which a colonic histological injury score was obtained, colonic gene expression analyzed using microarrays, and colon protein expression also measured (20). The authors reported reduced abundance of transcripts and proteins associated with immune and inflammatory response, which suggested that its anti-inflammatory activity is mediated by multiple molecular pathways.

Cruciferous vegetables, such as cabbage and broccoli, contain two types of sulfur-containing phytochemicals – glucosinolates and *S*-methyl cysteine sulfoxide. While these chemicals have a range of effects generally considered to be beneficial in the population at large, this group of food plants also polarizes individuals with CD. Laing and coworkers associated SNPs and the beneficial or adverse effects of the 10 most commonly eaten foods in this group (22). One of the SNPs that showed exceptionally beneficial properties to individuals consuming cruciferous vegetables was in the defensin (DEFA6) gene. Conversely, one SNP strongly associated with adverse effects was in the major histocompatibility complex, which characterizes one important group of CD patients (27).

The main polyphenol in green tea is (−)-epigallocatechin gallate (EGCG). Unno and coworkers showed this compound at levels of 0.6% to have strong effects on gut microbiota and biomarkers of colonic fermentation in rats (38). They found a significant reduction of *Clostridium* spp and an increased gut occupation by *Bacteroides*. Smaller changes were seen for *Bifidobacterium* and *Prevotella*. ECGC also has effects on genomic stability, suggesting that it could be protective against cancer development in IBD (7, 21).

Ellagic acid and ellagitannins are a class of hydrolyzable tannins found in some fruits and nuts. At least in *in vitro* studies, there is good evidence that these may have the potential to reduce inflammation in genetically modified cells (62). Boysenberries, a hybrid *Rubus* berry, are among the best food sources of ellagitannins, although chestnuts and pomegranates also have high concentrations. Nasef and coworkers found effects modulated through toll-like receptors 2 and 4 of extracts from blackcurrants and feijoa, which may have been associated with elligitannins (63). The significance of these results awaits confirmation by *in vivo* testing.

Resveratrol is another polyphenol that has been shown to protect against genomic instability. It acts as an antioxidant protecting against free radical-induced DNA damage and is likely to play a protective role in gut inflammation and progression to CRC (64).

Probiotics and Prebiotics

The diversity of microbiota appears as a key characteristic that may predict response to therapy in CD (65). In addition, it has been suggested that a discriminant score of intestinal microbiota may provide an index of disease activity in CD (66). While fecal transplants have received some recognition as therapy for CD generally, these are not sustainable over a long period (67). Additionally, a number of concerns have been raised about their true efficacy (68). Regulating the gut microbiota through diet may provide a more sustainable solution. There is no question but that diet plays a major role in modulating the colonic microbiota (69). Certain nutrients, especially vitamin D, may play an important role in this respect (70).

Probiotics have been defined by FAO/WHO as "live microorganisms that confer a health benefit to the host when administered in adequate amounts." Especially in combination with prebiotics (plant substrates that enable modulation of colonic microbiota), these may be especially beneficial to CD patients (35, 71). Probiotic bacteria have been shown to produce conjugated linoleic acid in the gut, which in turn plays a role in suppressing disease symptoms through targeting PPAR-γ gamma (42). Prebiotics are digested and fermented to form short-chain fatty acids, which themselves enhance the growth of certain important colonic bacteria (17).

Additionally, an increasing number of plant polyphenols are being identified to have effects on colonic microbiota identification and regulation (64). Innovative prebiotic/probiotic foods are also being developed and may have promise in a clinical context (71).

Methylation, miRNAs, and CD

Not only genetics *per se* but also certain epigenetic events are associated with susceptibility to CD, disease progression, and CRC development.

DNA methylation has been considered for its role in the development of CD. Whole-blood DNA methylation profiles were compared in treatment-naive children with CD and healthy controls, as measured using the Illumina 450 K platform (72). Sixty-five differentially methylated CpG sites were identified as reaching epigenome-wide significance. The most significantly differentially methylated region in patients with CD involves the transcription start site for microRNA (miR)-21.

microRNAs (miRNAs) are recognized as playing an essential role in the development and control of the innate and adaptive immune system. These are small non-coding RNA molecules that lead to post-translational gene silencing and control gene expression in diverse biological processes, including inflammation. miRNA genes occur within intronic sequences of protein-coding genes, within intronic or exonic regions of non-coding RNAs, are intergenic (72). As well as miR-21, various other miRNAs including miR-192, miR-122, miR-29, and miR-146a may be implicated in CD. Krissansen and coworkers found that increased circulating levels of miR-595 and miR-1246 related to a highly aggressive form of the disease (73). Because miRNAs play a major role in regulating gene expression, they are being looked at as therapeutic targets and also as biomarkers for aggressive disease development.

Effects of Selected Nutrient Classes on Gene Expression in CD

There are no published reports of individual nutrients affecting gene expression in CD from human studies because the definitive studies would be unethical to perform. However, there are a number of studies in animal models or tissue culture systems, which provide useful information.

Micronutrients

The active binding sites of 1,25-dihydroxyvitamin D3 (1,25D) are likely to regulate gene expression at the cellular level (74). This active form of vitamin D has been shown to interact with the epigenome through effects on DNA methylation, histone acetylation and miRNA, as well influencing pre-mRNA splicing. Genomic profiling has identified a set of 1,25D regulated genes that are especially relevant to cancer prevention. For example, Ma and coworkers compared 1,25D-regulated miRNA expression profiles in a human cancer cell line in comparison with a highly tumorigenic and metastatic variant, using miRNA qPCR panels (75). The two lines showed distinctly different miRNA expression profiles that the authors suggest are likely to influence the behavior of different tumor cells and are relevant to both the susceptibility to and subsequent behavior of cancer.

Human studies on vitamin D3 supplementation have enabled dissection of high from low responders in a pre-diabetic study population (76). Only around 60% of these subjects responded to supplementation. While VDR receptor gene polymorphisms played a role in this, other clinical parameters such as the level of parathyroid hormone were also important. The authors suggested that vitamin D3-induced changes in human peripheral blood mononuclear cells can be described by transcriptomics. Added to information from other serum biomarkers, these allow the identification of those subjects who will (or will not) respond to vitamin D supplementation.

Long-Chain Omega-3 PUFA

These compounds have sometimes been described as master regulators of gene expression (16). Serhan and coworkers have discussed the function of long-chain omega-3 PUFA as novel pro-resolving mediators in the resolution of acute inflammation. They have discovered a new genus of pro-resolving lipid mediators that is temporally produced by resolving exudates that enable a return to homeostasis. These not only have anti-inflammatory actions but also enhance microbial clearance. Such properties make the two long-chain omega-3 PUFAs, EPA and DHA, essential nutrients not only for protection against CD in the first place but also for reducing inflammation and thereby reducing disease progression.

Knoch and coworkers compared the effects of EPA supplementation with that of oleic acid (OA) as control in the *IL-10*$^{-/-}$ mouse model (77). They found that EPA reversed the decrease in colon fatty acid beta-oxidation gene expression observed in OA-fed mice. The mice fed with the OA diet showed a decrease in the expression of antioxidant enzyme genes, as well as those involved in detoxification, when compared with wild-type (C57Bl) mice on the same diet. In contrast, EPA up-regulated the expression of these same enzymes. The authors suggested that these results imply that EPA might have a potential anti-inflammatory effect on colon tissue. In support of this hypothesis was the observation that EPA also activated expression of the PPAR-α gene, which regulates the expression of proinflammatory and immunomodulatory genes.

Phytochemicals

Cytochrome P450s (CYPs) play an essential role in the metabolism of endogenous and exogenous molecules (78). They facilitate the biosynthesis of a number of essential molecules, including fatty acids, lipid-soluble vitamins, and steroid hormones. They also metabolize most pharmaceuticals as well as carcinogenic compounds. CYP gene expression is regulated by a number of polyphenols, such as EGCG. This molecule not only has antioxidant properties but can be pro-oxidant at very high levels, although generally beneficial in modulating the risk and progression of inflammatory diseases and cancer (79). The review by Korobkova extensively summarizes the effects of a range of polyphenols on CYP metabolism, with flow-on implications for gene expression (79). Aylissi and coworkers have also summarized the epigenetic effects of natural polyphenols, which have implications for the modulation of gene expression (80).

An extract of green tea, enriched in polyphenols (GrTP), was tested for effects on colonic inflammation in the MDRa$^{-/-}$ mouse model utilizing a transcriptomics approach (20). The histological injury score was significantly lower in the GrTP-fed mice than in the control group. Colon mRNA transcripts were assessed using microarrays. They revealed a reduced abundance of transcripts associated with immune and inflammatory response and an abundance of those associated with xenobiotic metabolism pathways, suggesting an anti-inflammatory effect mediated by diverse mechanisms. PPAR-a and signal activator of transcription 1 (STAT1) appear as two key regulatory molecules in these events.

Curcumin has been studied for effects on gene expression, using the N2A cell line (81). Treatment with this polyphenol leads to suppression of NF-kappaB and its downstream proinflammatory targets including COX-2 and iNOS. Resveratrol has also been shown to alter gene expression patterns in another cancer cell line (82). Microarray analysis showed effects on apoptosis-related genes. The green tea polyphenol, EGCG, has been used as an exemplar of a polyphenol showing effects on gene expression revealing other than antioxidant properties (39). In particular, EGCG regulates signal transduction pathways, transcription factors, DNA methylation, mitochondrial function, and autophagy, several of which effects are relevant to CD patients. ECGC has also been shown to attenuate the activation of STAT3, which again is an important pathway in CD (83). In an overview on the effects of polyphenols on gene expression, Joven and coworkers suggest that these provide an excellent illustration as to how we may be able to eat our way out of chronic diseases (84).

Dietary curcumin and rutin were compared for effects on colonic inflammation and gene expression in the MDR1$^{-/-}$ mouse model (44). Curcumin but not rutin significantly reduced the histological evidence of inflammation in this mouse model. Microarray and pathway analysis implied that the mechanism was

likely through up-regulation of xenobiotic metabolizing enzyme expression and a down-regulation of proinflammatory pathways.

Probiotics and Prebiotics

Plaza-Diaz and coworkers found evidence that a range of probiotics modulate the expression of immunity and inflammatory genes in the gut, which appear especially relevant to CD. They performed a systematic review to conclude that strains of *Bifidobacterium*, *Lactobacillus*, *Escherichia coli*, *Propionibacterium*, *Bacillus*, and *Saccharomyces* influence the gene expression of mucins, Toll-like receptors, caspases, NF-κB, and ILs, leading to an anti-inflammatory response in cultured enterocytes. Similar results were found in animal models ranging from fish to mice, rats, and piglets (85).

Prebiotics can also modulate host gene expression, with potentially beneficial flow-on effects relevant to CD (86). Sauer and coworkers studied the effects of products formed from an inulin derivative incubated with human gut flora (87). They considered the expression of 96 genes related to biotransformation using cDNA microarrays. The pattern of gene expression suggested various effects likely to protect the cells from carcinogenic compounds. Zenholm et al. used a Caco-2 gastrointestinal cancer cell model to study the effects of an oligosaccharide and a fructo-oligosaccharide in reducing the expression of IL-12p45, IL-8, and TNF-α (88).

Human Studies on the Benefits of this Combination of Nutrients on Gene Expression

Marlow and coworkers reported on gene expression effects of a specific diet containing examples of the nutrient classes under discussion (89). They developed a Mediterranean-inspired diet whose ingredients were delivered to participants each week over a 6-week intervention period along with recipes and tips for maintaining a healthy diet, and participants were also contacted for encouragement on a regular basis. The diet did not contain the amounts of whole grains and diary products common in the Mediterranean but had a seasonal range of fruits and vegetables (various vitamins and polyphenols), two meals of oily fish per week (omega-3 and vitamin D), green tea (EGCG), and probiotic capsules. Marlow and coworkers considered the effects on symptoms, biomarkers of inflammation, and the gut microbiota as well as classic measures of inflammation such as C-reactive protein (CRP), and they also used a transcriptomic end point. They found that adherence to the diet for 6 weeks reduced the established biomarkers of inflammation. However, transcriptomic analysis provided an important biomarker, showing that the expression of certain genes important in the etiology of CD was beneficially modulated. The diet also resulted in a trend of normalizing the microbiota.

Systems Biology Approaches to Validate the Efficacy of Dietary Approaches to Disease Control

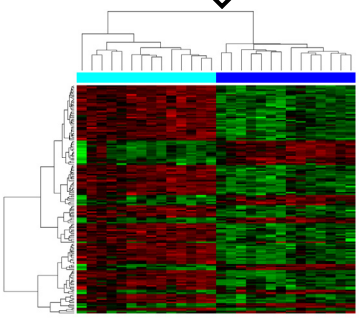
In previous sections, we have identified a number of nutrients that are especially important for CD development and progression. Other nutrients will undoubtedly play an additive role. Systems biology links the interactions among genes, gene products, and environmental factors. As Polytarchou et al. have suggested, there is reason to suggest that this is ready for prime time in CD research (90). It is being increasingly used to refine desirable nutrition for an individual and treat complex human diseases, such as CD. **Figure 2** illustrates the way in which these technologies have the potential to revolutionize conventional CD diagnosis and treatment by providing a strong scientific basis for nutritional therapy. As indicated in previous sections and **Figure 1**, genetic screening will inform the desirability of certain foods or nutrients that will become part of the regular diet (26, 27). The benefit of such a combination of foods may be tested, at an individuals or group level, using transcriptomic approaches (89), while the long-term benefits in protection against disease progression may be interrogated using proteomic and/or metabolomic approaches (91, 92). Changes in the gut microbiota will also be important (93).

Fiocchi has identified four components of IBD pathogenesis as environment, genetics, gut microbiota, and mucosal immune response, which can be defined by the terms "expososome," "genome," "microbiome," and "immunome" (94). None of these act independently, but interact (the "interactome"), resulting in the emergence of IBD. Polytarchou and coworkers (90) have stressed the way in which novel computational methodologies can now integrate high-throughput molecular data. They suggest that a systems biology approach could identify the central regulators in the IBD interactome. This work suggests that identification of key nutrients and their interactions with these central networks, possibly at an individual level, might lead to novel therapeutic approaches to CD.

Metabolomic approaches may provide important tools for understanding the differences between individuals in response to dietary components. For example, Lin and coworkers used a mouse model to investigate the effects of feeding kiwifruit-derived extracts from two different species (96). Not only did they find differences in anti-inflammatory activity between the green (*Actinidia deliciosa*) and gold-fleshed (*Actinidia chinensis*) kiwifruit extracts, but the efficacy of these extracts was modulated by the variant IL-10 genotype. This same mouse model and experimental approach revealed gradual changes in the metabolome as disease developed, particularly decreased levels of very low-density lipoprotein and increased low-density and very low-density lipoproteins and various PUFAs (97). The metabolome also interrelates with the microbiome, suggesting the possibility of utilizing targeted metabolomics for monitoring the consequences of therapeutically manipulating the microbial community as adjunct therapy in CD (98).

Much of the proof of concept of beneficial effects of certain diets in CD patients has been based on animal or tissue culture studies, rather than humans, since there are obvious ethical constraints in working with people. The classic biomarker of inflammation is considered to be CRP, while fecal calprotectin has gained considerable ground. However, as illustrated in the study by Marlow and coworkers, transcriptomics provides a more sensitive and more informative biomarker of beneficial effects of a selected diet in CD than CRP (89).

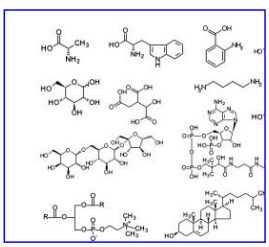
Genetics-enabled food selection

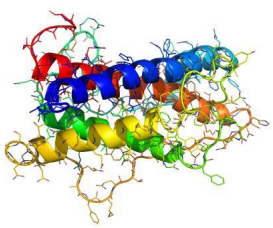


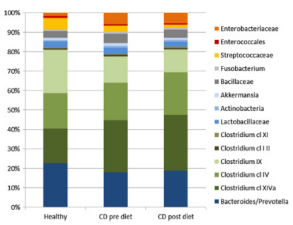
Transcriptomics-validated diet

Biomarkers of human response

Biomarkers of microbial response







Metabolomics Proteomics Metabonomics

FIGURE 2 | Flowchart showing how genetic and genomic technologies may inform optimal nutritional modulation of CD. Genetic characterization of human DNA and the nature of the associated gut microbiome enable selection of an individualized diet for CD patients. While long-chain omega-3 PUFA containing fish or dietary supplements may be appropriate for most individuals, the exact nature of the most desirable fruits, vegetables, and supplements will vary. Although we are now in a position to make informed guesses, the proof of efficacy of the dietary choice would and probably should be informed by genomic approaches. In particular, there is reason to believe that the inflammation associated with CD will itself consume the various nutrients at higher than average levels. These techniques are now sufficiently sensitive to allow testing the effect of the selected dietary strategy following short-term intervention studies. Blood, urine, and feces samples would be required, before and after the study period. Changes in gene expression as monitored by transcriptomic profiles from peripheral blood mononuclear cells show which CD-related genes have modulated activity (89). Urine or fecal samples can be used for metabolomic and proteomic techniques (91, 94). These may be utilized to monitor dietary compliance and also to provide biomarkers relevant to CD progression. The microbiota profile can be estimated from stool samples, and modulation of microbiota will provide important complementary information on whether the dietary selection has the desired effect on slowing disease progression (67, 89, 95). Images from Adobe stockimages and from Ref. (89).

Research into gastrointestinal cancer has highlighted the importance of biomarkers, especially miRNAs, usually taken from colonic endoscopies. There is considerable interest in the discovery that miRNAs are present in serum in a cell-free state, highlighting the possibility of their potential use as non-invasive biomarkers (99). At this time, extracellular miRNAs have been identified in most biological fluids, including serum, urine, saliva, and breast milk. They are being considered as novel biomarkers, with considerable potential for predicting disease course and response to therapy.

Metabolites reflect the physiological phenotype, providing a molecular readout of the cell status (95). Such measures, taken from various biological fluids, can lead to the identification of "marker metabolites" that differentiate health from disease. In particular, metabonomics (spectroscopy-based metabolic profiling) of fecal extracts has been used to differentiate active from inactive CD. This method comes across as a powerful non-invasive diagnostic tool able to characterize changes in the metabolic profile associated with malabsorption and dysbiosis (100). Specific metabolites that distinguished groups with active from those with inactive disease included N-acetylated compounds and phenylalanine, low-density lipoproteins, and very low-density lipoproteins (101). More generally, metabolomics is being used in human studies to monitor compliance with required dietary changes and also efficacy of the change in slowing disease progression (102).

Proteomics is defined as the large-scale study of proteins, particularly their structures and functions (103). This technique has been used to validate conclusions from other technologies in a series of mouse studies by Barnett and coworkers (20, 46, 104). In studies on green tea polyphenols, proteomic analysis supported the conclusions from microarrays regarding the mechanism of action of the polyphenol-enriched extract (20). Application of the same technology to studies of *n*-3 and *n*-6 PUFAs (arachidonic acid and EPA) showed that the proteomic analysis identified some actions that had not been apparent from transcriptomic studies (46). These authors emphasized the complementary nature of these two approaches and the importance of using the two in concert to better understand the nature of complex diseases such as CD.

The accurate diagnosis of CD has often lagged behind disease presentation. Proteomics is being increasingly used for early and accurate diagnosis of CD as well as for monitoring the course of the disease (92). By providing information on the nature of the disease process, such technology may inform appropriate treatment regimes. Although intestinal tissue will provide the most information, blood, urine, and stool are increasingly providing highly predictive substitutes that are considerably less invasive than the requirement for a colonoscopy.

The toolbox for studying microbiota has become more powerful in recent years, with metagenomics reflecting the study of DNA, metatranscriptomics the total transcribed RNA, and metaproteomics the protein associated with the microbiota. Draft genome sequences have been derived for more than 1,000 human-associated microorganisms derived from the gastrointestinal tract of more than 100 humans (70). Bioinformatics provides an essential tool to tie together these multi-omic analyses of IBD (105).

Conclusion

There is a great deal written about desirable intakes for various classes of food and nutrients. However, the genetic background of CD would appear to make certain nutrients more important than for the general population, albeit differing somewhat according to the genetics of the individual patient. Such nutrients will be especially important during a flare-up of the disease, but will also be crucial in slowing disease progression. Proof of principle of these assertions has been largely dependent on tissue culture or animal models to date. However, improvements in sensitivity of various technologies, especially transcriptomics, proteomics, and metabolomics, enable non-invasive sampling of humans from blood, urine, and/or stool samples, after relatively short-term human intervention studies. We suggest that there is justification for a paradigm shift in diagnostic tools and nutritional therapy for CD, involving a systems biology approach for implementation (106).

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