INTESTINAL HOMEOSTASIS AND DISEASE: A COMPLEX PARTNERSHIP BETWEEN IMMUNE CELLS, NON-IMMUNE CELLS AND THE MICROBIOME

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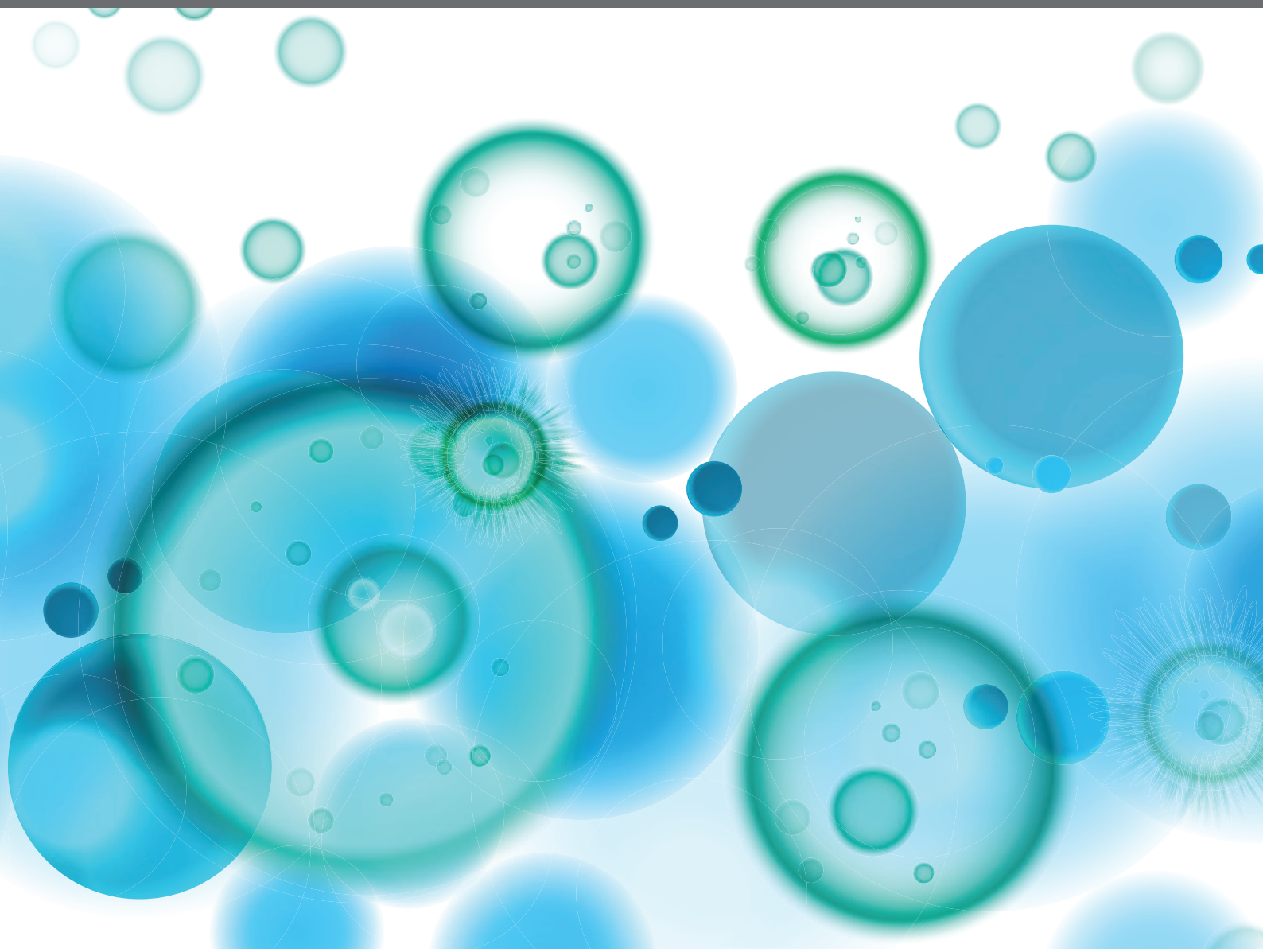

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INTESTINAL HOMEOSTASIS AND DISEASE: A COMPLEX PARTNERSHIP BETWEEN IMMUNE CELLS, NON-IMMUNE CELLS AND THE MICROBIOME

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EDITORIAL
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Editorial: Intestinal Homeostasis and Disease: A Complex Partnership Between Immune Cells, Non-Immune Cells, and the Microbiome

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Keywords: microbiome, gut microbiota, mucosal immunology, intestinal immunity, intestinal homeostasis, inflammatory bowel diseases, Crohn's diseases, ulcerative colitis

Editorial on the Research Topic

Intestinal Homeostasis and Disease: A Complex Partnership Between Immune Cells, Non-Immune Cells, and the Microbiome

INTRODUCTION

Epithelial cells, stromal cells, immune cells, and microbiota are fundamental components of the intestine, and all are importantin maintaining a healthy gut (1).

Risk factors that predispose an individual to loss of gut homeostasis include genetic variants and mutations, dysbiosis, immune dysregulation, and alterations in epithelial barrier function (2, 3). It is still unclear how these factors interrelate and lead to the development of inflammatory bowel diseases (IBD) in some individuals. Crohn's disease (CD) and ulcerative colitis (UC) are the most common types of IBD, affecting 1 in 250 individuals in the West. They are characterized by a relapsing and remitting progression of gut inflammation, which, if not treated with immune-suppression, leads to resection of inflamed tissue (4, 5).

The primary function of the gastrointestinal tract is to absorb nutrients and fluids to maintain health. Infectious agents take advantage of ingested food and water to either colonize the gut surface (e.g., *Vibrio cholera*) or invade the tissues (e.g., *Salmonella typhi*). The vulnerability of the gut is exacerbated by the fact that, in order to absorb nutrients, it is covered in a single layer of epithelial cells ∼30 μm thick. The epithelial layer is protected by mucus from goblet cells, especially in the colon, and Paneth cells, which secrete anti-microbial peptides and lysozyme.

In this special issue of Frontiers in Immunology, we have collected original works and reviews that provide new insights into the role of intestinal homeostasis and disease, with an emphasis on immune and non-immune cells and the microbiome.

A FOCUS ON EPITHELIAL CELLS AND FIBROBLASTS IN THE GUT

Curciarello et al. discuss the role of non-immune cells, such as epithelial cells and fibroblasts, in intestinal homeostasis and inflammation in the context of IBD. Epithelial cells are well-equipped to recognize infectious agents. They express Nod1 and Nod2 to recognize gram-positive and gram-negative infectious bacteria in the cytosol as well as TLRs on their apical and basolateral surfaces

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and in endosomes. Epithelial cells also express retinoic acid-inducible gene I to recognize RNA viruses. Additionally, they secrete cytokines that influence underlying cellular function, such as TGF-beta 1 and TSLP (6).

The fibroblasts that underlie the epithelium and that make up the stroma of the lamina propria maintain the structure of the lamina propria and make the matrix in which T cells, plasma cells, macrophages, and dendritic cells are embedded. In disease, however, when activated by pro-inflammatory cytokines, fibroblasts produce large amounts of interstitial collagenase and stromelysin, which degrade interstitial collagen and proteoglycans, respectively, leading to loss of epithelium and ulceration (7).

In the review by Wosen et al., emphasis is placed on how epithelial cells in the small intestine and lung mucosa constitutively express MHC class II. MHC II is not expressed in healthy colonic epithelium but is induced by pro-inflammatory cytokines in IBD. In diseases such as celiac disease, where there is very little TNF-alpha produced but a marked increase in interferon-gamma, MHC II increases on epithelial cells (it is well-known that interferon-gamma induces MHCII on non-professional antigen presenting cells). It is probable that antigen presentation mediated by MHC-II on epithelial cells is important in driving either tolerogenic or inflammatory responses.

Glal et al. show that activating transcription factor 3 (ATF3) is a key molecule needed to maintain intestinal integrity in health and inflammatory disease. ATF3-deficient mice die if given DSS-colitis, due to impaired epithelial regeneration. The authors suggest that IL-22 upregulates ATF3, leading to STAT3 phosphorylation by inhibiting phosphatase activity (SH-PTP2 and PTP-MEG2) and subsequent anti-microbial peptide production and epithelial fucosylation.

A FOCUS ON INNATE IMMUNE CELLS IN THE MUCOSA

Innate immune cells in intestinal mucosa are thought to be key modulators of tolerance and inflammatory responses. Stagg describes the heterogeneity of dendritic cells (DCs) in the gut and their role in regulatory and effector T cell-mediated responses in steady-state and inflammatory diseases. Bain and Schridde highlight the heterogeneity, ontogeny, origin, and inflammatory responses of intestinal macrophages. This is of great relevance given that, in contrast to other tissues such as the liver and brain where macrophages are yolk-sac derived and self-renewing, in the gut, for the most part, mucosal macrophages are bone marrow-derived.

Pantazi and Powell discuss the role of innate lymphoid cells (ILC) in the gut. They emphasize that Group 3 ILCs, which require RORγt for their development, are made up of diverse subpopulations. ILC3 has an important function in the response against pathogens, however dysregulated ILC3 activation may also promote inflammation, as implicated in IBD and/or colorectal cancer.

ADAPTIVE IMMUNE CELL RESPONSES IN THE MUCOSA

Cells and molecules associated with distinct effector profiles are induced in the immune response characterizing IBD (8). The evidence is now quite overwhelming that CD is caused by an excessive Th1 response to gut microbes (9). Although IL-17A is also markedly over-expressed in CD, treatment of patients with an anti-IL-17A antibody exacerbated disease (10). For UC, the situation is still unclear. It was proposed that it was driven by an unusual type of NKT cell that made IL-13, but subsequent studies have not shown elevated IL-13 in UC, and two clinical trials with anti-IL-13 antibody in UC were both negative (11). Prostaglandin E2 (PGE2) production is known to play a role in intestinal inflammation and exert both pro-inflammatory and regulatory effects (12), but the mechanisms by which it affects T cell function during colitis are not clear. In this issue, Maseda et al. evaluate the effect of autocrine vs. paracrine (non-lymphoid) secretion of PGE2 in a murine model of T cell-dependent colitis. They demonstrate that lymphocytes contribute to PGE2 production and that microsomal prostaglandin E synthase-1 (mPGES-1, which drives PGE2 production) deficient CD4+ T cells were less able to induce colitis. In contrast, the authors suggest that mPGES-1 deficiency in non-lymphoid cells impairs FoxP3+ regulatory cell development in mesenteric lymph nodes and increases total CD4+ cell infiltration into colonic lamina propria. Together, these data provide evidence that T cell PGE2 signaling is required to restrain colitis.

Sorini et al. discuss the role of commensal bacteria-specific CD4+ T cells, highlighting the factors involved in the generation of these cells and their contribution to intestinal immune homeostasis and disease. Actually, the microbiota has been shown to promote pathogenic T cell expansion in IBD patients. In addition, the authors discuss in overview the most commonly used experimental model to study the induction of commensal bacteria-specific CD4+ T cells in mice, including gnotobiotic mice, soluble peptide-MHC tetramers, and TCR transgenic mice.

Coronado et al. present a novel model of diet-induced gut inflammation in zebrafish larvae, identifying changes in immune cell populations using a fluorescent reporter for macrophages, neutrophils, and T cells. They found that myelocytes (young granulocytes normally found in hematopoietic tissues) and T cells orchestrate gut inflammation in response to dietary proteins.

Tregs have been identified as a significant immunosuppressive population critically involved in maintaining intestinal homeostasis (13). Butera et al. studied the frequency and contribution of different Treg cells (CD3+CD4+Foxp3+ and CD3+CD4+LAP+Foxp3–) to the extent of disease in UC patients and a colitis mouse model. The authors concluded that CD3+CD4+LAP+ Tregs are responsible for limiting the extension of inflammatory lesions in UC.

Intraepithelial lymphocytes (IELs) play a key role in maintaining intestinal immune homeostasis (14). Zhang et al. describe original research on the role of Fas-associated protein with death domain (FADD) in IEL development. Dominant-negative FADD (FADD-DN) mutant mice have a

selective deficiency of CD8αα+ TCRγδ+ cell IELs. Moreover, loss of γδ+ IELs in the gut of FADD-DN mice aggravates DSS-induced colitis, highlighting their important role in intestinal homeostasis.

Pararasa et al. studied memory B cells in blood and tissue samples of IBD patients and controls. The authors show a reduction in CD27-IgD- memory B cells in the blood of patients, while at the same time observing an increased frequency of this cell population in their mucosa.

THE ROLE OF MICROBIOTA AND DIETARY COMPONENTS IN INTESTINAL HOMEOSTASIS AND IMMUNE RESPONSE

The colon is colonized by more than 1×10^{13} bacterial cells, mostly of the phyla firmicutes and bacteroidetes (15). The microbiome shows major gradient differences along two axes, namely the mucosa-to-luminal axis and the longitudinal proximal intestine-to-distal intestine, with individuals showing substantial variations (15, 16).

Parada Venegas et al. discuss the production of short-chain fatty acids (SCFAs) by members of the commensal microbiota, their impact on epithelial and immune cells belonging to the innate and adaptive arms of the immune system, and their potential use as part of either pre- or probiotic therapies for attenuating gut inflammation.

Many factors have been shown to intervene with the gut microbiome, including age, genetics, diet, and drugs (15). The article by Siracusa et al. discusses the effect of the western diet (high intake of lipids, cholesterol, and salt) on the intestinal immune system, gut microbiota, and CD4+ T-associated cell differentiation into different effector sub-types. They suggest that a western diet increases the risk of intestinal and extra-intestinal inflammation and summarize the possible therapeutic effects of immune modulation using dietary supplementation with fiber, indoles, and vitamins.

Sugihara et al. summarize the role of different dietary nutrients in the maintenance or perturbation of immune intestinal homeostasis and highlight the relationship between nutrition and gut immune responses, including Th1/Th2/Th17/Treg cells, macrophages, and the microbiota.

EVOLUTIONARY BIOLOGY AND THE DEVELOPMENT OF IBD AND CANCER

Interdisciplinary research can greatly assist in understanding disease processes. Al Bakir et al. discuss the mechanisms underlying the connections between IBD and colon cancer risk from a mathematical/evolutionary perspective. They discuss the great opportunity to follow cancer evolution using genomic analyses over time of spatially separated colonic biopsies, from pre-malignancy to carcinoma, which show the key factors in the development of colorectal cancer (CRC). Clonal diversity and evolution could be useful for predicting progression risk, which will hopefully stimulate new thinking leading to novel early diagnosis and prevention strategies for IBD-associated CRC.

PERSPECTIVES

Immune responses in lymph nodes and the spleen occur in a controlled plasma-rich environment where the levels of nutrients, hormones, and cytokines are strictly controlled. In the gut, by virtue of the special needs of the tissue, immune responses take place in an environment rich in exogenous immunomodulating molecules. The list is long, but in this issue, we have highlighted the role of diet, the microbiota, short-chain fatty acids, prostaglandins, aryl hydrocarbon receptor ligands, and vitamins. In this relatively brief series of articles, it was not possible to capture the full breadth of activity in mucosal immunology and inflammation in humans, but there is no doubt that this is one of the most exciting areas of immunology, with many surprises ahead.

AUTHOR CONTRIBUTIONS

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

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REFERENCES

1. Turner JR. Intestinal mucosal barrier function in health and disease. *Nat Rev Immunol.* (2009) 9:799–809. doi: 10.1038/nri2653
2. Abraham C, Cho JH. Inflammatory bowel disease. *N Engl J Med.* (2009) 361:2066–78. doi: 10.1056/NEJMra0804647
3. Kaser A, Zeissig S, Blumberg RS. Inflammatory bowel disease. *Annu Rev Immunol.* (2010) 28:573–621. doi: 10.1146/annurev-immunol-030409-101225
4. Ramos GP, Papadakis KA. Mechanisms of disease: inflammatory bowel diseases. *Mayo Clin Proc.* (2019) 94:155–65. doi: 10.1016/j.mayocp.2018.09.013
5. Sica GS, Biancone L. Surgery for inflammatory bowel disease in the era of laparoscopy. *World J Gastroenterol.* (2013) 19:2445–8. doi: 10.3748/wjg.v19.i16.2445
6. Okumura R, Takeda K. Roles of intestinal epithelial cells in the maintenance of gut homeostasis. *Exp Mol Med.* (2017) 49:e338. doi: 10.1038/emm.2017.20
7. Valatas V, Filidou E, Drygiannakis I, Kolios G. Stromal and immune cells in gut fibrosis: the myofibroblast and the scarface. *Ann Gastroenterol.* (2017) 30:393–404. doi: 10.20524/aog.2017.0146
8. Maloy KJ, Powrie F. Intestinal homeostasis and its breakdown in inflammatory bowel disease. *Nature.* (2011) 474:298–306. doi: 10.1038/nature10208

9. Strober W, Fuss IJ. Proinflammatory cytokines in the pathogenesis of inflammatory bowel diseases. *Gastroenterology*. (2011) 140:1756–67. doi: 10.1053/j.gastro.2011.02.016
10. Hueber W, Sands BE, Lewitzky S, Vandemeulebroecke M, Reinisch W, Higgins PDR, et al. Secukinumab, a human anti-IL-17A monoclonal antibody, for moderate to severe Crohn's disease: unexpected results of a randomised, double-blind placebo-controlled trial. *Gut*. (2012) 61:1693–700. doi: 10.1136/gutjnl-2011-301668
11. Giuffrida P, Caprioli F, Facciotti F, Di Sabatino A. The role of interleukin-13 in chronic inflammatory intestinal disorders. *Autoimmun Rev*. (2019) 18:549–55. doi: 10.1016/j.autrev.2019.03.012
12. Nakanishi M, Rosenberg DW. Multifaceted roles of PGE2 in inflammation and cancer. *Semin Immunopathol*. (2013) 35:123–37. doi: 10.1007/s00281-012-0342-8
13. Zhou L, Sonnenberg GF. Essential immunologic orchestrators of intestinal homeostasis. *Sci Immunol*. (2018) 3:1–18. doi: 10.1126/sciimmunol.aao1605
14. Olivares-Villagómez D, Van Kaer L. Intestinal intraepithelial lymphocytes: sentinels of the mucosal barrier. *Trends Immunol*. (2018) 39:264–75. doi: 10.1016/j.it.2017.11.003
15. Maukonen J, Saarela M. Human gut microbiota: does diet matter? *Proc Nutr Soc*. (2015) 74:23–36. doi: 10.1017/s0029665114000688
16. Bäckhed F, Fraser CM, Ringel Y, Sanders ME, Sartor RB, Sherman PM, et al. Defining a healthy human gut microbiome: current concepts, future directions, and clinical applications. *Cell Host Microbe*. (2012) 12:611–22. doi: 10.1016/j.chom.2012.10.012

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ORIGINAL RESEARCH
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Dominant Negative FADD/MORT1 Inhibits the Development of Intestinal Intraepithelial Lymphocytes With a Marked Defect on CD8αα+TCRγδ+ T Cells

Xuerui Zhang[1,2†], Lina Huo[1†], Lulu Song[1], Zhaoqing Hu[1], Xinran Wang[1], Yuheng Han[1], Ying Wang[1,2], Peipei Xu[3], Jing Zhang[1,2] and Zi-Chun Hua[1,2,4*]*

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Intestinal intraepithelial lymphocytes (IELs) play a critical role in mucosal immune system, which differ from thymus-derived cells and develop locally in gut. Although the development of IELs has been studied in some detail, the molecular cues controlling their local development remain unclear. Here, we demonstrate that FADD, a classic adaptor protein required for death-receptor-induced apoptosis, is a critical regulator of the intestinal IEL development. The mice with a dominant negative mutant of FADD (FADD-DN) display an abnormal development of intestinal IELs with a marked reduction in the numbers of $CD8\alpha\alpha^{+}TCR\gamma\delta^{+}$ T cells. As a precursor for $CD8\alpha\alpha^{+}$ development, lamina propria lymphocytes in lin-negative expression (lin^{-} LPLs) were analyzed and the massive accumulation of $IL\text{-}7R^{-}lin^{-}$ LPLs was observed in FADD-DN mice. As IL-7R is one of Notch1-target genes, we further observed that the level of Notch1 expression was lower in Lin^{-} LPLs from FADD-DN mice compared with normal mice. The downregulation of Notch1 expression induced by FADD-DN overexpression was also confirmed in Jurkat T cells. Considering that IL-7 and its receptor IL7-R play a differentiation inducing role in the development of intestinal IELs, the influence of FADD via its DD domain on Notch1 expression might be a possible molecular signal involved in the early IELs development. In addition, loss of γδ T-IELs in FADD-DN mice aggravates DSS-induced colitis, suggesting that FADD is a relevant contribution to the field of mucosal immunology and intestinal homeostasis.

Keywords: FADD, mucosal immune system, γδ+ T-IELs, LPLs, DSS-induced colitis

INTRODUCTION

Intraepithelial lymphocytes (IELs), which are integral to the intestinal mucosal associated lymphoid system, play a key role in maintaining immune homeostasis of intestine. They constitute a constellation of barrier immune cells and contribute to the intestinal function by developing tolerance to food and microbial antigens in normal physiological state and controlling insults from pathogens and deleterious tissue inflammation during mucosal infections (1, 2). Studies conducted

to date have revealed that these T cells consist of two main subpopulations. One bears either CD4 or CD8αβ molecules and TCRαβ, mainly as thymus-dependent. The other bears CD8αα molecules and either TCRγδ or TCRαβ, which is also present in the athymic mice. These thymus-independent IELs like CD8αα$^+$ T cells gather mainly in the intestinal mucosa and develop locally (3, 4). The local development tends to facilitate the production of TCRγδ$^+$ T cells while it reduces the induction of TCRαβ rearrangements or pre-Tα chain expression. The pre-Tα chain as an essential component of the pre-TCR is responsible for the predominance of TCRαβ production by the thymus (5). Intestinal CD8 T-IELs have been proposed to develop locally from cryptopatch (CP) precursors, whereas the regulatory stages between CP and mature T-IELs remain unclear. There are striking differences in T cell differentiation process in the gut, when compared with T cell differentiation in the thymus, but far less is known about the molecules and signaling pathways that regulate the differentiation.

Fas-associated protein with death domain (FADD) is an adaptor protein critical for the death receptors (DRs) apoptotic signaling (6, 7). When extrinsic apoptosis is triggered, FADD interacts with death receptor (DR) like Fas, leading to the recruitment of procaspase-8 for its activation and then the consequent apoptosis (8–12). Besides being a main death adaptor molecule, FADD participates in other biological processes, such as embryogenesis (9), innate immunity (13), T cell activation and proliferation (14). FADD deficiency leads to inhibition of thymocyte development in a variety of transgenic mouse models including FADD$^{-/-}$ chimeras mice (FADD$^{-/-}\rightarrow$ RAG-1$^{-/-}$) and T-cell-specific FADD knockout mice (lck-cre FADD) (8, 15–20). In transgenic mice expressing a dominant negative FADD mutant (FADD-DN) under control of the lck promoter, there is also a defect in the progression of thymocytes from CD25$^+$CD44$^-$ to CD25$^-$CD44$^-$ phenotype, which is associated with pre-TCR expression. Several studies on FADD-DN transgenic mice have demonstrated its suppression role on T-cell proliferation and supported an acknowledgement of the functional FADD signaling essential for normal T cell development and T cell activation (21–23). But all these studies only focused on TCRαβ$^+$ T cells from thymus dependent, this is a meaningful question whether FADD also affects the development of TCRγδ$^+$ T cell, such as IELs which are thymus-independent.

Here we identify a novel role for FADD in the intestinal immune system. In FADD-DN transgenic mice, a significant decreased subset of CD8$^+$TCRγδ$^+$ T cell is observed in intestinal IELs. We provide evidences that FADD-DN expression inhibits the development of CD8$^+$TCRγδ$^+$ T through impeding the IL-7R expression in their precursors. Loss of CD8$^+$TCRγδ$^+$ T means an impaired intestinal immunologic barrier, so FADD-DN mice develop more severe inflammation in DSS-induced colitis.

RESULTS

Lack of γδ T Cells in FADD-DN Transgenic Mice

To test the role of FADD in the development of murine intestinal IELs, the transgenic mice expressing a dominant negative mutant of FADD/MORT1 (FADD-DN) under control of the mouse lck proximal promoter were used in the study. FADD-DN lacks the death effector domain (DED) which is required for recruiting and activating caspase-8 during apoptosis (**Figure 1A**). A 16 kDa FADD-DN protein in the T-cell specific expression was detected by western blotting (**Figure 1B**).

Murine intestinal T-IELs are developed by thymic and extrathymic pathways. About half of intestinal T-IELs derives from peripheral lymphoid tissues to the intestine (TCRαβ$^+$), and the other half differs from peripheral T cells, mostly expressing TCRγδ$^+$ (24). These γδ T-IELs mainly develop locally in the intestinal mucosa. Analysis of intestinal IELs in total number showed no obvious differences in matched wild type (WT) control and FADD-DN mice (**Figure 1C**). When the subsets of intestinal IELs were examined, TCRγδ$^+$ cells were missing in FADD-DN mice (**Figures 1D,E**). Statistical analysis showed that there were significant differences in TCRγδ$^+$ cells or CD8α$^+$TCRγδ$^+$ cells between WT and FADD-DN mice, while no significant changes in TCRαβ$^+$ cells were observed. By immunofluorescence assays in histological sections of the small intestines, the decreased numbers of γδ T-IELs were easier and more direct to be observed (**Figure 1F**).

A Selective Deficiency of CD8αα$^+$TCRγδ$^+$ T Cells Caused by FADD-DN Expression

T-IELs consist mainly of two populations of CD8$^+$ T cells. One bears CD8αβ$^+$TCRαβ$^+$; the other bears homodimeric α/α CD8 chains with TCRγδ$^+$ or TCRαβ$^+$, and it is mainly thymo-independent (25). Two subgroups of CD8αα$^+$ and CD8αβ$^+$ were gated respectively for testing the proportional changes of TCRγδ$^+$ or TCRαβ$^+$. The percentage of CD8αα$^+$ IELs from FADD-DN mice was significantly reduced compared with WT mice, while no significant differences were shown in CD8αβ$^+$ IELs (**Figure 2A**). In CD8αα$^+$ subset, over 40% of CD8αα$^+$ T expressing TCRγδ were observed in WT mice, but few γδ T cells were observed in FADD-DN mice (**Figure 2B**). The population of αβ T in CD8αβ$^+$ subset showed no obvious changes in both mice (**Figure 2C**). By comparison of statistic analysis, the effect of FADD-DN was mainly observed on the depletion of CD8αα$^+$ γδ T-IELs (**Figure 2D**). The properties of the IELs exhibit age-related changes. The total number of IELs are gradually increased until 8 weeks old and the development of CD8αα$^+$ T cell subsets also tends to be stable at 8 weeks, both in the γδ and αβ lineages (26, 27)**.** So we analyzed a group of FADD-DN and littermate control mice aged 3–8 weeks old and found that CD8αα$^+$TCRγδ$^+$ cells always maintained low number along the time-span in FADD-DN mice, and a similar kinetic on CD8αα$^+$TCRαβ$^+$ cells occurred in both mice (**Figures 2E,F**). These results suggest that FADD-DN expression inhibits the development of CD8αα$^+$ γδ T cells.

The Effect of FADD-DN on T-IELs Development Is Thymo-Independent

Intestinal T-IELs may originate from both thymus and extrathymus (24). To distinguish the pathway for FADD-DN to regulate the development of γδ T cells, we examined the percentage of CD4$^-$CD8$^-$ γδ T cells in the thymus by FACS.

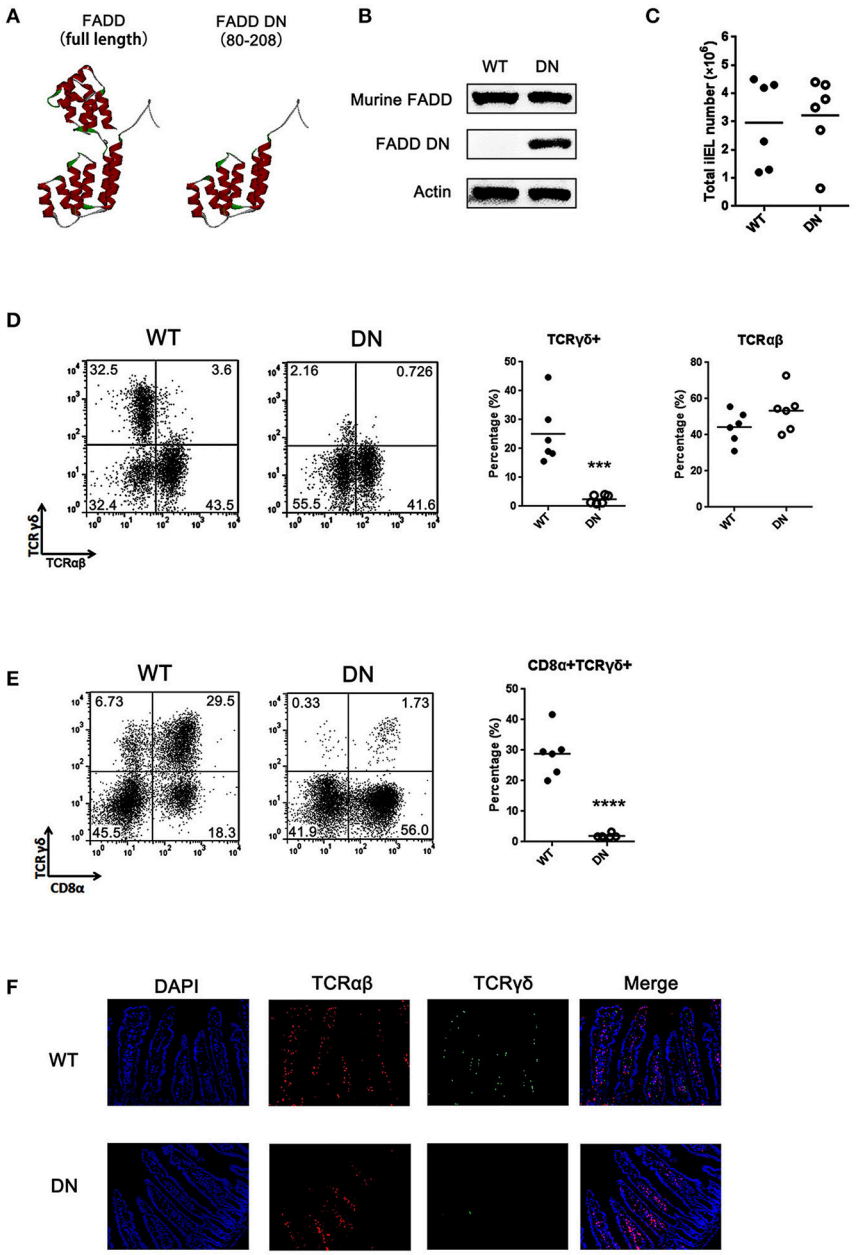

FIGURE 1 | Loss of γδ T cells in FADD-DN transgenic mice. **(A)** The three-dimensional structures of FADD [Protein Data Bank (PDB); accession number: 2GF5] and FADD-DN (death domain of FADD). **(B)** Western blot analysis of FADD expression in mesenteric lymph nodes from wild type (WT) and FADD-DN mice. **(C)** Total IEL numbers of wild type and FADD-DN mice, calculated from six mice per group. Each dot represents one mouse experimental of each group. The solid horizontal lines indicate mean value of each group. **(D)** IELs from WT and FADD-DN mice were stained by Abs to TCRαβ and TCRγδ. The percentage for γδ IELs ($TCR\gamma\delta^+$ $TCR\alpha\beta^-$) and αβ IELs ($TCR\gamma\delta^-$ $TCR\alpha\beta^+$) were displayed in the appropriate quadrant. Statistic analysis of γδ IELs and αβ IELs from indicated groups ($n = 6$ per group) on the right. Each dot represents one mouse of each experimental group. $^{***}P < 0.001$. **(E)** IELs from WT and FADD-DN mice were stained by Abs to CD8α and TCRγδ for FACS analysis. Statistic analysis of $CD8\alpha^+TCR\gamma\delta^+$ IELs from indicated groups (n = 6 per group) on the right. Each dot represents one mouse of each experimental group. $^{****}P < 0.0001$. **(F)** Immunofluorescent staining of γδ and αβ IELs in intestinal histological sections from WT and FADD-DN mice ($n > 3$ per group). Representative data from at least 3 mice per group is shown.

There were no significant differences in the thymic cellularity between FADD-DN mice and WT mice (**Figure 3A**). In the subsets of $CD4^-CD8^-$ T cells, the percentage of γδ T cells from FADD-DN mice was also similar to WT mice (**Figure 3B**), indicating that the development of γδ T cells in the thymus was not defective in FADD-DN mice.

In euthymic mice, there are some competitions for local cytokines between thymic αβ T cells and extrathymic T cell

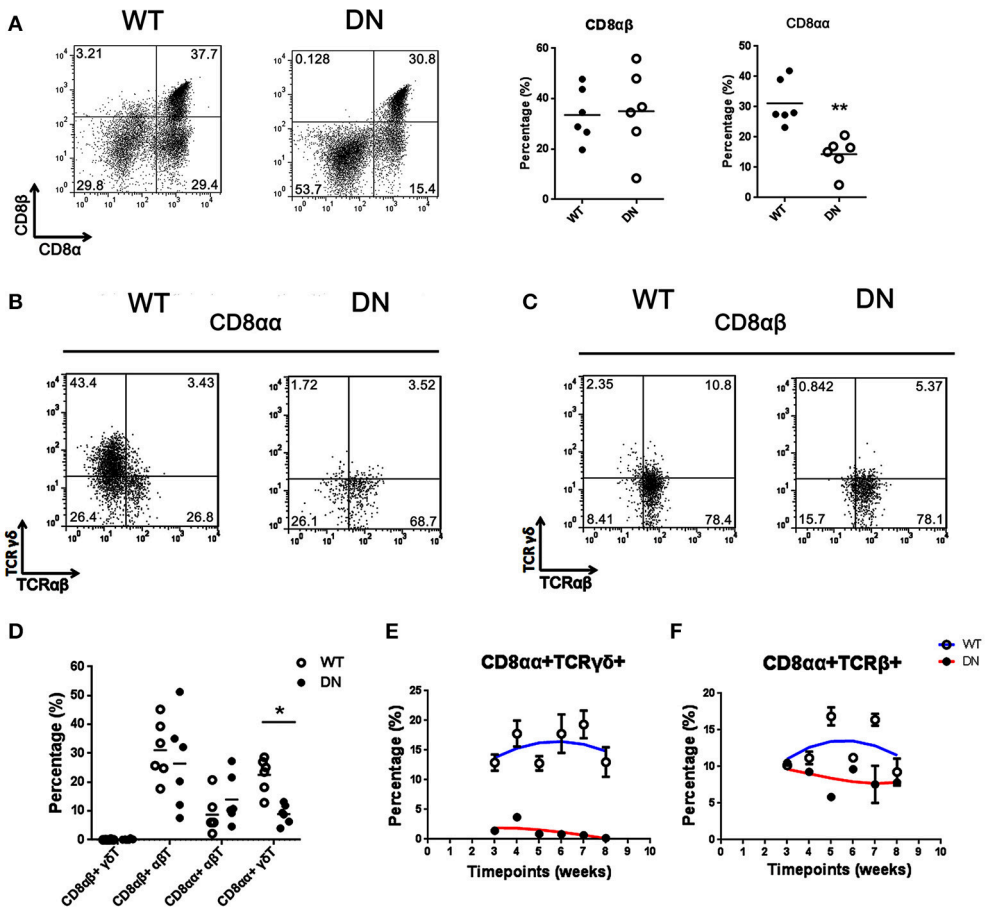

FIGURE 2 | Selective deficiency of CD8αα$^+$γδ T cells in FADD-DN mice. **(A)** Representative FACS analysis of CD8αα$^+$ (CD8α$^+$CD8β$^-$) and CD8αβ$^+$ (CD8α$^+$CD8β$^+$) populations in the IELs from WT and FADD-DN mice. Statistical analysis of CD8αα$^+$ and CD8αβ$^+$IELs from indicated groups ($n = 6$ per group) is on the right. Each dot represents one mouse of each experimental group. $^{**}P < 0.01$. **(B)** CD8αα$^+$ cells gated from **(A)** were analyzed for the expression of TCRαβ and TCRγδ, distinguished into two subsets: CD8αα$^+$ αβT (CD8αα$^+$TCRαβ$^+$TCRγδ$^-$) and CD8αα$^+$ γδT (CD8αα$^+$TCRαβ$^-$TCRγδ$^+$). **(C)** CD8αβ$^+$ cells gated from **(A)** were analyzed for the expression of TCRαβ and TCRγδ, distinguished into two subsets: CD8αβ$^+$αβT (CD8αβ$^+$TCRαβ$^+$TCRγδ$^-$) and CD8αβ$^+$ γδ T (CD8αβ$^+$TCRαβ$^-$TCRγδ$^+$). **(D)** Statistic analysis of the percentages of indicated subsets among total IELs from WT and FADD-DN mice ($n = 6$ per group). Each dot represents one mouse of each experimental group. $^*P < 0.05$. **(E)** Time-dependent changes of the percentage of CD8αα$^+$TCRγδ$^+$ subset in the IELs from WT and FADD-DN mice analyzed by FACS. Error bars reflect S.E.M. ($n = 3$ per group). **(F)** Time-dependent changes of the percentage of CD8αα$^+$TCRβ$^+$ subset in the IELs from WT and FADD-DN mice analyzed by FACS. Error bars reflect S.E.M. ($n = 3$ per group). Data was calculated from three mice per group.

progenitors, so the extrathymic development of T-IELs is severely repressed (28). In the athymic mice, the extrathymic lymphopoiesis emerges with a priority toward γδ T cells, which localizes mainly in the intestinal mucosa leading to the accumulation of T-IELs (29, 30). To confirm the effect of FADD-DN on the local development of intestinal IELs, the thymectomy was performed on both WT and FADD-DN mice. After 4 weeks, analysis of total intestinal IEL numbers in thymectomized mice showed a marked reduction (about 6-fold) in FADD-DN mice (**Figure 3C**), suggesting that there was a defect on IEL development. By comparison of the proportion of CD8α$^+$TCRγδ$^+$ T cells before or after thymectomy, more serious reductions of CD8α$^+$TCRγδ$^+$ T were observed in FADD-DN mice with thymectomy (**Figures 3D,E**). Thus, it is reasonable to speculate that the local γδ T-IELs are deficient in FADD-DN mice, and the few intestinal γδ T-IELs before thymectomy seem to mainly derive from thymus originally. Taken together, the deficiency of γδ T-IELs caused by FADD-DN might be a sign of stunting local development.

The Development of IELs Is Arrested at Stage of Lin$^-$ LPLs in FADD-DN Mice

Murine intestinal IELs originate from their own pre-existing stem cells present in the intestine (i.e., cryptopatches) (4, 31, 32) and appendix (33). Intestinal T cell precursors have specific phenotype: lineage markers negative (Lin$^-$) Thy$^+$c-kit$^+$IL-7R$^+$CD44$^+$CD25$^+$ (4). To determine at which point the differentiation of IELs was plagued by FADD-DN, we examined the Lin$^-$ cells prepared from intestinal IELs and lamina propria lymphocytes (LPLs). A mixture of mAbs was prepared to exclude

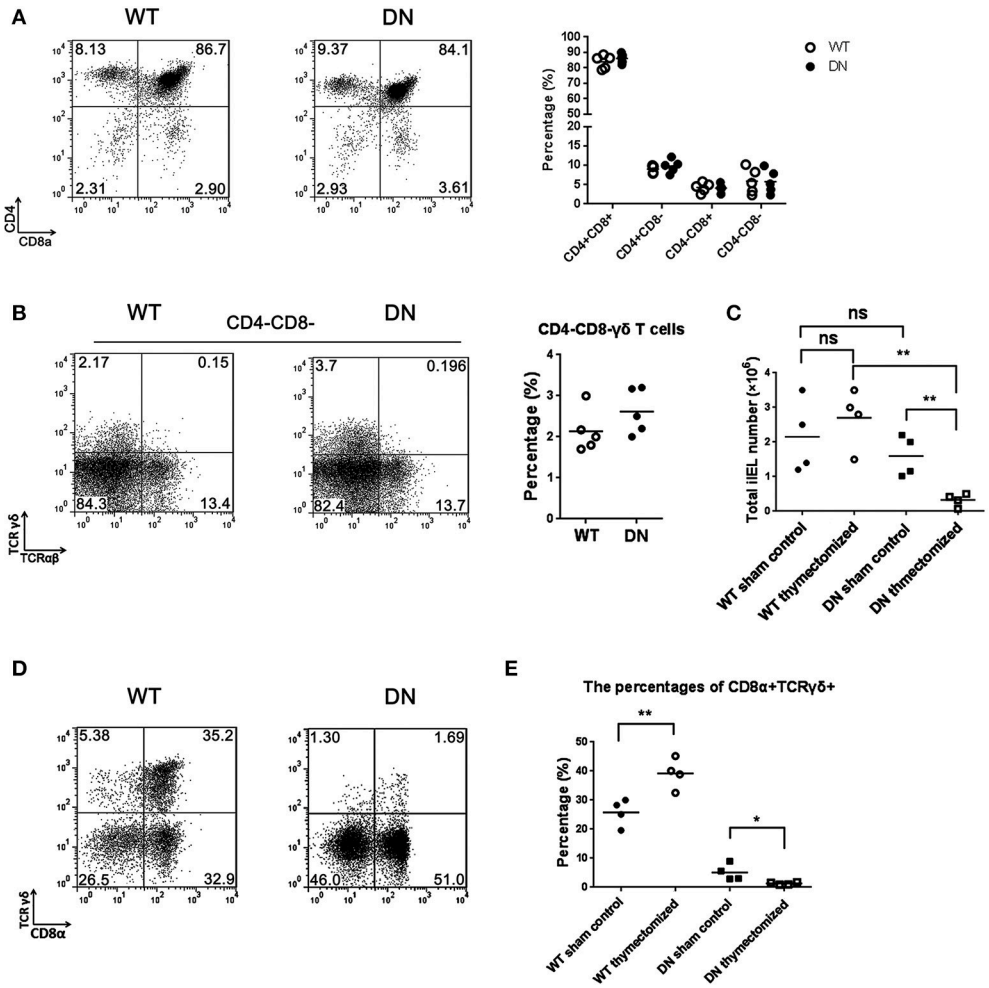

FIGURE 3 | The effect of FADD-DN on intestinal IELs development. **(A)** Representative FACS analysis of thymus from wild type and FADD-DN mice ($n = 5$ per group). Thymocytes were stained by Abs to CD4 and CD8. Statistical analysis of the subsets of $CD4^+CD8^+$, $CD4^+CD8^-$, $CD4^-CD8^+$, and $CD4^-CD8^-$ thymocytes from indicated groups is shown on the right. Each dot represents one mouse of each experimental group. **(B)** $CD4^-CD8^-$ T cells of thymus were gated from **(A)** and then analyzed for the markers of TCRγδ and TCRαβ. Representative FACS analysis on the left and statistical analysis of percentages of $CD4^-CD8^-$ γδ T cells ($CD4^-CD8^-TCR\alpha\beta^-TCR\gamma\delta^+$) on the right ($n = 5$ per group). Each dot represents one mouse of each experimental group. **(C)** Total IELs numbers of WT and FADD-DN mice before or after thymectomy. The changes of total IEL numbers between control and thymecomized mice are shown in the statistical analysis ($n = 4$ per group). Each dot represents one mouse of each experimental group. **(D)** IELs from WT and FADD-DN mice were obtained 4 weeks after thymectomy. Flow cytometry was performed to analyze the percentage of $CD8\alpha^+TCR\gamma\delta^+$ populations stained by Abs to CD8α and TCRγδ. **(E)** The percentages of $CD8\alpha^+TCR\gamma\delta^+$ subset among total IELs from indicated group($n = 4$ per group). Each dot represents one mouse of each experimental group. $^*P < 0.05$; $^{**}P < 0.01$.

mature cell types and isolate Lin^- cells (see section Materials and Methods).

By FACS analysis, there was about 25% Lin^- IELs in FADD-DN mice and about 12.5% in WT mice, and a significant difference between two groups (**Figure 4A**). Gating on Lin^- IELs for further analysis of CD8α expression, the percentage of Lin^-CD8^+ IELs was dramatically decreased in FADD-DN mice (**Figure 4B**), suggesting that the differentiation of intestinal T-IELs might be blocked in Lin^- $CD8^-$ stage. For a better understanding of intestinal T-IEL maturation in earlier events, we examined Lin^- LPLs which have been confirmed equal to precursors Lin^- CP. A massive accumulation of Lin^- LPLs was observed in FADD-DN mice, which is a clear hint on the attested stage of intestinal T-IEL development (**Figure 4C**). Our previous research found that FADD deficiency inhibits thymocyte development at the β-selection checkpoint by modulating Notch1 signaling (8). Since Notch1 is essential for T differentiation and specifying the cell fate, the expression of Notch1 in progenitor Lin^- LPLs was detected. Notably, there were two different groups of Notch1 expression: high expression as $Notch1^{high}$ and low expression as $Notch1^{low}$ in Lin^- LPLs from WT mice, but in FADD-DN mice, both groups of $Notch1^{high}$ and $Notch1^{low}$ were totally different from WT mice (**Figure 4D**). The proportion of $Notch1^{high}$ Lin^- LPLs was a noticeable decrease in FADD-DN mice. To reconfirmed the effect of FADD-DN on Notch1 expression, FADD-DN expression

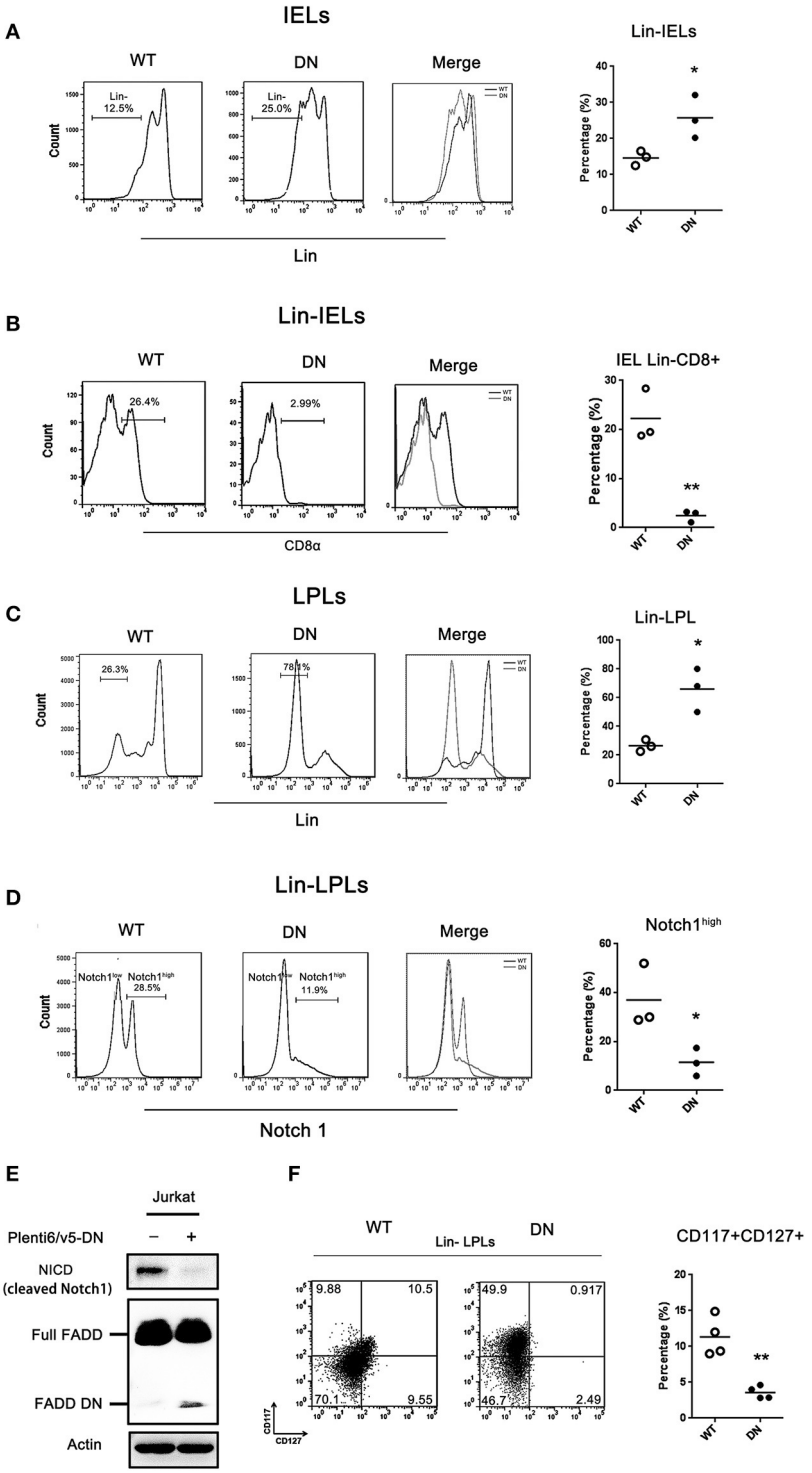

FIGURE 4 | The development of IELs arrested by FADD-DN in Lin$^-$ LPLs with IL-7R deficiency. **(A)** IELs from WT and FADD-DN mice were labeled with a mixture of mAbs recognizing mature hematopoietic cells to gate Lin$^-$ populations as described in methods. FACS plots show the gating strategy for the population of Lin$^-$ IELs. Statistic analysis of Lin$^-$ IELs from indicated groups ($n = 3$ per group) is shown on the right. Each dot represents one mouse of each experimental group. $^*P < 0.05$. **(B)** The Lin$^-$ IELs gated from **(A)** were labeled with antibody to CD8α. Histograms show the expression level of CD8α in Lin$^-$ IELs from indicated mice. Statistic analysis of CD8α$^+$ subset in Lin$^-$ IEL from indicated groups ($n = 3$ per group) is shown on the right, Each dot represents one mouse of each experimental group. $^{**}P < 0.01$. **(C)** LPLs from WT and FADD-DN mice were stained with antibodies against lineage markers (Lin).FACS plots show the gating strategy for Lin$^-$ LPL populations. Statistic analysis for Lin$^-$ LPLs on the right ($n = 3$ per group). Each dot represents one mouse of each experimental group. $^*P < 0.05$. **(D)** The Lin$^-$ LPLs

(Continued)

FIGURE 4 | gated from **(C)** were labeled with antibody to Notch1. Histograms show two levels of Notch1 expression in Lin^- LPLs indicated as $Notch1^{high}$ and $Notch1^{low}$.Statistic analysis for $Notch1^{high}$ Lin^- LPLs on the right ($n = 3$ per group). Each dot represents one mouse of each experimental group. $^*P < 0.05$. **(E)** Jurkat T cell were transiently transfected with FADD-DN expression vector. The level of NICD (cleaved Notch1) was detected by western blot. **(F)**The Lin^- LPLs gated from **(C)** were labeled with anti-CD117and anti-CD127 Abs. Note: CD117 as c-kit and CD127 as IL-7R. Statistical analysis of the percentages of $CD127^+$ LPLs on the right ($n = 4$ per group), Each dot represents one mouse of each experimental group. $^{**}P < 0.01$.

vector was transfected into Jurkat T cells. Consistent with *in vivo* study, overexpression of FADD-DN in Jurkat T cells also led to a significant decrease in Notch1 expression (**Figure 4E**).

IL-7R is one of Notch1-target genes and Notch1 controls T cell development in part by regulating the stage- and lineage-specific expression of IL-7R (34, 35). C-Kit is a tyrosine kinase receptor and extrathymus-derived IELs normally in older mice are c-Kit-dependent (4, 36). These two crucial markers: CD117 (c-kit) and CD127 (IL-7R) can be used to distinguish a lineage of T cells with unique developmental attributes. By comparisons of the subsets in Lin^- LPLs marked with CD117 and CD127, there was a distinct difference in $CD127^+$ (IL-7R expression) between WT mice and FADD-DN mice (**Figure 4F**). In FADD-DN mice, Lin^- LPL shared little expression of surface antigen IL-7R, just in line with the low expression of Notch1. The enterocyte-produced IL-7 plays a differentiation inducing role in the development of intestinal IELs (37, 38). In order to fully develop, the thymic-independent $TCR\gamma\delta^+$ IELs in an immature state must interact with their appropriate ligands *in situ*, so lack of IL-7R in Lin^- LPL cells from FADD-DN mice might provide a novel role for FADD in early intestinal T-IEL development.

The Role of FADD-DN in Intestinal Immunoregulation

As the first-line defense against infectious agents, γδ T-IELs are involved in intestinal immunoregulation (39). Depletion or deficiency in γδ T-IELs aggravates intestinal inflammation in almost every investigated model, especially DSS-induced colitis (40). Our finding about loss of γδ T-IELs in FADD-DN mice draws substantial interest in its pathological responses. DSS-induced colitis model was established in both WT and FADD-DN mice. Severe illness, which was characterized by diarrhea, intestinal bleeding, body weight loss, and shortened colon length, was observed at 4 days after DSS administration. Compared with WT group, FADD-DN mice exhibited severe colitis with a distinct reduction in colon length (**Figures 5A,B**). From day 3 to day 6 of DSS-induced colitis, a significant loss of body weight was observed in FADD-DN mice compared to WT mice (**Figure 5C**), and the disease activity index (DAI) of FADD-DN mice also increased more dramatically (**Figure 5D**). The pathological section with H&E staining showed more severe pathological changes in DSS-treated FADD-DN mice, including loss of goblet cells, distortion of crypts, mucosal damage and necrosis (**Figure 5E**). Correspondingly, histological score of colon sections was much higher in FADD-DN mice (**Figure 5F**). Consistent with these phenotypes, higher levels of TNF-α, IFN-γ, and IL-6 were detected in the serum of FADD-DN mice, while no significant differences in IL-12p40 and IL-1β (**Figure 5G**). It is worth mentioning that a higher level of several cytokines (TNF-α, IL-12p40, and IL-1β) was detected in sham group of FADD-DN mice, suggesting FADD-DN in the T-cell specific expression might affect basic immune response.

DISCUSSION

To date, a plethora of reports has clearly identified FADD as an essential adaptor between death receptors and caspase-8 (41, 42). Non-apoptotic functions of FADD have been implicated in T lymphocyte development and activation, mostly in thymic αβ T cells. To clarify the role of FADD in the development of γδ T cells, FADD-DN mice provide a useful tool to dissect T cell development in the mucosa for thymic and extrathymic pathways. Based on our findings, FADD plays an important role in intestinal IEL development, especially in γδ T-IELs which are thought to develop locally and largely via an extrathymic pathway.

T-IELs play a critical role in regulating intestinal mucosal immune responses. γδ T-IELs are a crucial protective T cell subset against colitis (43). Loss of intestinal γδ T-IELs in FADD-DN mice is a main phenotype. The decreased $CD8\alpha^+TCR\gamma\delta^+$ T cells will take responsibility for the reduced homeostasis in intestinal mucosal immune system, so FADD-DN mice show more severe inflammation in DSS-induced colitis model (**Figure 5**). To track the differentiation of intestinal $CD8\alpha^+TCR\gamma\delta^+$ T cells, we prepared intestinal T cell precursors referring to Lambolez's paper published on J. Exp. Med. (4). The Lin^- LPLs (or namely Lin^- WL) isolated by the reporting method has been fully demonstrated to be cryptopatch (CP) precursors. These Lin^- LPLs have specific phenotype: (Lin^-) $Thy^+c\text{-}kit^+IL\text{-}7R^+CD44^+CD25^+$. Analysis of Lin^- IELs and Lin^- LPLs revealed an arrest for $CD8\alpha^+TCR\gamma\delta^+$ T development at stage of $IL\text{-}7R^-c\text{-}kit^+Lin^-$ LPLs in FADD-DN mice. The IL-7R is expressed on lymphoid T and B precursors, and innate lymphoid cells. Its ligand IL-7 is integral to T and B cell development in primary lymphoid organs, so IL-7/IL-7R plays an essential role in supporting T cell development and homeostasis. The loss of IL-7R expression in intestinal precursors might be responsible for the defect on intestinal T-IEL development in FADD-DN mice, because the local development of IELs is IL-7-dependent. The possible molecular mechanism involved in the deficiency of IL-7R expression in Lin^- LPLs is the decreased Notch1 expression induced by FADD-DN overexpression (**Figure 4**).

We have reported previously that FADD regulates thymocyte development at the β-selection checkpoint by modulating Notch1 signaling (8). Notch1 mRNA increases from early thymocyte progenitor to the DN3a stage and markedly decreases at the DN3b stage, commensurate with pre-TCR signaling.

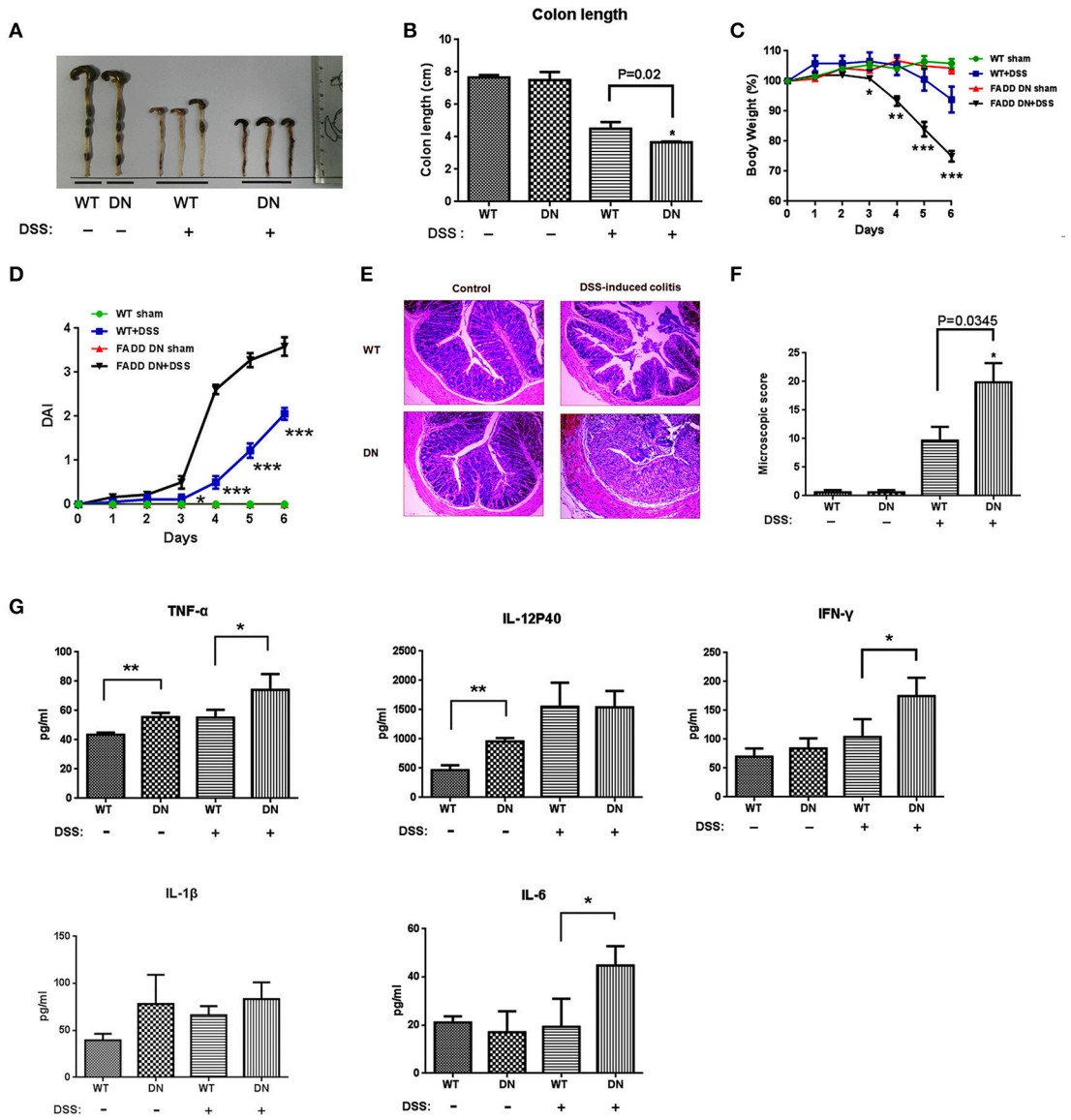

FIGURE 5 | More severe DSS-induced colitis in FADD-DN mice. Mice ($n = 6$ per group) were given 3% DSS in their drinking water for 5 days and then provided with water for 1 day before being sacrificed. Macroscopic appearances **(A)** and colon lengths **(B)** were measured in indicated groups. **(C)** Body weights of mice in each group ($n = 6$ per group) were recorded daily. The changes of body weights induced by DSS-induced colitis were shown in the percentage of the original body weight. **(D)** Disease activity index (DAI) was calculated as described in the material and methods section. **(E)** Colon sections of indicated groups with H&E staining. The original amplification was 100×. **(F)** Histological scores of colon sections were calculated as described in the Materials and methods. **(G)** Serum levels of cytokines in different groups detected by ELISA. Data shown here are from a representative experiment repeated three times with similar results. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. DSS-treated vehicle group.

Differential expression of Notch1 represents a distinct lineage of T cells with unique developmental and functional attributes (44). Consistent with the reports, we also have detected two states of Notch1 expression in intestinal T-IEL precursors, but the regulation for Notch1 expression is suppressed by FADD-DN expression (**Figure 4**). These results suggest that FADD is a part of signals for promoting intestinal T-IEL development through its DD domain. Of course, the signal that is inhibited by FADD-DN may or may not come from a DD-receptor signaling. It is possible that there are other binding molecules with FADD yet to be discovered. NKAP known as a Notch1 transcriptional repressor was previously identified by us to associate with FADD, implying a potential role of FADD in maintaining the stabilization of NKAP to inhibit Notch1 on transcriptional level. Besides being cytoplasmic, the FADD protein is also localized in the nucleus of many types of cells and nuclear FADD is probably implicated in a functional transcription factor complex to modulate Notch1 expression (8). The study of FADD function on intestine IEL

development by Notch1 still needs further investigation, which will give a comprehensive understanding of FADD function in the field of mucosal immunology.

In summary, the present study demonstrates a novel function of FADD in the development of intestinal T-IELs, especially on $CD8\alpha^{+}TCR\gamma\delta^{+}$ population. Several important immune functions have been reported for intestinal γδ T cells, including the first-line defense against pathogens and an immuno-down regulatory role during infection (45, 46). γδ T cell-deficient mice spontaneously develop colitis at 8 weeks of age (46). Decreased numbers of γδ T cells are observed in areas of Crohn's colitis (47) and depletion of γδ T cells aggravates TNBS colitis resulting in significant mortality (40, 48). Further investigation of FADD on mucosal immunology seems meaningful since the influence on $CD8\alpha^{+}TCR\gamma\delta^{+}$ subset mediated by FADD might be involved in the initiation and/or perpetuation of colitis.

MATERIALS AND METHODS

Cell Culture

Jurkat T cells (ATCC, Shanghai, China) were maintained in RPMI 1640 (Gibco-BRL, Basel, Switzerland) containing antibiotics and 10% FCS (Gibco-BRL). Human embryonic kidney (HEK)-293 T adherent cells (ATCC, Shanghai, China) were cultured in DMEM (Gibco-BRL) supplemented with antibiotics and 10% FCS (Gibco-BRL).

Mice

FADD DN mice were provided by the laboratory of Aster Winoto (University of California, Berkeley), which are generated and maintained on C57BL/6J background. Mice C57BL/6J were purchased from Beijing Animal Centre, and maintained in pathogen-free conditions. All animal experiments were approved by Nanjing University Animal Care and Use Committee, and we strictly followed the recommendation of the guidelines of the Animal Care Committee of Nanjing University.

Isolation of Intraepithelial Lymphocytes

Isolation of intestinal Intraepithelial lymphocytes (IELs) was performed as described previously (2). After washing the small intestine to remove fecal content, Peyer's patches and fat tissue were extirpated. The small intestine was opened longitudinally, cut into small pieces, and washed with ice-cold Ca^{2+}- and Mg^{2+}-free CMF-HBSS buffer containing 1 mM dithiothreitol (Sigma) three times. The intestines were then incubated with CMF-HBSS supplemented with penicillin, streptomycin, and 10% FCS and shaken at 220 rpm, 37°C. After filtrating the supernatants through a nylon mesh, IELs were collected by a 44–67% Percoll density gradient (Solarbio, Beijing, China) layered between the 44–67%. After washed by PBS, cells were counted and used for the following assays.

Isolation of Lamina Propria Lymphocytes (LPL)

After IELs isolation, tissues were digested in RPMI1640 supplemented with 200 U/ml collagenase type XI (Sigma, USA), 0.1 mg/ml DNase I (invitrogen), and 10% FCS at 220 rpm, 37°C for 60 min. Lamina propria lymphocytes (LPLs) were released and then isolated with 44–67% Percoll fractionation as described above.

Dextran Sodium Sulfate (DSS)-Induced Colitis

FADD DN or wild-type mice were fed with water charged with 3.0 % (W/V) DSS (dextran sulfate sodium salt, 36–50 kDa, 0216011080, MP Biomedicals) for 5 days and then with normal drinking water for 1 day till sacrificed. Mice given normal water served as control. Body weight of mice was recorded daily. Mice were sacrificed 6 days after DSS exposure. The length of the colons for each group were measured. After washing by PBS, colons tissue were cut and fixed in 4% (v/v) formaldehyde for histological analysis.

Evaluation of Disease Activity

During the experiment, body weight of mice for each group was recorded daily and feces were collected. Disease activity index(DAI) was determined by loss of weight, stool consistency, and fecal blood. The calculation of DAI was performed as previously (49).

Histological Analysis

The colons were fixed in formaldehyde, embedded in paraffin and cut into serial sections of 3-μm-thick for histological analysis. Stained with haematoxylin and eosin (H&E), tissues were analyzed by histological grading according to the criteria described previously (50).

Immunofluorescence Assay

The deparaffinized colon tissues underwent antigen retrieval and were blocked with 10% goat serum. Sections were incubated with anti-mouse TCRαβ and TCRγδ primary antibodies (1:50, Cell Signaling Technology (CST), Danvers, MA, USA) overnight at 4°C. After washing with PBS three times for 5 min, sections were incubated with appropriate secondary antibodies (1:1000; invitrogen) at room temperature for 30 min. Following washed with PBS three times for 5 min, slides were counterstained with DAPI (invitrogen). All images of the slides were visualized and captured by fluorescence microscope (Zeiss AX10, Carl Zeiss AG, Germany).

Construction and Transfection of FADD-DN Lentiviral Vector

The sequence of FADD-DN was cloned into a plenti6/v5-D-Topo expression vector (Invitrogen) using restriction enzyme BamHI and XhoI. Primers for amplifying the DNA sequence of FADD-DN: forward: 5′-CGGGATCCATGGACGACTTCGA-3′ and reverse: 5′-CCCTCGAGTCAGGACGCTTCGGAGGT-3′. Briefly, HEK-293T cells were co-tranfected with FADD-DN expression plasmid and lentiviral envelope plasmids (PL3, PL4, and PL5). The viruses were harvested by ultra-centrifugation on day 3 after transfection.

Jurkat T cells was replaced with transduction medium. 8 μg/ml polybrene (Santa Cruz Biotechnology) was added followed by lentiviral transduction. Virus was removed 24 h after

transduction and 10 μg/ml blasticidin was added for another 48 h. The cells were cultured for 72 h to examine the expression of FADD-DN by western blot.

Western Blotting Assay

Samples of protein lysates were separated by 10% SDS-PAGE and electro-transferred to PVDF membrane. After blocking with 5% non-fat milk for 2 h at room temperature, membranes were incubated with antibodies against mouse FADD (ab124812; Abcam, Cambridge, UK), human FADD C-terminus (610399; BD Pharmingen, Franklin Lake, NJ, USA), β-actin (AM1021B; Abgent, San Diego, CA, USA), and cleaved Notch1 (4147s; Cell Signaling Technology, Danvers, MA, USA) followed by suitable secondary antibody conjugated with horseradish peroxidase (Jackson Immunoresearch Laboratories, West Grove, PA, USA). Reactive bands were detected with enhanced chemiluminescence solution (Tanon, Shanghai, China) and visualized with an imaging program (Tanon, Shanghai, China).

Cytokine Measurement

Cytokines in serum samples including TNF-α, IL-6, IL-17, IL-1β, IL-12 were measured using ELISA kits (R&D Systems, US) according to the manufacturer's instructions.

Flow Cytometry Analysis

The IELs were stained with antibodies against lineage markers (Lin) as follows: anti-CD3 (145-2C11), anti-CD19 (1D3), anti-TCRαβ (H57-597), anti-TCRγδ (GL3), anti-erythroid cells (TER-119), anti-GR1 (8C5), anti–CD11b (M170), anti-IgM (LL41), and anti-CD8b (eBioH35-17.2). All the antibodies for FACS were purchased from BD Pharmingen (San Diego, CA). CD8αα cells were gated as $CD8\alpha^{+}CD8\beta^{-}$. Fluorescence was measured with a flow cytometer (FACS Calibur; BD Biosciences) equipped with Cell Quest software (BD Biosciences, Canada).

Thymectomy

Thymectomy was performed on 8-week-old mice as described before (32). A ventilator (V-100; Yuyan instruments, Shanghai, China) was used to keep mice breathing during the operation. Completeness of thymectomy was confirmed by visual inspection, both directly after removal of the organ and at the end of the experiment. Only fully thymectomized animals were included in this study.

Statistical Analysis

The experimental data were presented as mean ± SEM. The statistical significance of the differences between groups was evaluated by One-way ANOVA and Student's *t*-test ($P < 0.05$).

AUTHOR CONTRIBUTIONS

XZ and LH conceptualized the study, designed and carried out the experiments. XZ wrote the manuscript. LS, ZhH, and XW participated and analyzed the experiments. YH, YW and PX provided many materials and discussed the results. JZ and ZiH revised the manuscript and provided important advices. XZ and LH contributed equally to this work.

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REFERENCES

1. Cheroutre H, Lambolez F, Mucida D, The light and dark sides of intestinal intraepithelial lymphocytes. *Nat Rev Immunol.* (2011) 11:445–56. doi: 10.1038/nri3007
2. Wang X, Sumida H, Cyster JG, GPR18 is required for a normal CD8αα intestinal intraepithelial lymphocyte compartment. *J Exp Med.* (2014) 211:2351–9. doi: 10.1084/jem.20140646
3. Ettersperger J, Montcuquet N, Malamut G, Guegan N, Lopezlastra S, Gayraud S, et al. Interleukin 15-dependent T cell-like innate intraepithelial *Lymphocytes* develop in the intestine and transform into *Lymphomas* in celiac disease. *Immunity* (2016) 45:610–25. doi: 10.1016/j.immuni.2016.07.018
4. Lambolez F, Azogui O, Joret AM, Garcia C, von Boehmer H, Di Santo J, et al. Characterization of T cell differentiation in the murine gut. *J Exp Med.* (2002) 195:437–49. doi: 10.1084/jem.20010798
5. Mallis RJ, Bai K, Arthanari H, Hussey R. E, Handley M, Li Z, et al. Pre-TCR ligand binding impacts thymocyte development before αβTCR expression. *Proc Natl Acad Sci USA*. (2015) 112:8373–8. doi: 10.1073/pnas.1504971112
6. Lu JV, Weist BM, van Raam BJ, Marro BS, Nguyen LV, Srinivas P, et al. Complementary roles of Fas-associated death domain (FADD) and receptor interacting protein kinase-3 (RIPK3) in T-cell homeostasis and antiviral immunity. *Proc Natl Acad Sci USA*. (2011) 108:15312–7. doi: 10.1073/pnas.1102779108
7. Dowling JP, Nair A, Zhang J, A novel function of RIP1 in postnatal development and immune homeostasis by protecting against RIP3-dependent necroptosis and FADD-mediated apoptosis. *Front Cell Dev Biol.* (2015) 3:12. doi: 10.3389/fcell.2015.00012
8. Zhang X, Dong X, Wang H, Li J, Yang B, Zhang J, et al. FADD regulates thymocyte development at the β-selection checkpoint by modulating Notch signaling. *Cell Death Dis.* (2014) 5:e1273. doi: 10.1038/cddis.2014.198
9. Zhang H, Zhou X, Mcquade T, Li J, Chan FK, Zhang J. Functional complementation between FADD and RIP1 in embryos and lymphocytes. *Nature* (2011) 471:373–6. doi: 10.1038/nature09878
10. Kikuchi M, Kuroki S, Kayama M, Sakaguchi S, Lee KK, Yonehara S. Protease activity of procaspase-8 is essential for cell survival by inhibiting both apoptotic and nonapoptotic cell death dependent on receptor-interacting protein kinase 1 (RIP1) and RIP3. *J Biol Chem.* (2012) 287:41165. doi: 10.1074/jbc.M112.419747
11. Pennarun B, Meijer A, de Vries EG, Kleibeuker JH, Kruyt F, de Jong S. Playing the DISC: turning on TRAIL death receptor-mediated apoptosis in cancer. *Biochimica et Biophysica Acta* (2010) 1805:123–40. doi: 10.1016/j.bbcan.2009.11.004
12. Grunert M, Gottschalk K, Kapahnke J, Gündisch S, Kieser A, Jeremias I, The adaptor protein FADD and the initiator caspase-8 mediate activation of NF-κB by TRAIL. *Cell Death Dis.* (2012) 3:e414. doi: 10.1038/cddis.2012.154

13. Bonnet MC, Preukschat D, Welz PS, Van LG, Ermolaeva MA, Bloch W, et al. The adaptor protein FADD protects epidermal keratinocytes from necroptosis *in vivo* and prevents skin inflammation. *Immunity* (2011) 35:572–82. doi: 10.1016/j.immuni.2011.08.014
14. Bell BD, Leverrier S, Weist BM, Newton RH, Arechiga AF, Luhrs KA, et al. FADD and caspase-8 control the outcome of autophagic signaling in proliferating T cells. *Autophagy* (2008) 105:16677–82. doi: 10.1073/pnas.0808597105
15. Zhang JK, Cado D, Chen A, Kabra NH, Winoto A. Fas-mediated apoptosis and activation-induced T-cell proliferation are defective in mice lacking FADD/Mort1. *Nature* (1998) 392:296–300. doi: 10.1038/32681
16. Newton K, Harris AW, Strasser A. FADD/MORT1 regulates the pre-TCR checkpoint and can function as a tumour suppressor. *Embo J.* (2000) 19:931–41. doi: 10.1093/emboj/19.5.931
17. Zhang J, Kabra NH, Cado D, Kang C, Winoto A. FADD-deficient T cells exhibit a disaccord in regulation of the cell cycle machinery. *J Biol Chem*. (2001) 276:29815–18. doi: 10.1074/jbc.M103838200
18. Kabra NH, Kang CH, Hsing LC, Zhang JK, Winoto A. T cell-specific FADD-deficient mice: FADD is required for early T cell development. *P Natl Acad Sci USA*. (2001) 98:6307–12. doi: 10.1073/pnas.111158698
19. Zhang YH, Rosenberg S, Wang HM, Imtiyaz HZ, Hou YJ, Zhang JK. Conditional fas-associated death domain protein (FADD): GFP knockout mice reveal FADD is dispensable in thymic development but essential in peripheral T cell homeostasis. *J Immunol.* (2005) 175:3033–44. doi: 10.4049/jimmunol.175.5.3033
20. Osborn SL, Diehl G, Han SJ, Xue L, Kurd N, Hsieh K, et al. Fas-associated death domain (FADD) is a negative regulator of T-cell receptor-mediated necroptosis. *P Natl Acad Sci USA*. (2010) 107:13034–9. doi: 10.1073/pnas.1005997107
21. Newton K, Harris AW, Bath ML, Smith KG, Strasser A. A dominant interfering mutant of FADD/MORT1 enhances deletion of autoreactive thymocytes and inhibits proliferation of mature T lymphocytes. *Embo J.* (1998) 17:706–18. doi: 10.1093/emboj/17.3.706
22. Newton K, Kurts C, Harris AW, Strasser A. Effects of a dominant interfering mutant of FADD on signal transduction in activated T cells. *Curr Biol.* (2001) 11:273–6. doi: 10.1016/S0960-9822(01)00067-7
23. Walsh CM, Wen BG, Chinnaiyan AM, O'Rourke K, Dixit VM, Hedrick SM. A role for FADD in T cell activation and development. *Immunity* (1998) 8:439–49. doi: 10.1016/S1074-7613(00)80549-X
24. Peaudecerf L, dos Santos PR, Boudil A, Ezine S, Pardigon N, Rocha B, The role of the gut as a primary lymphoid organ: CD8Î±Î± intraepithelial T lymphocytes in euthymic mice derive from very immature CD44+ thymocyte precursors. *Mucosal Immunol.* (2011) 4:93–101. doi: 10.1038/mi.2010.47
25. Wurbel MA, Malissen M, Guygrand D, Malissen B, Campbell JJ. Impaired accumulation of antigen-specific CD8 *Lymphocytes* in chemokine CCL25-deficient intestinal epithelium and lamina propria. *J Immunol.* (2007) 178:7598–606. doi: 10.4049/jimmunol.178.12.7598
26. Helgeland L, Brandtzaeg P, Rolstad B, Vaage JT. Sequential development of intraepithelial gamma delta and alpha beta T lymphocytes expressing CD8 alpha beta in neonatal rat intestine: requirement for the thymus. *Immunology* (1997) 92 447–56. doi: 10.1046/j.1365-2567.1997.00379.x
27. Suzuki H, Jeong KI, Doi K. Age-related changes in the regional variations in the number and subsets of intraepithelial lymphocytes in mouse small intestine. *Dev Comp Immunol.* (2002) 26:589–95. doi: 10.1016/S0145-305X(02)00004-6
28. Jameson SC. Maintaining the norm: T-cell homeostasis. *Nat Rev Immunol.* (2002) 2:547–56. doi: 10.1038/nri853
29. Delphine GG, Orly A, Susanna C, Sylvie D, Nussenzweig MC, Philippe K, et al. Extrathymic T Cell *Lymphopoiesis*. *J Exp Med.* (2003) 197:333–41. doi: 10.1084/jem.20021639
30. Guy-Grand D, Vassalli P. Gut intraepithelial lymphocyte development. *Curr Opin Immunol.* (2002) 14:255–9. doi: 10.1016/S0952-7915(02)00330-8
31. Saran N, Łyszkiewicz M, Pommerencke J, Witzlau K, Vakilzadeh R, Ballmaier M, et al. Multiple extrathymic precursors contribute to T-cell development with different kinetics. *Blood* (2010) 115:1137–44. doi: 10.1182/blood-2009-07-230821
32. Herbrand H, Pabst O. Cryptopatches and Isolated Lymphoid Follicles: aspects of development, homeostasis and function, *Dev Biol Peripheral Lymphoid Organs* (2011) 107–17. doi: 10.1007/978-3-642-14429-5_10
33. Ford MS, Zhang ZX, Chen W, Zhang L. Double-negative T regulatory cells can develop outside the thymus and do not mature from CD8+ T cell precursors. *J Immunol.* (2006) 177:2803–9. doi: 10.4049/jimmunol.177.5.2803
34. Laky K, Lefrancois L, von Freeden-Jeffry U, Murray R, Puddington L. The role of IL-7 in thymic and extrathymic development of TCR gamma delta cells. *J Immunol.* (1998) 161:707–13.
35. Laky K, Lefrancois L, Ishikawa H, Olson S, Suzuki K, Ishimaru K, et al. Enterocyte expression of IL-7 induces development of TCR gamma delta cells and Peyer's patches. *Faseb J.* (1999) 13:A622.
36. Saito H, Kanamori Y, Takemori T, Nariuchi H, Kubota E, Takahashi-Iwanaga H, et al. Generation of intestinal T cells from progenitors residing in gut cryptopatches. *Science* (1998) 280:275–8. doi: 10.1126/science.280.5361.275
37. Shitara S, Hara T, Liang B, Wagatsuma K, Zuklys S, Holländer GA, et al. IL-7 produced by thymic epithelial cells plays a major role in the development of thymocytes and TCRγδ+ intraepithelial lymphocytes. *J Immunol.* (2013) 190:6173–9. doi: 10.4049/jimmunol.1202573
38. Cai YJ, Wang WS, Liang HY, Sun LH, Teitelbaum DH, Yang H. Keratinocyte growth factor up-regulates Interleukin-7 expression following intestinal ischemia/reperfusion *in vitro* and *in vivo*. *Int J Clin Exp Pathol*. (2012) 5:569–580.
39. Ismail AS, Severson KM, Vaishnava S, Behrendt CL, Yu X, Benjamin JL, et al. Gammadelta intraepithelial lymphocytes are essential mediators of host-microbial homeostasis at the intestinal mucosal surface. *Proc Natl Acad Sci USA*. (2011) 108:8743–8. doi: 10.1073/pnas.1019574108
40. Kuhl AA, Pawlowski NN, Grollich K, Loddenkemper C, Zeitz M, Hoffmann JC. Aggravation of intestinal inflammation by depletion/deficiency of gammadelta T cells in different types of IBD animal models. *J Leukocyte Biol.* (2007) 81:168–75. doi: 10.1189/jlb.1105696
41. Nogusa S, Thapa RJ, Dillon CP, Liedmann S, Oguin TH III, Ingram JP, et al. RIPK3 activates parallel pathways of MLKL-driven necroptosis and FADD-mediated apoptosis to protect against influenza A virus. *Cell Host Microbe* (2016) 20:13–24. doi: 10.1016/j.chom.2016.05.011
42. Wang PX, Ji YX, Zhang XJ, Zhao LP, Yan ZZ, Zhang P, et al. Targeting CASP8 and FADD-like apoptosis regulator ameliorates nonalcoholic steatohepatitis in mice and nonhuman primates. *Nat Med.* (2017) 23:439–49. doi: 10.1038/nm.4290
43. Meehan TF, Witherden DA, Kim CH, Sendaydiego K, Ye I, Garijo O, et al. Protection against colitis by CD100-dependent modulation of intraepithelial γδ T lymphocyte function. *Mucosal Immunol.* (2013) 7:134–42. doi: 10.1038/mi.2013.32
44. Ciofani M, Zuniga-Pflucker JC. Notch promotes survival of pre-T cells at the beta-selection checkpoint by regulating cellular metabolism. *Nat Immunol.* (2005) 6:881–8. doi: 10.1038/ni1234
45. Chen YP, Chou K, Fuchs E, Havran WL, Boismenu R. Protection of the intestinal mucosa by intraepithelial gamma delta T cells. *P Natl Acad Sci USA*. (2002) 99:14338–43. doi: 10.1073/pnas.212290499
46. Inagaki-Ohara K, Chinen T, Matsuzaki G, Sasaki A, Sakamoto Y, Hiromatsu K, et al. Mucosal T cells bearing TCRgammadelta play a protective role in intestinal inflammation. *J Immunol.* (2004) 173:1390–8. doi: 10.4049/jimmunol.173.2.1390
47. Nanno M, Shiohara T, Yamamoto H, Kawakami K, Ishikawa H. γδ T cells: firefighters or fire boosters in the front lines of inflammatory responses. *Immunol Rev.* (2007) 215:103–13. doi: 10.1111/j.1600-065X.2006.00474.x

48. Hoffmann JC, Peters K, Henschke S, Herrmann B, Pfister K, Westermann J, et al. Role of T lymphocytes in rat 2,4,6-trinitrobenzene sulphonic acid (TNBS) induced colitis: increased mortality after gamma delta T cell depletion and no effect of alpha beta T cell depletion. *Gut* (2001) 48:489–95. doi: 10.1136/gut.48.4.489
49. Saiz ML, Cibrian D, Ramírezhuesca M, Torralba D, Morenogonzalo O, Sánchezmadrid F. Tetraspanin CD9 limits mucosal healing in experimental colitis. *Front Immunol.* (2017) 8:1854. doi: 10.3389/fimmu.2017.01854
50. Zhang X, Wei L, Wang J, Qin Z, Wang J, Lu Y, et al. Suppression colitis and colitis-associated colon cancer by anti-S100a9 antibody in mice. *Front Immunol.* (2017) 8:1774. doi: 10.3389/fimmu.2017.01774

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REVIEW
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Epithelial MHC Class II Expression and Its Role in Antigen Presentation in the Gastrointestinal and Respiratory Tracts

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As the primary barrier between an organism and its environment, epithelial cells are well-positioned to regulate tolerance while preserving immunity against pathogens. Class II major histocompatibility complex molecules (MHC class II) are highly expressed on the surface of epithelial cells (ECs) in both the lung and intestine, although the functional consequences of this expression are not fully understood. Here, we summarize current information regarding the interactions that regulate the expression of EC MHC class II in health and disease. We then evaluate the potential role of EC as non-professional antigen presenting cells. Finally, we explore future areas of study and the potential contribution of epithelial surfaces to gut-lung crosstalk.

Keywords: epithelial cells, MHC class II, antigen presentation, intestine, respiratory

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INTRODUCTION

The epithelium serves as both a physical and chemical barrier as well as an absorptive surface. Our understanding of the function of the aerodigestive epithelium has gradually evolved from its role as a static barrier to a dynamic structure regulating multiple processes. In fact, ECs may have primary immune functions that affect the balance between tolerance and inflammation, as evidenced by expression of MHC class II on the EC surface, an area of exploration several decades ago. Recent evidence suggests there may also be communication with the lung from the gut directed by its microbiome. Because host-microbial interactions first occur at the epithelial surface, re-visiting the role of ECs in antigen processing and presentation is timely. This review aims to synthesize current findings on MHC class II expression in the gut and the lung, explore the role of ECs as non-professional antigen presenting cells (APCs) and discuss how this area may be further investigated as a target for potential diagnostic or therapeutic interventions.

Structure and Function of the Aerodigestive Epithelium

Though the digestive and respiratory systems are thought of as two entirely distinct anatomic cavities, they have a shared developmental origin from the primitive gut (1, 2). The digestive tube initially extends through the length of the body from which the respiratory tube outpouches, sharing a common embryonic chamber called the pharynx. The linings of these primitive tubes are comprised of embryonic endoderm. The digestive tube eventually differentiates into the components of the gastrointestinal tract including esophagus, stomach, small intestine and colon, due to the interaction of endodermal epithelium with regionally specific mesodermal mesenchyme. The respiratory tube bifurcates into two lungs, with laryngotracheal endoderm becoming the epithelial lining of the trachea, bronchi and lung parenchyma, similarly directed by the regional mesenchyme (3).

By birth, the terminal portion of the digestive tract has matured into the small intestine and large intestine. The small intestine is ∼5 m in length and composed of three separate segments—duodenum, jejunum, and ileum—that are exposed to dietary antigens and crucial for oral tolerance. Small intestinal epithelium is composed of finger-like projections called villi and invaginations called crypts of Lieberkühn. The large intestine, or colon, comprises the distal 1.5 m of the gastrointestinal tract and histologically lacks villi (4).

The intestinal mucosa is composed of simple columnar epithelium that comprises a surface area of 200–300 m^2 (1). This immense structure facilitates the absorption of nutrients in the small intestine and water in the large intestine, while also acting as a barrier and modulator of immunity (5). Intestinal epithelial cells (IECs) include multiple different specialized cells including enterocytes, goblet cells, Paneth cells, enteroendocrine cells, M cells and tuft cells, all of which have discrete functions (**Figure 1**). Enterocytes are the most abundant cell type in the gut epithelium and function to transcytose antimicrobial proteins and IgA as well as absorb nutrients (6). Goblet cells secrete mucus, resistin-like molecule β, which modifies T cell-mediated immunity, and trefoil factor, which promotes epithelial healing after injury; these cells have also been shown to participate in antigen delivery to dendritic cells (DCs) of the submucosa through specialized antigen passages (7, 8). Paneth cells in the small intestine secrete microbicidal proteins including α-defensins, C-type lectins, lysozyme and phospholipase A_2; they also sustain stem cells in the crypts of Lieberkühn to promote epithelial regeneration (6). Enteroendocrine cells secrete neurohormones including gastric inhibitory peptide, glucagon-like peptide, and vasoactive intestinal peptide in response to nutrients in order to regulate motility and digestion (6). M cells (microfold cells), a specialized EC subset derived from enterocytes, transcytose antigens to the underlying gut-associated lymphoid tissue (GALT), the complement of lymphocytes that is composed of intraepithelial and lamina propria lymphocytes (IELs, LPLs) (9–11). Because of their role in antigen uptake, there has been interest in whether M cells present antigens using the MHC class II pathway to facilitate adaptive immunity, though existing evidence is conflicting (12–16). Tuft cells, a rare EC with a distinctive tufted morphology, express chemosensory receptors and may have roles in type 2 immunity and mucosal immunity, but remain poorly understood (17, 18).

IECs have a known role in innate immune responses via expression of a variety of pattern recognition receptors (PRRs). PRRs trigger intracellular pathways that lead to cytokine and chemokine release. PRRs important in the gut include Toll-like receptors 1–9 (TLRs) and nucleotide-binding oligomerization domain-containing proteins (NODs) that recognize pathogen-associated molecular patterns (PAMPs) derived from microbial components (19–21). Interestingly, apical TLRs on the luminal side of the gut (TLR 1 and 2) appear to be hyporesponsive to PAMPs *in vitro*, while the other PRRs expressed in IECs are either endosomal (TLR3–4, 7–9), cytoplasmic (NOD1 and NOD2) or

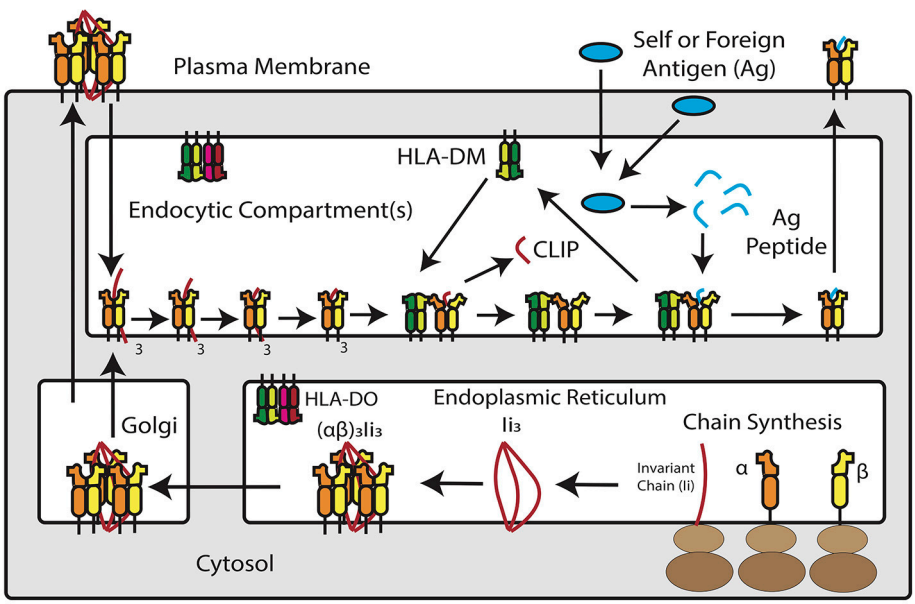

FIGURE 1 | Overview of MHC Class II Antigen Presentation Pathway. Newly synthesized MHC class II α and β chains assemble into heterodimers in the endoplasmic reticulum, where they are bound by trimers of invariant chain. MHC class II and invariant chain form nonamers–or, according to recent studies, pentamers and heptamers–that traffic into an acidic endosomal compartment. Within this compartment, invariant chain is degraded down to class II invariant chain-associated peptide (CLIP), which occupies the peptide binding groove of the MHC class II molecule. HLA-DM, a non-classical MHC protein, catalyzes the removal of CLIP in exchange for high-affinity peptide binders derived from extracellular or cytosolic antigens. In a subset of antigen presenting cells, HLA-DM is blocked by HLA-DO, which competitively binds to HLA-DM and prevents it from interacting with MHC class II. Once loaded with peptide, MHC class II molecules traffic to the plasma membrane for inspection by $CD4^+$ T cells.

only on the submucosal basolateral membrane (TLR4 and 5), supporting a role in tolerance to the gut microbiome (21–23).

The pseudostratified epithelium of the airway is ∼100 m^2 in surface area and composed of a variety of airway epithelial cells (AECs), many of which are specialized to the lung but homologous to the gut. The bronchial epithelium is composed of basal cells, columnar ciliated epithelial cells, mucous goblet cells, brush or tuft cells and Clara cells (24, 25). The alveolar epithelium, meanwhile, is composed mostly of Type I and Type II pneumocytes (24, 26). Neuroendocrine cells, similar to the gut, promote the vasomotor function of the airways (27). Basal cells, similar to Paneth cells, are important for epithelial regeneration and produce bioactive molecules including endopeptidase, 15-lipoxygenase products and cytokines (28, 29). Goblet cells, like those of the gut, secrete mucus in order to trap foreign particles and pathogens (24, 30). Columnar ciliated cells account for the majority of AECs in the bronchial lumen and are responsible mainly for mucus clearance (31). Clara cells produce surfactants and antiproteases including secretory leukocyte protease inhibitor and p450 mono-oxygenases (32). Type I pneumocytes are mainly responsible for gas exchange and make up the majority of the alveolar surface, though recent evidence suggests they may have additional roles in remodeling, regulation and defense (33). Type II pneumocytes are responsible for surfactant production and reuptake though they also act as progenitor cells and enhance immune responses (33, 34). Unlike the gut, an integrated mucosal immune system does not exist in the healthy adult human lung, though bronchus-associated lymphoid tissue (BALT) is present in young children and also develops in many disease states in adulthood (35, 36). AECs express a similar variety of PRRs as IECs including all known human TLRs, RIG-1-like receptors, NOD-like receptors, C-type lectins and surfactant proteins (26, 37–41).

Overview of MHC Class II and Costimulatory Molecules

MHC class II molecules are transmembrane αβ heterodimers. In humans, there are three MHC class II isotypes: HLA-DR, HLA-DP, and HLA-DQ, encoded by α and β chain genes within the Human Leukocyte Antigen (HLA) locus on chromosome 6. The expression of MHC class II antigen presentation machinery is tightly regulated by class II transactivator (CIITA), which recruits DNA-binding factors, chromatin modifying proteins, and transcription initiators to the MHC II locus. The class II pathway for processing and presenting antigen is complex but involves interaction with accessory molecules and trafficking through intracellular compartments (42–44). In the ER, nascent MHC class II molecules associate with invariant chain (CD74), a dedicated chaperone protein that directs MHC class II into a low-pH, late-stage endosomal compartment, known as the MHC II compartment (MIIC). Within MIIC, proteases cleave invariant chain and leave a nested set of invariant chain fragments known as class II invariant chain-associated peptides (CLIP) (44). CLIP temporarily occupies the peptide binding groove of the MHC class II molecule. A catalytic protein, HLA-DM, exchanges CLIP for peptides that bind MHC class II with high-affinity (45). Some APCs, including B cells, thymic epithelial cells and certain DCs, express a regulator of HLA-DM, known as HLA-DO, which competitively inhibits DM-MHC class II interaction (46). Once formed, peptide/MHC class II complexes traffic to the cell surface for interaction with $CD4^+$ T cells through the T-cell receptor, as the first signal to the lymphocyte required to elicit an antigen-specific adaptive response. The MHC class II pathway is shown schematically in **Figure 1**.

Efficient activation of naïve $CD4^+$ T cells requires a second signal to the lymphocyte in the form of co-stimulation to complete the APC-T cell interaction. Classical costimulatory signals include CD80 and CD86, members of the B7 family that interact with stimulatory CD28 or inhibitory CTLA-4 on T cells (47). These molecules are upregulated on professional APCs in response to PAMPs or damage associated molecular patterns (DAMPs), such as ATP (48). T cell recognition of peptide/MHC without sufficient co-stimulation induces a hyporesponsive, anergic state (49).

POTENTIAL ROLE OF ECS IN ANTIGEN PRESENTATION

MHC Class II in Health and Disease in Humans

Intestine

IECs have been described as capable of MHC class II expression for several decades (50–54). MHC class II, HLA-DM and invariant chain have been reproducibly detected in IECs throughout all segments of the small intestine (12, 52, 54–57). In humans, cell surface expression of class II is first detected around 18 weeks' gestation and increases through development (15, 58, 59). At homeostasis, MHC class II appears to be constitutively expressed on small intestinal enterocytes, most densely in the upper villus (15, 53, 56). Conversely, MHC class II is absent from small intestinal crypts as well as colonic epithelium under normal physiologic conditions but is upregulated in specimens obtained from patients with active inflammatory bowel disease (IBD), celiac disease, and graft vs. host disease (**Figure 2**) (55, 60–66). Exposure to inflammatory antigens, such as gliadin in celiac disease, has also been shown to cause the upregulation of cell surface MHC class II (62, 67). These changes are dependent on active disease; celiac patients in remission have IEC MHC class II levels comparable to those of non-celiac controls (68). IFNγ appears to be the key disease-elevated cytokine that regulates this process (69). In IBD, for example, increased surface MHC class II expression is correlated with increased tissue IFNγ levels (**Figure 2**) (70).

IECs are polarized, with brush border enzymes localized to the apical (luminal) surface to break down dietary antigens and poly-Ig receptors restricted to the basolateral surface to translocate IgA into the intestinal lumen (71). This polarity is important as peptide-presentation to the resident immune cells of the GALT is necessary for systemic crosstalk. Some early tissue staining studies in humans showed predominantly apical expression of MHC class II in IECs (53, 55, 72). However,

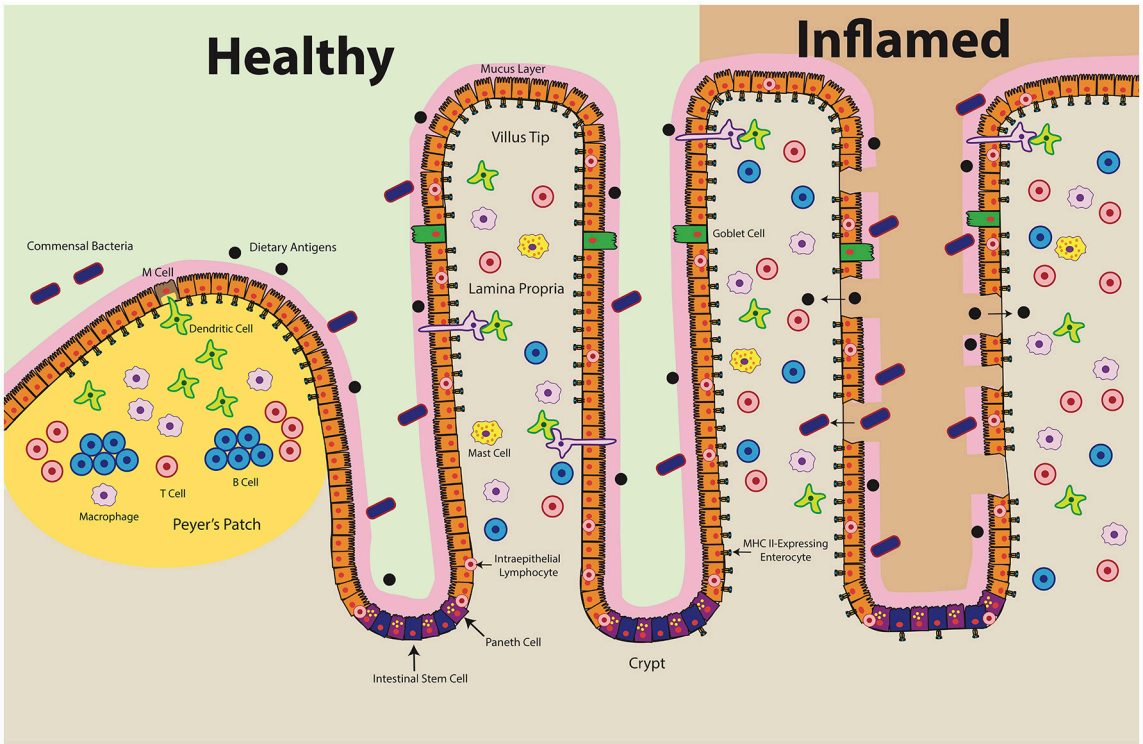

FIGURE 2 | Intestinal Epithelial Cell MHC Class II Expression in Health and Disease. At homeostasis (left), MHC class II is constitutively expressed in the upper villi of the small intestine. At the crypt base, intestinal stem cells self-renew and differentiate into specialized cell types: antimicrobial-producing Paneth cells, mucus-producing Goblet cells, hormone-producing enteroendocrine cells, and nutrient-absorptive enterocytes (200). Healthy crypts lack MHC class II expression. Intraepithelial lymphocytes, consisting of T cells and γδ T cells, likely play a key role in maintaining the baseline expression of MHC class II in ECs by producing IFNγ. During disease (right), MHC class II levels increase and extend into the crypts. Epithelial barrier integrity decreases, which may result in ECs encountering antigen along both the apical and basolateral surfaces. Organized lymphoid structures, known as Peyer's Patches, contain dense concentrations of professional antigen presenting cells (B cells, macrophages, dendritic cells). These cells encounter antigen delivered by microfold (M) cells, which transcytose luminal antigens. Whereas MHC class II expression has been shown in the Peyer's Patch epithelium, there are conflicting reports regarding MHC class II expression by M cells.

other reports, including a comparatively recent study, show lateral and basolateral MHC class II (73–75). These contradictory observations may be due to variability in methods of tissue processing and labeling, which has a significant effect on antigen stability and labeling efficiency (68, 76, 77). Notably, *in vitro* studies show expression of MHC class II along the basolateral surface and *in vivo* studies suggest that the amount of MHC class II along the basolateral surface of IECs is physiologically relevant (78–80).

Intestinal inflammation may also change MHC class II localization in IECs. Both conventional and electron microscopy have been used to show redistribution of IEC MHC class II from multivesicular bodies (late endosomes) to the basolateral membrane located on the submucosal side of the epithelial membrane in both celiac disease and IBD (74, 81). Increased trafficking of MHC class II to the cell surface likely requires downregulation of MARCH8 ubiquitin ligase, which drives MHC class II internalization and which IECs express at high levels (82). A similar pathway has been observed in DCs, where MARCH 1 is downregulated upon maturation stimulated by TLR ligands (83). Redistribution of MHC class II may allow IECs to influence immune responses during a pathogenic or inflammatory insult, by presenting peptides that promote immune clearance or induce tolerance.

Co-stimulatory molecules CD80 and CD86 are not expressed on IECs at baseline (57, 84, 85). Whether these molecules are expressed during inflammation is less clear. Some studies report that human IECs express neither CD80 nor CD86 during IBD, while others show selective expression of CD86 during active disease in biopsy specimens or with IFNγ-treatment in culture (85, 86). There is also evidence that the costimulatory molecule CD40, which interacts with CD40 ligand (CD40L) on T cells, is expressed by IECs during IBD in regions with visible pathology (87, 88). IECs may provide other forms of co-stimulation, such as CD58 (LFA-3), which interacts with CD2 on the surface of T cells (89). IECs express basolateral CD58 constitutively on surgically resected colonic epithelium and *in vitro* treatment with anti-CD58 antibody inhibits stimulation of antigen-specific $CD4^+$ T cell clones by antigen-pulsed IECs in a dose-dependent manner in humans (90).

Lung

Unlike the gut during ontogeny, fetal lung tissue does not appear to express MHC class II on AEC surfaces during gestation except in the case of active inflammation (91). Interestingly, invariant chain expression without co-expression of MHC class II has been detected on fetal alveolar epithelium by 12–14 weeks' gestational age in humans (92). Adult AECs, like small intestinal epithelium, were initially shown to constitutively express MHC class II on both bronchial and alveolar epithelium, specifically on type II pneumocytes and ciliated ECs (**Figure 3**) (93–95). However, additional studies utilizing clinical specimens have provided conflicting data, especially in primary bronchial EC cultures (96–99). Evidence in studies comparing germ-free to conventional rats supports constitutive surface expression of MHC II in lung parenchymal AECs, specifically Type II pneumocytes, but decreased expression in bronchial epithelium of germ-free rats, suggesting site-specific expression (100). Lung tissue obtained from patients with allergy or autoimmunity, including chronic bronchitis, asthma, idiopathic pulmonary fibrosis or lung transplant rejection, shows enhanced expression of MHC class II on AECs (96, 97, 101–103). Viral infection, including parainfluenza, have demonstrably increased AEC MHC class II expression, whereas bacterial infection appears to have the opposite effect in human lung specimens (91, 97, 104).

Co-stimulatory molecule expression appears to be region-specific in humans, as well. *in vitro* studies show baseline expression of CD86 on both bronchial and alveolar cells (A549 cell line), but baseline CD80 expression only on alveolar cells (98). Viral infection, specifically with rhinovirus, upregulates CD80 on alveolar cells and CD86 on bronchial cells (98). *In vivo* data obtained from lung biopsies in patients with a variety of autoimmune pathologies, including lung transplant rejection and idiopathic pulmonary fibrosis, shows increased expression of CD80 and CD86 on AEC from all segments of the respiratory tract (97, 105). In comparison, in bronchiolitis obliterans organizing pneumonia (now known as cryptogenic organizing pneumonia), an idiopathic interstitial lung disease believed to be secondary to epithelial damage, CD80 is upregulated in AECs without concurrent upregulation of CD86 or MHC class II expression (97, 106). Like gut, CD58 is constitutively expressed on alveolar ECs, though expression has not been demonstrated in isolated Type II pneumocytes (95).

In vitro Evidence for ECs as Antigen Presenting Cells

Studies utilizing human IEC lines (T84 and HT29) show that IFNγ-treated, protein antigen-pulsed IECs can stimulate antigen-specific immune responses in T cell hybridomas (107). T cell hybridomas do not need co-stimulation, which arguably mimics the reduced costimulatory requirements of the majority of T cells in the lamina propria, which are antigen-experienced memory cells (108, 109). Follow-up work in IECs found that generation of specific MHC II-restricted peptide epitopes differed if antigen was taken up from the apical or basolateral IEC surface (78). During disease, inflammatory signals including IFNγ and TNFα in the gut increase epithelial permeability (**Figure 2**) (110–112). When the epithelium is breached, IECs may interact with antigen along both the apical and basolateral surfaces, raising the possibility that novel peptide epitopes can be generated. Dotan et al. found that IECs isolated from surgically resected colon of Crohn's disease and ulcerative colitis patients induced $CD4^+$ T cells to proliferate and secrete more IFNγ than control IECs in a mixed lymphocyte reaction (65). This effect was blocked with an anti-HLA-DR antibody.

Another mechanism by which ECs may modulate antigen presentation is through exosomes. Exosomes, cell-derived vesicles laden with MHC class II, are released extracellularly when the limiting membrane of a multi-vesicular endosome

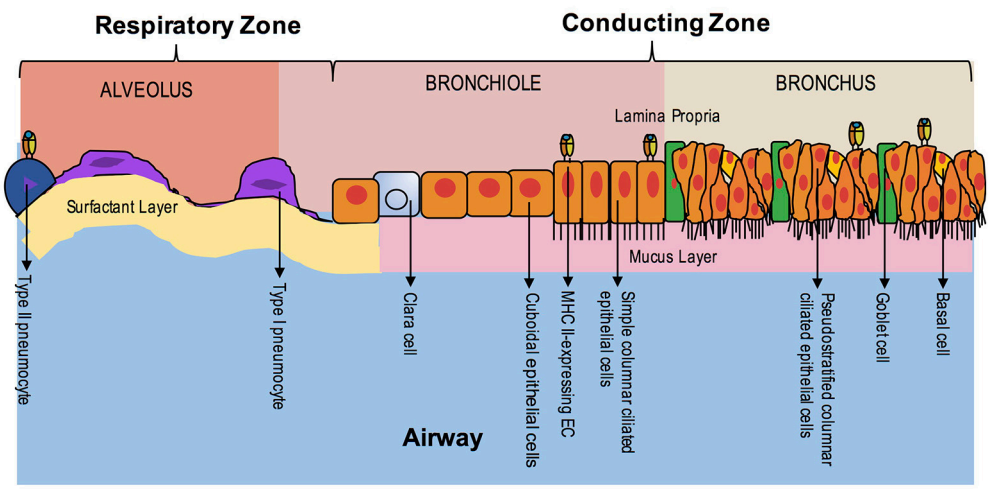

FIGURE 3 | EC MHC Class II Expression in the Lung During Homeostasis. The airway is composed of the upper airway conducting zone for humidifying and clearing particulates of inhaled air (bronchi and bronchioles) and lower airway respiratory zone for gas exchange (respiratory bronchioles and alveoli). At homeostasis, MHC class II expression has been seen in the ciliated ECs of the upper airway and in Type II pneumocytes of the alveoli. The polarity of class II expression is not well-defined. Unlike the intestine, organized lymphoid structures are not found in adulthood, except in disease states.

fuses with the plasma membrane (113). In fact, several studies show that exosomes from IFNγ-treated IECs express elevated MHC class II (114–116). These exosomes express late-endosomal markers, consistent with their origin in multi-vesicular bodies. Evidence suggests that IEC exosomes do not directly stimulate antigen-specific T cells but are first acquired by DCs. DCs primed with IEC exosomes require lower doses of antigen to stimulate T cell hybridomas (116). Exosomal transfer of peptide/MHC II complexes may promote rapid, primary adaptive immune responses by equipping DCs to stimulate naive T cells. Defining the relative contributions of direct IEC antigen presentation vs. exosome release to intestinal tolerance and immunity will require further investigation and may provide important insights into the communication between gut and lung.

AECs isolated from bronchial epithelium in humans and cultured with IFNγ have been shown to trigger proliferation of allogeneic lymphocytes as well (99). Additionally, investigations by Cunningham et al. demonstrated that the addition of anti-CD28 antibody as a co-stimulatory signal allows allogeneic $CD4^+$ T cells to proliferate in response to IFNγ-stimulated MHC class II^+ AECs (117). Further characterization by other groups shows that purified allogeneic T cells are stimulated in response to bronchial ECs, which is abolished by the addition of anti-DR antibody (118). Bronchial ECs have also been shown to present protein antigens to antigen-specific sensitized T cells, suggesting the ability of AECs to process and present foreign antigen to the underlying lymphoid tissue (119). Experiments utilizing electron microscopy verify that AECs stimulated with IFNγ are able to endocytose antigen and that, like IECs, uptake is polarized on the luminal side of tissue explant cultures. Co-localization studies further demonstrate the trafficking of these antigens through early and late endosomes to acid vesicles and lysosomes (120).

In vitro studies have important caveats. MHC class II is only expressed in the large intestine and potentially bronchi during disease, yet many commonly used intestinal cell lines (Caco-2, T84, and HT29) are colon-derived and pulmonary cell lines (BEAS-2B) bronchial in origin. Therefore, studies using these cell lines may be more representative of EC antigen presentation during inflammation rather than homeostasis. Colorectal cancer cells are also susceptible to genetic and epigenetic abnormalities, including changes in DNA methylation that affect CIITA expression (121). Small intestinal EC lines, such as HEC-6 and H4, exist, but are derived from fetal tissue and are more representative of crypt stem cells than fully differentiated ECs (122). Additionally, AECs are often derived from bronchoalveolar lavage brushings or fluid in patients with additional underlying pathologies, which are highly operator- and patient-dependent and may not be representative of the entire airway epithelium. Furthermore, *in vitro* experiments using peripheral blood T cells may not recapitulate interactions between ECs and organ-specific T cells. For instance, one study shows that IECs induce $CD4^+$ IELs to secrete IFNγ, but not $CD4^+$ T cells from the lamina propria or spleen (123). Moreover, $CD4^+$ and $CD8^+$ T cells found in adult human mucosa, including in both gut and lung, are largely memory cells, requiring different stimuli than naïve cells (124). Therefore, the complexity of the epithelium and the arrangements of the many cell types found within may not be well-represented in cultures of primary purified cell lines.

In vivo Evidence for ECs as Antigen Presenting Cells

Several *in vivo* studies of IEC antigen presentation have focused on IBD, where inflammatory responses to the gut microbiota are believed to elicit tissue damage, yet the role of IECs themselves remain poorly defined. Maggio-Price et al. induced colonic inflammation in $RAG2^{-/-}$ mice exclusively expressing MHC class II either on IECs or DCs. Animals with MHC class II^+ DCs developed severe colitis, whereas mice with MHC class II on IECs developed only mild inflammation (125). Additionally, mice lacking MHC class II on DCs appeared to develop intestinal inflammation due to lack of proper $CD4^+$ T cell-mediated adaptive immune responses to commensal bacteria, as gnotobiotic mice under the same conditions did not develop inflammation (126). In a different murine colitis model, Thelemann et al. showed that selectively knocking out MHC class II in IECs worsened colitis; additionally, mice without IEC MHC class II had higher IFNγ levels and a reduced proportion of Tregs (80). In this system, IECs failed to express CD40, CD80, or CD86 co-stimulatory molecules. Another elegant study targeted hemagglutinin (HA) expression to IECs in transgenic mice expressing an HA-specific T cell receptor. This resulted in the expansion of HA-specific Tregs and was not dependent on DCs acquiring antigen from apoptotic IECs. Isolated primary IECs directly stimulated Treg proliferation in an MHC class II-dependent manner (79). Interestingly, the authors ruled out TGF-β and retinoic acid as effectors—molecules known to skew naive T cells into induced Tregs (127). Together, this suggests a tolerogenic role for IECs that is not dependent on co-stimulation of CD80 or CD86. Similar *in vivo* data has not been collected in the respiratory tract of animal models, and effects on the lung epithelium were not evaluated in the above models.

Cytokine Regulation

Potential immune cell sources of IFNγ that upregulate MHC class II include natural killer (NK) and natural killer T (NKT) cells, group 1 ILCs (ILC1s), γδ T cells, $CD8^+$ T cells and subsets of $CD4^+$ T cells, which comprise the makeup of the GALT (128–132). The pIV isoform of the MHC class II transactivator CIITA is the main form expressed in non-hematopoietic cells in response to interferon gamma (IFNγ) and has been found in IECs (133, 134). Adoptive transfer of $CD4^+$ T cells into mice induces IEC MHC class II expression, whereas the transfer of IFNγ-knockout T cells does not (80). Direct treatment with IFNγ has been shown to increase AEC MHC II expression in rats both *in vitro* and *in vivo* (100, 119). These findings have been re-capitulated in human AECs *in vivo*, as well (99, 135). AECs may enhance MHC class II expression via a CIITA-independent pathway, at least when exposed to viral particles, though data appear conflicting (104, 136). A possible explanation for tissue-specific differences in cytokine regulation may be that IELs or other cell types that produce IFNγ to drive MHC class II in IECs, such as NK cells or ILC1, may be more abundant in the GALT compared to the airway, though further characterization is required (137–139).

To date, there is limited evidence that cytokines besides IFNγ induce MHC class II on non-hematopoietic cells during inflammation. One candidate is IL-27, an IL-12 superfamily cytokine released by activated DCs and elevated during intestinal inflammation (140). IL-27 elevates CIITA levels in colorectal cancer cells and stimulates endothelial cells *in vitro* to express HLA-DR, -DP, -DQ, -DM, and invariant chain (141, 142). The overlapping effects of IL-27 and IFNγ are consistent with the fact that both cytokines promote T helper 1 (Th1) $CD4^+$ T cell responses (143).

Another potential candidate is IL-18. IL-18, originally known as IFNγ inducing factor or IGIF, is a member of the IL-1 cytokine family and can mediate both Th1- and Th2-type responses (144–147). IL-18 has been shown to be elevated in autoimmune colitis including IBD and celiac disease and is a key mediator of intestinal homeostasis (144, 148–152). IEC appear to constitutively produce IL-18, at least *in vitro*, and promote increased production of IFNγ by T cells obtained from patients with active inflammation (153, 154). Interestingly, tissue explants from patients with active celiac disease show IL-18 expression only in the crypts (151). In a murine model of an NLRC4 inflammasome mutation, the intestinal epithelium appears solely responsible for the systemic elevation of IL-18 seen in macrophage activation syndrome and, furthermore, is associated with upregulation of IFNγ-induced genes and multiple genes associated with antigen presentation in the intestine (155). Recent evidence in humans shows that AEC also constitutively produce IL-18 *in vitro* in animal models (156, 157). IL-18 has been shown to directly upregulate MHC class II expression on IFNγ-stimulated keratinocytes, but this has not yet been explored in intestinal or airway epithelium (158). Whether EC-produced IL-18 is involved in paracrine MHC class II upregulation along the crypt-to-villus axis or through directing GALT-mediated IFNγ-production also remains unknown. Therefore, further investigation is needed to determine if these or other region-specific cytokines upregulate EC MHC class II expression.

Role of the Microbiome

Commensal bacteria reside within the lumen of the gut, reaching a density of up to 10^{12} cells per cm^3 in the large intestine (159). It is well-established that these microbes contribute to the development of the intestinal immune system; gnotobiotic mice, for example, do not form isolated lymphoid structures in the small intestine (160). Though the lung and gut share a common origin at the oropharynx, microbial populations are vastly different. The lung is not completely sterile but has a much lower bacterial burden without a characteristic microbiome like the gut; rather, lung flora tends to resemble oral flora and may change in response to a variety of stimuli and pathologies (161, 162).

Significantly, IECs of gnotobiotic mice lack MHC class II expression, while exposure to bacteria is found to increase IFNγ expression by γδ T cells and induce CIITA and MHC class II (163–165). There is limited but interesting evidence that specific classes of commensals, such as segmented filamentous bacteria, are sufficient to induce MHC class II in IECs (165). However, because γδ T cells compose a higher proportion of IELs in mice (∼50%) than in humans (∼10%), other cellular sources of class II inducing signals may be important in humans (166, 167). Additionally, the roles of viruses and fungi within the microbiome and their effects on EC MHC class II expression remain largely unexplored.

Recent evidence argues for a reciprocal effect of MHC class II in shaping the microbiome. Studies in natural fish populations link MHC class II allelic variation with the abundance of certain microbial taxa (168). These findings corroborate studies in laboratory mice, which show that MHC class II-linked changes in the microbiome mediate risk of enteric infection and autoimmune disease, such as type 1 diabetesc (169, 170). The precise mechanisms behind these effects remain poorly understood, though there is evidence that MHC class II polymorphisms control microbial populations through IgA phenotype and thus modify susceptibility to pathogens (169).

Exploration into the "gut-lung axis" in which the microbiome of the gut has direct impact on susceptibility to pulmonary disease is of key interest (171). The gut microbiome has been shown to affect lung susceptibility to infection with viral, fungal and bacterial pathogens (172–176). The severity of ozone-induced asthma in mice appears to be regulated by the gut microbiome through short chain fatty acid production (177). Segmented filamentous bacteria, a gut commensal that induces MHC class II on IECs as above, have independently been shown to provoke autoimmunity of the lung epithelium, but whether this is through affecting MHC class II expression on AECs is unknown (165, 176). The microbiome may even affect predisposition to lung cancer as evidenced through murine studies focused on probiotic use, though further mechanistic and human studies are still needed in this area, as well (171, 178).

DISCUSSION

Though much of the available evidence on MHC class II expression by ECs was obtained decades ago, this is an exciting time for research into the role of ECs in mucosal immunology. Renewed awareness of the role played by epithelial cells in homeostasis and disease and technical advances in different areas open up several new avenues for research and clinical applications.

Celiac disease, in which blunting of the villous tips on biopsy is pathognomonic, provides an example of a disease in which the role of the EC should be re-visited. Pathogenic $CD4^+$ T cells in celiac disease are DQ2- or DQ8-restricted, and T cell bound to DQ/gliadin tetramers are detectable using flow cytometry (179–182). Yet, most MHC class II studies examined in this review focus on HLA-DR. Cell surface HLA-DP and -DQ levels have been reported as lower, but T cells are remarkably sensitive, requiring as few as one to ten peptides per MHC II complexes for activation (183). Levels of MHC class II that are below the limit of detection by immunohistochemistry (used in many early papers) may therefore be sufficient to activate T cells. More sensitive techniques, such as flow cytometry or electron microscopy, are more informative, as evidenced by more recent papers. Another novel possibility is investigation utilizing multiplexed ion beam

imaging (MIBI) to visualize large panels of cell-surface proteins tagged with elemental metals that may allow improved detection of MHC II isoforms and co-localization of various co-stimulatory molecules on tissue sections (184). Using these technologies to study celiac disease, a model disease in which the inciting immunogen and the presenting MHC class II-molecules are known, may provide important insights into the role of ECs in antigen presentation.

The function of co-stimulatory molecules in this process is another area that requires more investigation. While some description of EC surface expression of classic B7 molecules, CD80 and CD86, is found in the literature (above), their roles during homeostasis and inflammation remain unclear. The lack of expression of CD86 found in the gut, compared to constitutive expression in the airway, may suggest a diminished role of IECs in interactions with Tregs (185). Another avenue of exploration are other members of the B7 family that appear to have novel roles on non-professional antigen presenting cells, including ICOS ligand, PD-L1 (B7-H1), PD-L2 (B7-DC), B7-H3, and B7-H4. Work on the ICOS co-stimulation pathway in the airway already has provided promising results, with anti-ICOS treatment leading to prevention of chronic lung transplant rejection and obliterative bronchiolitis as well as ICOS being shown as an important player in asthma (186, 187). However, the contribution of the aerodigestive epithelium in mediating these interactions remains to be explored.

Further delineation of the subsets and character of ECs are needed as well. The epithelium is composed of both stem cells and specialized subtypes as described above, many of which remain poorly understood. Both MHC II expression and antigen presenting capabilities and function may therefore differ among these cells. Work reviewed here has shown that, for example, M cells in the gut or type II pneumocytes in the lung may have roles in antigen presentation and expression of HLA-DR (9, 11, 16). Furthermore, the polarity and anatomic localization of intestinal and pulmonary ECs also likely bear significant implications for antigen uptake, processing and presentation and warrant further investigation (53, 55, 71–73, 78–80). Defining the roles of these various cell types and their locoregional interactions thus may provide additional important insights.

The conditions under which mucosal MHC class II contributes to inflammation vs. tolerance also remain to be clearly delineated. The work by Westendorf et al. in mouse models shows direct expansion of Tregs in response to ECs, arguing that mucosal MHC class II can function in a tolerogenic role, though the work by Dotan suggests that increased MHC class II on IECs from IBD patients may more efficiently activate effector T cell responses leading to inflammation (65, 79, 188). Moreover, it is plausible that MHC class II on ECs not only allows ECs to modulate immune responses, but also in fact allows the immune system to regulate the epithelium. Cytokines released by adjacent mucosal and intraepithelial immune cells in response to EC presentation of MHC class II-bound peptides may alter cell renewal, barrier integrity, cell type composition, and the innate immune functions of the epithelium. A promising approach to explore these questions is in organoids derived from stem cells or induced pluripotent stem cells that can differentiate into specialized cell types mimicking the physiological structure of the epithelium (189–192). Organoids offer a reductionist setting for testing the role of immune cells, cytokines, pathogens, and other regulatory factors on MHC class II in primary ECs in a way that appears to model organs physiologically. For example, mouse intestinal organoids treated with IFN have been shown to upregulate MHC class II (193). Intestinal organoids can be readily infected with human strains of enteric pathogens, such as rotavirus, norovirus, and *Salmonella* to allow exploration of MHC class II internalization and polarity during infection and inflammation (194–196). The study of lung organoids remains, comparatively, in its infancy, though work has been done to create structures resembling fetal lung buds in the second trimester of gestation for the study of respiratory syncytial virus (197). Organoids may provide a model system to study the aforementioned hypotheses to provide evidence more pertinent to humans.

Finally, current research is actively exploring the contributions of the microbiome to systemic immunity. However, how the microbiome changes EC structure and function, especially through MHC class II and co-stimulatory molecule expression, and whether this affects development of disease and ultimate outcomes are also key questions. Highlighting the importance of co-stimulatory molecules, recent clinical work has demonstrated that the efficacy of cancer immunotherapies targeting B7 molecules PD-L1 or CTLA-4 in epithelial cancers including non-small cell lung carcinoma as well as colon cancer appear keenly dependent on the gut microbiome; lack of or depletion of commensals using oral antibiotics appears to attenuate tumor response to these therapies (198, 199). Interestingly, blockade of CTLA-4 on IELs led to IEC apoptosis in intestinal organoids, also suggesting a bidirectional trophic communication between ECs and effector immune cells through co-stimulatory molecules (199). Valuable lessons may be learned by comparing MHC class II expression on ECs in the intestinal and respiratory tracts.

Ultimately, systems that integrate immunological, microbial, and environmental signals to study EC MHC class II expression and function are likely to advance our understanding of mucosal immunity and the epithelium of the aerodigestive tract. How these findings can be manipulated to affect infectious, autoimmune or even neoplastic diseases will likely be pursued in the coming years.

AUTHOR CONTRIBUTIONS

JW, DM, and EM were involved in planning the project. JW, DM, CM, and EM contributed to the writing of the final manuscript.

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REFERENCES

1. de Santa Barbara P, van den Brink GR, Roberts DJ. Molecular etiology of gut malformations and diseases. *Am J Med Genet.* (2002) 115:221–30. doi: 10.1002/ajmg.10978
2. Metzger R, Wachowiak R, Kluth D. Embryology of the early foregut. *Semin Pediatr Surg.* (2011) 20:136–44. doi: 10.1053/j.sempedsurg.2011.03.004
3. Gilbert S. *Developmental Biology*, 6th edn. Sunderland: Sinauer Associates (2000).
4. Mulholland M, Lillemoe KD, Doherty GM, Maier RV, Simeone DM, Upchurch Jr. GR. *Greenfield's Surgery*: *Scientific Principles and Practice*, 5th edn. Vol. 1. Philadelphia, PA: Wolters Kluwer Health (2011).
5. MacDonald TT, Monteleone G. Immunity, inflammation & allergy in the gut. *Science* (2005) 307:1920–5. doi: 10.1126/science.1106442
6. Hooper LV. Epithelial cell contributions to intestinal immunity. *Adv Immunol.* (2015) 126:129–72. doi: 10.1016/bs.ai.2014.11.003
7. McDole JR, Wheeler LW, McDonald KG, Wang B, Konjufca V, Knoop KA, et al. Goblet cells deliver luminal antigen to $CD103^{+}$ dendritic cells in the small intestine. *Nature* (2012) 483:345–9. doi: 10.1038/nature10863
8. Knoop KA, McDonald KG, McCrate S, McDole JR, Newberry RD. Microbial sensing by goblet cells controls immune surveillance of luminal antigens in the colon. *Mucosal Immunol.* (2015) 8:198–210. doi: 10.1038/mi.2014.58
9. Schulz O, Pabst O. Antigen sampling in the small intestine. *Trends Immunol.* (2013) 34:155–61. doi: 10.1016/j.it.2012.09.006
10. Niess JH, Brand S, Gu X, Landsman L, Jung S, McCormick BA, et al. CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. *Science* (2005) 307:254–8. doi: 10.1126/science.1102901
11. Kerneis S, Bogdanova A, Kraehenbuhl JP, Pringault E. Conversion by Peyer's patch lymphocytes of human enterocytes into M cells that transport bacteria. *Science* (1997) 277:949–52.
12. Mayrhofer G, Pugh CW, Barclay AN. The distribution, ontogeny and origin in the rat of Ia-positive cells with dendritic morphology and of Ia antigen in epithelia, with special reference to the intestine. *Eur J Immunol.* (1983) 13:112–22. doi: 10.1002/eji.1830130206
13. Spencer J, Finn T, Isaacson PG. Expression of HLA-DR antigens on epithelium associated with lymphoid tissue in the human gastrointestinal tract. *Gut* (1986) 27:153–7. doi: 10.1136/gut.27.2.153
14. Bjerke K, Brandtzaeg P. Lack of relation between expression of HLA-DR and secretory component (SC) in follicle-associated epithelium of human Peyer's patches. *Clin Exp Immunol.* (1988) 71:502–7.
15. MacDonald T, Weinel A, Spencer J. HLA-DR expression in human fetal intestinal epithelium. *Gut* (1988) 29:1342–8.
16. Nagura H, Ohtani H, Masuda T, Kimura M, Nakamura S. HLA-DR expression on M cells overlying Peyer's patches is a common feature of human small intestine. *Acta Pathol Jpn.* (1991) 41:818–23.
17. Gerbe F, Sidot E, Smyth DJ, Ohmoto M, Matsumoto I, Dardalhon V, et al. Intestinal epithelial tuft cells initiate type 2 mucosal immunity to helminth parasites. *Nature* (2016) 529:226–30. doi: 10.1038/nature16527
18. Banerjee A, McKinley ET, von Moltke J, Coffey RJ, Lau KS. Interpreting heterogeneity in intestinal tuft cell structure and function. *J Clin Invest.* (2018) 128:1711–9. doi: 10.1172/JCI120330
19. Okumura R, Takeda K. Roles of intestinal epithelial cells in the maintenance of gut homeostasis. *Exp Mol Med.* (2017) 49:e338. doi: 10.1038/emm.2017.20
20. Okumura R, Takeda K. Maintenance of intestinal homeostasis by mucosal barriers. *Inflamm Regen.* (2018) 38:5. doi: 10.1186/s41232-018-0063-z
21. Fukata M, Arditi M. The role of pattern recognition receptors in intestinal inflammation. *Mucosal Immunol.* (2013) 6:451–63. doi: 10.1038/mi.2013.13
22. Otte JM, Cario E, Podolsky DK. Mechanisms of cross hyporesponsiveness to Toll-like receptor bacterial ligands in intestinal epithelial cells. *Gastroenterology* (2004) 126:1054–70. doi: 10.1053/j.gastro.2004.01.007
23. Melmed G, Thomas LS, Lee N, Tesfay SY, Lukasek K, Michelsen KS, et al. Human intestinal epithelial cells are broadly unresponsive to Toll-like receptor 2-dependent bacterial ligands: implications for host-microbial interactions in the gut. *J Immunol.* (2003) 170:1406–15. doi: 10.4049/jimmunol.170.3.1406
24. Tam A, Wadsworth S, Dorscheid D, Man SF, Sin DD. The airway epithelium: more than just a structural barrier. *Ther Adv Respir Dis.* (2011) 5:255–73. doi: 10.1177/1753465810396539
25. Reid L, Meyrick B, Antony VB, Chang LY, Crapo JD, Reynolds HY. The mysterious pulmonary brush cell: a cell in search of a function. *Am J Respir Crit Care Med.* (2005) 172:136–9. doi: 10.1164/rccm.200502-203WS
26. Leiva-Juarez MM, Kolls JK, Evans SE. Lung epithelial cells: therapeutically inducible effectors of antimicrobial defense. *Mucosal Immunol.* (2018) 11:21–34. doi: 10.1038/mi.2017.71
27. Adriaensen D, Brouns I, Pintelon I, De Proost I, Timmermans JP. Evidence for a role of neuroepithelial bodies as complex airway sensors: comparison with smooth muscle-associated airway receptors. *J Appl Physiol. (1985)* (2006) 101:960–970. doi: 10.1152/japplphysiol.00267.2006
28. Hong KU, Reynolds SD, Watkins S, Fuchs E, Stripp BR. *In vivo* differentiation potential of tracheal basal cells: evidence for multipotent and unipotent subpopulations. *Am J Physiol Lung Cell Mol Physiol.* (2004) 286:L643–9. doi: 10.1152/ajplung.00155.2003
29. Knight DA, Holgate ST. The airway epithelium: structural and functional properties in health and disease. *Respirology* (2003) 8:432–446. doi: 10.1046/j.1440-1843.2003.00493.x
30. Jeffery PK. Morphologic features of airway surface epithelial cells and glands. *Am Rev Respir Dis.* (1983) 128:S14–20. doi: 10.1164/arrd.1983.128.2P2.S14
31. Spina D. Epithelium smooth muscle regulation and interactions. *Am J Respir Crit Care Med.* (1998) 158:S141–5. doi: 10.1164/ajrccm.158.supplement_2.13tac100a
32. De RW, Willems L, Van GM, Franken C, Fransen J, Dijkman J, et al. Ultrastructural localization of bronchial antileukoprotease in central and peripheral human airways by a gold-labeling technique using monoclonal antibodies. *Am Rev Respir Dis.* (1986) 133:882–90.
33. Dobbs LG, Johnson MD. Alveolar epithelial transport in the adult lung. *Respir Physiol Neurobiol.* (2007) 159:283–300. doi: 10.1016/j.resp.2007.06.011
34. Wright JR. Immunoregulatory functions of surfactant proteins. *Nat Rev Immunol.* (2005) 5:58–68. doi: 10.1038/nri1528
35. Hiller AS, Tschernig T, Kleemann WJ, Pabst R. Bronchus-associated lymphoid tissue (BALT) and larynx-associated lymphoid tissue (LALT) are found at different frequencies in children, adolescents and adults. *Scand J Immunol.* (1998) 47:159–62.
36. Tschernig T, Pabst R. Bronchus-associated lymphoid tissue (BALT) is not present in the normal adult lung but in different diseases. *Pathobiology* (2000) 68:1–8. doi: 10.1159/000028109
37. Gay NJ, Symmons MF, Gangloff M, Bryant CE. Assembly and localization of Toll-like receptor signalling complexes. *Nat Rev Immunol.* (2014) 14:546–58. doi: 10.1038/nri3713
38. Evans SE, Xu Y, Tuvim MJ, Dickey BF. Inducible innate resistance of lung epithelium to infection. *Annu Rev Physiol.* (2010) 72:413–35. doi: 10.1146/annurev-physiol-021909-135909
39. Opitz B, van Laak V, Eitel J, Suttorp N. Innate immune recognition in infectious and noninfectious diseases of the lung. *Am J Respir Crit Care Med.* (2010) 181:1294–309. doi: 10.1164/rccm.200909-1427SO
40. Heyl KA, Klassert TE, Heinrich A, Müller MM, Klaile E, Dienemann H, et al. Dectin-1 is expressed in human lung and mediates the proinflammatory immune response to nontypeable Haemophilus influenzae. *MBio* (2014) 5:e01492-01414. doi: 10.1128/mBio.01492-14.
41. Nayak A, Dodagatta-Marri E, Tsolaki AG, Kishore U. An insight into the diverse roles of surfactant proteins, SP-A and SP-D in innate and adaptive immunity. *Front Immunol.* (2012) 3:131. doi: 10.3389/fimmu.2012.00131
42. Jones PP, Murphy DB, Hewgill D, McDevitt HO. Detection of a common polypeptide chain in IA and IE sub-region immunoprecipitates. *Mol Immunol.* (1979) 16:51–60.
43. Cloutier M, Gauthier C, Fortin JS, Geneve L, Kim K, Gruenheid S, et al. ER egress of invariant chain isoform p35 requires direct binding to MHCII molecules and is inhibited by the NleA virulence factor of enterohaemorrhagic Escherichia coli. *Hum Immunol.* (2015) 76:292–6. doi: 10.1016/j.humimm.2015.02.002
44. Neefjes J, Jongsma MLM, Paul P, Bakke O. Towards a systems understanding of MHC class I and MHC class II antigen presentation. *Nat Rev Immunol.* (2011) 11:823–36. doi: 10.1038/nri3084

45. Mellins ED, Stern LJ. HLA-DM and HLA-DO, key regulators of MHC-II processing and presentation. *Curr Opin Immunol.* (2014) 26:115–22. doi: 10.1016/j.coi.2013.11.005
46. Guce AI, Mortimer SE, Yoon T, Painter CA, Jiang W, Mellins ED, et al. HLA-DO acts as a substrate mimic to inhibit HLA-DM by a competitive mechanism. *Nat Struct Mol Biol.* (2013) 20:90–8. doi: 10.1038/nsmb.2460
47. Greenwald RJ, Freeman GJ, Sharpe AH. The B7 family revisited. *Annu Rev Immunol.* (2005) 23:515–48. doi: 10.1146/annurev.immunol.23.021704.115611
48. Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, et al. A toll-like receptor recognizes bacterial DNA. *Nature* (2000) 408:740–5. doi: 10.1038/35047123
49. Alegre M-L, Frauwirth KA, Thompson CB. T-cell regulation by CD28 and CTLA-4. *Nat Rev Immunol.* (2001) 1:220–8. doi: 10.1038/35105024
50. Ramig RF. Pathogenesis of intestinal and systemic rotavirus infection. *J Virol.* (2004) 78:10213–20. doi: 10.1128/JVI.78.19.10213-10220.2004
51. Peterson LW, Artis D. Intestinal epithelial cells: regulators of barrier function and immune homeostasis. *Nat Rev Immunol.* (2014) 14:141–53. doi: 10.1038/nri3608
52. Wiman K, Curman B, Forsum U, Klareskog L, MalmnäS-Tjernlund U, Rask L, et al. Occurrence of Ia antigens on tissues of non-lymphoid origin. *Nature* (1978) 276:711–3.
53. Scott H, Solheim BG, Brandtzaeg P, Thorsby E. HLA-DR-like antigens in the epithelium of the human small intestine. *Scand J Immunol.* (1980) 12:77–82. doi: 10.1111/j.1365-3083.1980.tb00043.x
54. Parr EL, McKenzie IFC. Demonstration of Ia antigens on mouse intestinal epithelial cells by immunoferritin labeling. *Immunogenetics* (1979) 8:499–508. doi: 10.1007/bf01561459
55. Chiba M, Iizuka M, Masamune O. Ubiquitous expression of HLA-DR antigens on human small intestinal epithelium. *Gastroenterol Jpn.* (1988) 23:109–16. doi: 10.1007/bf02799021
56. Lin XP, Almqvist N, Telemo E. Human small intestinal epithelial cells constitutively express the key elements for antigen processing and the production of exosomes. *Blood Cells Mol Dis.* (2005) 35:122–8. doi: 10.1016/j.bcmd.2005.05.011
57. Byrne B, Madrigal-Estebas L, McEvoy A, Carton J, Doherty DG, Whelan A, et al. Human duodenal epithelial cells constitutively express molecular components of antigen presentation but not costimulatory molecules. *Hum Immunol.* (2002) 63:977–86. doi: 10.1016/S0198-8859(02)00436-6
58. Oliver AM, Thomson AW, Sewell HF, Abramovch DR. Major histocompatibility complex (MHC) class II antigen (HLA-DR, DQ & DP) expression in human fetal endocrine organs and gut. *Scand J Immunol.* (1988) 27:731–7. doi: 10.1111/j.1365-3083.1988.tb02407.x
59. Natali PG, De Martino C, Pellegrino MA, Ferrone S. Analysis of the expression of I-Ak-like antigens in murine fetal and adult tissues with the monoclonal antibody 10–2.16. *Scand J Immunol.* (1981) 13:541–6. doi: 10.1111/j.1365-3083.1981.tb00167.x
60. Koretz K, Momburg F, Otto HF, Moller P. Sequential induction of MHC antigens on autochthonous cells of ileum affected by Crohn's disease. *Am J Pathol.* (1987) 129:493–502.
61. Momburg F, Koretz K, Von Herbay A, Moller P. Nonimmune human cells can express MHC class II antigens in the absence of invariant chain–an immunohistological study on normal and chronically inflamed small intestine. *Clin Exp Immunol.* (1988) 72:367–72.
62. Fais S, Maiuri L, Pallone F, De Vincenzi M, De Ritis G, Troncone R, et al. Gliadin induced changes in the expression of MHC-class II antigens by human small intestinal epithelium. Organ culture studies with coeliac disease mucosa. *Gut* (1992) 33:472–5.
63. Kelly J, O'Farrelly C, O'Mahony C, Weir D, Feighery C. Immunoperoxidase demonstration of the cellular composition of the normal and coeliac small bowel. *Clin Exp Immunol.* (1987) 68:177.
64. Mason DW, Dallman M, Barclay AN. Graft-versus-host disease induces expression of Ia antigen in rat epidermal cells and gut epithelium. *Nature* (1981) 293:150–1.
65. Dotan I, Allez M, Nakazawa A, Brimnes J, Schulder-Katz M, Mayer L. Intestinal epithelial cells from inflammatory bowel disease patients preferentially stimulate $CD4^+$ T cells to proliferate and secrete interferon-γ. *Am J Physiol. Gastrointest Liver Physiol.* (2007) 292, G1630–40. doi: 10.1152/ajpgi.00294.2006
66. Mayer L, Eisenhardt D, Salomon P, Bauer W, Plous R, Piccinini L. Expression of class II molecules on intestinal epithelial cells in humans. Differences between normal and inflammatory bowel disease. *Gastroenterology* (1991) 100:3–12.
67. Zimmer KP, Poremba C, Weber P, Ciclitira PJ, Harms E. Translocation of gliadin into HLA-DR antigen containing lysosomes in coeliac disease enterocytes. *Gut* (1995) 36:703–9.
68. Arnaud-Battandier F, Cerf-Bensussan N, Amsellem R, Schmitz J. Increased HLA-DR expression by enterocytes in children with celiac disease. *Gastroenterology* (1986) 91:1206–12. doi: 10.1016/S0016-5085(86)80018-X
69. Colgan SP, Resnick MB, Parkos CA, Delp-Archer C, McGuirk D, Bacarra AE, et al. IL-4 directly modulates function of a model human intestinal epithelium. *J Immunol.* (1994) 153:2122–9.
70. Niessner M, Volk BA. Altered Th1/Th2 cytokine profiles in the intestinal mucosa of patients with inflammatory bowel disease as assessed by quantitative reversed transcribed polymerase chain reaction (RT-PCR). *Clin Exp Immunol.* (1995) 101:428–35. doi: 10.1111/j.1365-2249.1995.tb03130.x
71. Rojas R, Apodaca G. Immunoglobulin transport across polarized epithelial cells. *Nat Rev Mol Cell Biol.* (2002) 3:944–56. doi: 10.1038/nrm972
72. Daar AS, Fuggle SV, Fabre JW, Ting A, Morris PJ. The detailed distribution of class II antigens in normal human organs. *Transplantation* (1984) 38:293–8.
73. Hirata I, Austin LL, Blackwell WH, Weber JR, Dobbins III WO. Immunoelectron microscopic localization of HLA-DR antigen in control small intestine and colon and in inflammatory bowel disease. *Digest Dis Sci.* (1986) 31:1317–30.
74. Sarles J, Gorvel JP, Olive D, Maroux S, Mawas C, Giraud F. Subcellular localization of class I (A,B,C) and class II (DR and DQ) MHC antigens in jejunal epithelium of children with coeliac disease. *J Pediatr Gastroenterol Nutr.* (1987) 6:51–6.
75. Hundorfean G, Zimmer KP, Strobel S, Gebert A, Ludwig D, Büning J. Luminal antigens access late endosomes of intestinal epithelial cells enriched in MHC I and MHC II molecules: *in vivo* study in Crohn's ileitis. *Am J Physiol Gastrointest Liver Physiol.* (2007) 293:G798–808. doi: 10.1152/ajpgi.00135.2007
76. Arnold MM, Srivastava S, Fredenburgh J, Stockard CR, Myers RB, Grizzle WE. Effects of fixation and tissue processing on immunohistochemical demonstration of specific antigens. *Biotech Histochem.* (1996) 71:224–30.
77. Shi SR, Liu C, Pootrakul L, Tang L, Young A, Chen R, et al. Evaluation of the value of frozen tissue section used as "gold standard" for immunohistochemistry. *Am J Clin Pathol.* (2008) 129:358–66. doi: 10.1309/7cxuyxt23e5al8kq
78. Hershberg RM, Cho DH, Youakim A, Bradley MB, Lee JS, Framson PE, et al. Highly polarized HLA class II antigen processing and presentation by human intestinal epithelial cells. *J Clin Invest.* (1998) 102:792–803.
79. Westendorf AM, Fleissner D, Groebe L, Jung S, Gruber AD, Hansen W, et al. $CD4^+Foxp3^+$ regulatory T cell expansion induced by antigen-driven interaction with intestinal epithelial cells independent of local dendritic cells. *Gut* (2009) 58:211–9. doi: 10.1136/gut.2008.151720
80. Thelemann C, Eren RO, Coutaz M, Brasseit J, Bouzourene H, Rosa M, et al. Interferon-γ induces expression of MHC class II on intestinal epithelial cells and protects mice from colitis. *PLoS ONE* (2014) 9:e86844. doi: 10.1371/journal.pone.0086844
81. Bar F, Sina C, Hundorfean G, Pagel R, Lehnert H, Fellermann K, et al. Inflammatory bowel diseases influence major histocompatibility complex class I (MHC I) and II compartments in intestinal epithelial cells. *Clin Exp Immunol.* (2013) 172:280–9. doi: 10.1111/cei.12047
82. Uhlen M, Fagerberg L, Hallstrom BM, Lindskog C, Oksvold P, Mardinoglu A, et al. Tissue-based map of the human proteome. *Science* (2015) 347:1260419. doi: 10.1126/science.1260419
83. Walseng E, Furuta K, Goldszmid RS, Weih KA, Sher A, Roche PA. Dendritic cell activation prevents MHC class II ubiquitination and promotes MHC

class II survival regardless of the activation stimulus. *J Biol Chem.* (2010) 285:41749–54. doi: 10.1074/jbc.M110.157586
84. Sanderson IR, Ouellette AJ, Carter EA, Walker WA, Harmatz PR. Differential regulation of B7 mRNA in enterocytes and lymphoid cells. *Immunology* (1993) 79:434–8.
85. Nakazawa A, Watanabe M, Kanai T, Yajima T, Yamazaki M, Ogata H, et al. Functional expression of costimulatory molecule CD86 on epithelial cells in the inflamed colonic mucosa. *Gastroenterology* (1999) 117:536–45.
86. Bloom S, Simmons D, Jewell DP. Adhesion molecules intercellular adhesion molecule-1 (ICAM-1), ICAM-3 and B7 are not expressed by epithelium in normal or inflamed colon. *Clin Exp Immunol.* (1995) 101:157–63.
87. Borcherding F, Nitschke M, Hundorfean G, Rupp J, von Smolinski D, Bieber K, et al. The CD40-CD40L pathway contributes to the proinflammatory function of intestinal epithelial cells in inflammatory bowel disease. *Am J Pathol.* (2010) 176:1816–27. doi: 10.2353/ajpath.2010.090461
88. Cayabyab M, Phillips JH, Lanier LL. CD40 preferentially costimulates activation of $CD4^+$ T lymphocytes. *J Immunol.* (1994) 152:1523–31.
89. Leitner J, Herndler-Brandstetter D, Zlabinger GJ, Grubeck-Loebenstein B, Steinberger P. CD58/CD2 is the primary costimulatory pathway in human $CD28\text{-}CD8^+$ T cells. *J Immunol.* (2015) 195:477–87. doi: 10.4049/jimmunol.1401917
90. Framson PE, Cho DH, Lee LY, Hershberg RM. Polarized expression and function of the costimulatory molecule CD58 on human intestinal epithelial cells. *Gastroenterology* (1999) 116:1054–62.
91. Peters U, Papadopoulos T, Muller-Hermelink HK. MHC class II antigens on lung epithelial of human fetuses and neonates. Ontogeny and expression in lungs with histologic evidence of infection. *Lab Invest.* (1990) 63:38–43.
92. Badve S, Deshpande C, Hua Z, Logdberg L. Expression of invariant chain (CD 74) and major histocompatibility complex (MHC) class II antigens in the human fetus. *J Histochem Cytochem.* (2002) 50:473–82. doi: 10.1177/002215540205000404
93. Glanville AR, Tazelaar HD, Theodore J, Imoto E, Rouse RV, Baldwin JC, et al. The distribution of MHC class I and II antigens on bronchial epithelium. *Am Rev Respir Dis.* (1989) 139:330–4. doi: 10.1164/ajrccm/139.2.330
94. Rossi GA, Sacco O, Balbi B, Oddera S, Mattioni T, Corte G, et al. Human ciliated bronchial epithelial cells: expression of the HLA-DR antigens and of the HLA-DR alpha gene, modulation of the HLA-DR antigens by gamma-interferon and antigen-presenting function in the mixed leukocyte reaction. *Am J Respir Cell Mol Biol.* (1990) 3:431–9. doi: 10.1165/ajrcmb/3.5.431
95. Cunningham AC, Milne DS, Wilkes J, Dark JH, Tetley TD, Kirby JA. Constitutive expression of MHC and adhesion molecules by alveolar epithelial cells (type II pneumocytes) isolated from human lung and comparison with immunocytochemical findings. *J Cell Sci.* (1994) 107:443–9.
96. Kallenberg CG, Schilizzi BM, Beaumont F, Poppema S, De Leij L, The TH. Expression of class II MHC antigens on alveolar epithelium in fibrosing alveolitis. *Clin Exp Immunol.* (1987) 67:182–90.
97. Kaneko Y, Kuwano K, Kunitake R, Kawasaki M, Hagimoto N, Hara N. B7-1, B7-2 and class II MHC molecules in idiopathic pulmonary fibrosis and bronchiolitis obliterans-organizing pneumonia. *Eur Respir J.* (2000) 15:49–55. doi: 10.1034/j.1399-3003.2000.15a10.x
98. Papi A, Stanciu LA, Papadopoulos NG, Teran LM, Holgate ST, Johnston SL. Rhinovirus infection induces major histocompatibility complex class I and costimulatory molecule upregulation on respiratory epithelial cells. *J Infect Dis.* (2000) 181:1780–4. doi: 10.1086/315463
99. Tanaka H, Maeda K, Nakamura Y, Azuma M, Yanagawa H, Sone S. CD40 and IFN-gamma dependent T cell activation by human bronchial epithelial cells. *J Med Invest.* (2001) 48:109–17.
100. Steiniger B, Sickel E. Class II MHC molecules and monocytes/macrophages in the respiratory system of conventional, germ-free and interferon-gamma-treated rats. *Immunobiology* (1992) 184:295–310. doi: 10.1016/S0171-2985(11)80588-7
101. Sacco O, Lantero S, Scarso L, Galietta LJ, Spallarossa D, Silvestri M, et al. Modulation of HLA-DR antigen and ICAM-1 molecule expression on airway epithelial cells by sodium nedocromil. *Ann Allergy Asthma Immunol.* (1999) 83:49–54. doi: 10.1016/S1081-1206(10)63512-0
102. Chang SC, Hsu HK, Perng RP, Shiao GM, Lin CY. Increased expression of MHC class II antigens in rejecting canine lung allografts. *Transplantation* (1990) 49:1158–63.
103. Vignola AM, Campbell AM, Chanez P, Bousquet J, Paul-Lacoste P, Michel FB, et al. HLA-DR and ICAM-1 expression on bronchial epithelial cells in asthma and chronic bronchitis. *Am Rev Respir Dis.* (1993) 148:689–94. doi: 10.1164/ajrccm/148.3.689
104. Gao J, De BP, Banerjee AK. Human parainfluenza virus type 3 up-regulates major histocompatibility complex class I and II expression on respiratory epithelial cells: involvement of a STAT1- and CIITA-independent pathway. *J Virol.* (1999) 73:1411–8.
105. Elssner A, Jaumann F, Wolf WP, Schwaiblmair M, Behr J, Furst H, et al. Bronchial epithelial cell B7-1 and B7-2 mRNA expression after lung transplantation: a role in allograft rejection? *Eur Respir J.* (2002) 20:165–9. doi: 10.1183/09031936.02.00268102
106. King TE. *Cryptogenic Organizing Pneumonia* (2017). Available online at: https://www.uptodate.com/contents/cryptogenic-organizing-pneumonia.
107. Hershberg RM, Framson PE, Cho DH, Lee LY, Kovats S, Beitz J, et al. Intestinal epithelial cells use two distinct pathways for HLA class II antigen processing. *J Clin Invest.* (1997) 100:204–15.
108. London CA, Lodge MP, Abbas AK. Functional responses and costimulator dependence of memory $CD4^+$ T cells. *J Immunol.* (2000) 164:265–72. doi: 10.4049/jimmunol.164.1.265
109. Croft M, Bradley LM, Swain SL. Naive versus memory CD4 T cell response to antigen. Memory cells are less dependent on accessory cell costimulation and can respond to many antigen-presenting cell types including resting B cells. *J Immunol.* (1994) 152:2675–85.
110. Hollander D. Crohn's disease–a permeability disorder of the tight junction? *Gut* (1988) 29:1621–4. doi: 10.1136/gut.29.12.1621
111. Arnott ID, Kingstone K, Ghosh S. Abnormal intestinal permeability predicts relapse in inactive Crohn disease. *Scand J Gastroenterol.* (2000) 35:1163–9. doi: 10.1080/003655200750056637
112. Smecuol E, Bai JC, Vazquez H, Kogan Z, Cabanne A, Niveloni S, et al. Gastrointestinal permeability in celiac disease. *Gastroenterology* (1997) 112:1129–36.
113. Raposo G, Stoorvogel W. Extracellular vesicles: exosomes, microvesicles & friends. *J Cell Biol.* (2013) 200:373–83. doi: 10.1083/jcb.201211138
114. van Niel G, Raposo G, Candalh C, Boussac M, Hershberg R, Cerf-Bensussan N, et al. Intestinal epithelial cells secrete exosome-like vesicles. *Gastroenterology* (2001) 121:337–49. doi: 10.1053/gast.2001.26263
115. Van Niel G, Mallegol J, Bevilacqua C, Candalh C, Brugiere S, Tomaskovic-Crook E, et al. Intestinal epithelial exosomes carry MHC class II/peptides able to inform the immune system in mice. *Gut* (2003) 52:1690–7. doi: 10.1136/gut.52.12.1690
116. Mallegol J, Van Niel G, Lebreton C, Lepelletier Y, Candalh C, Dugave C, et al. T84-intestinal epithelial exosomes bear MHC class II/peptide complexes potentiating antigen presentation by dendritic cells. *Gastroenterology* (2007) 132:1866–76. doi: 10.1053/j.gastro.2007.02.043
117. Cunningham AC, Zhang JG, Moy JV, Ali S, Kirby JA. A comparison of the antigen-presenting capabilities of class II MHC-expressing human lung epithelial and endothelial cells. *Immunology* (1997) 91:458–63.
118. Kalb TH, Chuang MT, Marom Z, Mayer L. Evidence for accessory cell function by class II MHC antigen-expressing airway epithelial cells. *Am J Respir Cell Mol Biol.* (1991) 4:320–9. doi: 10.1165/ajrcmb/4.4.320
119. Suda T, Sato A, Sugiura W, Chida, K. Induction of MHC class II antigens on rat bronchial epithelial cells by interferon-gamma and its effect on antigen presentation. *Lung* (1995) 173:127–37.
120. Salik E, Tyorkin M, Mohan S, George I, Becker K, Oei E, et al. Antigen trafficking and accessory cell function in respiratory epithelial cells. *Am J Respir Cell Mol Biol.* (1999) 21:365–79. doi: 10.1165/ajrcmb.21.3.3529
121. Satoh A, Toyota M, Ikeda H, Morimoto Y, Akino K, Mita H, et al. Epigenetic inactivation of class II transactivator (CIITA) is associated with the absence of interferon-[gamma]-induced HLA-DR expression in colorectal and gastric cancer cells. *Oncogene* (2004) 23:8876–8886. doi: 10.1038/sj.onc.1208144
122. Cencič A, Langerholc T. Functional cell models of the gut and their applications in food microbiology—a review. *Int J Food Microbiol.* (2010) 141:S4–14. doi: 10.1016/j.ijfoodmicro.2010.03.026
123. Hatano R, Yamada K, Iwamoto T, Maeda N, Emoto T, Shimizu M, et al. Antigen presentation by small intestinal epithelial cells uniquely enhances IFN-gamma secretion from $CD4^+$ intestinal intraepithelial

lymphocytes. *Biochem Biophys Res Commun.* (2013) 435:592–6. doi: 10.1016/j.bbrc.2013.05.024
124. Thome JJ, Bickham KL, Ohmura Y, Kubota M, Matsuoka N, Gordon C, et al. Early-life compartmentalization of human T cell differentiation and regulatory function in mucosal and lymphoid tissues. *Nat Med.* (2016) 22:72–77. doi: 10.1038/nm.4008
125. Maggio-Price L, Seamons A, Bielefeldt-Ohmann H, Zeng W, Brabb T, Ware C, et al. Lineage targeted MHC-II transgenic mice demonstrate the role of dendritic cells in bacterial-driven colitis. *Inflamm Bowel Dis.* (2013) 19:174–84. doi: 10.1002/ibd.23000
126. Loschko J, Schreiber HA, Rieke GJ, Esterhazy D, Meredith MM, Pedicord VA, et al. Absence of MHC class II on cDCs results in microbial-dependent intestinal inflammation. *J Exp Med.* (2016) 213:517–34. doi: 10.1084/jem.20160062
127. Coombes JL, Siddiqui KR, Arancibia-Carcamo CV, Hall J, Sun CM, Belkaid Y, et al. A functionally specialized population of mucosal $CD103^+$ DCs induces $Foxp3^+$ regulatory T cells via a TGF-β-and retinoic acid–dependent mechanism. *J Exp Med.* (2007) 204:1757–64. doi: 10.1084/jem.20070590
128. Fuchs A, Vermi W, Lee JS, Lonardi S, Gilfillan S, Newberry RD, et al. Intraepithelial type 1 innate lymphoid cells are a unique subset of IL-12- and IL-15-responsive IFN-γ-producing cells. *Immunity* (2013) 38:769–81. doi: 10.1016/j.immuni.2013.02.010
129. Ferrick DA, Schrenzel MD, Mulvania T, Hsieh B. Differential production of interferon-gamma and interleukin-4 in response to Th1-and Th2-stimulating pathogens by gammadelta T cells *in vivo*. *Nature* (1995) 373:255.
130. Vivier E, Tomasello E, Baratin M, Walzer T, Ugolini S. Functions of natural killer cells. *Nat Immunol.* (2008) 9:503–10. doi: 10.1038/ni1582
131. Skeen MJ, Ziegler HK. Activation of gamma delta T cells for production of IFN-gamma is mediated by bacteria via macrophage-derived cytokines IL-1 and IL-12. *J Immunol.* (1995) 154:5832–41.
132. Loh L, Ivarsson M, Michaelsson J, Sandberg J, Nixon DF. Invariant natural killer T cells developing in the human fetus accumulate and mature in the small intestine. *Mucosal Immunol.* (2014) 7:1233–43. doi: 10.1038/mi.2014.13
133. Reith W, LeibundGut-Landmann S, Waldburger JM. Regulation of MHC class II gene expression by the class II transactivator. *Nat Rev Immunol.* (2005) 5:793–806. doi: 10.1038/nri1708
134. Sanderson IR, Bustin SA, Dziennis S, Paraszczuk J, Stamm DS. Age and diet act through distinct isoforms of the class II transactivator gene in mouse intestinal epithelium. *Gastroenterology* (2004) 127:203–212. doi: 10.1053/j.gastro.2004.04.014
135. Ibrahim L, Dominguez M, Yacoub M. Primary human adult lung epithelial cells *in vitro*: response to interferon-gamma and cytomegalovirus. *Immunology* (1993) 79:119–24.
136. Radosevich M, Ono SJ. MHC class II gene expression is not induced in HPIV3-infected respiratory epithelial cells. *Immunol Res.* (2004) 30:125–38. doi: 10.1385/IR:30:2:125
137. Mowat AM, Agace WW. Regional specialization within the intestinal immune system. *Nat Rev Immunol.* (2014) 14:667–85. doi: 10.1038/nri3738
138. Jarvinen TT, Collin P, Rasmussen M, Kyrönpalo S, Mäki M, Partanen J, et al. Villous tip intraepithelial lymphocytes as markers of early-stage coeliac disease. *Scand J Gastroenterol.* (2004) 39:428–33. doi: 10.1080/00365520310008773
139. Spits H, Bernink JH, Lanier L. NK cells and type 1 innate lymphoid cells: partners in host defense. *Nat Immunol.* (2016) 17:758–64. doi: 10.1038/ni.3482
140. Pflanz S, Timans JC, Cheung J, Rosales R, Kanzler H, Gilbert J, et al. IL-27, a Heterodimeric cytokine composed of EBI3 and p28 protein, induces proliferation of naive $CD4^+$ T cells. *Immunity* (2002) 16:779–90. doi: 10.1016/S1074-7613(02)00324-2
141. Diegelmann J, Olszak T, Goke B, Blumberg RS, Brand S. A novel role for interleukin-27 (IL-27) as mediator of intestinal epithelial barrier protection mediated via differential signal transducer and activator of transcription (STAT) protein signaling and induction of antibacterial and anti-inflammatory proteins. *J Biol Chem.* (2012) 287:286–98. doi: 10.1074/jbc.M111.294355
142. Feng XM, Chen XL, Liu N, Chen Z, Zhou YL, Han ZB, et al. Interleukin-27 upregulates major histocompatibility complex class II expression in primary human endothelial cells through induction of major histocompatibility complex class II transactivator. *Hum Immunol.* (2007) 68:965–72. doi: 10.1016/j.humimm.2007.10.004
143. Hunter CA. New IL-12-family members: IL-23 and IL-27, cytokines with divergent functions. *Nat Rev Immunol.* (2005) 5:521–31. doi: 10.1038/nri1648
144. Lopetuso LR, Chowdhry S, Pizarro TT. Opposing functions of classic and novel IL-1 family members in gut health and disease. *Front Immunol.* (2013) 4:181. doi: 10.3389/fimmu.2013.00181
145. Okamura H, Nagata K, Komatsu T, Tanimoto T, Nukata Y, Tanabe F, et al. A novel costimulatory factor for gamma interferon induction found in the livers of mice causes endotoxic shock. *Infect Immun.* (1995) 63:3966–72.
146. Kohno K, Kataoka J, Ohtsuki T, Suemoto Y, Okamoto I, Usui M, et al. IFN-gamma-inducing factor (IGIF) is a costimulatory factor on the activation of Th1 but not Th2 cells and exerts its effect independently of IL-12. *J Immunol.* (1997) 158:1541–50.
147. Nakanishi K, Yoshimoto T, Tsutsui H, Okamura H. Interleukin-18 is a unique cytokine that stimulates both Th1 and Th2 responses depending on its cytokine milieu. *Cytokine Growth Factor Rev.* (2001) 12:53–72. doi: 10.1016/S1359-6101(00)00015-0
148. Pizarro TT, Michie MH, Bentz M, Woraratanadharm J, Smith MF Jr., Foley E, et al. IL-18, a novel immunoregulatory cytokine, is up-regulated in Crohn's disease: expression and localization in intestinal mucosal cells. *J Immunol.* (1999) 162:6829–35.
149. Monteleone G, Trapasso F, Parrello T, Biancone L, Stella A, Iuliano R, et al. Bioactive IL-18 expression is up-regulated in Crohn's disease. *J Immunol.* (1999) 163:143–7.
150. Kanai T, Watanabe M, Okazawa A, Nakamaru K, Okamoto M, Naganuma M, et al. Interleukin 18 is a potent proliferative factor for intestinal mucosal lymphocytes in Crohn's disease. *Gastroenterology* (2000) 119:1514–23. doi: 10.1053/gast.2000.20260
151. Leon AJ, Garrote JA, Blanco-Quiros A, Calvo C, Fernandez-Salazar L, Del Villar A, et al. Interleukin 18 maintains a long-standing inflammation in coeliac disease patients. *Clin Exp Immunol.* (2006) 146:479–85. doi: 10.1111/j.1365-2249.2006.03239.x
152. Salcedo R, Worschech A, Cardone M, Jones Y, Gyulai Z, Dai RM, et al. MyD88-mediated signaling prevents development of adenocarcinomas of the colon: role of interleukin 18. *J Exp Med.* (2010) 207:1625–36. doi: 10.1084/jem.20100199
153. Maerten P, Shen C, Colpaert S, Liu Z, Bullens DA, van Assche G, et al. Involvement of interleukin 18 in Crohn's disease: evidence from *in vitro* analysis of human gut inflammatory cells and from experimental colitis models. *Clin Exp Immunol.* (2004) 135:310–7. doi: 10.1111/j.1365-2249.2004.02362.x
154. Kolinska J, Lisa V, Clark JA, Kozakova H, Zakostelecka M, Khailova L, et al. Constitutive expression of IL-18 and IL-18R in differentiated IEC-6 cells: effect of TNF-alpha and IFN-gamma treatment. *J Interferon Cytokine Res.* (2008) 28:287–96. doi: 10.1089/jir.2006.0130
155. Weiss ES, Girard-Guyonvarc'h C, Holzinger D, de Jesus AA, Tariq Z, Picarsic J. Interleukin-18 diagnostically distinguishes and pathogenically promotes human and murine macrophage activation syndrome. *Blood* (2018) 131:1442–55. doi: 10.1182/blood-2017-12-820852
156. Cameron LA, Taha RA, Tsicopoulos A, Kurimoto M, Olivenstein R, Wallaert B, et al. Airway epithelium expresses interleukin-18. *Eur Respir J.* (1999) 14:553–9.
157. Muneta Y, Goji N, Tsuji NM, Mikami O, Shimoji Y, Nakajima Y, et al. Expression of interleukin-18 by porcine airway and intestinal epithelium. *J Interferon Cytokine Res.* (2002) 22:883–9. doi: 10.1089/107999002760274908
158. Wittmann M, Purwar R, Hartmann C, Gutzmer R, Werfel T. Human keratinocytes respond to interleukin-18: implication for the course of chronic inflammatory skin diseases. *J Invest Dermatol.* (2005) 124:1225–33. doi: 10.1111/j.0022-202X.2005.23715.x

159. Englyst HN, Macfarlane GT. Breakdown of resistant and readily digestible starch by human gut bacteria. *J Sci Food Agric.* (1986) 37:699–706. doi: 10.1002/jsfa.2740370717
160. Lorenz RG, Chaplin DD, McDonald KG, McDonough JS, Newberry RD. Isolated lymphoid follicle formation is inducible and dependent upon lymphotoxin-sufficient B lymphocytes, lymphotoxin β receptor & TNF receptor I function. *J Immunol.* (2003) 170:5475–82. doi: 10.4049/jimmunol.170.11.5475
161. Charlson ES, Bittinger K, Haas AR, Fitzgerald AS, Frank I, Yadav A, et al. Topographical continuity of bacterial populations in the healthy human respiratory tract. *Am J Respir Crit Care Med.* (2011) 184:957–63. doi: 10.1164/rccm.201104-0655OC
162. Wu BG, Segal LN. Lung microbiota and its impact on the mucosal immune phenotype. *Microbiol Spectr.* (2017) 5. doi: 10.1128/microbiolspec.BAD-0005-2016
163. Matsumoto S, Setoyama H, Imaoka A, Okada Y, Amasaki H, Suzuki K, et al. Gamma delta TCR-bearing intraepithelial lymphocytes regulate class II major histocompatibility complex molecule expression on the mouse small intestinal epithelium. *Epithelial Cell Biol.* (1995) 4:163–70.
164. Matsumoto S, Nanno M, Watanabe N, Miyashita M, Amasaki H, Suzuki K, et al. Physiological roles of gammadelta T-cell receptor intraepithelial lymphocytes in cytoproliferation and differentiation of mouse intestinal epithelial cells. *Immunology* (1999) 97:18–25.
165. Umesaki Y, Okada Y, Matsumoto S, Imaoka A, Setoyama H. Segmented filamentous bacteria are indigenous intestinal bacteria that activate intraepithelial lymphocytes and induce MHC class II molecules and fucosyl asialo GM1 glycolipids on the small intestinal epithelial cells in the ex-germ-free mouse. *Microbiol Immunol.* (1995) 39:555–62.
166. Sydora BC, Mixter PF, Houlden B, Hershberg R, Levy R, Comay M, et al. T-cell receptor gamma delta diversity and specificity of intestinal intraepithelial lymphocytes: analysis of IEL-derived hybridomas. *Cell Immunol.* (1993) 152:305–22. doi: 10.1006/cimm.1993.1293
167. Jarry A, Cerf-Bensussan N, Brousse N, Selz F, Guy-Grand D. Subsets of $CD3^+$ (T cell receptor alpha/beta or gamma/delta) and $CD3^-$ lymphocytes isolated from normal human gut epithelium display phenotypical features different from their counterparts in peripheral blood. *Eur J Immunol.* (1990) 20:1097–103. doi: 10.1002/eji.1830200523
168. Bolnick DI, Snowberg LK, Caporaso JG, Lauber C, Knight R, Stutz WE. Major histocompatibility complex class IIb polymorphism influences gut microbiota composition and diversity. *Mol Ecol.* (2014) 23:4831–45. doi: 10.1111/mec.12846
169. Kubinak JL, Stephens WZ, Soto R, Petersen C, Chiaro T, Gogokhia L, et al. MHC variation sculpts individualized microbial communities that control susceptibility to enteric infection. *Nat Commun.* (2015) 6:8642. doi: 10.1038/ncomms9642
170. Silverman M, Kua L, Tanca A. Protective major histocompatibility complex allele prevents type 1 diabetes by shaping the intestinal microbiota early in ontogeny. *Proc Natl Acad Sci USA.* (2017) 114:9671–6. doi: 10.1073/pnas.1712280114
171. Bingula R, Filaire M. Desired turbulence? Gut-lung axis, immunity, and lung cancer. *J Oncol.* (2017) 2017:5035371. doi: 10.1155/2017/5035371
172. Tsay TB, Yang MC, Chen PH, Hsu CM, Chen LW. Gut flora enhance bacterial clearance in lung through toll-like receptors 4. *J Biomed Sci.* (2011) 18:68. doi: 10.1186/1423-0127-18-68
173. Fagundes CT, Amaral FA, Vieira AT, Soares AC, Pinho V, Nicoli JR, et al. Transient TLR activation restores inflammatory response and ability to control pulmonary bacterial infection in germfree mice. *J Immunol.* (2012) 188:1411–20. doi: 10.4049/jimmunol.1101682
174. Yitbarek A, Alkie T, Taha-Abdelaziz K, Astill J, Rodriguez-Lecompte JC, Parkinson J, et al. Gut microbiota modulates type I interferon and antibody-mediated immune responses in chickens infected with influenza virus subtype H9N2. *Benef Microbes* (2018) 9:417–27. doi: 10.3920/BM2017.0088
175. Tweedle JL, Deepe GS Jr. TNFalpha antagonism reveals a gut/lung axis that amplifies regulatory T cells in a pulmonary fungal infection. *Infect Immun.* (2018) 86. doi: 10.1128/IAI.00109-18
176. Bradley CP, Teng F, Felix KM, Sano T, Naskar D, Block KE, et al. Segmented filamentous bacteria provoke lung autoimmunity by inducing gut-lung axis Th17 cells expressing dual TCRs. *Cell Host Microbe* (2017) 22:697–704 e694. doi: 10.1016/j.chom.2017.10.007
177. Cho Y, Abu-Ali G, Tashiro H, Kasahara DI, Brown TA, Brand JD, et al. The microbiome regulates pulmonary responses to ozone in mice. *Am J Respir Cell Mol Biol.* (2018) 59:346–54. doi: 10.1165/rcmb.2017-0404OC
178. Gui QF, Lu HF, Zhang CX, Xu ZR, Yang YH. Well-balanced commensal microbiota contributes to anti-cancer response in a lung cancer mouse model. *Genet Mol Res.* (2015) 14:5642–51. doi: 10.4238/2015.May.25.16
179. Lundin KEA, Gjertsen HA, Scott H, Sollid LM, Thorsby E. Function of DQ2 and DQ8 as HLA susceptibility molecules in celiac disease. *Hum Immunol.* (1994) 41:24–7. doi: 10.1016/0198-8859(94)90079-5
180. Sarna VK, Skodje GI, Reims HM, Risnes LF, Dahal-Koirala S, Sollid LM, et al. HLA-DQ:gluten tetramer test in blood gives better detection of coeliac patients than biopsy after 14-day gluten challenge. *Gut* (2017) 67:1606–13. doi: 10.1136/gutjnl-2017-314461
181. Brottveit M, Raki M, Bergseng E, Fallang LE, Simonsen B, Lovik A, et al. Assessing possible celiac disease by an HLA-DQ2-gliadin tetramer test. *Am J Gastroenterol.* (2011) 106:1318–24. doi: 10.1038/ajg.2011.23
182. Sollid LM, Lie BA. Celiac disease genetics: current concepts and practical applications. *Clin Gastroenterol Hepatol.* (2005) 3:843–51. doi: 10.1016/S1542-3565(05)00532-X
183. Irvine DJ, Purbhoo MA, Krogsgaard M, Davis MM. Direct observation of ligand recognition by T cells. *Nature* (2002) 419:845–9. doi: 10.1038/nature01076
184. Angelo M, Bendall SC, Finck R, Hale MB, Hitzman C, Borowsky AD, et al. Multiplexed ion beam imaging of human breast tumors. *Nat Med.* (2014) 20:436–42. doi: 10.1038/nm.3488
185. Tang Q, Henriksen KJ, Boden EK, Tooley AJ, Ye J, Subudhi SK, et al. Cutting edge: CD28 controls peripheral homeostasis of $CD4^+CD25^+$ regulatory T cells. *J Immunol.* (2003) 171:3348–52. doi: 10.4049/jimmunol.171.7.3348
186. KleinJan A, Willart MA, Kuipers H, Coyle AJ, Hoogsteden HC, Lambrecht BN. Inducible costimulator blockade prolongs airway luminal patency in a mouse model of obliterative bronchiolitis. *Transplantation* (2008) 86:1436–44. doi: 10.1097/TP.0b013e3181886baa
187. Lombardi V, Singh AK, Akbari O. The role of costimulatory molecules in allergic disease and asthma. *Int Arch Allergy Immunol.* (2010) 151:179–89. doi: 10.1159/000242355
188. Westendorf AM, Bruder D, Hansen W, Buer J. Intestinal epithelial antigen induces $CD4^+$ T cells with regulatory phenotype in a transgenic autoimmune mouse model. *Ann N Y Acad Sci.* (2006) 1072:401–6. doi: 10.1196/annals.1326.035
189. Sato T, Vries RG, Snippert HJ, van de Wetering M, Barker N, Stange DE, et al. Single Lgr5 stem cells build crypt–villus structures *in vitro* without a mesenchymal niche. *Nature* (2009) 459:262–5. doi: 10.1038/nature07935
190. Ootani A, Li X, Sangiorgi E, Ho QT, Ueno H, Toda S, et al. Sustained *in vitro* intestinal epithelial culture within a Wnt-dependent stem cell niche. *Nat Med.* (2009) 15:701–6. doi: 10.1038/nm.1951
191. McCracken KW, Howell JC, Wells JM, Spence JR. Generating human intestinal tissue from pluripotent stem cells *in vitro*. *Nat Protoc.* (2011) 6:1920–8. doi: 10.1038/nprot.2011.410
192. Rouch JD, Scott A, Lei NY, Solorzano-Vargas RS, Wang J, Hanson EM, et al. Development of functional microfold (M) cells from intestinal stem cells in primary human enteroids. *PLoS ONE* (2016) 11:e0148216. doi: 10.1371/journal.pone.0148216
193. Farin HF, Karthaus WR, Kujala P, Rakhshandehroo M, Schwank G, Vries RG, et al. Paneth cell extrusion and release of antimicrobial products is directly controlled by immune cell–derived IFN-γ. *J Exp Med.* (2014) 211:1393–05. doi: 10.1084/jem.20130753
194. Ettayebi K, Crawford SE, Murakami K, Broughman JR, Karandikar U, Tenge VR, et al. Replication of human noroviruses in stem cell-derived human enteroids. *Science* (2016) 353:1387–93. doi: 10.1126/science.aaf5211
195. Saxena K, Blutt SE, Ettayebi K, Zeng XL, Broughman JR, Crawford SE, et al. Human intestinal enteroids: a new model to study human rotavirus

infection, host restriction & pathophysiology. *J Virol.* (2015) 90:43–56. doi: 10.1128/jvi.01930-15

196. Forbester JL, Goulding D, Vallier L, Hannan N, Hale C, Pickard D, et al. Interaction of salmonella enterica serovar typhimurium with intestinal organoids derived from human induced pluripotent stem cells. *Infect Immun.* (2015) 83:2926–34. doi: 10.1128/iai.00161-15
197. Chen YW, Huang SX, de Carvalho A, Ho SH, Islam MN, Volpi S, et al. A three-dimensional model of human lung development and disease from pluripotent stem cells. *Nat Cell Biol.* (2017) 19:542–9. doi: 10.1038/ncb3510
198. Routy B, Le Chatelier E. Gut microbiome influences efficacy of PD-1-based immunotherapy against epithelial tumors. *Science* (2018) 359:91–7. doi: 10.1126/science.aan3706
199. Vetizou M, Pitt JM, Daillere R, Lepage P, Waldschmitt N, Flament C, et al. Anticancer immunotherapy by CTLA-4 blockade relies on the gut microbiota. *Science* (2015) 350:1079–84. doi: 10.1126/science.aad1329
200. Van der Flier LG, Clevers H. Stem cells, self-renewal & differentiation in the intestinal epithelium. *Annu Rev Physiol.* (2009) 71:241–60. doi: 10.1146/annurev.physiol.010908.163145

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REVIEW
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From Colitis to Cancer: An Evolutionary Trajectory That Merges Maths and Biology

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Patients with inflammatory bowel disease have an increased risk of developing colorectal cancer, and this risk is related to disease duration, extent, and cumulative inflammation burden. Carcinogenesis follows the principles of Darwinian evolution, whereby somatic cells acquire genomic alterations that provide them with a survival and/or growth advantage. Colitis represents a unique situation whereby routine surveillance endoscopy provides a serendipitous opportunity to observe somatic evolution over space and time *in vivo* in a human organ. Moreover, somatic evolution in colitis is evolution in the 'fast lane': the repeated rounds of inflammation and mucosal healing that are characteristic of the disease accelerate the evolutionary process and likely provide a strong selective pressure for inflammation-adapted phenotypic traits. In this review, we discuss the evolutionary dynamics of pre-neoplastic clones in colitis with a focus on how measuring their evolutionary trajectories could deliver a powerful way to predict future cancer occurrence. Measurements of somatic evolution require an interdisciplinary approach that combines quantitative measurement of the genotype, phenotype and the microenvironment of somatic cells–paying particular attention to spatial heterogeneity across the colon–together with mathematical modeling to interpret these data within an evolutionary framework. Here we take a practical approach in discussing how and why the different "evolutionary ingredients" can and should be measured, together with our viewpoint on subsequent translation into clinical practice. We highlight the open questions in the evolution of colitis-associated cancer as a stimulus for future work.

Keywords: inflammatory bowel disease (IBD), colorectal cancer, cancer evolution, risk stratification, biomarker development, surveillance colonoscopy

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INFLAMMATORY BOWEL DISEASE AND COLORECTAL CANCER–THE CLINICAL BACKGROUND

Patients with inflammatory bowel disease (IBD) have an increased risk of developing colorectal cancer (CRC); this risk is related to disease duration, extent (1), and cumulative inflammation burden (2, 3). Patients with longstanding, extensive colonic inflammation develop colorectal cancer at a younger age compared to the general population, and are more likely to suffer from multifocal neoplasia. For this reason, across the world, patients are enrolled (4, 5) into surveillance programs that aim to detect early cancers and precursor dysplastic lesions with the hope of possible curative intervention before a symptomatic cancer develops. Current surveillance programs are plagued by

oversurveillance and overtreatment of low-risk patients, as well as interval cancers (that is, symptomatic cancers detected in between surveillance colonoscopies) in high risk patients (6). These challenges reflect our poor understanding of the molecular processes underpinning colorectal carcinogenesis in IBD. We propose the use of an evolutionary approach combining genetic, environmental, immune and microbiome parameters to better stratify IBD patients by CRC risk.

CANCER EVOLUTION–THE THEORY OF CARCINOGENESIS

The marked variability of cancers in terms of tissue origin, histopathological subtype, clinical progression and response to medical therapy, belies a shared mechanistic origin: namely, that all cancers are diseases caused by the acquisition of heritable genomic changes, which provide these mutant cells with a survival or growth advantage over their neighbors. During the stages of neoplastic initiation, promotion, progression and malignant conversion (**Figure 1**), the tissue microenvironment generates a (variable) selective pressure that defines those advantageous phenotypic traits (7, 8). In this respect, evolutionary principles, long utilized in the understanding a host of other biological phenomena, from the origin of new species to the emergence of drug-resistant pathogens, can also be applied to understand carcinogenesis (9). Measurements of the clonal composition of a neoplasm, namely the proportion of tumor cells bearing particular mutations, can be readily quantified and used to infer dynamics of the evolutionary process, including when clones emerged and how quickly they spread through the neoplasm (10).

EVIDENCE FOR LARGE SCALE TEMPOROSPATIAL CLONAL EVOLUTION IN IBD

"Field cancerization" was first described over 50 years ago to explain the presence of large regions of histopathologically abnormal pre-cancerous cells in the oral mucosa from which multiple squamous cancers arose (11); that cancerised fields arise from the clonal expansion(s) of (epi)genetically altered cells was shown later [see reviews (12, 13)]. Field cancerization has been described in the premalignant colon, and we have previously postulated that field cancerization can occur either as a single large clonal expansion, or via the parallel expansion of multiple large clones ("clonal mosaicism") (14). The two modes may co-occur, with small areas of mosaicism contained within a large expansion. One study analyzing the genomic abnormalities in individual crypts within different neoplastic lesions, demonstrated their monoclonality across most but not all lesions (15); in some lesions the adjacent phenotypically normal crypts shared the same driver mutation. Other studies have revealed field cancerization across large regions of the colon: Galandiuk et al. (16) undertook a chronological assessment of mutation status across multiple locations of the colon of

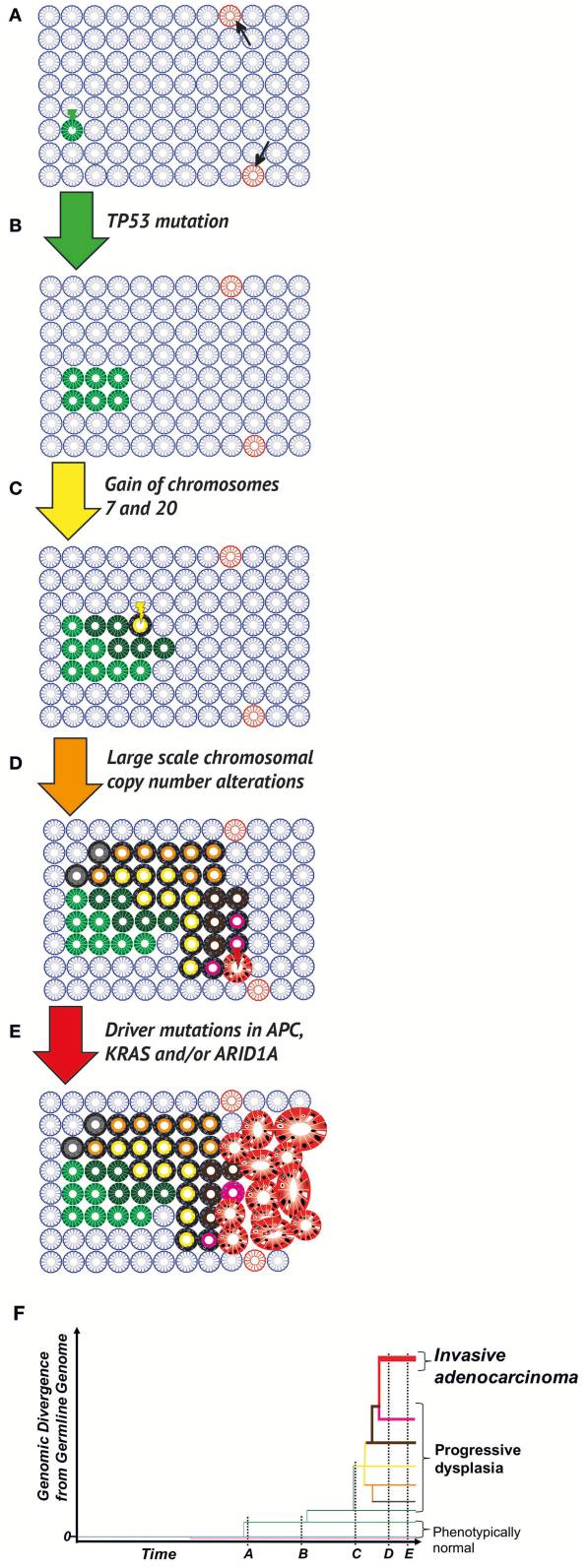

FIGURE 1 | Clonal evolution in the colitic bowel. **(A)** The normal colon. Wild type (blue), phenotypically normal epithelial cells within crypts acquire

(Continued)

FIGURE 1 | mutations occur through chance (DNA replication error at mitosis) or due to microenvironmental pressures (e.g., mutagens in the diet or microbiome). Most mutations (pink crypts, arrows) are evolutionary neutral (i.e., do not change phenotype), or in fact disadvantageous leading to eventual cell death, through the disruption of critical cellular mechanisms or the activation of senescence/apoptosis. Occasionally, a positively selected driver mutation will be acquired. Through either positive selection or neutral drift, the mutant cell population can grow and take over the whole population of cells within the crypt (green crypt). **(B)** Field cancerization. A crypt wholly populated by mutated cells may have an increased crypt fission rate and/or lowered crypt death rate (green crypt). This leads to the expansion of the mutant crypt through the epithelium (green patch), causing a "field" of crypts bearing the same base change, heritable epigenetic change or copy number alteration, which is disposed to subsequent neoplastic transformation. **(C)** Neoplastic initiation. IBD generates a selective pressure favoring clones capable of surviving repeated cycles of inflammation and more rapidly repopulating the healing mucosa during remission. These selected mutant clones, while remaining histopathologically unremarkable, continue to evolve and expand at varying rates (crypts in various shades of green). Expansion remains limited by environmental constraints (e.g., a reliance on paracrine signaling from the stroma or a limit to the tolerance of cell crowding). Within this cancerised field, a frankly dysplastic sub-clone (yellow crypt) eventually arises. **(D)** Neoplastic promotion. The dysplastic clone (yellow with black outline) grows at a much more rapid pace than non-dysplastic crypts, resulting in an accelerated rate of evolution (crypts with black outline and shades of orange, brown, gray or pink); this genetic progression may be accompanied by progression in dysplasia grading. Potential biological mechanisms are diverse and include a loss of dependence on morphogen gradients and an altered metabolism that is better adapted to a hypoxic and nutrient-poor environment. **(E)** Malignant transformation and progression. Transformation to a malignant phenotype (red gland) produces a clone that can undergo uncontrolled cell division and is capable of invasion. Further progression in clone size results in a symptomatic, clinically detectable cancer. Potential biological mechanisms for this malignant transformation include a loss of critical DNA repair mechanisms and escape from immune surveillance. **(F)** Phylogenetic representation. Through multi-region sequencing, quantification of genomic divergence, and mathematical modeling, the phylogenetic relationship between clones can be understood. Here the evolutionary history of an IBD-CRC and its precursor lesions is depicted, with branch lengths corresponding to the amount of genetic change from the bifurcation node of clone branches.

10 patients, and describe one case where at least 3 separate *TP53* mutations arise in non-neoplastic colon, each of which gives rise to neoplastic lesions several years later, with the most recently detected *TP53* mutant spreading over 3 years to involve the entire length of the colon. Similarly, Salk et al. (17, 18) used mutations of hypermutable polyguanine repeats as surrogate markers of clonal populations, and demonstrate a greater number of clonal populations in patients with concomitant high grade dysplasia (HGD) or CRC. Again, some of the clonal patches from wherein a cancer arose involve a large surface area of the colon, thereby providing further evidence for a "pre-cancerised" colonic epithelial field. Finally, Lai et al. (19) used comparative genomic hybridization to show that chromosomal instability in phenotypically normal mucosa increases with proximity to dysplasia and cancer. While chromosomal instability could be detected in regions extending more than 160 cm of the colon, the authors described clear evidence of clonal mosaicism, with copy number alterations varying greatly even between biopsies less than 2 cm apart.

COMMON SELECTIVE PRESSURES IN THE DEVELOPMENT OF INFLAMMATORY BOWEL DISEASE AND COLORECTAL CANCER

IBD is now recognized as a disease of multifactorial etiology, occurring in genetically predisposed individuals, and triggered by as-yet poorly defined environmental insults that generate an aberrant immune response toward an altered gut microbiome (20). One explanation for the increased neoplasia risk in IBD may be the shared etiological factors implicated in both IBD and colorectal carcinogenesis.

Shared Environmental Triggers

The rising IBD incidence worldwide over the last four decades has coincided with profound changes in diet and the adoption of a "Western" lifestyle. In terms of recognized environmental risk factors, both IBD and CRC are associated with sedentary populations that consume a poorly balanced diet, consisting of low plant fiber intake and excess consumption of processed red meat (21–23). A whole host of other lifestyle changes are also potentially implicated in both IBD and CRC, including the ingestion of emulsifiers (24, 25) that are found ubiquitously in processed foods, and low vitamin D levels (26, 27) associated with reduced dietary intake and sunlight exposure. The underlying mechanisms are diverse and are poorly understood, and it remains unclear whether the subsequent metabolome, microbiome and epithelial changes generated by a "Western" lifestyle simply represent associated but non-causative downstream effects of overall modern lifestyle habits, if colon neoplasia itself exerts pro-proliferative changes by modifying the luminal microenvironment, or vice versa.

One interesting commonality can be gleaned through the altered fecal metabolomic profile of both IBD and CRC, most notably the reduction of short chain fatty acids including butyrate and propionate, which are by-products of the bacterial fermentation of dietary fiber (28). Butyrate in particular plays a key role in the maintenance of intestinal homeostasis, namely as the preferred energy source of colonic epithelial cells (29). Butyrate exerts an anti-inflammatory impact both directly on resident innate immune cells (30, 31) and colonocytes (32), and indirectly through the maintenance of epithelial barrier integrity (33, 34) and subsequent reduction in bacterial translocation. Moreover, butyrate has also been shown to have anti-neoplastic properties in CRC cell lines, through the modulation of canonical Wnt signaling (35) and inhibition of histone deacetylation (36). This finding has been corroborated in murine models of IBD-CRC, where germ-free mice subjected to chemically-induced inflammation were partially protected from dysplasia and CRC formation when given high fiber diets with prior gut colonization by butyrate-producing *Butyrivibrio fibrisolvens* (37). No benefits were seen from a high fiber diet alone, *B. fibrisolvens* colonization alone, or a high fiber diet combined with colonization by a mutant strain of *B. fibrisolvens* incapable of producing significant quantities of butyrate. *In vitro* studies have demonstrated the antagonistic effect of bacterial fecal sulfides, which are related

to consumption levels of red meat and preservatives (38), on butyrate metabolism in colonocytes (39). These findings are corroborated by clinical observations in obesity, in IBD and in colorectal neoplasia. A high fat and/or low fiber diet are known to reduce fecal butyrate levels (40). Patients with active IBD demonstrate lower levels of fecal butyrate (41) and butyrate-producing bacterial species (42). Similarly, while healthy individuals had a higher intake of dietary fiber compared to patients with advanced sporadic colonic neoplasia (43), it is interesting to note that even the subgroup of patients with neoplasia and high fiber intake had a lower level of fecal butyrate and butyrate-producing bacteria compared to matched healthy controls.

Shared Microbiome Alterations

A driving role for the microbiome in the development of colitis-associated CRC is less clear, but nevertheless, there are a number of parallels between the microbiome changes in CRC and IBD, that extend beyond loss of overall microbiome diversity and reductions in butyrate-producing *Firmicutes* such as *Faecalibacterium prausnitzii* (44–46). Other notable shared changes are the increased mucosal abundance of *Enterobacter faecalis* and *Escherischia coli* [in particular adherent-invasive *E. coli* (47–49)], as well as *Fusobacterium* species (50, 51).

Fusobacterium nucleatum expresses unique adhesins that have been shown to promote CRC (but not non-neoplastic) cell line proliferation through modulation of Wnt signaling (52); *Fusobacterium* load within cancer specimens may even have adverse prognostic implications for patients with CRC (53). In practice, current assessments of species diversity may not fully capture the CRC-relevant microbiome changes in IBD: for example, *Fusobacterium nucleatum* is not only isolated more frequently in patients with IBD, but those isolates from inflamed IBD tissue show greater invasive potential than isolates from non-inflamed tissue of the same patient (54). Early evidence for the association of specific microbiome components with the earliest genomic changes seen in IBD-CRC comes from recent epigenetic studies, where *Fusobacterium* colonization is associated with pro-carcinogenic methylation changes in the non-neoplastic colonic mucosa of 86 patients; this association remained highly significant (OR 16.2, $p = 0.01$) in a multivariate analysis that included recognized clinical risk factors such as age, duration of disease and surrogate markers of inflammation severity (55). Nevertheless, it is remains unclear whether these microbiome changes represent a secondary consequence of disruption in the integrity and function of the epithelial barrier seen in inflammation and neoplasia (56), or are in fact truly independent drivers of inflammation and carcinogenesis.

Studies of adherent-invasive *E. coli* in IL10$^{-/-}$ IBD murine models demonstrate the potential complexity of the relationship between inflammation, the microbiome and carcinogenesis: inflammation does not promote the survival of adherent-invasive *E. coli*, but rather disrupts hitherto poorly defined processes for the natural negative selection of *Enterobacteriaceae* such as *E. Coli* (57). Adherent-invasive *E. coli* in turn drives colorectal carcinogenesis independently of inflammation activity. Interestingly, deletion of the *E. coli* gene coding for the toxin colibactin abrogates this increased cancer risk in IL10$^{-/-}$ IBD mouse models (58), whereas wild type mice colonized with colibactin-producing *E. coli* do not have an increased cancer risk. The carcinogenic effects of colibactin are therefore evident only in the setting of "exposed" epithelium as seen in IBD. *Enterobacter faecalis* on the other hand, while sharing the ability of adherent-invasive *E. Coli* to persist within innate immune cells (59, 60), has been shown to promote carcinogenesis by a more indirect mechanism. Murine macrophages colonized by *E. faecalis* are polarized toward a pro-inflammatory M1 phenotype. These M1 macrophages in turn produce 4-hydroxynonenal, an alkenal by-product of prostaglandin breakdown by cyclo-oxygenase 2 (COX-2) that disrupts mitotic spindle function in colonic epithelial cells, thereby inducing chromosomal instability and phenotypic transformation (61). Collectively, these findings confirm the ability of the microbiome to exert both direct and indirect carcinogenic effects on colonic epithelium in the context of compromised mucosal barrier integrity.

Immune Co-evolution in IBD-Associated Carcinogenesis Remains Poorly Understood

Active IBD generates a well-characterized cascade of pro-carcinogenic inflammatory changes [see (62, 63) for reviews]. These include but are not limited to TNF-α, which promotes tumor proliferation and invasion through macrophage recruitment and angiogenesis (64), as well as IL-6 and IL-22, with their pro-proliferative, anti-apoptotic effects mediated by epithelial STAT3 signaling (65, 66). As discussed in further detail in the rest of the review, it is clear that the altered immune microenvironment of IBD can have a direct (evolutionary selective) influence on the epithelial cells, by favoring the survival and expansion of some mutant clones over other mutants or non-mutant cells. However, the trajectory of epithelium-microenvironment co-evolution during carcinogenesis, and in particular the dynamic changes in the resident immune system's simultaneous host-protecting and tumor-promoting roles (67), remains poorly understood.

Current evolutionary approaches in carcinogenesis remain very "cancer cell" focused. In one revealing study, Galon et al. have demonstrated that simple measurements of CD3 and CD8 cell infiltration in the tumor core and invasive margin (68) of sporadic CRCs are superior to clinical TNM staging (69) and genetic microsatellite instability status (70) in predicting patient prognosis. The aberrant immune function that is characteristic of IBD may manifest itself in cancer immune-epithelium co-evolution: for example, IBD-CRC demonstrates greater lymphocyte infiltration compared to sporadic CRC, but with no associated prognostic improvement (71). This may be explained by impaired lymphocyte cytotoxicity in IBD, as reflected by reduced cancer cell apoptosis and lower granzyme B expression in lymphocytes infiltrating IBD-CRC compared to sporadic CRC.

Changes in the mucosal immune cell composition and function that may occur *prior to* malignant transformation

remain poorly characterized. A competent immune system plays a clear role in the surveillance for neoplastic growth and the elimination of entire neoplastic lesions. IBD low grade dysplasia (LGD) is thought to regress in the majority of patients (72), regression of sporadic colonic adenomas has also been demonstrated (73), and a role for immune clearance is feasible in both scenarios. Indeed, murine studies provide useful insight into potential immunoediting mechanisms at the earliest stages of neoplastic formation; for example, the transfer of CD4+ CD25+ T_{reg} cells from wild type mice into $APC^{Min/+}$ mice reduces adenoma burden in an IL-10 dependent fashion (74).

A key finding in IBD carcinogenesis is that a significant proportion of IBD LGD lesions demonstrate a level of aneuploidy comparable to that of established CRCs (75); even normal IBD epithelium can bear significant chromosomal copy number alterations (76). Aneuploidy is associated with mutations in genes involved in the DNA damage response (77); indeed *TP53* is the classical initiating mutation in IBD-CRC. Aneuploid cells are normally subjected to negative selection in healthy tissue because of the significant associated proteotoxic, metabolic and replicative stressors (78); therefore this finding in IBD suggests that certain patterns of copy number alterations increase fitness, such that these added "costs" are outweighed by cell-specific "benefits" within the context of the colitic microenvironment, such as less restricted replication and immune evasion. Along this line of thought, it is noteworthy that pan-cancer analyses demonstrate that increased tumor aneuploidy is associated with reduced expression of cytotoxic immune markers, M2 polarization of macrophages, and increased cell cycling and proliferation (77). The mechanisms for these alterations are unclear and the authors speculate that aneuploidy may also impair certain aspects of MHC class I antigen presentation, thereby promoting immune evasion. Evidence to support this hypothesis comes from studies of human lung and breast cancer xenografts in mice, demonstrating how aneuploid cells subvert lethal epithelial responses triggered by cytoplasmic translocation of DNA during mis-segregated mitosis (79) despite upregulation of various inflammatory responses, including the cGAS-STING pathway, which evolved to combat viral infection by detection of extranuclear DNA (80). In aneuploid cancers, activation of the cGAS-STING pathway does not generate the expected downstream canonical NFκB and type I interferon signaling, but rather drives the non-canonical NFκB signaling cascade more typically seen in myeloid-derived cells, which the authors speculate may represent a form of immune mimicry (79).

MHC class II molecules also play an important role in colonic neoplastic change. Normally restricted to traditional antigen presenting cells, MHC class II expression can be induced in transformed epithelial cells, increasing in frequency during the adenoma-carcinoma transition, with a corresponding increase in the density of tumor-infiltrating lymphocytes (81). Moreover, metastasis is associated with a loss of MHC class II expression across multiple solid cancers including CRC (81–83). An example of the role of MHC molecules in colitis-associated carcinogenesis is given by a case-control study of patients with ulcerative colitis, which demonstrates that HLA-DR17 expression (a particular serotype recognizing HLA molecule) is associated with increased CRC risk and methylation-induced HLA-DR silencing, while HLA-DR7 and HLA-DQ5 are associated with reduced CRC risk (84). The authors speculate that these patients may be particularly sensitive to the oncogenic effects of the altered microbiome in IBD (84); indeed MHC class II polymorphisms are known to be associated with susceptibility to infection-mediated cancers such as *Helicobacter pylori* associated gastric adenocarcinoma and HPV-associated cervical squamous cell cancer.

Shared Genetic Predisposition

In practice, the evidence for a shared genetic predisposition to both IBD and colorectal cancer remains very limited. In fact, a GWAS study comparing the 181 most common IBD susceptibility variants with those known to predispose to CRC in the general population demonstrate only one shared variant (rs11676348, which lies immediately upstream of *CXCR2*), that actually increases UC risk while *lowering* CRC risk (85). We note that there have been no GWAS studies within IBD patients that identify the subset of patients most at risk of developing dysplasia and cancer, and potentially "classical" GWAS for CRC risk may have included some IBD CRC cases. Nevertheless, these overall findings emphasize the greater importance of acquired (and in particular environmental) factors as the driver for both conditions, which (unlike genetic predisposition) are modifiable, and may therefore be targeted to alter the course of tumor evolution at its earliest stages for cancer prevention.

PATIENTS WITH IBD PROVIDE AN "IDEAL" HUMAN MODEL FOR THE EVOLUTIONARY STUDY OF COLORECTAL CARCINOGENESIS

IBD patients undergoing endoscopic surveillance offer an ideal human system for an evolutionary approach to studying the time course of colorectal carcinogenesis over a patient's lifetime. First, routinely collected biopsies at colonoscopy, years before a cancer is detected, form a rich tissue archive that is amenable to temporospatial evolutionary analysis. Similarly, the metachronous and synchronous nature of IBD neoplastic lesions provides another serendipitous opportunity for the assessment of clonal relationship between lesions separated by time and/or space. Finally, patients with IBD-CRC routinely undergo a complete resection of their colon and rectum rather than a limited segmental resection, thereby allowing for the detailed mapping of mutant populations in phenotypically normal colonic epithelium both proximal to and distal from a neoplasm (75), and facilitating correlation with local microenvironmental factors.

The Relapsing-Remitting Nature of Inflammatory Bowel Disease Drives Epithelial Clonal Evolution

In the following sections, we discuss the significant body of evidence (14) demonstrating that the recurrent cycles of ulceration and healing typical of IBD generate evolutionary pressures that:

(a) Increase the rates of epithelial stem cell mutations within crypts through more rapid cell cycling,
(b) Promote the survival of mutant clones that can tolerate an inflammatory environment during periods of disease activity, and
(c) Allow the expansion of mutant clones that can more rapidly repopulate the healing mucosa.

The Intestinal Crypt as the Evolutionary Unit of the Colon

The adult colon is lined by approximately 10 million crypts, which are single cell layer invaginations of the colon lining composed of 1,000–4,000 columnar epithelial cells (86), centered around an estimated five to seven rapidly cycling LGR5-positive stem cells at the crypt base (87–90). In mice, stem cells divide roughly daily (90); their progeny become differentiated as they migrate from the crypt base to the luminal surface over a period of 5–7 days (91) before their shedding into the intestinal lumen. In humans, the dynamics are less well quantified, but are likely a little slower (88). Any mutations that persist in the crypt must therefore have first arisen within a long-lived stem cell. As a result, disparate crypts, bearing stem cells with potentially differing stochastically accumulated mutations, can independently evolve in response to localized microenvironmental pressures; these pressures can vary significantly across the length of the colon (92). For mutant clone fixation to occur within the entire crypt, the "founder" mutant stem cell must first replace all other crypt base stem cells via symmetric divisions in a process referred to as niche succession (93). For those mutations that provide no survival advantage, niche succession through neutral drift within the human intestinal crypt is a thought to be a slow stochastic process that is due to low mutation rates and slow stem cell loss/replacement rates under homeostatic conditions, although reported rates remain under debate (88, 89). Stem cells bearing classical driver mutations for CRC subvert this process (89), with accelerated growth dynamics and likelihood of niche succession due to increased fitness advantages supplied by oncogenic mutations. The colonic crypt stem cells from which a future cancer arises are thought to have already acquired approximately half the somatic mutational burden of the future tumor prior to malignant transformation (94).

Lateral mutant clonal spread in the colon is driven by crypt fission (95) and/or crypt regeneration (96), the processes by which crypts grow. Crypt fission represents a key homeostatic mechanism that heals the mucosa in response to ulceration or other injury (97). Under normal conditions, colonic crypt fission is a surprisingly infrequent event, with fewer than 1% of all crypts dividing in a single year (89).

IBD Activity Provides a Survival Advantage for Mutant Clones That can Survive in a Hostile Inflammatory Environment

Evidence for the importance of the inflammatory microenvironmental context in selecting for crypt stem cells harboring key pro-oncogenic mutations comes from lineage tracing experiments in recombinant mouse models (98). Under normal conditions, stem cells bearing *TP53* mutations are no more likely to replace their neighboring wild type stem cells within the crypt base, while *APC* and *KRAS* mutations, which are more frequently encountered in sporadic CRC (75), offer a clear survival/growth advantage. However, in the context of chemically-induced colitis, the relative fitness of *TP53* mutant stem cells increases, as demonstrated by a 58% probability of mutant niche succession 21 days after colitis induction. This finding is consistent with studies on human IBD tissue showing that *TP53* mutations and loss of heterozygosity occur at a much higher frequency in IBD-CRC compared to sporadic CRC, and are an early event in IBD-driven carcinogenesis that can even be detected in non-dysplastic mucosa (75, 99, 100). This contrasts to sporadic colonic neoplasia, where *TP53* mutations are typically reported to be a late event in the adenoma-carcinoma sequence (101).

TP53 plays a critical role in inducing cell cycle arrest, senescence or apoptosis in cells with damaged genomes, as well as in cells exposed to severe metabolic and oxidative stressors (102) typically generated by severe IBD flares. On the other hand, colonic epithelial cells in the non-inflamed colon are not exposed to such a hostile microenvironment until the final stages of the adenoma-carcinoma transition, therefore the evolutionary pressure selecting for *TP53* mutations arises much later in sporadic CRC (103). Interestingly, *TP53* mutations are also able to generate an inflammatory response in their own right through oncogenic "gain-of-function" effects (104): human organoid studies and *in vivo* studies in $p53^{mut/+}$ mice demonstrate that epithelial cells bearing a *p53* mutation commonly observed in IBD (R273H) can exacerbate their local inflammatory microenvironment, by prolonging TNFα-induced NFκB activation, eventually generating flat dysplastic lesions with secondary cancers typical of those seen in IBD (105). Therefore, the early *TP53* mutations encountered in IBD may in fact act as independent drivers of IBD carcinogenesis through their downstream microenvironmental effects.

Oncogenic mutations that provide stem cells with a survival advantage within a crypt will often accelerate crypt fission as well. For example, data from murine (106) and human (89) studies confirm that the classical *KRAS* mutation seen in sporadic and to a lesser extent in IBD-associated neoplasia (G12D) increases crypt fission rates by at least one order of magnitude; murine studies also confirm accelerated crypt fission rates following *p53* mutation (107).

Colorectal Carcinogenesis in IBD Is Driven by Accelerated Cell Turnover and "Premature" Colonic Aging

Studies using Ki67, a cellular marker expressed during the active phases of the cell cycle, demonstrate an expanded proliferative zone in crypts from both regenerating IBD epithelium and early dysplasia when compared to normal colonic crypts (108). The precise stem cell population responsible for regenerating the human colonic epithelium in IBD is uncertain, with evidence from murine studies suggesting dynamic contributions

during the inflammation and regeneration phases. LGR5+ expression drops dramatically during the acute stages of colitis but increases dramatically during regeneration (109); other studies confirm that LGR5+ expressing stem cells may be very sensitive to intestinal injury (110) but nonetheless crucial to crypt regeneration (111). Inflammation has been shown to recruit long-lived and hitherto quiescent DCLK1+ tuft cells (112) from the crypt wall (113) that can acquire stem cell properties in the absence of LGR5+ stem cells, reconstituting the entire crypt, including the LGR5+ stem cell niche. Interestingly, these DCLK1+ cells did not proliferate or initiate neoplastic progression following conditional APC knockdown without the addition of an inflammatory stimulus (113).

It is thought that a rapid increase in stem cell numbers and subsequent clustering within the crypt base may act as a trigger for the initiation of crypt fission (114, 115). These observations may explain why active IBD, with an expanded stem cell proliferative zone, is associated with a crypt fission rate that is 30- to 70-fold higher than that of uninflamed mucosa (116), further accelerating cell turnover and clonal expansion.

These shifts in the properties of the stem cell niche in the context of injury and inflammation are in part driven by stromal cells that generate the necessary canonical and non-canonical Wnt signaling molecules necessary for epithelial reconstitution and subsequent maintenance of homeostasis (96). In mouse models, these stromal cells responsible for Wnt signaling have recently been identified as telocytes (117). Induction of acute colitis in mice by DSS ingestion results in an expansion of GLI1+ expressing telocytes (118). Of interest, a coding polymorphism in human *GLI1* that generates a variant protein with reduced transactivation is associated with predisposition to ulcerative colitis in populations of Northern European descent (119), further highlighting the role inflammation-modulated Wnt signaling in IBD pathogenesis. In addition, changes in the luminal metabolic and microbiome micro-environment induced by colitis may also play a role in promoting expansion of the stem cell niche. For example, intestinal intraluminal butyrate inhibits the proliferation of LGR5+ stem cells *in vitro*, and is thought to play a role in confining the stem cell niche to the crypt base, where differentiated colonocytes on the crypt walls have consumed the butyrate as their primary energy source (120). Reductions in butyrate associated with the microbiome dysbiosis of active IBD may therefore allow stem cells in injured crypts that are more directly exposed to luminal contents to continue dividing, thereby contributing to the accelerated cell turnover.

There now exists a substantial body of evidence demonstrating that the predominant mechanism for carcinogenesis in IBD is accelerated cell turnover and rapid colonic "aging," and that IBD-CRC is *not* a consequence of direct DNA damage from reactive oxygen and nitrogen species as traditionally thought (121). First, trinucleotide context mutational signature analysis (122) of IBD-CRC demonstrates a preponderance of C>T substitutions at NpCpG trinucleotides (123) (mutational signature 1) in keeping with aging driven by rapid cell cycling (124), with no detection of signatures typical of direct DNA exposure to a genotoxic environment (signatures 4, 7, 11, 22, 24, and 29). Second, multiple studies report telomere shortening, another surrogate marker of accelerated aging, in the colitic mucosa (18, 125–127). Finally, patients with ulcerative colitis bearing HGD or cancer demonstrate significant aging-related CpG island hypermethylation signatures in colitic mucosa far from the site of the neoplasia; these changes were not seen in UC mucosa of neoplasia-free patients or healthy controls (128).

The aforementioned finding of colonic aging through rapid cell cycling, combined with the heterogeneity of somatic mutations seen in IBD-associated CRCs (123, 129, 130), limits the extent to which any single animal model can replicate colitis-associated neoplasia formation (131). AOM-DSS mice remain the most utilized model, with mice exposed to the carcinogen azoxymethane (AOM), followed by repeated ingestion of dextran sulfate sodium (DSS) to induce inflammation. However, whole exome sequencing of these mouse cancers shows little overlap in terms of mutational landscape with human CRC, in particular the near absence of the most common IBD-CRC driver mutations such as *TP53*, *APC*, *KRAS,* and *PIK3CA*, no shared (132). Indeed, AOM-DSS mouse CRC are striking for the over-representation of C>T substitutions, which is more typical of DNA damage by alkylating agents like azoxymethane (132). Likewise, $IL10^{-/-}$ mice develop colitis-associated cancers that do not demonstrate the chromosomal instability (133) that is typically encountered in most human IBD-CRCs (134); recent studies suggest the need for additional microbial and immunological stressors to improve $IL10^{-/-}$ mouse model fidelity (61). In conclusion, the inability of current mouse models to replicate the diversity and dynamic shifts of the human microbiome, nor the cumulative effect of chronic inflammation and aging generated by colitis and time in patients, implies that assessment of IBD carcinogenesis requires study of the underlying process in patient-derived samples.

EVOLUTIONARY BIOMARKERS: A NOVEL APPROACH MERGING BIOLOGY AND MATHEMATICS

The stochastic nature of evolutionary changes (e.g., mutation accumulation, clonal expansion and selection) requires adequate time (years) for carcinogenesis to progress normal cell phenotype to malignancy in the human body. Using mathematical models, we can formulate mathematical expressions describing these evolutionary changes, and use the expressions to relate these evolutionary parameters to age-dependent epidemiological cancer incidence curves (135). In addition to the chronological age of the patient as an initial biomarker of CRC risk, biological aging of the colitic bowel itself may be considered as a potential marker of progression to neoplasia due to its prominence in the pathogenesis of IBD carcinogenesis explained above. The concept is based on measuring both the extent and speed of genetic evolution as a proxy of how "close to cancer" the cells have become (136). Tissue age is difficult to measure *in vivo,* but can be estimated with computational methods like Bayesian inference that have been used in molecular clocks applied to somatic epigenetic (137) and genomic (138) data from non-dysplastic Barrett's esophagus, a precursor of esophageal adenocarcinoma

that is characterized by intestinal metaplasia, driven by chronic acid- induced inflammation (139).

Partly due to accelerated cell turnover, IBD-CRCs show genomic diversity, both in terms of the inter-tumor permutations of genomic alterations leading to cancer formation, as well as the intra-tumor genomic heterogeneity in established cancers. IBD-CRCs may share only some of the same driver mutations (75), and some premalignant colonic adenomas do not have a detectable driver mutation in any of the 20 most frequent driver genes (140). For this reason, biomarkers of cancer risk that assay one molecular alteration or a single pathway will likely never be sensitive or specific enough to justify routine clinical use in cancer surveillance recommendations for patients with IBD.

Along with tissue age, other measures of *evolvability* may provide a more reliable marker of cancer risk in which the issue of intra-patient heterogeneity may be circumvented for clinical management. For example, IBD patients at risk of developing cancer may demonstrate high diversity in clonal composition that continues to change over time, while low risk patients may harbor few, if any, mutant clonal populations with size distributions that remain stable over time (14). A cornerstone principle of cancer evolution is that genomic diversity acts as the substrate for natural selection in the inflamed colonic bowel; the more diverse the colonic epithelial cell population, the more likely a well-adapted, "dangerous" clone will be present, outcompete other clones, and evolve toward a malignant phenotype. This concept of evolutionary biomarkers, defined in terms of ecological diversity measures, has been repeatedly demonstrated to be predictive of neoplastic progression in patients with Barrett's esophagus (138, 141, 142).

Rather than seeking a set pathway of necessary changes from IBD to IBD-CRC, we can quantify measures of evolvability that are "agnostic" to any specific oncological pathway, by capturing a range of distinct molecular processes that may be potentially driving an individual patient's cancer formation. By assaying the genomic alteration burden from spatially distributed biopsies, a wide range of evolutionary measures can be generated. For example, we could provide evidence of clonal sweeps by identifying shared genomic alterations across multiple individual biopsies, implying that they originated from a common founder. Similarly, clonal mosaicism can be assayed spatially between biopsies by measuring the genomic "distance" between the somatic mutations in these biopsies (where genomic distance is measured by the number of divergent mutations between two biopsies).

Finally, due to the extensive sampling of the IBD colon during routine surveillance, all assays can be measured over time in the same patient, with chronological rates of change potentially providing another measure of "evolvability." A simple measure of growth is the difference between the clone size at two time points (which in its coarsest resolution, could be represented by the number of equidistant biopsies bearing that clone) divided by the difference in time points of endoscopic screening examinations for precursor lesions in IBD. In this way, we can utilize the lifelong surveillance of IBD patients to collect the genomic measurements for more precise calculations of IBD-specific evolutionary parameters such as initiation (μ), birth (α), and death (β) rates of mutant clones in individual patients (**Figure 2**).

Indeed, temporal studies are vital when defining a "window of opportunity" for clinical intervention—i.e., when early pre-cancerous development may be observed/removed and further, when cancer risk can be reliably predicted (143, 144). Progression to IBD-CRC is driven by a series of rate-limiting evolutionary events (such as *TP53* mutation and critical copy number changes as described earlier in the review), while ongoing accumulation of heterogeneous genomic alterations occurs via genetic drift in crypts throughout patient lifetime. These salient rate-limiting events governing IBD evolution, which are not clearly understood but could also be assayed using a combination of particular mutations and evolutionary biomarkers, serve as the boundaries between windows of screening opportunity and may be reflective of histopathological stage. An evolutionary approach to IBD surveillance would aim to identify periods during a patient's lifetime when highly evolved and aberrant clones would be detectable during surveillance, and when an early intervention would be beneficial, in order to tailor surveillance screens accordingly. This method of monitoring patient-specific rates of evolutionary change (such as increasing levels of genetic diversity in clones) can be used to predict the age(s) at which clones will attain a threshold size and/or diversity (see **Figure 2**). We can then use this information to make dynamic, personalized recommendations for the most efficient next-surveillance-screen time; this approach of theoretically predicting biomarker value change in the future has been demonstrated in mathematical models for *Helicobacter pylori* driven gastric cancers (145).

Thus far, the modeling done in studies of premalignant risk stratification in Barrett's esophagus is typically standard survival analysis where the evolutionary biomarker (such as level of genetic diversity) is used as the predictor variable. More sophisticated models attempt to infer the temporal dynamics of clonal evolution, and extrapolate inferred trajectories to predict future disease state [for example, inference of time-dependent evolution model parameters for clone growth in Barrett's esophagus (138)].

Mathematical modeling of carcinogenesis in IBD is in its infancy. Several types of mathematical modeling approaches may be useful to incorporate measures of the evolutionary process into temporospatial models of IBD cancer evolution for calibration and prediction of patient-specific trajectories. Agent-based models for clone growth of the evolutionary process *in silico* can employ simulations of patient-specific parameters (such as the rate of clonal expansion of a particular clone in a particular individual, or the patient-specific mutation rate) and explore the effect of spatial tissue constraints and microenvironmental changes on disease progression. Such models require extensive and detailed biological data at the outset for parameterisation, though, meaning that they may be impractical to apply in practice. Continuum models of growth may be used to predict general tissue change that can evaluate the effects of spatial sampling bias (e.g., quadrant biopsy tissue removal) on detection of rare subclones, but the increased abstraction of these modeling formulations necessitates that some biological detail is neglected.

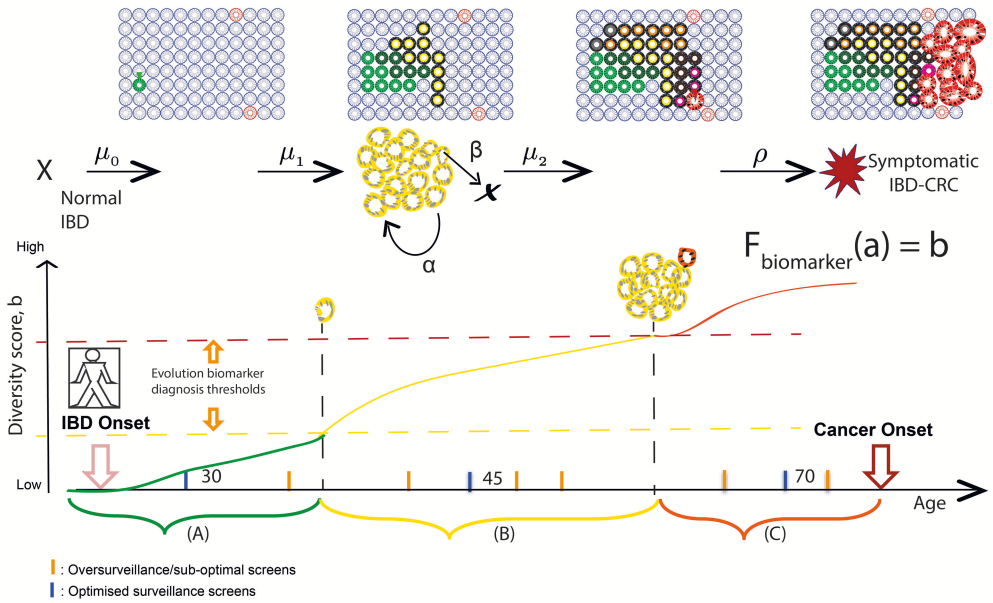

FIGURE 2 | Screening and surveillance intervals in IBD using an evolutionary approach. Patients in a population develop IBD at a known age; the aim for prevention is to provide a baseline endoscopic screen before some of those patients will have developed neoplasia. Hypothetical "windows of opportunities" for surveillance quantify timescales during which **(A)** pre-initiated cells gain alterations (early diagnosis), **(B)** initiated clones expand (promotion) and become more genetically diverse (early intervention), and **(C)** preclinical malignant clones evolve (progression) and could be thwarted (early detection). Levels of diversity increase in an evolving inflamed microenvironment and may act as a biomarker of evolutionary trajectory position. We aim to relate biomarker levels (b) measured during surveillance screens at specified ages (a) to multistage model parameters via variables in a theoretical function $F_{biomarker}$. As a theoretical example, if the relationship between diversity and size of premalignant clone (yellow) is known, then we can measure the rate of growth of the clone during surveillance and predict at what age the clone will be a certain size and likely harbor a detectable phenotype change of preclinical malignancy.

Population level models can also be used to incorporate epidemiological data (e.g., cancer incidence, premalignant prevalence) and thus describe cancer evolution in a population with IBD. An example would be multistage clonal expansion models, a family of cell-based stochastic models positing that cancer is caused by the accumulation of rare events that define the boundaries of the initiation-promotion-progression stages of carcinogenesis (135). This theoretical framework integrates time-varying risk factors into the analysis of cancer epidemiological data (such as incidence and multifocal sizes of pre-malignant lesions), wherein stages from normal to malignant transformation are defined by the occurrence of rate-limiting events (e.g., *TP53* mutation). Finally, hybrid models can combine the above techniques that are calibrated to multiple levels of data [see reference (137) for an example of modeling Barrett's esophagus clinical screening using agent-based tissue sampling *in silico* combined with cellular parameters calibrated to esophageal adenocarcinoma population data]. Choosing the appropriate model (and model type) is non-trivial, and depends upon the utility that is sought from the model.

By using the equations of the multistage mathematical model with estimated parameter values, we can then define such windows by solving for the probabilities (analytically and numerically) that an individual will most likely harbor a premalignant or malignant lesion of a screen-detectable size at endoscopic screening/diagnosis, and then use the outcome of each screen to benchmark the progress of evolution and iteratively predict the next window to recommend surveillance screen times.

At present, such temporospatial information about clonal evolution needed in these mathematical model predictions is generally lacking, and so consequently is not used in the design of IBD surveillance protocols. Candidate molecular markers that have been associated with progression in IBD (described earlier in the review) including aneuploidy, methylation assays, microsatellite instability and mutational panels of key driver genes in IBD-CRC (such as *TP53*, *APC*, *KRAS*, and *CDK2NA*). Of these, only aneuploidy, as measured using flow cytometry, has been shown to date to carry prognostic potential in IBD (76, 146, 147). Dynamical information using one or more of these markers could potentially enhance clinical practice beyond current *ad hoc* screening interval recommendations, which are based on crude clinical features (4, 5).

FROM COLITIS TO CANCER: TRANSLATIONAL IMPLICATIONS OF UTILIZING AN EVOLUTIONARY APPROACH

Pathogenic genomic alterations (e.g., point mutations and chromosomal copy number alterations) are known to occur in phenotypically normal epithelium many years before a cancer forms (16, 75, 146, 148). Enumeration of these mutations may

aid in risk stratifying patients who will more likely progress to IBD-CRC for more aggressive surveillance and treatment, while reducing surveillance requirements for lower risk patients. By using the aforementioned evolutionary approach, we envisage a more personalized approach to cancer risk assessment that combines patient demographic details and endoscopic features (149) with genomic assays.

At present, a significant proportion of patients with low grade dysplasia are advised to have a complete resection of their colon and rectum in light of the high risk of multifocal neoplasia (150). For these patients, the ability to define the extent of mutant clonal spread can justify a more limited surgical resection, with particular focus on the potential for rectal-sparing surgery, thereby avoiding the need for an ileo-anal pouch or permanent stoma.

A natural extension of this evolutionary approach to IBD carcinogenesis is that altering the inflammatory selection pressure may modify future cancer risk. At present, we are uncertain as to whether standard IBD anti-inflammatory or immunosuppressive therapies can halt (or possibly even reverse) the formation, expansion and/or evolution of mutant clones. 5-aminosalicylates, an anti-inflammatory class of drugs that form the first line of therapy for patients with UC, are thought to reduce the incidence of IBD-associated dysplasia and IBD-CRC (151). The precise mechanism remains unclear and is probably multi-faceted; 5-aminosalicylate use has been shown to reduce inflammation-generated β-catenin signaling in the mid- and upper crypt (152). β-catenin is a key transcription factor in the Wnt pathway, with aberrant constitutive Wnt signaling (through phosphorylation and nuclear translocation of β-catenin) extending beyond the crypt base stem cell being a common initiator in colorectal adenoma and carcinoma formation through the expansion of the crypt stem cell niche. In sporadic adenomas this is achieved through *APC* gene mutation; indeed, similar findings have been noted in $APC^{Min/+}$ mice, where non-steroidal anti-inflammatory drug (NSAID) use selectively increased the apoptosis rate in crypt stem cells with nuclear or phosphorylated β-catenin by over fivefold (153).

In practice, prospective randomized controlled trials that can assess the specific impact of the different medical therapies on IBD dysplasia and CRC risk will be challenging to conduct, and will probably be underpowered due to the required large patient cohort size (to handle inter-patient variability) and long follow-up time. Instead, a proxy for cancer-risk reduction will be the minimization of the cumulative inflammatory burden of the colitic bowel through the achievement of deep remission (154). An understanding of the evolutionary dynamics of carcinogenesis in IBD, and its evaluation *in vivo* in the presence and absence of chronic disease activity, may compensate for any limitations in our understanding of the precise anti-neoplastic mechanisms of anti-inflammatory IBD therapies. Indeed, experimental data on the feasibility of disease-modulating drugs to limit clonal expansion and progression in human tissue has emerged during the study of Barrett's esophagus. In Barrett's esophagus, NSAID use has been shown to modulate clonal evolution (155), with a reduction of both the burden and diversity of functional mutations affecting key cancer-associated pathways compared to matched controls (156).

Quantification of immune-epithelial cell co-evolution is an important area for future research. Both cell populations can be described quantitatively by complex system models (157, 158) with marked plasticity, resulting in a near infinite possible set of permutations (clonal and subclonal populations in the case of cancer, B cell and mucosal T cell receptor repertoire composition combined with microbiome diversity in IBD), and a susceptibility to sustained external selection pressures that can "promote" and "fix" clinically deleterious traits (e.g., loss of response to IBD therapy). As the "selfish" drive of the individual colon cancer cell to expand comes at the expense of the multicellular human host, so the same approach can be used to understand and model the conditions driving the "selfish" activation and/or expansion of aberrant immune cell populations.

Much like traditional cancer chemotherapies, current IBD therapies are the product of a reductionist approach that targets one or several pathways, which are of varying importance between patients (hence the variable and incomplete response rate to IBD therapies), and which have a minor impact on the long term course of the disease [as demonstrated by the persisting need for surgery (159) despite advances in medical therapy]. In cancer, evolutionary adaptive therapies, that aim to control tumor burden while simultaneously limiting the selection pressure driving the emergence of resistant mutant clones, offer a new paradigm in oncological management that already shows promise in pilot clinical trials (160). Similar treatment paradigms are needed in IBD patients: fecal microbiota transplantation shows promise as such an intervention in ulcerative colitis (161), with its concomitant alterations in microbiome diversity and composition. Indeed, fecal microbiota transplantation is most efficacious in patients with more recent diagnoses of ulcerative colitis (162), possibly because this intervention is performed prior to the irreversible "fixation" of aberrant adaptive immune clones in such patients.

CONCLUSION

Inflammatory bowel disease represents an ideal model for the study of human cancer using an evolutionary approach. Routine surveillance colonoscopies provide a serendipitous opportunity to observe somatic evolution over space and time *in vivo*. Moreover, somatic evolution is accelerated in IBD through the relapsing-remitting nature of disease flares. Direct and detailed temporospatial assays of clonal populations, together with their co-evolving immune and microbiome components of the mucosal microenvironment, now feasible using the latest sequencing technologies, can be leveraged toward the development of an "evolutionary" biomarker that can better predict an individual patient's cancer risk. Finally, an evolutionary systems approach (163), currently utilized in the study of carcinogenesis, may offer a novel paradigm for understanding the concomitant immunological evolution that is vital for escape from immune surveillance and promotion of tumor growth.

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REFERENCES

1. Ekbom A, Helmick C, Zack M, Adami HO. Ulcerative colitis and colorectal cancer. A population-based study. *N Engl J Med*. (1990) 323:1228–33. doi: 10.1056/NEJM199011013231802
2. Adami H, Bretthauer M, Emilsson L, Hernán MA, Kalager M, Ludvigsson JF, et al. The continuing uncertainty about cancer risk in inflammatory bowel disease. *Gut* (2016) 65:889–93. doi: 10.1136/gutjnl-2015-311003
3. Choi C-HR, Al Bakir I, Ding N-S (John), Lee G-H, Askari A, Warusavitarne J, et al. Cumulative burden of inflammation predicts colorectal neoplasia risk in ulcerative colitis: a large single-centre study. *Gut* (2017). doi: 10.1136/gutjnl-2017-314190. [Epub ahead of print].
4. Van Assche G, Dignass A, Bokemeyer B, Danese S, Gionchetti P, Moser G, et al. Second European evidence-based consensus on the diagnosis and management of ulcerative colitis Part 3: special situations. *J Crohn's Colitis*. (2013) 7:1–33. doi: 10.1016/j.crohns.2012.09.005
5. Shergill AK, Lightdale JR, Bruining DH, Acosta RD, Chandrasekhara V, Chathadi KV, et al. The role of endoscopy in inflammatory bowel disease. *Gastrointest Endosc*. (2015) 81:1101–21.e13. doi: 10.1016/j.gie.2014.10.030
6. Rutter MD, Riddell RH. Colorectal dysplasia in inflammatory bowel disease: a clinicopathologic perspective. *Clin Gastroenterol Hepatol*. (2014) 12:359–67. doi: 10.1016/j.cgh.2013.05.033
7. Gatenby RA, Gillies RJ. A microenvironmental model of carcinogenesis. *Nat Rev Cancer* (2008) 8:56–61. doi: 10.1038/nrc2255
8. DeGregori J. Evolved tumor suppression: why are we so good at not getting cancer? *Cancer Res*. (2011) 71:3739–44. doi: 10.1158/0008-5472.CAN-11-0342
9. Greaves M. Cancer's Darwinian dilemma: an evolutionary tale in three acts. *BMJ* (2015) 15:351. doi: 10.1136/bmj.h6581
10. Williams MJ, Werner B, Heide T, Curtis C, Barnes CP, Sottoriva A, et al. Quantification of subclonal selection in cancer from bulk sequencing data. *Nat Genet*. (2018) 28:50:895–903. doi: 10.1038/s41588-018-0128-6
11. Slaughter DP, Southwick HW, Smejkal W. "Field cancerization" in oral stratified squamous epithelium; Clinical implications of multicentric origin. *Cancer*. (1953) 6:963–8.
12. Braakhuis BJM, Tabor MP, Kummer JA, Leemans CR, Brakenhoff RH. A genetic explanation of slaughter's concept of field cancerization: evidence and clinical implications. *Cancer Res*. (2003) 63:1727–30. Available online at: http://cancerres.aacrjournals.org/content/63/8/1727.long
13. Curtius K, Wright NA, Graham TA. An evolutionary perspective on field cancerization. *Nat Rev Cancer* (2017) 18:19–32. doi: 10.1038/nrc.2017.102
14. Choi C-HR, Bakir I Al, Hart AL, Graham TA. Clonal evolution of colorectal cancer in IBD. *Nat Rev Gastroenterol Hepatol*. (2017) 14:218–29. doi: 10.1038/nrgastro.2017.1
15. Leedham SJ, Graham TA, Oukrif D, McDonald SAC, Rodriguez–Justo M, Harrison RF, et al. Clonality, founder mutations, and field cancerization in human ulcerative colitis–associated neoplasia. *Gastroenterology* (2009) 136:542–50.e6. doi: 10.1053/j.gastro.2008.10.086
16. Galandiuk S, Rodriguezjusto M, Jeffery R, Nicholson AM, Cheng Y, Oukrif D, et al. Field cancerization in the intestinal epithelium of patients with Crohn's ileocolitis. *Gastroenterology* (2012) 142:855–64.e8. doi: 10.1053/j.gastro.2011.12.004
17. Salk JJ, Salipante SJ, Risques RA, Crispin DA, Li L, Bronner MP, et al. Clonal expansions in ulcerative colitis identify patients with neoplasia. *Proc Natl Acad Sci USA*. (2009) 106:20871–6. doi: 10.1073/pnas.0909428106
18. Salk JJ, Bansal A, Lai LA, Crispin DA, Ussakli CH, Horwitz MS, et al. Clonal expansions and short telomeres are associated with neoplasia in early-onset, but not late-onset, ulcerative colitis. *Inflamm Bowel Dis*. (2013) 19:2593–602. doi: 10.1097/MIB.0b013e3182a87640
19. Lai LA, Risques RA, Bronner MP, Rabinovitch PS, Crispin D, Chen R, et al. Pan-colonic field defects are detected by CGH in the colons of UC patients with dysplasia/cancer. *Cancer Lett*. (2012) 320:180–8. doi: 10.1016/j.canlet.2012.02.031
20. de Souza HSP, Fiocchi C, Iliopoulos D. The IBD interactome: an integrated view of aetiology, pathogenesis and therapy. *Nat Rev Gastroenterol Hepatol*. (2017) 14:739–49. doi: 10.1038/nrgastro.2017.110
21. Tilg H, Kaser A. Diet and relapsing ulcerative colitis: take off the meat? *Gut* (2004) 53:1399–401. doi: 10.1136/gut.2003.035287
22. Larsson SC, Wolk A. Meat consumption and risk of colorectal cancer: a meta-analysis of prospective studies. *Int J Cancer*. (2006) 119:2657–64. doi: 10.1002/ijc.22170
23. Aune D, Chan DSM, Lau R, Vieira R, Greenwood DC, Kampman E, et al. Dietary fibre, whole grains, and risk of colorectal cancer: systematic review and dose-response meta-analysis of prospective studies. *BMJ* (2011) 2011:343. doi: 10.1136/bmj.d6617
24. Viennois E, Merlin D, Gewirtz AT, Chassaing B. Dietary emulsifier-induced low-grade inflammation promotes colon carcinogenesis. *Cancer Res*. (2017) 77:27–40. doi: 10.1158/0008-5472.CAN-16-1359
25. Chassaing B, Van De Wiele T, De Bodt J, Marzorati M, Gewirtz AT. Dietary emulsifiers directly alter human microbiota composition and gene expression *ex vivo* potentiating intestinal inflammation. *Gut* (2017) 66:1414–27. doi: 10.1136/gutjnl-2016-313099
26. Ananthakrishnan AN, Khalili H, Higuchi LM, Bao Y, Korzenik JR, Giovannucci EL, et al. Higher predicted vitamin D status is associated with reduced risk of Crohn's disease. *Gastroenterology* (2012) 142:482–9. doi: 10.1053/j.gastro.2011.11.040
27. McCullough ML, Zoltick ES, Weinstein SJ, Fedirko V, Wang M, Cook NR, et al. Circulating vitamin D and colorectal cancer risk: an international pooling project of 17 cohorts. *JNCI J Natl Cancer Inst.* (2018). doi: 10.1093/jnci/djy087. [Epub ahead of print].
28. Russell WR, Gratz SW, Duncan SH, Holtrop G, Ince J, Scobbie L, et al. High-protein, reduced-carbohydrate weight-loss diets promote metabolite profiles likely to be detrimental to colonic health. *Am J Clin Nutr*. (2011) 93:1062–72. doi: 10.3945/ajcn.110.002188
29. Donohoe DR, Garge N, Zhang X, Sun W, O'Connell TM, Bunger MK, et al. The microbiome and butyrate regulate energy metabolism and autophagy in the mammalian colon. *Cell Metab*. (2011) 13:517–26. doi: 10.1016/j.cmet.2011.02.018
30. Segain JP, Raingeard de la Blétière D, Bourreille A, Leray V, Gervois N, Rosales C, et al. Butyrate inhibits inflammatory responses through NFkappaB inhibition: implications for Crohn's disease. *Gut* (2000) 47:397–403. doi: 10.1136/gut.47.3.397
31. Chang PV, Hao L, Offermanns S, Medzhitov R. The microbial metabolite butyrate regulates intestinal macrophage function via histone deacetylase inhibition. *Proc Natl Acad Sci USA*. (2014) 111:2247–52. doi: 10.1073/pnas.1322269111
32. Yin L, Laevsky G, Giardina C. Butyrate suppression of colonocyte NF-kappa B activation and cellular proteasome activity. *J Biol Chem*. (2001) 276:44641–6. doi: 10.1074/jbc.M105170200
33. Kelly CJ, Zheng L, Campbell EL, Saeedi B, Scholz CC, Bayless AJ, et al. Crosstalk between microbiota-derived short-chain fatty acids and intestinal epithelial HIF augments tissue barrier function. *Cell Host Microbe*. (2015) 17:662–71. doi: 10.1016/j.chom.2015.03.005
34. Zheng L, Kelly CJ, Battista KD, Schaefer R, Lanis JM, Alexeev EE, et al. Microbial-derived butyrate promotes epithelial barrier function through IL-10 receptor–dependent repression of claudin-2. *J Immunol*. (2017) 199:2976–84. doi: 10.4049/jimmunol.1700105
35. Lazarova DL, Chiaro C, Bordonaro M. Butyrate induced changes in Wnt-signaling specific gene expression in colorectal cancer cells. *BMC Res Notes* (2014) 7:226. doi: 10.1186/1756-0500-7-226
36. Davie JR. Inhibition of histone deacetylase activity by butyrate. *J Nutr*. (2003) 133:2485S–93S. doi: 10.1093/jn/133.7.2485S

37. Donohoe DR, Holley D, Collins LB, Montgomery SA, Whitmore AC, Hillhouse A, et al. A gnotobiotic mouse model demonstrates that dietary fiber protects against colorectal tumorigenesis in a microbiota- and butyrate-dependent manner. *Cancer Discov*. (2014) 4:1387–97. doi: 10.1158/2159-8290.CD-14-0501
38. Magee EA, Richardson CJ, Hughes R, Cummings JH. Contribution of dietary protein to sulfide production in the large intestine: an *in vitro* and a controlled feeding study in humans. *Am J Clin Nutr*. (2000) 72:1488–94. doi: 10.1093/ajcn/72.6.1488
39. Roediger WE, Duncan A, Kapaniris O, Millard S. Reducing sulfur compounds of the colon impair colonocyte nutrition: implications for ulcerative colitis. *Gastroenterology* (1993) 104:802–9. doi: 10.1016/0016-5085(93)91016-B
40. O'Keefe SJD. Diet, microorganisms and their metabolites and colon cancer. *Nat Rev Gastroenterol Hepatol*. (2016) 13:691–706. doi: 10.1038/nrgastro.2016.165
41. Bjerrum JT, Wang Y, Hao F, Coskun M, Ludwig C, Günther U, et al. Metabonomics of human fecal extracts characterize ulcerative colitis, Crohn's disease and healthy individuals. *Metabolomics* (2014) 11:122–33. 10.1007/s11306-014-0677-3
42. Geirnaert A, Calatayud M, Grootaert C, Laukens D, Devriese S, Smagghe G, et al. Butyrate-producing bacteria supplemented *in vitro* to Crohn's disease patient microbiota increased butyrate production and enhanced intestinal epithelial barrier integrity. *Sci Rep*. (2017) 7:11450. doi: 10.1038/s41598-017-11734-8
43. Chen H-M, Yu Y-N, Wang J-L, Lin Y-W, Kong X, Yang C-Q, et al. Decreased dietary fiber intake and structural alteration of gut microbiota in patients with advanced colorectal adenoma. *Am J Clin Nutr*. (2013) 97:1044–52. doi: 10.3945/ajcn.112.046607
44. Chen W, Liu F, Ling Z, Tong X, Xiang C. Human intestinal lumen and mucosa-associated microbiota in patients with colorectal cancer. *PLoS ONE* (2012) 7:e39743. doi: 10.1371/journal.pone.0039743
45. Nakatsu G, Li X, Zhou H, Sheng J, Wong SH, Wu WKK, et al. Gut mucosal microbiome across stages of colorectal carcinogenesis. *Nat Commun*. (2015) 6:8727. doi: 10.1038/ncomms9727
46. Lopez-Siles M, Martinez-Medina M, Surís-Valls R, Aldeguer X, Sabat-Mir M, Duncan SH, et al. Changes in the abundance of faecalibacterium prausnitzii phylogroups i and II in the intestinal mucosa of inflammatory bowel disease and patients with colorectal cancer. *Inflamm Bowel Dis*. (2016) 22:28–41. doi: 10.1097/MIB.0000000000000590
47. Swidsinski A, Khilkin M, Kerjaschki D, Schreiber S, Ortner M, Weber J, et al. Association between intraepithelial *Escherichia coli* and colorectal cancer. *Gastroenterology* (1998) 115:281–6.
48. Prorok-Hamon M, Friswell MK, Alswied A, Roberts CL, Song F, Flanagan PK, et al. Colonic mucosa-associated diffusely adherent afaC+ *Escherichia coli* expressing lpfA and pks are increased in inflammatory bowel disease and colon cancer. *Gut* (2014) 63:761–70. doi: 10.1136/gutjnl-2013-304739
49. Palmela C, Chevarin C, Xu Z, Torres J, Sevrin G, Hirten R, et al. Adherent-invasive *Escherichia coli* in inflammatory bowel disease. *Gut* (2018) 67:574–87. doi: 10.1136/gutjnl-2017-314903
50. McCoy AN, Araujo-Perez F, Azcarate-Peril A, Yeh JJ, Sandler RS, Keku TO. Fusobacterium is associated with colorectal adenomas. *PLoS ONE* (2013) 8:e53653. doi: 10.1371/journal.pone.0053653
51. Gevers D, Kugathasan S, Denson LA, Vázquez-Baeza Y, Van Treuren W, Ren B, et al. The treatment-naive microbiome in new-onset Crohn's disease. *Cell Host Microbe*. (2014) 15:382–92. doi: 10.1016/j.chom.2014.02.005
52. Rubinstein MR, Wang X, Liu W, Hao Y, Cai G, Han YW. *Fusobacterium nucleatum* promotes colorectal carcinogenesis by modulating E-cadherin/β-catenin signaling via its fada adhesin. *Cell Host Microbe*. (2013) 14:195–206. doi: 10.1016/j.chom.2013.07.012
53. Mima K, Nishihara R, Qian ZR, Cao Y, Sukawa Y, Nowak JA, et al. *Fusobacterium nucleatum* in colorectal carcinoma tissue and patient prognosis. *Gut* (2016) 65:1973–80. doi: 10.1136/gutjnl-2015-310101
54. Strauss J, Kaplan GG, Beck PL, Rioux K, Panaccione R, Devinney R, et al. Invasive potential of gut mucosa-derived fusobacterium nucleatum positively correlates with IBD status of the host. *Inflamm Bowel Dis*. (2011) 17:1971–8. doi: 10.1002/ibd.21606
55. Tahara T, Hirata I, Nakano N, Tahara S. Potential link between *Fusobacterium* enrichment and DNA methylation accumulation in the inflammatory colonic mucosa in ulcerative colitis. *Oncotarget* (2017) 8:61917–26. doi: 10.18632/oncotarget.18716
56. Coleman OI, Haller D. Bacterial signaling at the intestinal epithelial interface in inflammation and cancer. *Front Immunol*. (2018) 8:1927. doi: 10.3389/fimmu.2017.01927
57. Arthur JC, Gharaibeh RZ, Mühlbauer M, Perez-Chanona E, Uronis JM, McCafferty J, et al. Microbial genomic analysis reveals the essential role of inflammation in bacteria-induced colorectal cancer. *Nat Commun*. (2014) 5:4724. doi: 10.1038/ncomms5724
58. Arthur JC, Perez-Chanona E, Mühlbauer M, Tomkovich S, Uronis JM, Fan T-J, et al. Intestinal inflammation targets cancer-inducing activity of the microbiota. *Science* (2012) 338:120–3. doi: 10.1126/science.1224820
59. Glasser AL, Boudeau J, Barnich N, Perruchot MH, Colombel JF, Darfeuille-Michaud A. Adherent invasive *Escherichia coli* strains from patients with Crohn's disease survive and replicate within macrophages without inducing host cell death. *Infect Immun*. (2001) 69:5529–37. doi: 10.1128/IAI.69.9.5529-5537.2001
60. Raisch J, Rolhion N, Dubois A, Darfeuille-Michaud A, Bringer M-A. Intracellular colon cancer-associated *Escherichia coli* promote protumoral activities of human macrophages by inducing sustained COX-2 expression. *Lab Investig*. (2015) 95:296–307. doi: 10.1038/labinvest.2014.161
61. Wang X, Yang Y, Huycke MM. Commensal bacteria drive endogenous transformation and tumour stem cell marker expression through a bystander effect. *Gut* (2015) 64:459–68. doi: 10.1136/gutjnl-2014-307213
62. Xavier RJ, Podolsky DK. Unravelling the pathogenesis of inflammatory bowel disease. *Nature* (2007) 448:427–34. doi: 10.1038/nature06005
63. Neurath MF. Cytokines in inflammatory bowel disease. *Nat Rev Immunol*. (2014) 14:329–42. doi: 10.1038/nri3661
64. Zins K, Abraham D, Sioud M, Aharinejad S. Colon cancer cell-derived tumor necrosis factor-a mediates the tumor growth-promoting response in macrophages by up-regulating the colony-stimulating factor-1 pathway. *Cancer Res*. (2007) 67:1038–45. doi: 10.1158/0008-5472.CAN-06-2295
65. Grivennikov S, Karin E, Terzic J, Mucida D, Yu GY, Vallabhapurapu S, et al. IL-6 and stat3 are required for survival of intestinal epithelial cells and development of colitis-associated cancer. *Cancer Cell* (2009) 15:103–13. doi: 10.1016/j.ccr.2009.01.001
66. Jiang R, Wang H, Deng L, Hou J, Shi R, Yao M, et al. IL-22 is related to development of human colon cancer by activation of STAT3. *BMC Cancer* (2013) 13:59. doi: 10.1186/1471-2407-13-59
67. Schreiber RD, Old LJ, Smyth MJ. Cancer immunoediting: Integrating immunity's roles in cancer suppression and promotion. *Science* (2011) 331:1565–70. doi: 10.1126/science.1203486
68. Galon J, Mlecnik B, Bindea G, Angell HK, Berger A, Lagorce C, et al. Towards the introduction of the "Immunoscore" in the classification of malignant tumours. *J Pathol*. (2014) 232:199–209. doi: 10.1002/path.428
69. Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pages C, et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science* (2006) 313:1960–4. doi: 10.1126/science.1129139
70. Mlecnik B, Bindea G, Angell HK, Maby P, Angelova M, Tougeron D, et al. Integrative analyses of colorectal cancer show immunoscore is a stronger predictor of patient survival than microsatellite instability. *Immunity* (2016) 44:698–711. doi: 10.1016/j.immuni.2016.02.025
71. Michael-Robinson JM, Pandeya N, Walsh MD, Biemer-Huttmann AE, Eri RD, Buttenshaw RL, et al. Characterization of tumour-infiltrating lymphocytes and apoptosis in colitis-associated neoplasia: comparison with sporadic colorectal cancer. *J Pathol*. (2006) 208:381–7. doi: 10.1002/path.1895
72. Zisman TL, Bronner MP, Rulyak S, Kowdley KV, Saunders M, Lee SD, et al. Prospective study of the progression of low-grade dysplasia in ulcerative colitis using current cancer surveillance guidelines. *Inflamm Bowel Dis*. (2012) 18:2240–6. doi: 10.1002/ibd.22912
73. Hofstad B, Vatn MH, Andersen SN, Huitfeldt HS, Rognum T, Larsen S, et al. Growth of colorectal polyps: redetection and evaluation of unresected polyps for a period of three years. *Gut* (1996) 39:449–56. doi: 10.1136/gut.39.3.449
74. Erdman SE, Sohn JJ, Rao VP, Nambiar PR, Ge Z, Fox JG, et al. CD4+CD25+ regulatory lymphocytes induce regression of intestinal tumors in ApcMin/+ mice. *Cancer Res*. (2005) 65:3998–4004. doi: 10.1158/0008-5472.CAN-04-3104

75. Baker A-M, Cross W, Curtius K, Al Bakir I, Choi C-HR, Davis HL, et al. Evolutionary history of human colitis-associated colorectal cancer. *Gut* (2018). doi: 10.1136/gutjnl-2018-316191. [Epub ahead of print].
76. Söderlund S, Tribukait B, Åst Ö, Broström O, Karlén P, Löfberg R, et al. Colitis-associated DNA aneuploidy and dysplasia in Crohn's disease and risk of colorectal cancer. *Inflamm Bowel Dis*. (2011) 17:1101–7. doi: 10.1002/ibd.21477
77. Davoli T, Uno H, Wooten EC, Elledge SJ. Tumor aneuploidy correlates with markers of immune evasion and with reduced response to immunotherapy. *Science* (2017) 355:eaaf8399. doi: 10.1126/science.aaf8399
78. Zhu J, Tsai HJ, Gordon MR, Li R. Cellular stress associated with aneuploidy. *Dev Cell* (2018) 44:420–31. doi: 10.1016/j.devcel.2018.02.002
79. Bakhoum SF, Ngo B, Laughney AM, Cavallo JA, Murphy CJ, Ly P, et al. Chromosomal instability drives metastasis through a cytosolic DNA response. *Nature* (2018) 553:467–72. doi: 10.1038/nature25432
80. Gulen MF, Koch U, Haag SM, Schuler F, Apetoh L, Villunger A, et al. Signalling strength determines proapoptotic functions of STING. *Nat Commun*. (2017) 8:427. doi: 10.1038/s41467-017-00573-w
81. Warabi M, Kitagawa M, Hirokawa K. Loss of MHC class II expression is associated with a decrease of tumor-infiltrating T cells and an increase of metastatic potential of colorectal cancer: immunohistological and histopathological analyses as compared with normal colonic mucosa and adenomas. *Pathol Res Pract*. (2000) 196:807–15. doi: 10.1016/S0344-0338(00)80080-1
82. Bernsen MR, Håkansson L, Gustafsson B, Krysander L, Rettrup B, Ruiter D, et al. On the biological relevance of MHC class II and B7 expression by tumour cells in melanoma metastases. *Br J Cancer* (2003) 88:424–31. doi: 10.1038/sj.bjc.6600703
83. Cromme FV, van Bommel PF, Walboomers JM, Gallee MP, Stern PL, Kenemans P, et al. Differences in MHC and TAP-1 expression in cervical cancer lymph node metastases as compared with the primary tumours. *Br J Cancer* (1994) 69:1176–81.
84. Garrity-Park MM, Loftus EV, Sandborn WJ, Bryant SC, Smyrk TC. MHC class II alleles in ulcerative colitis-associated colorectal cancer. *Gut* (2009) 58:1226–33. doi: 10.3748/wjg.v20.i44.16389
85. Khalili H, Gong J, Brenner H, Austin TR, Hutter CM, Baba Y, et al. Identification of a common variant with potential pleiotropic effect on risk of inflammatory bowel disease and colorectal cancer. *Carcinogenesis* (2015) 36:999–1007. doi: 10.1093/carcin/bgv086
86. Song JH, Huels DJ, Ridgway RA, Sansom OJ, Kholodenko BN, Kolch W, et al. The APC network regulates the removal of mutated cells from colonic crypts. *Cell Rep*. (2014) 7:94–103. doi: 10.1016/j.celrep.2014.02.043
87. Kozar S, Morrissey E, Nicholson AM, van der Heijden M, Zecchini HI, Kemp R, et al. Continuous clonal labeling reveals small numbers of functional stem cells in intestinal crypts and adenomas. *Cell Stem Cell*. (2013) 13:626–33. doi: 10.1016/j.stem.2013.08.001
88. Baker AM, Cereser B, Melton S, Fletcher AG, Rodriguez-Justo M, Tadrous PJ, et al. Quantification of crypt and stem cell evolution in the normal and neoplastic human colon. *Cell Rep*. (2014) 8:940–7. doi: 10.1016/j.celrep.2014.07.019
89. Nicholson AM, Olpe C, Hoyle A, Wilkinson M, Morrissey E, Winton DJ, et al. Fixation and spread of somatic mutations in adult human colonic epithelium. *Cell Stem Cell* (2018) 22:909–18.e8. doi: 10.1016/j.stem.2018.04.020
90. Barker N, van Es JH, Kuipers J, Kujala P, van den Born M, Cozijnsen M, et al. Identification of stem cells in small intestine and colon by marker gene Lgr5. *Nature* (2007) 449:1003–7. doi: 10.1038/nature06196
91. Clevers H. The intestinal crypt, a prototype stem cell compartment. *Cell* (2013) 154:274–84. doi: 10.1016/j.cell.2013.07.004
92. Dejea CM, Wick EC, Hechenbleikner EM, White JR, Mark Welch JL, Rossetti BJ, et al. Microbiota organization is a distinct feature of proximal colorectal cancers. *Proc Natl Acad Sci USA*. (2014) 111:18321–6. doi: 10.1073/pnas.1406199111
93. Kang H, Shibata D. Direct measurements of human colon crypt stem cell niche genetic fidelity: the role of chance in non-darwinian mutation selection. *Front Oncol*. (2013) 3:264. doi: 10.3389/fonc.2013.00264
94. Tomasetti C, Vogelstein B, Parmigiani G. Half or more of the somatic mutations in cancers of self-renewing tissues originate prior to tumor initiation. *Proc Natl Acad Sci USA*. (2013) 110:1999–2004. doi: 10.1073/pnas.1221068110
95. Greaves LC, Preston SL, Tadrous PJ, Taylor RW, Barron MJ, Oukrif D, et al. Mitochondrial DNA mutations are established in human colonic stem cells, and mutated clones expand by crypt fission. *Proc Natl Acad Sci USA*. (2006) 103:714–9. doi: 10.1073/pnas.0505903103
96. Miyoshi H, Ajima R, Luo CT, Yamaguchi TP, Stappenbeck TS. Wnt5a potentiates TGF-β signaling to promote colonic crypt regeneration after tissue injury. *Science* (2012) 338:108–13. doi: 10.1126/science.1223821
97. Seno H, Miyoshi H, Brown SL, Geske MJ, Colonna M, Stappenbeck TS. Efficient colonic mucosal wound repair requires Trem2 signaling. *Proc Natl Acad Sci USA*. (2009) 106:256–61. doi: 10.1073/pnas.0803343106
98. Vermeulen L, Morrissey E, Van Der Heijden M, Nicholson AM, Sottoriva A, Buczacki S, et al. Defining stem cell dynamics in models of intestinal tumor initiation. *Science* (2013) 342:995–8. doi: 10.1126/science.1243148
99. Brentnall TA, Crispin DA, Rabinovitch PS, Haggitt RC, Rubin CE, Stevens AC, et al. Mutations in the p53 gene: an early marker of neoplastic progression in ulcerative colitis. *Gastroenterology* (1994) 107:369–78.
100. Rosman-Urbach M, Niv Y, Birk Y, Smirnoff P, Zusman I, Morgenstern S, et al. A high degree of aneuploidy, loss of p53 gene, and low soluble p53 protein serum levels are detected in ulcerative colitis patients. *Dis Colon Rectum*. (2004) 47:304–13. doi: 10.1007/s10350-003-0048-z
101. Hao XP. The spectrum of p53 mutations in colorectal adenomas differs from that in colorectal carcinomas. *Gut* (2002) 50:834–9. doi: 10.1136/gut.50.6.834
102. Kruiswijk F, Labuschagne CF, Vousden KH. p53 in survival, death and metabolic health: a lifeguard with a licence to kill. *Nat Rev Mol Cell Biol*. (2015) 16:393–405. doi: 10.1038/nrm4007
103. Giatromanolaki A, Koukourakis MI, Koutsopoulos AV, Harris AL, Gatter KC, Sivridis E. Autophagy and hypoxia in colonic adenomas related to aggressive features. *Color Dis*. (2013) 15:e223–30. doi: 10.1111/codi.12147
104. Cooks T, Harris CC, Oren M. Caught in the crossfire: p53 in inflammation. *Carcinogenesis* (2014) 35:1680–90. doi: 10.1093/carcin/bgu134
105. Cooks T, Pateras IS, Tarcic O, Solomon H, Schetter AJ, Wilder S, et al. Mutant p53 prolongs NF-κB activation and promotes chronic inflammation and inflammation-associated colorectal cancer. *Cancer Cell* (2013) 23:634–46. doi: 10.1016/j.ccr.2013.03.022
106. Snippert HJ, Schepers AG, Van Es JH, Simons BD, Clevers H. Biased competition between Lgr5 intestinal stem cells driven by oncogenic mutation induces clonal expansion. *EMBO Rep*. (2014) 15:62–9. doi: 10.1002/embr.201337799
107. Pruitt SC, Freeland A, Kudla A. Cell cycle heterogeneity in the small intestinal crypt and maintenance of genome integrity. *Stem Cells* (2010) 28:1250–9. doi: 10.1002/stem.450
108. Andersen SN, Rognum TO, Bakka A, Clausen OP. Ki-67: a useful marker for the evaluation of dysplasia in ulcerative colitis. *Mol Pathol*. (1998) 51:327–32. doi: 10.1136/mp.51.6.327
109. Kim HS, Lee C, Kim WH, Maeng YH, Jang BG. Expression profile of intestinal stem cell markers in colitis-associated carcinogenesis. *Sci Rep*. (2017) 7:6533. doi: 10.1038/s41598-017-06900-x
110. Tian H, Biehs B, Warming S, Leong KG, Rangell L, Klein OD, et al. A reserve stem cell population in small intestine renders Lgr5-positive cells dispensable. *Nature* (2011) 478:255–9. doi: 10.1038/nature10408
111. Metcalfe C, Kljavin NM, Ybarra R, De Sauvage FJ. Lgr5+ stem cells are indispensable for radiation-induced intestinal regeneration. *Cell Stem Cell*. (2014) 14:149–59. doi: 10.1016/j.stem.2013.11.008
112. Gerbe F, Brulin B, Makrini L, Legraverend C, Jay P. DCAMKL-1 expression identifies Tuft cells rather than stem cells in the adult mouse intestinal epithelium. *Gastroenterology* (2009) 137:2179–80; author reply 2180-1. doi: 10.1053/j.gastro.2009.06.072
113. Westphalen CB, Asfaha S, Hayakawa Y, Takemoto Y, Lukin DJ, Nuber AH, et al. Long-lived intestinal tuft cells serve as colon cancer-initiating cells. *J Clin Invest*. (2014) 124:1283–95. doi: 10.1172/JCI73434
114. Loeffler M, Bratke T, Paulus U, Li YQ, Potten CS. Clonality and life cycles of intestinal crypts explained by a state dependent stochastic model of epithelial stem cell organization. *J Theor Biol*. (1997) 186:41–54. doi: 10.1006/jtbi.1996.0340
115. Langlands AJ, Almet AA, Appleton PL, Newton IP, Osborne JM, Näthke IS. Paneth cell-rich regions separated by a cluster of Lgr5+ cells initiate

crypt fission in the intestinal stem cell niche. *PLoS Biol.* (2016) 14:e1002491. doi: 10.1371/journal.pbio.1002491
116. Cheng H, Bjerknes M, Amar J, Gardiner G. Crypt production in normal and diseased human colonic epithelium. *Anat Rec.* (1986) 216:44–8. doi: 10.1002/ar.1092160108
117. Shoshkes-Carmel M, Wang YJ, Wangensteen KJ, Tóth B, Kondo A, Massassa EE, et al. Subepithelial telocytes are an important source of Wnts that supports intestinal crypts. *Nature* (2018) 557:242–6. doi: 10.1038/s41586-018-0084-4
118. Degirmenci B, Valenta T, Dimitrieva S, Hausmann G, Basler K. essential Wnt-secreting niche for colon stem cells. *Nature* (2018) 558:449–53. doi: 10.1038/s41586-018-0190-3
119. Lees CW, Zacharias WJ, Tremelling M, Noble CL, Nimmo ER, Tenesa A, et al. Analysis of germline GLI1 Variation Implicates Hedgehog Signalling in the Regulation of Intestinal Inflammatory Pathways. *PLoS Med*. (2008) 5:e239. doi: 10.1371/journal.pmed.0050239
120. Kaiko GE, Ryu SH, Koues OI, Pearce EL, Oltz EM, Stappenbeck TS, et al. The colonic crypt protects stem cells from microbiota-derived metabolites. *Cell* (2016) 165:1–13. doi: 10.1016/j.cell.2016.05.018
121. Dreher D, Junod AF. Role of oxygen free radicals in cancer development. *Eur J Cancer* (1996) 32A:30–8. doi: 10.1016/0959-8049(95)00531-5
122. Alexandrov LB, Nik-Zainal S, Wedge DC, Aparicio SAJR, Behjati S, Biankin A V, et al. Signatures of mutational processes in human cancer. *Nature* (2013) 500:415–21. doi: 10.1038/nature12477
123. Robles AI, Traverso G, Zhang M, Roberts NJ, Khan MA, Joseph C, et al. Whole-exome sequencing analyses of inflammatory bowel disease-associated colorectal cancers. *Gastroenterology* (2016) 150:931–43. doi: 10.1053/j.gastro.2015.12.036
124. Alexandrov LB, Jones PH, Wedge DC, Sale JE, Campbell PJ, Nik-Zainal S, et al. Clock-like mutational processes in human somatic cells. *Nat Genet*. (2015) 47:1402–7. doi: 10.1038/ng.3441
125. O'Sullivan JN, Bronner MP, Brentnall TA, Finley JC, Shen W-T, Emerson S, et al. Chromosomal instability in ulcerative colitis is related to telomere shortening. *Nat Genet*. (2002) 32:280–4. doi: 10.1038/ng989
126. Risques RA, Lai LA, Brentnall TA, Li L, Feng Z, Gallaher J, et al. Ulcerative colitis is a disease of accelerated colon aging: evidence from telomere attrition and DNA damage. *Gastroenterology* (2008) 135:410–8. doi: 10.1053/j.gastro.2008.04.008
127. Risques RA, Lai LA, Himmetoglu C, Ebaee A, Li L, Feng Z, et al. Ulcerative colitis-associated colorectal cancer arises in a field of short telomeres, senescence, and inflammation. *Cancer Res*. (2011) 71:1669–79. doi: 10.1158/0008-5472.CAN-10-1966
128. Issa JP, Ahuja N, Toyota M, Bronner MP, Brentnall TA. Accelerated age-related CpG island methylation in ulcerative colitis. *Cancer Res*. (2001) 61:3573–7.
129. Yaeger R, Shah MA, Miller VA, Kelsen JR, Wang K, Heins ZJ, et al. Genomic alterations observed in colitis-associated cancers are distinct from those found in sporadic colorectal cancers and vary by type of inflammatory bowel disease. *Gastroenterology* (2016) 151:278–287.e6. doi: 10.1053/j.gastro.2016.04.001
130. Fujita M, Matsubara N, Matsuda I, Maejima K, Oosawa A, Yamano T, et al. Genomic landscape of colitis-associated cancer indicates the impact of chronic inflammation and its stratification by mutations in the Wnt signaling. *Oncotarget* (2018) 9:969–81. doi: 10.18632/oncotarget.22867
131. Sussman D, Santaolalla R, Strobel S, Dheer R, Abreu MT. Cancer in inflammatory bowel disease: lessons from animal models. *Curr Opin Gastroenterol.* (2012) 28:327–33. doi: 10.1097/MOG.0b013e32835 4cc36
132. Pan Q, Lou X, Zhang J, Zhu Y, Li F, Shan Q, et al. Genomic variants in mouse model induced by azoxymethane and dextran sodium sulfate improperly mimic human colorectal cancer. *Sci Rep*. (2017) 7:25. doi: 10.1038/s41598-017-00057-3
133. Gerling M, Glauben R, Habermann JK, Kühl AA, Loddenkemper C, Lehr HA, et al. Characterization of chromosomal instability in murine colitis-associated colorectal cancer. *PLoS ONE* (2011) 6:e22114. doi: 10.1371/journal.pone.0022114
134. Gerling M, Meyer KF, Fuchs K, Igl BW, Fritzsche B, Ziegler A, et al. High frequency of aneuploidy defines ulcerative colitis-associated carcinomas: A prognostic comparison to sporadic colorectal carcinomas. *Ann Surg*. (2010) 252:74–83. doi: 10.1097/SLA.0b013e3181deb664
135. Luebeck EG, Curtius K, Jeon J, Hazelton WD. Impact of tumor progression on cancer incidence curves. *Cancer Res*. (2013) 73:1086–96. doi: 10.1158/0008-5472.CAN-12-2198
136. Risques RA, Kennedy SR. Aging and the rise of somatic cancer-associated mutations in normal tissues. *PLoS Genet*. (2018) 14:e1007108. doi: 10.1371/journal.pgen.1007108
137. Curtius K, Wong CJ, Hazelton WD, Kaz AM, Chak A, Willis JE, et al. A molecular clock infers heterogeneous tissue age among patients with barrett's esophagus. karchin R. *PLoS Comput Biol*. (2016) 12:e1004919. doi: 10.1371/journal.pcbi.1004919
138. Martinez P, Mallo D, Paulson TG, Li X, Sanchez CA, Reid BJ, et al. Evolution of Barrett's esophagus through space and time at single-crypt and whole-biopsy levels. *Nat Commun.* (2018) 9:794. doi: 10.1038/s41467-017-02621-x
139. Spechler SJ, Souza RF. Barrett's Esophagus. *N Engl J Med*. (2014) 371:836–45. doi: 10.1056/NEJMra1314704
140. Lin S-H, Raju GS, Huff C, Ye Y, Gu J, Chen J-S, et al. The somatic mutation landscape of premalignant colorectal adenoma. *Gut* (2017) 67:1299–305. doi: 10.1136/gutjnl-2016-313573
141. Merlo LMF, Shah NA, Li X, Blount PL, Vaughan TL, Reid BJ, et al. A comprehensive survey of clonal diversity measures in Barrett's esophagus as biomarkers of progression to esophageal adenocarcinoma. *Cancer Prev Res*. (2010) 3:1388–97. doi: 10.1158/1940-6207.CAPR-10-0108
142. Martinez P, Timmer MR, Lau CT, Calpe S, Sancho-Serra M del C, Straub D, et al. Dynamic clonal equilibrium and predetermined cancer risk in Barrett's oesophagus. *Nat Commun*. (2016) 7:12158. doi: 10.1038/ncomms12158
143. Li X, Galipeau PC, Paulson TG, Sanchez CA, Arnaudo J, Liu K, et al. Temporal and spatial evolution of somatic chromosomal alterations: a case-cohort study of Barrett's esophagus. *Cancer Prev Res (Phila)*. (2014) 7:114–27. doi: 10.1158/1940-6207.CAPR-13-0289
144. Weaver JMJ, Ross-Innes CS, Shannon N, Lynch AG, Forshew T, Barbera M, et al. Ordering of mutations in preinvasive disease stages of esophageal carcinogenesis. *Nat Genet*. (2014) 46:837–43. doi: 10.1038/ng.3013
145. Walker R, Mejia J, Lee JK, Pimiento JM, Malafa M, Giuliano AR, et al. Personalizing gastric cancer screening with predictive modeling of disease progression biomarkers. *Appl Immunohistochem Mol Morphol.* (2017). doi: 10.1097/PAI.0000000000000598. [Epub ahead of print].
146. Rubin CE, Haggitt RC, Burmer GC, Brentnall TA, Stevens AC, Levine DS, et al. DNA aneuploidy in colonic biopsies predicts future development of dysplasia in ulcerative colitis. *Gastroenterology* (1992) 103:1611–20. doi: 10.1016/0016-5085(92)91185-7
147. Tsai J-H, Rabinovitch PS, Huang D, Small T, Mattis AN, Kakar S, et al. Association of aneuploidy and flat dysplasia with development of high-grade dysplasia or colorectal cancer in patients with inflammatory bowel disease. *Gastroenterology* (2017) 153:1492–5.e4. doi: 10.1053/j.gastro.2017.08.031
148. Lofberg R, Brostrom O, Karlen P, Ost A, Tribukait B. DNA aneuploidy in ulcerative-colitis - reproducibility, topographic distribution, and relation to dysplasia. *Gastroenterology* (1992) 102:1149–54. doi: 10.1016/0016-5085(92)90751-J
149. Kabir M, Curtius K, Al-Bakir I, Choi C-HR, Graham T, Wilson A, et al. P127 developing a colorectal cancer risk prediction tool for patients with ulcerative colitis and low-grade dysplasia. *J Crohn's Colitis* (2018) 12(Suppl. 1):S157–8. doi: 10.1093/ecco-jcc/jjx180.254
150. Connelly TM, Koltun WA. The surgical treatment of inflammatory bowel disease-associated dysplasia. *Expert Rev Gastroenterol Hepatol.* (2013) 7:307–22. doi: 10.1586/egh.13.17
151. Bonovas S, Fiorino G, Lytras T, Nikolopoulos G, Peyrin-Biroulet L, Danese S. Systematic review with meta-analysis: use of 5-aminosalicylates and risk of colorectal neoplasia in patients with inflammatory bowel disease. *Aliment Pharmacol Ther*. (2017) 45:1179–92. doi: 10.1111/apt.14023
152. Brown JB, Lee G, Managlia E, Grimm GR, Dirisina R, Goretsky T, et al. 5-ASA inhibits epithelial beta-catenin activation in chronic ulcerative colitis. *Gastroenterology* (2010) 138:595. doi: 10.1053/j.gastro.2009.10.038
153. Qiu W, Wang X, Leibowitz B, Liu H, Barker N, Okada H, et al. Chemoprevention by nonsteroidal anti-inflammatory drugs eliminates oncogenic intestinal stem cells via SMAC-dependent apoptosis. *Proc Natl Acad Sci USA*. (2010) 107:20027–32. doi: 10.1073/pnas.1010430107

154. Pineton de Chambrun G, Blanc P, Peyrin-Biroulet L. Current evidence supporting mucosal healing and deep remission as important treatment goals for inflammatory bowel disease. *Exp Rev Gastroenterol Hepatol*. (2016) 10:915–27. doi: 10.1586/17474124.2016.1174064
155. Kostadinov RL, Kuhner MK, Li X, Sanchez CA, Galipeau PC, Paulson TG, et al. NSAIDs modulate clonal evolution in barrett's esophagus. *PLoS Genet*. (2013) 9:e1003553. doi: 10.1371/journal.pgen.1003553
156. Galipeau PC, Oman KM, Paulson TG, Sanchez CA, Zhang Q, Marty JA, et al. NSAID use and somatic exomic mutations in Barrett's esophagus. *Genome Med*. (2018) 10:17. doi: 10.1186/s13073-018-0520-y
157. Polytarchou C, Koukos G, Iliopoulos D. Systems biology in inflammatory bowel diseases: ready for prime time. *Curr Opin Gastroenterol*. (2014) 30:339–46. doi: 10.1097/MOG.0000000000000081
158. Werner HMJ, Mills GB, Ram PT. Cancer systems biology: a peek into the future of patient care? *Nat Rev Clin Oncol*. (2014) 11:167–76. doi: 10.1038/nrclinonc.2014.6
159. Ma C, Moran GW, Benchimol EI, Targownik LE, Heitman SJ, Hubbard JN, et al. Surgical rates for crohn's disease are decreasing: a population-based time trend analysis and validation study. *Am J Gastroenterol*. (2017) 112:1840–8. doi: 10.1038/ajg.2017.394
160. Zhang J, Cunningham JJ, Brown JS, Gatenby RA. Integrating evolutionary dynamics into treatment of metastatic castrate-resistant prostate cancer. *Nat Commun*. (2017) 8:1816. doi: 10.1038/s41467-017-01968-5
161. Shi Y, Dong Y, Huang W, Zhu D, Mao H, Su P. Fecal microbiota transplantation for ulcerative colitis: a systematic review and meta-analysis. *PLoS ONE* (2016) 11:e0157259. doi: 10.1371/journal.pone.0157259
162. Moayyedi P, Surette MG, Kim PT, Libertucci J, Wolfe M, Onischi C, et al. Fecal microbiota transplantation induces remission in patients with active ulcerative colitis in a randomized controlled trial. *Gastroenterology* (2015) 149:102–9. doi: 10.1053/j.gastro.2015.04.001
163. Loewe L. A framework for evolutionary systems biology. *BMC Syst Biol*. (2009) 3:27. doi: 10.1186/1752-0509-3-27

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CD3+CD4+LAP+Foxp3-Regulatory Cells of the Colonic Lamina Propria Limit Disease Extension in Ulcerative Colitis

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Background and Aims: In ulcerative colitis (UC), inflammation begins in the rectum and can extend proximally throughout the entire colon. The extension of inflammation is an important determinant of disease course, and may be limited by the action of regulatory T cells (Tregs). In this cross-sectional study, we evaluated the relationship between UC extension and the proportions of CD3+CD4+Foxp3+ and CD3+CD4+LAP+Foxp3- Tregs in the colonic lamina propria (LP) of 79 UC patients and 29 controls. The role of these cells in UC extension was also investigated in the murine oxazolone-induced colitis model.

Methods: Patients: Disease extension was classified according to the Montreal classification. Where possible, endoscopic biopsies of involved and uninvolved tissue were obtained from UC patients. Mouse model: Colitis was induced by intrarectal oxazolone administration. Lamina propria mononuclear cells were isolated from patient biopsies and mouse colon tissue using enzymatic method and the percentage of CD3+CD4+Foxp3+ and CD3+CD4+LAP+Foxp3-cells evaluated by immunofluorescence. Confocal microscopy was applied for the visualization and quantification of CD4+LAP+ cells on tissue histological sections.

Results: In UC patients with distal colitis the proportion of LP CD3+CD4+Foxp3+ Tregs was significantly higher in inflamed tissue than uninvolved tissue. As opposite, the proportion of LP CD3+CD4+LAP+ Tregs was significantly higher in uninvolved tissue than involved tissue. Both LP CD3+CD4+Foxp3+ and LP CD3+CD4+LAP+ Tregs proportion in involved tissue was significantly higher than in controls irrespective of the extension of inflammation. In mice with oxazolone-induced distal colitis, treatment with LAP-depleting antibody was associated with the development of extensive colitis.

Conclusions: Our findings suggest that CD3+CD4+LAP+Foxp3-Tregs limit the extension of inflammatory lesions in UC patients.

Keywords: CD3+CD4+LAP+Foxp3-regulatory cells, inflammation, immunity, oxazolone colitis, regulatory T cells, ulcerative colitis

INTRODUCTION

Ulcerative colitis (UC) is characterized by inflammation, typically restricted to the mucosal surface that begins in the rectum and can continuously extend proximally throughout the entire colon (1). A prospective study of Norwegian UC patients found that at the time of presentation, colitis was limited to the rectum (proctitis) in one third of patients, the colorectum distal to the splenic flexure (left-sided colitis) in another third, and the area proximal to the splenic flexure (extensive colitis) in the remaining third (2). In patients with distal colitis, inflammation progresses in 25–50% of patients, while regression is observed in about 16% of patients (3, 4). The anatomical extent of mucosal inflammation is one of the most important factors determining disease course. Patients with extensive colitis have a greater risk of colectomy than those with proctitis (5–7), as well as a greater risk of colorectal cancer (8, 9). Proximal disease extension is associated with increased disease severity upon diagnosis and greater likelihood of clinical relapse (10, 11). Moreover, extensive colitis is associated with an increased frequency of extraintestinal manifestations, a steroid-refractory disease course, and the need for immunosuppressive and immune-modulating medications and surgery (10, 12). Younger age at diagnosis and the presence of sclerosing cholangitis are pre-existing independent predictive factors for disease progression (12).

The biological factors that determine the extent of inflammation in UC are unknown. Tregs have been identified as a key immunosuppressive population that is critically involved in maintaining intestinal homeostasis. Therefore, we hypothesized that Tregs may be involved in limiting the extension of inflammation. To investigate this hypothesis, we evaluated the frequencies of different Treg types in the lamina propria (LP) of UC patients with varying degrees of disease extension, and examined the contribution of these cells to disease extension in a mouse model of UC. Specifically, we analyzed the frequencies of CD3+CD4+Foxp3+ cells, as well as another type of Treg, CD3+CD4+ latency associated peptide (LAP)+ Foxp3-regulatory cells. We previously demonstrated that the latter cell type is present in human LP (13), and is found at a higher frequency in the LP of endoscopically active UC patients, but not in Crohn's disease patients (14). Moreover, LAP^+ $Foxp3^-$ T cells have been described to infiltrate colorectal cancer exhibiting more potent immunosuppressive activity than $Foxp3^+$ regulatory T cells (15).

We found that in UC patients with proctitis and left-sided colitis the proportion of LP CD3+CD4+Foxp3+ Tregs was significantly higher in inflamed tissue than uninvolved tissue. As opposite, the proportion of LP CD3+CD4+LAP+ Tregs was significantly higher in uninvolved tissue than involved tissue. In a mouse model of distal colitis (16), we found that administration of an LAP-depleting antibody that has no effect on the frequency of CD4+Foxp3+ Tregs (17) was associated with the development of extensive colitis, suggesting that CD3+CD4+LAP+Foxp3-regulatory cells limit the extension of inflammatory lesions in UC.

MATERIALS AND METHODS

Patients

A total of 95 patients with endoscopically active UC who underwent colonoscopy for clinical flare-up at 2 tertiary centers (IBD, GE Unit, Sandro Pertini Hospital, Rome, and the Department of General Surgery, "P. Stefanini," Sapienza University, Rome) were included in the study. Disease activity was assessed using the endoscopic Mayo score (18). Patients with an endoscopic score $\geq$1, not on rectal 5-ASA and/or steroids in the last 3 months, were considered to have endoscopically active UC and were enrolled in the study. The control group consisted of 29 participants [14 men, 15 women; mean $\pm$ SE age, 55 $\pm$ 3 years; median (range) age, 56 (26–85)] undergoing colonoscopy for colonic cancer screening and suspected functional bowel disorders. In UC patients, disease extension, at time of endoscopy, was classified using the Montreal classification (19) as follows: proctitis, involvement limited to the rectum; left-sided colitis, involvement limited to a portion of the colorectum distal to the splenic flexure; extensive colitis, involvement extending proximal to the splenic flexure.

Biopsy Specimens

Multiple endoscopic mucosal biopsies were obtained. Biopsies were taken from endoscopically involved and uninvolved areas in UC patients, and from matched areas in controls. Diagnosis of UC was established based on established criteria and the localization and extension of the disease were confirmed by histology. Histopathology was quantified in H&E stained tissue sections using the Robarts histopathology index (RHI) (20). An RHI $\leq$6 in samples collected from involved tissue was considered indicative of remission, and the sample, together with the paired sample collected from uninvolved tissue, was excluded from the analysis. Accordingly, 16 patients with a Mayo endoscopic score $=$ 1 were excluded from the analysis. The remaining 79 patients were evaluated applying the aforementioned criteria. In these patients, RHI $<$ 3 were recorded for all biopsy samples collected from uninvolved tissue. Histology confirmed the absence of inflammatory changes in controls.

Some tissue sections were also analyzed by confocal microscopy. Three micrometer thick paraffin-embedded sections of human colon tissue from controls (CTR) and ulcerative colitis (UC) patients were stained after deparaffinization in xylene (5 min, two times), rehydratation by sequential washes in 100% ethanol (3 min), 95% ethanol (3 min), 80% ethanol (3 min), 70% ethanol, 50% ethanol, deionized water and antigen retrieval (5 min at 95°C in 10 mM sodium citrate, pH 6.0). Slides were saturated with blocking buffer (PBS, 0.05% tween 20, 4% BSA) for 1 h at room temperature. Specimens were stained with a polyclonal rabbit anti-human CD4 at 5 μg/ml, followed by donkey anti-rabbit-AlexaFluor-568, and a monoclonal mouse anti-human LAP followed by an goat anti-mouse AlexaFluor-647. After washing, slides were mounted in Prolong Gold anti-fade medium containing a DNA dye (DAPI). Confocal laser scanning microscopy (CLSM) observations were performed with a Leica TCS SP2 AOBS apparatus, using a 63x/1.40 NA oil

objective. Acquisition of images was performed by a Leica confocal software 2.6 (Leica, Germany).

Clinicopathological variables for all patients are shown in **Table 1**.

Isolation of Lamina Propria Mononuclear Cells (LPMCs)

LPMCs were isolated from freshly obtained biopsies using a previously described DDT-EDTA collagenase method (13, 14). Briefly, biopsies were washed in HBBS free of calcium and magnesium (HBSS-CMF; Hyclone, Europe LTD, Cramlington, United Kingdom), and then incubated for 5 min at room temperature in HBSS-CMF containing 1 mmol/l DTT (Sigma Chemical Co., St. Louis, MO, United States) and antibiotics (penicillin, 100 U/ml; streptomycin, 100 mg/ml; gentamicin, 50 mg/ml; and fungizone, 25 mg/ml). After washing 3 times in HBSS-CMF, the biopsies were cut into smaller pieces and incubated in HBSS-CMF containing 0.75 mmol/l EDTA, 10 mmol/l HEPES buffer, and antibiotics for 15 min at 37°C in a humid 5% CO_2 atmosphere to remove epithelial cells. After 2 washes, the tissue was incubated for a total of 2 h (2 × 1-h incubations) at 37°C in a humid 5% CO_2 atmosphere in complete medium (RPMI 1640 plus 10 mM HEPES buffer, 2 mM l-glutamine, 10% heat-inactivated FCS (Hyclone), and antibiotics) containing 25 U/ml collagenase V (Sigma-Aldrich, Milan, Italy) and 100 μg/ml of DNase (Roche Diagnostics, Mannheim, Germany). After incubation, the supernatant containing LMPCs was collected and washed twice in HBSS-CMF + antibiotics.

Antibodies And Reagents

APC-Cy7-labeled anti-CD3, PE-Cy7-labeled anti-IL-10, and isotype-matched control Igs were obtained from Biolegend (San Diego, CA, United States). PE-labeled anti IL-17A, and isotype-matched Ig control were obtained from eBioscience [San Diego, CA, United States]. FITC-labeled anti-CD4, PE-CF594-labeled anti-CD8 and isotype-matched control Ig were obtained from Becton Dickinson Horizon (San Jose, CA, United States). PerCP-labeled anti-LAP [TGF-β1] and isotype-matched control Ig were obtained from R&D Systems (Minneapolis, MN, United States). APC-labeled anti-Foxp3, the Foxp3 staining buffer set, and isotype-matched control Ig were obtained from eBioscience (San Diego, CA, United States). LIVE/DEAD ® Fixable Aqua Dead Cell Stain Kit was obtained from Life Technologies (Carlsbad, CA, United States). Phorbol-12-myristateacetate (PMA) and ionomycin were obtained from Sigma-Aldrich. FITC-labeled anti-CD4, isotype-matched control Igs, and Monensin solution (Golgi Stop) were obtained from BD Pharmingen (San Jose, CA, United States). For confocal microscopy imaging, polyclonal rabbit anti-CD4 (Novus, Colorado, United States), Donkey anti-rabbit-AlexaFluor-568 (Abcam, Cambridge, United Kingdom), monoclonal mouse anti-human LAP (R&D Systems) and goat anti-mouse AlexaFluor-647 (Abcam) were used.

Immunofluorescence Staining

Isolated LPMCs were incubated for 30 min with LIVE/DEAD® Fixable Aqua Dead Cell Stain. Next, cells were washed and stained with anti-human-CD3, anti-human-CD4, and anti-human LAP (TGF-β1). After incubation, cells were washed, fixed, and permeabilized with fixation/permeabilization solution for 40 min, and stained with anti-human Foxp3. In previous studies (14), we established that the percentages of LAP+ and Foxp3+ cells remained unchanged following PMA-ionomycin stimulation in the presence of Golgi Stop. Therefore, for evaluation of LAP and Foxp3 expression, together with that of the intracellular cytokine IL-10 and IL-17, LPMCs isolated from biopsies were incubated in X-VIVO15 medium and stimulated for 4 h with PMA (50 ng/ml) and ionomycin (1 μg/ml) in the presence of monensin (0.66 μl/ml Golgi Stop). After stimulation, cells were recovered and washed in PBS-1X, incubated for 30 min with LIVE/DEAD® Fixable Aqua Dead Cell Stain, and washed and labeled. Given the downregulation of CD4 expression that occurs following PMA-ionomycin stimulation, CD8 staining was preferred. Consequently, to assess

TABLE 1 | Patient clinicopathological variables.

Disease extension (*n*)	Age (years) mean ± SE median (range)	Sex M/F (*n*)/(*n*)	Disease duration since diagnosis (years) mean ± SE median (range)	Mayo endoscopic score mean ± SE median (range)	Therapy			
					CS	5-ASA agents	Immuno-modulators	Biological agents
Proctitis (21)	49 ± 3 52 (20–76)	17/14	10 ± 2 7 (0–35)	2 ± 0.1 2 (1–3)	5*+1	24	1	1
Left-sided Colitis (22)	53 ± 3 54 (23–82)	21/11	12 ± 2 12 (0–37)	2 ± 0.1 2 (1–3)	4*	22	6*	
Extensive Colitis (16)	43 ± 5 46 (19–70)	9/7	10 ± 2 15 (0–24)	2 ± 0.2 2 (1–3)	2*+1	10	1*+1	1*+1

*Patients with combined therapy.

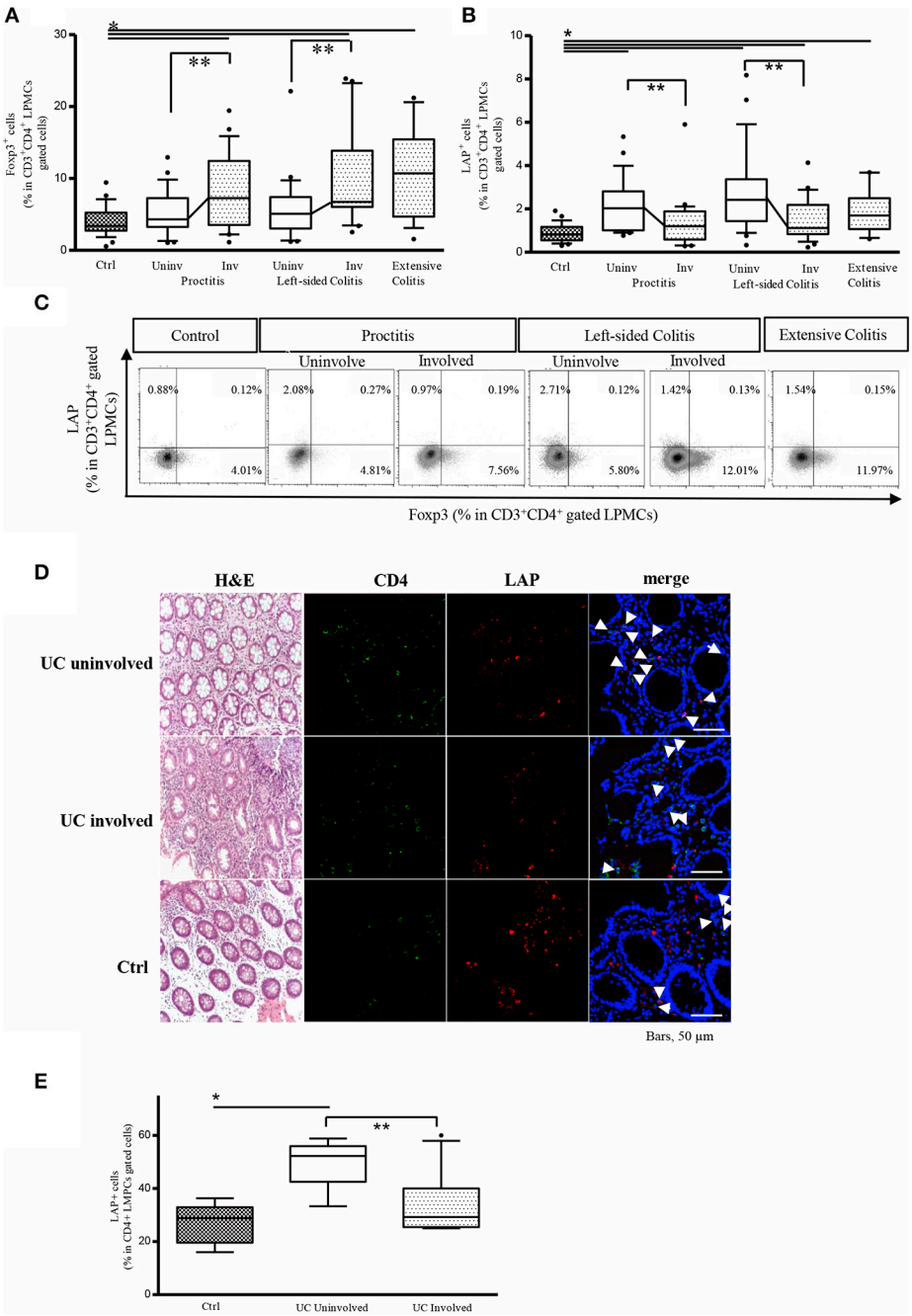

FIGURE 1 | The frequency of LP CD3+CD4+LAP+ cells is higher in uninvolved (Uninv) vs. involved (Inv) colon tissue from UC patients. **(A)** Frequency of LP Foxp3+ cells in the CD3+CD4+-gated LPMC population: *$P < 0.05$ (Mann-Whitney *U*-test) for controls (Ctrl) vs. proctitis (Uninv + Inv), left-sided colitis (Inv), and extensive colitis; **$P < 0.05$ (Wilcoxon signed-rank test) for uninvolved vs. involved tissue in proctitis and left-sided colitis. Data represent the mean ± SE of 79 UC patients and 29 controls. **(B)** Frequency of LP LAP+ cells in the CD3+CD4+-gated LPMC population: *$P < 0.05$ for Ctrl vs. proctitis (Uninv + Inv) and left-sided colitis (Inv); *$P < 0.05$ (Mann-Whitney *U*-test) for controls vs. left-sided colitis (Uninv + Inv) and extensive colitis; **$P < 0.05$ (Wilcoxon signed-rank test) for uninvolved vs. involved tissue in proctitis and left-sided colitis. Data represent the mean ± SE of 79 UC patients and 29 controls. **(C)** Representative density plots of Foxp3 and LAP expression in LP CD3+CD4+-gated cells. **(D)** Representative CD4+LAP+ T cells in colonic mucosa tissue of UC patients and controls. Confocal microscopy images of CD4 (green), LAP (red) and nuclei (blue) of matched involved and uninvolved colonic mucosa of a patient with UC, and a control subject (original magnification 630x). CD4+LAP+ double positive T cells are indicated by white arrows. For UC, 1 representative staining of 4 patients is shown. For control subjects, 1 representative staining of 2 subjects is shown. H&E stained sections of the corresponding subjects are also illustrated (original magnification 200x). **(E)** % of CD4+LAP+ cells in UC patients (involved and uninvolved tissue) and controls. For quantification at least 3 images for each patient ($n = 4$) or control subjects ($n = 2$) were assessed. Data represent mean ± SE, *$P < 0.05$ (Mann-Whitney *U*-test) for controls vs. uninvolved tissue; **$P < 0.05$ (Wilcoxon signed-rank test) for uninvolved vs. involved tissue.

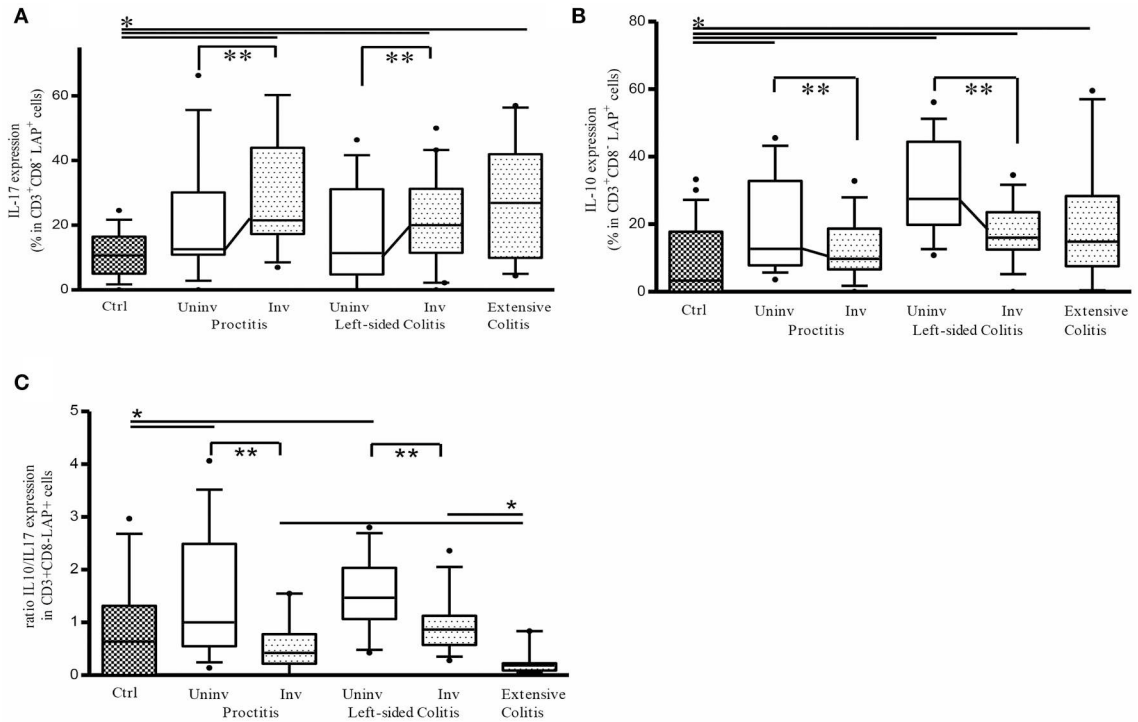

FIGURE 2 | Ratio between IL-10 and IL-17 expressing CD3+CD8-(CD4)LAP+ cells is significantly higher in uninvolved (Uninv) vs. involved (Inv) colon tissue from ulcerative colitis patients. **(A)** Frequency of LP LAP+ cells expressing intracellular IL-17 $^{*}P < 0.05$ (Mann-Whitney *U*-test) for controls vs. Involved (Inv) tissue in proctitis, left sided colitis and extensive colitis. $^{**}P < 0.05$ (Wilcoxon signed-rank test) for uninvolved vs. involved tissue in both proctitis and left-sided colitis. **(B)** Frequency of LP LAP+ cells expressing intracellular IL-10: $^{*}P < 0.05$ (Mann-Whitney *U*-test) for controls vs. proctitis (Uninv), left-sided colitis (Uninv + Inv), and extensive colitis; $^{**}P < 0.05$ (Wilcoxon signed-rank test) for uninvolved vs. involved tissue in both proctitis and left-sided colitis. **(C)** % LP LAP+ cells expressing intracellular IL-10 / % LP LAP+ cells expressing intracellular IL-17 (ratio). $^{**}P < 0.05$ (Wilcoxon signed-rank test) for uninvolved vs. involved tissue in proctitis, and left sided colitis. $^{*}P < 0.05$ (Mann-Whitney *U*-test) proctitis and left-sided colitis univolved tissue vs. controls $^{*}P < 0.05$ (Mann-Whitney *U*-test) Extensive colitis vs. proctitis and vs. left-sided colitis involved tissue. Data represent mean ± SE of 43 UC patients (proctitis, 16; left-sided colitis, 17; extensive colitis, 10) and 26 controls.

intracellular cytokine expression, cells were stained with anti-human CD3, anti-human CD8, and anti-human LAP (TGF-β1), and incubated for 30 min. After incubation, cells were washed, fixed, and permeabilized with fixation/permeabilization solution for 40 min. Cells were then washed in permeabilization buffer and incubated for 30 min with intracellular anti-human IL-10, anti-human IL-17 and anti-human Foxp3 or isotype-matched control Ig. Next, cells were washed twice with permeabilization buffer solution and fixed with 2% paraformaldehyde. CD4 cells were defined as those with a CD3+CD8-signature. The percentage of viable fluorescent cells was quantified using a Gallios™ Flow Cytometer (Beckman Coulter, Brea, CA, United States).

Induction of Experimental Colitis in Mice

Male BALB/c mice (Charles River Laboratories Italia, Calco, Italy) were housed in the Allevamenti Plaisant (Rome, Italy) animal facility in individually ventilated cages (IVC system) containing enrichment devices. Maintenance of pathogen-free conditions was ensured by monitoring every 6 months, in accordance with the Federation of European Laboratory Animal Science Associations (FELASA) recommendations. Experimental colitis was induced in 6–7 week-old male BALB/c mice by administering 6 mg of oxazolone [(4-ethoxymethylene-2-phenyl-2-oxazolin-5-one) Sigma Chemical Co., St. Louis, MO, United States] dissolved in 50% ethanol (total injection volume,150 μl), via a 3.5-F catheter inserted into the rectum of lightly anesthetized mice, as previously described (15). Control groups consisted of untreated mice and mice treated with 50% ethanol (total injection volume, 150 μl). Body weight was recorded at time zero (moment of intrarectal oxazolone/ethanol or ethanol administration), and on days 1 and 2 post-treatment. Mice were sacrificed on day 2 by cervical dislocation and colons collected for further analysis. In a preliminary experiment, we tested the ability of anti-LAP antibody (TW7-16B4 antibody, kindly donated by Professor HL Weiner, Harvard Medical School, Boston, MA, USA) to deplete the LP CD4+LAP+cells. To this end we treated two different groups of mice for 5 days with daily injection (40 μg, i.p.) of anti-LAP antibody or isotype control mouse IgG1 Clone MOPC-21 (BioXCell; DBA, Segrate, MI). Mice were sacrificed, colons were collected, and isolated LPMC cells were analyzed by immunofluorescence for LAP and Foxp3 expression. In additional experiments, different groups of mice received daily injections (40 μg, i.p.) of anti-LAP

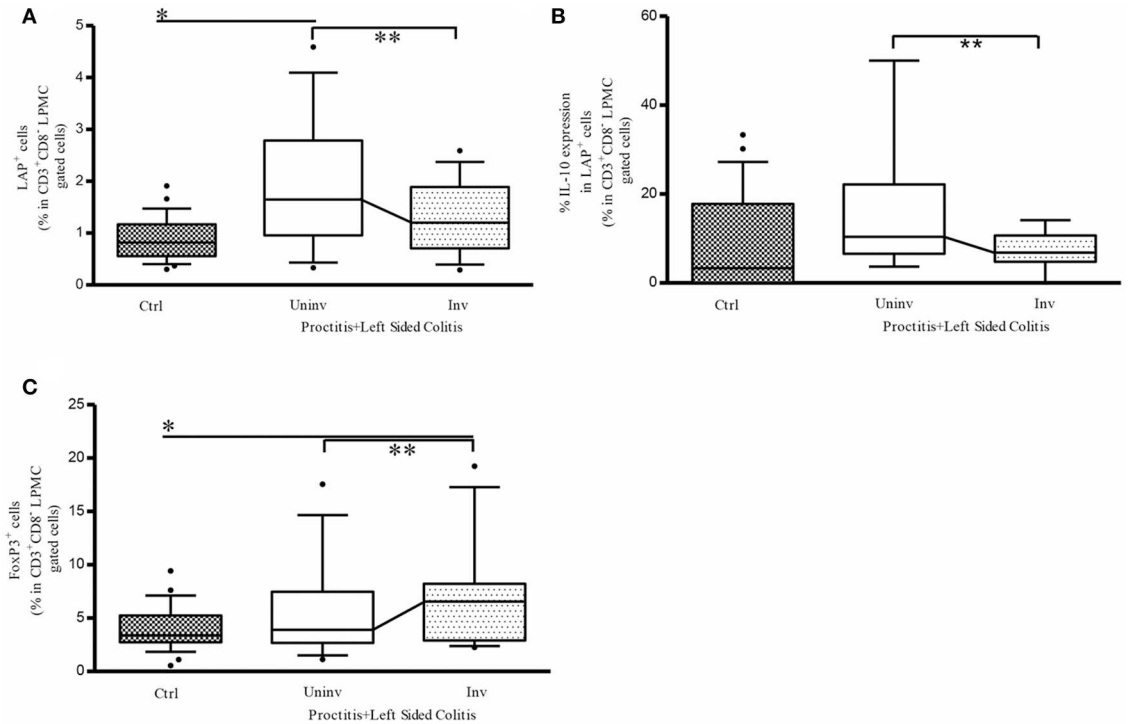

FIGURE 3 | Reduced disease extension is associated with a higher percentage of CD3+CD8-(CD4) LAP+ cells in uninvolved vs. involved colon tissue from UC patients. **(A)** Frequency of LP LAP+ cells in CD3+CD8- (CD4)-gated cells: $^{*}P < 0.05$ (Mann-Whitney *U*-test) for controls vs. uninvolved tissue in proctitis + left-sided colitis; $^{**}P < 0.05$ (Wilcoxon signed-rank test) for uninvolved vs. involved tissue in proctitis + left-sided colitis. **(B)** Frequency of LP LAP+ cells expressing intracellular IL-10: $^{**}P < 0.05$ (Wilcoxon signed-rank test) for uninvolved vs. involved tissue in proctitis + left-sided colitis. **(C)** Frequency of LP Foxp3+ cells in CD3+CD8-(CD4+)-gated cells: $^{**}P < 0.05$ (Wilcoxon signed-rank test) for uninvolved vs. involved tissue in proctitis + left-sided colitis. In all cases, data represent the mean ± SE of 26 controls and of 13 UC patients with a history of extensive colitis in whom inflammatory lesions were limited to the rectum or left colon at the moment of endoscopy. Patient clinicopathological variables of this UC subgroup are not different from the whole UC patients population [Age: 53 ± 3–53 (29–73) (years) mean ± SE-median (range); Sex: 7/6 M/F; Disease duration since diagnosis:14 ± 3.2–12 (1–32); (years) mean ± SE-median (range). Mayo endoscopic score: 2 ± 0.2–2 (1–3) mean ± SE-median (range)].

antibody (or isotype control), for 5 days before the induction of colitis.

Assessment of Colitis

Collected colons were macroscopically examined to assess the extension of colitis, and histological analysis of colitis was performed in colonic tissue samples that were fixed in 10% neutral buffered formalin solution (Sigma-Aldrich), embedded in paraffin, cut into tissue sections, and stained with hematoxylin and eosin. Multiple serial sections dividing the colon into three sections (proximal, medial and distal) were performed. Stained sections were examined by a pathologist [ISTOVET di Luca Crippa and C. S.A.S., Besana in Brianza (MB), Italy] and the extension of inflammatory lesions determined. Histopathologic grading of oxazolone-induced colitis was performed as previously described (23). Briefly, 5 criteria (hypervascularization, presence of mononuclear cells, epithelial hyperplasia, epithelial damage, presence of granulocytes and mucosal hemorrhages) were scored from 0 to 3 to produce a cumulative histopathologic score (HS) ranging from 0 (no colitis) to 15 (maximal colitis activity).

LPMCs Immunofluorescence Staining

Freshly isolated and washed LPMCs were subjected to Fc block with anti-CD16/CD32 (BD Pharmingen) and then labeled for 30 min with LIVE/DEAD® Fixable Aqua Dead Cell Stain. After washing, cells were stained by incubation for 30 min with APC-Cy7-labeled anti-CD3 (eBioscience), V450-labeled anti-CD4 (eBioscience), PE-labeled anti-LAP (BioLegend), or isotype control PE-labeled mouse IgG1 (R&D Systems). Intracellular Foxp3 expression was evaluated using the APC-anti-mouse/rat Foxp3 staining kit (eBioscience), following the manufacturer's protocol. The cells were then washed twice, and the percentage of fluorescent cells quantified using a Gallios™ Flow Cytometer (Beckman Coulter, Brea, CA, United States).

Some tissue sections were also analyzed by confocal microscopy, using the above-described procedure. Briefly, 3 μm-thick paraffin-embedded sections of unlesional and lesional colon tissues from mice treated as described in Induction of experimental colitis in mice paragraph were stained after deparaffinization, rehydratation, antigen retrieval and saturation with blocking buffer. Specimens were stained with a monoclonal FITC-conjugated rat anti-mouse CD4 at

5 μg/ml (BD Biosciences), and a monoclonal mouse anti-mouse LAP at 5 μg/ml (BioLegend) followed by an goat anti-mouse AlexaFluor-633. After washing, slides were mounted in Prolong Gold anti-fade medium containing a DNA dye (DAPI). Confocal laser scanning microscopy (CLSM) observations were performed with a Leica TCS SP2 AOBS apparatus, using a 63x/1.40 NA oil objective. Acquisition of images was performed by a Leica confocal software 2.6 (Leica, Germany).

Statistical Analysis

Data were analyzed using the two sided Mann-Whitney *U*-test and the two sided Wilcoxon signed-rank test in GraphPad Prism software (GraphPad Software, San Diego, CA, United States). Statistical significance was set at $p < 0.05$.

Human Study

All participants provided written informed consent prior to inclusion in the study. Ethical approval was provided by the Ethical Committee of the Istituto Superiore di Sanità (Reference: Pre-C-871/14, 25/11/2014).

Animal Studies

This study was carried out in accordance with the recommendations of Decreto Legislativo 4 marzo 2014, n. 26 according with 2010/63/UE(14G00036) direction. The protocol was approved by the Italian Ministry of Health (Reference: 16/2014-PR [DGSAF 12073-A, 05/06/2014], 03/10/2014).

RESULTS

The Frequency of LP CD3+CD4+LAP+ Cells Is Higher in Uninvolved vs. Involved Colon Tissue From Ulcerative Colitis Patients

Preliminary analyses of the percentage of CD3+CD4+LAP+ and CD3+CD4+Foxp3+ Tregs isolated from biopsies from different portions of control colons revealed no differences between colon regions (**Supplementary Figure 1**). We next evaluated the frequency of LP CD3+CD4+LAP+ and CD3+CD4+Foxp3+ Tregs in LPMCs isolated from biopsies from controls and from patients with endoscopically and histologically active UC with varying degrees of disease extension. In patients with either proctitis or left-sided colitis, the percentage of CD4+Foxp3+ cells was significantly higher in involved tissue than in uninvolved tissue (**Figure 1A**). As opposite % of CD4+LAP+ T regs was significantly higher in uninvolved vs. involved tissue (**Figure 1B**) In agreement with previous observations (14, 24, 25), the percentage of Foxp3+ Tregs in the LP CD3+CD4+ T-cell population was significantly higher in involved tissue, regardless of disease extension, than in controls (**Figure 1A**). Similarly, in line with previous reports (14), the percentage of CD4+LAP+ Tregs was significantly higher in involved tissue from patients with extensive colitis and left-sided colitis than in controls (**Figure 1B**). In uninvolved tissue, the percentage of CD4+Foxp3+ cells was comparable to that of controls, while the percentage of CD4+LAP+ Tregs tissue was significantly higher. As previously reported (14), the majority of LAP+ cells detected were Foxp3- (**Figure 1C**). Some biopsy specimens sections were also immunofluorescence stained for tissue assessment of CD4+LAP+ cells by confocal microscopy. As illustrated in **Figures 1D,E**, uninvolved tissue showed significantly more CD4+LAP+ double-fluorescent cells when compared with involved and control tissue.

Ratio Between IL-10 and IL-17 Expressing LAP+ Cells Is Significantly Higher in Uninvolved vs. Involved Colon Tissue From Ulcerative Colitis Patients

We have previously shown that in active UC patients, LP CD3+CD8- (CD4) LAP+ cells are enriched for IL-17 expression, showing reduced suppressor activity due the intracellular IL-17 expression (14). It has been recently reported that CD4+LAP+ cells expressing IL-10 exhibit regulatory activity (26, 27). We therefore evaluated IL-17 and IL-10 expression in LP CD3+CD8- (CD4) LAP+ cells. As shown in **Figure 2A**, the percentage of IL-17 expressing LP CD3+CD8- (CD4) LAP+ cells was significantly reduced in uninvolved vs. involved tissue while the % of IL-10-expressing LAP+ cells was significantly higher in uninvolved vs. involved tissue (**Figure 2B**). As a consequence, the ratio between IL-10 and IL-17 expressing LAP+ cells was significantly higher in uninvolved vs. involved tissue (**Figure 2C**). Notably, extensive colitis ratio was significantly lower than the ratio observed both in proctitis and left-sided colitis involved tissue. As previously reported and confirmed in the present study, % of IL-17 expressing LAP+ cells was significantly increased in involved tissues vs. controls (14). % of IL-10 expressing LAP+ cells was significantly increased in uninvolved tissues vs. controls.

Reduced Disease Extension Is Associated With a Higher Percentage of CD3+CD8– (CD4+) LAP+ Cells in Uninvolved vs. Involved Tissue From Ulcerative Colitis Patients

To determine whether the observed differences in Tregs frequencies were indeed linked to the extension of inflammatory lesions, we analyzed a subgroup of patients with a history of endoscopic assessed extensive colitis in whom inflammatory lesions were endoscopically limited to the distal colon (mainly rectum) at the moment of the entry in the present study. We found that the presence of distal inflammatory lesions was associated with a significantly higher frequency of LAP+ cells in LP cells isolated from uninvolved tissue vs. both control tissue and involved tissue from UC patients (**Figure 3A**). Within this cell population, the percentage of IL-10-expressing CD4+LAP+ cells was significantly higher in uninvolved vs. involved tissue (**Figure 3B**). The percentage of CD4+Foxp3+ Tregs was significantly higher in involved vs. uninvolved tissue, in which values were comparable to those detected in controls (**Figure 3C**). These findings suggest that the differences in the frequencies of regulatory cells between involved and uninvolved tissue are linked to the extension of inflammatory lesions.

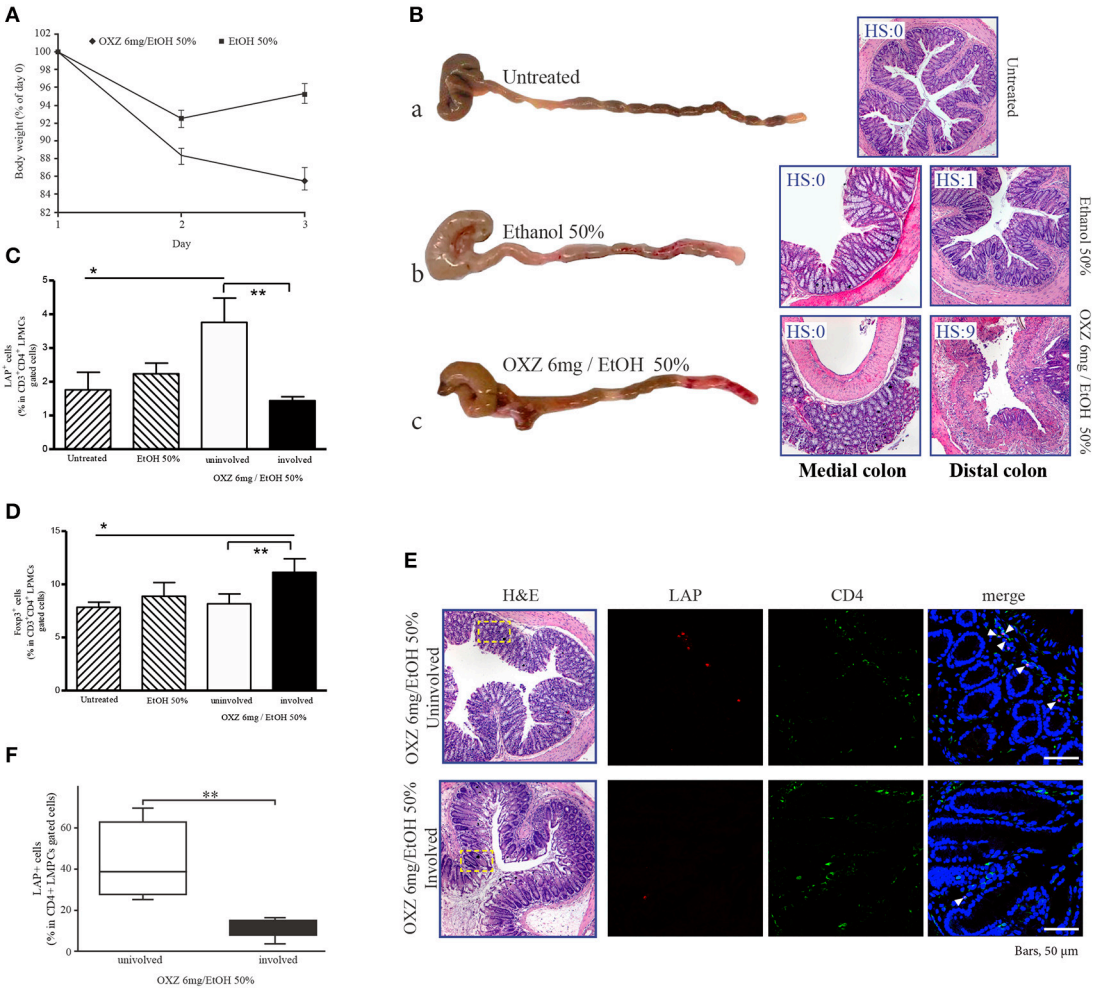

FIGURE 4 | Oxazolone-induced colitis reproduces the observations made in UC patients. **(A)** Weight changes in mice after intrarectal administration of oxazolone (6 mg/ethanol 50%). Each point represents the cumulative mean (± SE) weight from 3 separate experiments in which 5 mice per group were studied. *$P < 0.05$ (Student's *t*-test) for EtOH 50% vs. OXZ 6 mg/EtOH 50%. **(B)** Representative macroscopic (on the left) and corresponding microscopic (on the right) images of the colons from untreated (a), EtOH 50%-treated (b), and OXZ 6 mg /EtOH 50%-treated (c) mice, all of which were sacrificed 2 days post-treatment. H&E staining of distal (untreated), distal and medial colonic tract of EtOH 50%-treated and oxazolone-treated miceat 40× magnification. HS, histopathologic score (see methods). **(C)** Percentage of LAP+ cells among CD3+CD4+-gated LMPCs isolated from the colon of untreated, ethanol-treated and oxazolone/ethanol-treated mice: *$P < 0.05$ for untreated mice vs. OXZ-treated mice (uninvolved tissue); **$P < 0.05$ for uninvolved vs. involved tissue in OXZ-treated mice. Each point represents mean ± SE of pooled values derived from 3 experiments in which 5 mice per group were evaluated. **(D)** Percentage of Foxp3+ cells among CD3+CD4+-gated LMPCs isolated from the colon of untreated, ethanol-treated, and oxazolone/ethanol-treated mice: *$P < 0.05$ (Mann-Whitney *U*-test) for untreated mice vs. OXZ-treated mice (involved tissue); **$P < 0.05$ (Wilcoxon signed-rank test) for uninvolved vs. involved tissue in OXZ-treated mice. Each point represents the mean ± SE of pooled values derived from 3 experiments in which 5 mice/group were evaluated. **(E)** Representative CD4+LAP+ T cells in colonic mucosa of involved and uninvolved tissue of oxazolone-treated mice. Confocal microscopy images of CD4 (green), LAP (red) and nuclei (blue) of matched involved and uninvolved colonic mucosa of a oxazolone-treated mouse (original magnification 630x). CD4+LAP+ double positive T cells are indicated by white arrows. 1 representative staining of 3 mice is shown. H&E stained sections of the corresponding confocal images are also illustrated (original magnification 40x). The rectangles highlight the area shown in the confocal microscopy images. **(F)** % of CD4+LAP+ cells in colons of oxazolone-treated mice (involved and uninvolved tissue). For quantification at least 3 images for each colon ($n = 3$) were assessed. Data represent mean ± SE, **$P < 0.05$ (Wilcoxon signed-rank test) for uninvolved vs. involved tissue.

Depletion of LAP+ Cells Is Associated With Proximal Extension of Inflammatory Lesions in the Murine Oxazolone-Induced Colitis Model

To gain further insight into the roles of the two aforementioned Tregs subsets in limiting the extension of inflammatory lesions, we evaluated the effect of *in vivo* administration of an LAP-depleting antibody that has no effect on the frequency of CD4+Foxp3+ cells (17) in mice with oxazolone-induced experimental colitis (16). Intrarectal administration of oxazolone (6 mg in 50% ethanol) was associated with weight loss and the onset of distal colitis (**Figures 4A,B**), as previously described (16). This colitis was associated with significantly higher

TABLE 2 | Histopathologic score of colons of mice with oxazolone colitis administered the anti-LAP antibody or its isotype control.

Treatment	Mouse								Total
			A	B	C	D	E	F	(A+B+C+D+E+F)
			0–3	0–3	0–3	0–3	0–3	0–3	0–15
aLAPAb->OXZ 6mg	A	distal colonic tract	0	2	0	3	2	2	9
		medial colonic tract	0	2	0	3	2	0	7
		proximal colonic tract	0	0	0	0	0	0	0
aLAPAb->OXZ 6 mg	B	distal colonic tract	0	2	0	2	2	1	7
		medial colonic tract	0	1	0	2	1	1	5
		proximal colonic tract	0	0	0	0	0	0	0
aLAPAb->OXZ 6 mg	C	distal colonic tract	0	3	0	3	3	3	12
		medial colonic tract	0	2	0	2	3	2	9
		proximal colonic tract	0	0	0	0	0	0	0
IsoAb->OXZ 6 mg	D	distal colonic tract	0	3	0	3	3	3	12
		medial colonic tract	0	0	0	0	0	0	0
		proximal colonic tract	0	0	0	0	0	0	0
IsoAb->OXZ 6 mg	E	distal colonic tract	0	3	0	3	2	2	10
		medial colonic tract	0	0	0	0	0	0	0
		proximal colonic tract	0	0	0	0	0	0	0
IsoAb->OXZ 6 mg	F	distal colonic tract	0	2	0	3	3	1	9
		medial colonic tract	0	0	0	0	0	0	0
		proximal colonic tract	0	0	0	0	0	0	0
								Mean	**SE**
aLAPAb->OXZ 6 mg		distal colonic tract						9	1.8
		medial colonic tract						7	1.4
		proximal colonic tract						0	0.0
IsoAb->OXZ 6 mg		distal colonic tract						10	1.1
		medial colonic tract						0	0.0
		proximal colonic tract						0	0.0

Colons were divided into three sections (proximal, medial, and distal), multiple serial sections were done and slides were scored according with 5 criteria from 0 to 3 (A, hypervascularization; B, presence of mononuclear cells; C, epithelial hyperplasia; D, epithelial damage; E, presence of granulocytes; F, mucosal hemorrhages) to produce a cumulative histopathologic score (HS) ranging from 0 (no colitis) to 15 (maximal colitis activity).

percentages of LP CD3+CD4+LAP+ cells (**Figure 4C**) in uninvolved tissue as compared with both involved tissue and with tissue from untreated control mice, and a higher percentage of CD3+CD4+Foxp3+ cells in involved tissue as compared with both uninvolved tissue and control untreated mice (**Figure 4D**), thus reproducing the observations made in UC patients. In oxazolone colitis, the significant increased % of CD4+LAP+ cells in uninvolved vs. involved tissue was confirmed also by confocal microscopy (**Figures 4E,F**). After a preliminary validation of the ability of anti-LAP antibody administration to selectively deplete LP CD4+LAP+cell without affecting the % of LP CD4+Foxp3+ cells (**Supplementary Figure 2**), we administered the antibody or its isotype control in two groups of mice, before the induction of oxazolone colitis. Administration of anti-LAP antibody had no effect on weight loss, which was comparable to that observed in isotype-treated mice (**Figure 5A**), but was associated with more extensive colitis (**Figures 5B,C** and **Table 2**).

DISCUSSION

The present findings suggest that LP CD3+CD4+LAP+ Tregs are responsible for limiting the extension of inflammatory lesions in UC. We observed increases in both the percentage of CD3+CD4+LAP+ cells and the proportion of IL-10-expressing CD3+CD4+LAP+ cells associated with a reduction of IL-17-expressing CD3+CD4+LAP+ cells in uninvolved vs. involved tissue from UC patients. Previous studies have reported increases in the numbers of CD3+CD4+LAP+ cells (14) and CD3+CD4+Foxp3+ cells (24, 25) in involved UC tissue, the former were found to be functionally impaired *in vitro,* while the latter, although functional *in vitro,* were ineffective in counteracting inflammation *in vivo*. Indeed, in the present study, uninvolved tissue is characterized by a significant increase in the ratio between IL-10 and IL-17 expressing LAP+ cells suggesting that the increased % of CD3+CD4+LAP+ cells observed in uninvolved tissue is predominantly represented by functional active regulatory cells. This hypothesis is reinforced by the observations obtained in mice since selective depletion of LAP+ cells in a mouse model of distal colitis was associated with the extension of inflammatory lesions to the proximal colon. Taken together, these data strongly suggest that CD3+CD4+LAP+ regulatory cells play a key role in limiting the extension of colonic inflammatory lesions in UC. CD3+CD4+LAP+ cells (which express surface TGF-β linked to its latency associated peptide

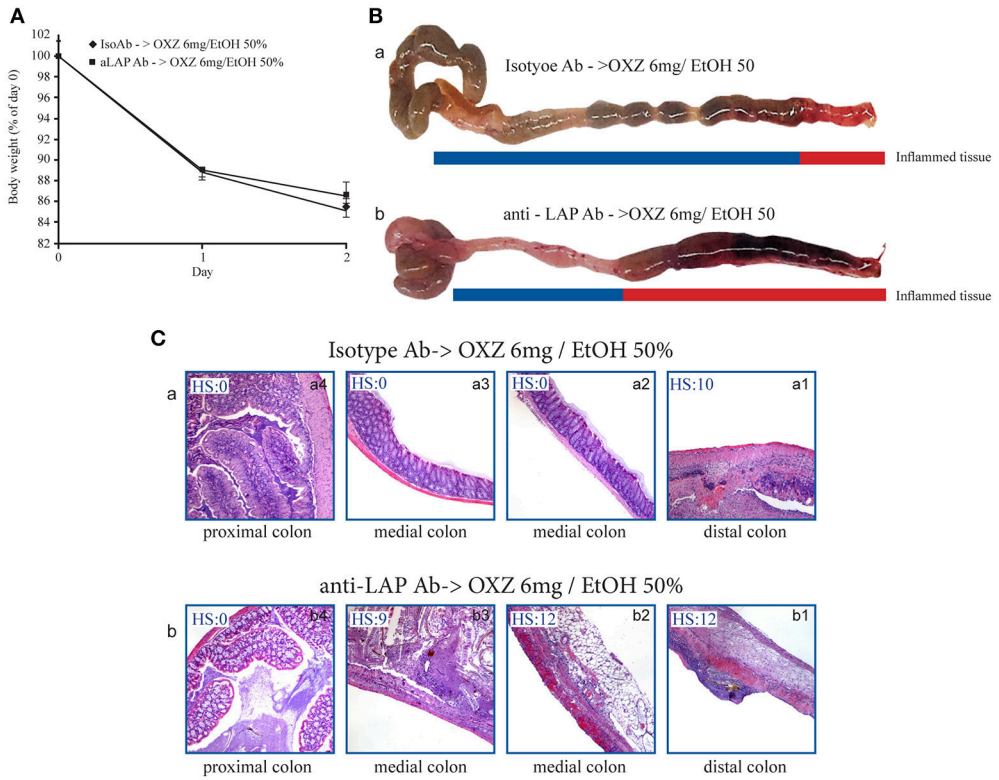

FIGURE 5 | Depletion of LAP+ cells is associated with proximal extension of inflammatory lesions in the mouse oxazolone-induced colitis model. **(A)** Weight changes in oxazolone-treated mice (6 mg/ethanol 50%) pretreated with either Isotype Ab or anti-LAP Ab. Each point represents the cumulative mean (± SE) weight from 3 separate experiments in which 5 mice per group were studied. **(B)** Representative macroscopic images of colons of oxazolone-treated mice pretreated with (a) Isotype Ab or (b) anti-LAP Ab. **(C)** H&E stained colonic specimens (40× magnification) of distal, medial and proximal colonic portions of oxazolone-treated mice pretreated with (a) Isotype Ab (a1-a4) or (b) anti-LAP Ab Isotype Ab (b1-b4). HS, histopathologic score (see methods).

[LAP]) have been shown to limit inflammation in the adoptive transfer mouse model of experimental colitis (28) and are crucial to ensure protection against murine TNBS colitis, since transfer of CD4+LAP+ depleted cells with intact CD4+Foxp3+ cells does not prevent TNBS colitis in recipient mice (29). In mice, the frequency of CD3+CD4+LAP+ cells is increased in conditions of increased intestinal permeability and during the homeostatic response to transient increases in intestinal permeability (29, 30). Furthermore, the frequency of these cells in the intestinal LP is increased in human patients with UC but not Crohn's disease (14), and their regulatory activity is significantly reduced in inflamed UC tissue due to an increase in the proportion of IL-17-expressing LAP+ cells (14). Studies in animal models of autoimmune diseases have shown that intracellular expression of IL-10 is linked to the ability of CD4+LAP+ cells to attenuate disease severity (26, 27). In the present study, we found that the proportion of CD3+CD8- (CD4) LAP+ cells expressing intracellular IL-10 was significantly higher in uninvolved vs. involved tissue from UC patients, suggesting a regulatory role of these cells, which may limit the extension of inflammatory lesions. Nasal administration of anti-CD3 in animal models induces suppressive IL-10-producing T cells (Tr1 cells), almost 75% of which exhibit cell surface expression of LAP (31). The link between the Tr1 cell subset and the CD3+CD4+LAP+ Foxp3-cells described in the present study remains unknown, and further studies will be necessary to clarify this relationship. However, our findings suggest that the suppressive capabilities of these cells are dependent on LAP expression, since depletion of LAP+ cells in mice with oxazolone-induced colitis was associated with proximal extension of colonic inflammatory lesions. Supporting this view, we previously demonstrated that blockade of TGF-β activity in oxazolone-induced colitis is associated with the development of extensive colitis (16).

We found that while the proportion of CD3+CD4+LAP+ cells was increased in uninvolved tissue, the proportion of CD3+CD4+Foxp3+ cells in the same tissue was comparable to that seen in controls, suggesting that the requirements for CD3+CD4+LAP+ cell expansion differ to those for CD3+CD4+Foxp3+ cell expansion. In mice, *in vivo* expansion of CD3+CD4+LAP+ cells is dependent on the presence of IL-10 and TLR2 (21, 29, 32), and the presence of TGF-β, acting in an autocrine fashion, is required for LAP expression independently of Foxp3 expression (22). It is therefore tempting to speculate that in uninvolved tissue, TGF-β levels facilitate the maintenance and expansion of CD3+CD4+LAP+ regulatory cells but are insufficient to induce CD3+CD4+Foxp3+ Tregs.

It is increasingly apparent that microbiota play an important role in modulating the intestinal immune system, influencing different immune-cell subsets and their soluble products. We previously reported that the expansion of CD3+CD4+LAP+ cells in mice is dependent on the presence of microbiota (29, 30), and showed that probiotic administration increases the percentage of LP CD3+CD4+LAP+ cells and protects mice from TNBS-induced colitis (32). Moreover, we have demonstrated that probiotic administration in patients with ileal pouch anal anastomosis for ulcerative colitis is associated with an increased percentage of CD3+CD4+LAP+ cells in the pouch LP and a decrease in pouch disease activity index (13). Taken together, these observations suggest that expansion of LP CD3+CD4+LAP+ cells may constitute a therapeutic strategy to limit and possibly prevent colonic inflammation in UC patients. Microbiota modulation might represent a useful tool to accomplish this task.

AUTHOR CONTRIBUTIONS

Study conception and design: MB and RP. Study supervision: MB. Acquisition of data: AB, AA. Immunofluorescence and experimental colitis: ND. Experimental colitis: NC. Flow cytometry analysis supervision: MS. Patients' enrollment, endoscopy, and biopsies collection: AP and RP. Clinical data collection: DD. Histology and histological score: FB. Confocal microscopy: RL and LF. Analysis and interpretation of data: AB, MS, FB, MB, AP, RP, RL, and LF. Drafting of manuscript: MB. Critical revision: AB, AP, RP, and MB. The final version of the manuscript has been approved by all authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02511/full#supplementary-material

REFERENCES

1. Ordás I, Eckmann L, Talamini M, Baumgart DC, Sandborn WJ. Ulcerative colitis. *Lancet* (2012) 380:1606–19. doi: 10.1016/S0140-6736(12)60150-0
2. Moum B, Ekbom A, Vatn MH, Elgjo K. Change in the extent of colonoscopic and histological involvement in ulcerative colitis over time. *Am J Gastroenterol*. (1999) 94:1564–69. doi: 10.1111/j.1572-0241.1999.01145.x
3. Langholz E, Munkholm P, Davidsen M, Nielsen OH, Binder V. Changes in extent of ulcerative colitis: a study on the course and prognostic factors. *Scand J Gastroenterol*. (1996) 31:260–66. doi: 10.3109/00365529609004876
4. Safroneeva E, Vavricka S, Fournier N, Seibold F, Mottet C, Nydegger A, et al. Systematic analysis of factors associated with progression and regression of ulcerative colitis in 918 patients. *Aliment Pharmacol Ther*. (2015) 42:540–8. doi: 10.1111/apt.13307
5. Solberg IC, Lygren I, Jahnsen J, Aadland E, Høie O, Cvancarova M, et al. Clinical course during the first 10 years of ulcerative colitis: results from a population-based inception cohort (IBSEN Study). *Scand J Gastroenterol*. (2009) 44:431–40. doi: 10.1080/00365520802600961
6. Hoie O, Wolters FL, Riis L, , Bernklev T, Aamodt G, Clofent J, et al. Low colectomy rates in ulcerative colitis in an unselected European cohort followed for 10 years. *Gastroenterology* (2007) 132:507–15. doi: 10.1053/j.gastro.2006.11.015
7. Dias CC, Rodrigues PP, da Costa-Pereira A, Magro F. Clinical predictors of colectomy in patients with ulcerative colitis: systematic review and meta-analysis of cohort studies. *J Crohns Colitis* (2015) 9:156–63. doi: 10.1093/ecco-jcc/jju016
8. Jess T, Loftus EV Jr, Velayos FS, Harmsen WS, Zinsmeister AR, Smyrk TC, et al. Risk of intestinal cancer in inflammatory bowel disease: a population-based study from Olmsted county, Minnesota. *Gastroenterology* (2006) 130:1039–46. doi: 10.1053/j.gastro.2005.12.037
9. Castaño-Milla C, Chaparro M, Gisbert JP. Systematic review with meta-analysis: the declining risk of colorectal cancer in ulcerative colitis. *Aliment Pharmacol Ther*. (2014) 39:645–59. doi: 10.1111/apt.12651
10. Kim B, Park SJ, Hong SP, Kim TI, Kim WH, Cheon JH. Proximal disease extension and related predicting factors in ulcerative proctitis. *Scand J Gastroenterol*. (2014) 49:177–83. doi: 10.3109/00365521.2013.867360
11. Pica R, Paoluzi OA, Iacopini F, Marcheggiano A, Crispino P, Rivera M, et al. Oral mesalazine (5-ASA) treatment may protect against proximal extension of mucosal inflammation in ulcerative proctitis. *Inflamm Bowel Dis*. (2004) 10:731–6. doi: 10.1097/00054725-200411000-00006
12. Etchevers MJ, Aceituno M, García-Bosch O, Ordás I, Sans M, Ricart E, et al. Risk factors and characteristics of extent progression in ulcerative colitis. *Inflamm Bowel Dis*. (2009) 15:1320–25. doi: 10.1002/ibd.20897
13. Pronio A, Montesani C, Butteroni C, Vecchione S, Mumolo G, Vestri A, et al. Probiotic administration in patients with ileal pouch-anal anastomosis for ulcerative colitis is associated with expansion of mucosal regulatory cells. *Inflamm Bowel Dis*. (2008) 14:662–8. doi: 10.1002/ibd.20369
14. D'Ambrosio A, Cossu A, Amendola A, Zandri A, Butera A, Sanchez M, et al. Lamina Propria CD4+LAP+ regulatory T cells are increased in active ulcerative colitis but show increased IL-17 expression and reduced suppressor activity. *J Crohns Colitis* (2016) 10:346–53. doi: 10.1093/ecco-jcc/jjv216
15. Scurr M, Ladell K, Besneux M, Christian A, Hockey T, Smart K, et al. Highly prevalent colorectal cancer-infiltrating LAP^{+} $Foxp3^{-}$ T cells exhibit more potent immunosuppressive activity than $Foxp3^{+}$ regulatory T cells. *Mucosal Immunol*. (2014) 7:428–39. doi: 10.1038/mi.2013.62
16. Boirivant M, Fuss IJ, Chu A, Strober W. Oxazolone colitis: a murine model of T helper cell type 2 colitis treatable with antibodies to interleukin 4. *J Exp Med*. (1998) 188:1929–39. doi: 10.1084/jem.188.10.1929
17. da Cunha AP, Wu HY, Rezende RM, Vandeventer T, Weiner HL. *In vivo* anti-LAP mAb enhances IL-17/IFN-γ responses and abrogates anti-CD3-induced oral tolerance. *Int Immunol*. (2015) 27:73–82. doi: 10.1093/intimm/dxu083
18. Rutgeerts P, Sandborn WJ, Feagan BG, Reinisch W, Olson A, Johanns J, et al. Infliximab for induction and maintenance therapy for ulcerative colitis. *N Engl J Med*. (2005) 353:2462–76. doi: 10.1056/NEJMoa50516

19. Silverberg MS, Satsangi J, Ahmad T, Arnott ID, Bernstein CN, Brant SR, et al. Toward an integrated clinical, molecular and serological classification of inflammatory bowel disease: Report of a Working Party of the 2005 Montreal World Congress of Gastroenterology. *Can J Gastroenterol.* (2005) 19(Suppl. A):5–36. doi: 10.1155/2005/269076
20. Mosli MH, Feagan BG, Zou G, Sandborn WJ, D'Haens G, Khanna R, et al. Development and validation of a histological index for UC. *Gut* (2017) 66:50–58. doi: 10.1136/gutjnl-2015-310393
21. Duan W, So T, Mehta AK, Choi H, Croft M. Inducible CD4+LAP+Foxp3- regulatory T cells suppress allergic inflammation. *J Immunol.* (2011) 187:6499–507. doi: 10.4049/jimmunol.1101398
22. Oida T, Weiner HL. TGF-β induces surface LAP expression on murine CD4 T cells independent of Foxp3 induction. *PLoS ONE* (2010) 5:e15523. doi: 10.1371/journal.pone.0015523
23. Neurath MF, Weigmann B, Finotto S, Glickman J, Nieuwenhuis E, Iijima H, et al. The transcription factor T-bet regulates mucosal T cell activation in experimental colitis and Crohn's disease *J Exp Med.* (2002) 195:1129–43. doi: 10.1084/jem.20011956
24. Yu QT, Saruta M, Avanesyan A, Fleshner PR, Banham AH, Papadakis KA. Expression and functional characterization of FOXP3+ CD4+ regulatory T cells in ulcerative colitis. *Inflamm Bowel Dis.* (2007) 13:191–9. doi: 10.1002/ibd.20053
25. Holmén N, Lundgren A, Lundin S, Bergin AM, Rudin A, Sjövall H, et al. Functional CD4+CD25high regulatory T cells are enriched in the colonic mucosa of patients with active ulcerative colitis and increase with disease activity. *Inflamm Bowel Dis.* (2006) 12:447–56. doi: 10.1097/00054725-200606000-00003
26. Wu HY, Maron R, Tukpah AM, Weiner HL. Mucosal anti-CD3 monoclonal antibody attenuates collagen-induced arthritis that is associated with induction of LAP+ regulatory T cells and is enhanced by administration of an emulsome-based Th2-skewing adjuvant. *J Immunol.* (2010) 185:3401–7. doi: 10.4049/jimmunol.1000836
27. Wu HY, Center EM, Tsokos GC, Weiner HL. Suppression of murine SLE by oral anti-CD3: inducible CD4+CD25-LAP+ regulatory T cells control the expansion of IL-17+ follicular helper T cells. *Lupus* (2009) 18:586–96. doi: 10.1177/0961203308100511
28. Oida T, Zhang X, Goto M, Hachimura S, Totsuka M, Kaminogawa S, et al. CD4+CD25-T cells that express latency associated peptide on the surface suppress CD4+CD45RBhigh-induced colitis by a TGF-beta-dependent mechanism. *J Immunol.* (2003) 170:2516–22. doi: 10.4049/jimmunol.170.5.2516
29. Boirivant M, Amendola A, Butera A, Sanchez M, Xu L, Marinaro M, et al. A transient breach in the epithelial barrier leads to regulatory T-cell generation and resistance to experimental colitis. *Gastroenterology* (2008) 135:1612–23. doi: 10.1053/j.gastro.2008.07.028
30. Amendola A, Butera A, Sanchez M, Strober W, Boirivant M. Nod2 deficiency is associated with an increased mucosal immunoregulatory response to commensal microorganisms. *Mucosal Immunol.* (2014) 7:391–404. doi: 10.1038/mi.2013.58
31. Wu HY, Quintana FJ, da Cunha AP, Dake BT, Koeglsperger T, Starossom SC, et al. *In Vivo* induction of Tr1 cells via mucosal dendritic cells and AHR signaling. *PLoS ONE* (2011) 6:e23618. doi: 10.1371/journal.pone.0023618
32. Di Giacinto C, Marinaro M, Sanchez M, Strober W, Boirivant M. Probiotics ameliorate recurrent Th1-mediated murine colitis by inducing IL-10 and IL-10-dependent TGF-beta-bearing regulatory cells. *J Immunol.* (2005) 174:3237–46. doi: 10.4049/jimmunol.174.6.3237

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ATF3 Sustains IL-22-Induced STAT3 Phosphorylation to Maintain Mucosal Immunity Through Inhibiting Phosphatases

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In gut epithelium, IL-22 transmits signals through STAT3 phosphorylation (pSTAT3) which provides intestinal immunity. Many components in the IL-22-pSTAT3 pathway have been identified as risk factors for inflammatory bowel disease (IBD) and some of them are considered as promising therapeutic targets. However, new perspectives are still needed to understand IL-22-pSTAT3 signaling for effective clinical interventions in IBD patients. Here, we revealed activating transcription factor 3 (ATF3), recently identified to be upregulated in patients with active IBD, as a crucial player in the epithelial IL-22-pSTAT3 signaling cascade. We found ATF3 is central to intestinal homeostasis and provides protection during colitis. Loss of ATF3 led to decreased crypt numbers, more shortened colon length, impaired ileal fucosylation at the steady state, and lethal disease activity during DSS-induced colitis which can be effectively ameliorated by rectal transplantation of wild-type colonic organoids. Epithelial stem cells and Paneth cells form a niche to orchestrate epithelial regeneration and host-microbe interactions, and IL-22-pSTAT3 signaling is a key guardian for this niche. We found ATF3 is critical for niche maintenance as ATF3 deficiency caused compromised stem cell growth and regeneration, as well as Paneth cell degeneration and loss of anti-microbial peptide (AMP)-producing granules, indicative of malfunction of Paneth/stem cell network. Mechanistically, we found IL-22 upregulates ATF3, which is required to relay IL-22 signaling leading to STAT3 phosphorylation and subsequent AMP induction. Intriguingly, ATF3 itself does not act on STAT3 directly, instead ATF3 regulates pSTAT3 by negatively targeting protein tyrosine phosphatases (PTPs) including SHP2 and PTP-Meg2. Furthermore, we identified ATF3 is also involved in IL-6-mediated STAT3 activation in T cells and loss of ATF3 leads to reduced capacity of Th17 cells to produce their signature cytokine IL-22 and IL-17A. Collectively, our results suggest that via IL-22-pSTAT3 signaling in the epithelium and IL-6-pSTAT3 signaling in Th17 cells, ATF3 mediates a cross-regulation in the barrier to maintain mucosal homeostasis and immunity.

Keywords: ATF3, IL-22, pSTAT3, PTPs, IBD, mucosal immunity

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INTRODUCTION

Inflammatory bowel disease (IBD) is a group of chronic relapsing inflammatory conditions characterized by impaired intestinal homeostasis and abnormal stress response (1). It is believed that breach or breakdown of the epithelial barrier sets the pre-disease stage of IBD, leading to microbial translocation, immune activation and chronic inflammation at the later stages (2). Not only functioning as a physical barrier, intestinal epithelial cells (IEC) also serve as a biological barrier for maintaining homeostasis between host and microbes (3), through IEC's microbial recognition and their regulatory function in innate and adaptive immunity (4). Therefore, epithelial barrier is the major platform where cross-regulation of host-microbe interactions takes place and is believed to have a predominant role in the initiation of IBD pathogenesis (5).

The cytokine IL-22 has a unique niche in the intestine due to exclusive expression of IL-22 receptor (IL-22R) by the IEC. Stimulation of IEC by IL-22 initiates signaling transduction of IL-22R-assoicated Jak2 and Tyk2 kinases, leading to phosphorylation and activation of STAT3 (6). It is well established that IL-22-STAT3 signaling controls epithelial stem cell regeneration (7), mucosal healing during colitis (8, 9), induction of anti-microbial, cytokine or chemokine genes, and production of mucins by IEC (10). More importantly, these IL-22-mediated biological processes are associated with host defense, intestinal inflammation, metabolic disorders (obesity, diabetes, or nephropathy), tumorigenesis, and even anti-TNF therapy for IBD (10–16). Clinical relevance of IL-22 in IBD pathogenesis has been established given that some pronounced IBD risk genes, such as *Il-23*, *Stat3, Jak2, Tyk2, Fut2*, are involved in IL-22-STAT3 signaling, and that enhancing IL-22 pathway can improve mucosal healing and ameliorate disease progression (10). Moreover, IL-22 is considered as an ideal IBD therapeutic target because of its specific actions on epithelial cells through constitutively activating (phosphorylating) epithelial STAT3 (10). Based on these facts, therefore, a better understanding of IL-22 signaling network and its upstream or downstream transcriptional modulators will potentially improve the efficacy and safety of the current IBD drugs, or lead to identification of novel therapeutic targets for IBD treatment.

Activating transcription factor 3 (ATF3) is a stress-inducible gene which encodes a member of ATF/cyclic adenosine monophosphate (cAMP) response element (CRE)-binding (CREB) family of transcription factors (17). ATF3 has been described as a hub for the cellular adaptive-response network that has a regulatory role in disease pathogenesis (17). The fact that ATF3 is induced by lipopolysaccharide (LPS) and involved in TLR-stimulated gene transcription pinpoints its role in innate immunity, inflammation and host defense (18). Correlated to this, several studies have linked ATF3 to epithelial barrier function. In a *Drosophila* model, ATF3 was shown to control JNK and STAT signaling to maintain intestinal barrier regeneration (19). In human enterocytes, ATF3 negatively regulates Nod2-induced pro-inflammatory response (20), further supporting ATF3 contributes to barrier immunity and IBD pathogenesis (Nod2 is an IBD risk factor). Clinical correlation of ATF3 in IBD has been reported, with ATF3 expression being significantly upregulated in patients with Crohn's disease (21). While ATF3 promotes IEC apoptosis via interacting with and regulating p53 in Crohn's disease (22), ATF3 also maintains IEC survival to provide cellular defense in response to chemical stress (23). Therefore, it is still unclear how ATF3 regulates epithelial barrier function in the context of homeostasis or during intestinal stress such as inflammation. Yet, it has not been investigated if ATF3-mediated stress response in IEC involves IL-22-STAT3 signaling. Furthermore, it remains to be determined whether loss of ATF3 (i.e., loss of stress control) in gut epithelial cells could be a trigger leading to IBD pathogenesis.

In skin cancer cells, activation of STAT3 by overexpressed ATF3 enhances cell proliferation while ATF3 knockdown abolishes this effect (24). In hepatocellular carcinoma (HCC), ATF3 cooperates with SPTBN1 and SMAD3 to inhibit STAT3 activity, thereby suppresses HCC development (25). The role of ATF3 in STAT3 activation in gut epithelial cells has not been studied, nor in the context of IL-22 signaling. Intriguingly, levels of ATF3, IL-22, and IL-22R were all upregulated in IBD patients (21, 26), suggesting that ATF3 could be associated with IL-22 signaling in gut inflamed tissues. Supporting this, we first identified that IL-22 stimulation of gut epithelial cells upregulates both mRNA and protein levels of ATF3. Given that IL-22 signaling uniquely induces STAT3 phosphorylation and activation in epithelial cells, it is likely that ATF3 is involved in IL-22-mediated STAT3 activation and its downstream function, such as IEC regeneration or anti-microbial peptide production. In this study here, for the first time, we provide evidence showing that ATF3 is actively involved in the IL-22-pSTAT3 signaling cascade to maintain intestinal homeostasis at the steady state and to protect against colon tissue damage during chemical-induced colitis, by a mechanism of suppressing PTP (protein tyrosine phosphatase)-mediated STAT3 inactivation. We also identified ATF3 is acting on IL-6-mediated pSTAT3 signaling in T cells, which affects intestinal Th17 function. We concluded ATF3 orchestrates IL-22/IL-6-pSTAT3 activation in epithelium and adaptive lymphoid cells to facilitate mucosal immunity across the barrier, including barrier regeneration and production of anti-microbial peptides and Th17 cytokines. Therefore, we propose that ATF3 is a multifaceted risk factor for IBD pathogenesis.

MATERIALS AND METHODS

Reagents and Antibodies

All reagents and antibodies used in this study were listed in **Tables 1A,B**, unless indicated elsewhere.

Mice

Global ATF3 knockout mice were kindly provided by Dr. Tsonwin Hai (Ohio State University, Columbus, United States) and obtained from Dr. Ching-Feng Cheng (Tzu Chi University, Taiwan). ATF3 knockout mice were backcrossed to C57BL/6J (B6) mice for at least seven generations before our study (27). $ATF3^{flox/flox}$ mice were also kindly provided by Dr. Tsonwin Hai and were backcrossed to C57BL/6J (B6) mice for six

TABLE 1 | List of the materials used in the study.

A. ANTIBODIES		
Antibodies (concentration)	**Technique**	**Source/Identifier**
Purified anti-mouse CD16/32 Antibody, clone 93, (1:500)	Flow cytometry	BioLegend (101301)
Anti-Mouse CD326 (EpCAM) Monoclonal Antibody (G8.8), eFluor 450, eBioscienseTM (1:400)	Flow cytometry	ThermoFisher Scientific (48-5791-80)
Anti-Mouse CD45 Monoclonal Antibody (30-F11), Alexa Fluor 700, eBioscienseTM	Flow cytometry	ThermoFisher Scientific (56-0451-82)
Anti-Mouse CD24 Monoclonal Antibody (M1/69), APC-eFluor 780, eBioscienseTM (1:400)	Flow cytometry	ThermoFisher Scientific (47-0242-80)
Anti-Mouse Ki-67 Monoclonal Antibody (SolA15), PE-Cyanine7, eBioscienseTM (1:400)	Flow cytometry	ThermoFisher Scientific (25-5698-80)
Phospho-STAT3 (Tyr705) Monoclonal Antibody (LUVNKLA), PE, eBioscienseTM	Flow cytometry	ThermoFisher Scientific/#12-9033-41
Mouse IgG2b kappa Isotype Control, PE, eBioscienseTM (clone eBMG2b) (1:100), Isotype control for pSTAT3	Flow cytometry	ThermoFisher Scientific (12-4732-81)
PE/Cy7 anti-mouse/human CD44, clone IM7	Flow cytometry	BioLegend (103029)
APC anti-mouse CD3 Antibody, clone 17A2 (1:400)	Flow cytometry	BioLegend (100235)
Anti-Human/Mouse IL-22 APC, clone IL22JOP, (1:200)	Flow cytometry	BioLegend (17-7222)
FITC anti-mouse IL-17A Antibody, clone TC11-18H10.1, (1:200)	Flow cytometry	BioLegend (506907)
CD11b Monoclonal Antibody (M1/70), PE-Cyanine7, eBioscienceTM (1:400)	Flow cytometry	ThermoFisher Scientific (25-0112-81)
CD11c Monoclonal Antibody (N418), PE-Cyanine5.5, eBioscienceTM (1:400)	Flow cytometry	ThermoFisher Scientific (35-0114-80)
Ly-6G/Ly-6C Monoclonal Antibody (RB6-8C5), PE-Cyanine7, eBioscienceTM (1:400)	Flow cytometry	ThermoFisher Scientific (25-5931-81)
CD19 Monoclonal Antibody [eBio1D3 (1D3)], PE-Cyanine7, eBioscienceTM (1:400)	Flow cytometry	ThermoFisher Scientific (25-0193-81)
CD45R (B220) Monoclonal Antibody (RA3-6B2), PE-Cyanine7, eBioscienceTM (1:400)	Flow cytometry	ThermoFisher Scientific (25-0452-82)
Rat IgG2a kappa Isotype Control, PE-Cyanine7, for Ki67	Flow Cytomtery	eBioscience (25-4321-81)
Rat IgG2a kappa Isotype Control, APC, for IL22	Flow cytometry	eBioscience (17-4321-81)
FITC Rat IgG1, κ Isotype Ctrl Antibody, for IL-17A	Flow cytometry	BioLegend (400405)
Rabbit monoclonal (IgG) Ki67 (1:150)	Tissue Immunofluorescence	Abcam (ab16667)/SP6
Anti-rabbit IgG (H+L), F(ab′)2 Fragment (Alexa Fluor® 594 Conjugate) (1:300)	Tissue Immunofluorescence	Cell Signaling Technology (CST#8889)
Ulex europaeus lectin (UEA-1-FITC) (20ug/ml)	Whole mount tissue Immunofluorescence	Sigma-Aldrich (L9006)
Wheat Germ Agglutinin, Alexa Fluor® 633 Conjugate (10 ug/ml)	Whole mount tissue Immunofluorescence	ThermoFisher Scientific (W21404)
Rabbit monoclonal (IgG) ATF3 (1:1,000)	Western blot/organoid Immunofluorescence	Abcam (ab207434)/ EPR19488
Rabbit monoclonal (IgG) pSTAT3 (Y705) (1:1,000)	Western blot	Cell signaling technology (CST#9145) /D3A7
Mouse monoclonal STAT3 (1:1,000)	Western blot	Cell signaling technology (CST#9139)/124H6
Mouse monoclonal (IgG_1) SH-PTP2 (1:500)	Western blot	Santa Cruz (sc-7384)/B-1
Mouse monoclonal (IgG_1) PTP-MEG2 (1:500)	Western blot	Santa Cruz (sc-271052)/D-5
Mouse monoclonal (IgG_{2a}) PAC-1 (1:500)	Western blot	Santa Cruz (sc-32776)/ 4O21
Mouse monoclonal β-actin (1:1000)	Western blot	Santa Cruz (SC-47778)/C4
Rabbit monoclonal Histone H3 (1:2,000)	Western blot	Cell signaling technology (CST#4499)/D1H2
Rabbit monoclonal GAPDH (1:1,000)	Western blot	Cell signaling technology (CST#5174)/D16H11
B. REAGENTS		
Reagents/Resources: *Organoid*	**Technique**	**Source/Identifier**
Corning® Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix, Phenol Red-free (10ml)	Organoid culture	Becton Dickinson (#356231)/Lot.5033306
Jagged−1 (188–204), Notch Ligand	Organoid culture	AnaSpec (AS-61298)
GibcoTM Advanced DMEM/F-12	Organoid culture media	ThermoFisher Scientific/12634010

(Continued)

TABLE 1 | Continued

Reagents/Resources: *Organoid*	**Technique**	**Source/Identifier**
GlutaMAX™ Supplement	Organoid culture media	Life Technologies (35050-061)
N-Acetyl-L-cysteine	Organoid culture media	Sigma Aldrich (A9165)
B-27	Organoid culture media	Life Technologies (17504-044)
N-2	Organoid culture media	Life Technologies (17502-048)
Bovine Albumin Fraction V Solution (7.5%)	Organoid culture media	Life Technologies (15260-037)
ROCK Inhibitor (Y-27632)	Organoid culture media	Sigma-Aldrich (Y0503)/Lot. 6123002
EGF Recombinant Mouse Protein	Organoid culture media	Life Technologies (PMG8041)/Lot. 5264009
Recombinant Mouse HGF Protein	Organoid culture media	R&D system (2207-HG-025)/Lot. 5313012
TGF-b inhibitor (A 83-01)	Organoid culture media	R&D system (2939)/Lot. 6214005
Recombinant Murine Wnt-3a	Organoid culture media	PeproTech (315-20)
Recombinant Murine Noggin	Organoid culture media	PeproTech (250-38)
Recombinant Mouse R-Spondin 1 Protein, CF	Organoid culture media	R&D systems (3474-RS-050)
HEPES (1 M)	Organoid culture media	Life Technologies (15630-080)
TrypLE™ Select (1X), no Phenol Red	Organoid culture	Life Technologies (12563-011)
Gentle Cell Dissociation Reagent	Organoid transfer/ICC staining	StemCell (#07174)
AERRANE (isoflurane, USP)	Organoid transfer /Inhalation anesthesia	Baxter Healthcare of Puerto Rico (#N029E423)
Reagents/Resources: *Kits*	**Technique**	**Source/Identifier**
in situ Cell Death Detection Kit, TMR red kit	TUNEL assay	Sigma-Aldrich/ 000000012156792910
iSript TM cDNA synthesis kit	Real Time PCR	Bio-Rad (1708890)
T-Pro Bradford Protein Assay kit	Western blot	OmicsBio (JB04-D002)
T-Pro LumiLong Plus Chemiluminescence Detection kit	Western blot	OmicsBio (JT96-K004M)
Pierce™ Biotin 3′ End DNA Labeling Kit	EMSA	ThermoFisher Scientific (89818)
LightShift™ Chemiluminescent EMSA Kit	EMSA	ThermoFisher Scientific(20148)
Reagents/Resources: *Common*	**Technique**	**Source/Identifier**
eBioscience™ IC Fixation Buffer	Flow cytometry	ThermoFisher Scientific (00-8222-49)
Mouse IL-22 Recombinant Protein, eBioscience™	Cell stimulation	ThermoFisher Scientific (14-8221-63)
Recombinant Mouse IL-6 (carrier-free)	Cell stimulation	BioLegend (575702)
Gibco™ RPMI 1640 Medium, Powder	LPL isolation/culture media	ThermoFisher Scientific (31800022)
Mouse IL-23 Recombinant Protein, eBioscience™	LPL stimulation	ThermoFisher Scientific (14-8231-63)
PHORBOL 12-MYRISTATE 13-ACETATE (PMA)	LPL stimulation	Sigma-Aldrich (P8139)
Ionomycin from Streptomyces conglobatus	LPL stimulation	Sigma-Aldrich (I9657)
Brefeldin A (BFA)	LPL culture	Sigma-Aldrich (B6542)
BD Cytofix/Cytoperm™	LPL flow cytometry intracellular staining	BD Biosciences (554722)
BD Perm/Wash™	LPL flow cytometry intracellular staining	BD Biosciences (554723)
Dulbecco's Modified Eagle's Medium—low glucose	Cell culture media	Sigma-Aldrich (D6046)
Fetal bovine serum (FBS)	Cell Organoid culture media	Life Technologies (10437-028)
PolyJet™ *in vitro* DNA Transfection Reagent	CRISPR	SigmaGen (SL100688)
Immobilon-P PVDF Membrane	Western blot	MERCK (IPVH00010)
Biodyne™ B Nylon Membrane, 0.45 μm, 8 × 12 cm	EMSA	ThermoFisher Scientific (77016)
ProLong® Diamond Antifade Mountant	Immunofluorescence	Molecular Probes (P36961)
MP Premium Dextran Sulfate Sodium Salt (DSS, 100 g/bottle)	*in vivo*/*in vitro* experiments	MP Biomedicals (0216011080)
LiberaseTM	LPL isolation	Roche (05401127001)
Deoxyribonuclease I (DNase I)	IECs/LPL isolation	Sigma-Aldrich (SI-DN25-1G)
TRIzol RNA Isolation Reagent	RNA extraction	ThermoFisher Scientific (15596018)
SYBR Green PCR Master Mix (5 ml × 10)	Real time PCR	ABI (4368708)
2x RIPA buffer I (pH = 7.4) 2x concentrate	Western blot	OmicsBio (RB4475)
Halt™ Protease and Phosphatase Inhibitor Cocktail, EDTA-free (100X)	Western blot/EMSA	ThermoFisher Scientific (78441)

(Continued)

TABLE 1 | Continued

Mouse Primers	Technique	Sequence
C.MOUSE PRIMERS SEQUENCE		
Mouse Primers	**Technique**	**Sequence**
ATF3	CRISPR	Forward: 5′CACCgCC ATCGGATGTCCTCTGCGC 3′
		Reverse: 5′AAACGCGCAGAGGACATCCGATGGc 3′
ATF3	Real time PCR	Forward: TTACCGTCA ACA ACA GAC CC
		Reverse: TCA GCT CAGCATTCACAC TC
Reg3g	Real time PCR	Forward: TCA GGT GCA AGG TGA AGT TG
		Reverse: GGCCACTGTTACCACTGC TT
S100A8	Real time PCR	Forward: TGT CCT CAG TTT GTG CAG AAT ATA AA
		Reverse: TCA CCA TCG CAA GGA ACT CC
S100A9	Real time PCR	Forward: GGT GGA AGC ACA GTT GGC A
		Reverse: TCC AGG TCCTCCATGATG
β-defensin 3	Real time PCR	Forward: GTC TCC ACC TGCAGC TTT TAG
		Reverse: AGG AAA GGA ACT CCA CAA CTG C
GRP78	Real time PCR	Forward: ACTTGGGGACCACCTATTCCT
		Reverse: ATCGCCAATCAGACGCTCC
sXBP1	Real time PCR	Forward: CTGAGTCCGAATCAGGTGCAG
		Reverse: GTCCATGGGAAGATGTTCTGG
CHOP	Real time PCR	Forward: CCACCACACCTGAAAGCAGAA
		Reverse: AGGTGAAAGGCAGGGACTCA
Cyclin D	Real time PCR	Forward: GCAAGCATGCACAGACCTT
		Reverse: GTTGTGCGGTAGCAGGAGA
C-Myc	Real time PCR	Forward: TAGTGCTGCATGAGGAGACA
		Reverse: GGTTTGCCTCTTCTCCACAG
TCF7	Real time PCR	Forward: ATCCTTGATGCTGGGATTCTG
		Reverse: CTTCTCTTGCCTTGGGTTCTG
Sox9	Real time PCR	Forward: CTGGAGGCTGCTGAACGAGAG
		Reverse: CGGCGGACCCTGAGATTGC
Fut2	Real time PCR	Forward: TGC ACT GGCCAG GAT GAA
		Reverse: GCGCTA GAG CGT TGT GCA T
IL-22	Real time PCR	Forward: TCG CCT TGA TCTCTCCAC TC
		Reverse: GCT CAGCTC CTG TCACAT CA
IL-22R1	Real time PCR	Forward: CTACGTGTGCCGAGTGAAGA
		Reverse: AAGCGTAGGGGTTGAAAGGT
IL-10R2	Real time PCR	Forward: GCCAGCTCTAGGAATGATTC
		Reverse: AATGTTCTTCAAGGTCCAC
IL-6	Real time PCR	Forward: ACA AGT CGG AGG CTT AAT TAC ACA T
		Reverse: TTG CCA TTG CAC AAC TCT TTT C
IL-6R1	Real time PCR	Forward: AAGCAGCAGGCAATGTTACC
		Reverse: CATAAATAGTCCCCAGTGTCG
gp130	Real time PCR	Forward: ATAGTCGTGCCTGTGTGCTTA
		Reverse: GGTGACCACTGGGCAATATG
IL-17A	Real time PCR	Forward: TCC AGA AGG CCC TCA GAC TA
		Reverse: TTC ATT GCG GTG GAG AGT C
L32	Real time PCR	Forward: GAA ACT GGC GGA AAC CCA
		Reverse: GGA TCTGGC CCT TGA ACC TT
GAPDH	Real time PCR	Forward: GTA TGA CTCCACTCA CGG CAA ATT

generations before our study (28). All mice were at the age of 2∼3 months old when analyzed and were maintained under standard conditions at Academia Sinica, Animal care and all experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the Institute of Biomedical Sciences, Academia Sinica, Taiwan.

DSS Induced Colitis

For survival studies, mice were given 2% dextran sodium sulfate (DSS) (MP Biologicals, molecular mass 36-40 kDa) in drinking water for two cycles over 20 days as follows: day-0 to day-5 (cycle 1) of 2% DSS followed by 5 days of normal drinking water, then switch back to 2% DSS (cycle 2, 5 days) then switched to normal drinking water (5 days). Disease activity index (DAI), slightly modified from previously described protocols (29, 30), was measured daily after the start of DSS treatment as a sum score of (a) weight loss percentage [scale: 0–4, no loss (0), 1–5% (1), 5–10% (2), 10–20% (3), >20% (4)], (b) stool consistency [scale: 0-4, hard (0), soft (2), very soft (3), diarrhea (4)], and (c) rectal blooding [scale: 0-4, no blood in feces (0), trace blood in feces (2), dark red colored feces (3), gross bleeding (4)].

Colonoscopy image: At day-8 of DSS intake, mice were anesthetized by isoflurane inhalation (one mouse at one time), then the mouse was moved to a chamber with cotton soaked with isoflurane for anesthesia maintenance while performing colonoscopy. Images were recorded as videos by using endoscopy system (TESALA AVS, Olympus, Tokyo, Japan) attached to an air pump to achieve regulated inflation of the colon, and endoscope was coated with a sterile thin layer of Vaseline as a lubricant to avoid mucosal irritation.

H&E staining: At day-8 of DSS intake, mice were sacrificed, colons were washed and cleaned with gentle rinsing by 1x PBS to remove all the feces, colon fragments (∼0.5 cm long) were fixed with 4% paraformaldehyde, and sent to the Pathology Core Lab at the Institute of Biomedical Sciences, Academia Sinica, Taiwan for H&E sections preparation. Histological scores were calculated as previously described (30), with some modifications as follows: inflammation severity (scale: 0–4), inflammation extent (scale: 0–4) (none, mucosa, submucosa, transmural), crypt loss (scale: 0–4) (0, 1/3, 2/3, surface epithelium present, damage of surface epithelium), percent of are affected (scale: 0–4) (0, 25, 50, 75, and 100%).

Isolation of Intestinal Epithelial Cells (IEC) and Flow Cytometry

IECs were isolated using a modified version of a previously described protocol (31). Intestine tissue was flushed by 1x PBS, cut open into small fragments (∼1 cm) which were washed in 50 ml falcon tube by hand shaking few times in 1x PBS. The tissues were transferred into a new pre-cold 50 ml falcon tube containing 30 mM EDTA/1x PBS and 1.5 mM DTT then incubated on ice for 20 min. Then the supernatants were discarded and the tissues were transferred to a new pre-warmed 50 ml falcon tube containing 30 mM EDTA/1x PBS and vortex for 30 s, the tissues were then incubated at 37°C for 10 min and vortex briefly 5 times to isolate epithelial cells. Supernatants were collected to a new 50 ml falcon tube and pelleted by centrifugation. The cells were then digested into single cells by incubating in 1x HBSS containing 0.3 U/ml Dispase at 37°C for 10 min. After 10 min, to stop digestion reaction, 100 ug DNase I and 5% FBS were added and the cells were pelleted, and diluted in 1 ml staining buffer (1x PBS with 2% FBS). 0.5 ml was used for quantitative PCR (QPCR) analysis, and the rest of the cells were used for flow cytometry analysis as follows: samples were incubated for 8 min with blocking antibody (anti-CD16/32) in staining buffer on ice, followed by washing in 1 ml staining buffer, centrifuged at high speed (12,000 × g) for 10 s, the pellets were then re-suspended in staining buffer containing surface antibody cocktail with the following fluorescent antibodies: EpCAM-eF450, CD45-AF700, CD24-APC-Cy7 for 20 min on ice. Next, cells were washed with 1 ml staining buffer and fixed in 4% PFA for 15 min before intracellular staining. The fixed single cells were incubated in 1x PBS containing 0.1% saponin and anti-Ki67 antibody for 30 min. Data were acquired using a LSRII flow cytometer (BD Biosciences) and analyzed with FlowJo software.

Immunofluorescence Staining of Tissue Sections

Mouse tissues were prepared as described above in the DSS-colitis section. Paraffin sections were first de-paraffinized by three times wash in xylene (5 min each), followed by two times wash of 100% ethanol (10 min each), then two times wash in 95% ethanol (10 min each), and then washed three times in ddH2O. Then the sections were dewaxed and heat-mediated antigen retrieval was performed by incubation in 10 mM sodium citrate buffer pH 6.0 for 15 min at a sub-boiling temperature. Slides were then allowed to cool down for 10 min at RT and then washed three times in ddH2O before being blocked with blocking buffer (1x PBS/5% normal goat serum) for 1 h, followed by permeabilization in 1x PBS/0.3% Triton X-100. After 15 min, the sections were incubated with primary rabbit anti-Ki67 (Abcam) in dilution buffer (1x PBS/1% BSA/0.3% Triton X-100) at 4°C overnight. Next day, the sections were washed three times with 1x PBS (5-10 min each) followed by incubating with fluorophore-conjugated with anti-rabbit Alexa Fluor-594 at room temperature for 2 h. To visualize the nucleus, slides were washed as before and counterstained with Hoechst 33342 (1 ug/mL in PBS) for 10 min and cover slips were mounted on the slides by using ProLong Gold Antifade Mountant (Invitrogen). Confocal images were obtained with a Carl Zeiss LSM 700 stage imaging system under a 20, 40, or 100x oil-immersion objectives.

Tunel Assay

Mouse tissues were prepared as mentioned above in the DSS-colitis section. Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) assay was performed according to instructions in *in situ* Cell Death Detection, TMR red kit (Roche). Paraffin-Embedded tissues sections were processed as mentioned above in the section of Immunofluorescence staining. After tissue permeabilization, the sections were incubated with TUNEL reaction mixture at dark in humidified atmosphere, 37°C for 1 h. To visualize the nucleus, slides were washed as before and counterstained with Hoechst 33342 (1 ug/mL in PBS) for 10 min and cover slips were mounted on the slides by using ProLong Gold Antifade Mountant (Invitrogen). Confocal images were obtained with a Carl Zeiss LSM 700 stage imaging system.

Whole Mount Tissue

Small sections of the terminal ileum (0.5–1 cm long) were cut, fixed immediately with 4% PFA when processing multiple sections at the same time, each section was then washed with 1x PBS and cleaned with feces and fat tissues removed. Each segment was cut open, washed again to remove the mucus, placed on tissue paper so that the luminal side faces upward and incubated overnight in 4% PFA at 4°C. Next day, tissues were washed in 1x PBS 3 times for 10 min with shaking at 60 rpm. A solution of fluorescence-conjugated UEA-1 (20 μg/mL) and WGA (10 μg/mL) in antibody dilution buffer was prepared. Each segment was mixed with 1 ml of antibody mixture and incubated on ice for 3 h for each antibody. After washing in 1x PBS, segments were placed on glass slides, with the apical luminal surface facing upward, residual PBS was removed gently by Kim wipes, then ProLong Gold Antifade Mountant (Invitrogen) was added and the tissue segments were sandwiched by placing cover slip over them. Confocal images were obtained with a Carl Zeiss LSM 700 stage imaging system.

Intestinal Crypt Isolation and Organoid Culture

Intestinal crypt isolation was based on the protocol by Sato et al with some modifications (32). Mice were euthanized with CO_2 and the abdomen was cut open to separate ∼12 cm of distal small intestine (whole Ileum) and ∼5 cm of distal large intestine (distal colon). Peyer's patches and fat tissues were removed and organs were cut opened longitudinally and washed with PBS. The colon was scratched with a cover slip a few times to remove the upper layer of epithelial cells. The intestines were then cut into 1 cm pieces and placed into 50 ml falcon tubes and washed vigorously by shaking in Hank's Balanced Salt Solution (HBSS) until the supernatant was clear. The tissues were then incubated in 30 mM EDTA/1x PBS with shaking at 150 rpm for 5 min in 37°C. The tissues were then transferred into a new 50 ml falcon tube containing cold 1x PBS and put horizontally under ice with shaking at 150 rpm for 15 min. The supernatant was then replaced with 10 ml wash buffer (1x PBS at pH 7.4, 1x penicillin/streptomycin, 50 ug/ml Gentamicin, 0.1% BSA), vortexed for 6 times and then the supernatant was transferred to a new 50 ml falcon tube on ice. This process was repeated 3–5 times. For ileum only, crypts were filtered through a 70 μm strainer to remove villi. After that, the collected supernatant was centrifuged at 50 g for 5 min at 4°C and the pellet was resuspended gently in 5–10 ml wash buffer. For colon only, colon crypts were mildly digested by incubating in 1x TrypLE Select Enzyme (Gibco) for 1 min at room temperature into small cluster of cells. The digestion was stopped by mixing the crypts with 10 ml wash buffer and pelleted by centrifuge at 300 g for 5 min at 4°C. The total crypt number was measured by plating 100 ul crypt suspension on petri dish and counted under a microscope. An aliquot of cell suspension containing approximately 500 crypts was centrifuged at 300 g for 5 min at 4°C and pellets were gently mixed with Matrigel (BD Biosciences) containing Jagged-1 peptide (50 ul Matrigel used for 500 crypts). The mixture was then plated in 24-well plate and incubated in 37°C for 10 min before adding organoid growth medium. Organoid growth medium was prepared (1:1 mixture of basal culture medium and WNR conditioned medium) and added to each well. Basal culture medium: (advanced DMEM/F-12 medium supplemented with 100 U/ml penicillin, 100 ug/ml streptomycin, 50 ug/ml Gentamicin, 2 mM GlutaMAX, 10 mM HEPES, 1 mM N-acetyl-L-cysteine, 1x B-27 Supplement, 1x N-2 Supplement, and 1% BSA). WNR conditioned medium: [WNT, R-spondin, Noggin medium, derived from stable cell lines, supplemented with 10 uM Y-27632 (ROCK inhibitor), 50 ng/ml mEGF, 0.5 uM A-83-01 (for ileum crypts), or 100 ng/ml murine Hepatocyte Growth Factor (mHGF) (for colon crypts)]. Medium was replaced every 2 days.

Organoid Transplantation

The procedure of colon organoid transplantation was based on the protocol described by Yui et al. with some modifications (33). Crypts were isolated from distal colon tissues of 8–12 weeks old mice as described above and organoids were cultured for 6 days and used as donor organoids. The recipient ATF3-deficient mice were fed with 5 days of 1.5% DSS water followed by 9 days of regular water. At day-6 and day-8 post DSS intake, Matrigel containing organoids were dissolved in cell recovery solution (Stem Cell Technologies) by pipetting and cell suspension was transferred to a 50 ml falcon tube, followed by shaking at 100 rpm on ice for 1 h and then centrifuged at 300 g for 5 min at 4°C. The pellet was washed twice in 10 ml of cold 1% BSA/1x PBS by spinning at 20 g for 5 min at 4°C. The pellets was then resuspended in 180 ul of cold PBS and transferred to an eppendorf, followed by adding 20 ul of Matrigel, 1 ul of Jagged-1 peptide, and 1 ul of Y-27632 inhibitor. The collected organoids (∼1,000 cells) were transferred to colon of the recipient mice (∼5 cm deep) through intra-rectal injection by using 1 ml syringe and a thin flexible catheter (35 cm in length and 1.2 mm in diameter, Terumo Inc.) at day-6 and day-8 post DSS administration. After infusion, the anal verge of recipient mouse was glued with Vetbond Tissue Adhesive (3 M Vetbond) for 6 h to prevent the injected organoids from being excreted from the colon. The survived recipient mice were sacrificed at day-14 for analysis.

Intracellular Organoid Staining of pSTAT3

Intracellular staining of STAT3 phosphorylation in organoids was performed as previously described (7). In brief, organoids were mechanically disrupted into crypt suspension in cell dissociation buffer (Stem Cell Technologies), by pipetting cell suspension in 50 ml falcon tube and rotating on ice at 100 rpm for 1 h, followed by centrifuging at 300 g for 5 min at 4°C. The pellet was then stimulated with 200 ul of organoid culture media containing rmIL-22 (20 ng/ml, eBioscience) for 20 min. After stimulation, the cells were fixed by adding an equal volume of intracellular fixation buffer (eBiosciense) and incubating in dark at RT for 1 h. Single cell suspension was made by rotating the cells in TrypLE (Gibco) at 100 rpm for 15 min at 37°C and then passed through a 70 μm strainer. Permeabilization was performed by adding 1 ml of ice-cold methanol for 30 min at 4°C. Cells were thoroughly washed with PBS before staining with a blocking

antibody (CD16/32) followed by intracellular staining with PE-pSTAT3 and PE-IgG2b isotype control antibodies (eBioscience). Samples were immediately analyzed by LSR-II flow cytometer.

Generation of ATF3-Deficient CMT-93 Cells by Crispr-Cas9

CMT-93 cells, a murine rectal-carcinoma cell line obtained from ATCC, were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL of penicillin, 100 ug/ml of streptomycin and 2 mM GlutaMAX (all reagents obtained from Gibco). Cells were passaged (1:10) after reaching 70∼80% confluence. sgRNA design and cloning were performed based on a protocol by Ran et al. (34), with a CRISPR design tool (http://crispr.mit.edu). The sequences of oligos were: Forward primer: 5′-CACCgC CATCGGATGTCCTCTGCGC-3′, Reverser primer: 5′AAACGC GCAGAGGACATCCGATGGC-3′. ATF3 targeting sequences were cloned into BbsI-digested pSpCas9(BB)-2A-GFP plasmid (Addgene, px458), which was a gift from Dr. Liuh-Yow Chen at Academia Sinica, Taiwan. The px458-based constructs were introduced into CMT-93 cells by PolyJet^{TM} *in vitro* DNA Transfection Reagent using advanced protocol (SignaGen). Single GFP^{+} cells were sorted into 96-well plate to obtain single cell clones by FACSAria cell sorter (BD Biosciences) at IBMS core facility, Academia Sinica, Taiwan. The ATF3-deficient cells were confirmed by Western blot analysis.

Silencing the Mouse PTPN11 (SHP2) Gene Expression in CMT93 Cells

The lentiviral RNAi system was provided by the National RNAi Core Facility, Academia Sinica, Taipei, Taiwan. To silence the Ptpn11 (NM_011202) gene expression, lentiviral vector pLKO_TRC005 encoding small hairpin RNA (shRNA) specific to Ptpn11 (Clone ID: TRCN0000327986) was used. The traget sequence for Ptpn11 was 5′-CGTGTTAGGAACGTCAAAGAA-3′ (nts 1,332–1,352), and the double-stranded oligonucleotide containing the following shRNA sequence 5′-CCGGCGTGT TAGGAACGTCAAAGAACTCGAGTTCTTT GACGTTCCT AACACGTTTTTG-3′ was introduced into the lentiviral vector. To produce recombinant lentiviruses, HEK293T cells were co-transfected with lentiviral vector carrying gene-specific shRNA (2.5 ug), Gag and Polymerase (RT) expression plasmid pCMV-ΔR8.91 (2.25 ug) and VSV-G envelope glycoprotein expression plasmid pMD.G (0.25 ug) by using PolyJet^{TM} *in vitro* DNA Transfection Reagent (SignaGen Laboratories #SL100688). After 72 h the medium was harvested, aliquoted and stored at −80°C. To generate Ptpn11 specific gene knockdown cells, CMT-93 ATF-3 KO cells were grown to 80% confluency and were infected with recombinant lentiviruses encoding Ptpn11 specific siRNAs with an MOI >6 in the presence of 8 ug/mL polybrene (Sigma #H9268) for 24 h. The cells were rinsed in DMEM and allowed to grow for another 36 h in the growth medium. Subsequently, the cells were selected in the growth medium containing 2 ug/mL of puromycin dihydrochloride (Gibco^{TM} #A11138) for 1 week. The puromycin-resistant cells were collected and gene knockdown in cells was determined by immunoblotting with anti-Ptpn11 antibody (Santa Cruz #sc-7384).

Wound Healing Assay

Approximately 1.2 × 10^{6} of wild-type or ATF3$^{-/-}$ CMT-93 cells were seeded in triplicate into a 6-well plate and grown for 12 h in complete DMEM. Cell monolayer was scraped to form a wide strip (∼1,100 μm) by using a standard 1,000 ul pipette tip. The wounded monolayers were washed to remove non-adherent cells. Each wound was photographed and measured at two pre-marked positions per well at time point of 0 h and time point of 24 h post wounding. Wound recovery was expressed as percentage of wound closure after 24 h relative to 0 h post scratch. Each dot shown in the figure represents one wound in each well and the data is representative of at least two independent experiments.

Cell Proliferation Assay

Approximately 0.2 × 10^{6} of wild-type or ATF3$^{-/-}$ CMT-93 cells were seeded, serum starved for 24 h and this was considered as day-0. Serum-free medium was replaced with complete DMEM containing 10% FBS after starvation and the cells were grown for another 24 h in complete DMEM with or without 5% DSS (day-1). DSS-treated cells were washed twice with PBS and allowed to recover in complete DMEM for another 24 h (day-2), whereas untreated cells were simply grown in complete DMEM until day-2. Total cell proliferation was calculated as percentage relative to the number of cells seeded. Cell number was determined by using 0.2% tryphan blue staining in cells and counted by Cellometer (Nexcelom Bioscience).

Isolation, Culture, and Stimulation of Lamina Propria Cells

Colon lamina propria (LP) cells were isolated based on a protocol by Moro et al. (35). In brief, dissected colons were washed with ice-cold 2% FBS/1x PBS wash buffer, cut into small pieces, and transferred to a 100 ml glass bottle containing 40 ml of depletion buffer (1 mM EDTA/1x PBS). Colon tissues were put for stirring at 37°C for 30 min, then washed in 1x PBS by hand shaking in a 50 ml falcon tube to ensure removal of epithelial cells. Next, colon pieces were minced by a scissor in eppendorf with 1 ml of digestion buffer, RPMI medium with 2% FBS, Liberase^{TM} (15 ug/ml, Roche), DNase1 (50 ug/ml, Sigma), then transferred to a 50 ml flask with 9 ml of digestion buffer and put for stirring at 37°C for 30 min. The supernatant was filtered through a 70 μm strainer into a new 50 ml falcon tube on ice and the tissues were homogenized through an 18G needle then again stirred in 10 ml of digestion buffer for another 30 min. The supernatant was centrifuged at 600 g for 6 min at 4°C and the pelleted LP cells were re-suspend in complete RPMI for cell counting, the cells were then divided into to 3 portions for RNA extraction, unstimulated or stimulated culture in V-bottom 96-well plate for flow cytometry analysis of cytokine production. After 3 h stimulation with IL23/PMA/Ionomycin cocktail, the cells with briefly re-suspended by multi-channel pipette before spinning down at 1,500 rpm for 5 min at 4°C and washed once with staining buffer. 50 ul staining buffer with blocking antibody (CD16/32) was added to the cells for 10 min before performing surface staining (EpCAM/CD45/Lineage markers) for 20 min in dark at 4°C. For intracellular staining, the cells were fixed in BD CytoFix/CytoPerm buffer for 30 min at 4°C, washed once with 200ul of BD Perm Wash Buffer followed

by spinning down, 1,800 rpm, 6 min for intracellular staining mixture (APC-IL22/FITC-IL17A) using 1x Wash Buffer. After washing in staining buffer, the cells were re-suspended in 150 ul staining buffer and immediately analyzed by LSR-II flow cytometer.

RNA Extraction and Quantitative Real-Time PCR Analysis

Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. One microgram of RNA was reverse-transcribed to cDNA with iScript™ cDNA synthesis kit (Bio-Rad). Transcript levels of the target genes were analyzed using Power SYBR® Green PCR Master Mix (Applied Biosystems) on a StepOnePlus Real-Time PCR System (Life Technologies). Primer sequences were listed in **Table 1C**.

Western Blot Analysis

Cells were cultured in complete DMEM till 70–80% confluence and serum starved for 5 h before stimulation. Medium was replaced with complete medium with or without recombinant mouse IL-22 (50 ng/ml, eBioscience) for 10 min stimulation. Cells were then washed with 1x PBS and immediately scraped from the plate into 1x RIPA lysis buffer containing protease/phosphatase inhibitors (Thermo Fisher). The lysates were then sonicated and centrifuged at 14,000 g for 15 min and the supernatants were collected and quantified with a Bio-rad protein assay kit for Western blot analysis. Protein samples were separated on SDS-PAGE gels and transferred to polyvinylidene fluoride membrane. Membranes were blocked with 5% skim dried milk in Tris buffered saline buffer (TBST: 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) and incubated overnight with the following primary antibodies: Anti-ATF3 (Abcam ab207434), pSTAT3 (CST#9145), STAT3 (CST#9139), PTP-SHP-2 (Santa Cruz #sc-7384), PTP-MEG2 (#sc-2271052), PAC-1 (#sc-32776), β-actin (#sc-47778), Histone H3 (CST#4499), GAPDH (CST#5174), α-tubulin (#abcam2791, Clone DM1A). After washing with TBST, blots were incubated with HRP conjugated secondary antibodies. Detection was achieved using a chemiluminescence substrate (Omics Bio) and images were acquired using ImageQuant™ LAS 4,000 camera.

Electrophoretic Mobility Shift Assay (EMSA)

Wild-type and ATF3$^{-/-}$ CMT93 cells were cultured till 80% confluence and serum starved for 5 h before IL-22 (50 ng/ml) stimulation. Cytoplasmic and nuclear extracts were isolated as previously described (36). After stimulation, cells were washed with 1x PBS, and immediately scraped from the plate and centrifuged at 300 g for 5 min at 4°C. Pellets were resuspended in cytoplasmic isolation buffer [10 mM HEPES, 60 mM KCl, 1 mM EDTA, 0.075% (v/v) NP40, at pH 7.6, 1 mM DTT and 1% (v/v) protease/phosphates inhibitor] on ice, incubated for 10 min, and then centrifuged for 5 min at maximum speed (∼14,000 g). The supernatant was transferred to a new pre-chilled tube containing 20% glycerol (cytoplasmic extract). The pellet was resuspended by vortex in nuclear isolation buffer [20 mM Tris Cl, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, at pH 8.0, 25% (v/v) glycerol, and 1% (v/v) protease/phosphates inhibitor] and incubated on ice for 1 h with agitation every 15 min. Samples were then centrifuged at high speed for 15 min, and the supernatants were collected into a new pre-chilled tubes (nuclear extract). Both cytoplasmic and nuclear extracts were stored in −80°C till use. Complementary oligonucleotides for the STAT3 promoter probe containing ATF/CRE binding site was synthesized and biotinylated using biotin labeling Kit (Thermo Fisher #89818). EMSAs were performed using EMSA kit (Thermo Fisher #20148). In brief, STAT3 promoter probe was incubated with 10 μg of nuclear extracts from unstimulated or IL-22-stimulated CMT93 cells in the presence of poly deoxy-inosinic-deoxy-cytidylic acid and protein-DNA complexes were resolved by native polyacrylamide gel electrophoresis.

Transmission Electron Microscopy (TEM)

Dissected and cleaned ileum tissues were washed with 1x HBSS, cut into small fragments (∼0.2 cm), and fixed in 1x PBS with 4% PFA/2.5% Glutaraldehyde. Tissue processing and image acquisition were performed by the TEM Core facility at Institute of Cellular and Organism Biology, Academia Sinica, Taiwan.

Statistical Analysis

Statistical analysis was performed most of the time using Multiple T-test when comparing two populations (wild-type & knockout) for only one variable (body weight, colon length, survival, etc.). Two-way analysis of variance (ANOVA), comparing the mean differences between and within populations (wild-type & knockout) that have been split into two independent variables (un-stimulated/stimulated), was used for experiments of multiple statistical comparisons. Survival curve was calculated using the Kaplan–Meier method and statistical significance was calculated using Log rank (Mantel-Cox) test. GraphPad Prism 6 software was used to run the analysis. All the data are presented as mean with standard deviation, and $p \leq 0.05$ was considered significant. The level of P value is expressed in asterisk as follow: $^{*}P \leq 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$.

RESULTS

ATF3 Is Essential for Balanced Homeostasis in the Intestine

Given the fact that increased ATF3 level is associated with active IBD patients (21) and in order to better understand the function of ATF3 in the intestine, we first sought to determine the tissue distribution of ATF3 in more details. For this purpose, we prepared samples from the intestine of mice for mRNA analysis, including intestine fragments, villus samples by scraping off the surface of epithelial layer, and crypt samples by shaking fragments in the EDTA solution (**Supplementary Figure 1A**). We found ATF3 is more expressed in epithelial scrapes isolated from duodenum and jejunum, and is more abundant in ileum and colon crypts (**Supplementary Figure 1B**). Moreover, intestinal epithelial cells express higher mRNA levels of ATF3 than those tissues rich in immune cells including Peyer's Patches, mesenteric lymph nodes, inguinal lymph nodes, and spleen (**Supplementary Figure 1B**), suggesting that ATF3 has a more important regulatory role in the IECs. This finding prompted us to examine the characteristics

of epithelial barrier integrity, determined macroscopically by the length of intestine, colon tissue histology, and the number of total crypts harvested from the intestine (30). Compared to wild-type littermates, we found that, at 3 months of age, naïve ATF3$^{-/-}$ mice showed more shortened colon length, marked reduction in total crypt numbers (**Figures 1A,B**), slightly reduced body weight, less proliferative crypt cells, and increased cell infiltration in colon tissues (**Supplementary Figures 1C,D**), indicative of spontaneous inflammation in the colon. However, in ATF3$^{-/-}$ mice, we did not observe any enlarged lymph nodes, splenomegaly or cellularity defects associated with other lymphoid organs at the steady state or during DSS colitis (data not shown).

Because most defects in ATF3$^{-/-}$ mice were associated with epithelial barrier, we next examined the proliferation capacity of epithelial cells. In freshly isolated crypts, we found there is defective proliferation or regeneration of ileum or colon crypts by Ki67 staining (**Figure 1C** and **Supplementary Figure 1E**). To confirm this phenotypical abnormality is cell intrinsic to epithelial cells, we used primary organoid culture for further investigation. Intestinal organoid is an *ex vivo* primary epithelial culture fueled with epithelial stem cells and their differentiated daughter cells that can transform into a mini-intestine while maintaining all the physiological and pathological features of the tissue from which they are derived (37). Notably, ATF3$^{-/-}$ organoids displayed slower growth rate, less budding (i.e., crypt formation), and less survival than wild-type organoids (**Figure 1D**). This growth impairment was also characterized by downregulation of the genes that control cellular proliferation and survival including Cyclin D, c-Myc, TCF7, and the stem cell marker Sox9 (**Figure 1E**). Together, we concluded ATF3 is essential for gut epithelial cell proliferation that directly controls crypt regeneration and barrier homeostasis.

Fucosylation is one of the most vital epithelial modifications involving adding oligosaccharides on glycoproteins or glycolipids. It plays a key role in regulating epithelial barrier, microbiota composition, and susceptibility to infection (38, 39). Epithelial fucosylation is catalyzed by fucosyltransferase enzymes, most notably the IBD risk gene fucosyltransferase 2 (*Fut2*) (5). We found that ileum fragments in naïve ATF3$^{-/-}$ mice have decreased Fut2 mRNA expression compared to wild-type mice (**Supplementary Figure 1F**). It was shown fucosylated epithelial cells can be more specifically marked by whole mount tissue immunofluorescence co-staining of α(1,2)-fucose-recognizing lectin (ulex europaeus agglutinin-1 or UEA-1) and wheat germ agglutinin (WGA) (40). We therefore used UEA-1/WGA staining to further confirm FUT2 downregulation in ATF3-defiecitnt epithelial cells (**Figure 1F**). Since *Fut2* is one of IL-22-pSTAT3 regulated host defense genes (38), the lack of FUT2 expression and diminished UEA-1/WGA signals in ATF3$^{-/-}$ IECs indicates that ATF3 is involved in epithelial IL-22-pSTAT3 fucosylation pathway which controls microbiota composition, host-commensal homeostasis and host defense against bacterial infection. Indeed, we found that ATF3$^{-/-}$ mice were more susceptible to intestinal infection by Citrobacter rodentium (**Supplementary Figure S2**). Collectively, our results demonstrate that epithelial ATF3 is intrinsically essential for mucosal homeostasis in the intestine.

Loss of ATF3 Disrupts Paneth Cell Homeostasis and Stem Cell Regeneration

Paneth cells, the maestros of epithelial anti-microbial immunity, are professional secretory cells with an apical clustering of secretory granules (41). Those granules within Paneth cells release key anti-microbial peptides (AMP) that help constitute a neat environment required for gut homeostasis (41). Because Paneth cells, together with stem cells, are located at the crypt base, the finding of compromised crypt homeostasis in ATF3$^{-/-}$ mice prompted us to further examine whether ATF3 has a role in the development or immunity of Paneth cells. Transmission electron microscopy (TEM) of Paneth cells revealed a dramatic depletion, degeneration, and reduction in numbers of Paneth cells and their granules in ATF3$^{-/-}$ mice (**Figures 2A,B**). Changes in inner cell structure and organization, such as fragmentation and disruption of the vacuolar system including endoplasmic reticulum (ER) and mitochondria, are hallmark morphological alterations occurring in apoptotic cells under TEM (42–44). Besides degeneration of Paneth secretory granules, fragmentation and abnormal distribution of ER, and swollen mitochondria with damaged cristae are typical morphological features of loss of cell homeostasis and cell death in Paneth/stem cell niche in ATF3$^{-/-}$ mice (**Figures 2C,D**). Paneth cells cohabitate with stem cells at the crypt base forming a mutual niche of all the essential signals for stem cell maintenance (45). Therefore, any Paneth cell abnormality eventually leads to stem cell dysfunction, as evidenced by impaired cell survival, proliferation, and regeneration of ATF3$^{-/-}$ IECs. This two-hit (Paneth/stem cells) abnormality phenomena due to ATF3 deficiency emphasizes the legitimacy of ATF3 to maintain the homeostasis and functionality of the crypt niche.

Next, we used a colon epithelial cell line, CMT93, to investigate whether ATF3 is involved in wound healing and epithelial regeneration in response to injury. Colitis-associated mucosal damage requires active and regulated processes of tissue healing and regeneration, which is facilitated by intestinal stem cells (ISCs). Accordingly, we performed wound healing assay in dextran sulfate sodium (DSS)-treated CMT93 cells, which mimics cell migration and tissue repair during regeneration *in vivo* after DSS-introduced epithelial damage. By monitoring wound closure after DSS exposure, we found that ATF3$^{-/-}$ CMT93 cells reseal the open wound much slower compared to wild-type cells in both untreated and DSS-treated conditions (**Figures 2E–G**). Cell proliferation rate is a critical factor that contributes to barrier homeostasis. Notably, we found proliferation rate of ATF3$^{-/-}$ CMT93 cells is significantly lower than wild-type cells in both untreated and DSS-treated conditions (**Figure 2H**), indicating that proliferative capability of ATF3$^{-/-}$ epithelial cells was compromised, consistent to those results observed in organoid culture (**Figures 1D,E**). Collectively, we concluded ATF3 regulates barrier maintenance which involves epithelial cell regeneration and tissue repair.

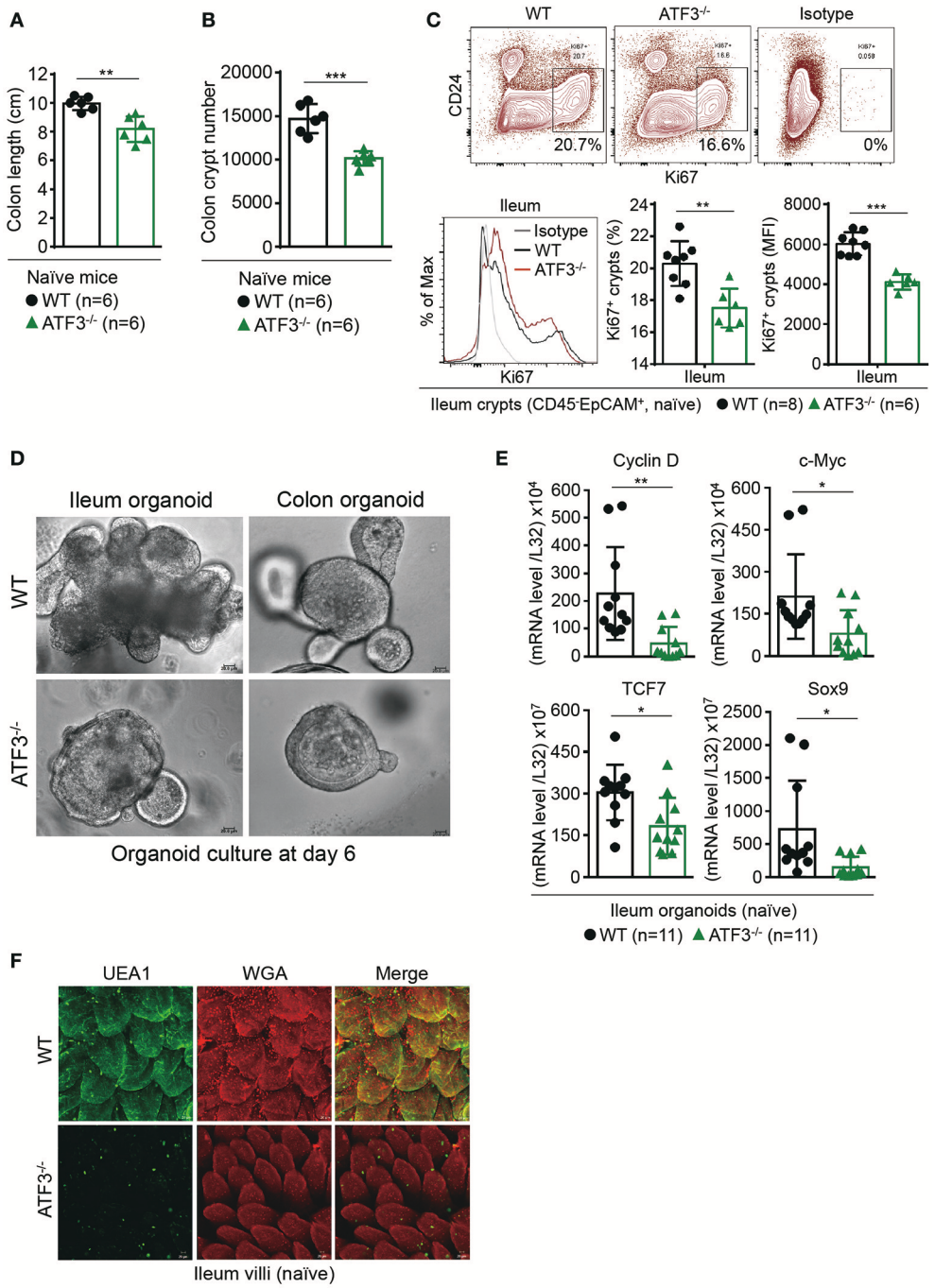

FIGURE 1 | ATF3 maintains intestinal homeostasis. **(A)** Comparison of colon length between naïve mice as indicated. **(B)** Colon crypts from mice were isolated by shaking colon fragments in EDTA and counted under light microscopy. **(C)** Flow cytometry analysis of Ki67 and CD24 expression in ileum crypts, gated on the $CD45^{-}EpCAM^{+}$ populations, from the indicated naive mice. **(D)** Representative micrographs showing intestinal organoids derived from naïve mice. **(E)** Quantitative real-time PCR analysis of cell cycle genes in naïve ileum organoids at day 6 of culture ("n" indicates organoids derived from 4 mice each group). **(F)** Representative confocal images of whole mount tissues with co-immunofluorescence staining of UEA-1 and WGA in naïve ileum villi. Results were from at least two independent experiments and "n" refers to the number of mice unless indicated otherwise. All mice were at the age of 2∼3 months old when analyzed. Statistical analysis was done using Multiple *T*-test on Prism software. $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$.

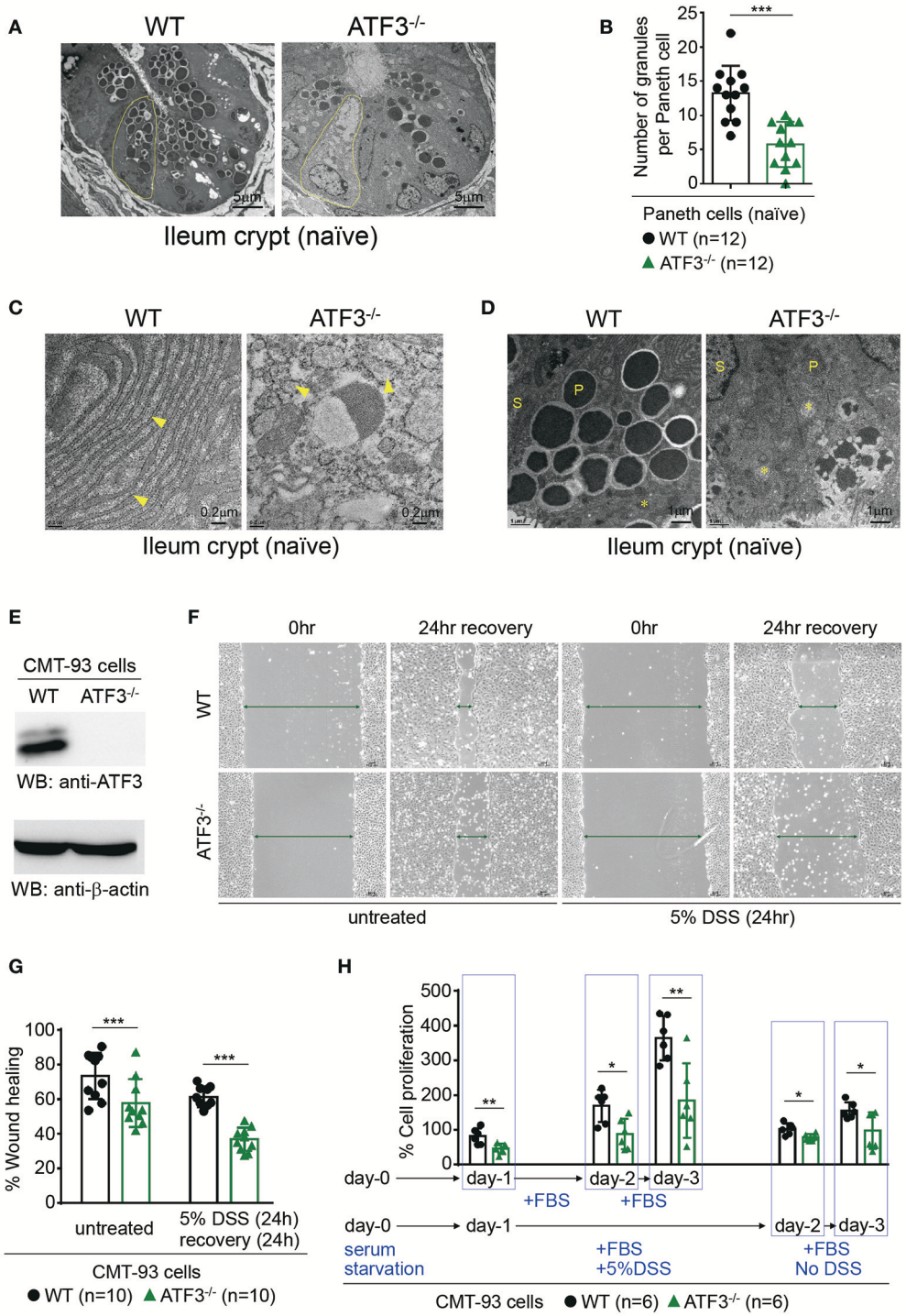

FIGURE 2 | ATF3 regulates Paneth cell homeostasis and promotes wound healing and proliferation of epithelial cells. **(A–D)** Transmission electron microscopy images of naïve ileum crypts showing the structural appearance of Paneth cells. **(A)** Degeneration of Paneth cells (outlined in yellow) and loss of their apical granules in ATF3$^{-/-}$ mice compared to wild-type mice. **(B)** Total number of granules in Paneth cells was counted manually in crypts ($n = 12$) imaged from 4 mice each group. **(C)** ER (indicated by arrowheads) fragmentation into cytoplasmic vesicles and (D) mitochondria (indicated by asterisks) swelling with damaged cristae in naïve ATF3$^{-/-}$ mice were shown. P= Paneth cells, S= Stem cells. **(E–H)** Assays of wound healing and proliferation in CMT93 cells and "n" refers to number of CMT93 samples. **(E)** Western blot analysis of ATF3 ablation in CMT93 cells. **(F,G)** Wound healing assay of CMT93 cells with or without DSS treatment for injury. Wound was introduced by scratch and % of wound closure was normalized and calculated after 24 h of recovery. **(H)** Cell proliferation assay of CMT93 cells. Cells were serum starved, then proliferation was compared with or without injury introduced by DSS treatment as indicated. Total cell proliferation was expressed as percentage relative to the number of cells seeded. Results were representative of two independent experiments. Statistical analysis was done using Multiple *T*-test on Prism software. $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$.

Epithelial ATF3 Is Protective During DSS-Induced Colitis

We next investigated whether disrupted Paneth/stem cell niche, due to loss of ATF3, will lead to clinical manifestation during intestinal stress such as inflammation. DSS-induced colitis was selected because it is well documented that DSS administration in mice causes epithelial barrier dysfunction which we believe ATF3 has a major regulatory role (46). Groups of wild-type and ATF3$^{-/-}$ mice were given two cycles of 2% DSS in drinking water (**Figure 3A**), and monitored daily for disease activity index (DAI), by measuring body weight, stool consistency, and rectal bleeding as previously described (29). While naïve ATF3$^{-/-}$ mice showed no signs of disease activity, notably, DSS-treated ATF3$^{-/-}$ mice developed more severe colitis with lethal disease activity leading to reduced survival rate and much shortened colon length compared to wild-type mice (**Figures 3B,C**, and **Supplementary Figures 3A,B**). Since increased shortening of the colon during DSS colitis suggests impaired epithelial regeneration, we sacrificed mice for further analysis at day-8 after the first DSS cycle when DAI was highest in mice. Consistent to clinical manifestation, we found colon crypts are less proliferative by Ki67 staining in ATF3$^{-/-}$ mice, which likely leads to decreased crypt numbers and increased colon tissue pathology (**Figure 3D**, and **Supplementary Figure 3C–E**). This indicates that wild-type mice can subdue DSS-induced inflammation by enhancing barrier repair through active epithelial cell proliferation, while ATF3$^{-/-}$ mice exhibit fatal damage of epithelial barrier in terms of loss of cellular proliferation accompanied by increased apoptosis, as evidenced by increased TUNEL^{+} cells (**Figure 3E**). Collectively, the observations that DSS treatment in ATF3$^{-/-}$ mice introduced more mucosal epithelial damage, extensive inflammation, loss of epithelial architecture, loss of crypts, and enhanced immune cell infiltrates, provide evidence that ATF3 is a pillar base for a balanced intestinal stress response to maintain mucosal integrity during colitis. Impaired AMP production and elevated levels of endoplasmic reticulum (ER) stress are believed to be prominently involved in the pathogenesis of IBD (41, 47). Notably, core intestinal AMPs, such as Reg3, defensin-β3, and the calcium binding proteins S100A8/A9, were markedly reduced in ATF3$^{-/-}$ mice (**Figure 3F**). Abnormally active ER machinery, as evidenced by increased mRNA levels of ER stress-related genes including GRP78, sXBP1 and CHOP, was also detected in the colons of ATF3$^{-/-}$ mice (**Figure 3G**). Taken together, we concluded ATF3$^{-/-}$ mice suffer lethal colitis due to impaired epithelial regeneration and immunity, as well as enhanced cellular stress.

To further strengthen our conclusion that epithelial ATF3 mediates protection during DSS colitis, we obtained ATF3 flox mice and generated epithelium-specific ATF3 conditional (Vil-Cre^{+}ATF3$^{F/F}$) knockout mice (28, 48). Similar to global ATF3$^{-/-}$ mice, at 3 months of age, naïve Vil-Cre^{+}ATF3$^{F/F}$ mice showed more shortened colon length and severe Paneth cell degeneration, compared to ATF3$^{F/F}$ littermate mice (**Figure 4A** and data not shown). We found epithelium-specific ATF3 conditional knockout mice also recapitulate most phenotypes of DSS colitis observed in global ATF3$^{-/-}$ mice, including increased disease activity index, more shortened colon length, reduced total crypt numbers and impaired epithelial regeneration (**Figures 4B–F**). Together, these results exclude the potential contribution of non-epithelial ATF3 from other cell types to colitis pathogenesis in our experimental model.

Rectal Organoid Transfer INTO DSS-Treated ATF3$^{-/-}$ Mice Ameliorates Colitis

To determine whether ATF3-mediated protection during colitis is intrinsic or cell-autonomous to epithelial cells, rectal organoid transplantation after DSS induction of colitis was performed as previously described (33). Colon organoids, derived from wild-type or ATF3$^{-/-}$ mice and cultured for 6 days, were used as donor cells (**Figure 5A**). Rectal transfer of donor organoids into ATF3$^{-/-}$ recipient mice was performed at day-6 and day-8 post DSS treatment. Notably, ATF3$^{-/-}$ recipient mice transplanted with wild-type, but not ATF3$^{-/-}$ donor organoids, showed ameliorated inflammatory conditions, including increased survival and decreased disease activity (**Figures 5B,C**). At the day-14 after two organoid transfers, we found that ATF3$^{-/-}$ mice receiving wild-type organoids recover from injury and improve colon integrity more rapidly, as evidenced by colon length and healthy appearance of both colon and cecum compared to those mice receiving ATF3$^{-/-}$ organoids (**Figure 5D**). Correlated to this, total colon crypt numbers in mice receiving wild-type organoids was significantly higher than those of mice with ATF3$^{-/-}$ organoid transfer (**Figure 5E**). Accordingly, colon pathology was decreased, likely due to improved tissue repair, in mice receiving wild-type organoids (**Figures 5F,G**). Together, these results indicate that restoring epithelial ATF3 signaling through wild-type organoid transplantation can effectively attenuate inflammatory conditions after DSS-induced colitis. These findings not only emphasize the protective role of intestinal epithelial barrier during colitis but also highlight the indispensable role for endogenous ATF3 in recovering and maintaining epithelial barrier functionality.

ATF3 Is a Downstream Target of Il-22 Signaling and Is Required for Il-22-Mediated AMP Production

In the intestine, IL-22 binds to IL-22 receptor (IL-22R) to transduce signaling exclusively in epithelial cells leading to STAT3 activation, epithelial proliferation and immunity (15). Direct stimulation of *ex vivo* cultured colon fragments with IL-22 induces AMP production that has been linked to host defense and protection of stem cell niche (11, 49). Nevertheless, the downstream IL-22-mediated genetic circuits are still largely unexplored. Although ATF3 is a stress-response molecule to extracellular stimuli such as DNA damage or Toll-like receptors (17), unexpectedly; we found ATF3 was upregulated in IL-22-stimulated ileum organoids or colon fragments (**Figures 6A–C**), indicating that ATF3 is a downstream target of IL-22 signaling.

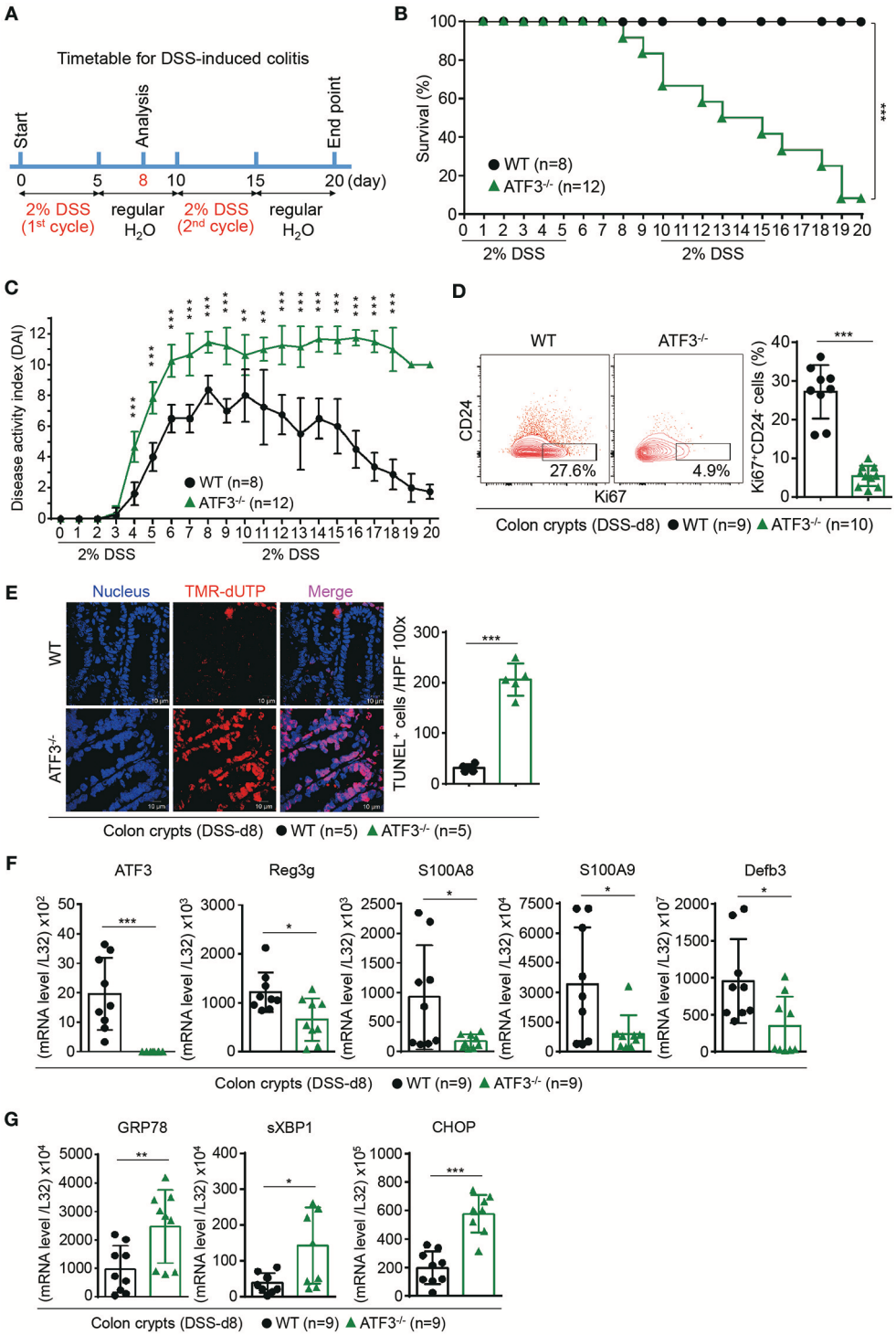

FIGURE 3 | ATF3 protects mice from DSS-induced colitis. **(A)** Experimental protocol of DSS-induced colitis was shown. **(B)** Survival rate in mice after DSS treatment. **(C)** Disease activity index (DAI), a composite measurement of weight loss percentage, stool consistency, and blood in stools, was indicated in each group of mice during DSS colitis. **(D–G)** Analysis of colitis severity at day-8 post DSS treatment. **(D)** Flow cytometry of $Ki67^{+}$ proliferating crypt cells in $CD24^{low/-}$ cell population. **(E)** TUNEL assay showing apoptotic cells in colon tissues. Magenta positive apoptotic cells were quantified per 100x high-power field (HPF) from 10 different views of colon section from each mouse. **(F–G)** Quantitative real-time PCR analysis of crypt cells at day-8 post DSS. **(F)** Expression of ATF3 and anti-microbial peptide-related genes. **(G)** Expression of ER stress-related genes. Results were from two independent experiments. "n" refers to the number of mice analyzed. Survival curve was calculated using the Kaplan–Meier method and statistical significance was calculated using Log rank (Mantel-Cox) test. Statistical analysis was done using Multiple *T*-test on Prism software. $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$.

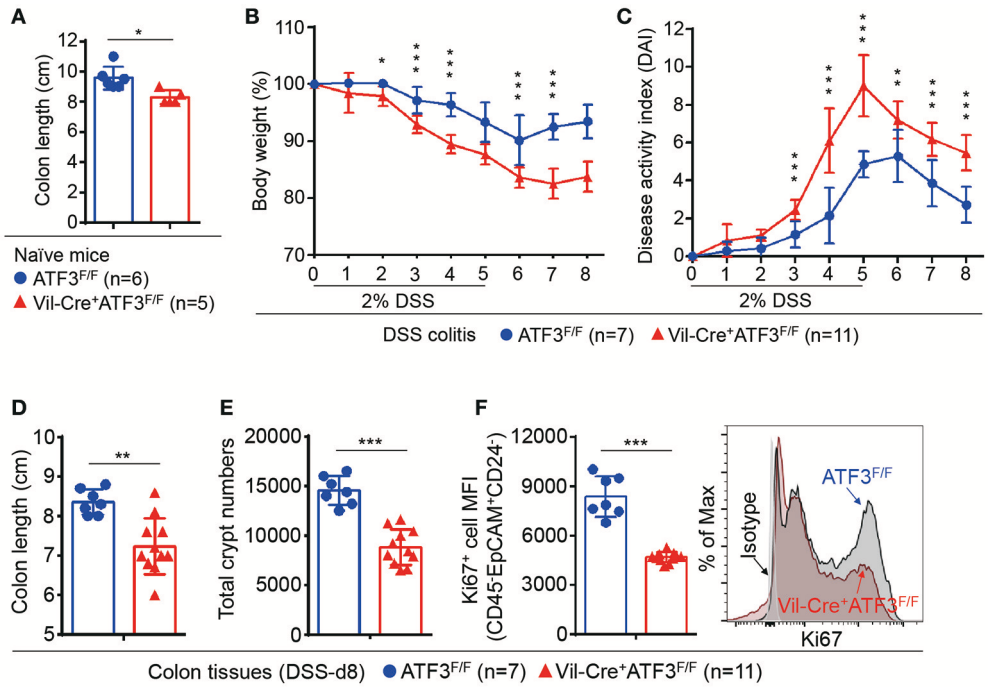

FIGURE 4 | Epithelial ATF3 is required for protection against DSS colitis. **(A)** Comparison of colon length between 3-month-old naïve mice as indicated. **(B-F)** Analysis of colitis severity during DSS treatment. **(B)** Percentage of body weight loss during DSS colitis. **(C)** Disease activity index (weight loss percentage, stool consistency, and blood in stools) was indicated in each group of mice during DSS colitis. **(D)** Colon length, **(E)** total colon crypt numbers, and **(F)** Ki67^{+} proliferating crypt cells by flow cytometry analysis, were measured at day-8 post DSS treatment. Results were from two independent experiments. "n" refers to the number of mice analyzed. Statistical analysis was done using Multiple *T*-test on Prism software. $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$.

This finding also pinpoints the unique role of ATF3 in epithelial cells since IL-22R signaling is exclusive to epithelium in the gut. To further investigate whether ATF3 is functionally involved in IL-22 signaling, we analyzed the IL-22-induced AMP production in colon fragments, freshly isolated from wild-type or global ATF3$^{-/-}$ mice (11). Notably, ATF3$^{-/-}$ colon fragments, compared to wild-type colon fragments, showed reduced AMP production (Reg3γ and S100A8) after IL-22 stimulation (**Figure 6D**). To address concerns that other immune cells in ATF3$^{-/-}$ colon fragments could also contribute to defective IL-22-induced AMP induction because of the use of global ATF3 knockout mice, we used colon fragments isolated from epithelium-specific ATF3 conditional knockout (Vil-Cre^{+}ATF3$^{F/F}$) mice or their littermates (ATF3$^{F/F}$) for AMP production analysis and confirmed the results (**Figure 6E**). Similar observation was also obtained using CMT93 colon epithelial cells (**Figure 6F**). The defects of IL-22-induced AMP production in Vil-Cre^{+}ATF3$^{F/F}$ colon fragments further support why Vil-Cre^{+}ATF3$^{F/F}$ mice were also more susceptible to DSS-induced colitis compared to ATF3$^{F/F}$ littermates (**Figure 4**). Taken together, we concluded that ATF3 is required to relay IL-22 signaling in epithelial cells for the induction of immunity and for epithelial regeneration as well, and that loss of ATF3 leads to compromised intestinal homeostasis and impaired recovery from mucosal damage due to lack of tissue-protective IL-22 signaling.

ATF3 Regulates Il-22-Induced STAT3 Phosphorylation via Targeting Phosphatases

In epithelial cells, STAT3 is the prime downstream target of IL-22 signaling and has been shown to be essential for intestinal organoid growth (7). IL-22 induces STAT3 phosphorylation on tyrosine-705, then subsequent dimerization and nuclear translocation of phosphorylated STAT3 initiate its transcriptional activity for programming sets of genes associated with AMP production, cell proliferation, tissue repair, and survival (8, 15). Mutations in STAT3 have been identified as susceptibility factors for IBD and loss of epithelial STAT3 rendered mice more susceptible to DSS colitis (9, 50, 51). Given that the expression of AMP such as Reg3β/γ is dependent on STAT3 signaling (9), we reasoned that ATF3-mediated AMP induction during IL-22 signaling might be through an action on STAT3 activation. Indeed, we found IL-22-induced STAT3 phosphorylation is dramatically abolished in the absence of ATF3, in freshly isolated ileum crypts, cultured ileum organoids, or CMT93 cells (**Figures 7A–C**). We further confirmed that IL-22-induced, ATF3-mediated STAT3 activation is epithelium-specific (**Figure 7D**). In addition, decreased IL-22 signaling was not due to downregulation of the IL-22 receptor complex, composed of IL-22R1 and IL-10R2 subunits (**Figures 7E,F**), in ATF3$^{-/-}$ epithelial cells, indicative of an intrinsic cellular defect.

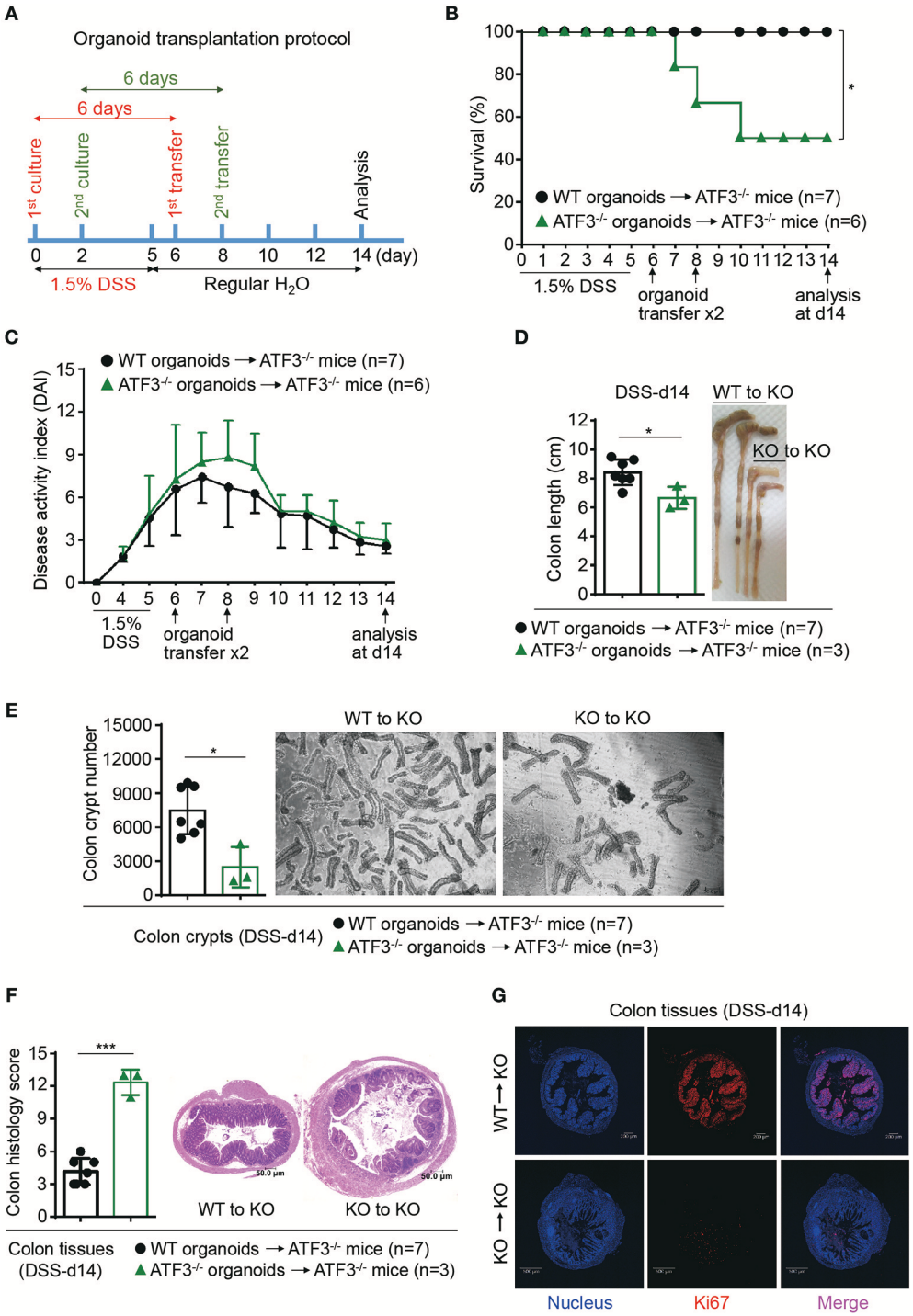

FIGURE 5 | Rectal transplantation of wild-type organoids ameliorates colitis in ATF3-deficient mice. **(A)** Experimental protocol of organoid transfer post DSS colitis was shown. **(B–G)** Analysis of DSS colitis severity after organoid transfer. **(B)** Survival rate of mice after organoid transfer. **(C)** Disease activity index was indicated in each group of mice after organoid transfer. **(D)** Colon length, **(E)** total colon crypt numbers, **(F)** colon tissue histology scores (images were taken and scored at magnification of 10x) based on hematoxylin and eosin (H and E) staining, and **(G)** confocal images of colon tissues immunostained with the Ki67 proliferation marker at day-14 post DSS treatment were shown. Results were from two independent experiments. "n" refers to the number of mice analyzed. Survival curve was calculated using the Kaplan–Meier method and statistical significance was calculated using Log rank (Mantel-Cox) test. Statistical analysis was done using Multiple *T*-test on Prism software. $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$.

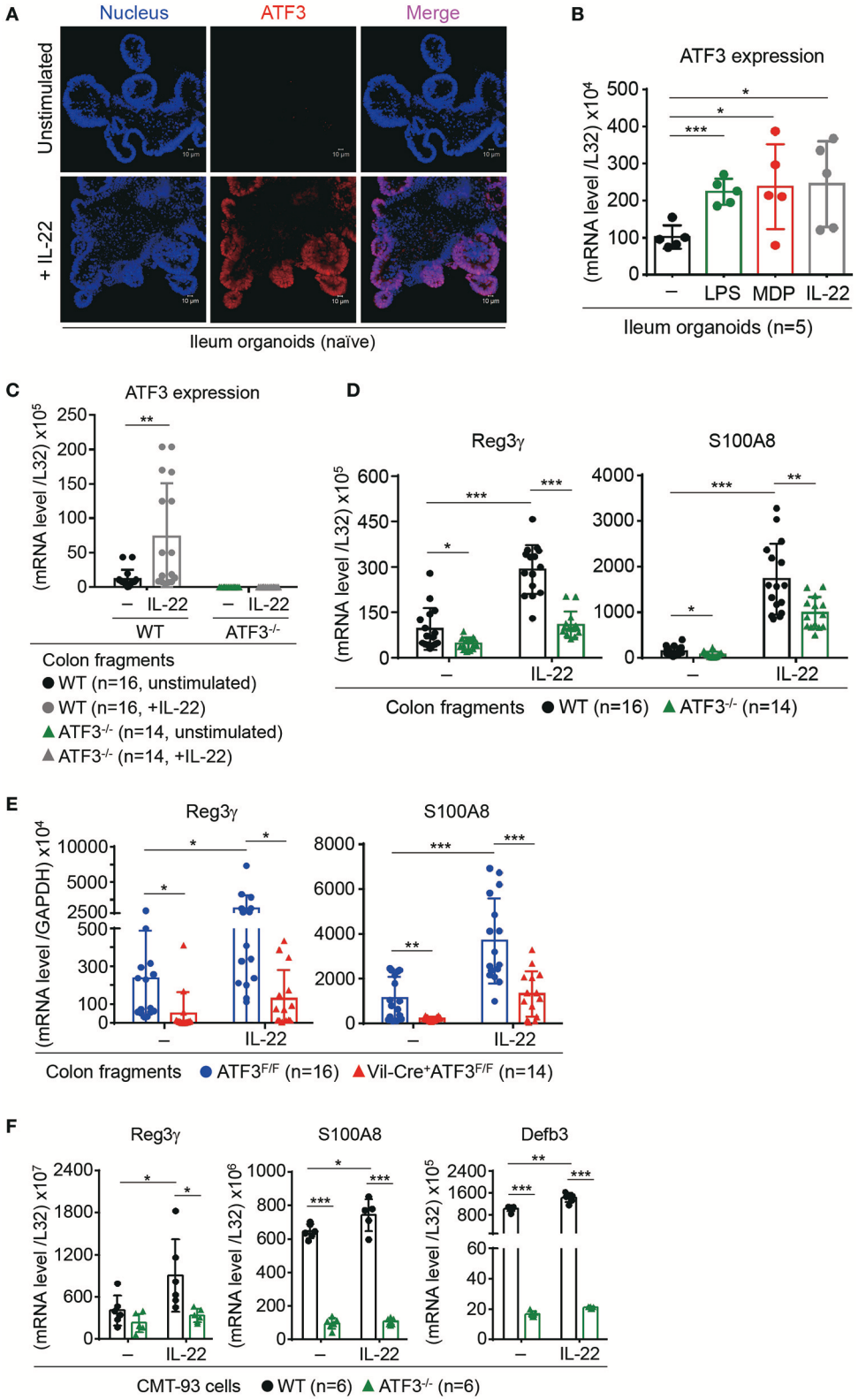

FIGURE 6 | ATF3 mediates IL-22-induced production of anti-microbial peptides in epithelial cells. **(A,B)** Induction of ATF3 by IL-22 in organoids. **(A)** Representative three-dimensional confocal images of ATF3 expression in ileum organoids at day 6 of culture with or without IL-22 stimulation for overnight. **(B)** Quantitative real-time

(Continued)

FIGURE 6 | PCR analysis of ATF3 mRNA levels in ileum organoids at day 6 of culture, stimulated with lipopolysaccharide (LPS), muramyl dipeptide (MDP), or IL-22. "n" indicates organoids derived from 5 wild-type mice. **(C–E)** Quantitative real-time PCR analysis of genes in IL-22-stimulated colon fragments. Pieces of colon fragments (∼0.5 cm) were cultured in complete DMEM with or without IL-22 for 5 h and mRNA levels of **(C)** ATF3, or **(D,E)** ant-microbial peptide Reg3γ and S100A8, were determined. "n" refers to the number of colon fragments obtained from 7 wild-type mice and 6 $ATF3^{-/-}$ mice **(C,D)**, or 8 $ATF3^{F/F}$ mice and 7 Vil-$Cre^{+}ATF3^{F/F}$ **(E)** mice. **(F)** Quantitative real-time PCR analysis of anti-microbial genes in IL-22-stimulated CMT93 cells. "n" refers to number of CMT93 samples analyzed. Results were from two independent experiments. Statistical analysis: Multiple *T*-test **B,C**, untreated control samples were used as the standard control for other stimulated samples), Two-way ANOVA test (**D–F**, for multiple comparison of samples). $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$.

We next sought to determine whether ATF3, as a transcription factor, can directly bind to the STAT3 promoter to regulate its expression or activation during IL-22 signaling. Biotinylated DNA probe containing ATF/CRE binding site within the STAT3 promoter was used for the electrophoretic mobility shift assay (EMSA) (**Supplementary Figure 4A**). Notably, we were not able to detect a shift of the DNA-protein complex consisting of nuclear ATF3 and the STAT3 promoter probe (**Supplementary Figure 4B**) (52), indicating that ATF3 does not target STAT3 directly during IL-22 signaling in epithelial cells. We then explored the possibility whether ATF3 targets negative regulators of STAT3, such as protein tyrosine phosphatases (PTPs), to promote STAT3 activation (53). We tested several PTPs and identified two PTPs, SHP2 and PTP-Meg2, both known to dephosphorylate STAT3 (54, 55). Notably, loss of ATF3 in CMT93 cells led to increased levels of SHP2 and PTP-Meg2 at the steady state and much higher levels after IL-22 stimulation (**Figure 7G**), suggesting ATF3 negatively regulates these PTPs. To provide evidence that PTPs are upstream regulators of STAT3 phosphorylation during IL-22-ATF3 signaling, SHP2 was knockdown ($SHP2^{KD}$) by shRNA in $ATF3^{-/-}$ cells (namely $ATF3^{-/-}SHP2^{KD}$). Notably, we found that impaired STAT3 phosphorylation in $ATF3^{-/-}$ cells was comparably restored in $ATF3^{-/-}SHP2^{KD}$ cells (**Figure 7H**), indicating that SHP2 is acting upstream of STAT3 as an inhibitor via de-phosphorylation in gut epithelial cells (53). Taken together, we concluded that ATF3 functions as a repressor to inhibit PTPs such that those PTPs are not functionally targeting STAT3 for suppression.

ATF3 Targets Intestinal Th17 Cell Functionality via Il-6-PSTAT3 Signaling

Because IL-6 could be produced by epithelial cells and it is also a strong STAT3 inducer, particularly in the context of inflammation (56, 57), we first examined whether IL-6 activates STAT3 in freshly isolated epithelial cells. Consistent to a study showing that loss of IL-6 did not affect epithelial STAT3 phosphorylation (9), we found IL-6 does not activate STAT3 in gut epithelial cells (**Figure 8A**). Intriguingly, IL-6 induces STAT3 activation in $CD45^{+}$ mononuclear cells and we found ATF3 also positively regulates IL-6-induced STAT3 activation in $CD45^{+}$ cells (**Figures 8A,B**). A recent study reported that in the gut, the dual-specificity phosphatase 2 (DUSP2) targets and catalyzes STAT3 de-phosphorylation leading to impaired Th17 cell development (58). Therefore, given that IL-6 drives Th17 cell differentiation whose regulation is tightly linked to IBD pathogenesis (1, 59), we next investigated whether loss of ATF3 in mice affects intestinal Th17 cell development. Notably, while mRNA and protein levels of both IL-22 and IL-17A were not much changed at the steady state, the capability of lamina propria Th17 cells to produce IL-22 or IL-17A, after ionomycin and PMA stimulation, was significantly compromised in $ATF3^{-/-}$ cells (**Figures 8C,D**). In addition, we found in $ATF3^{-/-}$ mice that mRNA levels of IL-6, IL-6R1, and gp130 (the signaling subunit of the IL-6 receptor complex) were not altered in various intestinal tissues (**Supplementary Figure 5**), excluding the possibility that compromised Th17 accumulation in the gut was due to decreased IL-6 or IL-6 receptor complex. Because increased expression of IL-6 and IL-22 are associated with IBD in patients (60, 61), our findings here pinpoint a cross-regulation of ATF3 for intestinal immunity maintenance, between IL-22-induced STAT3 activation in gut epithelial cells and IL-6-induced STAT3 activation in $CD45^{+}$ cells especially Th17 cells (summarized in **Figure 8E**).

DISCUSSION

In light of a recent microarray analysis showing up-regulation of ATF3 in patients with active IBD (21), while lacking evidence supporting a role for ATF3 in intestinal homeostasis and IBD pathogenesis, we have performed in-depth analyses here, using primary intestinal organoids and animal models, to reveal how ATF3 is acting as a critical downstream regulator of the IL-22 signaling cascade in intestinal epithelial cells. We further identified a novel IL-22-mediated circuit in epithelial cells where ATF3 relays IL-22 signaling to inhibit PTPs to prevent them from inactivating STAT3 phosphorylation. Given that IL-22 itself could also induce ATF3, our results therefore illustrate a delicate mechanism where ATF3 facilitates functionality and amplification of IL-22 signaling. In addition, we provide evidence that ATF3 influences IL-6-mediated STAT3 activation which might be involved in intestinal Th17 accumulation, survival or development. As both IL-6 and IL-22 controls STAT3 activation which is associated with cell homeostasis, host defense, inflammation and tumorigenesis (56), our findings of ATF3-mediated cross-regulation between lymphoid cells and epithelial cells in the intestine support ATF3 as a novel and critical gatekeeper for intestinal immunity.

The intestinal barrier is composed of a versatile and dynamic layer of epithelial cells which is, to a great extent, maintained by a niche between intestinal stem cells and Paneth cells located at the crypt base (45, 62). Intriguingly, loss of ATF3 disrupts this niche leading to decreased $Ki67^{+}$ proliferating transit-amplifying (TA) cells and total crypt numbers (i.e., regeneration capability) at the steady state or during colitis. The disrupted Paneth/stem

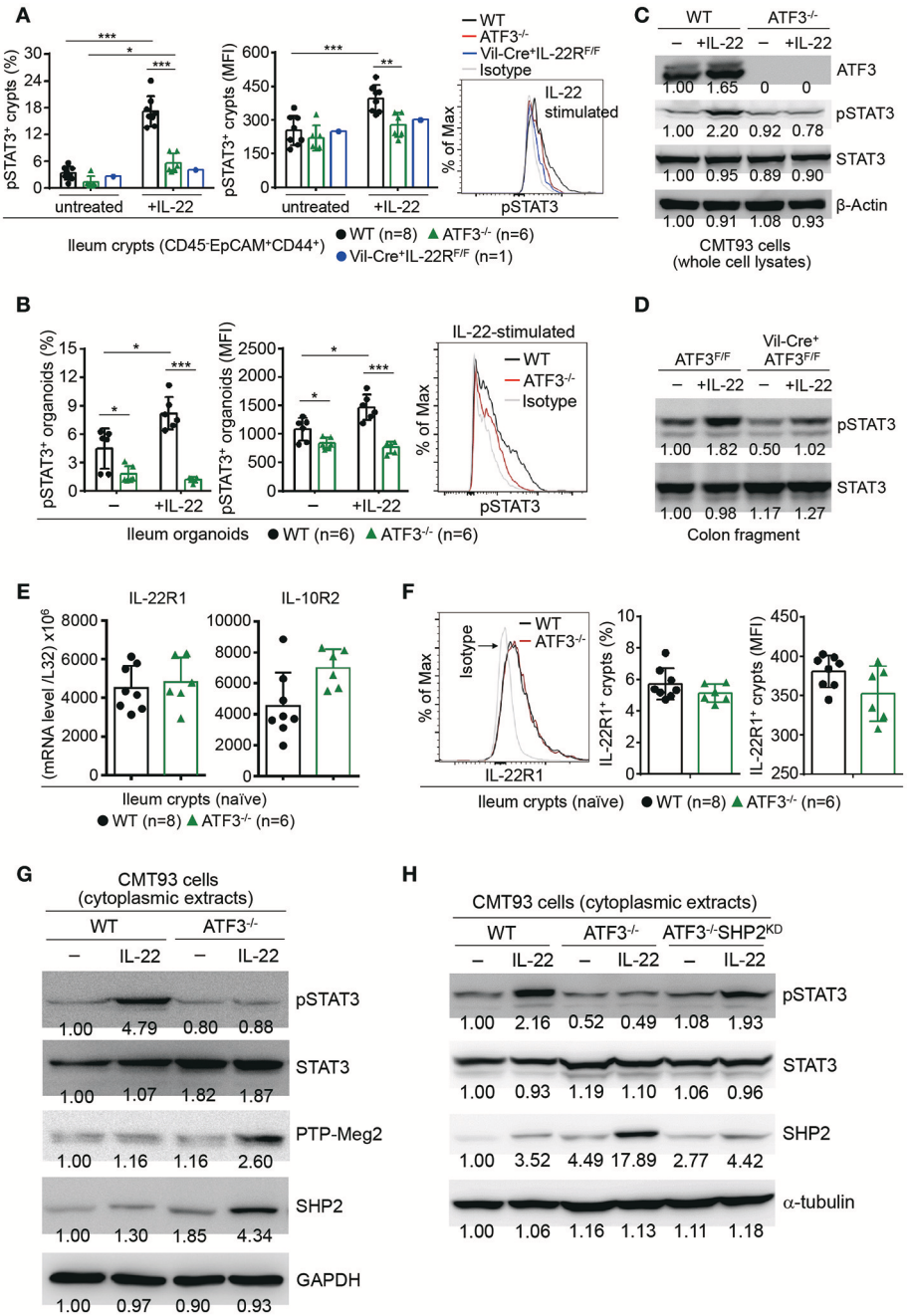

FIGURE 7 | ATF3 promotes IL-22-induced STAT3 phosphorylation by suppressing phosphatases. **(A)** Freshly isolated ileum crypts, or **(B)** ileum organoids at day 6 of culture, were stimulated with IL-22, followed by fixation and intracellular staining of phospho-STAT3, and analyzed by flow cytometry. Western blot analysis of **(C)** IL-22-stimulated CMT93 cells, or **(D)** IL-22-stimuated colon fragments isolated from the indicated mice, for the expression of the indicated proteins. **(E)** Quantitative real-time PCR analysis of IL-22R1 and IL-10R2 mRNA levels in freshly isolated ileum crypts from mice. **(F)** Flow cytometry analysis of IL-22R1 in freshly isolated ileum crypt cells gated on the $CD45^{-}EpCAM^{+}$ population. **(G,H)** Western blot analysis of unstimulated or IL-22-stimulated CMT93 cells for the indicated proteins. $ATF3^{-/-}$ CMT93 cells with SHP2 knockdown ($ATF3^{-/-}SHP2^{KD}$) were indicated. Images were representative of four independent experiments **(G–H)**. Results were from two independent experiments **(A–F)**. "n" refers to the number of mice analyzed **(A,B,E,F)**. Statistical analysis was done by multiple comparison in Two-way ANOVA test using Prism software. $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$.

cell homeostasis is likely due to compromised IL-22-induced STAT3 activation in $ATF3^{-/-}$ stem cells, as is also supported by a recent study showing IL-22 activates STAT3 signaling in intestinal organoids and promotes stem cell regeneration (7). Notably, while IL-22 does not enhance STAT3 phosphorylation in Paneth cells (7), we found that ATF3 deficiency *in vivo*

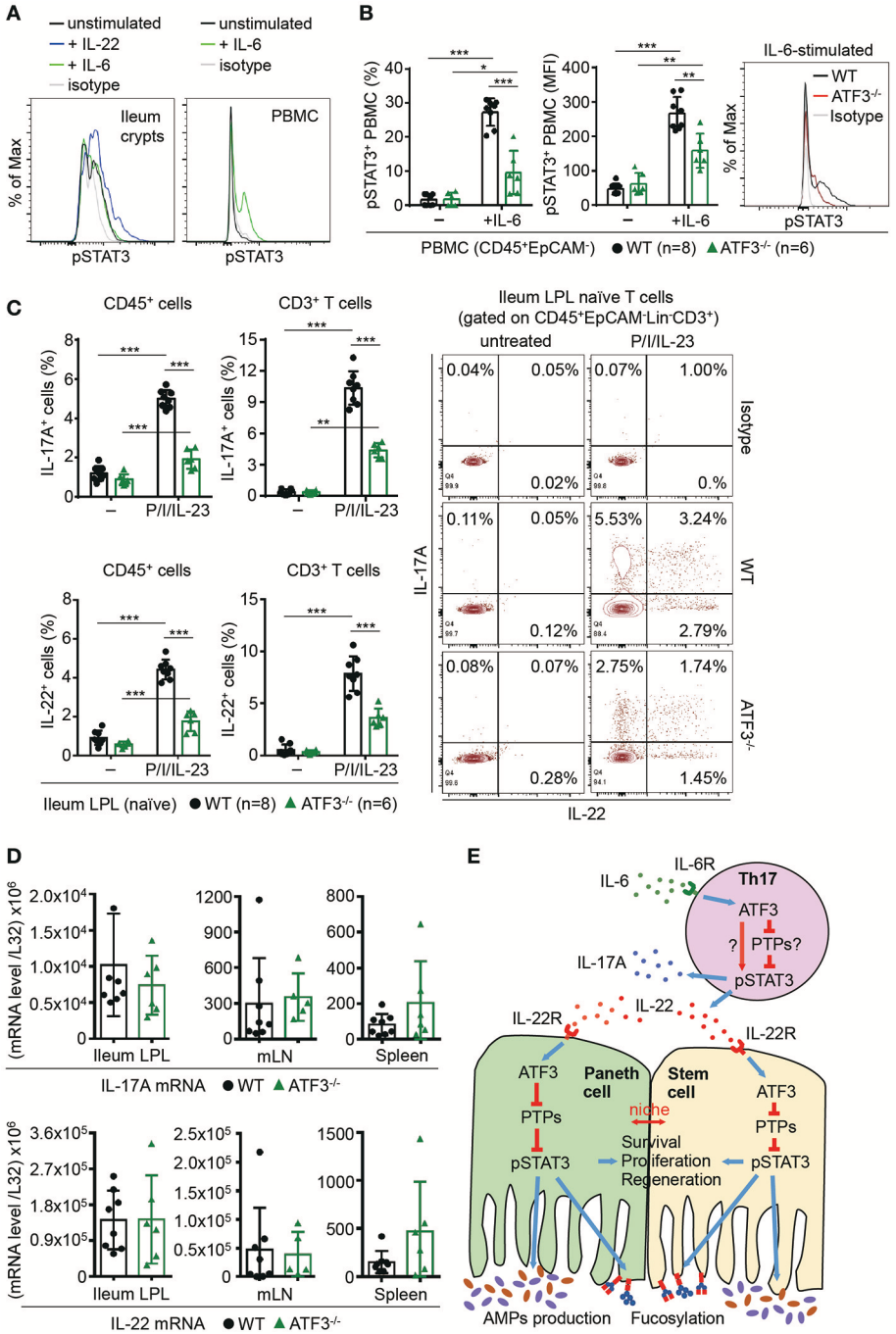

FIGURE 8 | ATF3 regulates IL-6-pSTAT3 signaling in intestinal Th17 cells. Flow cytometry analysis of phospho-STAT3 in **(A)** IL-6 or IL-22 stimulated freshly isolated ileum crypts or IL-6-stimulated peripheral blood mononuclear cell (PBMC) from wild-type mice, or in **(B)** IL-6-stimulated PBMC from wild-type and ATF3-deficient mice. **(C)** Flow cytometry analysis of intracellular IL-17A and IL-22 expression in naïve lamina propria T cells from the indicated mice. Cells were treated with PMA, ionomycin and IL-23 in the presence of BFA for 4 h before analysis and gated on live $CD45^+EpCAM^-Lin^-CD3^+$ population as shown. **(D)** Quantitative real-time PCR analysis of IL-17A and IL-22 mRNA levels in freshly isolated lamina propria (LPL) cells, mesenteric lymph nodes (mLN), or splenocytes. **(E)** Model of ATF3-mediated mucosal immunity via cross-regulation between IL-22-pSTAT3 signaling in epithelium (associated with AMP production and epithelial fucosylation) and IL-6-pSTAT3 signaling in Th17 cells (associated with signature IL-17A and IL-22 production). "n" refers to the number of mice analyzed. Statistical analysis was done by multiple comparison in Two-way ANOVA test using Prism software. $^*P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$.

leads to Paneth cell degeneration featured by loss of AMP-producing granules. Although genetic depletion of Paneth cells *in vivo* results in the concomitant loss of $Lgr5^+$ stem cells (45), we reason that ATF3 is primarily targeting stem cell homeostasis and regeneration, but not Paneth cells in the niche, as organoid transplantation effectively ameliorates colitis and restores total

crypt numbers in DSS-treated ATF3$^{-/-}$ mice. Thus, at the cellular level, ATF3-regulated epithelial niche homeostasis (but not ATF3-regulated Th17 cell functionality, see below) primarily determines intestinal immunity and susceptibility to colitis, while at the molecular level, ATF3-regulated IL-22-STAT3 activation determines full functionality of intestinal stem cells.

Clinical relevance of IL-22 and IL-6 signaling to IBD pathogenesis has been established (1, 10, 26, 61). STAT3 activation could be induced by IL-6R, IL-23R, or IL-22R signaling in Th17 cells (for IL-6R, IL-23R) and epithelial cells (for IL-22R) (1, 9, 56, 63). Components within these three signaling pathways are mostly overlapping and associated with IBD signaling modules by genome-wide association studies (1, 51). While we revealed a role for ATF3 in the IBD gene network of IL-22-STAT3, a function for ATF3 in IL-6-STAT3 or even IL-23-STAT3 activation in Th17 cell network has also been observed in our study. As an upstream regulator, ATF3 could suppress IL-6 transcription or even IL-22 transcription via NF-kB (18, 64). However, as a downstream regulator, we showed ATF3 is required to relay IL-6 and IL-22 signaling for the induction of STAT3 phosphorylation. Intriguingly, loss of ATF3 *in vivo* appears to compromise epithelial function via IL-22-ATF3-STAT3 and impair Th17 function via IL-6-ATF3-STAT3, without affecting overall levels of IL-6 and IL-22 in the gut. Thus, ATF3 seems to play a more dominant role in the downstream of IL-22 signaling circuit *in vivo*, compared to a role in IL-6 signaling, as global ATF3$^{-/-}$ mice were more susceptible to DSS colitis and Citrobacter infection. In light of this cross-regulation by ATF3 in different cell types, more dedicated genetic studies remain needed to untangle complicated ATF3-mediated function in intestinal cell activation and disease pathogenesis.

Toll-like receptor 4 (TLR4) signaling by LPS in macrophages induces ATF3-mediated IL-6 suppression via ATF3 binding to the ATF/CRE site of STAT3 promoter in the IL-6 gene (18). Binding of ATF3 to the STAT3 promoter for gene inactivation has also been shown in human hepatocellular carcinoma (25). However, we found ATF3 itself does not bind to the STAT3 promoter directly, at least in CMT93 epithelial cells, excluding the possibility of direct suppression of STAT3 by ATF3. Protein tyrosine phosphatases (PTPs) are known negative regulators of STAT3 signaling (53). A recent study showed phosphatase DUSP2 (PAC1) interacts with STAT3 and catalyzes de-phosphorylation of STAT3 (58). During our screening of PTPs for potential ATF3 targets, we found loss of ATF3 in CMT93 cells does not affect DUSP2 levels (data not shown), while levels of PTP-SHP2 and PTP-Meg2 are increased. Although restoration of STAT3 phosphorylation in ATF3$^{-/-}$SHP2^{KD} cells confirms IL-22-induced, ATF3-mediated SHP2 is acting upstream of STAT3 in epithelial cells, the same signaling cascade (i.e., IL-6-ATF3-PTP-STAT3) was not validated in IL-6-induced, ATF3-mediated STAT3 activation in T cells. Whether ATF3 targets STAT3 directly, or indirectly via PTPs in Th17 cells, needs further investigation, as requirement for transcriptional regulation by ATF3 is cell type-dependent. Collectively, our study here revealed unique and protective roles of ATF3 in intestinal immunity, where ATF3 links IL-22 signaling to STAT3 activation via targeting PTPs for epithelial immunity, while ATF3 links IL-6 signaling to STAT3 activation for Th17-mediated immunity.

ETHICS STATEMENT

John T. Kung, Ph.D., IACUC Chair, The Institutional Animal Care and Use Committee of Academia Sinica All animal studies described in the manuscript were approved and adhered to the guidelines and policies of the Institutional Animal Care and Use Committee of Academia Sinica (AS IACUC). *In vivo* animal studies were designed and performed based on the 3R (to replace, to reduce, to refine) principles and animal rights and welfare defined by the IACUC of Academia Sinica.

For experimental ethics, the authors claimed all data reported in the manuscript has no research misconduct, including fabricating or falsifying the results, plagiarism, or data duplication. We agree all original results described in the manuscript, once published, would be open for data sharing to the public. Conduct of research is in full compliance to the guidelines and policies of the Institution of Biomedical Sciences, Academia Sinica in Taiwan.

AUTHOR CONTRIBUTIONS

DG designed, performed the experiments, analyzed the data, and wrote the manuscript. JS, M-CL, C-FC, and J-WS helped in generating ideas, designing the experiments, and troubleshooting for this study. H-HL and H-YC helped in designing and performing critical experiments for revised manuscript. Y-CL helped mouse husbandry and genotyping. J-WS wrote the manuscript and did the critical revision of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02522/full#supplementary-material

Supplementary Figure 1 | ATF3 regulates intestinal homeostasis. **(A)** Isolation of ileum villi by scraping and crypts by fragment shaking in 30 mM EDTA for 30 min. **(B)** Quantitative real-time PCR analysis of ATF3 mRNA levels in freshly isolated tissues from different intestinal compartments and abdominal organs. **(C)** Comparison of body weight of the indicated mice at the age of 2∼3 months old. **(D)** Flow cytometry analysis of Ki67 and CD24 expression in colon crypts, gated

on the $CD45^{-}EpCAM^{+}$ populations, from the indicated naive mice. **(E)** H&E sections of naïve colon tissues showing healthy colon structure in WT mice while increased cell infiltration in ATF3-deficient colon. **(F)** Quantitative real-time PCR analysis of Fut2 mRNA levels in naïve ileum fragments ("n" refers to the number of fragments obtained from 7 wild-type mice and 7 $ATF3^{-/-}$ mice). Results were from two independent experiments and "n" refers to the number of mice **(B–D)**, unless indicated otherwise. Statistical analysis was done using Multiple *T*-test on Prism software. $*P < 0.05$, $**P < 0.005$, $***P < 0.0005$.

Supplementary Figure 2 | $ATF3^{-/-}$ mice were more susceptible to Citrobacter infection. Groups of mice were infected with a single dose (8×10^8 CFU) of Citrobacter rodentium by oral gavage. **(A)** Fecal colony-forming unit (CFU) was measured and compared at the indicated days post Citrobacter infection. **(B)** Colonoscopy view showing ulceration/bleeding in the colon of $ATF3^{-/-}$ mice at day 7 (Citro-d7) post infection. **(C)** Colon CFU and **(D)** colon length at day 12 post infection were measured and compared. Results were representative of two independent experiments. "n" refers to the number of mice used for analysis. Statistical analysis was done using Multiple *T*-test on Prism software. $*P < 0.05$, $**P < 0.005$.

Supplementary Figure 3 | $ATF3^{-/-}$ mice were more susceptible to DSS colitis. Analysis of colitis severity during DSS treatment. **(A)** Percentage of body weight loss during DSS colitis. **(B)** Colon length, **(C)** total colon crypt numbers, **(D)** colon tissue histology scores based on hematoxylin and eosin (H and E) staining, and **(E)** colonoscopic appearance were analyzed at the indicated day post DSS treatment. Results shown were from two independent experiments and "n" refers to the number of mice used for analysis. Statistical analysis was done using Multiple *T*-test on Prism software. $*P < 0.05$, $**P < 0.005$, $***P < 0.0005$.

Supplementary Figure 4 | ATF3 does not target the STAT3 promoter during IL-22 signaling in CMT93 epithelial cells. **(A)** Sequence of the mouse STAT3 promoter. Oligonucleotide probe (underlined), containing ATF/CRE binding site (shown in red) and STAT-binding element (SBE, shown in green) in the STAT3 promoter, was used for EMSA experiment. CTG (indicated in purple) is the transcriptional initiation site. GC box (shown in blue) is indicated. **(B)** EMSA assay, control system: Lane #1, only biotin-labeled 60 bp duplex bearing the EBNA-1 binding sequence showing only free DNA. Lane #2, biotin-labeled 60 bp duplex bearing the EBNA-1 binding sequence and EBNA extract showing DNA-protein complex shift. In assay with CMT93 cells, EMSA was performed with biotinylated STAT3 promoter probe and nuclear extracts prepared from WT or $ATF3^{-/-}$ CMT93 cells with or without IL-22 stimulation (50 ng/ml, 10 min after 5 h of serum starvation). EBNA: Epstein-Barr Nuclear Antigen. Results shown were representative of two independent experiments.

Supplementary Figure 5 | ATF3 deficiency in mice does not affect mRNA levels of IL-6, IL-6R1 and gp130 in intestinal compartments. Quantitative real-time PCR analysis of **(A)** IL-6, **(B)** IL-6R1, and **(C)** gp130 mRNA levels in freshly isolated tissues from different intestinal compartments and abdominal organs. Samples of mesenteric lymph nodes (mLN) and spleen were used for comparison. Results shown were combined from two independent experiments and "n" refers to the number of mice used for analysis. No statistical difference between wild-type and $ATF3^{-/-}$ mice was detected.

REFERENCES

1. Khor B, Gardet A, Xavier RJ. Genetics and pathogenesis of inflammatory bowel disease. *Nature* (2011) 474:307–17. doi: 10.1038/nature1020
2. Neurath MF. Cytokines in inflammatory bowel disease. *Nat Rev Immunol.* (2014) 14:329–42. doi: 10.1038/nri3661
3. Okumura R, Takeda K. Maintenance of intestinal homeostasis by mucosal barriers. *Inflamm Regen.* (2018) 38:5. doi: 10.1186/s41232-018-0063-z
4. Peterson LW, Artis D. Intestinal epithelial cells:regulators of barrier function and immune homeostasis. *Nat Rev Immunol.* (2014) 14:141–53. doi: 10.1038/nri3608
5. Jostins L, Ripke S, Weersma RK, Duerr RH, McGovern DP, Hui KY, et al. Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. *Nature* (2012) 491:119–24. doi: 10.1038/nature1158
6. Dudakov JA, Hanash AM, van den Brink MR. Interleukin-22:immunobiology and pathology. *Annu Rev Immunol.* (2015) 33:747–85. doi: 10.1146/annurev-immunol-032414-112123
7. Lindemans CA, Calafiore M, Mertelsmann AM, O'Connor MH, Dudakov JA, Jenq RR, et al. Interleukin-22 promotes intestinal-stem-cell-mediated epithelial regeneration. *Nature* (2015) 528:560–4. doi: 10.1038/nature16460
8. Mizoguchi A. Healing of intestinal inflammation by IL-22. *Inflammatory Bowel Dis.* (2012) 18:1777–84. doi: 10.1002/ibd.22929
9. Pickert G, Neufert C, Leppkes M, Zheng Y, Wittkopf N, Warntjen M, et al. STAT3 links IL-22 signaling in intestinal epithelial cells to mucosal wound healing. *J Exp Med.* (2009) 206:1465–72. doi: 10.1084/jem.20082683
10. Mizoguchi A, Yano A, Himuro H, Ezaki Y, Sadanaga T, Mizoguchi E. Clinical importance of IL-22 cascade in IBD. *J Gastroenterol.* (2018) 53:465–74. doi: 10.1007/s00535-017-140
11. Zheng Y, Valdez PA, Danilenko DM, Hu Y, Sa SM, Gong Q, et al. Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. *Nat Med.* (2008) 14:282–9. doi: 10.1038/nm1720
12. Hasnain SZ, Borg DJ, Harcourt BE, Tong H, Sheng YH, Ng CP, et al. Glycemic control in diabetes is restored by therapeutic manipulation of cytokines that regulate beta cell stress. *Nat Med.* (2014) 20:1417–26. doi: 10.1038/nm.3705
13. Grivennikov SI, Wang K, Mucida D, Stewart CA, Schnabl B, Jauch D, et al. Adenoma-linked barrier defects and microbial products drive IL-23/IL-17-mediated tumour growth. *Nature* (2012) 491:254–8. doi: 10.1038/nature11465
14. Suh JS, Cho SH, Chung JH, Moon A, Park YK, Cho BS. A polymorphism of interleukin-22 receptor alpha-1 is associated with the development of childhood IgA nephropathy. *J Interferon cytokine Res.* (2013) 33:571–7. doi: 10.1089/jir.2012.0097
15. Li LJ, Gong C, Zhao MH, Feng BS. Role of interleukin-22 in inflammatory bowel disease. *World J Gastroenterol.* (2014) 20:18177–88. doi: 10.3748/wjg.v20.i48.18177
16. Pelczar P, Witkowski M, Perez LG, Kempski J, Hammel AG, Brockmann L, et al. A pathogenic role for T cell-derived IL-22BP in inflammatory bowel disease. *Science* (2016) 354:358–62. doi: 10.1126/science.aah5903
17. Hai T, Wolford CC, Chang YS. ATF3, a hub of the cellular adaptive-response network, in the pathogenesis of diseases:is modulation of inflammation a unifying component? *Gene Expr.* (2010) 15:1–11. doi: 10.3727/105221610X12819686555015
18. Gilchrist M, Thorsson V, Li B, Rust AG, Korb M, Roach JC, et al. Systems biology approaches identify ATF3 as a negative regulator of Toll-like receptor 4. *Nature* (2006) 441:173–8. doi: 10.1038/nature04768
19. Zhou J, Edgar BA, Boutros M. ATF3 acts as a rheostat to control JNK signalling during intestinal regeneration. *Nat Commun.* (2017) 8:14289. doi: 10.1038/ncomms14289
20. Park SH, Do KH, Choi HJ, Kim J, Kim KH, Park J, et al. Novel regulatory action of ribosomal inactivation on epithelial Nod2-linked proinflammatory signals in two convergent ATF3-associated pathways. *J Immunol.* (2013) 191:5170–81. doi: 10.4049/jimmunol.1301145
21. Pierdomenico M, Stronati L, Costanzo M, Vitali R, Di Nardo G, Nuti F, et al. New insights into the pathogenesis of inflammatory bowel disease:transcription factors analysis in bioptic tissues from pediatric patients. *J Pediatr Gastroenterol Nutr.* (2011) 52:271–9. doi: 10.1097/MPG.0b013e3182034d08
22. Gu L, Ge Z, Wang Y, Shen M, Zhao P. Activating transcription factor 3 promotes intestinal epithelial cell apoptosis in Crohn's disease. *Pathol Res Pract.* (2018) 214:862–70. doi: 10.1016/j.prp.2018.04.013
23. Yang H, Park SH, Choi HJ, Moon Y. Epithelial cell survival by activating transcription factor 3 (ATF3) in response to chemical ribosome-inactivating stress. *Biochem pharmacol.* (2009) 77:1105–15. doi: 10.1016/j.bcp.2008.11.028
24. Hao Z-F, Ao J-H, Zhang J, Su Y-M, Yang R-Y. ATF3 Activates Stat3 phosphorylation through Inhibition of p53 expression in

skin cancer cells. *Asian Pacific J Cancer Prev.* (2013) 14:7439–44. doi: 10.7314/apjcp.2013.14.12.7439
25. Lin L, Yao Z, Bhuvaneshwar K, Gusev Y, Kallakury B, Yang S, et al. Transcriptional regulation of STAT3 by SPTBN1 and SMAD3 in HCC through cAMP-response element-binding proteins ATF3 and CREB2. *Carcinogenesis* (2014) 35:2393–403. doi: 10.1093/carcin/bgu163
26. Sekikawa A, Fukui H, Suzuki K, Karibe T, Fujii S, Ichikawa K, et al. Involvement of the IL-22/REG Ialpha axis in ulcerative colitis. *Lab Invest.* (2010) 90:496–505. doi: 10.1038/labinvest.2009.147
27. Lai PF, Cheng CF, Lin H, Tseng TL, Chen HH, Chen SH. ATF3 Protects against LPS-induced inflammation in mice via inhibiting HMGB1 expression. *Evid Based Comple Alternat Med.* (2013) 2013:716481. doi: 10.1155/2013/716481
28. Kalfon R, Koren L, Aviram S, Schwartz O, Hai T, Aronheim A. ATF3 expression in cardiomyocytes preserves homeostasis in the heart and controls peripheral glucose tolerance. *Cardiovasc Res.* (2017) 113:134–46. doi: 10.1093/cvr/cvw228
29. Zaki MH, Boyd KL, Vogel P, Kastan MB, Lamkanfi M, Kanneganti TD. The NLRP3 inflammasome protects against loss of epithelial integrity and mortality during experimental colitis. *Immunity* (2010) 32:379–91. doi: 10.1016/j.immuni.2010.03.003
30. Erben U, Loddenkemper C, Doerfel K, Spieckermann S, Haller D, Heimesaat MM, et al. A guide to histomorphological evaluation of intestinal inflammation in mouse models. *Int J Clin Exp Pathol.* (2014) 7:4557–76.
31. Gracz AD, Fuller MK, Wang F, Li L, Stelzner M, Dunn JC, et al. Brief report:CD24 and CD44 mark human intestinal epithelial cell populations with characteristics of active and facultative stem cells. *Stem Cells* (2013) 31:2024–30. doi: 10.1002/stem.1391
32. Sato T, Stange DE, Ferrante M, Vries RG, Van Es JH, Van den Brink S, et al. Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. *Gastroenterology* (2011) 141:1762–72. doi: 10.1053/j.gastro.2011.07.050
33. Yui S, Nakamura T, Sato T, Nemoto Y, Mizutani T, Zheng X, et al. Functional engraftment of colon epithelium expanded *in vitro* from a single adult Lgr5(+) stem cell. *Nat Med.* (2012) 18:618–23. doi: 10.1038/nm.2695
34. Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F. Genome engineering using the CRISPR-Cas9 system. *Nat Protoco.* (2013) 8:2281–308. doi: 10.1038/nprot.2013.143
35. Moro K, Ealey KN, Kabata H, Koyasu S. Isolation and analysis of group 2 innate lymphoid cells in mice. *Nat Proto.* (2015) 10:792–806. doi: 10.1038/nprot.2015.047
36. Holden NS, Tacon CE. Principles and problems of the electrophoretic mobility shift assay. *J Pharmacol Toxicol Methods* (2011) 63:7–14. doi: 10.1016/j.vascn.2010.03.002
37. Zachos NC, Kovbasnjuk O, Foulke-Abel J, In J, Blutt SE, de Jonge HR, et al. Human enteroids/colonoids and intestinal organoids functionally recapitulate normal intestinal physiology and pathophysiology. *J Biol Chem.* (2016) 291:3759–66. doi: 10.1074/jbc.R114.635995
38. Pham TA, Clare S, Goulding D, Arasteh JM, Stares MD, Browne HP, et al. Epithelial IL-22RA1-mediated fucosylation promotes intestinal colonization resistance to an opportunistic pathogen. *Cell Host Microbe* (2014) 16:504–16. doi: 10.1016/j.chom.2014.08.017
39. Ramanan D, Cadwell K. Intrinsic defense mechanisms of the intestinal epithelium. *Cell Host Microbe* (2016) 19:434–41. doi: 10.1016/j.chom.2016.03.003
40. Goto Y, Obata T, Kunisawa J, Sato S, Ivanov, II, Lamichhane A, et al. Innate lymphoid cells regulate intestinal epithelial cell glycosylation. *Science* (2014) 345:1254009. doi: 10.1126/science.1254009
41. Clevers HC, Bevins CL. Paneth cells:maestros of the small intestinal crypts. *Annu Rev Physiol.* (2013) 75:289–311. doi: 10.1146/annurev-physiol-030212-183744
42. Bottone MG, Santin G, Aredia F, Bernocchi G, Pellicciari C, Scovassi AI. Morphological features of organelles during apoptosis:an overview. *Cells* (2013) 2:294–305. doi: 10.3390/cells2020294
43. Saraste A, Pulkki K. Morphologic and biochemical hallmarks of apoptosis. *Cardiovascu Res.* (2000) 45:528–537. doi: 10.1016/S0008-6363(99)00384-3
44. Ziegler U, Groscurth P. Morphological features of cell death. *Physiology* (2004) 19:124–8. doi: 10.1152/nips.01519.2004
45. Sato T, van Es JH, Snippert HJ, Stange DE, Vries RG, van den Born M, et al. Paneth cells constitute the niche for Lgr5 stem cells in intestinal crypts. *Nature* (2011) 469:415–8. doi: 10.1038/nature09637
46. Perse M, Cerar A. Dextran sodium sulphate colitis mouse model:traps and tricks. *J Biomed Biotechnol.* (2012) 2012:718617. doi: 10.1155/2012/718617
47. Kaser A, Blumberg RS. Endoplasmic reticulum stress and intestinal inflammation. *Mucosal Immunol.* (2010) 3:11–6. doi: 10.1038/mi.2009.122
48. el Marjou F, Janssen KP, Chang BH, Li M, Hindie V, Chan L, et al. Tissue-specific and inducible Cre-mediated recombination in the gut epithelium. *Genesis* (2004) 39:186–93. doi: 10.1002/gene.20042
49. Diefenbach A. Interleukin-22, the guardian of the intestinal stem cell niche? *Immunity* (2012) 37:196–8. doi: 10.1016/j.immuni.2012.08.007
50. Bollrath J, Phesse TJ, von Burstin VA, Putoczki T, Bennecke M, Bateman T, et al. gp130-mediated Stat3 activation in enterocytes regulates cell survival and cell-cycle progression during colitis-associated tumorigenesis. *Cancer Cell* (2009) 15:91–102. doi: 10.1016/j.ccr.2009.01.002
51. Anderson CA, Boucher G, Lees CW, Franke A, D'Amato M, Taylor KD, et al. Meta-analysis identifies 29 additional ulcerative colitis risk loci, increasing the number of confirmed associations to 47. *Nat Genet.* (2011) 43:246–52. doi: 10.1038/ng.764
52. Kato K, Nomoto M, Izumi H, Ise T, Nakano S, Niho Y, et al. Structure and functional analysis of the human STAT3 gene promoter:alteration of chromatin structure as a possible mechanism for the upregulation in cisplatin-resistant cells. *Biochim Biophys Acta* (2000) 1493:91–100. doi: 10.1016/S0167-4781(00)00168-8
53. Kim M, Morales LD, Jang IS, Cho YY, Kim DJ. Protein tyrosine phosphatases as potential regulators of STAT3 signaling. *Int J Mol Sci.* (2018) 19:E2708. doi: 10.3390/ijms19092708
54. Su F, Ren F, Rong Y, Wang Y, Geng Y, Wang Y, et al. Protein tyrosine phosphatase Meg2 dephosphorylates signal transducer and activator of transcription 3 and suppresses tumor growth in breast cancer. *Breast Cancer Res.* (2012) 14:R38. doi: 10.1186/bcr3134
55. Kim DJ, Tremblay ML, Digiovanni J. Protein tyrosine phosphatases, TC-PTP, SHP1, and SHP2, cooperate in rapid dephosphorylation of Stat3 in keratinocytes following UVB irradiation. *PLoS ONE* (2010) 5:e10290. doi: 10.1371/journal.pone.0010290
56. Camporeale A, Poli V. IL-6, IL-17 and STAT3:a holy trinity in auto-immunity? *Front Biosci.* (2012) 17:2306–26. doi: 10.2741/4054
57. Mirlekar B, Ghorai S, Khetmalas M, Bopanna R, Chattopadhyay S. Nuclear matrix protein SMAR1 control regulatory T-cell fate during inflammatory bowel disease (IBD). *Mucosal Immunol.* (2015) 8:1184–200. doi: 10.1038/mi.2015.42
58. Lu D, Liu L, Ji X, Gao Y, Chen X, Liu Y, et al. The phosphatase DUSP2 controls the activity of the transcription activator STAT3 and regulates TH17 differentiation. *Nat Immunol.* (2015) 16:1263–73. doi: 10.1038/ni.3278
59. Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM, Stockinger B. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity* (2006) 24:179–89. doi: 10.1016/j.immuni.2006.01.001
60. Brand S, Beigel F, Olszak T, Zitzmann K, Eichhorst ST, Otte JM, et al. IL-22 is increased in active Crohn's disease and promotes proinflammatory gene expression and intestinal epithelial cell migration. *Am J Physiol Gastrointest Liver Physiol.* (2006) 290:G827–38. doi: 10.1152/ajpgi.00513.2005
61. Reinecker HC, Steffen M, Witthoeft T, Pflueger I, Schreiber S, MacDermott RP, et al. Enhanced secretion of tumour necrosis factor-alpha, IL-6, and IL-1 beta by isolated lamina propria mononuclear cells from patients with ulcerative colitis and Crohn's disease. *Clin Exp Immunol.* (1993) 94:174–81. doi: 10.1111/j.1365-2249.1993.tb05997.x

62. Barker N. Adult intestinal stem cells:critical drivers of epithelial homeostasis and regeneration. *Nat Rev Mol Cell Biol.* (2014) 15:19–33. doi: 10.1038/nrm3721
63. McGeachy MJ, Chen Y, Tato CM, Laurence A, Joyce-Shaikh B, Blumenschein WM, et al. The interleukin 23 receptor is essential for the terminal differentiation of interleukin 17-producing effector T helper cells *in vivo*. *Nat Immunol.* (2009) 10:314–24. doi: 10.1038/ni.1698
64. Victor AR, Nalin AP, Dong W, McClory S, Wei M, Mao C, et al. IL-18 Drives ILC3 Proliferation and Promotes IL-22 Production via NF-kappa. *J Immunol B.* (2017) 199:2333–42. doi: 10.4049/jimmunol.1601554

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REVIEW
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Commensal Bacteria-Specific CD4$^+$ T Cell Responses in Health and Disease

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Over the course of evolution, mammalian body surfaces have adapted their complex immune system to allow a harmless coexistence with the commensal microbiota. The adaptive immune response, in particular CD4$^+$ T cell-mediated, is crucial to maintain intestinal immune homeostasis by discriminating between harmless (e.g., dietary compounds and intestinal microbes) and harmful stimuli (e.g., pathogens). To tolerate food molecules and microbial components, CD4$^+$ T cells establish a finely tuned crosstalk with the environment whereas breakdown of these mechanisms might lead to chronic disease associated with mucosal barriers and beyond. How commensal-specific immune responses are regulated and how these molecular and cellular mechanisms can be manipulated to treat chronic disorders is yet poorly understood. In this review, we discuss current knowledge of the regulation of commensal bacteria-specific CD4$^+$ T cells. We place particular focus on the key role of commensal-specific CD4$^+$ T cells in maintaining tolerance while efficiently eradicating local and systemic infections, with a focus on factors that trigger their aberrant activation.

Keywords: commensals, microbiota, CD4$^+$ T cells, inflammatory bowel disease (IBD), bacteria–host interaction, immune education, intestinal immunity, autoimmunity

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INTRODUCTION

The human body is composed of a myriad of microbes that outnumber the whole body human cells (1, 2). The highest density of commensal bacteria is located in the gastrointestinal tract, which is the largest immune organ in the body (3). The vast capabilities of the microbiota have led scientists to call for a "second human genome project" to account for the effect of microbial genes in human health (4). Commensal bacteria serve various functions, from catabolizing certain food molecules to promoting tissue repair. Among other functions, the microbiota controls enteric infections by competing for common resources as well as inducing the production of antimicrobial peptides by gut epithelial cells (5, 6). Importantly, the gut microbiota shapes both local and systemic immune responses. This is most evident in the case of chronic inflammation in the intestines, as this can lead to an unbalance of immune cells as well as dysbiosis of commensals and can result in various diseases, including local inflammatory bowel disease (IBD) and systemic diseases such as diabetes, asthma, and cancer (7). While many immune cells play a role in maintaining homeostasis with the microbiota (8), both effector and regulatory CD4$^+$ T cells are highly enriched in intestinal tissues and represent a major immune cell in mediating homeostatic responses. Moreover, CD4$^+$ T cells are believed to be the main drivers of pathogenicity in IBD and as such are considered a main therapeutic target (9).

Commensals have been shown to play a crucial role in driving physiological CD4$^+$ T cell differentiation at barrier sites. T cells recognize immunological epitopes through engagement of their T cell receptors (TCRs) with cognate antigens presented on major histocompatibility (MHC) molecules on antigen-presenting cells (APCs) (10). Unlike T cells specific for self-antigens expressed by thymic epithelial cells, commensal-specific T cells do not undergo negative selection in the thymus and are present in healthy individuals despite the constant presence of their cognate antigens (11). Importantly, T cell transfer studies have shown the ability of these cells to drive pathogenesis. In this review, we will therefore discuss the multifaceted role of commensal bacteria-specific CD4$^+$ T cells with special emphasis on the regulation and fate of these cells. More in detail, we will illustrate the mechanisms at the basis of commensal-host mutualism and how their disturbance affects local and systemic health. A detailed description of the state-of-the-art models available for the study of microbiota-specific T cell responses will provide major insights into the variety of CD4$^+$ T cell responses that are elicited by selected commensal bacterial species.

Commensal-Host Mutualism

The relationship between humans and microbes is multifaceted; while several bacterial species can result in pathogenesis upon colonization, the vast majority are innocuous and even beneficial to the host (12). Mutualism between hosts and microbes benefits both parties but relies on a delicate balance between the antimicrobial and tolerogenic effector functions of the immune system as well as the control of pathogenicity of commensals. Commensals aid their hosts by inactivating toxins and catabolizing host-indigestible nutrients, such as some complex carbohydrates, to useful metabolites such as short chain fatty acids (SCFAs) (13–15). The commensal bacteria also control pathogen overgrowth through competition for the same biological niches and release of molecular mediators (16–22). Beyond nutrient breakdown and protection from infections, commensal bacteria also play a role in the development of anatomical structures in the intestines, angiogenesis, maturation of the epithelial layer, and leukocyte imprinting (5, 23, 24).

The gut microbiota has a vast effect on the local immune system. Accordingly, antibiotic-mediated clearance of commensal microbes has been associated with susceptibility to infections, likely due to an unfit immune system (25). Importantly, commensal bacteria can induce both pro- and anti-inflammatory CD4$^+$ T cell responses via different mechanisms. For example, segmented filamentous bacteria (SFB) efficiently induce ileal IL-17 producer T helper (T_H17) cell expansion, whereas the production of SCFAs by colonic bacteria promotes the differentiation of colon-resident regulatory T cells (26). The delicate balance between pro-inflammatory and regulatory T helper cells is believed to play a pivotal role for the maintenance of the symbiotic relationship between commensal bacteria and their host.

Regulation of Bacteria-Host Interaction by the Mucosal Barrier

The maintenance of a symbiotic relationship between commensal microbes and their host relies both on physical segregation of the microbiota to the intestinal lumen and on active sampling of bacteria by the immune system under steady-state conditions. This physical segregation is achieved through a single layer of columnar intestinal epithelial cells (IECs) kept together by tight junctions, that regulate paracellular permeability. In addition, the epithelium is covered by mucous layers of varying thickness and structure along the intestinal tract (27, 28). Disturbance of the inner colonic mucus layer structure, which can be achieved by genetic depletion of Muc2 or a short-term treatment with dextran sulfate sodium (DSS), is sufficient to allow bacteria to relocate to the epithelial layer and cause gut inflammation in mice (29–31). In CA-MLCK mice, which are characterized by constitutive tight junction barrier loss due to transgenic expression of constitutively active myosin light chain kinase, expansion of intestinal CD4$^+$ T cells depends on the presence of commensal bacteria. This response resulted in protection against early *Salmonella Typhimurium* infection but exacerbated inflammation during chronic infection (32). Similarly, defective mucin production and aberrant expression of epithelial junctional proteins associated with early colorectal neoplastic lesions promoted permeability to commensal bacteria in humans, furthering inflammation and tumorigenesis (33).

The mucosal barrier is far from being a passive defense mechanism against microbial translocation. Immunoglobulins A (IgA), the most abundant immunoglobulin class in the body, are produced by B cells and plasma cells that reside in the Peyer's patches and intestinal lamina propria, respectively. Functional importance of this molecule in limiting commensal-specific T cell activation has been demonstrated in studies using the CBir1 TCR transgenic mouse model (**Table 1**). Activation of adoptively-transferred CBir1 Tg cells in response to orally-administered CBir1 flagellin was specifically blocked in WT mice, while selective impairment of IgA production or mucosal secretion unleashed CBir1 antigen-dependent T cell proliferation (48). Interestingly, IgA-mediated compartmentalization of the mucosal T cell response to the commensal microbiota does not apply to all bacteria, as activation of SFB or *Helicobacter hepaticus*-specific T cell clones occurs in immunocompetent mice (39, 42) (**Figure 1**). Furthermore, by the secretion of microbial peptides, epithelial cells actively contribute to the segregation of selected commensal bacteria to the intestinal lumen. These peptides are critical regulators of bacterial immunity and their impaired production is associated with intestinal and systemic inflammation. Mutations in Nod2, which are highly correlated with Crohn's disease, were found to negatively affect the production of a subgroup of intestinal anti-microbial peptides known as cryptidins (56). RegIII lectins are produced by Paneth cells in response to MyD88-dependent recognition of gut microbial patterns (57). Their depletion in RegIII$\gamma^{-/-}$ and RegIII$\beta^{-/-}$ mice resulted in increased colonization of the intestinal epithelial surface and bacterial translocation to the liver, respectively, with consequent extra-intestinal inflammation (58, 59). Interestingly, depletion of retinoic acid (RA) receptor alpha specifically in epithelial cells resulted in increased numbers of goblet and Paneth cells and increased RegIIIγ production. In agreement with the role of RegIIIγ in regulating bacterial colonization at the epithelium vicinity, these mice did not have any detectable bacteria as seen by 16S FISH staining (60).

TABLE 1 | Models to study commensal-specific T cell responses.

Model	Description	References
Gnotobiotic mice	• Germ-free (GF) mice, which lack commensal microbes, have immunological defects that extend beyond the intestinal mucosa, with hypoplastic lymphoid tissues lacking B and T-cell compartmentalization	(23, 34)
	• GF mice colonized with defined bacterial species are useful to study the effects of selected microbes on the development of the immune system	(35)
	• GF mice colonized at birth with rat or human microbiota maintain an immature gut immune system compared to specific pathogen-free (SPF) mice. The immune-educating effect of the microbiota is host-specific	(36)
Tetramers	• Soluble MHC/peptide multimers with the capability of binding selected TCRs, useful for the *ex vivo* study of low-frequency endogenous antigen-specific $CD4^+$ or $CD8^+$ T cell populations	(37, 38)
	• I-Ab/3340-A6 tetramer allows recognition of segmented filamentous bacteria (SFB)-specific T cells	(39, 40)
	• I-Ab-CBir1p tetramer selectively stains cells that recognize CBir1 flagellin, an immunodominant microbiota antigen	(41)
	• HH1713172–86 and HH1713230–44 tetramers stain *Helicobacter*-specific T cells	(42)
TCR transgenic mice	• SFB, a unique member of Clostridiales that inhabits the terminal ileum, has been shown to specifically induce T_H17 cell differentiation *in vivo* • In **7B8 TCR transgenic mice**, CD4 T cells recognize SFB-specific antigens • Upon transfer of naïve 7B8 cells to SFB-colonized congenic mice, these cells proliferate and are detected in the small intestinal lamina propria as T_H17 cells	(39, 43, 44)
	• Commensal Clostridia induce the accumulation of T_{reg} cells in the intestine • **CT2 and CT6 mouse lines** express microbiota-dependent colonic T_{reg} TCRs • Upon adoptive transfer into young immunocompetent hosts, these naïve TCR Tg cells spontaneously acquired a T_{reg} cell phenotype	(45–47)
	• Increased antibodies against microbial antigens were detected in both Crohn's disease patients and animal models of gut inflammation. The Cbir1 epitope of bacterial flagellin protein was immunodominant in both settings • **CBir1 TCR transgenic mice** have $CD4^+$ T cells capable of recognizing bacterial flagellin and other commensal microbes • Activation of adoptively transferred naïve Cbir1 TCR cells does not occur under steady-state conditions but can be achieved via disturbance of the intestinal mucosal barrier or lack of immunoregulatory mechanisms	(48–52)
	• **HH7-2tg and HH5-1tg mouse strains** are transgenic for clonotypic TCRs that recognize *Helicobacter hepaticus* • HH7-2tg cells acquired a $Foxp3^+ROR\gamma t^+$ regulatory phenotype under steady state conditions • Intestinal inflammation skews the *H. hepaticus*-specific response toward a pro-inflammatory T_H17/T_H1 cell phenotype	(42, 53)

Altogether, these data demonstrate that tight regulation of the mucosal barrier is crucial in maintaining microbial homeostasis.

Regulation of $CD4^+$ T Cell Responses Against Commensal Bacteria

$CD4^+$ T cells orchestrate the immune response through the release of pro- and anti-inflammatory cytokines and expression of co-stimulatory molecules. To this end, they play crucial roles in driving or repressing the response of macrophages, $CD8^+$ T cells, and B cells toward both pathogens and autoimmune antigens [reviewed in (61)]. $CD4^+$ T cells can differentiate into various T helper (T_H) subsets with differing effector functions [reviewed in (62, 63)]. The most extensively characterized T_H subsets include: T_H1 cells, which are characterized by the production of interferon gamma (IFNγ), tumor necrosis factor alpha (TNFα), and expression of the transcription factor T-box expressed in T cells (T-bet); T_H2 cells, which produce IL-4 and IL-13 and express the transcription factor GATA-binding protein 3 (GATA-3); and T_H17 cells, which express IL-17A/F and IL-22 and the transcription factor RA receptor-related orphan nuclear receptor RORγt. Anti-inflammatory T cell subsets include "natural" $CD4^+CD25^+FoxP3^+$ regulatory (T_{reg}) cells that develop in the thymus as well as "inducible" regulatory cells, such as $FoxP3^+$ T_{reg} and $FoxP3^-$ T_R1 cells, which arise in the periphery (64–66). In addition, Bcl6-expressing T follicular helper (T_{FH}) cells reside in germinal centers and coordinate B cells responses through regulation of B cell recruitment, expansion, survival, antibody class-switching, and somatic hypermutation [reviewed in (67)]. Differentiation of T cells into certain T_H subsets can be fostered by specific features of the microenvironment. *In vitro* studies have shown that neutralization of IFNγ reduces the development of T_H1 cells, while transforming growth factor beta (TGFβ) promotes the differentiation of T_H17 and T_{reg} cells (61, 68).

Adherence of selective microbes to the gut epithelium or intestinal damage can expose commensal bacterial antigens to APCs, which can then initiate commensal-specific T cell responses. Several subsets of APCs inhabit the intestinal lamina propria and have been shown to respond to fluctuations of the commensal microbiota composition (69, 70). For instance, $CX3CR1^{hi}$ mononuclear phagocytes residing in the small

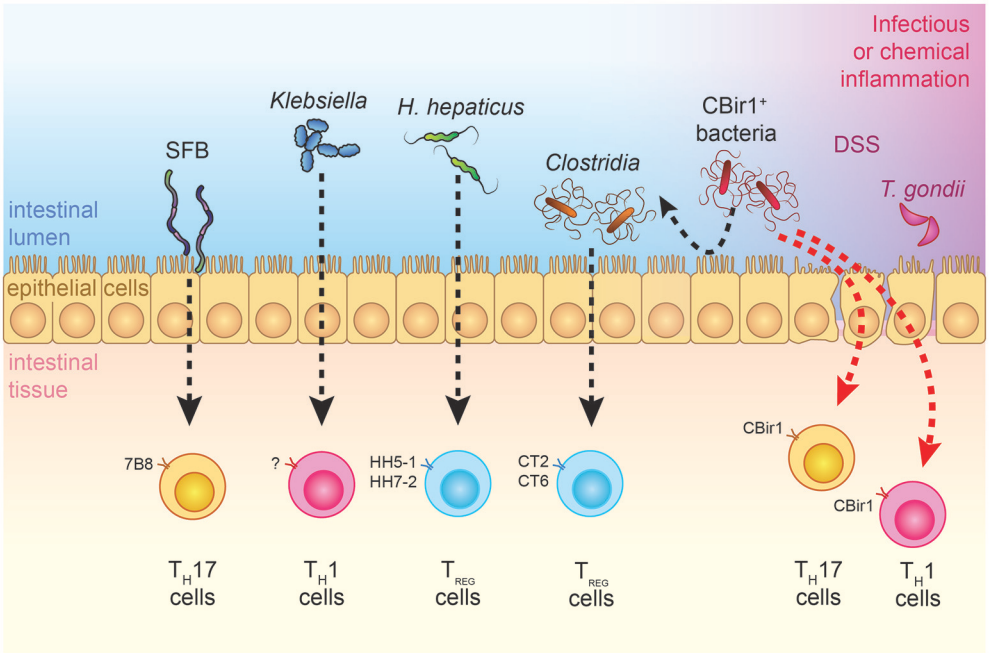

FIGURE 1 | Effects of commensal bacteria on T cell differentiation during steady-state and inflammatory conditions. TCR transgenic models that are available for the study of bacteria-specific immune responses are depicted. SFB-specific 7B8 cells are preferentially skewed toward a T_H17 cell phenotype, while *H. hepaticus*-specific TCR transgenic cells and *Clostridia*-specific CT2-CT6 cells acquire a T_{reg} fate under steady-state conditions (39, 42, 47). Physiological encounter of CBir1 T cells with their cognate antigen does not occur in adult individuals but can be triggered by different infectious or barrier-disrupting events, which shape the type of $CD4^+$ T cell response (48, 49, 54). Ectopic colonization of oral *Klebsiella* in the intestines has T_H1-inducing and pro-inflammatory effects on the gut, although antigen specificity has yet to be investigated (55).

intestine were reported to express tight junction proteins that allow them to extend dendrites through the intact intestinal epithelium and sample microbial antigens (71, 72). Moreover, despite being non-migratory under steady state, these APCs were able to migrate to the MLNs and trigger *Salmonella*-specific T cell responses upon dysbiotic conditions (73). Both IRF4-dependent $CD103^+CD11b^+$ dendritic cells (DCs) and CX_3CR1^+ intestinal macrophages have been shown to play a role in the induction of intestinal T_H17 cells in response to epithelium-associated SFB (74, 75). A number of reports demonstrate the ability of $CD103^+CD11b^+$ DCs to produce pro-inflammatory cytokines IL-6 and IL-23 in response to detection of microbial patterns, therefore making them good candidates for the induction of T_H17 cells (74, 76). Cytokine production by innate immune cells may synergize with or be neutralized by TCR specificity in determining the fate of the T_H17 cell response. For instance, joint colonization with SFB and T_H1-inducer *Listeria monocytogenes* does not prevent SFB-specific T cells from acquiring T_H17 features (39).

Intestinal innate lymphoid cells (ILCs) represent another innate immune population with a high degree of functional compartmentalization that can be finely shaped by the composition of the commensal microbiota (77). It has been recently demonstrated that some group 3 ILCs (ILC3s) have the capacity to present antigens through MHC class II molecules, and this feature allows them to regulate the commensal-specific T cell response (78). Loss of MHCII expression within this ILC subset resulted in the accumulation and pro-inflammatory activation of commensal microbiota-specific CBir1 Tg T cells in the MLN and colonic lamina propria. In addition, the regulatory function of ILC3 was shown to rely on MHCII- and antigen-dependent induction of CBir1 T cell apoptosis, thus uncovering a novel regulatory mechanism of the commensal-specific T cell response (40).

T Cell Tolerance Toward Commensals

Activation of T_H cells in the gut-associated lymphoid tissues is subjected to a delicate control, whereby T cells specific for commensal bacteria are either ignorant of their cognate antigens (e.g., CBir1 flagellin-specific cells) or physiologically skewed toward a regulatory function (e.g., CT2 and CT6 commensal-specific T cells) (45, 49) (**Figure 1**). Understanding the physiological mechanisms that are in place to maintain T cell homeostasis toward commensal antigens is therefore fundamental to understand why inflammation occurs and to potentially revert pathogenic activation of commensal-specific T cells.

The peculiar microenvironment present at mucosal surfaces—largely influenced by microbial metabolic products and soluble factors either digested of produced by IECs—promotes tolerance toward foreign antigens (26, 60, 79). Peripheral tolerance prevents the development of immune-mediated inflammatory diseases (IMIDs) (11, 80) and can be achieved by different mechanisms. The high frequency of T_{reg} cells in the gut,

which has been shown to depend at least in part on TCR-mediated recognition of some bacteria residing in the colonic mucosa, namely *Clostridia*, provides an important mechanism for the maintenance of tolerance to commensals (45). $CD4^+$ regulatory T cells dampen inflammation through the release of cytokines such as interleukin 10 (IL-10) and TGFβ. Several non-$CD4^+$ T cell subsets such as $CD8^+$ T cells and double negative T regulatory cells can also promote tolerance through the expression of anti-inflammatory cytokines or through direct killing of effector cells (81, 82). All of these regulatory cell types can be induced in the context of oral tolerance, which is defined as the active establishment of local and systemic unresponsiveness to antigens acquired via the oral route (83).

The gut-draining mesenteric lymph nodes (MLNs) and $CD103^+$ DCs play a critical role in the induction of antigen-specific T_{reg} cells (84, 85). One mechanism of T_{reg} cell induction involves the ability of $CD103^+$ DCs to metabolize dietary vitamin A to RA (86–90). The difference in T_H cell subsets, and in particular percentages of T_{reg} cells, along the intestinal tract could therefore, be ascribed to a differential uptake of vitamin A and other nutrients in selected anatomical locations, as well as to the presence of different consortia of commensal bacteria (45, 46, 91). While the process of tolerance toward food antigens shares some features with tolerance to commensals, the two mechanisms are fundamentally different. Indeed, tolerance to microbes is largely limited to the gut whereas oral tolerance has systemic consequences (92).

Regulatory T cells can be implicated in the maintenance of the host-microbiota commensalism at different levels. First of all, T cells that are specific for selected bacteria, i.e., *Clostridium* species or *H. hepaticus*, acquire a T_{reg} phenotype under steady-state conditions (42, 45, 47). For instance, the preferential conversion of *H. hepaticus*-specific HH7-2tg cells into functional $ROR\gamma t^+Foxp3^+$ iT_{reg} cells depends on the intrinsic expression of the transcription factor c-MAF, which is known to promote a cellular anti-inflammatory program including production of the cytokine IL-10. Selective depletion of c-MAF in T_{reg} cells resulted in the accumulation of both polyclonal and *H. hepaticus*-specific T_H17 cells in the large intestine (42). Additionally, T_{reg} cells participate indirectly in the restriction of CBir1 flagellin-specific T cell responses by providing help to B cells which, in turn, produce CBir1-specific IgA molecules (48). Thus, T_{reg} cells contribute to tolerance of commensals by various mechanisms.

Commensal-Specific T Cells in Homeostasis

While the general effect of commensals on the immune system is established, it is only of recent that focus has been directed toward the specific T cells that recognize them. Antibiotic treatment has not only been shown to affect T_{reg} cell populations as a whole, but also to alter the TCR repertoire of T_{reg} cells (93). Moreover, enhanced T_{reg} suppressive activity has been observed in the presence of their cognate antigen (93). Indeed, the existence of colonic T_{reg} cells appears to be regulated by TCR specificity and antigen availability, and *in vitro* stimulation assays of colonic T_{reg}-associated TCRs with host-derived commensals show that many of the local antigens these cells react to are microbiota-derived (45–47). Despite this, the microbiota does not appear to be essential to induce T_{reg} differentiation, since germ-free (GF) mice, which are devoid of microbiota, do not lack this T cell subset. The variable ontogeny of T_{reg} cells inhabiting the intestinal mucosa, either thymic-derived or peripherally induced, might explain the controversial observations regarding the effects of the presence of commensals on T_{reg} cell frequencies [reviewed in (94)]. The effect of the microbiota on T_H1 and T_H17 induction is more evident, as their numbers are greatly reduced in GF animals (95, 96). In particular, it has been demonstrated that the majority of T_H17 cells in the intestinal lumen are specific for commensals (43, 46, 97).

Mouse studies reveal that commensal-specific T cells in the gut mucosa typically develop into either T_H17 or T_{reg} cells during steady state and these subsets collectively help maintain homeostasis in these tissues. The fate of commensal-specific T cells is dependent on the microbe they are specific to. For instance, the presence of SFB, a commensal that resides in the ileum, has been shown to drive T_H17 differentiation, whereas T cells that are specific to some *Clostridia* species develop into colonic T_{reg} cells under homeostatic conditions (43, 45, 46, 98). A comprehensive analysis of T cells from human peripheral blood and mucosal samples recently revealed that commensal-specific T cells are similarly present in healthy individuals, mainly have a memory phenotype and are capable to express different cytokines according to their immune specificities (9). In memory phase, commensal-specific $CD4^+$ T cells behave like pathogen-specific $CD4^+$ T cells, persisting after clearance of infection but declining in numbers over time (49, 99). Understanding the mechanisms behind the reduction of commensal-specific T cell numbers despite the persistence of their cognate antigen could shed light on the different capacity of individuals to recover from intestinal challenges (100).

Interestingly, despite the large antigenic load of commensal bacteria present within the intestinal lumen, T cells specific for certain bacterial antigens remain naïve in mice under homeostatic conditions (48). Upon disturbance of the barrier-mediated segregation of bacteria to the intestinal lumen, however, activation of T cells toward such antigens may take place. Whether activation of $CD4^+$ T cell responses toward such antigens is directly contributing to the pathogenesis of inflammation or represents a mere epiphenomenon in the broader context of barrier disruption is still an open question.

PATHOGENESIS OF COMMENSAL-SPECIFIC T CELLS

Local passage of either pathogenic or normally tolerated bacteria through the mucosal barrier and their systemic translocation can cause disease. As described above, despite the fact that commensal antigens are non-self, their coexistence within the host is finely regulated via multiple immune tolerance

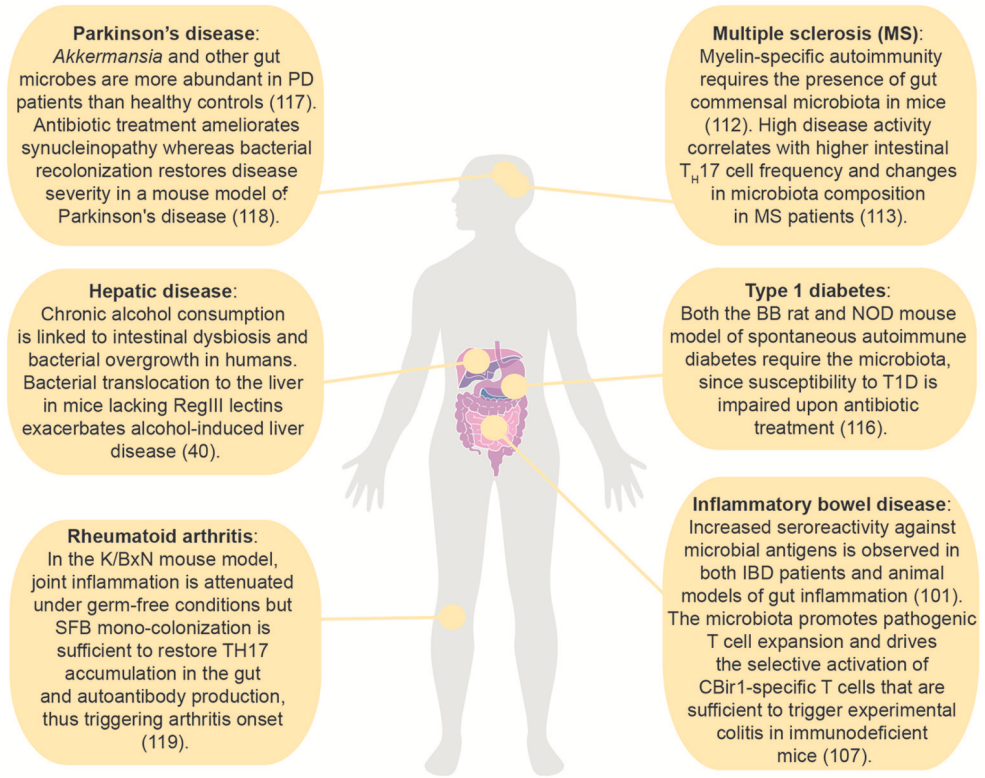

FIGURE 2 | Effects of the intestinal microbiota on immune mediated diseases within and outside the gastrointestinal tract. (50); (113); (114); (115); (116); (59); (117); (51); (118).

mechanisms, spanning from an efficient epithelium and mucus-mediated segregation to the secretion of antimicrobial peptides, and specific antibodies (101–103). Contact between host epithelial cells and commensals within the intestinal lumen is not in itself problematic since some bacterial species have been described to colonize the epithelial surface and induce protective immune responses in an adherence-dependent manner (43, 104).

Mice with genetic defects in the barrier develop spontaneous intestinal inflammation (105, 106) but gut-resident macrophages have been shown to efficiently clear invading pathogens thus preventing their systemic translocation (107). Intestinal immune responses are normally limited to the intestines and associated lymphoid tissue, since DCs that pick up commensal antigens migrate from the gut mucosa to the gut-draining MLNs, but not further into the body (108). However, antibodies against skin- and gut-resident commensals have been identified in humans, suggesting that we are challenged throughout life with commensal antigens and some of these immune responses have systemic effects (109). As a result of a mucosal firewall-breaching pathogen, commensal-specific T cells are activated and expand in the gastrointestinal tract (49, 110, 111) and this may result in the persistent disruption of migratory DC trafficking and impairment of tissue-specific adaptive immunity and tolerance (112). Furthermore, an acute enteric infection model has been shown to result in the differentiation of normally quiescent commensal-specific T cells into pro-inflammatory T_H1 cells (49).

Emerging evidence points toward the intestinal microbiota as a major factor in the establishment, persistence and/or resolution of intestinal, and extra-intestinal immune diseases (**Figure 2**). Although the definition of a "healthy" microbiota is still under discussion, a balanced microbiota composition is necessary to maintain intestinal immune homeostasis (36). By contrast, dysbiosis has been associated with disease by promoting immune dysregulation and inflammation in the gut and beyond. This unbalance is reflected by modifications of the immune response to physiologically-recognized bacterial antigens and novel recognition of bacteria to which the immune system is normally ignorant. For instance, pro-inflammatory T cell responses are observed toward classical T_{reg}-inducer *Clostridium* species during gastrointestinal infection (49). Furthermore, immunoglobulins reactive to commensal antigens can be found in patients with active gut inflammation (50).

While the microbiota plays an important role in the determination of T cell fate, its effects are buffered by the genetic background of the host. For example, T_{reg} induction following the introduction of altered Schaedler flora (ASF) was shown to be mouse strain-dependent (119). Indeed, while IBD is recognized as a disease caused by an aberrant immune response against commensals in the intestinal mucosa, it is unknown why some

people develop chronic inflammation upon barrier damage while others recover (50, 100).

Intestinal Disorders

The intestinal mucosa and its immune system, by providing the major interface between gut bacteria and the host, are susceptible to a number of different pathological conditions upon disturbance of their delicate symbiotic relationship. IBD is considered an intestinal disorder characterized by an aberrant immune response against the commensal microbiota in genetically-susceptible hosts, most likely triggered by the environment. Commensal-specific T cells are thought to play a direct role in IBD pathogenesis. For instance, transfer of naïve T cells in lymphopenic mice is sufficient to induce chronic intestinal inflammation in a microbiota-dependent manner (120, 121). Furthermore, increased seroreactivity against a commensal-derived flagellin expressed by a subset of *Clostridia* (CBir1) was found to induce a systemic adaptive immune response in both Crohn's disease patients and colitic mice, suggesting a microbiota-specific immune response (50, 122, 123). Indeed, the microbiota has been shown to promote pathogenic T cell expansion and drive the selective activation of CBir1-specific T cells that are sufficient to trigger experimental colitis in immunodeficient mice (51). Recent studies suggest that during colitis an effector T cell response is specifically activated against other microbial antigens, e.g., those provided by the mucosal-associated *Helicobacter* spp. that elicit T_{reg} responses during homeostatic conditions (53, 122). Today, several pathogenic T cells have been identified in mouse and humans, such as the IL-17 and IFNγ double-producer T cells as well as the recently described IL-22BP-producer T cells (124, 125). Whether these T cells are microbiota-specific needs to be demonstrated.

Evidence also suggests that commensal-specific T cells may play a role in the pathogenesis of colorectal cancer (CRC) and dietary conditions. Epidemiological studies reveal that chronic inflammation, such as the one resulting from IBD, increases the risk of CRC (126). The gut microbiota was shown to play a role in both stages of CRC-associated inflammation. Studies in IL-10 knockout mice showed that alteration of the microbiota by introduction of probiotic *Lactobacilli* can reduce the incidence of both mucosal inflammation and spontaneous CRC development in these mice (127). In addition, commensals have been linked to dietary conditions. For example, through their distinct metabolic patterns, some commensal bacteria are able to modify immunogenicity of dietary proteins, thus triggering gluten-specific T-cell responses and celiac disease (128). Whether bacteria-targeted therapy is effective in treating or preventing these conditions remains to be determined.

Extra-Intestinal Disorders

Remarkably, gut commensal-specific T cells circulate systemically (9) and the gut microbiota affects the balance between pro-inflammatory and anti-inflammatory T cell responses also in extra-intestinal autoimmune diseases. For example, myelin-specific autoimmunity requires the presence of gut commensal microbiota, as confirmed by lack of spontaneous EAE in GF compared to SPF susceptible mice (113). Similarly, in multiple sclerosis patients, high disease activity correlated with higher intestinal T_H17 cell frequency, and changes in microbiota composition (114). Furthermore, recent evidence unveiled the importance of the microbiota for the development of spontaneous RA (129, 130). The association between the intestinal environment and type 1 diabetes (T1D) relies on a constantly increasing number of studies both in T1D patients and mouse models of the disease. The requirement for the gut microbiota in the pathogenesis of T1D has been proven in both the BB rat and NOD mouse model of spontaneous autoimmune diabetes, whose susceptibility to the disease is impaired upon antibiotic treatment (117). Unlike in MS and RA models, however, the effect of the microbiota on the induction of pathogenic or protective immune cell responses is not unanimously accepted in T1D. However, since the antigen-specificity of microbiota-induced T cells has been poorly characterized in these disease settings, further studies are required to understand the role of commensal-specific T cells in tissue-specific autoimmune diseases. A functional link between the microbiota and Parkinson's disease (PD), a neurodegenerative disorder, was also recently delineated. *Akkermansia* and other gut microbes were more abundant in PD patients than healthy controls (115). Antibiotic treatment was capable of ameliorating synucleinopathy (motor dysfunctions derived from the aggregation of the protein α-synuclein) in a mouse model of Parkinson's disease, and recolonization of adult mice with commensal bacteria derived from PD patients or administration of selected bacterial metabolites, namely SCFAs, was sufficient to restore disease penetrance (116). Whether or not reestablishment of a normal microbiota composition can revert PD and other neurogenerative conditions remains to be determined.

CONCLUDING REMARKS

The use of experimental mouse models has provided key insights into the complex regulation of intestinal immune homeostasis by the microbiota. Descriptive studies comparing IBD patients and healthy individuals have implicated $CD4^+$ T cell-mediated immune responses against commensal bacteria as a potential key factor in the progression of the disease. Moreover, emerging evidence points toward far-reaching consequences of altered microbiota-mediated T cell responses originated in the gut. For instance, aberrant T cell differentiation in the gut have shown effects in skin tumor growth, the onset of T1D as well as MS. Hence, it is broadly accepted that, besides its gut-centric effects, the microbiota has also extra-intestinal repercussions on its host. The role of commensal-specific T cells in various disease settings is yet to be determined and only with deeper understanding of the molecular and cellular mechanism driving commensal specific T cell differentiation and function will the goal of targeting T cell responses to cure IBD become feasible. Toward this goal, the advances in microbiota analysis, use of gnotobiotic mice and TCR transgenic experimental models provides the potential to discern such mechanisms. Moreover, progress in available tools to track commensal specific T cells in humans

are urgently needed. Accomplishing these goals will require tight collaboration between researchers and the clinics.

AUTHOR CONTRIBUTIONS

CS, RFC, and EJV outlined and wrote the manuscript. CS and EJV designed and made the figures. NG revised the manuscript. All authors approved the manuscript for publication.

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REFERENCES

1. Ley RE, Peterson DA, Gordon JI. Ecological and evolutionary forces shaping microbial diversity in the human intestine. *Cell* (2006) 124:837–48. doi: 10.1016/j.cell.2006.02.017
2. O'Hara AM, Shanahan F. The gut flora as a forgotten organ. *EMBO Rep.* (2006) 7:688–93. doi: 10.1038/sj.embor.7400731
3. Hill DA, Artis D. Intestinal bacteria and the regulation of immune cell homeostasis. *Annu Rev Immunol.* (2009) 28:623–67. doi: 10.1146/annurev-immunol-030409-101330
4. Relman DA, Falkow S. The meaning and impact of the human genome sequence for microbiology. *Trends Microbiol.* (2001) 9:206–8. doi: 10.1016/S0966-842X(01)02041-8
5. Sekirov I, Finlay BB. The role of the intestinal microbiota in enteric infection. *J Physiol.* (2009) 587:4159–67. doi: 10.1113/jphysiol.2009.172742
6. Kamada N, Chen GY, Inohara N, Núñez G. Control of pathogens and pathobionts by the gut microbiota. *Nat Immunol.* (2013) 14:685–90. doi: 10.1038/ni.2608
7. Lebeer S, Vanderleyden J, De Keersmaecker SC. Host interactions of probiotic bacterial surface molecules: comparison with commensals and pathogens. *Nat Rev Microbiol.* (2010) 8:171–84. doi: 10.1038/nrmicro2297
8. Hooper LV, Macpherson AJ. Immune adaptations that maintain homeostasis with the intestinal microbiota. *Nat Rev Immunol.* (2010) 10:159–69. doi: 10.1038/nri2710
9. Hegazy AN, West NR, Stubbington MJT, Wendt E, Suijker KIM, Datsi A, et al. Circulating and tissue-resident CD4(+) T cells with reactivity to intestinal microbiota are abundant in healthy individuals and function is altered during inflammation. *Gastroenterology* (2017) 153:1320–37.e16. doi: 10.1053/j.gastro.2017.07.047
10. Rudolph MG, Stanfield RL, Wilson IA. How TCRs bind MHCs, peptides, and coreceptors. *Annu Rev Immunol.* (2006) 24:419–66. doi: 10.1146/annurev.immunol.23.021704.115658
11. Klein L, Kyewski B, Allen PM, Hogquist KA. Positive and negative selection of the T cell repertoire: what thymocytes see (and don't see). *Nat Rev Immunol.* (2014) 14:377–91. doi: 10.1038/nri3667
12. Dethlefsen L, McFall-Ngai M, Relman DA. An ecological and evolutionary perspective on human-microbe mutualism and disease. *Nature* (2007) 449:811–8. doi: 10.1038/nature06245
13. Flint HJ. Polysaccharide breakdown by anaerobic microorganisms inhabiting the mammalian gut. *Adv Appl Microbiol.* (2004) 56:89–120. doi: 10.1016/S0065-2164(04)56003-3
14. LeBlanc JG, Chain F, Martin R, Bermudez-Humaran LG, Courau S, Langella P. Beneficial effects on host energy metabolism of short-chain fatty acids and vitamins produced by commensal and probiotic bacteria. *Microb Cell Fact* (2017) 16:79. doi: 10.1186/s12934-017-0691-z
15. Pool-Zobel B, Veeriah S, Böhmer F. Modulation of xenobiotic metabolising enzymes by anticarcinogens—focus on glutathione S-transferases and their role as targets of dietary chemoprevention in colorectal carcinogenesis. *Mut Res.* (2005) 591:74–92. doi: 10.1016/j.mrfmmm.2005.04.020
16. Flint HJ, Duncan SH, Scott KP, Louis P. Interactions and competition within the microbial community of the human colon: links between diet and health. *Environ Microbiol.* (2007) 9:1101–11. doi: 10.1111/j.1462-2920.2007.01281.x
17. Fukuda S, Toh H, Hase K, Oshima K, Nakanishi Y, Yoshimura K, et al. Bifidobacteria can protect from enteropathogenic infection through production of acetate. *Nature* (2011) 469:543–7. doi: 10.1038/nature09646
18. Benson A, Pifer R, Behrendt CL, Hooper LV, Yarovinsky F. Gut commensal bacteria direct a protective immune response against *Toxoplasma gondii*. *Cell Host Microbe* (2009) 6:187–96. doi: 10.1016/j.chom.2009.06.005
19. Reid G, Bruce AW. Probiotics to prevent urinary tract infections: the rationale and evidence. *World J Urol.* (2006) 24:28–32. doi: 10.1007/s00345-005-0043-1
20. Brook I. The role of bacterial interference in otitis, sinusitis and tonsillitis. *Otolaryngol Head Neck Surg.* (2005) 133:139–46. doi: 10.1016/j.otohns.2005.03.012
21. Servin AL. Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens. *FEMS Microbiol Rev.* (2004) 28:405–40. doi: 10.1016/j.femsre.2004.01.003
22. Tilman D. Niche tradeoffs, neutrality, and community structure: a stochastic theory of resource competition, invasion, and community assembly. *Proc Natl Acad Sci USA.* (2004) 101:10854–61. doi: 10.1073/pnas.0403458101
23. Bauer H, Horowitz RE, Levenson SM, Popper H. The response of the lymphatic tissue to the microbial flora. Studies on germfree mice. *Am J Pathol.* (1963) 42:471–83.
24. Esterhazy D, Canesso MC, Muller PA, Lockhart A, Mesin L, Faria AM, et al. Compartmentalized lymph node drainage dictates intestinal adaptive immune responses. *BioRxiv [Preprint]* (2018). doi: 10.1101/299628
25. Sekirov I, Tam NM, Jogova M, Robertson ML, Li Y, Lupp C, et al. Antibiotic-induced perturbations of the intestinal microbiota alter host susceptibility to enteric infection. *Infect Immun.* (2008) 76:4726–36. doi: 10.1128/IAI.00319-08
26. Maslowski KM, Vieira AT, Ng A, Kranich J, Sierro F, Yu D, et al. Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43. *Nature* (2009) 461:1282–6. doi: 10.1038/nature08530
27. Neunlist M, Van Landeghem L, Mahe MM, Derkinderen P, des Varannes SB, Rolli-Derkinderen M. The digestive neuronal-glial-epithelial unit: a new actor in gut health and disease. *Nat Rev Gastroenterol Hepatol.* (2013) 10:90–100. doi: 10.1038/nrgastro.2012.221
28. Pelaseyed T, Bergstrom JH, Gustafsson JK, Ermund A, Birchenough GM, Schutte A, et al. The mucus and mucins of the goblet cells and enterocytes provide the first defense line of the gastrointestinal tract and interact with the immune system. *Immunol Rev.* (2014) 260:8–20. doi: 10.1111/imr.12182
29. Velcich A, Yang W, Heyer J, Fragale A, Nicholas C, Viani S, et al. Colorectal cancer in mice genetically deficient in the mucin Muc2. *Science* (2002) 295:1726–9. doi: 10.1126/science.1069094
30. Johansson ME, Phillipson M, Petersson J, Velcich A, Holm L, Hansson GC. The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. *Proc Natl Acad Sci USA.* (2008) 105:15064–9. doi: 10.1073/pnas.0803124105
31. Johansson ME, Gustafsson JK, Holmen-Larsson J, Jabbar KS, Xia L, Xu H, et al. Bacteria penetrate the normally impenetrable inner colon mucus layer in both murine colitis models and patients with ulcerative colitis. *Gut* (2014) 63:281–91. doi: 10.1136/gutjnl-2012-303207
32. Edelblum KL, Sharon G, Singh G, Odenwald MA, Sailer A, Cao S, et al. The microbiome activates CD4 T-cell-mediated immunity to compensate for increased intestinal permeability. *Cell Mol Gastroenterol Hepatol.* (2017) 4:285–97. doi: 10.1016/j.jcmgh.2017.06.001

33. Grivennikov SI, Wang K, Mucida D, Stewart CA, Schnabl B, Jauch D, et al. Adenoma-linked barrier defects and microbial products drive IL-23/IL-17-mediated tumour growth. *Nature* (2012) 491:254–8. doi: 10.1038/nature11465
34. Bauer H, Horowitz RE, Watkins KC, Popper H. Immunologic competence and phagocytosis in germfree animals with and without stress. *JAMA* (1964) 187:715–8. doi: 10.1001/jama.1964.03060230043011
35. Martin R, Bermudez-Humaran LG, Langella P. Gnotobiotic rodents: an *in vivo* model for the study of microbe-microbe interactions. *Front Microbiol.* (2016) 7:409. doi: 10.3389/fmicb.2016.00409
36. Chung H, Pamp SJ, Hill JA, Surana NK, Edelman SM, Troy EB, et al. Gut immune maturation depends on colonization with a host-specific microbiota. *Cell* (2012) 149:1578–93. doi: 10.1016/j.cell.2012.04.037
37. Altman JD, Moss PA, Goulder PJ, Barouch DH, McHeyzer-Williams MG, Bell JI, et al. Phenotypic analysis of antigen-specific T lymphocytes. *Science* (1996) 274:94–6. doi: 10.1126/science.274.5284.94
38. Sims S, Willberg C, Klenerman P. MHC–peptide tetramers for the analysis of antigen-specific T cells. *Expert Rev Vaccines* (2010) 9:765–74. doi: 10.1586/erv.10.66
39. Yang Y, Torchinsky MB, Gobert M, Xiong H, Xu M, Linehan JL, et al. Focused specificity of intestinal TH17 cells towards commensal bacterial antigens. *Nature* (2014) 510:152–6. doi: 10.1038/nature13279
40. Hepworth MR, Fung TC, Masur SH, Kelsen JR, McConnell FM, Dubrot J, et al. Immune tolerance. Group 3 innate lymphoid cells mediate intestinal selection of commensal bacteria-specific CD4(+) T cells. *Science* (2015) 348:1031–5. doi: 10.1126/science.aaa4812
41. Feng T, Cao AT, Weaver CT, Elson CO, Cong Y. Interleukin-12 converts Foxp3+ regulatory T cells to interferon-gamma-producing Foxp3+ T cells that inhibit colitis. *Gastroenterol.* (2011) 140:2031–43. doi: 10.1053/j.gastro.2011.03.009
42. Xu M, Pokrovskii M, Ding Y, Yi R, Au C, Harrison OJ, et al. c-MAF-dependent regulatory T cells mediate immunological tolerance to a gut pathobiont. *Nature* (2018) 554:373–7. doi: 10.1038/nature25500
43. Ivanov II, Atarashi K, Manel N, Brodie EL, Shima T, Karaoz U, et al. Induction of intestinal Th17 cells by segmented filamentous bacteria. *Cell* (2009) 139:485–98. doi: 10.1016/j.cell.2009.09.033
44. Sczesnak A, Segata N, Qin X, Gevers D, Petrosino JF, Huttenhower C, et al. The genome of th17 cell-inducing segmented filamentous bacteria reveals extensive auxotrophy and adaptations to the intestinal environment. *Cell Host Microbe* (2011) 10:260–72. doi: 10.1016/j.chom.2011.08.005
45. Lathrop SK, Bloom SM, Rao SM, Nutsch K, Lio CW, Santacruz N, et al. Peripheral education of the immune system by colonic commensal microbiota. *Nature* (2011) 478:250–4. doi: 10.1038/nature10434
46. Atarashi K, Tanoue T, Shima T, Imaoka A, Kuwahara T, Momose Y, et al. Induction of colonic regulatory T cells by indigenous Clostridium species. *Science* (2011) 331:337–41. doi: 10.1126/science.1198469
47. Nutsch K, Chai JN, Ai TL, Russler-Germain E, Feehley T, Nagler CR, et al. Rapid and efficient generation of regulatory T cells to commensal antigens in the periphery. *Cell Rep.* (2016) 17:206–20. doi: 10.1016/j.celrep.2016.08.092
48. Cong Y, Feng T, Fujihashi K, Schoeb TR, Elson CO. A dominant, coordinated T regulatory cell-IgA response to the intestinal microbiota. *Proc Natl Acad Sci USA.* (2009) 106:19256–61. doi: 10.1073/pnas.0812681106
49. Hand TW, Dos Santos LM, Bouladoux N, Molloy MJ, Pagan AJ, Pepper M, et al. Acute gastrointestinal infection induces long-lived microbiota-specific T cell responses. *Science* (2012) 337:1553–6. doi: 10.1126/science.1220961
50. Lodes MJ, Cong Y, Elson CO, Mohamath R, Landers CJ, Targan SR, et al. Bacterial flagellin is a dominant antigen in Crohn disease. *J Clin Invest.* (2004) 113:1296–306. doi: 10.1172/JCI200420295
51. Feng T, Wang L, Schoeb TR, Elson CO, Cong Y. Microbiota innate stimulation is a prerequisite for T cell spontaneous proliferation and induction of experimental colitis. *J Exp Med.* (2010) 207:1321–32. doi: 10.1084/jem.20092253
52. Chiaranunt P, Tometich JT, Ji J, Hand TW. T cell proliferation and colitis are initiated by defined intestinal microbes. *J Immunol.* (2018) 201:243–50. doi: 10.4049/jimmunol.1800236
53. Chai JN, Peng Y, Rengarajan S, Solomon BD, Ai TL, Shen Z, et al. Helicobacter species are potent drivers of colonic T cell responses in homeostasis and inflammation. *Sci Immunol.* (2017) 2:eaal5068. doi: 10.1126/sciimmunol.aal5068
54. Knoop KA, Gustafsson JK, McDonald KG, Kulkarni DH, Coughlin PE, McCrate S, et al. Microbial antigen encounter during a preweaning interval is critical for tolerance to gut bacteria. *Sci Immunol.* (2017) 2:eaao1314. doi: 10.1126/sciimmunol.aao1314
55. Atarashi K, Suda W, Luo C, Kawaguchi T, Motoo I, Narushima S, et al. Ectopic colonization of oral bacteria in the intestine drives TH1 cell induction and inflammation. *Science* (2017) 358:359–65. doi: 10.1126/science.aan4526
56. Kobayashi KS, Chamaillard M, Ogura Y, Henegariu O, Inohara N, Nunez G, et al. Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract. *Science* (2005) 307:731–4. doi: 10.1126/science.1104911
57. Vaishnava S, Behrendt CL, Ismail AS, Eckmann L, Hooper LV. Paneth cells directly sense gut commensals and maintain homeostasis at the intestinal host-microbial interface. *Proc Natl Acad Sci USA.* (2008) 105:20858–63. doi: 10.1073/pnas.0808723105
58. Vaishnava S, Yamamoto M, Severson KM, Ruhn KA, Yu X, Koren O, et al. The antibacterial lectin RegIIIgamma promotes the spatial segregation of microbiota and host in the intestine. *Science* (2011) 334:255–8. doi: 10.1126/science.1209791
59. Wang L, Fouts DE, Starkel P, Hartmann P, Chen P, Llorente C, et al. Intestinal REG3 lectins protect against alcoholic steatohepatitis by reducing mucosa-associated microbiota and preventing bacterial translocation. *Cell Host Microbe* (2016) 19:227–39. doi: 10.1016/j.chom.2016.01.003
60. Jijon HB, Suarez-Lopez L, Diaz OE, Das S, De Calisto J, Yaffe MB, et al. Intestinal epithelial cell-specific RARalpha depletion results in aberrant epithelial cell homeostasis and underdeveloped immune system. *Mucosal Immunol.* (2018) 11:703–15. doi: 10.1038/mi.2017.91
61. Zhu J, Yamane H, Paul WE. Differentiation of effector CD4 T cell populations (*). *Annu Rev Immunol.* (2010) 28:445–89. doi: 10.1146/annurev-immunol-030409-101212
62. Hirahara K, Nakayama T. CD4+ T-cell subsets in inflammatory diseases: beyond the Th1/Th2 paradigm. *Int Immunol.* (2016) 28:163–71. doi: 10.1093/intimm/dxw006
63. Askar M. T helper subsets & regulatory T cells: rethinking the paradigm in the clinical context of solid organ transplantation. *Int J Immunogenet.* (2014) 41:185–94. doi: 10.1111/iji.12106
64. Peterson RA. Regulatory T-cells: diverse phenotypes integral to immune homeostasis and suppression. *Toxicol Pathol.* (2012) 40:186–204. doi: 10.1177/0192623311430693
65. Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. *Cell* (2008) 133:775–87. doi: 10.1016/j.cell.2008.05.009
66. Gagliani N, Magnani CF, Huber S, Gianolini ME, Pala M, Licona-Limon P, et al. Coexpression of CD49b and LAG-3 identifies human and mouse T regulatory type 1 cells. *Nat Med.* (2013) 19:739–46. doi: 10.1038/nm.3179
67. Crotty S. A brief history of T cell help to B cells. *Nat Rev Immunol.* (2015) 15:185–9. doi: 10.1038/nri3803
68. Oh SA, Li MO. TGF-beta: guardian of T cell function. *J Immunol.* (2013) 191:3973–9. doi: 10.4049/jimmunol.1301843
69. Watchmaker PB, Lahl K, Lee M, Baumjohann D, Morton J, Kim SJ, et al. Comparative transcriptional and functional profiling defines conserved programs of intestinal DC differentiation in humans and mice. *Nat Immunol.* (2014) 15:98–108. doi: 10.1038/ni.2768
70. Joeris T, Muller-Luda K, Agace WW, Mowat AM. Diversity and functions of intestinal mononuclear phagocytes. *Mucosal Immunol.* (2017) 10:845–64. doi: 10.1038/mi.2017.22
71. Rescigno M, Urbano M, Valzasina B, Francolini M, Rotta G, Bonasio R, et al. Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nat Immunol.* (2001) 2:361–7. doi: 10.1038/86373
72. Chieppa M, Rescigno M, Huang AY, Germain RN. Dynamic imaging of dendritic cell extension into the small bowel lumen in response to epithelial cell TLR engagement. *J Exp Med.* (2006) 203:2841–52. doi: 10.1084/jem.20061884

73. Diehl GE, Longman RS, Zhang JX, Breart B, Galan C, Cuesta A, et al. Microbiota restricts trafficking of bacteria to mesenteric lymph nodes by CX(3)CR1(hi) cells. *Nature* (2013) 494:116–20. doi: 10.1038/nature 11809
74. Persson EK, Uronen-Hansson H, Semmrich M, Rivollier A, Hagerbrand K, Marsal J, et al. IRF4 transcription-factor-dependent CD103(+)CD11b(+) dendritic cells drive mucosal T helper 17 cell differentiation. *Immunity* (2013) 38:958–69. doi: 10.1016/j.immuni.2013.03.009
75. Panea C, Farkas AM, Goto Y, Abdollahi-Roodsaz S, Lee C, Koscso B, et al. Intestinal monocyte-derived macrophages control commensal-specific Th17 responses. *Cell Rep.* (2015) 12:1314–24. doi: 10.1016/j.celrep.2015.07.040
76. Kinnebrew MA, Buffie CG, Diehl GE, Zenewicz LA, Leiner I, Hohl TM, et al. Interleukin 23 production by intestinal CD103(+)CD11b(+) dendritic cells in response to bacterial flagellin enhances mucosal innate immune defense. *Immunity* (2012) 36:276–87. doi: 10.1016/j.immuni.2011.12.011
77. Gury-BenAri M, Thaiss CA, Serafini N, Winter DR, Giladi A, Lara-Astiaso D, et al. The spectrum and regulatory landscape of intestinal innate lymphoid cells are shaped by the microbiome. *Cell* (2016) 166:1231–46.e13. doi: 10.1016/j.cell.2016.07.043
78. Hepworth MR, Monticelli LA, Fung TC, Ziegler CG, Grunberg S, Sinha R, et al. Innate lymphoid cells regulate CD4+ T-cell responses to intestinal commensal bacteria. *Nature* (2013) 498:113–7. doi: 10.1038/nature 12240
79. Weiner HL, da Cunha AP, Quintana F, Wu H. Oral tolerance. *Immunol Rev.* (2011) 241:241–59. doi: 10.1111/j.1600-065X.2011.01017.x
80. Legoux FP, Lim JB, Cauley AW, Dikiy S, Ertelt J, Mariani TJ, et al. CD4+ T cell tolerance to tissue-restricted self antigens is mediated by antigen-specific regulatory T cells rather than deletion. *Immunity* (2015) 43:896–908. doi: 10.1016/j.immuni.2015.10.011
81. Ligocki AJ, Niederkorn JY. Advances on non-CD4 + Foxp3+ T regulatory cells: CD8+, type 1, and double negative T regulatory cells in organ transplantation. *Transplantation* (2015) 99:1553–9. doi: 10.1097/TP.0000000000000813
82. Josefowicz SZ, Lu LF, Rudensky AY. Regulatory T cells: mechanisms of differentiation and function. *Annu Rev Immunol.* (2012) 30:531–64. doi: 10.1146/annurev.immunol.25.022106. 141623
83. Commins SP. Mechanisms of oral tolerance. *Pediatr Clin North Am.* (2015) 62:1523–9. doi: 10.1016/j.pcl.2015.07.013
84. Johansson-Lindbom B, Svensson M, Pabst O, Palmqvist C, Marquez G, Forster R, et al. Functional specialization of gut CD103+ dendritic cells in the regulation of tissue-selective T cell homing. *J Exp Med.* (2005) 202:1063–73. doi: 10.1084/jem.20051100
85. Worbs T, Bode U, Yan S, Hoffmann MW, Hintzen G, Bernhardt G, et al. Oral tolerance originates in the intestinal immune system and relies on antigen carriage by dendritic cells. *J Exp Med.* (2006) 203:519–27. doi: 10.1084/jem.20052016
86. Sun CM, Hall JA, Blank RB, Bouladoux N, Oukka M, Mora JR, et al. Small intestine lamina propria dendritic cells promote *de novo* generation of Foxp3 T reg cells via retinoic acid. *J Exp Med.* (2007) 204:1775–85. doi: 10.1084/jem.20070602
87. Jaensson E, Uronen-Hansson H, Pabst O, Eksteen B, Tian J, Coombes JL, et al. Small intestinal CD103+ dendritic cells display unique functional properties that are conserved between mice and humans. *J Exp Med.* (2008) 205:2139–49. doi: 10.1084/jem.20080414
88. Cassani B, Villablanca EJ, De Calisto J, Wang S, Mora JR. Vitamin A and immune regulation: role of retinoic acid in gut-associated dendritic cell education, immune protection and tolerance. *Mol Aspects Med.* (2012) 33:63–76. doi: 10.1016/j.mam.2011.11.001
89. Hadis U, Wahl B, Schulz O, Hardtke-Wolenski M, Schippers A, Wagner N, et al. Intestinal tolerance requires gut homing and expansion of FoxP3+ regulatory T cells in the lamina propria. *Immunity* (2011) 34:237–46. doi: 10.1016/j.immuni.2011.01.016
90. Villablanca EJ. Retinoic acid-producing DCs and gut-tropic FOXP3(+) regulatory T cells in the induction of oral tolerance. *Oncoimmunology* (2013) 2:e22987.doi: 10.4161/onci.22987
91. Atarashi K, Tanoue T, Oshima K, Suda W, Nagano Y, Nishikawa H, et al. Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota. *Nature* (2013) 500:232–6. doi: 10.1038/nature 12331
92. Pabst O, Mowat AM. Oral tolerance to food protein. *Mucosal Immunol.* (2012) 5:232–9. doi: 10.1038/mi.2012.4
93. Cebula A, Seweryn M, Rempala GA, Pabla SS, McIndoe RA, Denning TL, et al. Thymus-derived regulatory T cells contribute to tolerance to commensal microbiota. *Nature* (2013) 497:258–62. doi: 10.1038/nature 12079
94. Ai TL, Solomon BD, Hsieh C. T-cell selection and intestinal homeostasis. *Immunol Rev.* (2014) 259:60–74. doi: 10.1111/imr.12171
95. Niess JH, Leithäuser F, Adler G, Reimann J. Commensal gut flora drives the expansion of proinflammatory CD4 T cells in the colonic lamina propria under normal and inflammatory conditions. *J Immunol.* (2008) 180:559–68. doi: 10.4049/jimmunol.180.1.559
96. Asseman C, Read S, Powrie F. Colitogenic Th1 cells are present in the antigen-experienced T cell pool in normal mice: control by CD4 regulatory T cells and IL-10. *J Immunol.* (2003) 171:971–8. doi: 10.4049/jimmunol.171.2.971
97. Omenetti S, Pizarro TT. The Treg/Th17 axis: a dynamic balance regulated by the gut microbiome. *Front Immunol.* (2015) 6:639. doi: 10.3389/fimmu.2015.00639
98. Ivanov II, de Llanos Frutos R, Manel N, Yoshinaga K, Rifkin DB, Sartor RB, et al. Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine. *Cell Host Microbe* (2008) 4:337–49. doi: 10.1016/j.chom.2008. 09.009
99. Homann D, Teyton L, Oldstone MB. Differential regulation of antiviral T-cell immunity results in stable CD8 but declining CD4 T-cell memory. *Nat Med.* (2001) 7:913–9. doi: 10.1038/90950
100. Lee SH, Cho M. Immunological pathogenesis of inflammatory bowel disease. *Intestinal Res.* (2018) 16:26–42. doi: 10.5217/ir.2018.16.1.26
101. Palm NW, Medzhitov R. Pattern recognition receptors and control of adaptive immunity. *Immunol Rev.* (2009) 227:221–33. doi: 10.1111/j.1600-065X.2008.00731.x
102. Meyer-Hoffert U, Hornef MW, Henriques-Normark B, Axelsson LG, Midtvedt T, Putsep K, et al. Secreted enteric antimicrobial activity localises to the mucus surface layer. *Gut* (2008) 57:764–71. doi: 10.1136/gut.2007.141481
103. Hooper LV, Littman DR, Macpherson AJ. Interactions between the microbiota and the immune system. *Science* (2012) 336:1268–73. doi: 10.1126/science.1223490
104. Atarashi K, Tanoue T, Ando M, Kamada N, Nagano Y, Narushima S, et al. Th17 *Cell* induction by adhesion of microbes to intestinal epithelial cells. *Cell* (2015) 163:367–80. doi: 10.1016/j.cell.2015.08.058
105. Hermiston ML, Gordon JI. Inflammatory bowel disease and adenomas in mice expressing a dominant negative N-cadherin. *Science* (1995) 270:1203–7. doi: 10.1126/science.270.5239.1203
106. Heazlewood CK, Cook MC, Eri R, Price GR, Tauro SB, Taupin D, et al. Aberrant mucin assembly in mice causes endoplasmic reticulum stress and spontaneous inflammation resembling ulcerative colitis. *PLoS Med.* (2008) 5:e54. doi: 10.1371/journal.pmed.0050054
107. Schenk M, Mueller C. Adaptations of intestinal macrophages to an antigen-rich environment. *Semin Immunol.* (2007) 19:84–93. doi: 10.1016/j.smim.2006.09.002
108. Macpherson AJ, Uhr T. Compartmentalization of the mucosal immune responses to commensal intestinal bacteria. *Ann NY Acad Sci.* (2004) 1029:36–43. doi: 10.1196/annals.1309.005
109. Zimmermann K, Haas A, Oxenius A. Systemic antibody responses to gut microbes in health and disease. *Gut Microbes* (2012) 3:42–7. doi: 10.4161/gmic.19344
110. Heimesaat MM, Bereswill S, Fischer A, Fuchs D, Struck D, Niebergall J, et al. Gram-negative bacteria aggravate murine small intestinal Th1-type immunopathology following oral infection with *Toxoplasma gondii*. *J Immunol.* (2006) 177:8785–95. doi: 10.4049/jimmunol.177. 12.8785
111. Meinzer U, Barreau F, Esmiol-Welterlin S, Jung C, Villard C, Léger T, et al. Yersinia pseudotuberculosis effector YopJ subverts the Nod2/RICK/TAK1 pathway and activates caspase-1 to induce intestinal barrier dysfunction. *Cell Host Microbe* (2012) 11:337–51. doi: 10.1016/j.chom.2012.02.009

112. Fonseca DM, Hand TW, Han SJ, Gerner MY, Glatman Zaretsky A, Byrd AL, et al. Microbiota-dependent sequelae of acute infection compromise tissue-specific immunity. *Cell* (2015) 163:354–66. doi: 10.1016/j.cell.2015.08.030
113. Berer K, Mues M, Koutrolos M, Rasbi ZA, Boziki M, Johner C, et al. Commensal microbiota and myelin autoantigen cooperate to trigger autoimmune demyelination. *Nature* (2011) 479:538–41. doi: 10.1038/nature10554
114. Cosorich I, Dalla-Costa G, Sorini C, Ferrarese R, Messina MJ, Dolpady J, et al. High frequency of intestinal TH17 cells correlates with microbiota alterations and disease activity in multiple sclerosis. *Sci Adv.* (2017) 3:e1700492. doi: 10.1126/sciadv.1700492
115. Heintz-Buschart A, Pandey U, Wicke T, Sixel-Doring F, Janzen A, Sittig-Wiegand E, et al. The nasal and gut microbiome in Parkinson's disease and idiopathic rapid eye movement sleep behavior disorder. *Mov Disord.* (2018) 33:88–98. doi: 10.1002/mds.27105
116. Sampson TR, Debelius JW, Thron T, Janssen S, Shastri GG, Ilhan ZE, et al. Gut microbiota regulate motor deficits and neuroinflammation in a model of Parkinson's Disease. *Cell* (2016) 167:1469.e12–80.e12. doi: 10.1016/j.cell.2016.11.018
117. Brugman S, Klatter FA, Visser JT, Wildeboer-Veloo AC, Harmsen HJ, Rozing J, et al. Antibiotic treatment partially protects against type 1 diabetes in the bio-breeding diabetes-prone rat. Is the gut flora involved in the development of type 1 diabetes? *Diabetologia* (2006) 49:2105–8. doi: 10.1007/s00125-006-0334-0
118. Wu HJ, Ivanov II, Darce J, Hattori K, Shima T, Umesaki Y, et al. Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells. *Immunity* (2010) 32:815–27. doi: 10.1016/j.immuni.2010.06.001
119. Geuking MB, Cahenzli J, Lawson MA, Ng DC, Slack E, Hapfelmeier S, et al. Intestinal bacterial colonization induces mutualistic regulatory T cell responses. *Immunity* (2011) 34:794–806. doi: 10.1016/j.immuni.2011.03.021
120. Powrie F, Correa-Oliveira R, Mauze S, Coffman RL. Regulatory interactions between CD45RBhigh and CD45RBlow CD4+ T cells are important for the balance between protective and pathogenic cell-mediated immunity. *J Exp Med.* (1994) 179:589–600. doi: 10.1084/jem.179.2.589
121. Stepankova R, Powrie F, Kofronova O, Kozakova H, Hudcovic T, Hrncir T, et al. Segmented filamentous bacteria in a defined bacterial cocktail induce intestinal inflammation in SCID mice reconstituted with CD45RBhigh CD4+ T cells. *Inflamm Bowel Dis.* (2007) 13:1202–11. doi: 10.1002/ibd.20221
122. Duck LW, Walter MR, Novak J, Kelly D, Tomasi M, Cong Y, et al. Isolation of flagellated bacteria implicated in Crohn's disease. *Inflamm Bowel Dis.* (2007) 13:1191–201. doi: 10.1002/ibd.20237
123. Wu W, Liu HP, Chen F, Liu H, Cao AT, Yao S, et al. Commensal A4 bacteria inhibit intestinal Th2-cell responses through induction of dendritic cell TGF-beta production. *Eur J Immunol.* (2016) 46:1162–7. doi: 10.1002/eji.201546160
124. Annunziato F, Cosmi L, Santarlasci V, Maggi L, Liotta F, Mazzinghi B, et al. Phenotypic and functional features of human Th17 cells. *J Exp Med.* (2007) 204:1849–61. doi: 10.1084/jem.20070663
125. Huber S, Gagliani N, Zenewicz LA, Huber FJ, Bosurgi L, Hu B, et al. IL-22BP is regulated by the inflammasome and modulates tumorigenesis in the intestine. *Nature* (2012) 491:259–63. doi: 10.1038/nature11535
126. Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. *Nature* (2008) 454:436–44. doi: 10.1038/nature07205
127. O'Mahony L, Feeney M, O'Halloran S, Murphy L, Kiely B, Fitzgibbon J, et al. Probiotic impact on microbial flora, inflammation and tumour development in IL-10 knockout mice. *Aliment Pharmacol Ther.* (2001) 15:1219–25. doi: 10.1046/j.1365-2036.2001.01027.x
128. Caminero A, Galipeau HJ, McCarville JL, Johnston CW, Bernier SP, Russell AK, et al. Duodenal bacteria from patients with celiac disease and healthy subjects distinctly affect gluten breakdown and immunogenicity. *Gastroenterology* (2016) 151:670–83. doi: 10.1053/j.gastro.2016.06.041
129. Taurog JD, Richardson JA, Croft JT, Simmons WA, Zhou M, Fernandez-Sueiro JL, et al. The germfree state prevents development of gut and joint inflammatory disease in HLA-B27 transgenic rats. *J Exp Med.* (1994) 180:2359–64. doi: 10.1084/jem.180.6.2359
130. Yoshitomi H, Sakaguchi N, Kobayashi K, Brown GD, Tagami T, Sakihama T, et al. A role for fungal {beta}-glucans and their receptor Dectin-1 in the induction of autoimmune arthritis in genetically susceptible mice. *J Exp Med.* (2005) 201:949–60. doi: 10.1084/jem.20041758

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REVIEW
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Origin, Differentiation, and Function of Intestinal Macrophages

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Macrophages are increasingly recognized as essential players in the maintenance of intestinal homeostasis and as key sentinels of the intestinal immune system. However, somewhat paradoxically, they are also implicated in chronic pathologies of the gastrointestinal tract, such as inflammatory bowel disease (IBD) and are therefore considered potential targets for novel therapies. In this review, we will discuss recent advances in our understanding of intestinal macrophage heterogeneity, their ontogeny and the potential factors that regulate their origin. We will describe how the local environment of the intestine imprints the phenotypic and functional identity of the macrophage compartment, and how this changes during intestinal inflammation and infection. Finally, we highlight key outstanding questions that should be the focus of future research.

Keywords: macrophage, monocyte, intestine, inflammation, colitis, ontogeny

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INTRODUCTION

The gastrointestinal tract faces an unrivaled exposure to foreign antigens and, as a result, is home to the largest compartment of the immune system. This includes a network of mononuclear phagocytes (MPs), including macrophages and conventional dendritic cells (cDCs), that play distinct yet complementary roles in discriminating between innocuous antigens and potential pathogens, ensuring that the appropriate response is mounted to each. While this is a highly efficient process, it can break down in some individuals, leading to the development of chronic inflammation, such as inflammatory bowel disease (IBD) in which inappropriate immune responses are mounted against the commensal microbiota. Thus, there is great interest in understanding the biology of intestinal MPs. The role of macrophages in health and disease has attracted particular attention, as their plasticity and wound healing capabilities make them attractive targets for potential novel therapies to treat IBD. In this article, we will first discuss the current understanding of macrophage heterogeneity in the gut wall, before describing the roles macrophages play in intestinal homeostasis and how this may depend on their anatomical positioning. We will then review the recent developments in intestinal macrophage ontogeny, discussing how the local environment of the gut imprints the phenotypic and functional identity of macrophages, before finally describing the changes that occur when homeostasis is perturbed by inflammation.

IDENTIFYING MACROPHAGES IN THE GUT WALL

One of the major issues that has stifled our progress on understanding the immunobiology of intestinal macrophages is their inaccurate identification. For instance, although murine macrophages have traditionally been identified based on their expression of the pan-macrophage

marker F4/80 (1), it is clear that other cells, such as conventional dendritic cells (cDCs) and eosinophils can express F4/80 to some extent (2, 3). Furthermore, many macrophages, including those in the intestine, express high levels of CD11c and MHCII, markers that have classically been used to identify cDCs (3). Thus, the identification of intestinal macrophages requires a multi-parameter approach. The Mer tyrosine kinase (MerTK) and the high affinity FcγR1 (CD64), have emerged as superior markers for the identification of macrophages across different tissues (4–6), the latter also being useful across species (7–10). When used in combination with CD11c and MHCII, CD64 expression distinguishes macrophages from *bona fide* cDC in the gut wall (5, 10, 11). This is corroborated by the distinct growth factor dependency and migration patterns of CD64-defined MPs. Whereas $CD64^+$ MPs are highly dependent on colony stimulating factor 1 (CSF1; also known as M-CSF) for their development and/or survival, $CD64^-$ $CD11c^+$ $MHCII^+$ MPs, but not $CD64^+$ MPs, are highly dependent on the cDC-specific growth factor Flt3L (10, 11). Consistently, $CD64^-$ $CD11c^+MHCII^+$ MPs have been shown to continually migrate to the mesenteric lymph nodes in a CCR7-dependent manner to participate in T cell priming (10, 12–14), defining features of cDC. In contrast, $CD64^+$ MPs are non-migratory and display characteristic macrophage morphology, with abundant cytoplasm and cytoplasmic vacuoles (9, 15, 16). Thus, by multiple criteria, $CD64^-$ $CD11c^+$ $MHCII^+$ MPs and $CD64^+$ MPs fit the definition of cDC and macrophages, respectively. One additional feature of murine intestinal macrophages that distinguishes them from cDC is their high expression of the chemokine receptor CX3CR1 (9, 15, 17–20). Indeed, by using *Cx3cr1*$^{+/gfp}$ knock-in mice (21), mature $CX3CR1^{hi}$ macrophages can be visualized throughout the lamina propria (LP), the large layer of connective tissue underlying the epithelium, as well as in the deeper layers of the gut wall, such as the submucosa and muscularis (17, 19, 20). Macrophages in these distinct sites are reported to express differential levels of CD11c, with $CD11c^+$ and $CD11c^{-/lo}$ $CX3CR1^{hi}$ macrophages enriched in the LP and muscularis, respectively (18, 20, 22). As discussed in more detail below, additional heterogeneity has been unmasked recently by transcriptional profiling, with discrete subsets of $CX3CR1^{hi}$ macrophages identifiable based on their expression of CD4 and Tim4 (23, 24).

The recent advances in multi-parameter analysis have also led to the much-needed alignment of analysis of murine and human tissue macrophages. The use of markers such as CD64 and CD14 has meant that the same cells can be characterized across species (7, 9, 10, 25). This has highlighted similarities, but also important differences between mouse and man. For instance, expression of CD4, CD163, CD172a (SIRPα), and CD206 are conserved features of intestinal macrophages across species (7, 9, 23, 24, 26). However, mature intestinal macrophages in humans express only low levels of the CX3CR1 and CD11c markers found on their murine equivalents. Very recent work has also described potential phenotypic heterogeneity between human LP and muscularis macrophages, with the latter expressing higher levels of CD14 and CD11b (7).

FUNCTIONS OF MACROPHAGES IN INTESTINAL HOMEOSTASIS

Macrophages play a variety of roles to maintain intestinal homeostasis (**Figure 1**). Like their counterparts in other tissues, macrophages in the gut wall are avidly phagocytic. However, while being highly bactericidal, phagocytosis by intestinal macrophages does not result in an overt inflammatory response in both mouse and man (see below)(7, 9, 25, 27, 28). Consistent with this role, intestinal macrophages display high expression of genes associated with phagocytosis, such as *Mertk*, *Cd206*, *Gas6*, *Axl*, *Cd36*, *Itgav,* and *Itgb5* (23, 29). Integrins αv and β5 dimerise to form αvβ5, which is involved in the uptake of apoptotic cells (29), a process known as efferocytosis (30). Notably, *Lys2*-directed deletion of integrin αv results in the accumulation of apoptotic cells in the intestine (31), and *Itgb5* deficiency predisposes to increased susceptibility to DSS-induced colitis (29), highlighting a particularly important role for this pathway in this process.

The sub-epithelial positioning of LP macrophages means they are ideally placed to capture and eliminate any bacteria that cross the epithelial barrier. In addition, murine studies have shown that they are able to sample luminal bacteria, involving the formation of transepithelial dendrites (TEDs), cellular processes that cross the epithelial barrier without perturbing tight junctions and epithelial integrity and depend on the CX3CL1-CX3CR1 axis (32–34). A similar process may allow mature $CX3CR1^{hi}$ macrophages in the upper small bowel to capture dietary materials and is suggested to be involved in the generation of oral tolerance to dietary antigens (35). This requires the induction of antigen specific Tregs in the gut draining mesenteric lymph nodes with gut homing properties (36, 37). Given that $CX3CR1^{hi}$ macrophages do not migrate to draining lymph nodes under normal conditions and naïve T cells are essentially absent in the LP (13, 15, 38), they are unlikely to play a major role in this process. However, they may contribute to the induction of oral tolerance through antigen transfer to migratory $CD103^+$ DC via connexin-43-dependent gap junctions (35). Indeed, mice that lack connexin-43 in $CD11c^+$ cells fail to develop oral tolerance (35). Macrophages have also been proposed to regulate oral tolerance development by supporting Treg maintenance locally in the mucosa (37, 39, 40). This is thought to involve macrophage-derived IL10, as *Cx3cr1*-mediated deletion of IL10 reduces antigen specific Treg frequencies in a model of oral tolerance (37, 40). Interestingly however, deletion of IL10 in macrophages appears to have no impact on the overall abundance of endogenous Treg (40, 41). In addition to Tregs, macrophages may also support the induction/maintenance of commensal-specific Th17 cells through IL1β secretion (42, 43). Notably, whether maintenance of mucosal T cells also involves cognate interactions remains to be determined with certainty. Although macrophages are very poor in activating naive T cells compared to DCs (15), their high expression of MHCII suggests that they might be involved in antigen presentation to previously activated T cells locally in the intestine. By doing so, they could maintain or promote further differentiation of antigen-specific T

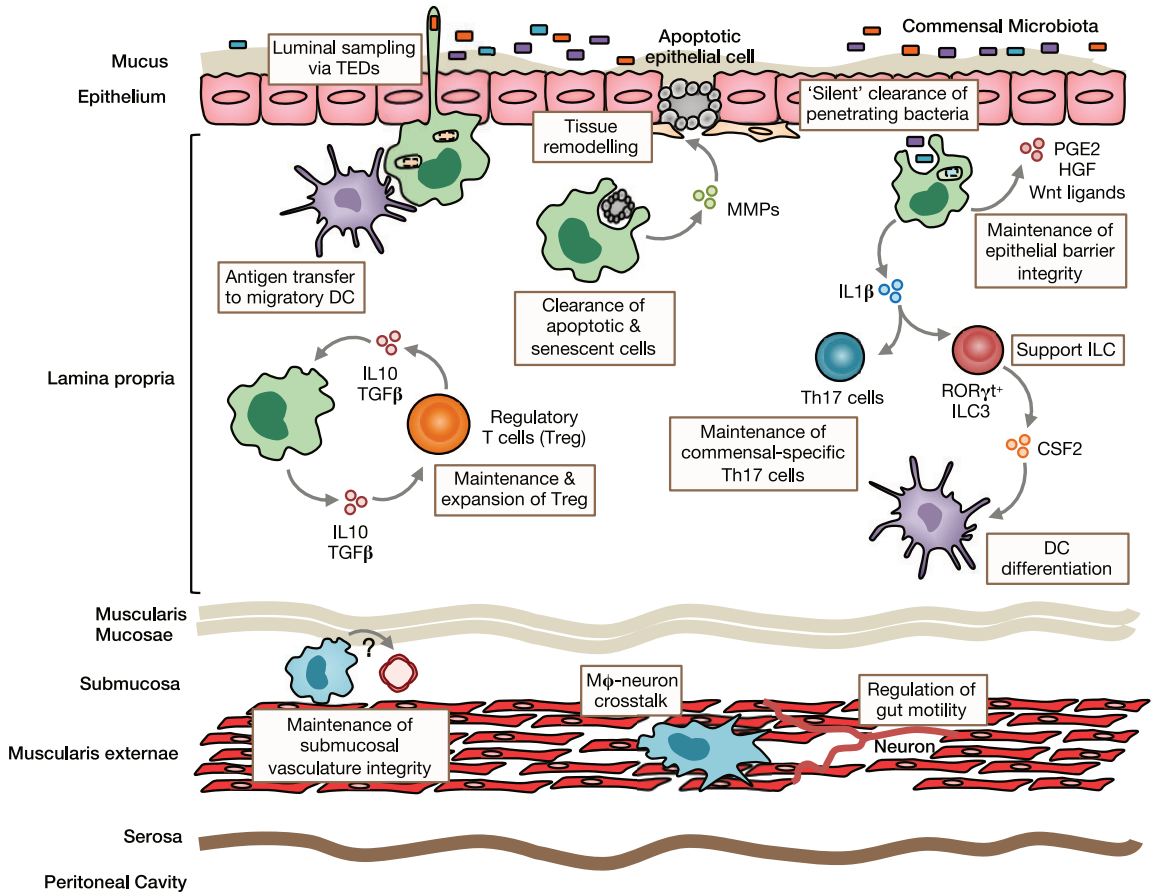

FIGURE 1 | Homeostatic functions of intestinal macrophages. Intestinal lamina propria (LP) macrophages are highly phagocytic and are responsible for clearing apoptotic and senescent epithelial cells. Through their expression of tissue-remodeling metalloproteinases and secretion of factors that stimulate epithelial stem cell renewal, such as prostaglandin E2 (PGE2), hepatocyte growth factor (HGF) and Wnt ligands, they promote epithelial integrity. Their position under the epithelial monolayer and their bactericidal activity, mean LP macrophages are ideally placed to capture and destroy any bacteria that breach the barrier. They may also send cellular processes across the epithelial barrier to sample luminal contents. Macrophages can transfer acquired antigen to migratory dendritic cells (DCs) for presentation to T cells in the draining mesenteric lymph nodes. Through their production of immunoregulatory cytokines, such as IL10 and TGFβ, they maintain and facilitate secondary expansion of regulatory T cells (Tregs) locally in the LP. In a similar manner, they support Th17 cells and ILC3s through their production of IL1β, which is induced by exposure to the microbiota or its derivatives. Macrophages are also present in deeper layers of the gut wall, including the submucosa and muscularis externae. Submucosa macrophages are thought to support the integrity of the submucosal vasculature, although the factors involved in this interaction remain unclear. Muscularis macrophages participate in bidirectional crosstalk with sympathetic neurons of the enteric nervous system and influence gut motility.

cells. Consistent with this, *Cx3cr1*-mediated deletion of MHCII retards the generation/maintenance of antigen-specific Treg after feeding of ovalbumin (OVA) (40). Macrophages may also influence T cell priming indirectly through their effects on cDC differentiation. For instance, secretion of IL1β has been shown to enhance ILC3 production of CSF2 (44), which is known to control cDC differentiation in the intestinal LP (45). Notably, most functional analyses have been performed in mice and whether human intestinal macrophages carry out the same roles remains unclear.

While it has been known for many years that macrophages are present in deeper layers of the gut wall (46), only recently has work begun to interrogate their role in intestinal homeostasis. Macrophages in the muscularis are intimately associated with the enteric nervous system and, in mice, appear morphologically and transcriptionally distinct (47). There is bidirectional crosstalk between muscularis macrophages and neurons, where macrophage-derived bone morphogenic protein 2 (BMP2) acts on the BMP receptor (BMPR) expressed by enteric neurons to induce secretion of CSF1, which maintains the muscularis macrophage compartment and stimulates further BMP2 expression (20, 22). These interactions regulate smooth muscle contractions, thereby controlling peristalsis, and can be disrupted by broad spectrum antibiotics (22), suggesting the microbiota may regulate gut motility to some extent (48). Macrophages are also found in the submucosa and recent depletion studies have revealed a role for these cells in maintaining the integrity of the submucosal vasculature (47).

Thus, macrophages fulfill niche-specific functions to meet the local demands of their microenvironment.

ORIGIN OF INTESTINAL MACROPHAGES

There have been major developments in our understanding of macrophage ontogeny in recent years [see (49) for review]. Traditionally, it was proposed that tissue macrophages were derived from blood monocytes that were replenished in turn by highly proliferative bone marrow (BM) progenitors as part of a linear mononuclear phagocyte system (MPS) (50, 51). Geissmann et al. (52) then refined this model demonstrating that the murine monocyte compartment, like its human counterpart (53), is heterogeneous, with subsets defined on the basis of Ly6C expression. In this scheme, Ly6C^{hi} monocytes were shown to preferentially enter tissues under inflammatory conditions, leading to them being described as "inflammatory" monocytes (52). However, as discussed below, Ly6C^{hi} monocytes can also be found in healthy tissues and tend to fulfill the functions classically ascribed to monocytes, therefore these are now referred to as "classical" monocytes. Because they did not enter inflamed tissues, it was proposed that Ly6C^{lo} monocytes were the precursors of tissue resident macrophages (52). However, adoptively transferred Ly6C^{lo} monocytes rarely enter healthy tissues, even following diphtheria toxin (DT)-mediated depletion of resident macrophages (9, 17). Moreover, recent work has shown that a major function of 'non-classical' Ly6C^{lo} monocytes is to patrol the vasculature and scavenge necrotic endothelial cells (54) rather than acting as a circulating intermediate. Thus, in some respects, Ly6C^{lo} monocytes could be thought of as macrophages of the circulatory system.

Rather than deriving from blood monocytes, recent elegant fate mapping techniques have shown that many tissue macrophages exist independently from conventional haematopoiesis and instead derive from embryonic precursors arising from the yolk sac and/or fetal liver (55–59). For instance, microglia of the central nervous system and epidermal Langerhans cells appear to maintain themselves autonomously through intrinsic longevity and *in situ* self-renewal throughout adult life (60–64). In contrast, we have shown that although the intestine is initially seeded by embryo-derived macrophages, these are subsequently displaced with age by cells deriving from conventional haematopoiesis (16). Consistently, colonic macrophages, but not microglia or Langerhans cells, are labeled in genetic fate mapping studies exploiting *Flt3* or *Kit* expression to fate map cells deriving from haematopoietic stem cells (HSCs) (16, 55, 59, 65). Moreover, intestinal macrophages are largely replaced by donor cells in the setting of parabiosis and in tissue-protected bone marrow chimeric mice, unlike many other tissue macrophages (16, 56, 66–69). The finding that macrophage numbers are reduced in the gut wall of unmanipulated adult *Ccr2*$^{-/-}$ mice (9), in whom classical Ly6C^{hi} monocyte egress from BM is defective (70), implies that Ly6C^{hi} and not Ly6C^{lo} monocytes are the main precursors of intestinal macrophages in adulthood. In line with this, adoptively transferred classical Ly6C^{hi} monocytes, but not Ly6C^{lo} monocytes, give rise to fully mature intestinal macrophages (9, 17, 19, 71). Furthermore, intestinal macrophages are eliminated by repetitive administration of DT to CCR2-DTR transgenic mice (43, 72), again indicating that the macrophage pool relies on CCR2-dependent replenishment. Notably, as well as its role in BM egress, homeostatic extravasation of Ly6C^{hi} monocytes from the bloodstream into the intestinal mucosa relies on the CCL2-CCR2 axis. This is demonstrated by the failure of both WT monocytes to enter the colonic mucosa of *Ccl2*-deficient mice and *Ccr2*-deficient monocytes to enter the mucosa of WT mice in mixed BM chimeras or in the setting of parabiosis (5, 16, 73).

It is now clear that a monocyte to macrophage differentiation continuum exists in the intestinal LP, a process that has become known as the monocyte "waterfall" (5, 9) (**Figure 2**). At one end are Ly6C^{hi} CX3CR1^{int} MHCII^{-} ("P1") monocytes that appear phenotypically and morphologically similar to their counterparts in blood. Indeed, monocytes in the mucosa retain expression of molecules involved in chemotaxis and extravasation from the circulation, such as CCR2, CD62L, VLA-1, LFA-1, and of course Ly6C (23). These monocytes first acquire MHCII expression ('P2' monocytes), before downregulating Ly6C, and the other markers of extravasation ("P3"macrophages), and finally upregulating CX3CR1 to give rise to fully mature ("P4") macrophages; this process takes around 5–6 days and involves major gene expression changes (5, 9, 23). Importantly, there is mounting evidence that an analogous "waterfall" is present in the human intestinal mucosa, with classical CD14^{hi}CCR2^{+}CD11c^{hi} monocytes at one end and mature CD14^{lo}CCR2^{-}CD11c^{lo} macrophages at the other (7, 9, 25) (**Figure 2**). In support of this, Bujko et al. have recently used HLA-mismatched duodenal transplants to measure turnover of intestinal macrophages in man, showing that donor CD14^{hi}CCR2^{+}CD11c^{hi} cells in the graft, which are analogous to P1/P2 cells in mouse, are rapidly replaced by recipient cells. Mature macrophages are also replaced by recipient-derived cells, albeit at slower rates (7). This contrasts markedly with Langerhans cells of the skin epidermis, which have been shown to remain of graft origin in transplanted skin for at least up to 10 years (74). Similarly, alveolar macrophages persist for up to 2 years in transplanted lungs (75). Thus, the limited data available from human transplant studies support the findings from fate mapping studies in mice (64, 65, 69, 76).

Despite the evidence that intestinal macrophages are derived from continuous replenishment by extravasating monocytes, this idea may need to be refined on the basis of very recent findings that long-lived macrophages may be present in the adult intestine (24, 47). Longitudinal fate-mapping using *Cx3cr1*-based strategies and tissue-protected BM chimeric mice have identified macrophages that persist for longer than 8 months in the gut wall (47). Two independent studies showed that long-lived macrophages can be identified by Tim4 and CD4 expression (24, 47), which are unaffected by *Ccr2* deficiency, unlike most of their Tim4^{-} counterparts (24). This is consistent with the long-lived nature of Tim4-expressing macrophages in other tissues, such as the liver and the peritoneal cavity (69, 77). Notably, De Schepper and colleagues (47) showed that long-lived macrophages were predominantly found in the deeper

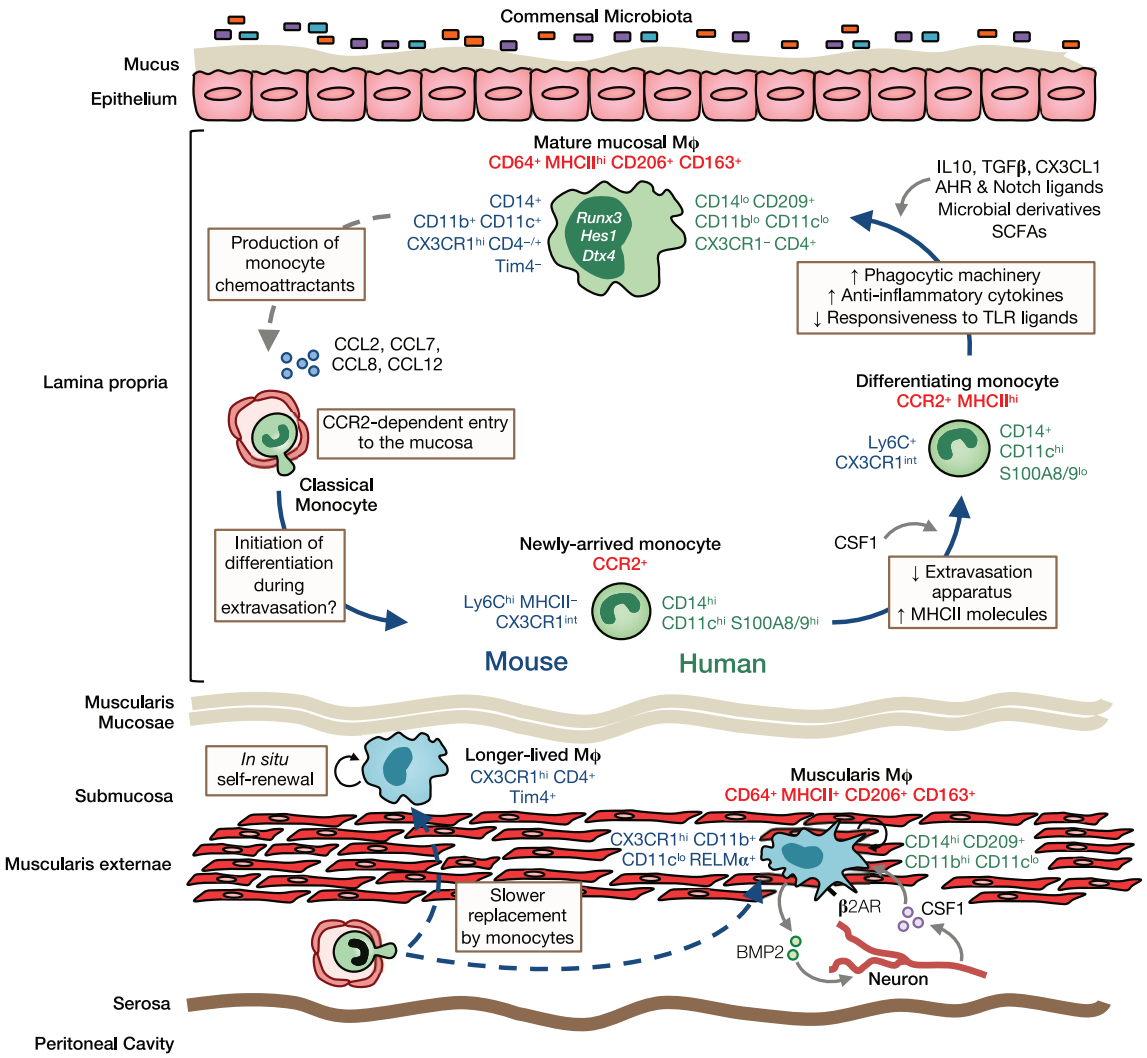

FIGURE 2 | Heterogeneity, origin and differentiation of intestinal macrophages. The majority of mucosal macrophages are replenished by classical monocytes that enter the mucosa in a CCR2-dependent manner and differentiate through a series of intermediaries (mouse-and human-specific markers denoted in blue and green, respectively) to give rise to mature macrophages, which can be identified in both mouse and man as CD64^{+}MHCII^{hi} CD206^{+}CD163^{+} cells (common markers denoted in red). In addition, high levels of CD11b, CD11c, CD14, and CX3CR1 are characteristic features of murine LP macrophages. In contrast, human LP macrophages express only low levels of most of these markers but express high levels of CD209. Once in the mucosa and under cues from the local environment, monocytes first upregulate MHCII and downregulate molecules involved in extravasation, such as CCR2, LFA-1 and CD62L. They then upregulate phagocytic receptors and increase their production of anti-inflammatory cytokines, as well as becoming hyporesponsive to stimulation through e.g., TLRs. Studies in mice have identified IL10, TGFβ, and CX3CL1 as key factors in promoting macrophage differentiation in the healthy mucosa. Furthermore, exposure to the microbiota and its metabolites is known to influence macrophage differentiation and the rate of their turnover in the LP. Mature macrophages may also regulate their own turnover through secretion of monocyte chemoattractants, such as CCL2, CCL7, CCL8, and CCL12. Longer-lived macrophages may also exist in the murine intestinal mucosa and submucosa, and can be identified by their expression of the phagocytic receptor Tim4. While sharing certain features with their LP counterparts, such as CD64, MHCII, CD206, and CD163 expression, muscularis macrophages have a relatively distinct phenotype. In mouse, they express low levels of CD11c but high levels of the immunoregulatory cytokine RELMα, whereas in man, they have high levels of CD14 and CD11b. Muscularis macrophages are acutely dependent on CSF1 and norepinephrine signaling by sympathetic neurons via β2 adrenergic receptors (β2AR) shapes their differentiation. Monocytes also replenish macrophages of the muscularis, although the rate of replenishment is slower than in the mucosa and a larger proportion of these macrophages are long-lived.

layers of the gut wall, such as the muscularis and submucosa, whereas mucosal macrophages showed high levels of turnover from BM. Importantly, this group also showed that long-lived macrophages derived from both embryonic and BM-derived cells, demonstrating that intrinsic longevity is not an exclusive property of embryo-derived macrophages (47). Thus, in light of these findings, it is clear that the origin of intestinal macrophages is highly dynamic, with embryonic and BM-derived macrophages present alongside one another in each layer of the gut wall, the proportions of which change markedly with age and microbial colonization in a niche-specific manner (see below). Indeed, this brings the gut into line with other tissues, such as the heart

(58, 78, 79), lung (65, 69, 80), dermis (67), and the peritoneal cavity (69, 81, 82) where short-lived and long-lived macrophages co-exist.

Here it should also be noted that it has never been shown definitely that patrolling Ly6C^{lo} monocytes cannot contribute to gut macrophage replenishment. One approach has been to assess macrophage abundance in the gut wall of mice in whom Ly6C^{lo} blood monocytes are markedly reduced, for instance those deficient in *Cx3cr1* (37, 83). However, different groups have reached discordant conclusions on the effect of *Cx3cr1* deficiency on intestinal macrophage numbers (37, 83). Furthermore, given the high expression of CX3CR1 by intestinal macrophages themselves, effects of *Cx3cr1* deficiency may be due to altered differentiation and/or survival of mature macrophages rather than indicating derivation from Ly6C^{lo} monocytes (see below). More recent work has identified *Nr4a1* as a master regulator of Ly6C^{lo} monocyte differentiation and survival (84). New tools that specifically target *Nr4a1* deficiency to monocytes, while sparing its roles in macrophage function, such as those described recently by Hedrick and colleagues (85), will be critical to assess the role of Ly6C^{lo} monocytes in tissue macrophage replenishment under normal physiological conditions and if this changes in the context of disease.

What Controls the Origin of Intestinal Macrophages?

The exact factors that determine why different tissues contain macrophages of distinct origins remain very poorly understood. Specifically, it is unknown how and why embryonic-derived macrophages persist in the CNS and the epidermis, but fail to persist in significant numbers in the gut mucosa. It has been proposed that this could simply reflect niche accessibility and availability (86) and indeed, there is free accessibility to the mucosa throughout life, whereas the brain and epidermis are separated from the vasculature during development by the blood brain barrier and basement membrane, respectively. However, other tissue macrophages that are not separated from the vasculature by a physical barrier, such as liver Kupffer cells, also exist relatively autonomously, suggesting that tissue accessibility may not be the main factor influencing replacement by blood monocytes (65). In the intestine, monocyte recruitment may be driven by the "physiological inflammation" generated by exposure to antigenic material from the diet or commensal bacteria (87). Indeed, there are now several lines of evidence to demonstrate a key role for the microbiota in influencing macrophage population dynamics in the mucosa. First, major changes in the colonic macrophage compartment are seen following microbial colonization, particularly at the point of weaning where monocyte differentiation through the monocyte "waterfall" becomes established (16). Secondly, macrophage turnover can be reduced by administration of broad spectrum antibiotics, further indicating a role of the commensal microbiota in controlling macrophage turnover (16). Moreover, fewer macrophages are found in the gut wall of germ free mice compared with their SPF counterparts(16, 24, 88). The mucosal microenvironment may actually programme macrophages to orchestrate their own replacement. This is supported by the findings that as intestinal macrophages mature, they progressively upregulate monocyte chemoattractants, such as CCL7, CCL8, and CCL12 (23). As noted above, the microbiota may constitute one stimulus for this differentiation and additional possibilities could include dietary metabolites or the continual mechanical stress generated by peristalsis. Mechanical stress has been suggested to explain the replacement of embryo-derived macrophages in the heart (89) and as well as generating low grade "inflammation," it could simply prevent long term macrophage residence. That differential turnover rates of macrophages are observed in distinct anatomical locales of the gut wall could reflect the fact that particular niches do not support macrophage self-renewal. However, whether distinct macrophage subpopulations display differential rates of proliferation has not been tested experimentally. Thus, while some progress has been made in understanding macrophage turnover dynamics, more work is needed to identify the factors that govern this process.

ENVIRONMENTAL PROGRAMMING OF INTESTINAL MACROPHAGES

While it is clear that monocytes progress through a defined series of intermediaries to replenish the majority of macrophages in the gut, the molecular factors in the gut environment that imprint the unique phenotypic and functional profile of intestinal macrophages are only starting to be understood. CSF1 is clearly involved in the differentiation and/or survival of intestinal macrophages, as demonstrated by their reduction in *Csf1*$^{op/op}$ mice, which have a naturally occurring inactivating mutation in the CSF1 gene (90, 91), their inability to arise from *Csf1r*$^{-/-}$ precursors in a competitive BM chimeric setting (11) and their depletion by anti-CSF1R antibody treatment (22, 92, 93). As described above, upregulation of MHCII is one of the first features of monocyte differentiation in the mucosa of mouse and man (5, 7, 9). Although it is clear that this occurs independently of the IFNγ-STAT1 pathway (94), the exact factors that drive upregulation of MHCII remain unclear. Given that this appears to be a common feature of monocytes entering a variety of tissues (16, 59, 67, 68, 95), it is plausible that MHCII expression may be triggered by extravasation through the vascular endothelium (68). Indeed monocytes in the colonic mucosa already display major transcriptional differences compared with their counterparts in blood (23), despite appearing phenotypically similar. This is tissue specific, because recently arrived colonic monocytes are also transcriptionally distinct from their phenotypic counterparts in the dermis (23). Once in the mucosa, we have shown that TGFβR signaling is essential for the terminal differentiation of macrophages. In particular, upregulation of genes associated with the homeostatic profile of gut macrophages, such as CX3CR1, IL10, and αvβ5 integrin relies on the TGFβ-TGFβR axis (23). Consistent with this, expression of the Runt-related transcription factor 3 (RUNX3), which regulates TGFβ signaling, is a unique feature of intestinal macrophages (96). The TGFβ-TGFβR axis may

also regulate macrophage turnover by dampening expression of the monocyte chemoattractant CCL8 by colonic macrophages (23). Although many sources of TGFβ exist in the mucosa, macrophages themselves may be important, since efferocytosis is known to induce TGFβ expression in macrophages (97) and, at least in man, macrophages may activate TGFβ through their expression of integrin β8 (98). Indeed, uptake of apoptotic epithelial cells induces an anti-inflammatory programme in intestinal macrophages (99). The epithelium may also support macrophage differentiation through expression of Notch ligands, such as Delta-like and Jagged family members, as mature macrophages express high levels of *Hes1* (23), a downstream target of Notch signaling, and their differentiation is disrupted when Notch signaling is ablated (100).

A characteristic feature of mature intestinal macrophages is their hyporesponsiveness to exogenous stimulation (9, 27, 101–105), a functional adaptation that allows these cells to exist in this microbe-rich environment. Interestingly, TGFβ does not appear to be responsible for the unresponsiveness of intestinal macrophages to TLR stimulation in mice (23), whereas this is proposed to be a key role of TGFβ in the human mucosa (102, 105). In contrast, the IL10-IL10R axis plays a fundamental role in the control of macrophage responsiveness in both species. Colonic macrophages from mice in which this axis has been disrupted, either globally or specifically in myeloid cells, have heightened expression of proinflammatory mediators, such as iNOS, IL23, and IL12. As a result, they display overt responsiveness to TLR stimulation and an altered metabolic profile, leading to the development of spontaneous intestinal inflammation (41, 103, 106–109) that can be rescued by rendering macrophages unresponsive to TLR stimulation through cell specific deletion of the TLR adaptor molecule MyD88 (110). Early onset IBD occurs in patients with polymorphisms in *IL10RA* and *IL10RB* genes (111), and *in vitro* generated monocyte-derived macrophages from these patients respond aggressively to LPS stimulation (108). The exaggerated pro-inflammatory responses in the absence of IL10R signaling may result from a failure to downregulate inflammation potentiating molecules such as TREM-1 and STAT1 (41, 112) and/or altered accessibility to pro-inflammatory genes that is normally restricted by IL10-dependent chromatin remodeling (113, 114). IL10 can also limit pro-inflammatory responses by inducing expression of negative regulators of NF-kB, such as IBNS (115).

The high expression of CX3CR1 by murine intestinal macrophages and their positioning adjacent to CX3CL1-producing epithelial cells suggests that the CX3CL1-CX3CR1 axis could also control macrophage differentiation. For instance, CX3CR1 is indispensable for the formation of TEDs that permit luminal sampling by LP macrophages (32–34). Furthermore, *Cx3cr1*-deficient macrophages produce less IL10 (37), suggesting the CX3CL1-CX3CR1 axis promotes the regulatory features of gut macrophages. In line with this, *Cx3cr1*-deficient mice have been shown to be more susceptible to chemically-induced colitis (83), although this has been contested by other reports showing that *Cx3cr1* deficiency suppresses DSS-induced and T cell transfer colitis (32, 34). However, as noted above, human intestinal macrophages do not express CX3CR1, raising questions about the general significance of its role.

In addition to influencing their turnover (16, 24), the microbiota is required for optimal production of IL1β (42) and IL10 by intestinal macrophages(40, 71, 103), with the latter proposed to rely on autocrine type 1 IFNs (116). Microbial colonization may also contribute to the anergic phenotype of colonic macrophages, since some studies have shown them to display TLR hyperresponsiveness when isolated from germ free mice (103), although this is disputed by others (104). The microbiota may act directly on macrophages, for example through the release of as yet unidentified polysaccharides, such as that recently identified by the Powrie group to be released by *H. hepaticus* (117) or via metabolism of dietary fiber to provide short chain fatty acids (SCFAs), which are known to have wide ranging effects on immune cell function (118). In particular, the SCFA butyrate can repress *Il6, Il12b,* and *Nos2* expression by colonic macrophages (119) and alter their metabolic profile (120), while propionate can dampen macrophage activation *in vitro* (121). Aryl hydrocarbon receptor (Ahr) ligands derived from the microbiome or the diet may also control macrophage behavior. Consistent with this idea, $CD11c^{Cre}$-$Ahr^{fl/fl}$ mice display heightened susceptibility to DSS-induced colitis, which is attributed to altered Wnt ligand expression by AhR-deficient macrophages and impaired epithelial barrier integrity (122). Thus, multiple environmental factors act in concert to control macrophage differentiation and function in the mucosa.

The equivalent factors controlling macrophage differentiation in the muscularis remain relatively unexplored, although it is clear they are acutely dependent on CSF1R signaling (22). In addition, norepinephrine signaling by sympathetic neurons via β2 adrenergic receptors on muscularis macrophages has been reported to shape their tissue protective phenotype (20). Notably, the abundance and patterning of macrophages in the muscularis is not dependent on neuronal signals because they are normal in $Ret^{-/-}$ mice, which lack an enteric nervous system, as well as in patients with Hirschsprung disease (HSCR), where the enteric nervous system is absent from the distal bowel (123).

MONOCYTES AND MACROPHAGES IN INTESTINAL INFLAMMATION

The monocyte/macrophage compartment alters markedly in both CD and UC, with accumulation of $CD14^{hi}CD11c^{hi}$ monocytes/immature macrophages that come to outnumber $CD64^{+}HLA\text{-}DR^{hi}CD14^{lo}$ resident macrophages (9, 27, 124–127). In contrast to their homeostatic counterparts, these $CD14^{hi}$ cells in the gut produce pro-inflammatory cytokines and chemokines, such as TNFα, IL1β, IL6, IL12, IL23, and CCL11 (125, 127), display respiratory burst activity (128) and respond in an aberrant manner to commensal bacteria (125). In addition, they express high levels of TREM1, which can potently amplify pro-inflammatory responses (129). Importantly, mucosal healing in IBD patients receiving anti-TNF has been shown to be accompanied by loss of these $CD14^{hi}$ cells and accumulation of $CD206^{+}$ macrophages, which are thought to

be pro-reparative (130). Although anti-TNF (adalimumab) has been shown to bind membrane-bound TNF on $CD14^+$ intestinal macrophages in CD patients (131, 132), whether this triggers a phenotypic switch of existing pro-inflammatory macrophages or if these are replaced by $CD206^+$ macrophages remains unclear. Thus, much of the recent work in this field has focussed on understanding the relationship between homeostatic and pro-inflammatory macrophages and the nature of their precursors, with the ultimate aim of identifying novel therapeutic targets.

Several models of intestinal inflammation, including T cell transfer, *Helicobacter hepaticus*-induced and DSS-induced colitis have been used to dissect these processes experimentally. As in humans, all these models show intense accumulation of classical ($Ly6C^{hi}$) monocytes, together with their immediate progeny that express intermediate levels of CX3CR1 (P1, P2, P3 subsets—see above) (5, 9, 19, 71, 93, 133–135). These cells respond in a highly pro-inflammatory manner to TLR stimulation, express reactive oxygen intermediates, produce high levels of IL1β, IL6, IL12, IL23, and TNFα and express high levels of TREM1 (5, 9, 17, 19, 71, 135), again mirroring the processes seen in human IBD. In contrast to these effects on monocytes, the $CX3CR1^{hi}$ resident macrophages that persist in colitis retain their anti-inflammatory signature (9, 19, 134), suggesting they may continue to play an immunoregulatory role even during inflammation (136).

Multiple lines of evidence indicate that $Ly6C^{hi}$ monocytes and their derivatives are of crucial importance in the intestinal pathology. Firstly, neutralization of IL1β, which is thought to arise predominantly from elicited monocytes, reduces susceptibility to chemically-induced colitis (137). Secondly, colitis development is reduced by selective ablation of *Tnfa* in $Ly6C^{hi}$ monocytes (17). Whether this reflects direct effects of monocyte-derived TNFα in tissue pathology is uncertain, as monocyte survival appears to require autocrine TNFα (138), suggesting that reduced monocyte accumulation in the gut may be the mechanism underlying protection by depletion of TNFα. Finally, mice in whom monocyte recruitment to the inflamed mucosa is defective due to deletion or neutralization of CCL2, CCR2 or β_7 integrin are protected from DSS-induced colitis (9, 19, 73, 133, 139, 140). Importantly, the CCL2-CCR2 axis may also govern monocyte migration in man, where classical monocytes also express CCR2 (141) and elevated levels of its ligands CCL2 and CCL4 are found in IBD mucosa (142). Furthermore, radio-labeled $CD14^{hi}$ classical monocytes have been shown to migrate to actively inflamed regions of IBD mucosa (124). As in the healthy gut, resident macrophages may contribute to this recruitment of monocytes through the release of CCR2 ligands. Nevertheless, it is important to note that CCR2 may not govern monocyte migration in all contexts, as accumulation of $Ly6C^{hi}$ monocytes and their progeny is unaffected by CCR2 deficiency in *H. hepaticus* induced colitis (110) and CCR1 plays a key role in monocyte migration during acute toxoplasmosis (143). Moreover, circulating monocytes in mouse and man express CCR5 (144), which is known to navigate monocytes in certain contexts of inflammation (145), and CCR5 deficient mice develop less inflammation when administered DSS (146). In addition, a unique $CD169^+$ subset of $CX3CR1^{hi}$ macrophages, located preferentially around intestinal crypts, is expanded during experimental colitis and is important for pathogenesis via its ability to recruit monocytes through secretion of the CCR2/CCR3/CCR5 ligand CCL8 (147, 148).

As well as direct effects of elicited monocytes and their products, these cells can recruit and support other innate and adaptive immune effector cells that are important in pathology. For instance, $CD14^{hi}$ monocytes/macrophages in the IBD mucosa are thought to support pathogenic T cell function through IL23 production and their expression of CD40 and CD80 (125, 149, 150). Consistent with this idea, $CX3CR1^{int}$ monocyte/macrophage-derived IL23 supports effector T cell differentiation during *H. hepaticus*-induced colitis (93, 110, 134), assisting the generation of highly pathogenic Th17 cells that co-express IFNγ (93, 151). Elicited monocytes/macrophages may also recruit eosinophils to the inflamed mucosa through the production of CCL11, although whether these play a pro-inflammatory or pro-resolution function remains unclear (127, 152–154).

Macrophages in Intestinal Infection

Despite their pathogenic role in sterile intestinal inflammation, $Ly6C^{hi}$ monocytes and their progeny are vital for protective immunity against enteric pathogens. For instance, $Ccr2^{-/-}$ mice are more susceptible to infection with *Citrobacter rodentium,* a model of enteropathogenic and enterohaemorrhagic *E. coli* infection in man, and the protozoan parasite *Toxoplasma gondii* (155, 156). This can be restored by transfer of wild type $Ly6C^{hi}$ monocytes. Although depletion of $CCR2^+$ cells in the CCR2-DTR mouse leads to enhanced susceptibility to *C. rodentium* (157), it should be noted that this approach deletes both elicited and resident macrophages in the intestine (43, 72, 157), meaning the specific roles of these individual subsets cannot be distinguished in this model. Nevertheless, macrophages play an important protective role in *C. rodentium* infection via the production of IL1β, IL23, and TNF-like ligand 1A (TL1A), triggering IL22 production by ILC3s, which in turn augments local production of the anti-microbial proteins RegIIIβ and RegIIIγ (157–159), known to be necessary for *C. rodentium* clearance (160). Moreover, through their production of IL12 and IL23, macrophages support the differentiation of IFNγ and IL17-producing effector T cells (161). Whether this occurs exclusively in the mucosa or if $Ly6C^{hi}$ monocyte-derived cells leave the mucosa to contribute to T cell priming in the lymph nodes remains a matter of debate and may depend on the nature of the inflammatory insult (162, 163). In addition to their pro-inflammatory roles, elegant work from the Belkaid lab has shown that $Ly6C^{hi}$ monocytes can also exert regulatory functions. During acute toxoplasmosis, elicited $Ly6C^{hi}$ monocytes respond to the microbiota by producing PGE2 and IL10 that protect against immunopathology by inhibiting neutrophil function (164). As a result, $Ccr2^{-/-}$ mice show enhanced susceptibility to this model of infection (156). Thus, it is clear that monocytes play a multifaceted role in the inflamed mucosa.

Until now, we have considered macrophage function in Th1 and/or Th17-dominated forms of inflammation, but they also participate in the Th2-mediated protective immune responses generated against intestinal helminth parasites. However, the

exact role macrophages play may depend on the parasite in question. For instance, while arginase producing, alternatively activated macrophages are critical for the expulsion of the gastrointestinal nematode *Heligmosomoides polygyrus bakeri* (165–167), inhibition of arginase has no effect on expulsion of *Trichuris muris* (168). Instead, macrophages are considered to play a more central role in the tissue repair that occurs after *T. muris* has been expelled. The role of alternatively activated macrophages in expulsion of the nematode *Nippostrongylus brasiliensis* also remains contentious (169, 170). Interestingly, although macrophage accumulation in Th2 type settings in other tissues is now typically thought to involve *in situ* proliferation of resident cells under the control of IL4 (171), accumulation of 'alternatively activated' macrophages in the gut of mice with *T. muris* is dependent on monocyte infiltration (172). Thus, regardless of the nature of the insult, monocyte recruitment appears to be the principal mechanism of bolstering the macrophage reservoir in the gut mucosa.

Monocyte Differentiation in the Inflamed Mucosa

Why monocytes accumulate during colitis and do not differentiate into anti-inflammatory macrophages as they do in healthy tissue remains unclear. Based on adoptive transfer studies in the DSS-induced model of colitis, we proposed that immature monocytes accumulate due to a breakdown in the normal differentiation process (9). The exact cause of this remains elusive, but may reflect both a loss of factors that normally promote monocyte differentiation, such as IL10 and TGFβ, together with increased levels of pro-inflammatory cytokines that block this process or reduce monocyte half-life. Indeed, high levels of IFNγ are found in the IBD mucosa and have been shown to promote the pro-inflammatory features of $CD14^+$ monocyte/macrophages (125). Consistent with this, deletion of IFNγR1 or its downstream signaling molecule STAT1 in mice limits the differentiation of pro-inflammatory $Ly6C^+MHCII^+$ monocytes in the colon and provides relative protection from DSS-induced colitis (94). IFNγ may also act by upregulating negative regulators of the TGFβR pathway, such as Smad7 (173), thus disrupting the pathway by which monocytes normally differentiate into mature, anti-inflammatory macrophages (see above). Finally, the hypoxic nature of the inflamed mucosa may support the differentiation of pro-inflammatory monocytes/macrophages, as myeloid-specific deletion of the hypoxia inducible factor (HIF)-1α also protects mice from DSS-induced colitis (174).

As well as local programming by the intestinal microenvironment, there is increasing evidence that monocytes arriving in the inflamed mucosa may be inherently different to those during health. Monocytosis is a feature of human and experimental IBD (175, 176), and monocytes arriving in the inflamed mucosa already have higher expression of TNFα, iNOS, IL6 and STAT1 compared with their homeostatic counterparts (9, 94). This may involve "priming" of monocytes in the BM by IFNγ derived from NK cells responding to IL12 released from the inflamed intestine, as has been shown to occur in acute toxoplasmosis (177), or through as yet unidentified pathways. Thus, the inflamed mucosal environment may control monocyte fate both locally and through long-range conditioning of BM precursors.

MONOCYTES/MACROPHAGES DURING RESOLUTION OF INFLAMMATION

Experimental models of colitis have also allowed characterization of the monocyte/macrophage compartment during the resolution of pathology. Cessation of DSS administration is accompanied by major changes in the macrophage pool, with a massive reduction in $CX3CR1^{int}$ monocytes/macrophages and restoration of the $CX3CR1^{hi}$ macrophage subset, together with loss of granulocytes (19). A similar contraction of inflammatory cells is seen following the infectious phase of *H. hepaticus*-induced colitis, although interestingly, eosinophils persist at elevated levels in this model, suggesting they may play a pro-resolution role (134). Resident intestinal macrophages promote mucosal healing, as colitis is worsened by their depletion (136) or if they are rendered unresponsive to anti-inflammatory cytokines, such as TGFβ (178). However, whether macrophages elicited by an inflammatory agent also play a pro-restorative role following removal its clearance remains unclear. Interestingly, resolution of inflammation in post-operative ileus is delayed in $Ccr2^{-/-}$ mice, suggesting that recruited $Ly6C^{hi}$ monocytes and their derivatives are important for restoration of homeostasis in the muscularis (179).

The fate of the monocytes elicited during inflammation in the repairing mucosa is unclear, although it is assumed that they are cleared by apoptosis, as in other tissues (180). This would be consistent with the increased numbers of apoptotic $CD68^+$ cells seen in the healing mucosa of CD and UC patients treated with infliximab (181). An alternative fate of elicited monocytes is that they subsequently convert into mature resident macrophages under the guidance of local cues. While this has been shown to occur during the resolution of inflammation in other tissues, such as the peritoneal cavity (57), it is not known whether it occurs in the repairing mucosa. Moreover, just like in the setting of infection described above, long-range conditioning of monocytes may also occur during inflammation resolution meaning the nature of the monocytes arriving at the repairing mucosa may be intrinsically-distinct. Consistent with this idea, Ikeda et al. (182) have recently shown that a specific subset of $Ly6C^{hi}$ monocytes expressing the regulatory molecule Ym1 can be found during the resolution phase of DSS-induced colitis and their depletion hinders effective mucosal healing.

CONCLUSIONS AND FUTURE PERSPECTIVES

Several major advances have been made over the last few years in our understanding of intestinal macrophage ontogeny and development, including the identification of some of environmental signals that regulate tissue-specific phenotypes

and functions. Nevertheless, many aspects of intestinal macrophage biology remain poorly understood. For instance, our understanding of heterogeneity within the intestinal macrophage compartment remains incomplete. The application of single cell technologies, such as single cell RNA sequencing, will continue to provide further insights into macrophage heterogeneity in both mouse and man. This should also allow for further alignment of the ways in which murine and human MPs are characterized and lead to better translation between systems. With the discovery of macrophage subpopulations, it will be important to determine the environmental signals that shape the phenotype, function and longevity of these niche-specific macrophages. Why do some niches promote the longevity of macrophages (e.g., the muscularis externa) whereas others (e.g., the LP) mainly rely on the constant replenishment by BM-derived monocytes? Understanding the cellular interactions between macrophages and their neighboring cells (e.g., stromal cells), the environmental challenges (e.g., antigenic exposure) in particular niches, as well as niche accessibility will be pivotal in answering this question. Importantly, macrophage longevity is not an exclusive property of embryo-derived macrophages and it remains to be determined whether long-lived embryo-derived and BM-derived macrophages perform analogous functions or if they have discrete roles in intestinal homeostasis. Another area warranting further investigation is how differences along the intestinal tract, for instance antigenic exposure and commensal microbiota composition, might impact macrophage development and function. A major effort must be placed on understanding how the monocyte/macrophage compartment changes during acute and chronic inflammation, as well as during inflammation resolution. Do long-lived macrophages persist during and following an inflammatory insult? If so, do they perform specific roles? Given that accumulation of pro-inflammatory monocytes/macrophages is a characteristic feature of IBD, it is vital to understand the precise nature of the molecular factors controlling monocyte/macrophage differentiation under normal physiological conditions, how these change during disease and the relative contribution of local conditioning vs. long-range effects on haematopoiesis. Providing answers to these questions will be vital if macrophages are to be realized as therapeutic targets in IBD.

AUTHOR CONTRIBUTIONS

Both authors made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REFERENCES

1. Hume DA, Perry VH, Gordon S. The mononuclear phagocyte system of the mouse defined by immunohistochemical localisation of antigen F4/80: macrophages associated with epithelia. *Anat Rec.* (1984) 210:503–12. doi: 10.1002/ar.1092100311
2. McGarry MP, Stewart CC. Murine eosinophil granulocytes bind the murine macrophage-monocyte specific monoclonal antibody F4/80. *J Leuk Biol.* (1991) 50:471–8.
3. Pabst O, Bernhardt G. The puzzle of intestinal lamina propria dendritic cells and macrophages. *Eur J Immunol.* (2010) 40:2107–11. doi: 10.1002/eji.201040557
4. Langlet C, Tamoutounour S, Henri S, Luche H, Ardouin L, Gregoire C, et al. CD64 expression distinguishes monocyte-derived and conventional dendritic cells and reveals their distinct role during intramuscular immunization. *J Immunol.* (2012) 188:1751–60. doi: 10.4049/jimmunol.1102744
5. Tamoutounour S, Henri S, Lelouard H, de Bovis B, de Haar C, van der Woude CJ, et al. CD64 distinguishes macrophages from dendritic cells in the gut and reveals the Th1-inducing role of mesenteric lymph node macrophages during colitis. *Eur J Immunol.* (2012) 42:3150–66. doi: 10.1002/eji.201242847
6. Gautier EL, Shay T, Miller J, Greter M, Jakubzick C, Ivanov S, et al. Gene-expression profiles and transcriptional regulatory pathways that underlie the identity and diversity of mouse tissue macrophages. *Nat Immunol.* (2012) 13:1118–28. doi: 10.1038/ni.2419
7. Bujko A, Atlasy N, Landsverk OJB, Richter L, Yaqub S, Horneland R, et al. Transcriptional and functional profiling defines human small intestinal macrophage subsets. *J Exp Med.* (2018) 215:441–58. doi: 10.1084/jem.20170057
8. Bharat A, Bhorade SM, Morales-Nebreda L, McQuattie-Pimentel AC, Soberanes S, Ridge K, et al. Flow cytometry reveals similarities between lung macrophages in humans and mice. *Am J Respir Cell Mol Biol.* (2016) 54:147–9. doi: 10.1165/rcmb.2015-0147LE
9. Bain CC, Scott CL, Uronen-Hansson H, Gudjonsson S, Jansson O, Grip O, et al. Resident and pro-inflammatory macrophages in the colon represent alternative context-dependent fates of the same Ly6Chi monocyte precursors. *Mucosal Immunol.* (2013) 6:498–510. doi: 10.1038/mi.2012.89
10. Scott CL, Bain CC, Wright PB, Sichien D, Kotarsky K, Persson EK, et al. CCR2(+)CD103(-) intestinal dendritic cells develop from DC-committed precursors and induce interleukin-17 production by T cells. *Mucosal Immunol.* (2015) 8:327–39. doi: 10.1038/mi.2014.70
11. Schlitzer A, McGovern N, Teo P, Zelante T, Atarashi K, Low D, et al. IRF4 transcription factor-dependent CD11b+ dendritic cells in human and mouse control mucosal IL-17 cytokine responses. *Immunity* (2013) 38:970–83. doi: 10.1016/j.immuni.2013.04.011
12. Johansson-Lindbom B, Svensson M, Pabst O, Palmqvist C, Marquez G, Forster R, et al. Functional specialization of gut CD103+ dendritic cells in the regulation of tissue-selective T cell homing. *J Exp Med.* (2005) 202:1063–73. doi: 10.1084/jem.20051100
13. Cerovic V, Houston SA, Scott CL, Aumeunier A, Yrlid U, Mowat AM, et al. Intestinal CD103(-) dendritic cells migrate in lymph and prime effector T cells. *Mucosal Immunol.* (2013) 6:104–13. doi: 10.1038/mi.2012.53
14. Coombes JL, Siddiqui KR, Arancibia-Carcamo CV, Hall J, Sun CM, Belkaid Y, et al. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. *J Exp Med.* (2007) 204:1757–64. doi: 10.1084/jem.20070590
15. Schulz O, Jaensson E, Persson EK, Liu X, Worbs T, Agace WW, et al. Intestinal CD103+, but not CX3CR1+, antigen sampling cells migrate in lymph and serve classical dendritic cell functions. *J Exp Med.* (2009) 206:3101–14. doi: 10.1084/jem.20091925
16. Bain CC, Bravo-Blas A, Scott CL, Gomez Perdiguero E, Geissmann F, Henri S, et al. Constant replenishment from circulating monocytes maintains

the macrophage pool in the intestine of adult mice. *Nat Immunol.* (2014) 15:929–37. doi: 10.1038/ni.2967
17. Varol C, Vallon-Eberhard A, Elinav E, Aychek T, Shapira Y, Luche H, et al. Intestinal lamina propria dendritic cell subsets have different origin and functions. *Immunity* (2009) 31:502–12. doi: 10.1016/j.immuni.2009.06.025
18. Bogunovic M, Ginhoux F, Helft J, Shang L, Hashimoto D, Greter M, et al. Origin of the lamina propria dendritic cell network. *Immunity* (2009) 31:513–25. doi: 10.1016/j.immuni.2009.08.010
19. Zigmond E, Varol C, Farache J, Elmaliah E, Satpathy AT, Friedlander G, et al. Ly6C hi monocytes in the inflamed colon give rise to proinflammatory effector cells and migratory antigen-presenting cells. *Immunity* (2012) 37:1076–90. doi: 10.1016/j.immuni.2012.08.026
20. Gabanyi I, Muller PA, Feighery L, Oliveira TY, Costa-Pinto FA, Mucida D. Neuro-immune interactions drive tissue programming in intestinal macrophages. *Cell* (2016) 164:378–91. doi: 10.1016/j.cell.2015.12.023
21. Jung S, Aliberti J, Graemmel P, Sunshine MJ, Kreutzberg GW, Sher A, et al. Analysis of fractalkine receptor CX(3)CR1 function by targeted deletion and green fluorescent protein reporter gene insertion. *Mol Cell Biol.* (2000) 20:4106–14. doi: 10.1128/MCB.20.11.4106-4114.2000
22. Muller PA, Koscsó B, Rajani GM, Stevanovic K, Berres M-L, Hashimoto D, et al. Crosstalk between muscularis macrophages and enteric neurons regulates gastrointestinal motility. *Cell* (2014) 158:300–13. doi: 10.1016/j.cell.2014.04.050
23. Schridde A, Bain CC, Mayer JU, Montgomery J, Pollet E, Denecke B, et al. Tissue-specific differentiation of colonic macrophages requires TGFβ receptor-mediated signaling. *Mucosal Immunol.* (2017) 10:1387–99. doi: 10.1038/mi.2016.142
24. Shaw TN, Houston SA, Wemyss K, Bridgeman HM, Barbera TA, Zangerle-Murray T, et al. Tissue-resident macrophages in the intestine are long lived and defined by Tim-4 and CD4 expression. *J Exp Med.* (2018) 215:1507–18. doi: 10.1084/jem.20180019
25. Bernardo D, Marin AC, Fernández-Tomé S, Montalban-Arques A, Carrasco A, Tristán E, et al. Human intestinal pro-inflammatory CD11chighCCR2+CX3CR1+ macrophages, but not their tolerogenic CD11c-CCR2-CX3CR1- counterparts, are expanded in inflammatory bowel disease. *Mucosal Immunol.* (2018) 11:1114–26. doi: 10.1038/s41385-018-0030-7
26. Hume DA, Allan W, Hogan PG, Doe WF. Immunohistochemical characterisation of macrophages in human liver and gastrointestinal tract: expression of CD4, HLA-DR, OKM1, and the mature macrophage marker 25F9 in normal and diseased tissue. *J Leuk Biol.* (1987) 42:474–84.
27. Rugtveit J, Nilsen EM, Bakka A, Carlsen H, Brandtzaeg P, Scott H. Cytokine profiles differ in newly recruited and resident subsets of mucosal macrophages from inflammatory bowel disease. *Gastroenterology* (1997) 112:1493–505.
28. Mahida YR, Wu KC, Jewell DP. Respiratory burst activity of intestinal macrophages in normal and inflammatory bowel disease. *Gut* (1989) 30:1362–70.
29. Kumawat AK, Yu C, Mann EA, Schridde A, Finnemann SC, Mowat AM. Expression and characterization of αvβ5 integrin on intestinal macrophages. *Eur J Immunol.* (2018) 48:1181–7.doi: 10.1002/eji.201747318
30. Henson PM. Cell removal: efferocytosis. *Annu Rev Cell Dev Biol.* (2017) 33:127–44. doi: 10.1146/annurev-cellbio-111315-125315
31. Lacy-Hulbert A, Smith AM, Tissire H, Barry M, Crowley D, Bronson RT, et al. Ulcerative colitis and autoimmunity induced by loss of myeloid alphav integrins. *Proc Natl Acad Sci USA.* (2007) 104:15823–8. doi: 10.1073/pnas.0707421104
32. Niess JH, Brand S, Gu X, Landsman L, Jung S, McCormick BA, et al. CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. *Science* (2005) 307:254–8. doi: 10.1126/science.1102901
33. Chieppa M, Rescigno M, Huang AY, Germain RN. Dynamic imaging of dendritic cell extension into the small bowel lumen in response to epithelial cell TLR engagement. *J Exp Med.* (2006) 203:2841–52. doi: 10.1084/jem.20061884
34. Kim KW, Vallon-Eberhard A, Zigmond E, Farache J, Shezen E, Shakhar G, et al. *In vivo* structure/function and expression analysis of the CX3C chemokine fractalkine. *Blood* (2011) 118:e156–67. doi: 10.1182/blood-2011-04-348946
35. Mazzini E, Massimiliano L, Penna G, Rescigno M. Oral tolerance can be established via gap junction transfer of fed antigens from CX3CR1(+) Macrophages to CD103(+) dendritic cells. *Immunity* (2014) 40:248–61. doi: 10.1016/j.immuni.2013.12.012
36. Worbs T, Bode U, Yan S, Hoffmann MW, Hintzen G, Bernhardt G, et al. Oral tolerance originates in the intestinal immune system and relies on antigen carriage by dendritic cells. *J Exp Med.* (2006) 203:519–27. doi: 10.1084/jem.20052016
37. Hadis U, Wahl B, Schulz O, Hardtke-Wolenski M, Schippers A, Wagner N, et al. Intestinal tolerance requires gut homing and expansion of FoxP3+ regulatory T cells in the lamina propria. *Immunity* (2011) 34:237–46. doi: 10.1016/j.immuni.2011.01.016
38. MacDonald TT, Pender SL. Lamina propria T cells. *Chem Immunol.* (1998) 71:103–17.
39. Murai M, Turovskaya O, Kim G, Madan R, Karp CL, Cheroutre H, et al. Interleukin 10 acts on regulatory T cells to maintain expression of the transcription factor Foxp3 and suppressive function in mice with colitis. *Nat immunol.* (2009) 10:1178–84. doi: 10.1038/ni.1791
40. Kim M, Galan C, Hill AA, Wu W-J, Fehlner-Peach H, Song HW, et al. Critical role for the microbiota in CX3CR1+ intestinal mononuclear phagocyte regulation of intestinal T cell responses. *Immunity* (2018) 49:151–63.e5. doi: 10.1016/j.immuni.2018.05.009
41. Zigmond E, Bernshtein B, Friedlander G, Walker CR, Yona S, Kim K-W, et al. Macrophage-restricted interleukin-10 receptor deficiency, but not IL-10 deficiency, causes severe spontaneous colitis. *Immunity* (2014) 40:720–733. doi: 10.1016/j.immuni.2014.03.012
42. Shaw MH, Kamada N, Kim Y-G, Núñez G. Microbiota-induced IL-1β, but not IL-6, is critical for the development of steady-state TH17 cells in the intestine. *J Exp Med.* (2012) 209:251–8. doi: 10.1084/jem.20111703
43. Panea C, Farkas AM, Goto Y, Abdollahi-Roodsaz S, Lee C, Koscsó B, et al. Intestinal monocyte-derived macrophages control commensal-specific Th17 responses. *Cell Rep.* (2015) 12:1314–24. doi: 10.1016/j.celrep.2015.07.040
44. Mortha A, Chudnovskiy A, Hashimoto D, Bogunovic M, Spencer SP, Belkaid Y, et al. Microbiota-dependent crosstalk between macrophages and ILC3 promotes intestinal homeostasis. *Science* (2014) 343:1249288. doi: 10.1126/science.1249288
45. Greter M, Helft J, Chow A, Hashimoto D, Mortha A, Agudo-Cantero J, et al. GM-CSF controls nonlymphoid tissue dendritic cell homeostasis but is dispensable for the differentiation of inflammatory dendritic cells. *Immunity* (2012) 36:1031–46. doi: 10.1016/j.immuni.2012.03.027
46. Mikkelsen HB, Rumessen JJ. Characterization of macrophage-like cells in the external layers of human small and large intestine. *Cell Tissue Res.* (1992) 270:273–9.
47. De Schepper S, Verheijden S, Aguilera-Lizarraga J, Viola MF, Boesmans W, Stakenborg N, et al. Self-maintaining gut macrophages are essential for intestinal homeostasis. *Cell* (2018) 175:400–15.e13. doi: 10.1016/j.cell.2018.07.048
48. Yang M, Fukui H, Eda H, Kitayama Y, Hara K, Kodani M, et al. Involvement of gut microbiota in the association between gastrointestinal motility and 5-HT expression/M2 macrophage abundance in the gastrointestinal tract. *Mol Med Rep.* (2017) 16:3482–8. doi: 10.3892/mmr.2017.6955
49. Ginhoux F, Guilliams M. Tissue-resident macrophage ontogeny and homeostasis. *Immunity* (2016) 44:439–49. doi: 10.1016/j.immuni.2016.02.024
50. van Furth R, Cohn ZA. The origin and kinetics of mononuclear phagocytes. *J Exp Med.* (1968) 128:415–35.
51. van Furth R, Cohn ZA, Hirsch JG, Humphrey JH, Spector WG, Langevoort HL. The mononuclear phagocyte system: a new classification of macrophages, monocytes, and their precursor cells. *Bull World Health Organ.* (1972) 46:845–52.
52. Geissmann F, Jung S, Littman DR. Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity* (2003) 19:71–82.
53. Ziegler-Heitbrock HW, Passlick B, Flieger D. The monoclonal antimonocyte antibody My4 stains B lymphocytes and two distinct monocyte subsets in human peripheral blood. *Hybridoma* (1988) 7:521–7.
54. Carlin LM, Stamatiades EG, Auffray C, Hanna RN, Glover L, Vizcay-Barrena G, et al. Nr4a1-dependent Ly6C(low) monocytes monitor

endothelial cells and orchestrate their disposal. *Cell* (2013) 153:362–75. doi: 10.1016/j.cell.2013.03.010
55. Schulz C, Gomez Perdiguero E, Chorro L, Szabo-Rogers H, Cagnard N, Kierdorf K, et al. A lineage of myeloid cells independent of Myb and hematopoietic stem cells. *Science* (2012) 336:86–90. doi: 10.1126/science.1219179
56. Hashimoto D, Chow A, Noizat C, Teo P, Beasley MB, Leboeuf M, et al. Tissue-resident macrophages self-maintain locally throughout adult life with minimal contribution from circulating monocytes. *Immunity* (2013) 38:792–804. doi: 10.1016/j.immuni.2013.04.004
57. Yona S, Kim K-W, Wolf Y, Mildner A, Varol D, Breker M, et al. Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis. *Immunity* (2013) 38:79–91. doi: 10.1016/j.immuni.2012.12.001
58. Epelman S, Lavine KJ, Beaudin AE, Sojka DK, Carrero JA, Calderon B, et al. Embryonic and adult-derived resident cardiac macrophages are maintained through distinct mechanisms at steady state and during inflammation. *Immunity* (2014) 40:91–104. doi: 10.1016/j.immuni.2013.11.019
59. Sheng J, Ruedl C, Karjalainen K. Most Tissue-resident macrophages except microglia are derived from fetal hematopoietic stem cells. *Immunity* (2015) 43:382–93. doi: 10.1016/j.immuni.2015.07.016
60. Alliot F, Godin I, Pessac B. Microglia derive from progenitors, originating from the yolk sac, and which proliferate in the brain. *Brain Res Dev Brain Res.* (1999) 117:145–52.
61. Ajami B, Bennett JL, Krieger C, Tetzlaff W, Rossi FM. Local self-renewal can sustain CNS microglia maintenance and function throughout adult life. *Nat Neurosci.* (2007) 10:1538–43. doi: 10.1038/nn2014
62. Ginhoux F, Greter M, Leboeuf M, Nandi S, See P, Gokhan S, et al. Fate mapping analysis reveals that adult microglia derive from primitive macrophages. *Science* (2010) 330:841–5. doi: 10.1126/science.1194637
63. Hoeffel G, Wang Y, Greter M, See P, Teo P, Malleret B, et al. Adult Langerhans cells derive predominantly from embryonic fetal liver monocytes with a minor contribution of yolk sac-derived macrophages. *J Exp Med.* (2012) 209:1167–81. doi: 10.1084/jem.20120340
64. Hoeffel G, Chen J, Lavin Y, Low D, Almeida FF, See P, et al. C-Myb(+) erythro-myeloid progenitor-derived fetal monocytes give rise to adult tissue-resident macrophages. *Immunity* (2015) 42:665–78. doi: 10.1016/j.immuni.2015.03.011
65. Gomez Perdiguero E, Klapproth K, Schulz C, Busch K, Azzoni E, Crozet L, et al. Tissue-resident macrophages originate from yolk-sac-derived erythro-myeloid progenitors. *Nature* (2015) 518:547–51. doi: 10.1038/nature13989
66. Guilliams M, De Kleer I, Henri S, Post S, Vanhoutte L, De Prijck S, et al. Alveolar macrophages develop from fetal monocytes that differentiate into long-lived cells in the first week of life via GM-CSF. *J Exp Med.* (2013) 210:1977–92. doi: 10.1084/jem.20131199
67. Tamoutounour S, Guilliams M, Montanana Sanchis F, Liu H, Terhorst D, Malosse C, et al. Origins and functional specialization of macrophages and of conventional and monocyte-derived dendritic cells in mouse skin. *Immunity* (2013) 39:925–38. doi: 10.1016/j.immuni.2013.10.004
68. Jakubzick C, Gautier EL, Gibbings SL, Sojka DK, Schlitzer A, Johnson TE, et al. Minimal differentiation of classical monocytes as they survey steady-state tissues and transport antigen to lymph nodes. *Immunity* (2013) 39:599–610. doi: 10.1016/j.immuni.2013.08.007
69. Bain CC, Hawley CA, Garner H, Scott CL, Schridde A, Steers NJ, et al. Long-lived self-renewing bone marrow-derived macrophages displace embryo-derived cells to inhabit adult serous cavities. *Nat Commun.* (2016) 7:ncomms11852. doi: 10.1038/ncomms11852
70. Serbina NV, Pamer EG. Monocyte emigration from bone marrow during bacterial infection requires signals mediated by chemokine receptor CCR2. *Nat Immunol.* (2006) 7:311–7. doi: 10.1038/ni1309
71. Rivollier A, He J, Kole A, Valatas V, Kelsall BL. Inflammation switches the differentiation program of Ly6Chi monocytes from antiinflammatory macrophages to inflammatory dendritic cells in the colon. *J Exp Med.* (2012) 209:139–55. doi: 10.1084/jem.20101387
72. Kinnebrew MA, Buffie CG, Diehl GE, Zenewicz LA, Leiner I, Hohl TM, et al. Interleukin 23 production by intestinal CD103(+)CD11b(+) dendritic cells in response to bacterial flagellin enhances mucosal innate immune defense. *Immunity* (2012) 36:276–87. doi: 10.1016/j.immuni.2011.12.011
73. Takada Y, Hisamatsu T, Kamada N, Kitazume MT, Honda H, Oshima Y, et al. Monocyte chemoattractant protein-1 contributes to gut homeostasis and intestinal inflammation by composition of IL-10-producing regulatory macrophage subset. *J Immunol.* (2010) 184:2671–6. doi: 10.4049/jimmunol.0804012
74. Kanitakis J, Morelon E, Petruzzo P, Badet L, Dubernard J-M. Self-renewal capacity of human epidermal Langerhans cells: observations made on a composite tissue allograft. *Exp Dermatol.* (2011) 20:145–6. doi: 10.1111/j.1600-0625.2010.01146.x
75. Eguíluz-Gracia I, Schultz HHL, Sikkeland LIB, Danilova E, Holm AM, Pronk CJH, et al. Long-term persistence of human donor alveolar macrophages in lung transplant recipients. *Thorax* (2016) 16:2300–11. doi: 10.1136/thoraxjnl-2016-208292
76. Misharin AV, Morales-Nebreda L, Reyfman PA, Cuda CM, Walter JM, McQuattie-Pimentel AC, et al. Monocyte-derived alveolar macrophages drive lung fibrosis and persist in the lung over the life span. *J Exp Med.* (2017) 214:2387–404. doi: 10.1084/jem.20162152
77. Scott CL, Zheng F, De Baetselier P, Martens L, Saeys Y, De Prijck S, et al. Bone marrow-derived monocytes give rise to self-renewing and fully differentiated Kupffer cells. *Nat Commun.* (2016) 7:10321. doi: 10.1038/ncomms10321
78. Heidt T, Courties G, Dutta P, Sager HB, Sebas M, Iwamoto Y, et al. Differential contribution of monocytes to heart macrophages in steady-state and after myocardial infarction. *Circ Res.* (2014) 115:284–95. doi: 10.1161/CIRCRESAHA.115.303567
79. Molawi K, Wolf Y, Kandalla PK, Favret J, Hagemeyer N, Frenzel K, et al. Progressive replacement of embryo-derived cardiac macrophages with age. *J Exp Med.* (2014) 211:2151–8. doi: 10.1084/jem.20140639
80. Gibbings SL, Thomas SM, Atif SM, McCubbrey AL, Desch AN, Danhorn T, et al. Three unique interstitial macrophages in the murine lung at steady State. *Am J Respir Cell Mol Biol.* (2017) 57:66–76. doi: 10.1165/rcmb.2016-0361OC
81. Cain DW, O'Koren EG, Kan MJ, Womble M, Sempowski GD, Hopper K, et al. Identification of a tissue-specific, C/EBPβ-dependent pathway of differentiation for murine peritoneal macrophages. *J Immunol.* (2013) 191:4665–75. doi: 10.4049/jimmunol.1300581
82. Kim K-W, Williams JW, Wang Y-T, Ivanov S, Gilfillan S, Colonna M, et al. MHC II+ resident peritoneal and pleural macrophages rely on IRF4 for development from circulating monocytes. *J Exp Med.* (2016) 213:1951–9. doi: 10.1084/jem.20160486
83. Medina-Contreras O, Geem D, Laur O, Williams IR, Lira SA, Nusrat A, et al. CX3CR1 regulates intestinal macrophage homeostasis, bacterial translocation, and colitogenic Th17 responses in mice. *J Clin Invest.* (2011) 121:4787–95. doi: 10.1172/JCI59150
84. Hanna RN, Carlin LM, Hubbeling HG, Nackiewicz D, Green AM, Punt JA, et al. The transcription factor NR4A1 (Nur77) controls bone marrow differentiation and the survival of Ly6C- monocytes. *Nat Immunol.* (2011) 12:778–85. doi: 10.1038/ni.2063
85. Thomas GD, Hanna RN, Vasudevan NT, Hamers AA, Romanoski CE, McArdle S, et al. Deleting an Nr4a1 super-enhancer subdomain ablates Ly6Clow monocytes while preserving macrophage gene function. *Immunity* (2016) 45:975–87. doi: 10.1016/j.immuni.2016.10.011
86. Guilliams M, Scott CL. Does niche competition determine the origin of tissue-resident macrophages? *Nat Rev.* (2017) 17:451–60. doi: 10.1038/nri.2017.42
87. Fiocchi C. What is "physiological" intestinal inflammation and how does it differ from "pathological" inflammation? *Inflamm Bowel Dis.* (2008) 14 (Suppl. 2):S77–8. doi: 10.1002/ibd.20618
88. Niess JH, Adler G. Enteric flora expands gut lamina propria CX3CR1+ dendritic cells supporting inflammatory immune responses under normal and inflammatory conditions. *J Immunol.* (2010) 184:2026–37. doi: 10.4049/jimmunol.0901936
89. Lambrecht B, Guilliams M. Monocytes find a new place to dwell in the niche of heartbreak hotel. *J Exp Med.* (2014) 211:2136. doi: 10.1084/jem.21111insight1
90. Dai X-M, Zong X-H, Sylvestre V, Stanley ER. Incomplete restoration of colony-stimulating factor 1 (CSF-1) function in CSF-1-deficient Csf1op/Csf1op mice by transgenic expression of cell surface CSF-1. *Blood* (2004) 103:1114–23. doi: 10.1182/blood-2003-08-2739

91. Ryan GR, Dai XM, Dominguez MG, Tong W, Chuan F, Chisholm O, et al. Rescue of the colony-stimulating factor 1 (CSF-1)-nullizygous mouse (Csf1(op)/Csf1(op)) phenotype with a CSF-1 transgene and identification of sites of local CSF-1 synthesis. *Blood* (2001) 98:74–84. doi: 10.1182/blood.V98.1.74
92. MacDonald KP, Palmer JS, Cronau S, Seppanen E, Olver S, Raffelt NC, et al. An antibody against the colony-stimulating factor 1 receptor depletes the resident subset of monocytes and tissue- and tumor-associated macrophages but does not inhibit inflammation. *Blood* (2010) 116:3955–63. doi: 10.1182/blood-2010-02-266296
93. Arnold IC, Mathisen S, Schulthess J, Danne C, Hegazy AN, Powrie F. CD11c(+) monocyte/macrophages promote chronic Helicobacter hepaticus-induced intestinal inflammation through the production of IL-23. *Mucosal Immunol.* (2015) 9:352–63. doi: 10.1038/mi.2015.65
94. Nakanishi Y, Sato T, Takahashi K, Ohteki T. IFN-γ-dependent epigenetic regulation instructs colitogenic monocyte/macrophage lineage differentiation *in vivo*. *Mucosal Immunol.* (2018) 11:871–80. doi: 10.1038/mi.2017.104
95. Movahedi K, Laoui D, Gysemans C, Baeten M, Stange G, Van den Bossche J, et al. Different tumor microenvironments contain functionally distinct subsets of macrophages derived from Ly6C(high) monocytes. *Cancer Res.* (2010) 70:5728–39. doi: 10.1158/0008-5472.CAN-09-4672
96. Lavin Y, Winter D, Blecher-Gonen R, David E, Keren-Shaul H, Merad M, et al. Tissue-resident macrophage enhancer landscapes are shaped by the local microenvironment. *Cell* (2014) 159:1312–26. doi: 10.1016/j.cell.2014.11.018
97. Fadok VA, Bratton DL, Konowal A, Freed PW, Westcott JY, Henson PM. Macrophages that have ingested apoptotic cells *in vitro* inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. *J Clin Invest.* (1998) 101:890–8. doi: 10.1172/JCI1112
98. Kelly A, Gunaltay S, McEntee CP, Shuttleworth EE, Smedley C, Houston SA, et al. Human monocytes and macrophages regulate immune tolerance via integrin αvβ8-mediated TGFβ activation. *J Exp Med.* (2018) 215:2725–36. doi: 10.1084/jem.20171491
99. Cummings RJ, Barbet G, Bongers G, Hartmann BM, Gettler K, Muniz L, et al. Different tissue phagocytes sample apoptotic cells to direct distinct homeostasis programs. *Nature* (2016) 539:565–9. doi: 10.1038/nature20138
100. Ishifune C, Maruyama S, Sasaki Y, Yagita H, Hozumi K, Tomita T, et al. Differentiation of CD11c+ CX3CR1+ cells in the small intestine requires Notch signaling. *Proc Natl Acad Sci USA.* (2014) 111:5986–91. doi: 10.1073/pnas.1401671111
101. Smythies LE, Sellers M, Clements RH, Mosteller-Barnum M, Meng G, Benjamin WH, et al. Human intestinal macrophages display profound inflammatory anergy despite avid phagocytic and bacteriocidal activity. *J Clin Invest.* (2005) 115:66–75. doi: 10.1172/JCI19229
102. Smythies LE, Shen R, Bimczok D, Novak L, Clements RH, Eckhoff DE, et al. Inflammation anergy in human intestinal macrophages is due to Smad-induced IkappaBalpha expression and NF-kappaB inactivation. *J Biol Chem* (2010) 285:19593–604. doi: 10.1074/jbc.M109.069955
103. Ueda Y, Kayama H, Jeon SG, Kusu T, Isaka Y, Rakugi H, et al. Commensal microbiota induce LPS hyporesponsiveness in colonic macrophages via the production of IL-10. *Int Immunol.* (2010) 22:953–62. doi: 10.1093/intimm/dxq449
104. Franchi L, Kamada N, Nakamura Y, Burberry A, Kuffa P, Suzuki S, et al. NLRC4-driven production of IL-1β discriminates between pathogenic and commensal bacteria and promotes host intestinal defense. *Nat immunol.* (2012) 13:449–56. doi: 10.1038/ni.2263
105. Maheshwari A, Kelly DR, Nicola T, Ambalavanan N, Jain SK, Murphy-Ullrich J, et al. TGF-beta2 suppresses macrophage cytokine production and mucosal inflammatory responses in the developing intestine. *Gastroenterology* (2011) 140:242–53. doi: 10.1053/j.gastro.2010.09.043
106. Kuhn R, Lohler J, Rennick D, Rajewsky K, Muller W. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* (1993) 75:263–74.
107. Takeda K, Clausen BE, Kaisho T, Tsujimura T, Terada N, Forster I, et al. Enhanced Th1 activity and development of chronic enterocolitis in mice devoid of Stat3 in macrophages and neutrophils. *Immunity* (1999) 10:39–9.
108. Shouval DS, Biswas A, Goettel JA, McCann K, Conaway E, Redhu NS, et al. Interleukin-10 receptor signaling in innate immune cells regulates mucosal immune tolerance and anti-inflammatory macrophage function. *Immunity* (2014) 40:706–19. doi: 10.1016/j.immuni.2014.03.011
109. Girard-Madoux MJH, Ober-Blöbaum JL, Costes LMM, Kel JM, Lindenbergh-Kortleve DJ, Brouwers-Haspels I, et al. IL-10 control of CD11c+ myeloid cells is essential to maintain immune homeostasis in the small and large intestine. *Oncotarget* (2016) 7:32015–30. doi: 10.18632/oncotarget.8337
110. Hoshi N, Schenten D, Nish SA, Walther Z, Gagliani N, Flavell RA, et al. MyD88 signalling in colonic mononuclear phagocytes drives colitis in IL-10-deficient mice. *Nat Commun.* (2012) 3:1120. doi: 10.1038/ncomms2113
111. Glocker EO, Kotlarz D, Boztug K, Gertz EM, Schaffer AA, Noyan F, et al. Inflammatory bowel disease and mutations affecting the interleukin-10 receptor. *N Engl J Med.* (2009) 361:2033–45. doi: 10.1056/NEJMoa0907206
112. Schenk M, Bouchon A, Birrer S, Colonna M, Mueller C. Macrophages expressing triggering receptor expressed on myeloid cells-1 are underrepresented in the human intestine. *J Immunol.* (2005) 174:517–24. doi: 10.4049/jimmunol.174.1.517
113. Kobayashi T, Matsuoka K, Sheikh SZ, Russo SM, Mishima Y, Collins C, et al. IL-10 regulates Il12b expression via histone deacetylation: implications for intestinal macrophage homeostasis. *J Immunol.* (2012) 189:1792–99. doi: 10.4049/jimmunol.1200042
114. Simon JM, Davis JP, Lee SE, Schaner MR, Gipson GR, Weiser M, et al. Alterations to chromatin in intestinal macrophages link IL-10 deficiency to inappropriate inflammatory responses. *Eur J Immunol.* (2016) 46:1912–25. doi: 10.1002/eji.201546237
115. Hirotani T, Lee PY, Kuwata H, Yamamoto M, Matsumoto M, Kawase I, et al. The nuclear IkappaB protein IkappaBNS selectively inhibits lipopolysaccharide-induced IL-6 production in macrophages of the colonic lamina propria. *J Immunol.* (2005) 174:3650–7. doi: 10.4049/jimmunol.174.6.3650
116. Kole A, He J, Rivollier A, Silveira DD, Kitamura K, Maloy KJ, et al. Type I IFNs regulate effector and regulatory T cell accumulation and anti-inflammatory cytokine production during T cell-mediated colitis. *J Immunol.* (2013) 191:2771–9. doi: 10.4049/jimmunol.1301093
117. Danne C, Powrie F. Helicobacter hepaticus polysaccharide induces an anti-inflammatory response in intestinal macrophages. *Microb Cell* (2018) 5:208–11. doi: 10.15698/mic2018.04.626
118. Corrêa-Oliveira R, Fachi JL, Vieira A, Sato FT, Vinolo MAR. Regulation of immune cell function by short-chain fatty acids. *Clin Transl Immunol.* (2016) 5:e73. doi: 10.1038/cti.2016.17
119. Chang PV, Hao L, Offermanns S, Medzhitov R. The microbial metabolite butyrate regulates intestinal macrophage function via histone deacetylase inhibition. *Proc Natl Acad Sci USA.* (2014) 111:2247–52. doi: 10.1073/pnas.1322269111
120. Scott NA, Andrusaite A, Andersen P, Lwason M, Alcon-Giner C, Leclaire C, et al. Antibiotics induce sustained dysregulation of intestinal T cell immunity by perturbing macrophage homeostasis. *Sci Transl Med.* (2018). 464:eaao4755. doi: 10.1126/scitranslmed.aao4755
121. Ciarlo E, Heinonen T, Herderschee J, Fenwick C, Mombelli M, Le Roy D, et al. Impact of the microbial derived short chain fatty acid propionate on host susceptibility to bacterial and fungal infections *in vivo*. *Sci Rep.* (2016) 6:37944. doi: 10.1038/srep37944
122. Chng SH, Kundu P, Dominguez-Brauer C, Teo WL, Kawajiri K, Fujii-Kuriyama Y, et al. Ablating the aryl hydrocarbon receptor (AhR) in CD11c+ cells perturbs intestinal epithelium development and intestinal immunity. *Sci Rep.* (2016) 6:23820. doi: 10.1038/srep23820
123. Avetisyan M, Rood JE, Huerta Lopez S, Sengupta R, Wright-Jin E, Dougherty JD, et al. Muscularis macrophage development in the absence of an enteric nervous system. *Proc Natl Acad Sci USA.* (2018) 115:4696–701. doi: 10.1073/pnas.1802490115
124. Grimm MC, Pullman WE, Bennett GM, Sullivan PJ, Pavli P, Doe WF. Direct evidence of monocyte recruitment to inflammatory bowel disease mucosa. *J Gastroenterol Hepatol.* (1995) 10:387–95.
125. Kamada N, Hisamatsu T, Okamoto S, Chinen H, Kobayashi T, Sato T, et al. Unique CD14 intestinal macrophages contribute to the pathogenesis of

Crohn disease via IL-23/IFN-gamma axis. *J Clin Invest.* (2008) 118:2269–80. doi: 10.1172/JCI34610
126. Thiesen S, Janciauskiene S, Uronen-Hansson H, Agace W, Hågerkorp C-M, Spee P, et al. CD14hiHLA-DRdim macrophages, with a resemblance to classical blood monocytes, dominate inflamed mucosa in Crohn's disease. *J Leukoc Boil.* (2013) 95:531–41. doi: 10.1189/jlb.0113021
127. Lampinen M, Waddell A, Ahrens R, Carlson M, Hogan SP. CD14+CD33+ myeloid cell-CCL11-eosinophil signature in ulcerative colitis. *J Leukoc Boil.* (2013) 94:1061–70. doi: 10.1189/jlb.1212640
128. Mahida YR, Patel S, Gionchetti P, Vaux D, Jewell DP. Macrophage subpopulations in lamina propria of normal and inflamed colon and terminal ileum. *Gut* (1989) 30:826–34.
129. Schenk M, Bouchon A, Seibold F, Mueller C. TREM-1–expressing intestinal macrophages crucially amplify chronic inflammation in experimental colitis and inflammatory bowel diseases. *J Clin Invest.* (2007) 117:3097–106. doi: 10.1172/JCI30602
130. Vos ACW, Wildenberg ME, Arijs I, Duijvestein M, Verhaar AP, de Hertogh G, et al. Regulatory macrophages induced by infliximab are involved in healing *in vivo* and *in vitro*. *Inflamm Bowel Dis.* (2012) 18:401–8. doi: 10.1002/ibd.21818
131. Bloemendaal FM, Koelink PJ, van Schie KA, Rispens T, Peters CP, Buskens CJ, et al. TNF-anti-TNF immune complexes inhibit IL-12/IL-23 secretion by inflammatory macrophages via an fc-dependent mechanism. *J Crohns Colitis.* (2018) 12:1122–30. doi: 10.1093/ecco-jcc/jjy075
132. Atreya R, Neumann H, Neufert C, Waldner MJ, Billmeier U, Zopf Y, et al. *In vivo* imaging using fluorescent antibodies to tumor necrosis factor predicts therapeutic response in Crohn's disease. *Nat Med.* (2014) 20:313–18. doi: 10.1038/nm.3462
133. Platt AM, Bain CC, Bordon Y, Sester DP, Mowat AM. An independent subset of TLR expressing CCR2-dependent macrophages promotes colonic inflammation. *J Immunol.* (2010) 184:6843–54. doi: 10.4049/jimmunol.0903987
134. Bain CC, Oliphant CJ, Thomson CA, Kullberg MC, Mowat AM. Proinflammatory role of monocyte-derived CX3CR1int macrophages in Helicobacter hepaticus-Induced Colitis. *Infect Immun.* (2018) 86:IAI.00579-17. doi: 10.1128/IAI.00579-17
135. Weber B, Saurer L, Schenk M, Dickgreber N, Mueller C. CX3CR1 defines functionally distinct intestinal mononuclear phagocyte subsets which maintain their respective functions during homeostatic and inflammatory conditions. *Eur J Immunol.* (2011) 41:773–9. doi: 10.1002/eji.201040965
136. Qualls JE, Kaplan AM, Van Rooijen N, Cohen DA. Suppression of experimental colitis by intestinal mononuclear phagocytes. *J Leukoc Boil.* (2006) 80:802–15. doi: 10.1189/jlb.1205734
137. Seo S-U, Kamada N, Muñoz-Planillo R, Kim Y-G, Kim D, Koizumi Y, et al. Distinct commensals induce interleukin-1β via Nlrp3 inflammasome in inflammatory monocytes to promote intestinal inflammation in response to injury. *Immunity* (2015) 42:744–55. doi: 10.1016/j.immuni.2015.03.004
138. Wolf Y, Shemer A, Polonsky M, Gross M, Mildner A, Yona S, et al. Autonomous TNF is critical for *in vivo* monocyte survival in steady state and inflammation. *J. Exp Med.* (2017) 214:905–17. doi: 10.1084/jem.20160499
139. Schippers A, Muschaweck M, Clahsen T, Tautorat S, Grieb L, Tenbrock K, et al. β7-Integrin exacerbates experimental DSS-induced colitis in mice by directing inflammatory monocytes into the colon. *Mucosal Immunol.* (2016) 9:527–38. doi: 10.1038/mi.2015.82
140. Becker F, Kurmaeva E, Gavins FNE, Stevenson EV, Navratil AR, Jin L, et al. A critical role for monocytes/macrophages during intestinal inflammation-associated Lymphangiogenesis. *Inflamm Bowel Dis.* (2016) 22:1326–45. doi: 10.1097/MIB.0000000000000731
141. Ingersoll MA, Spanbroek R, Lottaz C, Gautier EL, Frankenberger M, Hoffmann R, et al. Comparison of gene expression profiles between human and mouse monocyte subsets. *Blood* (2010) 115:e10–9. doi: 10.1182/blood-2009-07-235028
142. Smith PD, Smythies LE, Shen R, Greenwell-Wild T, Gliozzi M, Wahl SM. Intestinal macrophages and response to microbial encroachment. *Mucosal Immunol.* (2010) 4:31-42. doi: 10.1038/mi.2010.66
143. Schulthess J, Meresse B, Ramiro-Puig E, Montcuquet N, Darche S, Bègue B, et al. Interleukin-15-dependent NKp46+ innate lymphoid cells control intestinal inflammation by recruiting inflammatory monocytes. *Immunity* (2012) 37:108–21. doi: 10.1016/j.immuni.2012.05.013
144. Nieto JC, Zamora C, Cantó E, Garcia-Planella E, Gordillo J, Ortiz MA, et al. CSF-1 regulates the function of monocytes in Crohn's disease patients in remission. *Sci Rep.* (2017) 7:92. doi: 10.1038/s41598-017-00145-4
145. Tacke F, Alvarez D, Kaplan TJ, Jakubzick C, Spanbroek R, Llodra J, et al. Monocyte subsets differentially employ CCR2, CCR5, and CX3CR1 to accumulate within atherosclerotic plaques. *J Clin Invest.* (2007) 117:185–94. doi: 10.1172/JCI28549
146. Andres PG, Beck PL, Mizoguchi E, Mizoguchi A, Bhan AK, Dawson T, et al. Mice with a selective deletion of the CC chemokine receptors 5 or 2 are protected from dextran sodium sulfate-mediated colitis: lack of CC chemokine receptor 5 expression results in a NK1.1+ lymphocyte-associated Th2-type immune response in the intestine. *J Immunol.* (2000) 164:6303–12. doi: 10.4049/jimmunol.164.12.6303
147. Li Q, Wang D, Hao S, Han X, Xia Y, Li X, et al. CD169 Expressing macrophage, a key subset in mesenteric lymph nodes promotes mucosal inflammation in dextran sulfate sodium-induced colitis. *Front Immunol.* (2017) 8:669. doi: 10.3389/fimmu.2017.00669
148. Asano K, Takahashi N, Ushiki M, Monya M, Aihara F, Kuboki E, et al. Intestinal CD169(+) macrophages initiate mucosal inflammation by secreting CCL8 that recruits inflammatory monocytes. *Nat Commun.* (2015) 6:7802. doi: 10.1038/ncomms8802
149. Carlsen HS, Yamanaka T, Scott H, Rugtveit J, Brandtzaeg P. The proportion of CD40+ mucosal macrophages is increased in inflammatory bowel disease whereas CD40 ligand (CD154)+ T cells are relatively decreased, suggesting differential modulation of these costimulatory molecules in human gut lamina propria. *Inflamm Bowel Dis.* (2006) 12:1013–24. doi: 10.1097/01.mib.0000234135.43336.72
150. Rugtveit J, Bakka A, Brandtzaeg P. Differential distribution of B7.1 (CD80) and B7.2 (CD86) costimulatory molecules on mucosal macrophage subsets in human inflammatory bowel disease (IBD). *Clin Exp Immunol.* (1997) 110:104–13.
151. Morrison PJ, Bending D, Fouser LA, Wright JF, Stockinger B, Cooke A, et al. Th17-cell plasticity in Helicobacter hepaticus-induced intestinal inflammation. *Mucosal Immunol.* (2013) 6:1143–56. doi: 10.1038/mi.2013.11
152. Ahrens R, Waddell A, Seidu L, Blanchard C, Carey R, Forbes E, et al. Intestinal macrophage/epithelial cell-derived CCL11/eotaxin-1 mediates eosinophil recruitment and function in pediatric ulcerative colitis. *J Immunol.* (2008) 181:7390–9. doi: 10.4049/jimmunol.181.10.7390
153. Waddell A, Ahrens R, Steinbrecher K, Donovan B, Rothenberg ME, Munitz A, et al. Colonic eosinophilic inflammation in experimental colitis is mediated by Ly6C(high) CCR2(+) inflammatory monocyte/macrophage-derived CCL11. *J Immunol.* (2011) 186:5993–6003. doi: 10.4049/jimmunol.1003844
154. Griseri T, Arnold IC, Pearson C, Krausgruber T, Schiering C, Franchini F, et al. Granulocyte macrophage colony-stimulating factor-activated eosinophils promote interleukin-23 driven chronic colitis. *Immunity* (2015) 43:187–99. doi: 10.1016/j.immuni.2015.07.008
155. Kim YG, Kamada N, Shaw MH, Warner N, Chen GY, Franchi L,et al. The Nod2 sensor promotes intestinal pathogen eradication via the chemokine CCL2-dependent recruitment of inflammatory monocytes. *Immunity* (2011) 34:769–80. doi: 10.1016/j.immuni.2011.04.013
156. Dunay IR, Damatta RA, Fux B, Presti R, Greco S, Colonna M, et al. Gr1(+) inflammatory monocytes are required for mucosal resistance to the pathogen Toxoplasma gondii. *Immunity* (2008) 29:306–17. doi: 10.1016/j.immuni.2008.05.019
157. Seo S-U, Kuffa P, Kitamoto S, Nagao-Kitamoto H, Rousseau J, Kim Y-G, et al. Intestinal macrophages arising from CCR2(+) monocytes control pathogen infection by activating innate lymphoid cells. *Nat Commun.* (2015) 6:8010. doi: 10.1038/ncomms9010
158. Manta C, Heupel E, Radulovic K, Rossini V, Garbi N, Riedel CU, et al. CX(3)CR1(+) macrophages support IL-22 production by innate lymphoid cells during infection with Citrobacter rodentium. *Mucosal Immunol.* (2013) 6:177–88. doi: 10.1038/mi.2012.61
159. Longman RS, Diehl GE, Victorio DA, Huh JR, Galan C, Miraldi ER, et al. CX3CR1+ mononuclear phagocytes support colitis-associated innate

lymphoid cell production of IL-22. *J. Exp Med.* (2014) 211:1571–83. doi: 10.1084/jem.20140678
160. Zheng Y, Valdez PA, Danilenko DM, Hu Y, Sa SM, Gong Q, Abbas AR, Modrusan Z, Ghilardi N, de Sauvage FJ, et al. Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. *Nat Med.* (2008) 14:282–9. doi: 10.1038/nm1720
161. Schreiber HA, Loschko J, Karssemeijer RA, Escolano A, Meredith MM, Mucida D, et al. Intestinal monocytes and macrophages are required for T cell polarization in response to *Citrobacter rodentium*. *J Exp Med.* (2013) 210:2025–39. doi: 10.1084/jem.20130903
162. Cerovic V, Bain CC, Mowat AM, Milling SWF. Intestinal macrophages and dendritic cells: what's the difference? *Trends Immunol.* (2014) 35:270–7. doi: 10.1016/j.it.2014.04.003
163. Bain CC, Mowat AM. Macrophages in intestinal homeostasis and inflammation. *Immunol Rev.* (2014) 260:102–17. doi: 10.1111/imr.12192
164. Grainger JR, Wohlfert EA, Fuss IJ, Bouladoux N, Askenase MH, Legrand F, et al. Inflammatory monocytes regulate pathologic responses to commensals during acute gastrointestinal infection. *Nat Med.* (2013) 19:713–21. doi: 10.1038/nm.3189
165. deSchoolmeester ML, Little MC, Rollins BJ, Else KJ. Absence of CC chemokine ligand 2 results in an altered Th1/Th2 cytokine balance and failure to expel *Trichuris muris* infection. *J Immunol.* (2003) 170:4693–4700. doi: 10.4049/jimmunol.170.9.4693
166. Filbey KJ, Grainger JR, Smith KA, Boon L, van Rooijen N, Harcus Y, et al. Innate and adaptive type 2 immune cell responses in genetically controlled resistance to intestinal helminth infection. *Immunol Cell Biol.* (2014) 92:436–48. doi: 10.1038/icb.2013.109
167. Schmidt S, Hoving JC, Horsnell WGC, Mearns H, Cutler AJ, Brombacher TM, et al. Nippostrongylus-induced intestinal hypercontractility requires IL-4 receptor alpha-responsiveness by T cells in mice. *PLoS ONE* (2012) 7:e52211. doi: 10.1371/journal.pone.0052211
168. Bowcutt R, Bell LV, Little M, Wilson J, Booth C, Murray PJ, et al. Arginase-1-expressing macrophages are dispensable for resistance to infection with the gastrointestinal helminth *Trichuris muris*. *Parasite Immunol.* (2011) 33:411–20. doi: 10.1111/j.1365-3024.2011.01300.x
169. Herbert DR, Hölscher C, Mohrs M, Arendse B, Schwegmann A, Radwanska M, et al. Alternative macrophage activation is essential for survival during schistosomiasis and downmodulates T helper 1 responses and immunopathology. *Immunity* (2004) 20:623–635. doi: 10.1016/S1074-7613(04)00107-4
170. Zhao A, Urban JF, Anthony RM, Sun R, Stiltz J, van Rooijen N, et al. Th2 cytokine-induced alterations in intestinal smooth muscle function depend on alternatively activated macrophages. *Gastroenterology* (2008) 135:217–25.e1. doi: 10.1053/j.gastro.2008.03.077
171. Jenkins SJ, Ruckerl D, Cook PC, Jones LH, Finkelman FD, Van Rooijen N, et al. Local macrophage proliferation, rather than recruitment from the blood, is a signature of TH2 inflammation. *Science* (2011) 332:1284–8. doi: 10.1126/science.1204351
172. Little MC, Hurst RJM, Else KJ. Dynamic changes in macrophage activation and proliferation during the development and resolution of intestinal inflammation. *J Immunol.* (2014) 193:4684–95. doi: 10.4049/jimmunol.1400502
173. Monteleone G, Boirivant M, Pallone F, MacDonald TT. TGF-beta1 and Smad7 in the regulation of IBD. *Mucosal Immunol.* (2008) 1(Suppl. 1):S50–3. doi: 10.1038/mi.2008.55
174. Bäcker V, Cheung F-Y, Siveke JT, Fandrey J, Winning S. Knockdown of myeloid cell hypoxia-inducible factor-1α ameliorates the acute pathology in DSS-induced colitis. *PLoS ONE* (2017) 12:e0190074. doi: 10.1371/journal.pone.0190074
175. Mee AS, Berney J, Jewell DP. Monocytes in inflammatory bowel disease: absolute monocyte counts. *J Clin Pathol.* (1980) 33:917–20.
176. Trottier MD, Irwin R, Li Y, McCabe LR, Fraker PJ. Enhanced production of early lineages of monocytic and granulocytic cells in mice with colitis. *Proc Natl Acad Sci USA.* (2012) 109:16594–9. doi: 10.1073/pnas.1213854109
177. Askenase MH, Han S-J, Byrd AL, Morais da Fonseca D, Bouladoux N, Wilhelm C, et al. Bone-marrow-resident NK cells prime monocytes for regulatory function during Infection. *Immunity* (2015) 42:1130–42. doi: 10.1016/j.immuni.2015.05.011
178. Rani R, Smulian AG, Greaves DR, Hogan SP, Herbert DR. TGF-beta limits IL-33 production and promotes the resolution of colitis through regulation of macrophage function. *Eur J Immunol.* (2011) 41:2000–9. doi: 10.1002/eji.201041135
179. Farro G, Stakenborg M, Gomez-Pinilla PJ, Labeeuw E, Goverse G, Di Giovangiulio M, et al. CCR2-dependent monocyte-derived macrophages resolve inflammation and restore gut motility in postoperative ileus. *Gut* (2017) 66:2098–109. doi: 10.1136/gutjnl-2016-313144
180. Gautier EL, Ivanov S, Lesnik P, Randolph GJ. Local apoptosis mediates clearance of macrophages from resolving inflammation in mice. *Blood* (2013) 122:2714–22. doi: 10.1182/blood-2013-01-478206
181. Caprioli F, Bosè F, Rossi RL, Petti L, Viganò C, Ciafardini C, et al. Reduction of CD68+ macrophages and decreased IL-17 expression in intestinal mucosa of patients with inflammatory bowel disease strongly correlate with endoscopic response and mucosal healing following infliximab therapy. *Inflamm Bowel Dis.* (2013) 19:729–39. doi: 10.1097/MIB.0b013e318280292b
182. Ikeda N, Asano K, Kikuchi K, Uchida Y, Ikegami H, Takagi R, et al. Emergence of immunoregulatory Ym1+Ly6Chi monocytes during recovery phase of tissue injury. *Sci Immunol.* (2018) 3:eaat0207. doi: 10.1126/sciimmunol.aat0207

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Intestinal Dendritic Cells in Health and Gut Inflammation

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Dendritic cells (DCs) mediate tolerance to food antigens, limit reactivity to the gut microbiota and are required for optimal response to intestinal pathogens. Intestinal DCs are heterogeneous but collectively generate both regulatory and effector T cell responses. The balance of outcomes is determined by the activity of functionally distinct DC subsets and their modulation by environmental cues. DCs constantly sample luminal content to monitor for pathogens; the significance of the various pathways by which this occurs is incompletely understood. Intestinal DC have distinctive properties shaped by local host, dietary and microbial signals. These properties include the ability to produce all-trans retinoic acid (RA) and imprint gut tropism on T cells they activate. In the steady-state, subsets of intestinal DC are potent generators of inducible Treg, aided by their ability to activate TGFβ and produce RA. However, responses induced by steady-state intestinal DCs are not exclusively regulatory in nature; effector T cells with specificity for commensal bacterial can be found in the healthy mucosa and these can be locally controlled to prevent inflammation. The ability of intestinal DCs to enhance effector responses in infection or sustain inflammation in disease is likely to involve both modulation of the local DC population and recruitment of additional populations. Immune pathways in the pathogenesis of inflammatory bowel disease can be mapped to DCs and in inflamed intestinal tissue, DCs show increased expression of microbial recognition machinery, activation, and production of key immunological mediators. Intestinal DCs may be targeted for disease therapy or to improve vaccine responses.

Keywords: Dendritic cells, intestinal inflammation, antigen sampling, lymphocyte homing, inflammatory bowel disease

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INTRODUCTION

Dendritic cells (DCs) are bone marrow-derived antigen presenting cells which comprise two major subsets: conventional (or classical) DCs (cDCs) and plasmacytoid DC (pDC). They are developmentally distinct from both tissue resident macrophages and monocyte-derived populations (1) but share many phenotypic markers with these populations. Historically this has led to confusion and apparently conflicting data in analyses of DCs in the intestine. However, recent development of better strategies for DC identification, together with improved cell isolation techniques that help maintain DCs in their native state (2, 3), and genetic tools that enable specific deletion of DC populations *in vivo*, have enabled a clearer picture of the role of DCs in the intestine to emerge.

cDCs play critical roles in immune regulation in the intestine and are the focus of this review. The reader is referred to a recent review article (4) for more information on the role of pDC in the

intestine. cDCs are required for induction of oral tolerance (5) and the generation of regulatory T cells (Treg) recognizing soluble antigens (5) and commensal microbes (6, 7). They are also required for optimal protective immune responses against diverse pathogens (8–12). The balance of regulatory and effector responses is influenced by the contribution of functionally distinct cDC subsets as well as their modulation by environmental cues.

INTESTINAL DENDRITIC CELLS IN THE STEADY-STATE

Heterogeneity of Intestinal DCs

Intestinal cDCs are found within organized lymphoid tissue, including Peyer's patches (PP) (13) and draining lymph nodes, as well as in the lamina propria (LP) of small intestine (SI) and colon (14–16). As in other tissues, LP cDCs comprise cDC1 and cDC2 which can be defined in mice and humans by expression of X-C motif chemokine receptor 1 (XCR1) or signal regulatory protein α (SIRPα/CD172a), respectively, (1, 17).

Additional cell surface markers are used in particular species to define intestinal cDC subsets further. In mice, cDC1 are CD103+CD11b–, whereas cDC2 comprise CD103–CD11b+ cells and a gut-specific CD103+CD11b+ population (18) (**Figure 1**). The two CD11b+ populations are closely related; CD103–CD11b+ cDC give rise to CD103+CD11b+cells under the influence of TGFβ (23). There are more CD103+CD11b+ in the SI than in the colon (24). Equivalent cDC populations are present in the human intestine (25) and are often identified based on expression of CD103 in conjunction with SIRPα rather than CD11b.

LP DC populations are dependent on FLT3L for development and are derived from a committed pre-cDC progenitor (**Figure 1**). Some pre-cDC can acquire expression of α4β7 integrin and thereby commit to an intestinal cDC fate (16, 19, 20). LP cDCs express the cDC-specific transcription factor Zbtb46 (11) but not the macrophage marker CD64. Critically, all have the capacity to migrate in a CCR7-dependent manner to draining LN to interact with recirculating T cells (26, 27). LP cDC subsets require different transcription factors for development and their selective deletion has enabled some of their functions to be defined (**Figure 1**). Detailed discussion of these experiments is beyond the scope of this review but the reader is referred to other recent authoritative articles (21, 22).

Antigen Sampling

To protect against luminal pathogens and establish regulatory response to innocuous antigens, DCs continuously sample intestinal contents (28, 29). In PP, microfold (M) cells in the follicle associated epithelium internalize bacteria and other particulates and deliver them to underlying DCs [reviewed in (30)]. PP cDCs can also capture translocated IgA immune complexes (31) and extend dendrites through M cell specific transcellular pores (32). cDCs cross-present viral antigen captured from infected epithelial cells (33, 34). Ileal CD103+ cDCs in the epithelium (**Figure 1**) sample soluble and particulate antigen (35). Transport of low molecular weight soluble material by SI goblet cells (36) and retro-transport of IgG immune complexes across the epithelium (37) can also deliver antigen to cDCs. Non-migratory CX3CR1+ macrophages in the SI sample antigens via trans-epithelial processes (38–41) and hand on to migratory CD103+ cDCs (38). The functional significance of different modes of sampling and the impact of inflammation are poorly understood.

Imprinting of Gut Tropism

Lymphocytes activated in gut lymphoid tissue traffic to the intestinal mucosa because they express specific adhesion molecules and chemokine receptors. The integrin α4β7 binds MAdCAM-1 expressed by intestinal vascular endothelium and facilitates entry to both the colon and the SI. The chemokine receptor CCR9 is required for entry to the SI, where its ligand CCL25 is expressed (42). GPR15 is implicated in homing to the colon but there may be important differences between mice and humans with regard to its role in the trafficking of effector vs. regulatory T cell populations (43–45). These mechanisms facilitate lymphocyte homing to the intestine in the absence of overt inflammation and enable intestinal responses to be regulated independently of the systemic response. In mice, cDCs that have migrated from LP to mesenteric lymph nodes induce expression of α4β7 and CCR9 on T cells they activate (46–49) via production of all-trans retinoic acid (RA) (50). Only cDC from the intestine express the enzyme RALDH2 (encoded by *aldh1a2*), required for the generation of RA from dietary vitamin A, explaining the site-specific induction of gut tropism (50). Initial data suggested that only CD103+ cDC had imprinting activity (15, 46, 51, 52) but it has subsequently been detected in mouse CD103– cDCs (26). Also, both CD103+ and CD103– cDCs in the human colon express *ALDH1A2* (53). Stromal cells in mesenteric lymph nodes can also produce RA to reinforce the imprinting activity of migratory intestinal cDCs (54–56).

Induction of Regulatory and Effector T Cell Responses

In the steady-state, intestinal DCs can induce Treg. In the mouse SI, induction of gut tropic Treg directed against soluble antigens, by both CD103+CD11b+ and CD103+CD11b– DCs, occurs in the mesenteric LN (52) and underlies the long-recognized phenomenon of oral tolerance generated to such antigens (57). The ability of SI CD103+ cDC to generate Treg is dependent on their expression of the integrin αvβ8, which activates latent TGFβ, and is enhanced by their production of RA (58–62). PD-L1 and PD-L2 have also been implicated in generation of Treg by MLN cDC (63). It is notable that induction of tolerance to colonic antigens differs from tolerance in the SI in that it is induced in the iliac, not mesenteric, nodes, is mediated by CD103–CD11b+ cDC and is independent DC-generated RA (16). The generation of Treg directed against commensal bacteria has been less studied. Nonetheless, in a cell transfer model, the rapid generation of Treg from naïve commensal-reactive transgenic CD4 T cells required Notch2-dependent but not Batf3-dependent cDC, suggesting that SIRPα+ cDC2, possibly CD103+CD11b+ cells, play a dominant role (7).

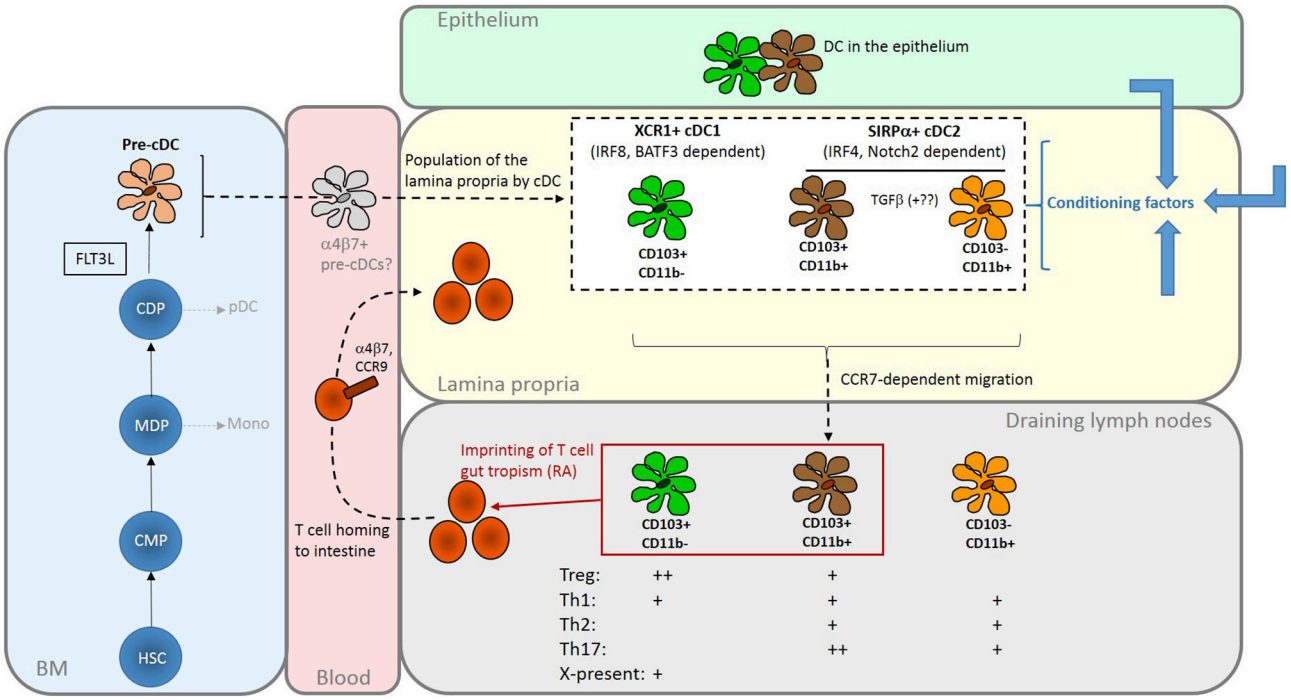

FIGURE 1 | Summary of the origins and properties of intestinal DC. FLT3L-dependent development of cDC gives rise to two major subsets via CDP and committed pre-cDC in the BM. cDC1 are XCR1+ and depend on transcription factors including IRF8 and BATF3; cDC2 are SIRPα+ and depend on transcription factors that include IRF4. Population of the intestinal mucosa may be via an intermediate gut-committed α4β7+ precursor [sometimes termed pre-mucosal (μ) DC] stage (19, 20). In the mouse LP, the cDC2 population comprises two closely related CD11b+ subsets that can be distinguished by the presence or absence of CD103; CD11b+CD103+, found predominately in the SI, are probably derived from the CD11b+CD103– cells under the influence of TGFβ. The cDC1 population is CD11b–CD103+. Similar populations in the human intestine can be identified on the basis of CD103 and SIRPα expression. In the LP they are exposed to conditioning factors (shown as blue arrows) from epithelial cells and other sources which further shape their functional properties. All three subsets have the capacity to migrate to draining lymph nodes to interact with recirculating T cells. Here, production of RA by LP- derived DCs, particularly those expressing CD103, imprints gut tropism on T cells they activate by inducing expression of α4β7 integrin and the chemokine receptor CCR9. Although DCs can induce both Treg and effector T cells, with some specialism across DC subsets, the balance of these outcomes favors regulation in the steady-state. The reader is referred to recent reviews for a more in-depth discussion of intestinal DC subsets (21, 22). HSC, haematopoietic stem cell; CMP, common myeloid progenitor; MDP, monocyte dendritic cell precursor; CDP, common dendritic cell precursor; pDC, plasmacytoid DC; Mono, monocyte; X-present, cross-presentation to CD8+ T cells.

T cell responses stimulated by DCs in the steady-state are not exclusively regulatory. Effector T cells are present in the lamina propria of healthy mice and humans; although some of these may reflect past pathogen encounter others are specific for the commensal microbiota (64, 65). Effector cells in the healthy intestine enhance the epithelial barrier (66) and protect against translocation of pathogens (67). Their activity can be locally controlled by regulatory CX3CR1^{hi} mucosal myeloid populations (68), anti-inflammatory cytokines such as TGFβ (69) as well as Treg. CD103– cDC migrating from the mouse SI can prime effector T cells in the absence of stimulation (26) indicating one mechanism by which these responses can be generated.

Conditioning of Intestinal DC

The ability of intestinal cDC to generate RA and promote tolerance requires active Wnt/β-catenin signaling with the cDCs (70) and is determined in part by local environment cues (71). Epithelial cells promote the ability of DC to generate both regulatory (72, 73) and Type 2 responses (74). In the mouse, epithelial TSLP, with IL-25 and IL-33, inhibits IL-12 production by DCs and promotes their ability to generate Th2 responses that clear *Trichuris muris* infection (74). RA and TGFβ from human epithelial cells promote regulatory DC function (72). Exposure to RA can induce characteristics of SI DCs *in vitro* (75) and is required for *aldh1a2* expression (76). Sources of RA include epithelial cells (77), LP stromal cells (78), and bile retinoids (79). In contrast, prostaglandin E2 has been reported to negatively regulate the expression of RA generating enzymes in DC (80). Dietary and microbial products, including ligands of the aryl hydrocarbon receptor [AhR (81)] and butyrate (82), also affect intestinal DCs.

INTESTINAL DENDRITIC CELLS IN THE PROMOTION OF EFFECTOR FUNCTION AND INFLAMMATION

Promotion of Effector Function

The balance of responses induced by DC can change in the context of infection to favor effector mechanisms. Signaling

through p38 MAPK in CD103+ mouse DC regulates the balance of Treg and Th1 development from naïve T cells (83); in its absence, expression of RALDH2 and generation of Treg are reduced but Th1 responses enhanced.

A change in the balance of T cell responses induced by intestinal cDC may result from direct modulation by danger signals, altered conditioning of the resident cDC population or recruitment of distinct pro-inflammatory DC (**Figure 2**). Intestinal cDC express pattern recognition receptors and respond to microbial products (84, 85). Subsets of mouse and human DC differentially express Toll-like receptors (TLRs) suggesting specialization for direct recognition of particular microbes (25, 86). Mouse CD103+CD11b+ cDCs express TLR5 and their ability to induce Th17 responses is enhanced following activation with flagellin (24, 85, 87, 88). Activation of CD103+CD11b+ cDC results in increased production of IL-6 and IL-23 which promote Th17 development and production of the anti-microbial peptide RegIIIγ (85, 89). Administration of a TLR7 agonist *in vivo* results in activation of a CD103+CD11b− cDC migratory subset with the ability to generate effector CD8+ T cell responses to cross-presented antigen (90).

In most tissues, exposure to microbial products is sufficient to convert immature cDCs to mature cells which generate potent effector responses. However, exposure to PAMPs from the commensal microbiota is likely to be a common occurrence in the healthy intestine and therefore a second signal may also be required. Indeed, mouse CD103+ SI cDCs can induce Treg even in the presence of the high level of costimulatory molecule expression characteristic of mature cDC (58). The nature of this second signal is not known but IgA-containing immune complexes, normally restricted to the lumen, but present in significant quantities in damaged tissues, can enhance the pro-inflammatory activity of DCs (91).

Intestinal cDCs reside in the mucosa for a few days (92) during which time they are conditioned to acquire regulatory properties. cDCs that escape conditioning in the steady-state may facilitate the generation of "tonic" protective effector T cell responses. An increase in turnover following exposure to TLR ligands (93, 94) or inflammatory cytokines could shorten residence time, reduce exposure to conditioning factors and increase cDC-generated effector responses.

Intestinal cDC function may also be influenced remotely during their development in the bone marrow. Intestinal inflammation alters hematopoiesis to influence the development of monocytes and subsequently the intestinal populations derived from them (95). These concepts remain to be explored for cDCs but changes in blood DCs have been described in IBD (96, 97).

Alternative precursors may also be recruited into the tissue under inflammatory conditions to provide cells with the ability to generate effector responses. Monocytes can give rise to DC-like cells (monocyte-derived DC; moDC) under inflammatory conditions (98, 99). In healthy mice, monocytes recruited into the intestine differentiate into anti-inflammatory macrophages (100–103). However, under inflammatory conditions this differentiation process is interrupted, generating a population with some DC-like properties including the ability to activate naïve T cells and generate Th1 cells (102). Similarly, cells expressing the monocyte marker CD14 accumulate in the inflamed mucosa of inflammatory bowel disease (IBD) patients (104, 105) and these too have a high capacity to naive stimulate T cells and generate gut tropic Th1 cells and Th17 cells (104). However, it is unclear if monocyte derived-cells can migrate to lymphnodes or act solely within the mucosa.

Effector cells generated by DCs could also be released from local control within the tissues where diverse antigen presenting cell (APC) populations exert a local influence (68, 106–110). In the context of chronic inflammation, T cells lose responsiveness to regulatory TGFβ due to over-expression of SMAD7, an inhibitor of TGFβR signaling (69). In addition, inflammatory cytokines can change the repertoire of peptides presented by DCs (111) and the emergence of "cryptic" determinants may allow escape from Treg control.

Irrespective of the combination of mechanisms which allow effector responses to be enhanced to deal with infections, a key unanswered question remains how these responses are confined to the pathogen and do not normally extend to co-sampled commensal organisms.

DC and Inflammatory Intestinal Disease

Many immune pathways key to IBD pathogenesis, highlighted by the identification of genetic variants associated with disease susceptibility, can be mapped to DCs. Nucleotide-binding oligomerization domain–containing-2 (NOD2), loss of function variants of which are associated with Crohn's disease (CD), is a bacterial sensor in DCs; its engagement impacts upon bacterial handling, cytokine production and antigen presentation (112, 113). DCs that express CD-associated variants are defective in these pathways (112, 113). The IL-23 axis is implicated in CD and DCs are a major source of bacterially driven intestinal IL-23 (85, 87, 89). Moreover, expression of genes associated with variation in CD prognosis, rather than susceptibility, can also be mapped to DCs (114).

Mouse models of colitis provide direct evidence for the importance of DCs. Transfer of bone marrow derived DCs increases inflammation whereas depletion of DCs reduces it (115, 116). Colitis develops in mice in which TGFβ receptor signaling is non-functional in DCs (23, 117) and, in T cell deficient mice, administration of an agonistic anti-CD40 antibody activates DCs and induces IL-23-dependent intestinal inflammation (118).

DCs isolated from inflamed intestinal tissue show evidence of enhanced microbial recognition and heightened activation. More colonic cDCs from inflamed tissue of IBD patients express TLR2 and TLR4 (119, 120) and they also express higher levels of activation-associated CD40 (119). In CD, mature CCR7+ DCs, retained in the mucosa by locally produced ligands, cluster with proliferating T cells (121).

In CD, more colonic LP cDCs produce IL-12/23p40 and IL-6 but the proportion of cDCs that produce IL-10 is similar to healthy controls (119). Production of pro-inflammatory cytokines by colonic cDCs in CD correlates with disease activity, levels of inflammation and aspects of the intestinal microbiota (122). In UC, the frequency of cDCs producing IL-12/23p40 and IL-10 has been reported to be greater (122) or similar (119) to healthy controls in different studies. Production of IL-6 by cDC

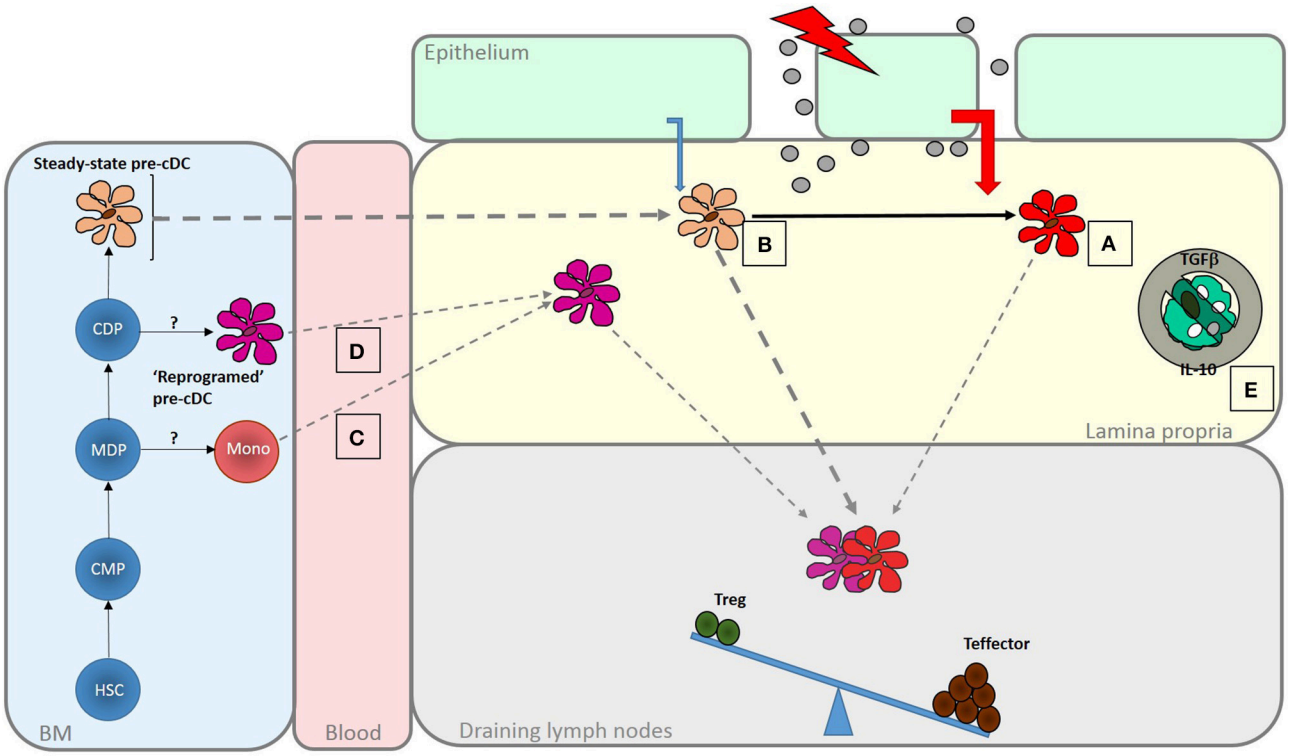

FIGURE 2 | Enhanced generation of effector T cells in response in infection and inflammatory disease. A number of mechanisms act to shift the balance of response induced by intestinal DC to favor the generation of effector responses. These are important for pathogen clearance but if dysregulated may lead to inflammatory disease. Increased exposure to PAMPS and DAMPS, as the result of loss of barrier integrity or tissue penetration by pathogens may be sensed by LP DC populations leading to their activation **(A)**. Altered microbial sensing by epithelial cells due, for instance, to engagement of basolateral PRRs, may change the nature of conditioning factors they produce. Increased DC turnover may reduce exposure to those conditioning factors that normally promote regulatory function in intestinal DC **(B)**. Alternatively, different types of DC, specialized to promote effector responses, may be recruited to the intestine under these conditions. These could be DC-like cells derived from monocytes **(C)** or possibly "reprogrammed" DC arise as a result of altered differentiation in the BM **(D)**. There may also be a release from the regulatory mechanisms that normally limit effector function within the mucosa, due, for instance to a loss of response to regulatory cytokines like IL-10 or TGFβ or a failure to interact with CX3CR1+ cells that promote regulation **(E)**. The role of DCs on determining responsiveness to these local control mechanisms is not known.

from UC patients was not increased in either study. In coeliac disease, activated DC producing Th1-promoting cytokines accumulate in the duodenal mucosa (123). In the MLN of CD patients, DCs release more IL-23 upon bacterial stimulation and CD4+ T cells produce more IL-17 and IFNγ (124).

MicroRNAs are short non-coding RNA molecules that function in RNA silencing and post-transcriptional regulation of gene expression. Expression of microRNA (miR)-10a, which acts in DC to limit their ability to produce IL-23/23p40 and generate Th1 and Th17 responses, is reduced in the inflamed mucosa of IBD patients (125). The IBD-associated reduction in mir-10a is accompanied by increased expression of IL-12/23. In UC, immature colonic DCs are poorly stimulatory but induce an atypical T cell response characterized by increased IL-4 but reduced IFNγ and IL-22 (120, 126) possibly indicating a reduced ability to induce barrier protective T cell responses.

Numbers of CD103+ cDCs are reduced in mice with chronic ileitis (127) and in the colon of UC patients (128). Whether these differences reflect a change in phenotype or altered cDC populations is not clear. Unlike equivalent cDCs from normal human colon, CD103+ colonic cDCs from UC patients do not generate Foxp3+ Treg but do generate IFNγ-, IL-13-, and IL-17-producing CD4 T cells. CD103+ cDCs from UC patients also have higher expression of IL-6, IL-12p40, IL-12p35, and TNFα (128).

RA is required for optimal Treg induction by intestinal CD103+ cDCs in the steady-state but these cells have reduced expression of RA-generating enzymes in mice with colitis (127, 129). The effects of RA on the immune system are context dependent [reviewed in (130, 131)] and it can promote pro-inflammatory as well as regulatory responses (132). In the presence of IL-15, RA induces the release of pro-inflammatory IL-12 and IL-23 by cDC and promotes intestinal inflammation (133). There is conflicting data on the production of RA by DC in the inflamed human colon: increased (53) and decreased (134) expression of RA-generating enzymes have both been reported in IBD. It should be borne in mind that neither study measured RA itself nor assessed other factors that regulate RA availability, such as expression of CYP26 enzymes that degrade RA (135).

CONCLUDING REMARKS

Antibodies which target α4β7, an integrin which can be imprinted by intestinal DCs to facilitate T cell entry to the intestine, are already used to treat IBD. DCs also control the balance between regulatory and effector T cell responses in the intestine. Understanding the mechanisms involved and their regulation will facilitate rational manipulation of DCs to promote effector responses in the context of infection and vaccination or to re-establish regulation in the context of inflammatory disease.

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

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REFERENCES

1. Guilliams M, Ginhoux F, Jakubzick C, Naik SH, Onai N, Schraml BU, et al. Dendritic cells, monocytes and macrophages: a unified nomenclature based on ontogeny. *Nat Rev Immunol.* (2014) 14:571–8. doi: 10.1038/nri3712
2. Scott CL, Bain CC, Mowat AM. Isolation and identification of intestinal myeloid cells. *Methods Mol Biol.* (2017) 1559:223–39. doi: 10.1007/978-1-4939-6786-5_15
3. Scott CL, Wright PB, Milling SW, Mowat AM. Isolation and identification of conventional dendritic cell subsets from the intestine of mice and men. *Methods Mol Biol.* (2016) 1423:101–18. doi: 10.1007/978-1-4939-3606-9_7
4. Lombardi VC, Khaiboullina SF. Plasmacytoid dendritic cells of the gut: relevance to immunity and pathology. *Clinical immunology* (2014) 153:165–77. doi: 10.1016/j.clim.2014.04.007
5. Esterhazy D, Loschko J, London M, Jove V, Oliveira TY, Mucida D. Classical dendritic cells are required for dietary antigen-mediated induction of peripheral Treg cells and tolerance. *Nat Immunol.* (2016) 17:545–55. doi: 10.1038/ni.3408
6. Loschko J, Schreiber HA, Rieke GJ, Esterhazy D, Meredith MM, Pedicord VA, et al. Absence of MHC class II on cDCs results in microbial-dependent intestinal inflammation. *J Exp Med.* (2016) 213:517–34. doi: 10.1084/jem.20160062
7. Nutsch K, Chai JN, Ai TL, Russler-Germain E, Feehley T, Nagler CR, et al. Rapid and efficient generation of regulatory T cells to commensal antigens in the periphery. *Cell Rep.* (2016) 17:206–20. doi: 10.1016/j.celrep.2016.08.092
8. Gao Y, Nish SA, Jiang R, Hou L, Licona-Limon P, Weinstein JS, et al. Control of T helper 2 responses by transcription factor IRF4-dependent dendritic cells. *Immunity* (2013) 39:722–32. doi: 10.1016/j.immuni.2013.08.028
9. Mayer JU, Demiri M, Agace WW, MacDonald AS, Svensson-Frej M, Milling SW. Different populations of CD11b$^+$ dendritic cells drive Th2 responses in the small intestine and colon. *Nat Commun.* (2017) 8:15820. doi: 10.1038/ncomms15820
10. Meredith MM, Liu K, Darrasse-Jeze G, Kamphorst AO, Schreiber HA, Guermonprez P, et al. Expression of the zinc finger transcription factor zDC (Zbtb46, Btbd4) defines the classical dendritic cell lineage. *J Exp Med.* (2012) 209:1153–65. doi: 10.1084/jem.20112675
11. Satpathy AT, Briseno CG, Lee JS, Ng D, Manieri NA, Kc W, et al. Notch2-dependent classical dendritic cells orchestrate intestinal immunity to attaching-and-effacing bacterial pathogens. *Nat Immunol.* (2013) 14:937–48. doi: 10.1038/ni.2679
12. Sun T, Rojas OL, Li C, Ward LA, Philpott DJ, Gommerman JL. Intestinal Batf3-dependent dendritic cells are required for optimal antiviral T-cell responses in adult and neonatal mice. *Mucosal Immunol.* (2017) 10:775–88. doi: 10.1038/mi.2016.79
13. Kelsall BL, Strober W. Distinct populations of dendritic cells are present in the subepithelial dome and T cell regions of the murine Peyer's Patch. *J Exp Med.* (1996) 183:237–47. doi: 10.1084/jem.183.1.237
14. Chirdo FG, Millington OR, Beacock-Sharp H, Mowat AM. Immunomodulatory dendritic cells in intestinal lamina propria. *Eur J Immunol.* (2005) 35:1831–40. doi: 10.1002/eji.200425882
15. Jaensson E, Uronen-Hansson H, Pabst O, Eksteen B, Tian J, Coombes JL, et al. Small intestinal CD103+ dendritic cells display unique functional properties that are conserved between mice and humans. *J Exp Med.* (2008) 205:2139–49. doi: 10.1084/jem.20080414
16. Veenbergen S, van Berkel LA, du Pre MF, He J, Karrich JJ, Costes LM, et al. Colonic tolerance develops in the iliac lymph nodes and can be established independent of CD103(+) dendritic cells. *Mucosal Immunol.* (2016) 9:894–906. doi: 10.1038/mi.2015.118
17. Gurka S, Hartung E, Becker M, Kroczek RA. Mouse conventional dendritic cells can be universally classified based on the mutually exclusive expression of XCR1 and SIRPα. *Front Immunol.* (2015) 6:35. doi: 10.3389/fimmu.2015.00035
18. Becker M, Guttler S, Bachem A, Hartung E, Mora A, Jakel A, et al. Ontogenic, phenotypic, and functional characterization of XCR1(+) dendritic cells leads to a consistent classification of intestinal dendritic cells based on the expression of XCR1 and SIRPα. *Front Immunol.* (2014) 5:326. doi: 10.3389/fimmu.2014.00326
19. Villablanca EJ, De Calisto J, Torregrosa Paredes P, Cassani B, Nguyen DD, Gabrielsson S, et al. β7 integrins are required to give rise to intestinal mononuclear phagocytes with tolerogenic potential. *Gut* (2014) 63:1431–40. doi: 10.1136/gutjnl-2013-305386
20. Zeng R, Oderup C, Yuan R, Lee M, Habtezion A, Hadeiba H, et al. Retinoic acid regulates the development of a gut-homing precursor for intestinal dendritic cells. *Mucosal Immunol.* (2013) 6:847–56. doi: 10.1038/mi.2012.123
21. Bekiaris V, Persson EK, Agace WW. Intestinal dendritic cells in the regulation of mucosal immunity. *Immunol Rev.* (2014) 260:86–101. doi: 10.1111/imr.12194
22. Joeris T, Muller-Luda K, Agace WW, Mowat AM. Diversity and functions of intestinal mononuclear phagocytes. *Mucosal Immunol.* (2017) 10:845–64. doi: 10.1038/mi.2017.22
23. Bain CC, Montgomery J, Scott CL, Kel JM, Girard-Madoux MJH, Martens L, et al. TGFβR signalling controls CD103$^+$CD11b$^+$ dendritic cell development in the intestine. *Nat Commun.* (2017) 8:620. doi: 10.1038/s41467-017-00658-6
24. Denning TL, Norris BA, Medina-Contreras O, Manicassamy S, Geem D, Madan R, et al. Functional specializations of intestinal dendritic cell and macrophage subsets that control Th17 and regulatory T cell responses are dependent on the T cell/APC ratio, source of mouse strain, and regional localization. *J Immunol.* (2011) 187:733–47. doi: 10.4049/jimmunol.1002701
25. Watchmaker PB, Lahl K, Lee M, Baumjohann D, Morton J, Kim SJ, et al. Comparative transcriptional and functional profiling defines conserved programs of intestinal DC differentiation in humans and mice. *Nat Immunol.* (2014) 15:98–108. doi: 10.1038/ni.2768
26. Cerovic V, Houston SA, Scott CL, Aumeunier A, Yrlid U, Mowat AM, et al. Intestinal CD103(-) dendritic cells migrate in lymph and prime effector T cells. *Mucosal Immunol.* (2012) 6:104–13. doi: 10.1038/mi.2012.53
27. Jang MH, Sougawa N, Tanaka T, Hirata T, Hiroi T, Tohya K, et al. CCR7 is critically important for migration of dendritic cells in intestinal lamina propria to mesenteric lymph nodes. *J Immunol.* (2006) 176:803–10. doi: 10.4049/jimmunol.176.2.803

28. Liu LM, Macpherson GG. Lymph-borne (veiled) dendritic cells can acquire and present intestinally administered antigens. *Immunology* (1991) 73:281–6.
29. Liu LM, MacPherson GG. Antigen acquisition by dendritic cells: intestinal dendritic cells acquire antigen administered orally and can prime naive T cells *in vivo*. *J Exp Med.* (1993) 177:1299–307. doi: 10.1084/jem.177.5.1299
30. Schulz O, Pabst O. Antigen sampling in the small intestine. *Trends Immunol.* (2012) 34:151–61. doi: 10.1016/j.it.2012.09.006
31. Kadaoui KA, Corthesy B. Secretory IgA mediates bacterial translocation to dendritic cells in mouse Peyer's patches with restriction to mucosal compartment. *J Immunol.* (2007) 179:7751–7. doi: 10.4049/jimmunol.179.11.7751
32. Lelouard H, Fallet M, de Bovis B, Méresse S, Gorvel JP. Peyer's Patch dendritic cells sample antigens by extending dendrites through m cell-specific transcellular pores. *Gastroenterology* (2012) 142:592–601.e593. doi: 10.1053/j.gastro.2011.11.039
33. Fleeton MN, Contractor N, Leon F, Wetzel JD, Dermody TS, Kelsall BL. Peyer's patch dendritic cells process viral antigen from apoptotic epithelial cells in the intestine of reovirus-infected mice. *J Exp Med.* (2004) 200:235–45. doi: 10.1084/jem.20041132
34. Huang FP, Platt N, Wykes M, Major JR, Powell TJ, Jenkins CD, et al. A discrete subpopulation of dendritic cells transports apoptotic intestinal epithelial cells to t cell areas of mesenteric lymph nodes. *J Exp Med.* (2000) 191:435–44. doi: 10.1084/jem.191.3.435
35. Farache J, Koren I, Milo I, Gurevich I, Kim KW, Zigmond E, et al. Luminal bacteria recruit CD103+ dendritic cells into the intestinal epithelium to sample bacterial antigens for presentation. *Immunity* (2013) 38:581–95. doi: 10.1016/j.immuni.2013.01.009
36. McDole JR, Wheeler LW, McDonald KG, Wang B, Konjufca V, Knoop KA, et al. Goblet cells deliver luminal antigen to CD103+ dendritic cells in the small intestine. *Nature* (2012) 483:345–9. doi: 10.1038/nature10863
37. Yoshida M, Claypool SM, Wagner JS, Mizoguchi E, Mizoguchi A, Roopenian DC, et al. Human neonatal Fc receptor mediates transport of IgG into luminal secretions for delivery of antigens to mucosal dendritic cells. *Immunity* (2004) 20:769–83. doi: 10.1016/j.immuni.2004.05.007
38. Mazzini E, Massimiliano L, Penna G, Rescigno M. Oral tolerance can be established via gap junction transfer of fed antigens from $CX3CR1^{+}$ macrophages to $CD103^{+}$ dendritic cells. *Immunity* (2014) 40:248–61. doi: 10.1016/j.immuni.2013.12.012
39. Niess JH, Brand S, Gu X, Landsman L, Jung S, McCormick BA, et al. CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. *Science* (2005) 307:254–8. doi: 10.1126/science.1102901
40. Rescigno M, Urbano M, Valzasina B, Francolini M, Rotta G, Bonasio R, et al. Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nat Immunol.* (2001) 2:361–7. doi: 10.1038/86373
41. Vallon-Eberhard A, Landsman L, Yogev N, Verrier B, Jung S. Transepithelial pathogen uptake into the small intestinal lamina propria. *J Immunol.* (2006) 176:2465–9. doi: 10.4049/jimmunol.176.4.2465
42. Mora JR, Von Andrian UH. T-cell homing specificity and plasticity: new concepts and future challenges. *Trends Immunol.* (2006) 27:235–43. doi: 10.1016/j.it.2006.03.007
43. Fischer A, Zundler S, Atreya R, Rath T, Voskens C, Hirschmann S, et al. Differential effects of α4β7 and GPR15 on homing of effector and regulatory T cells from patients with UC to the inflamed gut *in vivo*. *Gut* (2016) 65:1642–64. doi: 10.1136/gutjnl-2015-310022
44. Kim SV, Xiang WV, Kwak C, Yang Y, Lin XW, Ota M, et al. GPR15-mediated homing controls immune homeostasis in the large intestine mucosa. *Science* (2013) 340:1456–9. doi: 10.1126/science.1237013
45. Nguyen LP, Pan J, Dinh TT, Hadeiba H, O'Hara Iii E, Ebtikar A, et al. Role and species-specific expression of colon T cell homing receptor GPR15 in colitis. *Nat Immunol.* (2015) 16:207–13. doi: 10.1038/ni.3079
46. Johansson-Lindbom B, Svensson M, Pabst O, Palmqvist C, Marquez G, Forster R, et al. Functional specialization of gut $CD103^{+}$ dendritic cells in the regulation of tissue-selective T cell homing. *J Exp Med.* (2005) 202:1063–73. doi: 10.1084/jem.20051100
47. Johansson-Lindbom B, Svensson M, Wurbel MA, Malissen B, Marquez G, Agace W. Selective generation of gut tropic T cells in gut-associated lymphoid tissue (GALT): requirement for GALT dendritic cells and adjuvant. *J ExpMed.* (2003) 198:963–9. doi: 10.1084/jem.20031244
48. Mora JR, Bono MR, Manjunath N, Weninger W, Cavanagh LL, Rosemblatt M, et al. Selective imprinting of gut-homing T cells by Peyer's patch dendritic cells. *Nature* (2003) 424:88–93. doi: 10.1038/nature01726
49. Stagg AJ, Kamm MA, Knight SC. Intestinal dendritic cells increase T cell expression of α4β7 integrin. *EurJ Immunol.* (2002) 32:1445–54. doi: 10.1002/1521-4141(200205)32:5<1445::AID-IMMU1445>3.0.CO;2-E
50. Iwata M, Hirakiyama A, Eshima Y, Kagechika H, Kato C, Song SY. Retinoic acid imprints gut-homing specificity on T cells. *Immunity* (2004) 21:527–38. doi: 10.1016/j.immuni.2004.08.011
51. Annacker O, Coombes JL, Malmstrom V, Uhlig HH, Bourne T, Johansson-Lindbom B, et al. Essential role for CD103 in the T cell-mediated regulation of experimental colitis. *J Exp Med.* (2005) 202:1051–61. doi: 10.1084/jem.20040662
52. Welty NE, Staley C, Ghilardi N, Sadowsky MJ, Igyarto BZ, Kaplan DH. Intestinal lamina propria dendritic cells maintain T cell homeostasis but do not affect commensalism. *J Exp Med.* (2013) 210:2011–24. doi: 10.1084/jem.20130728
53. Sanders TJ, McCarthy NE, Giles EM, Davidson KL, Haltalli ML, Hazell S, et al. Increased production of retinoic acid by intestinal macrophages contributes to their inflammatory phenotype in patients with Crohn's disease. *Gastroenterology* (2014) 146:1278–88. doi: 10.1053/j.gastro.2014.01.057
54. Hammerschmidt SI, Ahrendt M, Bode U, Wahl B, Kremmer E, Forster R, et al. Stromal mesenteric lymph node cells are essential for the generation of gut-homing T cells *in vivo*. *J Exp Med.* (2008) 205:2483–90. doi: 10.1084/jem.20080039
55. Molenaar R, Greuter M, van der Marel AP, Roozendaal R, Martin SF, Edele F, et al. Lymph node stromal cells support dendritic cell-induced gut-homing of T cells. *J Immunol.* (2009) 183:6395–402. doi: 10.4049/jimmunol.0900311
56. Molenaar R, Knippenberg M, Goverse G, Olivier BJ, de Vos AF, O'Toole T, et al. Expression of retinaldehyde dehydrogenase enzymes in mucosal dendritic cells and gut-draining lymph node stromal cells is controlled by dietary vitamin A. *J Immunol.* (2011) 186:1934–42. doi: 10.4049/jimmunol.1001672
57. Mowat AM. To respond or not to respond - a personal perspective of intestinal tolerance. *Nat Rev Immunol.* (2018) 18:405–15. doi: 10.1038/s41577-018-0002-x
58. Benson MJ, Pino-Lagos K, Rosemblatt M, Noelle RJ. All-trans retinoic acid mediates enhanced T reg cell growth, differentiation, and gut homing in the face of high levels of co-stimulation. *J Exp Med.* (2007) 204:1765–74. doi: 10.1084/jem.20070719
59. Coombes JL, Siddiqui KRR, Arancibia-Carcamo CV, Hall J, Sun CM, Belkaid Y, et al. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGFβ- and retinoic acid-dependent mechanism. *J Exp Med.* (2007) 204:1757–64. doi: 10.1084/jem.20070590
60. Mucida D, Park Y, Kim G, Turovskaya O, Scott I, Kronenberg M, et al. Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid. *Science* (2007) 317:256–60. doi: 10.1126/science.1145697
61. Sun CM, Hall JA, Blank RB, Bouladoux N, Oukka M, Mora JR, et al. Small intestine lamina propria dendritic cells promote *de novo* generation of Foxp3 T reg cells via retinoic acid. *J Exp Med.* (2007) 204:1775–85. doi: 10.1084/jem.20070602
62. Worthington JJ, Czajkowska BI, Melton AC, Travis MA. Intestinal dendritic cells specialize to activate transforming growth factor-β and induce Foxp3+ regulatory T cells via integrin αvβ8. *Gastroenterology* (2011) 141:1802–12. doi: 10.1053/j.gastro.2011.06.057
63. Fukaya T, Takagi H, Sato Y, Sato K, Eizumi K, Taya H, et al. Crucial roles of B7-H1 and B7-DC expressed on mesenteric lymph node dendritic cells in the generation of antigen-specific CD4+Foxp3+ regulatory T cells in the establishment of oral tolerance. *Blood* (2010) 116:2266–76. doi: 10.1182/blood-2009-10-250472
64. Hegazy AN, West NR, Stubbington MJT, Wendt E, Suijker KIM, Datsi A, et al. Circulating and tissue-resident CD4+ T cells with reactivity to intestinal microbiota are abundant in healthy individuals and function is

altered during inflammation. *Gastroenterology* (2017) 153:1320–1337.e1316. doi: 10.1053/j.gastro.2017.07.047
65. Ivanov II, Frutos Rde L, Manel N, Yoshinaga K, Rifkin DB, Sartor RB, et al. Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine. *Cell Host Microbe* (2008) 4:337–49. doi: 10.1016/j.chom.2008.09.009
66. Muzaki AR, Tetlak P, Sheng J, Loh SC, Setiagani YA, Poidinger M, et al. Intestinal CD103(+)CD11b(-) dendritic cells restrain colitis via IFN-γ-induced anti-inflammatory response in epithelial cells. *Mucosal Immunol.* (2016) 9:336–51. doi: 10.1038/mi.2015.64
67. Edelblum KL, Sharon G, Singh G, Odenwald MA, Sailer A, Cao S, et al. The Microbiome Activates CD4 T-cell mediated immunity to compensate for increased intestinal permeability. *Cell Mol Gastroenterol Hepatol.* (2017) 4:285–97. doi: 10.1016/j.jcmgh.2017.06.001
68. Kayama H, Ueda Y, Sawa Y, Jeon SG, Ma JS, Okumura R, et al. Intestinal CX3C chemokine receptor 1(high) [CX3CR1(high)] myeloid cells prevent T-cell-dependent colitis. *Proc Natl Acad Sci USA*. (2012) 109:5010–5. doi: 10.1073/pnas.1114931109
69. Monteleone G, Kumberova A, Croft NM, McKenzie C, Steer HW, MacDonald TT. Blocking Smad7 restores TGF-β1 signaling in chronic inflammatory bowel disease. *J Clin Invest.* (2001) 108:601–9. doi: 10.1172/JCI12821
70. Manicassamy S, Reizis B, Ravindran R, Nakaya H, Salazar-Gonzalez RM, Wang YC, et al. Activation of β-catenin in dendritic cells regulates immunity versus tolerance in the intestine. *Science* (2010) 329:849–53. doi: 10.1126/science.1188510
71. Cassani B, Villablanca EJ, De Calisto J, Wang S, Mora JR. Vitamin A and immune regulation: role of retinoic acid in gut-associated dendritic cell education, immune protection and tolerance. *Mol Aspects Med.* (2012) 33:63–76. doi: 10.1016/j.mam.2011.11.001
72. Iliev ID, Mileti E, Matteoli G, Chieppa M, Rescigno M. Intestinal epithelial cells promote colitis-protective regulatory T-cell differentiation through dendritic cell conditioning. *Mucosal Immunol.* (2009) 2:340–50. doi: 10.1038/mi.2009.13
73. Iliev ID, Spadoni I, Mileti E, Matteoli G, Sonzogni A, Sampietro GM, et al. Human intestinal epithelial cells promote the differentiation of tolerogenic dendritic cells. *Gut* (2009) 58:481–9. doi: 10.1136/gut.2008.175166
74. Zaph C, Troy AE, Taylor BC, Berman-Booty LD, Guild KJ, Du Y, et al. Epithelial-cell-intrinsic IKK-beta expression regulates intestinal immune homeostasis. *Nature* (2007) 446:552–6. doi: 10.1038/nature05590
75. Zeng R, Bscheider M, Lahl K, Lee M, Butcher EC. Generation and transcriptional programming of intestinal dendritic cells: essential role of retinoic acid. *Mucosal Immunol.* (2016) 9:183–93. doi: 10.1038/mi.2015.50
76. Villablanca EJ, Wang S, de Calisto J, Gomes DC, Kane MA, Napoli JL, et al. MyD88 and retinoic acid signaling pathways interact to modulate gastrointestinal activities of dendritic cells. *Gastroenterology* (2011) 141:176–85. doi: 10.1053/j.gastro.2011.04.010
77. McDonald KG, Leach MR, Brooke KW, Wang C, Wheeler LW, Hanly EK, et al. Epithelial expression of the cytosolic retinoid chaperone cellular retinol binding protein II is essential for *in vivo* imprinting of local gut dendritic cells by lumenal retinoids. *Am J Pathol.* (2012) 180:984–97. doi: 10.1016/j.ajpath.2011.11.009
78. Vicente-Suarez I, Larange A, Reardon C, Matho M, Feau S, Chodaczek G, et al. Unique lamina propria stromal cells imprint the functional phenotype of mucosal dendritic cells. *Mucosal Immunol.* (2015) 8:141–51. doi: 10.1038/mi.2014.51
79. Jaensson-Gyllenback E, Kotarsky K, Zapata F, Persson EK, Gundersen TE, Blomhoff R, et al. Bile retinoids imprint intestinal CD103$^+$ dendritic cells with the ability to generate gut-tropic T cells. *Mucosal Immunol.* (2011) 4:438–47. doi: 10.1038/mi.2010.91
80. Stock A, Booth S, Cerundolo V. Prostaglandin E2 suppresses the differentiation of retinoic acid-producing dendritic cells in mice and humans. *J Exp Med*. (2011) 208:761–73 doi: 10.1084/jem.20101967
81. Chng SH, Kundu P, Dominguez-Brauer C, Teo WL, Kawajiri K, Fujii-Kuriyama Y, et al. Ablating the aryl hydrocarbon receptor (AhR) in CD11c+ cells perturbs intestinal epithelium development and intestinal immunity. *Sci Rep.* (2016) 6:23820. doi: 10.1038/srep23820
82. Singh N, Gurav A, Sivaprakasam S, Brady E, Padia R, Shi H, et al. Activation of Gpr109a, receptor for niacin and the commensal metabolite butyrate, suppresses colonic inflammation and carcinogenesis. *Immunity* (2014) 40:128–39. doi: 10.1016/j.immuni.2013.12.007
83. Huang G, Wang Y, Chi H. Control of T cell fates and immune tolerance by p38α signaling in mucosal CD103+ dendritic cells. *J Immunol.* (2013) 191:650–9. doi: 10.4049/jimmunol.1300398
84. Cerovic V, Jenkins CD, Barnes AG, Milling SW, MacPherson GG, Klavinskis LS. Hyporesponsiveness of intestinal dendritic cells to TLR stimulation is limited to TLR4. *J Immunol.* (2009) 182:2405–15. doi: 10.4049/jimmunol.0802318
85. Kinnebrew MA, Buffie CG, Diehl GE, Zenewicz LA, Leiner I, Hohl TM, et al. Interleukin 23 production by intestinal CD103(+)CD11b(+) dendritic cells in response to bacterial flagellin enhances mucosal innate immune defense. *Immunity* (2012) 36:276–87. doi: 10.1016/j.immuni.2011.12.011
86. Fujimoto K, Karuppuchamy T, Takemura N, Shimohigoshi M, Machida T, Haseda Y, et al. A new subset of CD103+CD8alpha+ dendritic cells in the small intestine expresses TLR3, TLR7, and TLR9 and induces Th1 response and CTL activity. *J Immunol.* (2011) 186:6287–95. doi: 10.4049/jimmunol.1004036
87. Liu H, Chen F, Wu W, Cao AT, Xue X, Yao S, et al. TLR5 mediates CD172α+ intestinal lamina propria dendritic cell induction of Th17 cells. *Sci Rep.* (2016) 6:22040. doi: 10.1038/srep22040
88. Uematsu S, Fujimoto K, Jang MH, Yang B-G, Jung Y-J, Nishiyama M, et al. Regulation of humoral and cellular gut immunity by lamina propria dendritic cells expressing toll-like receptor 5. *Nat Immunol.* (2008) 9:769–76. doi: 10.1038/ni.1622
89. Persson EK, Uronen-Hansson H, Semmrich M, Rivollier A, Hagerbrand K, Marsal J, et al. IRF4 transcription-factor-dependent CD103(+)CD11b(+) dendritic cells drive mucosal T helper 17 cell differentiation. *Immunity* (2013) 38:958–69. doi: 10.1016/j.immuni.2013.03.009
90. Cerovic V, Houston SA, Westlund J, Utriainen L, Davison ES, Scott CL, et al. Lymph-borne CD8α$^+$ dendritic cells are uniquely able to cross-prime CD8$^+$ T cells with antigen acquired from intestinal epithelial cells. *Mucosal Immunol*. (2014) 8:38–48. doi: 10.1038/mi.2014.40
91. Hansen IS, Krabbendam L, Bernink JH, Loayza-Puch F, Hoepel W, van Burgsteden JA, et al. FcαRI co-stimulation converts human intestinal CD103+ dendritic cells into pro-inflammatory cells through glycolytic reprogramming. *Nat Commun.* (2018) 9:863. doi: 10.1038/s41467-018-03318-5
92. Pugh CW, MacPherson GG, Steer HW. Characterization of nonlymphoid cells derived from rat peripheral lymph. *J Exp Med.* (1983) 157:1758–79. doi: 10.1084/jem.157.6.1758
93. Turnbull EL, Yrlid U, Jenkins CD, MacPherson GG. Intestinal dendritic cell subsets: differential effects of systemic TLR4 stimulation on migratory fate and activation *in vivo*. *J Immunol.* (2005) 174:1374–84. doi: 10.4049/jimmunol.174.3.1374
94. Yrlid U, Milling SWF, Miller JL, Cartland S, Jenkins CD, MacPherson GG. Regulation of intestinal dendritic cell migration and activation by plasmacytoid dendritic cells, TNF-alpha and type 1 IFNs after feeding a TLR7/8 ligand. *J Immunol.* (2006) 176:5205–12. doi: 10.4049/jimmunol.176.9.5205
95. Askenase MH, Han SJ, Byrd AL, Morais da Fonseca D, Bouladoux N, Wilhelm C, et al. Bone-marrow-resident NK cells prime monocytes for regulatory function during infection. *Immunity* (2015) 42:1130–42. doi: 10.1016/j.immuni.2015.05.011
96. Baumgart DC, Thomas S, Przesdzing I, Metzke D, Bielecki C, Lehmann SM, et al. Exaggerated inflammatory response of primary human myeloid dendritic cells to lipopolysaccharide in patients with inflammatory bowel disease. *Clin Exp Immunol.* (2009) 157:423–36. doi: 10.1111/j.1365-2249.2009.03981.x
97. Peake ST, Bernardo D, Knight SC, Hart AL. Homing marker expression on circulating dendritic cells correlates with different phenotypes of Crohn's disease. *J Crohns Colitis* (2013) 7:594–6. doi: 10.1016/j.crohns.2012.10.002
98. Jakubzick CV, Randolph GJ, Henson PM. Monocyte differentiation and antigen-presenting functions. *Nat Rev Immunol.* (2017) 17:349–62. doi: 10.1038/nri.2017.28

99. Menezes S, Melandri D, Anselmi G, Perchet T, Loschko J, Dubrot J, et al. The heterogeneity of Ly6C^{hi} monocytes controls their differentiation into iNOS+ macrophages or monocyte-derived dendritic cells. *Immunity* (2016) 45:1205–18. doi: 10.1016/j.immuni.2016.12.001
100. Bain CC, Bravo-Blas A, Scott CL, Gomez Perdiguero E, Geissmann F, Henri S, et al. Constant replenishment from circulating monocytes maintains the macrophage pool in the intestine of adult mice. *Nat Immunol.* (2014) 15:929–37. doi: 10.1038/ni.2967
101. Bain CC, Scott CL, Uronen-Hansson H, Gudjonsson S, Jansson O, Grip O, et al. Resident and pro-inflammatory macrophages in the colon represent alternative context-dependent fates of the same Ly6Chi monocyte precursors. *Mucosal Immunol.* (2013) 6:498–510. doi: 10.1038/mi.2012.89
102. Rivollier A, He J, Kole A, Valatas V, Kelsall BL. Inflammation switches the differentiation program of Ly6Chi monocytes from antiinflammatory macrophages to inflammatory dendritic cells in the colon. *J Exp Med.* (2012) 209:139–55. doi: 10.1084/jem.20101387
103. Tamoutounour S, Henri S, Lelouard H, de Bovis B, de Haar C, van der Woude CJ, et al. CD64 distinguishes macrophages from dendritic cells in the gut and reveals the Th1-inducing role of mesenteric lymph node macrophages during colitis. *Eur J Immunol.* (2012) 42:3150–66. doi: 10.1002/eji.201242847
104. Kamada N, Hisamatsu T, Honda H, Kobayashi T, Chinen H, Kitazume MT, et al. Human CD14+ macrophages in intestinal lamina propria exhibit potent antigen-presenting ability. *J Immunol.* (2009) 183:1724–31. doi: 10.4049/jimmunol.0804369
105. Kamada N, Hisamatsu T, Okamoto S, Chinen H, Kobayashi T, Sato T, et al. Unique CD14 intestinal macrophages contribute to the pathogenesis of Crohn disease via IL-23/IFN-gamma axis. *J Clin Invest.* (2008) 118:2269–80. doi: 10.1172/JCI34610
106. Cassani B, Villablanca EJ, Quintana FJ, Love PE, Lacy-Hulbert A, Blaner WS, et al. Gut-tropic T cells that express integrin α4β7 and CCR9 are required for induction of oral immune tolerance in mice. *Gastroenterology* (2011) 141:2109–18. doi: 10.1053/j.gastro.2011.09.015
107. Hadis U, Wahl B, Schulz O, Hardtke-Wolenski M, Schippers A, Wagner N, et al. Intestinal tolerance requires gut homing and expansion of FoxP3+ regulatory T cells in the lamina propria. *Immunity* (2011) 34:237–46. doi: 10.1016/j.immuni.2011.01.016
108. Hepworth MR, Fung TC, Masur SH, Kelsen JR, McConnell FM, Dubrot J, et al. Group 3 innate lymphoid cells mediate intestinal selection of commensal bacteria–specific CD4+ T cells. *Science* (2015) 348:1031–5. doi: 10.1126/science.aaa4812
109. Laouar A, Haridas V, Vargas D, Zhinan X, Chaplin D, van Lier RAW, et al. CD70+ antigen-presenting cells control the proliferation and differentiation of T cells in the intestinal mucosa. *Nat Immunol.* (2005) 6:698–706. doi: 10.1038/ni1212
110. Tyler CJ, McCarthy NE, Lindsay JO, Stagg AJ, Moser B, Eberl M. Antigen-presenting human gammadelta T cells promote intestinal CD4+ T cell expression of IL-22 and mucosal release of calprotectin. *J Immunol.* (2017) 198:3417–25. doi: 10.4049/jimmunol.1700003
111. Drakesmith H, O'Neil D, Schneider SC, Binks M, Medd P, Sercarz E, et al. *In vivo* priming of T cells against cryptic determinants by dendritic cells exposed to interleukin 6 and native antigen. *Proc Natl Acad Sci USA.* (1998) 95:14903–8. doi: 10.1073/pnas.95.25.14903
112. Cooney R, Baker J, Brain O, Danis B, Pichulik T, Allan P, et al. NOD2 stimulation induces autophagy in dendritic cells influencing bacterial handling and antigen presentation. *Nat Med.* (2009) 16:90–7. doi: 10.1038/nm.2069
113. van Beelen AJ, Zelinkova Z, Taanman-Kueter EW, Muller FJ, Hommes DW, Zaat SA, et al. Stimulation of the intracellular bacterial sensor NOD2 programs dendritic cells to promote interleukin-17 production in human memory T cells. *Immunity* (2007) 27:660–9. doi: 10.1016/j.immuni.2007.08.013
114. Lee JC, Biasci D, Roberts R, Gearry RB, Mansfield JC, Ahmad T, et al. Genome-wide association study identifies distinct genetic contributions to prognosis and susceptibility in Crohn's disease. *Nat Genet.* (2017) 49:262–8. doi: 10.1038/ng.3755
115. Abe K, Nguyen KP, Fine SD, Mo JH, Shen C, Shenouda S, et al. Conventional dendritic cells regulate the outcome of colonic inflammation independently of T cells. *Proc Natl Acad Sci USA.* (2007) 104:17022–7. doi: 10.1073/pnas.0708469104
116. Berndt BE, Zhang M, Chen GH, Huffnagle GB, Kao JY. The role of dendritic cells in the development of acute dextran sulfate sodium colitis. *J Immunol.* (2007) 179:6255–62. doi: 10.4049/jimmunol.179.9.6255
117. Ramalingam R, Larmonier CB, Thurston RD, Midura-Kiela MT, Zheng SG, Ghishan FK, et al. Dendritic cell-specific disruption of TGF-β receptor II leads to altered regulatory T cell phenotype and spontaneous multiorgan autoimmunity. *J Immunol.* (2012) 189:3878–93. doi: 10.4049/jimmunol.1201029
118. Uhlig HH, McKenzie BS, Hue S, Thompson C, Joyce-Shaikh B, Stepankova R, et al. Differential activity of IL-12 and IL-23 in mucosal and systemic innate immune pathology. *Immunity* (2006) 25:309–18. doi: 10.1016/j.immuni.2006.05.017
119. Hart AL, Al Hassi HO, Rigby RJ, Bell SJ, Emmanuel AV, Knight SC, et al. Characteristics of intestinal dendritic cells in inflammatory bowel diseases. *Gastroenterology* (2005) 129:50–65. doi: 10.1053/j.gastro.2005.05.013
120. Mann ER, Bernardo D, Ng SC, Rigby RJ, Al-Hassi HO, Landy J, et al. Human gut dendritic cells drive aberrant gut-specific t-cell responses in ulcerative colitis, characterized by increased IL-4 production and loss of IL-22 and IFNgamma. *Inflamm Bowel Dis.* (2014) 20:2299–307. doi: 10.1097/MIB.0000000000000223
121. Middel P, Raddatz D, Gunawan B, Haller F, Radzun HJ. Increased number of mature dendritic cells in Crohn's disease: evidence for a chemokine mediated retention mechanism. *Gut* (2006) 55:220–7. doi: 10.1136/gut.2004.063008
122. Ng SC, Benjamin JL, McCarthy NE, Hedin CR, Koutsoumpas A, Plamondon S, et al. Relationship between human intestinal dendritic cells, gut microbiota, and disease activity in Crohn's disease. *Inflamm Bowel Dis.* (2010) 17:2027–37. doi: 10.1002/ibd.21590
123. Di Sabatino A, Pickard KM, Gordon JN, Salvati V, Mazzarella G, Beattie RM, et al. Evidence for the role of interferon-alfa production by dendritic cells in the Th1 response in celiac disease. *Gastroenterology* (2007) 133:1175–87. doi: 10.1053/j.gastro.2007.08.018
124. Sakuraba A, Sato T, Kamada N, Kitazume M, Sugita A, Hibi T. Th1/Th17 immune response is induced by mesenteric lymph node dendritic cells in Crohn's disease. *Gastroenterology* (2009) 137:1736–45. doi: 10.1053/j.gastro.2009.07.049
125. Wu W, He C, Liu C, Cao AT, Xue X, Evans-Marin HL, et al. miR-10a inhibits dendritic cell activation and Th1/Th17 cell immune responses in IBD. *Gut* (2015) 64:1755–64. doi: 10.1136/gutjnl-2014-307980
126. Al-Hassi HO, Mann ER, Sanchez B, English NR, Peake ST, Landy J, et al. Altered human gut dendritic cell properties in ulcerative colitis are reversed by *Lactobacillus plantarum* extracellular encrypted peptide STp. *Mol Nutr Food Res.* (2014) 58:1132–43. doi: 10.1002/mnfr.201300596
127. Collins CB, Aherne CM, Kominsky D, McNamee EN, Lebsack MDP, Eltzschig H, et al. Retinoic acid attenuates ileitis by restoring the balance between T-helper 17 and T regulatory cells. *Gastroenterology* (2011) 141:1821–31. doi: 10.1053/j.gastro.2011.05.049
128. Matsuno H, Kayama H, Nishimura J, Sekido Y, Osawa H, Barman S, et al. CD103+ Dendritic cell function is altered in the colons of patients with ulcerative colitis. *Inflamm Bowel Dis.* (2017) 23:1524–34. doi: 10.1097/MIB.0000000000001204
129. Laffont S, Siddiqui KR, Powrie F. Intestinal inflammation abrogates the tolerogenic properties of MLN CD103+ dendritic cells. *Eur J Immunol.* (2010) 40:1877–83. doi: 10.1002/eji.200939957
130. Brown CC, Noelle RJ. Seeing through the dark: new insights into the immune regulatory functions of vitamin A. *Eur J Immunol.* (2015) 45:1287–95. doi: 10.1002/eji.201344398
131. Erkelens MN, Mebius RE. Retinoic acid and immune homeostasis: a balancing act. *Trends Immunol.* (2017) 38:168–80. doi: 10.1016/j.it.2016.12.006
132. Hall JA, Cannons JL, Grainger JR, Dos Santos LM, Hand TW, Naik S, et al. Essential role for retinoic acid in the promotion of CD4^{+} T cell effector responses via retinoic acid receptor alpha. *Immunity* (2011) 34:435–47. doi: 10.1016/j.immuni.2011.03.003

133. DePaolo RW, Abadie V, Tang F, Fehlner-Peach H, Hall JA, Wang W, et al. Co-adjuvant effects of retinoic acid and IL-15 induce inflammatory immunity to dietary antigens. *Nature* (2011) 47:220–4. doi: 10.1038/nature09849
134. Magnusson MK, Brynjolfsson SF, Dige A, Uronen-Hansson H, Borjesson LG, Bengtsson JL, et al. Macrophage and dendritic cell subsets in IBD: ALDH+ cells are reduced in colon tissue of patients with ulcerative colitis regardless of inflammation. *Mucosal Immunol.* (2016) 9:171–82. doi: 10.1038/mi.2015.48
135. Takeuchi H, Yokota A, Ohoka Y, Iwata M. Cyp26b1 regulates retinoic acid-dependent signals in T cells and its expression is inhibited by transforming growth factor-beta. *PLoS ONE* (2011) 6:e16089. doi: 10.1371/journal.pone.0016089

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mPGES-1-Mediated Production of PGE_2 and EP4 Receptor Sensing Regulate T Cell Colonic Inflammation

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PGE_2 is a lipid mediator of the initiation and resolution phases of inflammation, as well as a regulator of immune system responses to inflammatory events. PGE_2 is produced and sensed by T cells, and autocrine or paracrine PGE_2 can affect T cell phenotype and function. In this study, we use a T cell-dependent model of colitis to evaluate the role of PGE_2 on pathological outcome and T-cell phenotypes. $CD4^+$ T effector cells either deficient in mPGES-1 or the PGE_2 receptor EP4 are less colitogenic. Absence of T cell autocrine mPGES1-dependent PGE_2 reduces colitogenicity in association with an increase in $CD4^+ROR\gamma t^+$ cells in the lamina propria. In contrast, recipient mice deficient in mPGES-1 exhibit more severe colitis that corresponds with a reduced capacity to generate $FoxP3^+$ T cells, especially in mesenteric lymph nodes. Thus, our research defines how mPGES-1-driven production of PGE_2 by different cell types in distinct intestinal locations impacts T cell function during colitis. We conclude that PGE_2 has profound effects on T cell phenotype that are dependent on the microenvironment.

Keywords: IBD–inflammatory bowel diseases, T cell, PGE2, colitis, Th17 & Tregs cells, inflammation immunomodulation, Th17 activation, Treg = regulatory T cell

INTRODUCTION

Prostaglandin E_2 (PGE_2) is an eicosanoid that modulates diverse physiologic and pathologic functions. To avoid undesired effects associated with dysregulated inflammation, PGE_2 tissue concentrations are tightly regulated by expression of constitutive (COX1) and inducible (COX2, mPGES1) biosynthetic enzymes, as well as degradative enzymes (15-PGDH) (1, 2) and the transporters multiple drug resistance-associated protein 4 (MRP4) and prostaglandin transporter (PGT). The COX1 and COX2 enzymes are responsible for the catabolism of arachidonic acid into PGH_2, the precursor of PGI_2, PGD_2, PGF_{2a}, TxA_2, and PGE_2. COX1 and 2 expression differs strongly among different tissues, and distinct inflammatory triggers markedly upregulate the inducible isoform, COX2 (2, 3). mPGES1, also an inducible enzyme often co-regulated with COX-2, acts downstream of COX enzymes to specifically generate PGE_2, and does not directly generate other prostaglandins that are derived from the shared PGH_2 intermediate metabolite. Part of the variety of effects that can be triggered by PGE_2 are due to its 4 known receptors (EP1-4), which display different PGE_2 binding affinities and a range of tissue and cell-specific expression

patterns (2, 4). PGE_2 signaling in T cells is almost exclusively transduced through the EP2 and EP4 receptors (5, 6).

Immune responses can be highly sensitive to PGE_2, as it acts in a pleiotropic manner affecting many cells of the immune system. With respect to the innate immune system, PGE_2 promotes neutrophil, macrophage, and mast cell activation and migration into inflamed sites, and can also influence dendritic cells (DC) with both pro- and anti-inflammatory results (7–9). Cells belonging to the adaptive immune system can also be influenced by PGE_2, and T cells display an array of diverse responses to PGE_2 that include effects on T cell lineage commitment and cytokine secretion (10–13). In general, PGE_2 can differentially expand Th1 and Th17 cells via expression of the PGE_2 receptors EP2 and EP4 when IL1β and IL-23 are part of the inflammatory response. However, some of the reported effects are contradictory, as PGE_2 can either increase IL-17 and reduce IFNγ production in human memory T cells (14) and inhibit Th1 responses (15), or enhance antigen-specific Th1 function and expansion (11). We recently found that production and sensing of PGE_2 in $CD4^+$ T cells controls antigen-specific regulatory T cell (Treg) and Th17 expansion, and that PGE_2 can re-direct T cells undergoing Th17 polarization to inhibit IL-17A production in lieu of IFNγ (13).

In the gastrointestinal tract, PGE_2 is implicated in the modulation of autoimmune and inflammatory diseases as well as in the control of carcinogenesis (16–18). Expression of COX-2 is greatly increased during active phases in patients with ulcerative colitis (19), and non-steroidal anti-inflammatory drugs (NSAIDs) are associated with significant gastrointestinal adverse events and exacerbate symptoms of inflammatory bowel disease (IBD) (20). COX-2 is partially responsible for intestinal damage in the DSS colitis model in mice, with presence of COX-2 being required specifically in myeloid and endothelial cells but not epithelial cells (21) to increase intestinal pathology. However, although PGE_2 is generally regarded as a pro-inflammatory molecule, it is also critical for the resolution of inflammation and restoration of tissue homeostasis (2, 22).

It is unclear how PGE_2 affects pathogenic T cell responses during colitis, and what the relative contribution of T cell intrinsic or extrinsic sources might be to clinical disease. Given the effects of PGE_2 on T cells, PGE_2 could constitute a critical regulator of Th17 cells in the colon, which can either enhance protective responses from gut pathogens and epithelial regeneration, but also potentially serving to mediate IBD (23–25). In this study we evaluated the impact of mPGES1-driven PGE_2 and PGE_2 sensing through EP4 in a T-cell driven colitis model. We found that signaling through EP4 on $CD4^+$ cells strongly controlled colitogenicity and colonic T cell expansion. Moreover, our studies comparing the origin of PGE_2 production in T cells compared with non-lymphoid cells demonstrate that T cell autocrine mPGES1-mediated PGE_2 contributes to colitogenesis by reducing a protective $CD4^+ROR\gamma t^+$ cell response, while paracrine mPGES-1-driven PGE_2 inhibits colon inflammation by favoring the expansion of $FoxP3^+$ Treg cells. We conclude that site-specific PGE_2 production and PGE_2 sensing by $CD4^+$ T cells play important roles in intestinal inflammation.

MATERIALS AND METHODS

Mice and Colitis Induction

WT and $mPGES1^{-/-}$ mice in a BL/6 background were bred in house and maintained under SPF conditions in the same MCN II facility and room at Vanderbilt University. mPGES-1 mice were originally obtained from Pfizer and their generation has been previously described (26). $EP4^{fl/fl}$ transgenic mice were a kind gift of Dr. Richard Breyer (27), and they were crossed with either C57BL/6 or C57BL/6 $CD4^{Cre}$ mice obtained from Jackson Laboratories. $Rag1^{-/-}$ mice were obtained from Jackson Laboratories and crossed with the $mPGES1^{-/-}$ line to obtain double knock-outs. All mice were bred in a specific pathogen-free barrier facility and used at 8–14 weeks of age. The Vanderbilt University Animal Care and Use Committee approved all studies performed for the preparation of this manuscript.

Colitis was induced by adaptive transfer of 1×10^6 purified (>99% purity) $CD4^+CD25^-CD45RB^{hi}$ cells i.p., and in the indicated cases co-injection of 0.5×10^6 $CD4^+CD25^-$ cells was performed to study Treg function. Spleen and lymph nodes suspensions were used first to purify untouched $CD4^+$ cells using magnetic bead cell separation with a StemCell Kit and these cells were stained with anti-CD4, anti-CD25, and anti-CD45RB for further flow sorting using a FACS Diva flow cytometer (Becton-Dickinson) with purities over 95% of the indicated populations. Mice that received adoptive transfers of different cell genotypes were always cohoused in the same cages to avoid differences due to microbiota composition divergence during colitis development.

Cell Preparation and Flow Cytometry

Single cell suspensions were prepared from spleen, colon LP, and mesenteric lymph nodes, and stained on ice using predetermined optimal concentrations of each antibody (Ab) for 20–30 min, washed, and fixed using 1.5% PFA or eBioscience FoxP3 fixation reagent. Colon lamina propria was obtained as previously described (28). Cells with the light scatter properties of singlet lymphocytes were analyzed by multicolor immunofluorescence staining and a BD FACS Fortessa II flow cytometer (Becton Dickinson, San Jose, CA). Fcγ receptors blockade was performed (2.4G2; BD PharMingen) prior to surface staining of cell surface markers. The anti-mouse mAbs used in this study included CD4-PE.Cy7/FITC (GK1.5), CD45.1-AF700/Pacific Blue (A20), CD45RB-AF647 (C363-16A) from BioLegend, RORγt-PE (Q31-378) from BD PharMingen, FoxP3-APC (FJK-16s) from eBioscience, and CD45.2-PE (104) from Tonbo. The LIVE/DEAD® fixable cell death stain kit (Invitrogen) was used to remove dead cells from all analysis and avoid background staining noise of dead cells. All flow cytometry analysis and data display were performed using FlowJo software.

Tissue Culture and PGE_2 Measure

For all *in vitro* experiments IMDM medium was supplemented with 10% FCS, Pen/Strep at 50 UI/ml and 50 μg/ml respectively, and 2-beta-ME at 10 μM. Colon explant cultures were performed

in 48-well round-bottom plates and supernatants were collected 12 h after initiation, spin down at >12.000 g in Eppendorf tubes, and clear supernatants used for further analysis. NS-398 was purchased from Cayman Chemicals, and stored aliquots were freshly reconstituted before every use.

The PGE_2 ELISA Kit from Cayman chemical was used to evaluate PGE_2 supernatant concentrations.

Histology and Pathological Scoring

Colon Swiss rolls were generated from mice undergoing colitis at the indicated time-points. Fresh colon tissue was washed with cold PBS, cut longitudinally to prepare Swiss rolls and fixed in 10% Formaldehyde for 3 days before transfer to 70% Ethanol. Paraffin blocks were generated with these fixed samples and 10 μm sections placed in slides for further H&E processing. Pathological severity and features were evaluated according to the following scoring system: Lamina Propria Infiltrate (LPI, 0–3), Neutrophilic Infiltrate (NI, 0–2), Goblet Cell Loss (GCL, 0–3), Abnormal Crypts (Ab.Cr., 0–3), Crypt Abscesses (Cr. Ab., 0–1), Erosion and Ulcers (Er.+Ulc, 0–2), and Depth of Inflammation (DOI, 0–3). Scale bars on the images correspond to 100 μM length. For detection of COX2 and mPGES-1 in colon tissue, we used rabbit polyclonal anti-mouse COX2 ab52237 and anti-mouse mPGES-1 ab62050 from Abcam following manufacturer's instructions.

Microscopy Analysis, Immunofluorescence and Signal Quantification

Paraffin-embedded colonic tissues were sectioned (5 μm) prior to deparaffinization, rehydration, and antigen retrieval using a citrate buffer (pH 6.0) for 20 min in a pressure cooker at 105°C, followed by a 20 min cool down at room temperature (RT). Endogenous background signal was quenched by incubating tissue slides in 3% hydrogen peroxide for 10 min at RT. Tissues were blocked in 3% BSA/10% donkey serum for 1 h before primary Ab staining. Antibodies used for immunofluorescence were: rat anti-FoxP3-APC (1:100, eBioScience FJK-16a), mouse anti-RORγt-PE (1:100, BD Q31-378), goat anti-CD3ε (1:100, Santa Cruz M-20), rabbit anti-pSTAT3 (Tyr705) (1:100, Cell Signaling D3A7), and AF-647-conjugated secondary antibodies (Life Technologies). Sequential staining and fluorescent dye inactivation was performed as previously described (29, 30). Immunofluorescent imaging was performed using an Olympus X81 inverted microscope with an UPlanSAPO UIS2 (20X/0.75NA) objective lens and filter sets specific for DAPI, GFP, CY3, CY5, and Cy7. Images were acquired at 20X magnification and image exposure for each Ab stain was set manually (<800 ms). Initial surveying of the tissue was performed at 2X magnification on the DAPI channel to establish 10–15 regions per Swiss roll for subsequent analysis. Primary Ab staining was performed overnight at RT and secondary Ab staining for 1 h at RT before slide imaging. Complete inactivation of fluorochromes was performed as described previously (29). Final image processing and layering was performed using ImageJ.

Microscopy Imaging Processing, Single-Cell Quantification, and Data Analysis

Images acquired for each stain round were processed as previously described (29). For each stain round, DAPI images were aligned to those from the first round using rigid transformation. Autofluorescence removal and correction was performed by background subtraction of registered images. Autofluorescence removed images for each stain were used for single-cell segmentation using Mathworks MATLAB software. Expression values of transcription factors were quantified by median intensity levels within a given mask-generated nuclear segmentation using combination of all nuclear markers available. $CD3\varepsilon^+$ cell numbers were estimated by the total area of coverage per field of the $CD3\varepsilon^+$ mask, divided by the average area of a single $CD3\varepsilon^+$ cell. This estimate was verified manually by counting $CD3\varepsilon^+$ cells in selected fields of view and comparing to estimated values. All analyses were performed in a blind fashion without phenotype identifiers. Cytobank was utilized to analyze single-cell intensity values and quantify cell populations.

RESULTS

Production of PGE_2 in the Colon Is Regulated by mPGES-1 With the Contribution of T and B Cells

The intestines are well-known to generate PGE_2, and inhibition of COX enzymes reduces PGE_2. However, it has not been investigated to which extent different components of the intestines contribute to this PGE2 pool. To address this and to evaluate how baseline production of PGE_2 is influenced by both biosynthetic enzymes and by the presence of lymphocytes, we cultured colon explants of untreated $Rag1^{-/-}$, mPGES-$1^{-/-}$, or $Rag1^{-/-}$ × mPGES-$1^{-/-}$ double knockout mice overnight and their supernatants were assessed for PGE_2 concentration. As expected, colons of WT animals produced the highest amounts of PGE_2. Absence of mPGES-1 significantly impaired PGE_2 production, but so did too the lack of adaptive immune cells in the $Rag1^{-/-}$ group (**Figure 1A**). Interestingly, lack of both the mPGES-1 enzyme and lymphocytes demonstrated an additive effect, with $Rag1^{-/-}$ × mPGES-$1^{-/-}$ double knockouts exhibiting the lowest levels of PGE_2. Specific inhibition of COX-2 activity using NS-398 in these explant cultures resulted in a reduction of nearly half of the secreted PGE_2 under most conditions. However, a significant decrease in PGE_2 was not observed following COX-2 inhibition in colons of $Rag1^{-/-}$ × mPGES-$1^{-/-}$ double knockout mice likely due to the already low basal levels produced.

We hypothesized that colonic inflammation would increase the PGE_2 production capacity in the colon. To test this, we assessed PGE_2 concentration in colon explants of WT untreated mice or $Rag1^{-/-}$ mice undergoing colitis induced by adoptive transfer of effector $CD4^+$ cells. Colons of mice undergoing colitis at week 10 post-transfer depicted a 5-fold increase of PGE_2 compared to healthy untreated colons (**Figure 1B**).

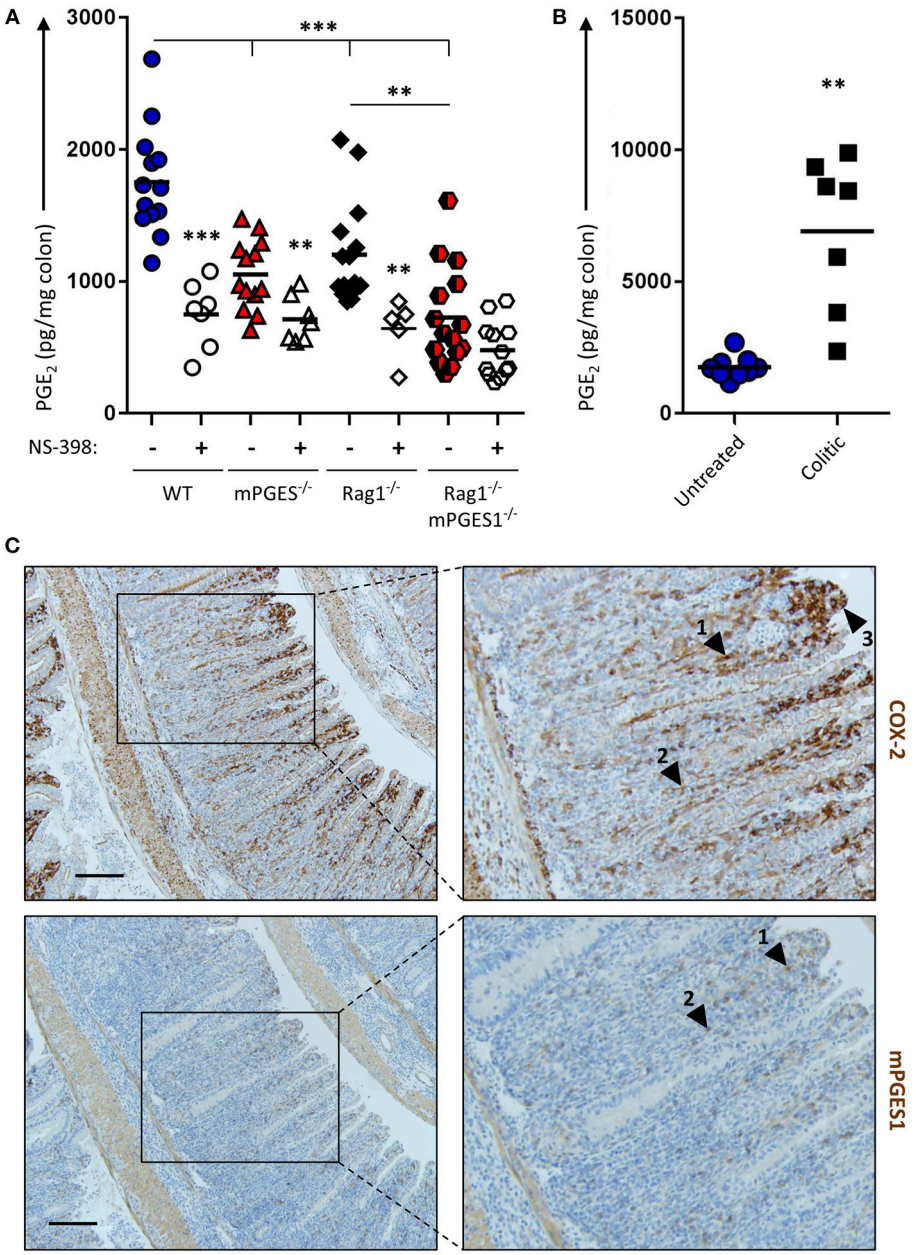

FIGURE 1 | Basal production of PGE_2 in the colon is regulated by COX-2 and mPGES-1 with significant contribution from cells of the adaptive immune system. **(A)** Colon explants from WT (blue), mPGES-$1^{-/-}$ (red), $Rag1^{-/-}$ (black) or $Rag1^{-/-}$ × mPGES-$1^{-/-}$ (red/black) mice were cultured for 12 h and their supernatants were analyzed for basal PGE_2 production. The specific COX2 inhibitor NS-398 was added to evaluate the differential contribution of COX2 and COX1 to the PGH_2 pool prior to PGE_2 biosynthesis. **(B)** PGE_2 production in colon explants from untreated WT mice ("Untreated," blue circles) or $Rag1^{-/-}$ mice undergoing WT Teff cell-driven colitis at week 10 post-transfer ("Colitic," black squares). ** indicates a P value < 0.05 using a 2-tailed heteroscedastic Student's T-test. **(C)** Immunohistochemistry of COX2 (upper row) and mPGES-1 (lower row) in the colon of mice that transferred with WT Teff cells ($CD4^+CD25^-CD45RB^{hi}$). Scale bar is 100 μm long. Arrowheads 1 and 2 point to localization within the LP, while arrowhead 3 indicates localization in the brush border. ** Significant at $P < 0.05$ and *** at $P < 0.01$ in a one-way ANOVA with Welch's correction.

Previous studies have shown that the COX-2 and mPGES-1 enzymes are especially abundant in monocyte/macrophage cells, and COX-2 expression is restricted in the absence of inflammation to the kidney, gastrointestinal tract, brain, and thymus (31). To better understand the increase of PGE_2 observed during colitis, we collected, fixed and stained colitic colons to detect both enzymes. COX-2 expression was maximal in the lamina propria (LP) at the apical side of hyperplastic villi, and mPGES-1 expression was found to mimic that pattern to some extent (**Figure 1C**, arrowheads #1-2), although strong COX-2,

but not mPGES-1 expression, was observed in the brush border of the villi (arrowhead #3).

CD4$^+$ Effector T Cells Lacking mPGES-1 Have Impaired Colitogenic Potential

We have recently demonstrated that T cell autocrine mPGES-1 expression in CD4$^+$ cells contributes to the cytokine profile that CD4$^+$ cells can acquire during antigen-specific stimulation. This T cell intrinsic effect is synergic with the ability to secrete PGE_2 by interacting APC cells, and it impacts especially IL-17A and IFNγ production by CD4$^+$ cells (13). We asked ourselves if this T cell intrinsic mPGES-1-driven PGE_2 effect was also contributing to the phenotype and pathogenic potential of T cells in a colitis model. Rag1$^{-/-}$ mice received an adoptive transfer of CD4$^+$CD25$^-$CD45B^{hi} cells (Teff) from WT or mPGES-1$^{-/-}$ donor mice, and were monitored and evaluated for colitis progression for 10 subsequent weeks after transfer. Mice that received mPGES-1$^{-/-}$ Teff CD4$^+$ cells demonstrated a less severe weight loss than mice receiving WT Teff CD4$^+$ cells at the latter phases of disease (**Figure 2A**). Co-transfer of WT Treg cells with WT Teff cells resulted in suppression of colitis as expected (**Figure 2A**, empty circles), and mPGES-1$^{-/-}$ Tregs also displayed full protective function indistinguishable from WT Tregs in terms of weight loss (empty squares). Analysis of the pathological characteristics in the colons of both groups transferred with Teff cells revealed that mice that received mPGES-1$^{-/-}$ Teff CD4$^+$ cells lost less goblet cells and developed less crypt abscesses (**Figure 2B**). To investigate if the attenuation of colitis was due to an altered phenotype of the transferred T cells, we analyzed by flow cytometry the expression of FoxP3 and RORγt in CD4$^+$ cells in the mesenteric lymph nodes (mLN) and colonic lamina propria (cLP) of the mice belonging to the same colitis cohorts described above. mPGES-1$^{-/-}$ Teff CD4$^+$ cells were able to generate moderately but significantly increased numbers of CD4$^+$RORγt$^+$ T cells in the mLN and the cLP (**Figures 2C,D**) with the percent being increased only in the cLP. CD4$^+$FoxP3$^+$ cells and total CD4$^+$ cells were not altered.

To determine if the reduced colitogenic potential observed in absence of mPGES-1 Teff was due to a reduced proliferation advantage, we co-transferred Teff cells from CD45.1$^+$ WT and CD45.2$^+$ mPGES-1$^{-/-}$ animals in a 1:1 ratio into Rag1$^{-/-}$ recipient animals. Ten weeks after transfer, the mLN (**Figure 3A**) and cLP (**Figure 3B**) were analyzed. Under these competitive circumstances, WT T cells were clearly able to expand more than T cells deficient in mPGES-1. This translated into significantly more CD4$^+$RORγt$^+$ cells derived from WT T cells. However, we observed that mPGES-1$^{-/-}$ Teff CD4$^+$ cells were capable of generating a proportion of RORγt^{hi} T cells in the LP that was absent in the WT donor T cells (**Figure 3B**, shaded box). It is known that colonic CD4$^+$ cells display unique characteristics like high co-expression of RORγt in their FoxP3$^+$ population (32, 33). This prompted us to investigate if mPGES-1 sufficiency would impact RORγt expression in Tregs in the colon. For this purpose, a mix of either CD45.1$^+$ WT Teff and CD45.2$^+$ mPGES-1$^{-/-}$ Treg cells or a reciprocal CD45.1$^+$ WT Treg and CD45.2$^+$ mPGES-1$^{-/-}$ Teff cells mix were injected in a 2:1 (Teff:Treg) ratio into Rag1$^{-/-}$ recipient animals, and mLN and cLP were examined as previously described. Total WT Treg cells were found in much larger proportions than mPGES-1$^{-/-}$ Treg in the mLN, independently of whether they expressed RORγt or not (**Figure 3C**). In stark contrast, mPGES-1$^{-/-}$ Treg cells showed a much larger proportion of RORγt$^+$ cells in the cLP. Furthermore, mPGES-1$^{-/-}$ Treg cells showed greater relative expression levels of RORγt than WT Tregs in the cLP (**Figure 3C** shaded boxes). In summary, we demonstrate that mPGES-1 deficiency in T cells reduces their expansion capacity and colitogenic potential. Additionally, absence of T cell intrinsic mPGES-1 strongly promotes the localization of Tregs into the cLP to the detriment of the draining mLN, and this effect is simultaneous with acquisition of high expression levels of RORγt. These data suggest unique characteristics of mPGES-1 deficient T cells that protect from colitis.

Inability to Sense PGE_2 Through EP4 by CD4$^+$ Effector T Cells Impairs Their Colitogenicity

Detection of PGE_2 through EP4 increases Th1 responses when IL-12 is present (12) but also amplifies Th17 expansion in synergy with IL1β/IL-23 (10, 11). To examine if sensing of PGE_2 produced during inflammation of the intestines was mediated through EP4 on T cells, we injected either EP4$^{fl/fl}$ (virtual WT) and CD4^{Cre}xEP4$^{fl/fl}$ (EP4$^{\Delta CD4}$) Teff cells into Rag1$^{-/-}$ recipient animals and evaluated colitis development and T cell phenotype as previously described. Mice that received Teff cells deficient in EP4 were significantly protected from colitis development, with little to no weight loss, and significantly less overall LP infiltration and neutrophils (**Figures 4A,B**). Examination of mLN and cLP infiltrates by microscopy and flow cytometry revealed a decrease in total CD4$^+$ numbers that directly translated into less CD4$^+$FoxP3$^+$ and CD4$^+$RORγt$^+$ cells, but with no alteration of CD4$^+$ subsets proportions (**Figures 4C,D**) on either examined anatomical location. Microscopic analysis of CD3ε, RORγt, and FoxP3 distribution within the cLP revealed that WT Teff cells accumulated in larger conglomerates compared to EP4$^{\Delta CD4}$ Teff cells (**Figure 4E**). We conclude that EP4 expression in CD4$^+$ Teff cells is critical for their proliferative capacity without T cell subset specificity.

Paracrine mPGES-1-deficiency in Non-lymphoid Cells Facilitates Colitis by Inhibiting Generation of CD4$^+$FoxP3$^+$ Cells

Non-lymphoid cells have the ability to produce large amounts of PGE_2 in a COX-2-dependent manner (8, 13, 34). The net effect of PGE_2 during intestinal inflammation is however hard to discern, as PGE_2 has been reported to display both pro-inflammatory effects, and also result in protection from intestinal damage. We sought to determine whether production of PGE_2 mediated by mPGES-1 in the cLP non-lymphoid cell compartment would alter T cell pathogenicity. For that purpose, we transferred WT Teff CD4$^+$ cells into either Rag1$^{-/-}$ or Rag1$^{-/-}$ × mPGES-1$^{-/-}$ recipients. Recipient mice lacking mPGES-1 developed colitis faster, lost more weight, and displayed more overall cell

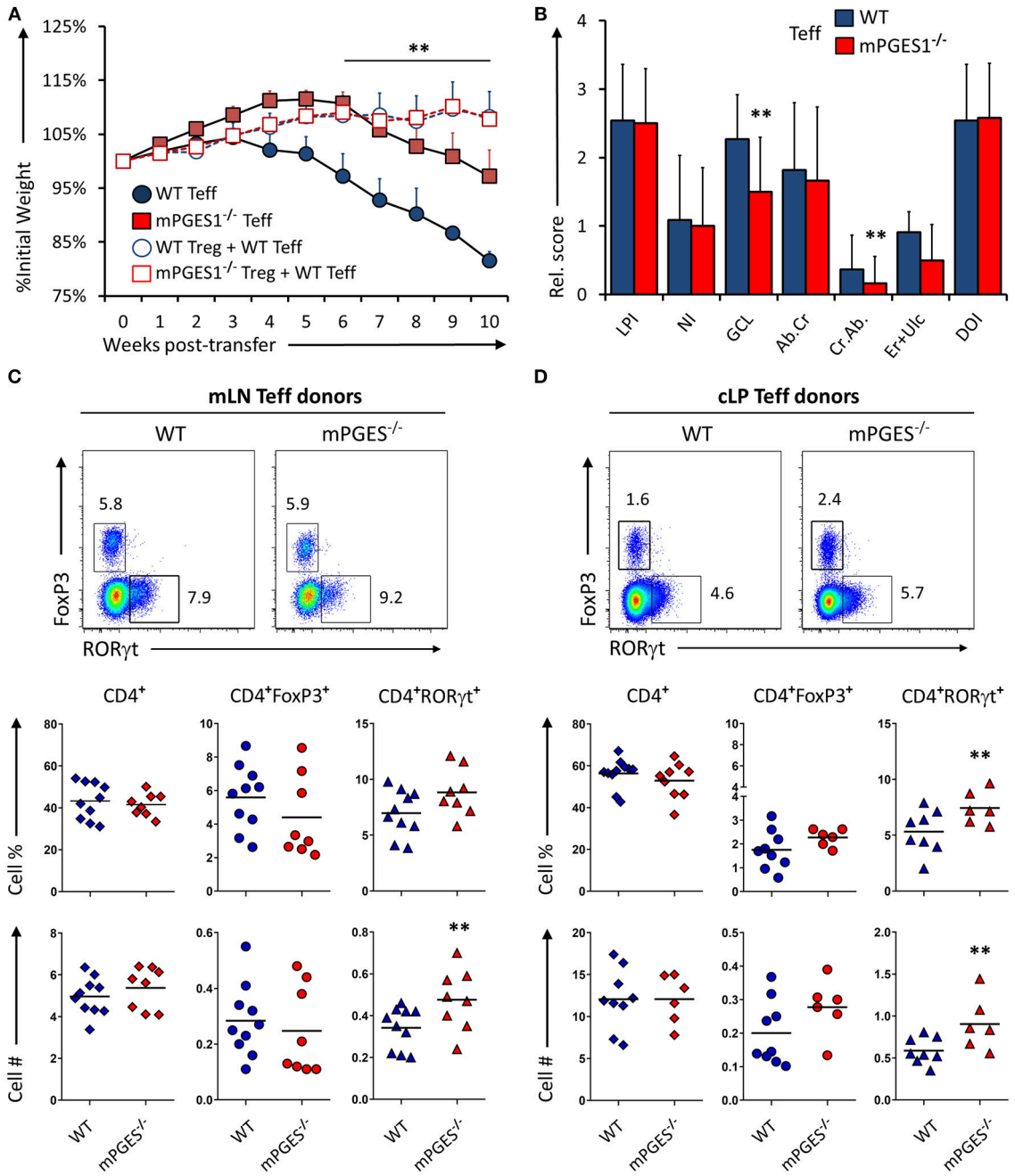

FIGURE 2 | mPGES-1-deficiency in T effector cells protects against colitis. **(A)** Weight loss in Rag1$^{-/-}$ mice that received transfer of 1 × 10^6 CD4$^+$CD25$^-$CD45RB^{hi} T cells (Teff) from WT or mPGES-1$^{-/-}$ donors. The dashed lines correspond to mice that received WT Teff and WT or mPGES-1$^{-/-}$ CD4$^+$CD25$^+$ (Treg) co-transfers. **(B)** Colon pathology scores from cohorts receiving transfers of WT or mPGES-1$^{-/-}$ Teff cells. Flow cytometry analysis of the **(C)** mesenteric lymph nodes (mLN) and **(D)** colon lamina propria (cLP) CD4$^+$ populations at the end of the experiment (week 10), with representative dot plots indicating intracellular expression of RORγt and FoxP3 and graphs below indicating summarized results from 4 experiments. ** indicates a significant difference with $P < 0.05$ using a two-tailed heteroscedastic Student's *T*-test between the WT and mPGES-1$^{-/-}$ Teff donor groups.

infiltration and neutrophilic content (**Figures 5A,B**). Analysis of the mLN and cLP of colitic mice revealed that absence of non-lymphoid mPGES-1 impaired the generation of CD4$^+$FoxP3$^+$ T cells in both tissues (**Figures 5C–E**), but more strikingly in the mLN where CD4$^+$FoxP3$^+$ T cell proportions and numbers were markedly decreased. The proportion of CD4$^+$RORγt$^+$ cells was also significantly reduced in absence of mPGES-1 in the mLN. However, in the cLP, total CD4$^+$ and RORγt$^+$CD4$^+$

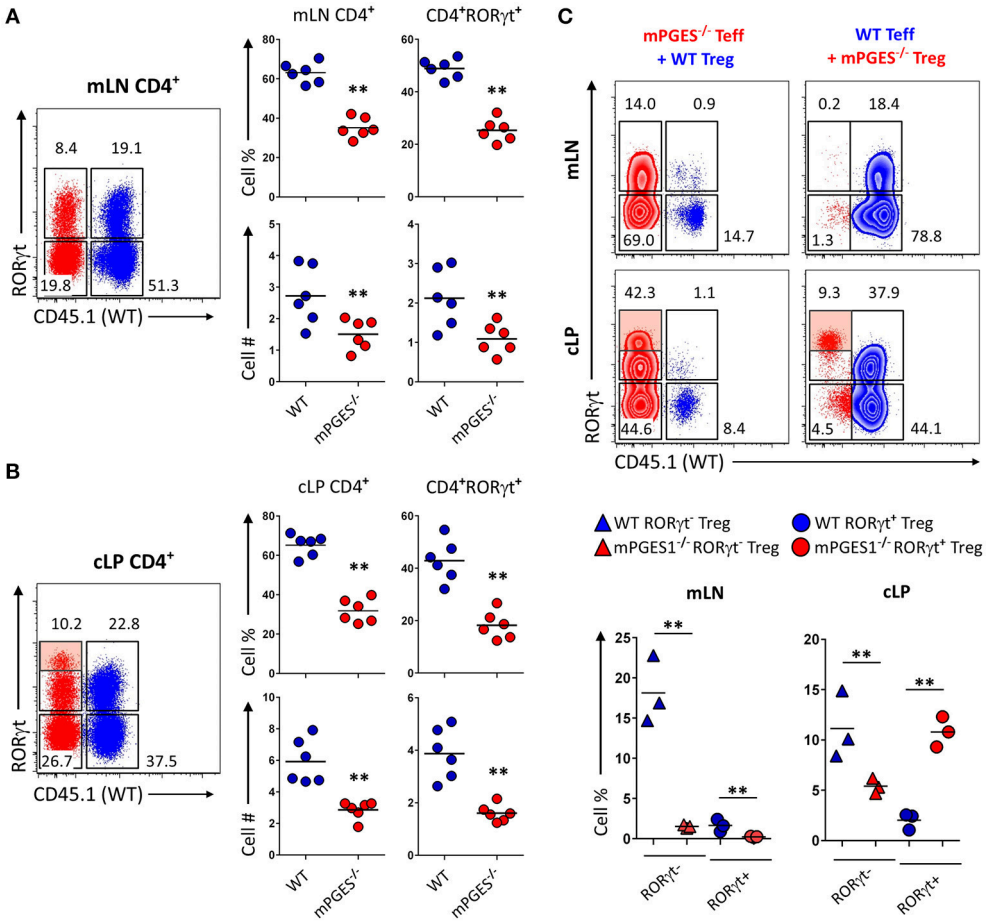

FIGURE 3 | Deficiency in CD4-intrinsic mPGES-1 impairs Teff $CD4^+$ cell expansion but enhances Treg localization and RORγt expression in the colonic lamina propria. **(A,B)** $Rag1^{-/-}$ recipient mice received a co-transfer of a 1:1 mix of $CD45.1^+$ WT (blue) and $CD45.2^+$ mPGES-$1^{-/-}$ (red) Teff cells. Flow cytometric analysis of the **(A)** mLN and **(B)** cLP $CD4^+$ populations, with representative dot plots indicating intracellular expression of CD45.1 or CD45.2 congenic marker expression together with RORγt. In the cLP plot **(B)**, the shaded box indicates a unique $RORγt^{hi}$ population of mPGES-1 deficient cells in the cLP. Graphs on the right indicate the proportions and total numbers for each group. **(C)** Co-transfer of either $CD45.1^+$ WT Treg with $CD45.2^+$ mPGES-$1^{-/-}$ Teff cells or $CD45.2^+$ mPGES-$1^{--/-}$ Treg cells with $CD45.1^+$ WT Teff into $Rag1^{-/-}$ recipients. Transfers were always performed with a 2:1 Teff:Treg ratio. In the cLP, mPGES-$1^{-/-}$ $CD4^+$ T cells are able to acquire higher RORγt expression than WT cells (shaded boxes). These $CD4^+RORγt^{hi}$ cells arise from both mPGES-$1^{-/-}$ Teff cells and mature Treg cells. Graphs on the bottom show the proportions of WT or mPGES-$1^{-/-}$ Treg cells that are either $RORγt^-$ or $RORγt^+$ in the mLN or the cLP. $^{**}P < 0.05$ using in a one-way ANOVA with Welch's correction.

cells numbers were increased in the mPGES-1-deficient $Rag1^{-/-}$ mice. Additionally, $CD4^+$ cells from the cLP of $Rag1^{-/-}$ × mPGES-$1^{-/-}$ recipients were found to form more densely aggregated foci than in $Rag1^{-/-}$ recipients (**Figure 5E**). We conclude that deficiency of mPGES-1 in non-lymphoid tissues enhances colitis by reducing the generation of $CD4^+FoxP3^+$ T cells in the mLN and increasing pathogenic $CD4^+$ T cells in the cLP. These data suggest that paracrine mPGES-1-derived PGE_2 may help to limit immune-mediated colitis by facilitating generation of Tregs in mLN and reducing pathogenic T cells in the cLP.

PGE_2 Controls Phosphorylation of STAT3 in Colon $CD4^+$ Cells During Colitis

We have previously described that PGE_2 can diminish phosphorylation of STAT3 during Th17 polarization (13). This led us to hypothesize that phosphorylation of STAT3 in colonic T cells could be altered by local PGE_2. We quantified the numbers of $pSTAT3^+$ cells in colon samples of our previous experiments to determine the relative contribution of either PGE_2 signaling through EP4 ($EP4^{\Delta CD4}$, group 2) or host non-lymphoid mPGES-1-mediated PGE_2 ($Rag1^{-/-}$ × mPGES-$1^{-/-}$ recipients, group 3). We focused on these groups as they displayed the biggest differences when compared to a standard colon pathogenic response induced by WT Teff (group 1).

We first validated the quantification of different T cell populations by microscopy and computational analysis in whole colon tissue samples (**Figures 6A,B**). The percentages of $CD3ε^+$ cells, as well as the $CD3ε^+FoxP3^+$, and $CD3e^+RORγt^+$ subsets, were consistent with the previous data observed by flow cytometry analysis of the cLP. Thus, the percentage of $CD3ε^+$ cells was lowest when EP4 null cells were used as donor Teff and was highest when recipient mice were deficient in mPGES-1 (**Figure 6A**). Additionally, there were significantly

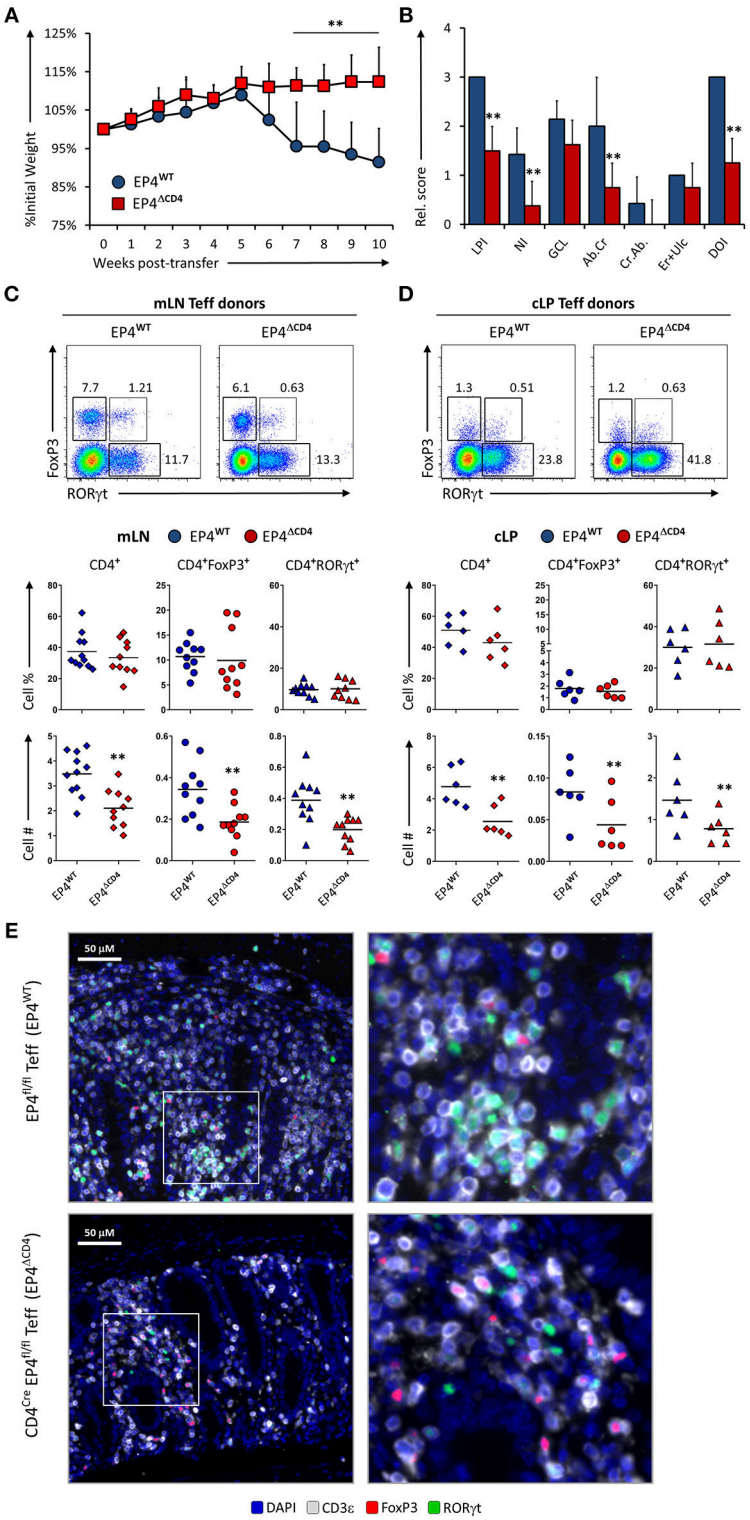

FIGURE 4 | EP4 deficient CD4$^+$ T effector cells have severely blunted colitogenic potential due to impaired proliferative capacity**. (A)** Weight loss in Rag1$^{-/-}$ mice that received transfer of Teff cells from EP4$^{fl/fl}$ (EP4^{WT}) or CD4^{Cre} × EP4$^{fl/fl}$ (EP4$^{\Delta CD4}$) donor mice. **(B)** Colon pathology scores from both cohorts. Flow cytometry analysis of the **(C)** mLN and **(D)** cLP CD4$^+$ populations at the end of the experiment (week 10), indicating intracellular expression of RORγt and FoxP3 in summarized results from 4 experiments. **(E)** Colon images detailing lamina propria T cell infiltrates in both groups, with magnified inserts on the right-hand side. Blue = DAPI, Gray = CD3ε, Green = RORγt, and Red = FoxP3. $^{**}P < 0.01$ using in a one-way ANOVA with Welch's correction.

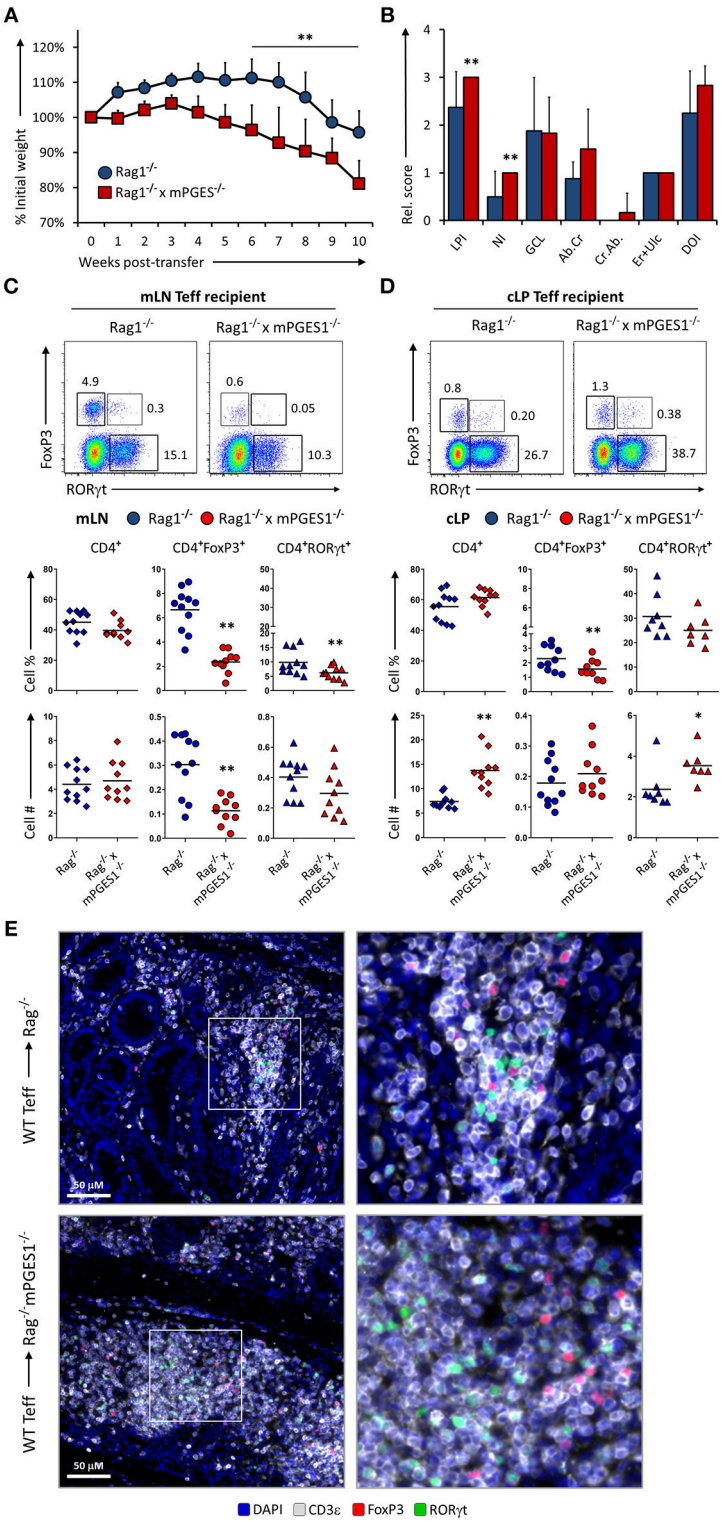

FIGURE 5 | Paracrine mPGES-1-deficiency in non-lymphoid cells facilitates colitis by inhibiting generation of $CD4^+FoxP3^+$ cells. **(A)** Weight loss in $Rag1^{-/-}$ or $Rag1^{-/-}$ x mPGES-$1^{-/-}$ mice that received a transfer of WT Teff donor cells. **(B)** Segregated colon pathology scores from both cohorts. Flow cytometry analysis of **(C)** mLN and **(D)** cLP $CD4^+$ populations at the end of the experiment (10 weeks), indicating intracellular expression of RORγt and FoxP3 in summarized results from 3 experiments. **(E)** Fluorescence microscopy analysis of colon sections denoting $CD4^+$ cell infiltrates. Blue = DAPI, Gray = CD3ε, Green = RORγt, and Red = FoxP3. $^{**}P < 0.01$ and $^{*}P < 0.05$ using in a one-way ANOVA with Welch's correction.

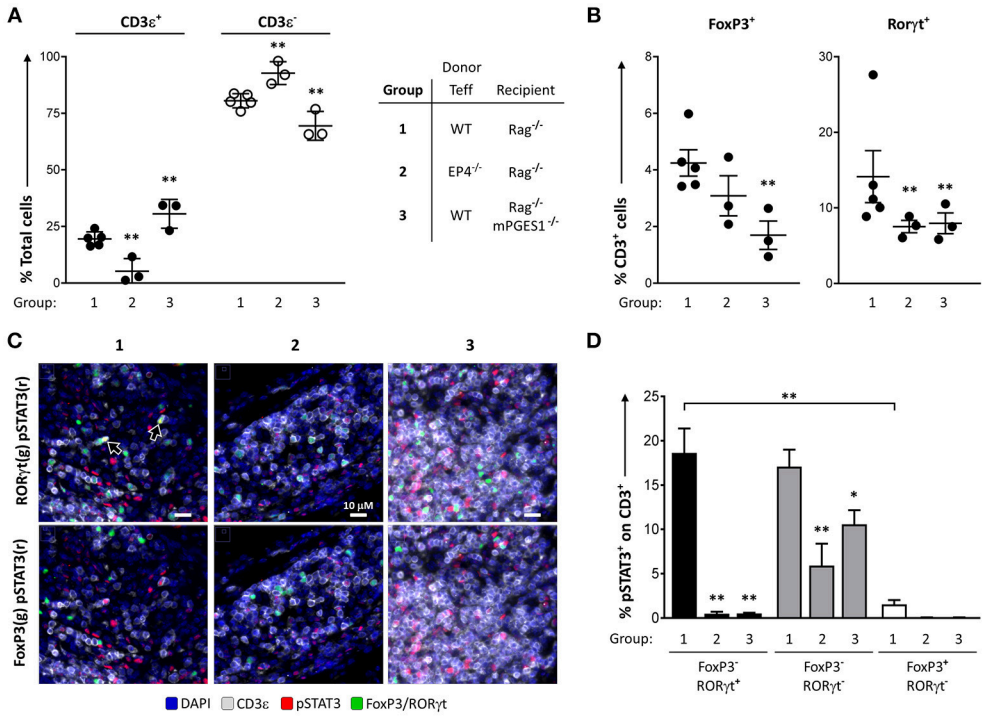

FIGURE 6 | PGE_2 controls phosphorylation of STAT3 in colon-infiltrating T cells. Individual colons from the 3 different groups of colitic mice indicated (1= WT Teff $\rightarrow Rag1^{-/-}$, 2= $EP4^{\Delta CD4}$ Teff $\rightarrow Rag1^{-/-}$, 3 = WT Teff $\rightarrow Rag1^{-/-}$ x mPGES-$1^{-/-}$) were processed and stained for fluorescence microscopy evaluation DAPI, CD3ε, RORγt, FoxP3, and pSTAT3. **(A)** Quantification of total $CD3\varepsilon^+$ and $CD3\varepsilon^-$ cells in 15 regions/colon containing a total of 15–30K cells. **(B)** Quantification of total $CD3\varepsilon^+FoxP3^+$ and $CD3\varepsilon^+ROR\gamma t^+$ cells in the same groups. **(C)** Representative colon sections depicting co-localization of RORγt (green), pSTAT3 (red) and CD3 (gray) in the upper row, or of FoxP3 (green), pSTAT3 (red) with CD3 (gray) in the lower row in the same 3 groups. **(D)** Summary of the quantification of $pSTAT3^+$ cells within the indicated $CD3\varepsilon^+$ subsets (single $ROR\gamma t^+$, $ROR\gamma t^-FoxP3^-$ or single $FoxP3^+$). $^*P < 0.05$ and $^{**}P < 0.01$ using a one-way ANOVA with Welch's correction.

lower $CD3\varepsilon^+FoxP3^+$ cell percentages in mPGES-1-deficient recipient mice compared to WT recipient mice, and lower $CD3e^+ROR\gamma t^+$ percentages with EP4 deficiency transferred Teff (**Figure 6B**). Of note, the percentages of $CD3e^+ROR\gamma t^+$ in the colon were not fully consistent when we compare the flow cytometry data (**Figures 4C**, **5C**) with the results obtained with microscopy fluorescent quantification (**Figure 6B**). We explain this discrepancy due to a combination of differences in sample size (number of cells and coverage/colon), sample preparation-antigen reactivity, and technique (microscopy vs. flow cytometry). Co-expression of pSTAT3 was observed with RORγt (**Figure 6C**, arrowheads), but it was almost fully absent in $FoxP3^+$ cells. Additionally, pSTAT3 expression was harder to detect in absence of EP4 on transferred Teff cells (group 2) or in absence of mPGES-1 in the recipient colon (group 3). We then quantified pSTAT3 positivity within each of the different T cell subsets that were previously evaluated in all the T cells present in 10 combined sections per colon (**Figure 6D**). WT (group 1) $CD4^+ROR\gamma t^+$ and RORγt- showed similar proportions of $pSTAT3^+$ cells, while $CD4^+FoxP3^+$ cells showed a very reduced percentage comparatively (**Figure 6D**). Interestingly, both lack of T cell intrinsic EP4 deficiency and mPGES-1-derived PGE_2 in the recipient colon niche abrogated almost all pSTAT3 in $CD4^+ROR\gamma t^+$ and $CD4^+FoxP3^+$ cells (black and white bars, **Figure 6D**). However, $CD4^+ROR\gamma t^-FoxP3^-$ cells exhibited different sensitivities to PGE_2: Inability to detect PGE_2 with EP4 decreased phosphorylation of STAT3 by more than 50% (group 2), while absence of paracrine PGE_2 more modestly reduced pSTAT3 percentages (group 3) (gray bars, **Figure 6D**). We conclude that upregulation of pSTAT3 in colonic T cells is strongly influenced by PGE_2, and abrogating either EP4 sensing or paracrine PGE_2 production inhibits STAT3 phosphorylation.

DISCUSSION

This study is the first to demonstrate the divergent effects of T cell-derived PGE_2 compared with non-lymphocyte-derived PGE_2 on T cell phenotypes during colitis. Using a T cell dependent model of colitis, we show that deficient production or sensing of PGE_2 reduces the colitogenic potential of transferred $CD4^+$ T cells. Deficient production of PGE_2 due to deletion of mPGES-1 in transferred T cells reduces colitis in association with developing $CD4^+$ cells expressing high levels of RORγt. Deficient sensing by deletion of the EP4 receptor almost completely abolishes clinical colitis likely due to reduced T cell proliferation in the colonic lamina propria. In contrast, transfer of WT T effector cells into recipient mice deficient in mPGES-1 results in

a marked increase in colitogenesis. mPGES-1 deficiency in non-lymphoid cells impairs the development of FoxP3$^+$ cells in mLN and increases infiltration of total CD4$^+$ cells in the cLP. This finding suggests that PGE_2 is required for proper development of Th17 and Treg cells that restrain colitis.

PGE_2 has been classically regarded as a driver and perpetuator of inflammation (2, 16, 35), but it is also clear that it has potent protective roles (36). In the intestines, the COX1, COX2, and mPGES-1 enzymes control PGE_2 levels, but they differ in their tissue expression pattern. Indeed, indiscriminate inhibition of COX enzymatic activity with piroxicam accelerates colitis in both IL10$^{-/-}$ mice (37) and during transfer of CD4$^+$ cells from IL10$^{-/-}$ mice into Rag1$^{/-}$ mice (38). However, the role of mPGES-1 during intestinal inflammation has not been as deeply studied. mPGES-1-deficient animals are partially protected from dextran sulfate sodium (DSS)-induced colitis (35), and PGE_2 treatment alleviates mucosal injury and induces EP4 expression during DSS-induced colitis in WT mice (39). These data suggest a protective role of mPGES-1-derived PGE_2 during intestinal injuries, but the mechanisms describing how mPGES-1 competence on tissue damaging cells like lymphocytes affects colitis is unknown. In the current study we show that in absence of inflammatory stimuli, nearly 50% of production of PGE_2 in colon explants relies on mPGES-1 competence (**Figure 1A**). Lymphocytes present in the colon contribute less dramatically to this basal PGE_2 pool, but colons void of lymphocytes that also lack mPGES-1 (Rag1$^{-/-}$ × mPGES-1$^{-/-}$) show further reduced PGE_2 concentrations, indicating some synergy of environmental mPGES-1 expression with lymphocytes. A significant proportion of this basal PGE_2 can be inhibited by specifically inhibiting COX-2 when either mPGES-1 and/or lymphocytes are present, but not when colons lack both components.

Dysregulation of T cell activity during colitis leads to harmful responses incapacitating Tregs at the same time that pathogenic Th1 and Th17 responses are heightened (40, 41). The pathogenic potential of Th1 and Th17 cells actually depends on the colitis model studied (42, 43), and it has recently been shown that IFNγ production is necessary in Th17 cells to cause colitis (24). Indeed, although Th17 cells are generally associated with a pro-inflammatory response in autoimmunity, IL-17A can have very different effects during colitis (44, 45). The potential protective function of Th17-driven IL-17A in IBD is also exemplified by the recent failure of mAb therapies that target IL-17 such as secukinumab (46). In this study, we show that T cell intrinsic mPGES-1 deficiency confers partial protection from colitis development, and this is associated with increased RORγt-expressing cells in the mLN and cLP (**Figures 2C–E**). Additionally, mPGES-1 deficiency also impairs the capacity of CD4$^+$ Teff cells to more efficiently expand compared to WT CD4$^+$ Teff cells (**Figures 3A,B**). This aspect of T cell biology contrasts with the lack of T cell-intrinsic mPGES-1 effect that we recently found during a proinflammatory T cell responses in a mouse model of collagen-induced arthritis (CIA) (13). These differences are likely due to several factors related to the characteristics of each model. Most of the T cell expansion in a Rag-deficient mouse are due to homeostatic expansion that is not controlled by Tregs, while the CIA model involves a more moderate and antigen-specific and adjuvant-boosted expansion. Furthermore, the T cell responses we observed in the CIA model happened early on, while T cell homeostatic expansion in the Rag-deficient model takes weeks to develop, and additionally, the specific anatomical locations where expansion occurs are very different (draining lymph nodes vs. lamina propria). Interestingly, we found that the frequencies of CD4$^+$FoxP3$^+$ cells were consistently reduced in the cLP compared to the mLN when only Teff cells were transferred, while CD4$^+$RORγt$^+$ cells were generally increased, as it would be expected by a preferential microbiota-driven migration and expansion of Th17 cells in the intestines. It is hence important to reflect on the fact that T cell expansion can strongly vary due to the distinct inflammatory conditions and microenvironment-imposed constraints, and thus the impact of local concentrations of PGE_2 mediated by mPGES-1 or other enzymes contributing to the PGE_2 pool can also render different results.

Our results demonstrate the paradoxical finding that mPGES-1$^{-/-}$ Teff cells are partially protective compared to WT Teff, associated with an increase in CD4$^+$RORγt$^+$ cells (**Figure 2**), while EP4$^{-/-}$ Teff cells also exert a protective effect, but in this case with a decrease in CD4$^+$RORγt$^+$ cells (**Figure 4**). Whether the phenotype or the expansion of T cells are more affected by PGE_2 and how this is related to colitis is a complex question. PGE_2 sensing through EP4 controls *in vivo* expansion of the total numbers of T cells. This is a straightforward result that we interpret as related to T cell numbers regardless of their phenotype. mPGES-1-mediated PGE_2 production has differing effects depending on whether the source is autocrine, examined here by transfer of mPGES-1-deficient Teff cells (**Figures 2**, **3**), or paracrine, examined here using Rag1 and mPGES-1 double deficient recipients of WT Teff cells (**Figures 5**, **6**). Autocrine T cell intrinsic PGE_2 production appears to control the balance of T cell phenotypes while paracrine PGE_2 appears to be protective of colitis and may facilitate development of Treg cells. Therefore, depending on the specific inflammatory response and location, PGE_2 can induce T cell expansion through EP4 but also control intestinal damage by altering Th17 and Treg phenotypes. The effect of PGE_2 on T cell phenotype is likely to be complex and dependent on the overall inflammatory milieu. Our results also point to the plastic nature of Th17 and Treg responses, and how these classically defined phenotypes should be constantly re-evaluated based on context.

mPGES1$^{-/-}$ CD4$^+$ Teff and Treg cells can acquire higher levels of RORγt expression exclusively in the cLP (**Figures 3B,C**), a phenotype that was especially apparent when mPGES-1-deficient Tregs were transferred (**Figure 3C**). It has previously been shown that a proportion of intestinal FoxP3$^+$ Treg cells co-express RORγt, and their presence is dependent on intestinal microbiota (33, 47). The suppressive capacities of CD4$^+$FoxP3$^+$RORγt$^+$ cells have been reported to be superior to their FoxP3$^+$RORγt$^-$ counterparts (32), and generally geared toward controlling intestinal Th2 and Th17 responses (48, 49). It is tempting to speculate that the RORγt^{hi} cells we observed might be responsible for the reduction in clinical colitis when mPGES-1-deficient cells were transferred. STAT3 is necessary for the Th17 lineage to develop (48), and is also a master regulator of Treg

and Th17 lineage commitment (50). STAT3 phosphorylation mediates resistance of human T cells to regulatory T cell suppression (51). We have previously reported that exogenous addition of PGE_2 can downregulate pSTAT3 of $CD4^+$ cells during Th17 polarization *in vitro* (13). We now show that during the late phases of induced colitis, phosphorylation of STAT3 is present on nearly 20% of all cLP $CD3^+FoxP3^-ROR\gamma t^+$ and $CD3^+FoxP3^-ROR\gamma t^-$ cells (**Figures 6C,D**), while it is barely expressed in $CD3^+FoxP3^+$ $ROR\gamma t^-$ regulatory T cells. Moreover, ablation of mPGES-1 in recipient mice or EP4 expression in Teff cells dramatically reduced pSTAT3 signal specifically in $CD3^+FoxP3^-ROR\gamma t^+$ cells, while this reduction was significant but more modest in the $CD3^+FoxP3^-ROR\gamma t^-$ compartment. These data imply that the context of exposure to different PGE_2 levels and signals is critically important for phosphorylation of STAT3 in different T cell populations.

We have also identified that deficiency of paracrine mPGES1-driven PGE_2 exacerbates T cell-driven colitis by selectively decreasing $CD4^+FoxP3^+$ cells in the mLN (**Figure 5C**), with a concomitant increase of total number of $CD4^+$ infiltration in the cLP (**Figure 5D**). Classical *ex vivo* suppression assays with WT or mPGES-deficient Treg showed no difference in suppression capacity (**Figure S1**) suggesting that effects of PGE_2 are related to changes in differentiation to Treg or migration to the colon. Additionally, no differences in disease progression were found when comparing WT and mPGES1-deficient Treg-intrinsic suppressive capacity *in vivo* (**Figure 2A**, discontinuous lines). The specific effects that we observed in mLN or cLP due to mPGES1-derived PGE_2 in recipient mice could be explained by differences in gut tropism, which we are currently investigating. In this regard, PGE_2 has been shown to inhibit the production of retinoic acid by intestinal $CD103^+$ DCs and their capacity to express CCR9 upon T cell priming (52).

Signaling through EP2 can induce proliferation and cytokine secretion in Th17 cells (10), but human Th17 cells show specific downregulation of EP2 expression through RORC-dependent silencing (6). In the context of IBD, certain polymorphisms have been found in patients with Crohn's Disease that lead to increased expression of EP4 (53). From all EPs, only EP4 is critical to prevent mucosal damage in murine colitis induced with DSS (5). EP4 signaling is also critical for *in vitro* generation of Th1 cells and for expansion of Th17 cells upon IL-23 exposure (11), but surprisingly EP4 is downregulated during murine Th17 polarization in absence of IL-23 (13). Hemizigosity in EP4 during T cell induced colitis in the adoptive T cell transfer colitis model is partially protective, and also affects IFNγ and IL2 production by MLN $CD4^+$ cells (12). In our study we show that EP4 controls expansion of all phenotypes derived from Teff transfers during colitis (**Figure 4**), and therefore EP4-deficiency confers protection.

Th17 cell generation relies on sensing IL-23 signals generated during intestinal inflammation (54), and IL-23 also restrains Treg cells (55). PGE_2 can stimulate IL-23 production by DCs, myeloid cells, and other cells present in the intestines (2, 10, 36). It is hence possible that PGE_2 potentiates IL-23-mediated intestinal inflammation through enhancing EP4-dependent pathogenic $CD4^+ROR\gamma t^+$ responses, while in other instances and locations (like the mLN) it can induce $CD4^+FoxP3^+$ cells that protect from colitis development. However, Treg and Th17 cell lineage commitment can display significant plasticity, a feature especially patent in mucosal sites (24, 56, 57). In this context, PGE_2 could act inducing shifts in T cell lineage commitment, either directly on T cells or by altering the cytokine milieu generated by surrounding APCs: PGE_2 could hence act in mature Tregs by contributing to acquire FoxP3 expression in first place, but also to gain RORγt expression on $FoxP3^+$ cells. PGE_2 can also inhibit IL-17A and induce IFNγ production *in vitro* during Th17 polarization (13), so a different effect of PGE_2 could be its impact on promoting IFNγ in effector T cells.

In summary, we provide evidence that supports both pro- and anti-inflammatory effects of PGE_2 on T cells and colitis. We show that PGE_2 can exert opposite effects on T cell colitogenicity depending on the source of such PGE_2 and how it is sensed. Our results imply that extreme caution should be considered when using drugs that modulate PGE_2 production in a non-cell specific manner, as they can have disparate net effects. Our research also suggests that cell specificity and spatio-temporal considerations of PGE_2 production within the colon can be exploited to promote regulatory vs. pathogenic T cell function.

AUTHOR CONTRIBUTIONS

DM, KL, and LC were responsible for experimental design, data interpretation, and manuscript preparation. DM, AB, and EJ performed the experiments. DM, AB, HK, KL, and LC contributed to data analysis, MW performed the patho-histological scoring. DM and AB performed the preparation, labeling, and imaging of colon tissues. AB and HK performed the colon fluorescent signal quantifications.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02954/full#supplementary-material

Figure S1 | *In vitro* T reg suppression assays with WT and mPGES-1-deficient Tregs Conventional $CD4^+$ cells (Tconv, $CD4^+CD25^-$) were cocultured with either WT or mPGES1-deficient Tregs (Treg, $CD4^+CD25^+$) isolated and pooled from 3 different mice in the presence of plate bound anti-CD3ε (0.5 mg/ml) and soluble anti-CD28 (0.5 mg/ml) for 4 days in different Tconv:Treg ratios to address the Treg suppressive capacities. **(A)** Representative histogram depicting a CFSE dilution assay to evaluate Tconv proliferation in presence of either WT or mPGES-1-deficient Treg cells (1:2 Tconv:Treg ratio) and Tconv alone. **(B)** Summary of proliferating percentages at different Tconv:Treg ratios. Shown is one of two representative experiments.

REFERENCES

1. Park JY, Pillinger MH, Abramson SB. Prostaglandin E2 synthesis and secretion: the role of PGE2 synthases. *Clin Immunol.* (2006) 119:229–40. doi: 10.1016/j.clim.2006.01.016
2. Kalinski P. Regulation of immune responses by prostaglandin E2. *J Immunol.* (2012) 188:21–8. doi: 10.4049/jimmunol.1101029
3. Williams CS, Mann M, DuBois RN. The role of cyclooxygenases in inflammation, cancer, and development. *Oncogene* (2000) 18:7908–16. doi: 10.1038/sj.onc.1203286
4. Dey I, Lejeune M, Chadee K. Prostaglandin E2 receptor distribution and function in the gastrointestinal tract. *Br J Pharmacol.* (2006) 149:611–23. doi: 10.1038/sj.bjp.0706923
5. Kabashima K, Saji T, Murata T, Nagamachi M, Matsuoka T, Segi E, et al. The prostaglandin receptor EP4 suppresses colitis, mucosal damage and CD4 cell activation in the gut. *J Clin Invest.* (2002) 109:883–93. doi: 10.1172/JCI0214459
6. Kofler DM, Marson A, Dominguez-Villar M, Xiao S, Kuchroo VK, Hafler DA. Decreased RORC-dependent silencing of Prostaglandin receptor EP2 induces autoimmune Th17 cells. *J Clin Invest.* (2014) 124:2513–22. doi: 10.1172/JCI72973
7. Muthuswamy R, Mueller-Berghaus J, Haberkorn U, Reinhart TA, Schadendorf D, Kalinski P. PGE2 transiently enhances DC expression of CCR7 but inhibits the ability of DCs to produce CCL19 and attract naive T cells. *Blood* (2010) 116:1454–9. doi: 10.1182/blood-2009-12-258038
8. Monrad SU, Kojima F, Kapoor M, Kuan EL, Sarkar S, Randolph GJ, et al. Genetic deletion of mPGES-1 abolishes PGE2 production in murine dendritic cells and alters the cytokine profile, but does not affect maturation or migration. *Prostaglandins Leukot Essent Fat Acids* (2011) 84:113–21. doi: 10.1016/j.plefa.2010.10.003
9. Duffin R, OConnor RA, Crittenden S, Forster T, Yu C, Zheng X, et al. Prostaglandin E2 constrains systemic inflammation through an innate lymphoid cell-IL-22 axis. *Science* (2016) 351:1333–8. doi: 10.1126/science.aad9903
10. Boniface K, Bak-Jensen KS, Li Y, Blumenschein WM, McGeachy MJ, McClanahan TK, et al. Prostaglandin E2 regulates Th17 cell differentiation and function through cyclic AMP and EP2/EP4 receptor signaling. *J Exp Med.* (2009) 206:535–48. doi: 10.1084/jem.20082293
11. Yao C, Sakata D, Esaki Y, Li Y, Matsuoka T, Kuroiwa K, et al. Prostaglandin E2–EP4 signaling promotes immune inflammation through TH1 cell differentiation and TH17 cell expansion. *Nat Med.* (2009) 15:633–40. doi: 10.1038/nm.1968
12. Yao C, Hirata T, Soontrapa K, Ma X, Takemori H, Narumiya S. Prostaglandin E2 promotes Th1 differentiation via synergistic amplification of IL-12 signalling by cAMP and PI3-kinase. *Nat Commun.* (2013) 4:1685. doi: 10.1038/ncomms2684
13. Maseda D, Johnson EM, Nyhoff LE, Baron B, Kojima F, Wilhelm AJ, et al. mPGES1-dependent Prostaglandin E2 (PGE2) controls antigen-specific Th17 and Th1 responses by regulating T autocrine and paracrine PGE2 production. *J Immunol.* (2017) 200:725–36. doi: 10.4049/jimmunol.1601808
14. Napolitani G, Acosta-Rodriguez EV, Lanzavecchia A, Sallusto F. Prostaglandin E2 enhances Th17 responses via modulation of IL-17 and IFN-ɣ production by memory $CD4^+$ T cells. *Eur J Immunol.* (2009) 39:1301–12. doi: 10.1002/eji.200838969
15. Fabricius D, Neubauer M, Mandel B, Schütz C, Viardot A, Vollmer A, et al. Prostaglandin E2 inhibits IFN-alpha secretion and Th1 costimulation by human plasmacytoid dendritic cells via E-prostanoid 2 and E-prostanoid 4 receptor engagement. *J Immunol.* (2010) 184:677–84. doi: 10.4049/jimmunol.0902028
16. Nakanishi M, Rosenberg DW. Multifaceted roles of PGE2 in inflammation and cancer. *Semin Immunopathol.* (2013) 35:123–37. doi: 10.1007/s00281-012-0342-8
17. Schumacher Y, Aparicio T, Ourabah S, Baraille F, Martin A, Wind P, et al. Dysregulated CRTC1 activity is a novel component of PGE2 signaling that contributes to colon cancer growth. *Oncogene* (2016) 35:2602–14. doi: 10.1038/onc.2015.283
18. Sasaki Y, Nakatani Y, Hara S. Role of microsomal prostaglandin E synthase-1 (mPGES-1)-derived prostaglandin E2 in colon carcinogenesis. *Prostaglandins Other Lipid Mediat.* (2015) 121:42–5. doi: 10.1016/j.prostaglandins.2015.06.006
19. Vong L, Ferraz JGP, Panaccione R, Beck PL, Wallace JL. A pro-resolution mediator, prostaglandin D2, is specifically up-regulated in individuals in long-term remission from ulcerative colitis. *Proc Natl Acad Sci USA.* (2010) 107:12023–7. doi: 10.1073/pnas.1004982107
20. Long MD, Kappelman MD, Martin CF, Chen W, Anton K, Sandler RS. Role of nonsteroidal anti-inflammatory drugs in exacerbations of inflammatory bowel disease. *J Clin Gastroenterol.* (2016) 50:152–6. doi: 10.1097/MCG.0000000000000421
21. Ishikawa TO, Oshima M, Herschman HR. Cox-2 deletion in myeloid and endothelial cells, but not in epithelial cells, exacerbates murine colitis. *Carcinogenesis* (2011) 32:417–26. doi: 10.1093/carcin/bgq268
22. Roulis M, Nikolaou C, Kotsaki E, Kaffe E, Karagianni N, Koliaraki V, et al. Intestinal myofibroblast-specific Tpl2-Cox-2-PGE2 pathway links innate sensing to epithelial homeostasis. *Proc Natl Acad Sci USA.* (2014) 111:E4658–67. doi: 10.1073/pnas.1415762111
23. Wang Z, Friedrich C, Hagemann SC, Korte WH, Goharani N, Cording S, et al. Regulatory T cells promote a protective Th17-associated immune response to intestinal bacterial infection with C. rodentium. *Mucosal Immunol.* (2014) 7:1290–301. doi: 10.1038/mi.2014.17
24. Harbour SN, Maynard CL, Zindl CL, Schoeb TR, Weaver CT. Th17 cells give rise to Th1 cells that are required for the pathogenesis of colitis. *Proc Natl Acad Sci USA.* (2015) 112:7061–6. doi: 10.1073/pnas.1415675112
25. Kempski J, Brockmann L, Gagliani N, Huber S. TH17 cell and epithelial cell crosstalk during inflammatory bowel disease and carcinogenesis. *Front Immunol.* (2017) 8:1373. doi: 10.3389/fimmu.2017.01373
26. Trebino CE, Stock JL, Gibbons CP, Naiman BM, Wachtmann TS, Umland JP, et al. Impaired inflammatory and pain responses in mice lacking an inducible prostaglandin E synthase. *Proc Natl Acad Sci USA.* (2003) 100:9044–9. doi: 10.1073/pnas.1332766100
27. Schneider A, Guan YF, Zhang Y, Magnuson MA, Pettepher C, Loftin CD, et al. Generation of a conditional allele of the mouse prostaglandin EP4receptor. *Genesis* (2004) 40:7–14. doi: 10.1002/gene.20048
28. Weigmann B, Tubbe I, Seidel D, Nicolaev A, Becker C, Neurath MF. Isolation and subsequent analysis of murine lamina propria mononuclear cells from colonic tissue. *Nat Protoc.* (2007) 2:2307–11. doi: 10.1038/nprot.2007.315
29. Gerdes MJ, Sevinsky CJ, Sood A, Adak S, Bello MO, Bordwell A, et al. Highly multiplexed single-cell analysis of formalin-fixed, paraffin-embedded cancer tissue. *Proc Natl Acad Sci USA.* (2013) 110:11982–7. doi: 10.1073/pnas.1300136110
30. McKinley ET, Sui Y, Al-Kofahi Y, Millis BA, Tyska MJ, Roland JT, et al. Optimized multiplex immunofluorescence single-cell analysis reveals tuft cell heterogeneity. *JCI Insight* (2017) 2:93487. doi: 10.1172/jci.insight.93487
31. Kirkby NS, Chan MV, Zaiss AK, Garcia-Vaz E, Jiao J, Berglund LM, et al. Systematic study of constitutive cyclooxygenase-2 expression: role of NF-κB and NFAT transcriptional pathways. *Proc Natl Acad Sci USA.* (2016) 113:434–9. doi: 10.1073/pnas.1517642113
32. Yang B-H, Hagemann S, Mamareli P, Lauer U, Hoffmann U, Beckstette M, et al. Foxp3+ T cells expressing RORγt represent a stable regulatory T-cell effector lineage with enhanced suppressive capacity during intestinal inflammation. *Mucosal Immunol.* (2015) 205:1381–93. doi: 10.1038/mi.2015.74
33. Solomon BD, Hsieh C-S. Antigen-specific development of mucosal Foxp3+RORγt+ T cells from regulatory T cell precursors. *J Immunol.* (2016) 197:3512–9. doi: 10.4049/jimmunol.1601217
34. Newson J, Motwani MP, Kendall AC, Nicolaou A, Muccioli GG, Alhouayek M, et al. Inflammatory resolution triggers a prolonged phase of immune suppression through COX-1/mPGES-1-derived Prostaglandin E2. *Cell Rep.* (2017) 20:3162–75. doi: 10.1016/j.celrep.2017.08.098
35. Montrose DC, Nakanishi M, Murphy RC, Zarini S, McAleer JP, Vella AT, et al. The role of PGE2 in intestinal inflammation and tumorigenesis. *Prostaglandins Other Lipid Mediat.* (2015) 116–7:26–36. doi: 10.1016/j.prostaglandins.2014.10.002
36. Chinen T, Komai K, Muto G, Morita R, Inoue N, Yoshida H, et al. Prostaglandin E2 and SOCS1 have a role in intestinal immune tolerance. *Nat Commun.* (2011) 2:190. doi: 10.1038/ncomms1181
37. Holgersen K, Kvist PH, Markholst H, Hansen AK, Holm TL. Characterisation of enterocolitis in the piroxicam-accelerated interleukin-10 knock out mouse

- A model mimicking inflammatory bowel disease. *J Crohns Colitis* (2014) 8:147–60. doi: 10.1016/j.crohns.2013.08.002
38. Blum AM, Metwali A, Elliott DE, Berg DJ, Weinstock JV. CD4+ T cells from IL-10-deficient mice transfer susceptibility to NSAID-induced Rag colitis. *Am J Physiol Gastrointest Liver Physiol.* (2004) 287:G320–5. doi: 10.1152/ajpgi.00527.2003
39. Peng X, Li J, Tan S, Xu M, Tao J, Jiang J, et al. COX-1/PGE2/EP4 alleviates mucosal injury by upregulating β-arr1-mediated Akt signaling in colitis. *Sci Rep.* (2017) 7:1055. doi: 10.1038/s41598-017-01169-6
40. Shale M, Schiering C, Powrie F. CD4+ T-cell subsets in intestinal inflammation. *Immunol Rev.* (2013) 252:164–82. doi: 10.1111/imr.12039
41. Kanai T, Mikami Y, Sujino T, Hisamatsu T, Hibi T. RORγt-dependent IL-17A-producing cells in the pathogenesis of intestinal inflammation. *Mucosal Immunol.* (2012) 5:240–7. doi: 10.1038/mi.2012.6
42. Giles EM, Sanders TJ, McCarthy NE, Lung J, Pathak M, MacDonald TT, et al. Regulation of human intestinal T-cell responses by type 1 interferon-STAT1 signaling is disrupted in inflammatory bowel disease. *Mucosal Immunol.* (2016) 10:184–93. doi: 10.1038/mi.2016.44
43. Zimmermann J, Kühl AA, Weber M, Grün JR, Löffler J, Haftmann C, et al. T-bet expression by Th cells promotes type 1 inflammation but is dispensable for colitis. *Mucosal Immunol.* (2016) 9:1487–99. doi: 10.1038/mi.2016.5
44. Lee JS, Tato CM, Joyce-Shaikh B, Gulan F, Cayatte C, Chen Y, et al. Interleukin-23-independent IL-17 production regulates intestinal epithelial permeability. *Immunity* (2015) 43:727–38. doi: 10.1016/j.immuni.2015.09.003
45. Maxwell JR, Zhang Y, Brown WA, Smith CL, Byrne FR, Fiorino M, et al. Differential roles for interleukin-23 and interleukin-17 in intestinal immunoregulation. *Immunity* (2015) 43:739–50. doi: 10.1016/j.immuni.2015.08.019
46. Neurath MF. Current and emerging therapeutic targets for IBD. *Nat Rev Gastroenterol Hepatol.* (2017) 14:269–78. doi: 10.1038/nrgastro.2016.208
47. Lochner M, Peduto L, Cherrier M, Sawa S, Langa F, Varona R, et al. *In vivo* equilibrium of proinflammatory IL-17+ and regulatory IL-10+ Foxp3+ RORγ t+ T cells. *J Exp Med.* (2008) 205:1381–93. doi: 10.1084/jem.20080034
48. Ohnmacht C, Park J, Cording S, Wing JB, Atarashi K, Obata Y, et al. The microbiota regulates type 2 immunity through RORγt+ T cells. *Science* (2015) 349:989–93. doi: 10.1126/science.aac4263
49. Sefik E, Geva-Zatorsky N, Oh S, Konnikova L, Zemmour D, McGuire AM, et al. MUCOSAL IMMUNOLOGY. Individual intestinal symbionts induce a distinct population of RORγ(+) regulatory T cells. *Science* (2015) 349:993–7. doi: 10.1126/science.aaa9420
50. Chaudhry A, Rudra D, Treuting P, Samstein RM, Liang Y, Kas A, et al. CD4+ regulatory T cells control TH17 responses in a Stat3-dependent manner. *Science* (2009) 326:986–91. doi: 10.1126/science.1172702
51. Goodman WA, Young AB, McCormick TS, Cooper KD, Levine AD. Stat3 phosphorylation mediates resistance of primary human T cells to regulatory T cell suppression. *J Immunol.* (2011) 186:3336–45. doi: 10.4049/jimmunol.1001455
52. Stock A, Booth S, Cerundolo V. Prostaglandin E2 suppresses the differentiation of retinoic acid–producing dendritic cells in mice and humans. *J Exp Med.* (2011) 208:761–73. doi: 10.1084/jem.20101967
53. Prager M, Büttner J, Büning C. PTGER4 modulating variants in Crohn's disease. *Int J Colorectal Dis.* (2014) 29:909–15. doi: 10.1007/s00384-014-1881-3
54. Krausgruber T, Schiering C, Adelmann K, Harrison OJ, Chomka A, Pearson C, et al. T-bet is a key modulator of IL-23-driven pathogenic CD4+ T cell responses in the intestine. *Nat Commun.* (2016) 7:11627. doi: 10.1038/ncomms11627
55. Ahern PP, Izcue A, Maloy KJ, Powrie F. The interleukin-23 axis in intestinal inflammation. *Immunol Rev.* (2008) 226:147–59. doi: 10.1111/j.1600-065X.2008.00705.x
56. Miyao T, Floess S, Setoguchi R, Luche H, Fehling HJ, Waldmann H, et al. Plasticity of Foxp3+T cells reflects promiscuous Foxp3 expression in conventional T cells but not reprogramming of regulatory T cells. *Immunity* (2012) 36:262–75. doi: 10.1016/j.immuni.2011.12.012
57. Gagliani N, Vesely MCA, Iseppon A, Brockmann L, Xu H, Palm NW, et al. Th17 cells transdifferentiate into regulatory T cells during resolution of inflammation. *Nature* (2015) 523:221–5. doi: 10.1038/nature 14452

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REVIEW
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The Role of Dietary Nutrients in Inflammatory Bowel Disease

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Inflammatory bowel disease (IBD) is a chronic and relapsing inflammatory disease of the gastrointestinal tract. Although the precise etiology of IBD remains incompletely understood, accumulating evidence suggests that various environmental factors, including dietary nutrients, contribute to its pathogenesis. Dietary nutrients are known to have an impact on host physiology and diseases. The interactions between dietary nutrients and intestinal immunity are complex. Dietary nutrients directly regulate the immuno-modulatory function of gut-resident immune cells. Likewise, dietary nutrients shape the composition of the gut microbiota. Therefore, a well-balanced diet is crucial for good health. In contrast, the relationships among dietary nutrients, host immunity and/or the gut microbiota may be perturbed in the context of IBD. Genetic predispositions and gut dysbiosis may affect the utilization of dietary nutrients. Moreover, the metabolism of nutrients in host cells and the gut microbiota may be altered by intestinal inflammation, thereby increasing or decreasing the demand for certain nutrients necessary for the maintenance of immune and microbial homeostasis. Herein, we review the current knowledge of the role dietary nutrients play in the development and the treatment of IBD, focusing on the interplay among dietary nutrients, the gut microbiota and host immune cells. We also discuss alterations in the nutritional metabolism of the gut microbiota and host cells in IBD that can influence the outcome of nutritional intervention. A better understanding of the diet-host-microbiota interactions may lead to new therapeutic approaches for the treatment of IBD.

Keywords: inflammatory bowel disease (IBD), diet, microbiota, immunity, intestinal barrier

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INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic and relapsing inflammatory condition of the gastrointestinal tract. Crohn's disease (CD) and ulcerative colitis (UC) are the two principal types of IBD. The prevalence of IBD has been increasing worldwide with the highest incidence found in Western countries (1). Although the precise etiology of IBD remains unclear, interactions between genetic and environmental factors are associated with its pathogenesis (2, 3). Advances in next-generation gene sequencing technology have led to the identification of over 160 genetic polymorphisms associated with the risk for IBD (4). Since the number of IBD patients in developing countries has rapidly increased in the past several decades, in concert with industrialization and westernization of lifestyle, it is unlikely that susceptibility genes are the primary driver of rising rates of IBD (5). Therefore, it is likely that environmental exposure is the most significant risk factor in IBD.

Among environmental factors, accumulating evidence suggests that dietary nutrients contribute to the pathogenesis of IBD (6, 7). Specifically, diets rich in fat and protein, common in the western world and countries with similar lifestyles, have been identified as risk factors for the development of IBD. Hence, nutritional intervention, which aims to reduce the intake of potential nutritional hazards, is a treatment option in IBD that induces and extends disease remission (8). Moreover, some dietary nutrients can potentiate the host immune system and intestinal barrier function, which in turn protect the host from disease. Thus, providing beneficial nutrients, while limiting nutritional hazards, is a key strategy for successful dietary therapies designed for the treatment of IBD. In addition to affecting host immunity and intestinal barrier function, dietary nutrients have an impact on the composition and function of the gut microbiota. The altered gut microbiota can, in turn, influence host physiology and disease. Furthermore, the metabolism of host immune and non-immune cells, as well as that of the gut microbiota, are known to change during inflammation. Hence, the demand for certain nutrients by the host and/or the microbiota may be changed in IBD (9–11). A more thorough understating of the complex interplay among dietary nutrients, host immunity, and the gut microbiota is required to increase the effectiveness of dietary interventions used to treat IBD. Herein, we review the current knowledge of the role of dietary nutrients in the development and the treatment of IBD.

DIETARY AMINO ACIDS AND IBD

Dietary amino acids act as key regulatory factors in cellular and microbial metabolic pathways. They also play important roles in gut homeostasis. Intestinal inflammation affects several metabolic pathways and disturbances in amino acid metabolism are observed in IBD patients. Amino acid metabolic profiles in the blood, urine, feces, and intestinal tissues are also altered in IBD patients and correlate with the severity of disease (**Table 1**). Additionally, metagenomic studies have revealed that amino acid biosynthesis genes are downregulated and amino acid transporter genes are upregulated in the gut microbiome of IBD patients, indicating that the gut microbiota lessens the production of amino acids and increases the rate of their utilization (11, 25). In addition to bacteria, host immune cells also utilize amino acids differently during inflammation. For instance, certain amino acids are critical for T cell effector function as well as the proliferation of macrophages (26, 27). Thus, the demand for certain amino acids by host cells and the gut microbiota may increase as a result of inflammation.

Tryptophan

Tryptophan is an essential amino acid and a common constituent of protein-based foods, such as fish, meat, and cheese. It is utilized in the synthesis of nicotinamide derivatives, indole derivatives and serotonin (28). Tryptophan metabolites, such as kynurenine, indole-3-aldehyde, and indole-3-acetic acid, can act as ligands for the aryl hydrocarbon receptor (AhR), a critical regulator of immunity and inflammation involved in adaptive immunity and intestinal barrier function (**Figure 1**) (29, 30). In a murine model of colitis, mice fed a tryptophan-deficient diet showed exacerbation of colitis accompanied by increased weight loss and reduced levels of antimicrobial peptides (31). Conversely, dietary supplementation with tryptophan and tryptophan metabolites ameliorated intestinal inflammation in experimental colitis (31–35).

Indoleamine 2,3 dioxygenase-1 (IDO1) is ubiquitously expressed in epithelial cells, dendritic cells and macrophages. IDO1 is the first step in the kynurenine pathway, a major route for tryptophan catabolism (28). IDO1 regulates the differentiation and maturation of adaptive immune cells (36). Kynurenine is an initial metabolite of IDO1-mediated tryptophan catabolism and the kynurenine/tryptophan ratio is a surrogate marker of IDO1 activity. A recent clinical study has shown that serum tryptophan levels are lower and the kynurenine/tryptophan ratio is elevated in IBD patients compared to healthy controls (37). Additionally, IDO1 mRNA expression in colonic tissues is significantly higher in IBD and correlates with disease severity, suggesting the kynurenine pathway is upregulated in IBD. The expression of IDO1 is also higher in murine models of experimental colitis and has been shown to regulate inflammatory response (38). Since kynurenine is known to have anti-inflammatory properties, elevated levels of IDO1 in IBD may be a counter reaction to inflammation. Indeed, in the 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis model, disease severity is exacerbated in IDO1 deficient mice or mice receiving an IDO inhibitor (38, 39). IDO1 also attenuates intestinal inflammation in other models of colitis, including graft vs. host disease (40) and the *Citrobacter rodentium* infection model (41). Kynurenine promotes the differentiation of regulatory T cell (Treg) and induces IL-10 production by Treg cells through AhR activation (30, 42). Likewise, kynurenine-mediated AhR activation increases expression of the IL-10 receptor in intestinal epithelial cells (35). IL-10 signaling regulates mucosal wound repair through WNT1-inducible signaling pathway protein 1, suggesting that kynurenine plays a role in mucosal wound repair (43). Additionally, kynurenine supplementation ameliorates body weight loss, intestinal permeability and histology in the model of dextran sodium sulfate (DSS)-induced colitis (35).

Peripheral immune activation can affect the systemic metabolism of tryptophan and emotional behavior. Activation of T cells in *Pdcd1*$^{-/-}$ mice, which lack the inhibitory programmed cell death protein 1 (PD-1), has been shown to affect the blood metabolic profile (44). Specifically, it results in an increase of amino acid uptake and intracellular accumulation of free amino acids, resulting in the reduction of amino acid levels (especially tryptophan) in the blood. Tryptophan is essential for the synthesis of the neurotransmitter serotonin, which regulates many aspects of animal behavior, including anxiety, aggression and fear (45). Brain serotonin levels are lower in *Pdcd1*$^{-/-}$ mice. Accordingly, these animals are prone to anxiety and exhibit enhanced fear responses. Collectively, this evidence suggests that activation of T cells causes a systemic metabolic shift, resulting in abnormal emotional behavior.

TABLE 1 | Comparison of amino acid levels between healthy subjects and IBD patients.

Sample	Patients	Increase	Decrease	References
Serum	HC: 60, UC: 60		UC: Asn, Asp, Gln, His, Trp, Val, Ile, Thr, Pro, Ser, Met, Glu, Tyr, Lys,	(10)
Serum	HC: 20, UC: 20, CD: 20		CD: Leu, Lys, Val, Arg, Ser, Gln	(12)
Serum	HC: 40, UC: 20, CD: 20	UC: Lys, Ile	UC: Tyr, Val, Ser	(13)
		CD: Pro, Arg, Gly, Ile	CD: Val	
Serum	HC: 17, UC: 24, CD: 19	IBDa: Leu, Ile, Gly, Phe,	IBDa: His	(14)
Serum	HC: 17, UC: 22, CD: 21	UC: Asp, Gly	UC: Asn, Gln, Glu, His, Trp	(15)
		CD: Ala, Asp, Gly, Met, Pro	CD: Gln, His, Trp	
Serum	HC: 29, UC: 25, CD: 26	UC: Arg, Ile, Ser, Pro	UC: Trp	(9)
		CD: Arg, Ile, Ser, Pro	CD: Trp	
Plasma	HC: 102, UC: 102, CD: 102	UC: Pro	UC: Val, Leu, Met, His, Trp, Phe, Asn, Tyr	(16)
			CD: Val, Leu, Ile, Thr, Lys, Met, His, Phe, Asn, Gln, Gly, Tyr	
Urine	HC: 21, IBD: 21	IBD: Gly, Asp, Cys, Glu, Ile, Met, Pro, Val		(17)
Urine	HC: 60, UC: 30, CD: 30		IBD: His, Lys, Asp	(18)
Urine	HC: 40, UC: 20, CD: 20	UC: Trp, Thr, Arg	UC: Ser, Phe	(13)
		CD: Thr		
Urine	HC: 17, UC: 24, CD: 19	IBDa: Gly	IBDa: Ala	(14)
Feces	HC: 29, UC: 25, CD: 26	UC: Asp, Gly, Trp, Ser, Thr, Asn, His, Phe, Ala	CD: Asp, Thr, Asn, His	(9)
		CD: Gly, Trp, Ser, Phe, Ala		
Feces	UC: 15, CD: 15, HV: 15	UC: Ala, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp, Tyr, Val		(19)
		CD: Ala, His, Leu, Phe, Trp, Tyr, Val		
Feces	HC: 13, UC: 10, CD: 10	UC: Gln, Lys		(20)
		CD: Ala, Ile, Leu, Lys, Val		
Feces	HC: 21, UC: 41, CD: 44	UCa: Ile, Leu, Val, Lys, Ala		(21)
		CDa: Ile, Leu, Val, Lys, Ala, Tyr, Phe, Gly		
Feces	HC: 51, UC: 82, CD: 50	UC: Gly, Phe	UC: Glu	(22)
		CD: Ala, Phe		
Colonic biopsies	HC: 17, UC: 22, CD: 21		Inflamed: Ala, Asp, Glu, Gln, Gly, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Tyr, Val	(15)
Colonic biopsies	HC: 26, UC: 31, CD: 26	UCa: Arg/Leu/Lys	UCa: Ile/Leu/Val, Ala, Glu/Gln	(23)
			CDa: Ile/Leu/Val, Ala, Glu/Gln	
Colonic biopsies	HC: 25, UC: 68	UCa: Glu, Gln, Asp		(24)

HC, healthy control; IBD, inflammatory bowel disease; UC, ulcerative colitis; CD, Crohn's disease; UCa, active ulcerative colitis; CDa, active Crohn's disease.

Importantly, dietary supplementation of tryptophan ameliorates these behavioral abnormalities. Anxiety and depression are more common in patients with IBD and symptoms stemming from these conditions tend to be more severe during periods of active disease (46). Thus, tryptophan is an important nutrient that plays a role in the regulation of inflammation and maintenance of mental health.

In addition to tryptophan metabolism in the host, tryptophan metabolites generated by the gut microbiota also contribute to the regulation of mucosal homeostasis (47). A genome-wide association study has found that caspase recruitment domain-containing protein 9 (CARD9), an adaptor protein involved in apoptosis and antifungal immunity, is a susceptibility gene for IBD (4). CARD9 deficient mice produce reduced amounts of IL-22, a cytokine with an important role in maintaining mucosal immunity and integrity (48), and are more susceptible to colitis (49). Interestingly, the gut microbiota of CARD9 deficient mice lack certain bacteria, such as *Lactobacillus reuteri* and *Allobaculum*, that are capable of catabolizing tryptophan into indole derivatives (49). Hence, dysbiotic microbiotas in CARD9 deficient mice do not generate indole derivatives, which promote mucosal IL-22 production through AhR activation (49). Consistently, reduced levels of AhR ligands are also typically observed in the microbiotas found in IBD patients, particularly in those with the CARD9 risk alleles associated with IBD (49). Thus, gut dysbiosis in the context of IBD may lead to compromised

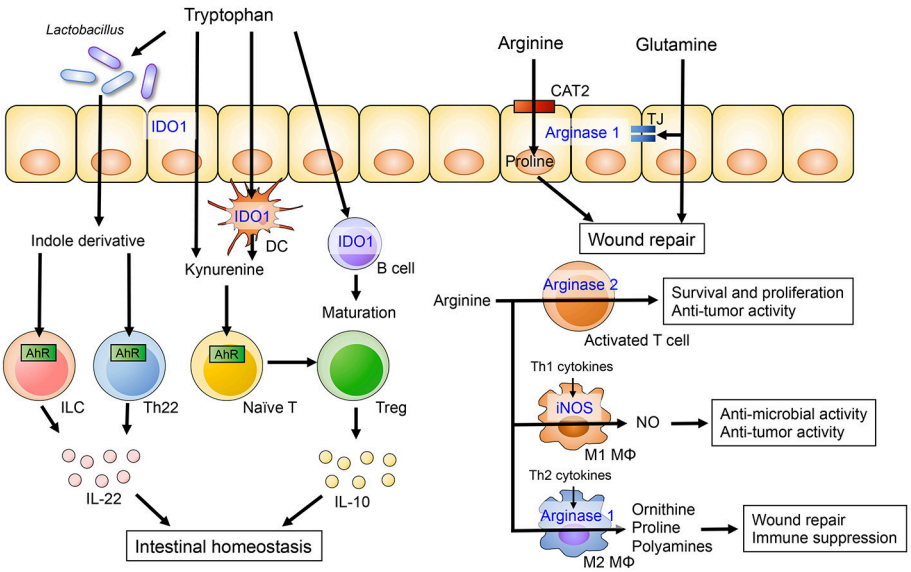

FIGURE 1 | The role of dietary amino acids in intestinal homeostasis. Dietary tryptophan is metabolized to kynurenine or indole derivatives by host cells or the gut microbiota, respectively. Kynurenine promotes the differentiation of Treg and induces IL-10 production by Treg cells through AhR. *Lactobacillus* species are capable of catabolizing tryptophan into indole derivatives that are ligands for the AhR. Activation of AhR in gut-resident T cells and ILC enhances production of IL-22, which in turn potentiates mucosal barrier integrity. Arginine and glutamine metabolism in intestinal epithelial cells is associated with epithelial barrier and intestinal wound repair. Arginine also plays an important role of the immune system. Arginine is catabolized by iNOS in M1 macrophages and by arginase II in M2 macrophages. The arginine metabolisms regulate anti-microbial and -tumor activity and immune suppression in M1 and M2 macrophages, respectively.

tryptophan catabolism, which in turn influences IL-22-mediated mucosal protection.

Arginine

Arginine is a semi-essential amino acid and a substrate for 4 enzymes including arginases, nitric oxide synthases (NOS), arginine-glycine amidinotransferase and arginine decarboxylase (50). Arginases have two isoforms, arginase I and arginase II, both of which metabolize L-arginine to NO and citrulline. Arginase I is highly expressed in the liver, whereas arginase II is expressed in the brain, kidneys, mammary glands and intestine. nitric oxide synthases (NOS) catalyzes the synthesis of NO from arginine. There are three NOS isoenzymes: neuronal nitric oxide synthase (nNOS), inducible nitric oxide synthase (iNOS), and endothelial nitric oxide synthase (eNOS). Arginine uptake into cells is mainly mediated by the cationic amino acid transporter (CAT) family of proteins encoded by the solute carrier (SLC).

Alterations in arginine metabolism have been reported in animal models of colitis as well as IBD patients, and arginine supplementation has been shown to ameliorate experimental colitis (51–53). A prospective cohort study has demonstrated that colonic arginine levels are decreased in UC patients and they correlate with disease severity (51). Altered levels of arginine in the colonic tissue are associated with increased mRNA expression of the arginine metabolic enzymes arginase II and iNOS. Although previous animal and clinical studies have revealed contradictory findings regarding arginine transporter expression during inflammation, arginine transport is critical for the maintenance of gut homeostasis (51–53). Wounding of intestinal epithelial cells has been shown to alter the expression of genes related to arginine transport and metabolism *in vitro* (52). CAT2, unlike CAT1, mediates intestinal wound repair and cell migration (54). Therefore, a CAT2 deficiency renders mice susceptible to DSS-induced colitis (53). Arginine catabolism also promotes wound repair. L-arginine metabolites, including proline and ornithine, are associated with restitution of intestinal epithelial integrity. Hence, supplementation of arginine during the recovery phase of DSS-induced colitis alleviates intestinal inflammation and promotes the migration of colonic epithelial cells (52). Thus, arginine metabolism plays a crucial role in the resolution of inflammation and, therefore, arginine supplementation could be used to promote mucosal healing in IBD.

Arginine has also been suggested to be crucial for immune cell activation and function. Arginase II is up-regulated in activated T cell, resulting in enhanced $CD4^+$ and $CD8^+$ T cell survival and anti-tumor activity (55). Depletion of extracellular arginine has been found to impair aerobic glycolysis, T cell proliferation and cytokine production (56–58). Additionally, the deletion of argininosuccinate 1 (ASS1), an enzyme utilized in the *de novo* synthesis of arginine, blunts Th1 and Th17 cell polarization even in the presence of extracellular arginine (59). Similarly, arginine metabolism may play a role in the regulation of macrophage functions. It has been reported that macrophages can undergo polarization toward the M1 or M2 phenotype based on environmental polarization cues. Stimulation with LPS plus IFN-γ induces M1 polarization, whereas Th2 cytokines, such as IL-4, IL-10, and IL-13, induce M2 polarization (60,

61). Interestingly, these two types of macrophages catabolize arginine differently. M1 macrophages express iNOS, which converts arginine to NO and citrulline. Both of these arginine metabolites are involved in the elimination of intracellular pathogens and tumor cells (62). In contrast, M2 macrophages express arginase I, which regulates the synthesis of proline and polyamines from ornithine, and is critical for wound healing and immune suppression (63). While a number of studies have demonstrated the importance of arginine metabolism in immune cells, the role of arginine in intestinal immunity is poorly understood. Thus, more research is necessary to understand how arginine metabolism shapes intestinal immune response during inflammation.

Glutamine

Glutamine is considered a conditionally essential amino acid because its consumption is increased during conditions of catabolic stress, including trauma, sepsis and post-surgery recovery (64). Glutamine is the most important fuel for enterocytes and immune cells, and has beneficial effects on clinical outcomes (65). Activation of lymphocytes results in a metabolic reprogramming that switches energy metabolism from oxidative phosphorylation to aerobic glycolysis and glutaminolysis, the process by which cells convert glutamine into TCA cycle metabolites (66, 67). Thus, glutamine metabolism and the demand for glutamine during inflammation may be increased in certain cells. Indeed, glutamine levels in the colonic tissue of IBD patients in remission are decreased compared to control subjects. This difference is even more considerable during active disease (15, 68). In addition to the colonic tissue, blood glutamine levels are also diminished in IBD patients and experimental animal models of colitis (10, 69). Oral or rectal supplementation of glutamine attenuates intestinal inflammation, intestinal fibrosis, and colitis-associated colon tumorigenesis in various models of colitis (69–73). Glutamine treatment significantly improves histology results, reduces oxidative stress and cytokine production via downregulation of the NF-κB and STAT signaling pathways (72). Glutamine also regulates epithelial integrity. Glutamine has been implicated in the preservation of gut barrier function, maintenance of epithelial tight junction integrity (74) and modulating paracellular permeability (75). In contrast, glutamine deprivation or inhibition of glutamine synthase significantly increase paracellular permeability and decrease the expression of tight junction proteins (76, 77). Glutamine is also associated with the proliferation of Lgr5-positive intestinal stem cell and subsequent crypt expansion. In intestinal organoid culture, the deprivation of glutamine suppresses epithelial proliferation (78). Replenishment of culture medium with supplementation of glutamine rescues the proliferation of epithelial cells via the activation of mammalian target of rapamycin (mTOR) (78). However, glutamine deprivation does not affect the proportions of Paneth and goblet cells, indicating that certain amino acids may support the differentiation and proliferation of intestinal epithelial cells in a lineage-specific manner. Although glutamine supplementation has been shown to be beneficial in murine models of experimental colitis, the utility of this amino acid in IBD patients remains poorly understood. Clinical studies showed that glutamine-supplemented enteral nutrition and total parenteral nutrition did not improve therapeutic outcomes for pediatric CD, adult CD, and adult UC patients (79, 80).

Other Amino Acids

Metabolome analyses have shown that the levels of other amino acids, such as histidine, glycine, and threonine, are altered in the serum and intestinal tissues of IBD patients (10, 13, 15). These amino acids have also been reported to be beneficial to IBD patients and in models of experimental colitis. Histidine is a natural amino acid and is one of conditionally essential amino acids. Histidine acts as a scavenger of hydroxyl radicals and singlet oxygen (81), and hence can suppress oxidative stress in intestinal epithelial cells (82). Moreover, dietary histidine ameliorates intestinal inflammation in the IL-10-deficient cell transfer model of colitis through the inhibition of NF-κB activation, following the down-regulation of pro-inflammatory cytokine production in macrophages (83). In IBD patients, the levels of histidine in the blood and intestinal tissues are markedly decreased compared to control subjects (10, 15). A recent clinical study has shown that the plasma histidine level is a predictor of relapse risk in UC patients (84). Glycine has been reported to be protective against various kinds of organ injuries as well as intestinal inflammation. Dietary glycine improves histology and dampens the expression of pro-inflammatory cytokines in TNBS- and DSS-induced colitis (85). Threonine is an essential amino acid with roles in maintaining intestinal homeostasis. It is important for mucosal barrier function, which prevents luminal bacteria from penetrating the mucosal tissue (86). Defects in intestinal mucosal barrier function are observed in IBD patients and experimental colitis, enabling gut bacteria to penetrate the intestinal mucosa (87, 88). Mucins contain high levels of threonine, serine and proline (89). Consequently, mucin synthesis requires robust amounts of these amino acids and restriction of dietary threonine impairs intestinal mucin synthesis (89). In contrast, dietary supplementation of threonine, serine, proline and cysteine ameliorates intestinal damage in DSS-treated rats, presumably by improving mucosal barrier function (90).

Intestinal inflammation changes the metabolic requirements of host cells and the gut microbiota. The up-regulation of amino acid metabolic pathways is one of the major inflammation-related metabolic shifts in the host and the microbiota (11, 44, 55). In other words, host cells and resident microbes have an increased demand for certain amino acids in the context of IBD. Thus, dietary supplementation of these sought-after amino acids is a key strategy for the treatment of IBD. However, it is noteworthy to mention that other amino acids may fuel pro-inflammatory responses in IBD and restriction of certain amino acids can attenuate intestinal inflammation. Amino acid deficiency is sensed by a serine/threonine-protein kinase called general control non-derepressible 2 (GCN2) (91). GCN2 suppresses pathogenic Th17 responses by inhibiting ROS-mediated activation of the inflammasome (92). Therefore, when GCN2 is deleted in a tissue-specific manner from intestinal epithelial cells and $CD11c^+$ antigen-presenting cells,

the animals experienced more severe weight loss and intestinal inflammation, indicating that both intestinal epithelial cells and antigen-presenting cells mediate the protective effects of GCN2 during colitis. Notably, amino acid starvation, induced by the consumption of a low-protein diet or a leucine-deficient diet, can suppress DSS-induced colitis through the activation of GCN2 (92). Since deprivation of other amino acids, such as tryptophan, tends to enhance intestinal inflammation (31), it is likely that each amino acid plays a distinct role in the promotion and/or suppression of intestinal inflammation. Thus, a more thorough understanding of the specific roles different amino acids play in IBD and supplying the optimal amount of anti-inflammatory amino acids while limiting the consumption of pro-inflammatory amino acids can lead to the development of effective amino acid-based dietary interventions.

CARBOHYDRATE

Carbohydrates are an important source of nutrients for both host cells and the gut bacteria. However, host cells largely lack the capacity to digest complex poly-saccharides (93). Rather, dietary fibers and other carbohydrate polymers are degraded and fermented by the gut microbiota into mono-saccharides as well as various by-products. Recent accumulating evidence indicates these gut microbial byproducts can modulate the host immune system and intestinal barrier function, thus playing a crucial role in intestinal homeostasis (94). Therefore, the intake of indigestive carbohydrates (dietary fibers) benefits the host. On the other hand, gut dysbiosis may compromise the metabolic activities of the gut microbiota, resulting in impaired generation of the protective microbial byproducts. Furthermore, high intake of undigested and fermentable carbohydrates, such as mono- and di-saccharides, induces various gastrointestinal symptoms. In this section, we summarize the roles of dietary fiber and fermentable carbohydrates in intestinal homeostasis and the management of IBD symptoms.

Fibers

Dietary fibers are complex carbohydrates consisting of both soluble and insoluble components. Dietary fibers are not digested or absorbed by host cells, as mammalian cells largely lack the enzymes necessary to degrade them. Instead, dietary fibers are subjected to bacterial fermentation in the gastrointestinal tract. Although a wide range of bacteria ferment dietary fibers, each bacterium has a substrate preference based on its enzymatic activity. For example, the human gut symbionts *Bacteroides thetaiotaomicron* and *B. ovatus* can degrade a wide variety of glycans, whereas some *Bacteroides* species are restricted to one or only a few types (95). Thus, dietary intervention can remodel the gut microbial composition by customizing the content of dietary fibers. Also, bacterial byproducts generated through the fermentation of dietary fibers have various effects on host immune cells and intestinal barrier function. Short-chain fatty acids (SCFAs), such as acetate, propionate and butyrate, are the major end products of microbial fermentation of dietary fibers (**Figure 2**) (93). Dietary fiber-derived SCFAs are key energy substrates utilized by colonocytes (96). Additionally, SCFAs act as signaling molecules via G-protein-coupled receptors (GPRs), such as GPR41, GPR43, and GPR109a. SCFAs can regulate the differentiation of immune cells and immune responses through GPRs. SCFAs have been shown to promote anti-inflammatory responses through the activation of GPRs, as mice deficient in GPR43 and GPR109a develop more severe DSS-induced colitis (97, 98). Furthermore, GPR109a signaling promotes anti-inflammatory properties in colonic macrophages and dendritic cells as well as inducing the differentiation of regulatory type T cells, such as Treg cells and IL-10-producing T cells (97). Since SCFAs are generated by the gut microbiota through fermentation of dietary fibers, germ-free (GF) mice display lower levels of colonic $Foxp3^+$ Treg cells (99). Butyrate is known to regulate histone acetylation, which in turn up-regulates the expression of Foxp3. Transcriptional factor Foxp3 is a lineage specification factor of Tregs that has been shown to prevent the development of colitis in a T cell transfer model of colitis (100). Collectively, SCFAs are important immunomodulatory molecules that exert numerous beneficial effects on host metabolism and immunity.

In addition to their regulatory function, SCFAs and, by extension, dietary fibers are also important for mucosal barrier function. Butyrate enhances intestinal epithelial barrier function via hypoxia inducible factor-1 (HIF-1) that regulates the integrity of epithelial tight junctions (101, 102). Likewise, butyrate treatment in mice facilitates the assembly of tight junction proteins and increases the synthesis of MUC2 protein, the main component of intestinal mucus (103, 104). Thus, a lack of dietary fibers may compromise epithelial integrity and mucus production due to insufficient SCFA generation, resulting in impaired intestinal barrier function (100, 101). In contrast, butyrate is known to suppress the proliferation of intestinal stem cells. Butyrate inhibits histone acetylation and enhances the promoter activity for the negative cell-cycle regulator Foxo3 in intestinal stem cells (105). Increased expression of Foxo3 in intestinal stem cells results in delayed wound repair after mucosal injuries (105). Notably, a recent report has unveiled that consumption of a low fiber diet leads to the disruption of intestinal barrier function through a mechanism that is independent of SCFAs. In the absence of dietary fibers, some commensal bacteria, such as *Akkermansia muciniphila*, utilize host mucus glycans to meet their energy needs (106). As a result, these mucolytic bacteria become the predominant species within the gut microbiota (106). Importantly, a bloom of mucolytic bacteria results in the degradation of the colonic mucus layer that renders the host susceptible to enteric pathogens, such as *C. rodentium* (106).

Gut dysbiosis observed in IBD patients is primarily characterized by reduced bacterial diversity, the enrichment of the phylum of *Proteobacteria* and a lower abundance of *Firmicutes* and *Bacteroidetes* phyla (107). IBD-associated gut dysbiosis is accompanied by functional changes in the gut microbiota that affect its ability to ferment dietary fibers. The abundance of butyrate-producing bacteria, such as *Roseburia hominis* and *Faecalibacterium prausnitzii*, is decreased in IBD patients (11, 108, 109) and, therefore, fecal SCFAs levels are lower in IBD patients compared to controls (20). On

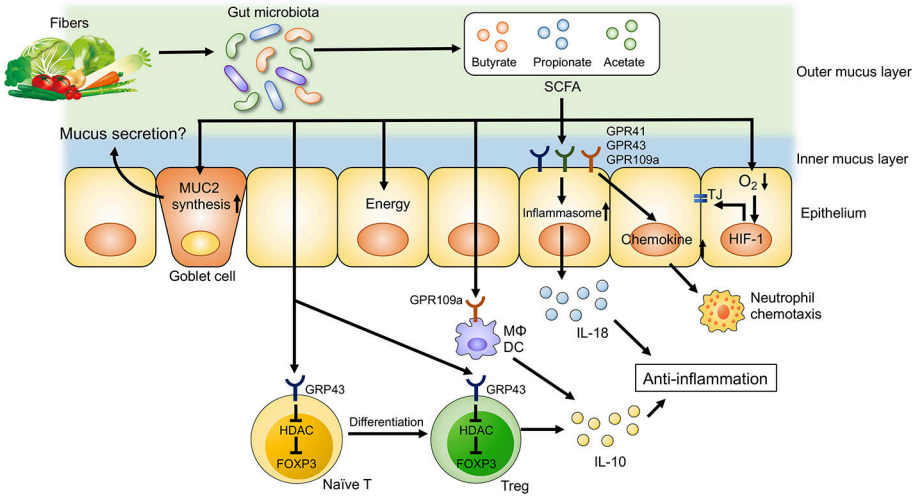

FIGURE 2 | Dietary fiber-derived SCFAs regulate intestinal homeostasis. Dietary fiber-derived SCFAs serve as energy substrates for colonocytes. Likewise, SCFAs regulate intestinal barrier function and immune system through GPCRs signaling. SCFAs promote the differentiation into Treg cells and the production of IL-10 from Treg cells through GPR43. SCFA facilitate inflammasome activation in colonic epithelial cells through GPR43, resulting in an IL-18 production that is critical for anti-inflammation and epithelial repair. SCFAs also regulate intestinal barrier function via enhancing the expression of tight junction proteins and the synthesis of MUC2.

the other hand, recent clinical trials did not demonstrate overt therapeutic benefits associated with butyrate enemas in UC patients (110, 111). These results suggest that IBD patients might have functional impairments involving butyrate utilization in addition to its impaired generation, likely due to gut dysbiosis. For instance, several studies have reported that butyrate oxidation and the expression of genes involved in butyrate oxidation are diminished in the intestinal mucosa of IBD patients (112–115), indicating that butyrate cannot be used properly in the inflamed gut. Moreover, butyrate uptake by colonocytes is impaired in IBD patients. Although butyrate uptake is mediated by monocarboxylate transporter 1 (MCT1), the transcription of *Mct1* is downregulated upon stimulation with IFN-γ and TNF-α, thereby reducing the uptake of butyrate (116). Consistently, expression levels of *Mct1* mRNA negatively correlate with the degree of intestinal inflammation in IBD (116).

Although numerous animal studies have reported that dietary fiber supplementation attenuates intestinal inflammation (117–119), only limited evidence is available from clinical trials involving IBD patients (120). Prebiotic fibers might promote the growth of protective members of the gut microbiota. However, there is no study that provides statistically significant evidence for the efficacy of prebiotic fibers as treatment for IBD (121). It implies that fiber treatment is not effective for active IBD. Regarding this notion, it has been reported that intestinal inflammation down-regulates carbohydrate metabolism in the gut microbiota (11, 25). Thus, it is possible that gut microbes are not able to utilize supplied fibers efficiently in the inflamed gut. Consistently, consumption of a high fiber diet does not attenuate colitis, while pretreatment with the same diet can prevent the development of colitis (119). Thus, metabolic activities of the microbiota, which might be altered as a result of disease, may determine the usefulness of administrating prebiotic fibers to IBD patients. In addition to disease status, the initial composition of the microbiota may influence the efficacy of dietary fiber treatment. For example, dietary fiber can improve postprandial glucose metabolism in healthy individuals, but they are divided into responders and non-responders (122). The abundance of *Prevotella*, a bacterial genus capable of digesting fibers, is higher in responders compared to non-responders. In other words, a high fiber diet is not beneficial in individuals who do not have sufficient numbers of fiber-degrading bacteria in their gut microbiota. Taken together, personalized nutritional management will likely be a key component of any successful therapeutic approach for IBD since intestinal inflammation results in both compositional and functional changes of the gut microbiota.

Elimination of Fermentable Carbohydrate

Several dietary therapies that eliminate specific carbohydrates have been developed and evaluated for induction of remission, maintenance and improvement of functional symptoms in IBD. Symptoms of IBD commonly include abdominal pain, discomfort, rectal bleeding, and a change in stool consistency and frequency during active periods. However, similar gastrointestinal symptoms can also be observed during periods of remission, even in the absence of clinical inflammation (123). These functional-like gastrointestinal symptoms are negatively correlated with the quality of life (QOL) experienced by IBD patients (124). Naturally, a more effective control of functional-like gastrointestinal symptoms is needed to improve the QOL of IBD patients. Dietary therapies for these functional-like gastrointestinal symptoms have recently been developed. These therapies include a low Fermentable Oligo-saccharides, Di-saccharides, Mono-saccharides And Polyols (FODMAP) diet and a Specific Carbohydrate Diet (SCD) (8). We will review the

effects of a low-FODMAP diet on IBD symptoms, because there is emerging clinical evidence suggesting the usefulness of this approach.

A diet low in FODMAP is used to manage symptoms in patients with irritable bowel syndrome (IBS) (125). A randomized, double-blind, placebo-controlled, cross-over study has revealed that the administration of fructans, unlike galacto-oligosaccharides or sorbitol, exacerbates functional-like gastrointestinal symptoms in quiescent IBD (126). A recent meta-analysis has shown that a low FODMAP diet can reduce gastrointestinal symptoms in patients with quiescent IBD (127). Although the precise mechanism by which FODMAPs promotes functional-like gastrointestinal symptoms is unclear, FODMAP intake is associated with increased luminal water content and colonic gas production (125). Whereas, most carbohydrates are completely digested and absorbed in the small intestine, some carbohydrates are not. Unabsorbed carbohydrates, such as fructose, polyols, and lactose, alter intestinal osmolality and increase water content in the small intestine. Other unabsorbed carbohydrates, including fructans and galacto-oligosaccharides, are fermented by the bacteria in the colon, leading to gas production. High levels of luminal water and gas result in luminal distension, thereby causing functional-like gastrointestinal symptoms. In an animal study, a high FODMAP diet increased intestinal permeability and altered the composition of microbial communities, leading to increased luminal levels of LPS (128). Inhibition of Toll-like receptor (TLR) activation by LPS ameliorates intestinal barrier function and visceral nociception. Moreover, since fecal LPS is higher in IBS patients compared to healthy subjects, a low-FODMAP diet improves IBS symptoms by reducing the levels of fecal LPS. Dietary intervention is an exciting topic in the current IBD research. Further studies are needed to unravel the mechanism of action and the long-term effects of a low FODMAP diet.

FATTY ACIDS

The Western diet is characterized by high intake of fats. There is evidence that links increased fat intake to the pathogenesis of IBD. Dietary fat comprises saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), and polyunsaturated fatty acids (PUFAs). Most fatty acids, except for linoleic acid and alpha-linolenic acid which are essential fatty acids, can synthesize in the body. Fatty acids are important energy sources and membrane constituents, and their biological activities influence both the immune system and the gut microbiota. A number of studies have revealed that high-fat diets change the composition of the gut microbiota and impair intestinal barrier function, resulting in intestinal and systemic inflammation (129–131). Furthermore, consumption of a diet containing high levels of fat and sugar promotes the colonization of adherent-invasive *E. coli* (AIEC), an IBD-associated pathobiont (132). A recent systematic literature review of epidemiological data has shown that high dietary intakes of total fats, SFAs, omega-6 (n-6) and PUFAs are associated with an increased risk of both CD and UC (133). Similar to amino acids and carbohydrates, abnormal fatty acid profiles and metabolism are observed in the blood and intestinal mucosa of IBD patients (134). On the other hand, dietary supplementation with specific fatty acids can improve perturbed fatty acids profiles and intestinal inflammation (135). We summarize the roles of fatty acids in the pathogenesis and treatment of IBD.

Saturated Fatty Acids (SFAs)

SFAs, mainly myristic acid, palmitic acid, and stearic acid, are found in animal fat-containing products, such as meat, butter, whole milk, and other dairy products. Although a connection between saturated fats and the risk of IBD has been uncovered by small case–control studies, prospective cohorts have not identified a statistical association, suggesting the relationship is more complex. In animal studies, a diet high in SFAs induces colonic inflammation, including increased expression of pro-inflammatory cytokines and enhanced intestinal permeability in a microbiota- or TLR4-dependent manner (129–131). Similarly, a diet high in SFAs exacerbates intestinal inflammation in experimental models of colitis (130, 136). A milk-derived diet high in saturated fats, unlike a diet high in polyunsaturated fats, promotes the expansion of a sulphite-reducing pathobiont, *Bilophila wadsworthia*, and aggravates Th1-mediated colitis in IL-10 deficient mice (136). Milk-derived fat promotes taurine conjugation of hepatic bile acids, thereby leading to the accumulation of sulfur that aids the growth of *B. wadsworthia*.

In a murine model of DSS-induced colitis, the level of a saturated fat 1-stearoyl-sn-glycero-3-phosphorylcholine (stearoyl-LPC) in the blood is increased compared to control mice, whereas the unsaturated fat 1-oleoyl-sn-glycero-3-phosphorylcholine (oleoyl-LPC) is decreased following DSS treatment. Thus, intestinal inflammation shifts the balance between saturated LPCs and monounsaturated LPCs (135). The perturbation of the lipid serum profile is caused by decreased expression of stearoyl-CoA desaturase 1 (SCD1) in the liver. SCD1 is an enzyme found in the endoplasmic reticulum. It is responsible for the biosynthesis of oleic acid (18:1) and palmitoleic acid (16:1) from stearic acid (18:0), and palmitic acid (16:0), respectively (137). Saturated LPCs, unlike their monounsaturated counterparts, increase IL-1β production in human monocytes (138). Consistently, SCD1-deficient mice are more susceptible to DSS and exacerbated colitis can be ameliorated by means of oleic acid supplementation (135).

Monounsaturated Fatty Acids (MUFAs)

MUFAs are classified as fatty acids containing a single double bond. Oleic acid, the most common omega-9 MUFA, is found in various vegetable oils, such as olive oil and canola oil. The levels of MUFAs, including oleic acid, are significantly reduced in IBD patients compared to control subjects, both systemically (blood) and locally (intestinal mucosa) (139, 140). Epidemiologically, the Mediterranean diet containing high levels of MUFAs, especially oleic acid, is well-recognized for its beneficial effects on metabolic syndrome and cardiovascular health (141). The effects of MUFAs on IBD remain unresolved. There are conflicting reports regarding the therapeutic potential of MUFAs in IBD. A large prospective cohort study has demonstrated that dietary

oleic acid intake is inversely associated with UC development (142). In contrast, other studies have shown that a high intake of MUFAs increases the risk for the development of UC and CD. Several animal studies have consistently demonstrated that supplementation of oleic acid or olive oil attenuates intestinal inflammation in DSS-induced colitis (135, 143).

Polyunsaturated Fatty Acids (PUFAs)

PUFAs are classified into omega-3 (n-3) and n-6 families. These two families are thought to have different effects on inflammation. Conventionally, n-6 PUFAs are considered to be pro-inflammatory. Linoleic acid, the major PUFA in the diet, is converted to arachidonic acid, a precursor of inflammatory mediators such as prostaglandins and leukotrienes (134). N-6 PUFAs are incorporated into phospholipids found in cellular membranes. Multiple members of the phospholipase family of enzymes, such as phospholipase A2 (PLA2), hydrolyze n-6 PUFAs during inflammation. Arachidonic acid is a primary target for PLA2 and is converted to inflammatory mediators involved in pro-inflammatory cell signaling events. The levels of PLA2 are elevated in the mucosal biopsies of CD and UC patients (144), resulting in increased hydrolysis of arachidonic acid and the subsequent generation of inflammatory mediators. In contrast, n-3 PUFAs appear to be critical for the suppression of inflammation. α-linolenic acid, an essential n-3 PUFA, is a precursor of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), both of which are contained in fish oils. N-3 PUFAs compete with n-6 PUFAs in the substrate pool of the lipoxygenase pathway, inhibiting the production of inflammatory mediators downstream of n-6 PUFAs (134). Likewise, pro-resolving mediators are generated from n-3 fatty acids via several enzymatic reactions (145). Resolvin E1 (RvE1) is an anti-inflammatory lipid mediator derived from EPA through aspirin-acetylated cyclooxygenase-2 (COX2) and microbial cytochrome P450 (146, 147). RvE1 inhibits leukocyte infiltration and IL-12 production by dendritic cells and macrophages (148, 149). Consistent with the anti-inflammatory effects on immune cells, the administration of RvE1 attenuates TNBS- and DSS-induced colitis in mice (146, 149). Moreover, n-3 PUFAs can directly inhibit the TLR4 and NF-κB signaling pathways, leading to the down-regulation of pro-inflammatory genes, such as COX2 and TNF-α (150, 151). Thus, an appropriate balance of n-6/n-3 PUFAs is important for the regulation of inflammation.

A Western diet typically contains high levels of n-6 PUFAs and low levels of n-3 PUFAs (152). The skewed ratio between the intake of n-6 PUFAs and n-3 PUFAs is considered a risk factor for IBD (133). Although previous studies have examined the effect of n-3 fatty acid supplementation in prevention and treatment of IBD, the outcomes were inconsistent in both human IBD and animal models of colitis. In the animal studies, supplementation with n-3 fatty acids found in fish oil attenuated intestinal inflammation (153, 154), whereas another study showed that n-3 fatty acids exacerbated colitis (155). This disparity may have been caused by various confounding factors, such as control diet, administration period and sensitivity to oxidation. Another report demonstrated that fat-1 transgenic mice are protected against DSS-induced colitis (156). Since fat-1 transgenic mice have increased endogenous levels of n-3 PUFAs, the protective phenotype observed in this mouse strain may be due to n-3 PUFAs (156). In human IBD, a systematic review does not support the notion that supplementation of n-3 fatty acids can induce and maintain remission of IBD (157). The latest Cochrane review has reached a similar conclusion (158). Interestingly, several studies have demonstrated that different genotypes may be associated with the variable response to nutritional intervention with n-3 PUFAs. For example, genetic polymorphisms of TNF-α and PPARα have been associated with an altered response to nutritional intervention with n-3 PUFAs (159, 160). In IBD, genetic polymorphisms, such as CYP4F3 and Caspase 9+93C/T, modify the association between dietary fatty acid intake and risk of IBD (161, 162). Since fatty acid utilization may vary depending on the genetic background, personalized nutritional management is likely required to maximize the efficacy of n-3 PUFAs supplementation in IBD.

PHYTOCHEMICALS

Phytochemicals are a wide range of natural compounds found in plants. Accumulating evidence suggest that phytochemicals have beneficial effect on several chronic diseases (163). A number of polyphenols, including flavonoids, phenolic acids, stilbenes, and lignans, have been shown to influence the gut microbiota and intestinal immunity. For example, curcumin, a hydrophobic polyphenol derived from *Curcuma longa*, displays a wide variety of biological functions, such as anti-inflammatory, anti-cancer, and anti-oxidant activities (164). Curcumin suppresses cytokine production by macrophages and intestinal epithelial cells via the inhibition of NF-κB activation (165, 166), and thus administration of curcumin ameliorates DSS- and TNBS-induced colitis (167, 168). Polyphenols including curcumin are not efficiently absorbed from the small intestine, and therefore polyphenol complexes contained in diet have limited bioavailability (169). Given this evidence, a large portion of unabsorbed polyphenols are likely transported to the large intestine where they interact with the colonic gut microbiota. Of note, the gut resident bacteria are capable of catabolizing polyphenols and degrading them into small fragments (170). For instance, curcumin is converted into tetrahydrocurcumin by curcumin-converting enzyme purified from *E. coli* (171). Curcumin-derived tetrahydrocurcumin can prevent colitis and colitis-associated colon cancer in mice (167, 172). Recent epidemiological study reported a potential association of polyphenol in CD (173). Moreover, clinical trials show that curcumin supplementation is effective for the induction and maintenance of remission in UC patients (174, 175).

FOOD ADDITIVES

Recent widespread use of processed food increases consumption of food additives. Emulsifiers are complex molecules that are incorporated into most processed foods to enhance texture and stability. It has been implied that the increased consumption of emulsifier is associated with IBD pathogenesis

(176). In recent studies, the consumption of dietary emulsifier disrupted host-microbiota interaction and promoted intestinal inflammation and colon carcinogenesis (177, 178). Although precise mechanism remains incompletely understood, dietary emulsifiers foster the blooms of mucolytic bacteria, such as *Ruminococcus gnavus* and *Akkermansia muciniphila* (177). Importantly, the negative impact of dietary emulsifiers is not observed in GF mice, suggesting that the compositional and functional modulation of the gut microbiota by emulsifiers play a key role in their adverse effect (177). Other food additives, for example artificial sweeteners, also induce gut dysbiosis (179, 180). The consumption of artificial sweeteners promotes the expansion of *Proteobacteria* and increases the infiltration of bacteria into the ileal lamina propria in CD-like ileitis model mice (180). Moreover, dietary phosphate, the main component of many food additives, enhances intestinal inflammation through the activation of NF-κB in macrophages (181). These studies suggest that many food additives may be associated with the risk of IBD. In fact, epidemiological and clinical studies reported that processed meats and beverages are risk factors for the onset and relapse of IBD (182, 183). However, it is difficult to ascertain whether food additives are risk factors for developing IBD, because most food frequency questionnaires cannot fully evaluate the consumption of food additives.

CONCLUSION AND FUTURE DIRECTION

The relationship between dietary nutrients and intestinal homeostasis is complex. It is influenced by a myriad of interactions between host immunity, the intestinal epithelium and the gut microbiota. Moreover, intestinal inflammation changes cellular and microbial metabolisms, adding another layer of complexity to an already complex system (**Table 2**). The demand for certain nutrients necessary for the maintenance of intestinal homeostasis is likely altered in IBD. The scarcity or overabundance of some nutrients can disturb intestinal homeostasis even further, thus exacerbating the disease. Genetic polymorphisms also influence the efficacy of nutritional intervention. Thus, an in-depth knowledge of a patient's genetic predispositions, gut microbial compositions, disease activity

TABLE 2 | Changes of dietary nutrient availavility in the host and gut microbiota of IBD.

Host or gut microbiota	Nutrient availability	Mediator	IBD	Related nutrient	References
Host	Metabolic enzyme	IDO1	UCa, CDa > UCia, CDia, Control	Tryptophan	(37)
		Arginase II, iNOS	UCa > UCia, Control	Arginine	(51)
		ACSM3, ACADS, ECHS1	UC, CD > Control	SCFA	(113, 115)
	Transporter	MCT1 (SLC16A1)	UC, CD < Control	SCFA	(116)
		B0AT1 (SLC6A19)	UCa, CDa < UCia, CDia, Control	Tryptophan, Neutral amino acid	(37)
		CAT2 (SLC7A2)	UCa < Control	Arginine, Cationic amino acids	(51)
	SNP	CARD9	UC, CD	Tryprophan	(49)
		CYP4F3	UC	Fatty acids	(162)
		Caspase 9	CD	Fatty acids	(161)
		PPAR-γ	CD	Fatty acids	(161)
		Fas ligand	CD	Fatty acids	(161)
Gut microbiota	Microbial composition	Butyrate producung bacteria	UC, CDa < Control	Fiber, SCFA	(25, 108, 109)
	Metabolic pathway	Carbohydrate metabolism	CDi > Control	Carbohydrates	(11)
			UC Inflamed < UC normal	Carbohydrates	(25)
		Amino acid metabolism	UC Inflamed > UC normal	Amino acids	(25)
		Lysine/Arginine biosynthesis	UC, CD < Control	Amino acids	(11)
		Lipid metabolism	CDi < Control	Fatty acids	(11)
			UC Inflamed > UC normal	Fatty acids	(25)
		Butyrate and propionate metabolism	CDi < Control	SCFA	(11)
	Transport system	Carbohydrate transport	CDi > Control	Amino acids	(11)
		Lysine/arginine/ornithine transport	UC, CD > Control	Amino acids	(11)

UCa, active ulcerative colitis; UCia, inactive ulcerative colitis; CDa, active Crohn's disease; CDia, inactive Crohn's disease; CDi, CD with ileal; UC inflamed, tissues of inflamed lesion from UC; UC tissues of normal lesion from UC; Normal DO1, Indoleamine 2,3 dioxygenase-1; iNOS, inducible nitric oxide synthase; ACSM3, acyl-CoA synthetase medium chain family member 3; ACADS, Acyl-CoA dehydrogenase; ECHS1, enoyl-CoA hydratase, short chain 1; MCT1, monocarboxylic acid transporter 1; B0AT1, systemB(0) neutral amino acid transporter 1; CAT2, cationic amino acid transporter 2; CARD9, caspase recruitment domain 9; PPAR-γ, Peroxisome Proliferator-Activated Receptor gamma.

and type (i.e., ileal, colonic, etc.) will be required to maximize the effectiveness of nutritional intervention approaches in IBD.

AUTHOR CONTRIBUTIONS

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

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REFERENCES

1. Cosnes J, Gowerrousseau C, Seksik P, Cortot A. Epidemiology and natural history of inflammatory bowel diseases. *Gastroenterology* (2011) 140:1785–94. doi: 10.1053/j.gastro.2011.01.055
2. Ananthakrishnan AN. Epidemiology and risk factors for IBD. *Nat Rev Gastroenterol Hepatol.* (2015) 12:205–17. doi: 10.1038/nrgastro.2015.34
3. Loddo I, Romano C. Inflammatory bowel disease: genetics, epigenetics, and pathogenesis. *Front Immunol.* (2015) 6:6–11. doi: 10.3389/fimmu.2015.00551
4. Jostins L, Ripke S, Weersma RK, Duerr RH, McGovern DP, Hui KY, et al. Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. *Nature* (2012) 491:119–24. doi: 10.1038/nature11582
5. Molodecky NA, Soon IS, Rabi DM, Ghali WA, Ferris M, Chernoff G, et al. Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. *Gastroenterology* (2012) 142:46–54.e42. doi: 10.1053/j.gastro.2011.10.001
6. Lee D, Albenberg L, Compher C, Baldassano R, Piccoli D, Lewis JD, et al. Diet in the pathogenesis and treatment of inflammatory bowel diseases. *Gastroenterology* (2015) 148:1087–106. doi: 10.1053/j.gastro.2015.01.007
7. Spooren CEGM, Pierik MJ, Zeegers MP, Feskens EJM, Masclee AAM, Jonkers DMAE. Review article: the association of diet with onset and relapse in patients with inflammatory bowel disease. *Aliment Pharmacol Ther.* (2013) 38:1172–87. doi: 10.1111/apt.12501
8. Sigall-Boneh R, Levine A, Lomer M, Wierdsma N, Allan P, Fiorino G, et al. Research gaps in diet and nutrition in inflammatory bowel disease. a topical review by D-ECCO Working Group (Dietitians of ECCO). *J Crohns Colitis.* (2017) 1407–19. doi: 10.1093/ecco-jcc/jjx109
9. Kolho KL, Pessia A, Jaakkola T, de Vos WM, Velagapudi V. Faecal and serum metabolomics in paediatric inflammatory bowel disease. *J Crohns Colitis.* (2017) 11:321–34. doi: 10.1093/ecco-jcc/jjw158
10. Kohashi M, Nishiumi S, Ooi M, Yoshie T, Matsubara A, Suzuki M, et al. A novel gas chromatography mass spectrometry-based serum diagnostic and assessment approach to ulcerative colitis. *J Crohns Colitis.* (2014) 8:1010–21. doi: 10.1016/j.crohns.2014.01.024
11. Morgan X, Tickle T, Sokol H, Gevers D, Devaney K, Ward D. Dysfunction of the intestinal microbiome in inflammatory bowel disease and treatment. *Genome Biol.* (2012) 13:R79. doi: 10.1186/gb-2012-13-9-r79
12. Scoville EA, Allaman MM, Brown CT, Motley AK, Horst SN, Williams CS, et al. Alterations in lipid, amino acid, and energy metabolism distinguish Crohn's disease from ulcerative colitis and control subjects by serum metabolomic profiling. *Metabolomics* (2018) 14:1–12. doi: 10.1007/s11306-017-1311-y
13. Schicho R, Shaykhutdinov R, Ngo J, Nazyrova A, Schneider C, Panaccione R, et al. Quantitative metabolomic profiling of serum, plasma, and urine by 1H NMR spectroscopy discriminates between patients with inflammatory bowel disease and healthy individuals. *J Proteome Res.* (2012) 11:3344–57. doi: 10.1021/pr300139q
14. Dawiskiba T, Deja S, Mulak A, Zabek A, Jawien E, Pawełka D, et al. Serum and urine metabolomic fngerprinting in diagnostics of inflammatory bowel diseases. *World J Gastroenterol.* (2014) 20:163–74. doi: 10.3748/wjg.v20.i1.163
15. Ooi M, Nishiumi S, Yoshie T, Shiomi Y, Kohashi M, Fukunaga K, et al. GC/MS-based profiling of amino acids and TCA cycle-related molecules in ulcerative colitis. *Inflamm Res.* (2011) 60:831–40. doi: 10.1007/s00011-011-0340-7
16. Hisamatsu T, Okamoto S, Hashimoto M, Muramatsu T, Andou A, Uo M, et al. Novel, objective, multivariate biomarkers composed of plasma amino acid profiles for the diagnosis and assessment of inflammatory bowel disease. *PLoS ONE* (2012) 7:e31131. doi: 10.1371/journal.pone.0031131
17. Martin FP, Su MM, Xie GX, Guiraud SP, Kussmann M, Godin JP, et al. Urinary metabolic insights into host-gut microbial interactions in healthy and IBD children. *World J Gastroenterol.* (2017) 23:3643–54. doi: 10.3748/wjg.v23.i20.3643
18. Stephens NS, Siffledeen J, Su X, Murdoch TB, Fedorak RN, Slupsky CM. Urinary NMR metabolomic profiles discriminate inflammatory bowel disease from healthy. *J Crohns Colitis.* (2013) 7:e42–8. doi: 10.1016/j.crohns.2012.04.019
19. Bosch S, Struys EA, Van Gaal N, Bakkali A, Jansen EW, Diederen K, et al. Fecal amino acid analysis can discriminate de novo treatment-naïve pediatric inflammatory bowel disease from controls. *J Pediatr Gastroenterol Nutr.* (2018) 66:773–8. doi: 10.1097/MPG.0000000000001812
20. Marchesi JR, Holmes E, Khan F, Kochhar S, Scanlan P, Shanahan F, et al. Rapid and noninvasive metabonomic characterization of inflammatory bowel disease. *J Proteome Res.* (2007) 6:546–51. doi: 10.1021/pr060470d
21. Bjerrum JT, Wang Y, Hao F, Coskun M, Ludwig C, Günther U, et al. Metabonomics of human fecal extracts characterize ulcerative colitis, Crohn's disease and healthy individuals. *Metabolomics* (2014) 11:122–33. doi: 10.1007/s11306-014-0677-3
22. Santoru ML, Piras C, Murgia A, Palmas V, Camboni T, Liggi S, et al. Cross sectional evaluation of the gut-microbiome metabolome axis in an Italian cohort of IBD patients. *Sci Rep.* (2017) 25:9523. doi: 10.1038/s41598-017-10034-5
23. Balasubramanian K, Kumar S, Singh R, Sharma U, Ahuja V, Makharia G, et al. Metabolism of the colonic mucosa in patients with inflammatory bowel diseases: an *in vitro* proton magnetic resonance spectroscopy study. *Magn Reson Imag.* (2009) 27:79–86. doi: 10.1016/j.mri.2008.05.014
24. Bjerrum JT, Nielsen OH, Hao F, Tang H, Nicholson JK, Wang Y, et al. Metabonomics in ulcerative colitis: diagnostics, biomarker identification, and insight into the pathophysiology. *J Proteome Res.* (2010) 9:954–62. doi: 10.1021/pr9008223
25. Davenport M, Poles J, Leung JM, Wolff MJ, Abidi WM, Ullman T, et al. Metabolic alterations to the mucosal microbiota in inflammatory bowel disease. *Inflamm Bowel Dis.* (2014) 20:723–31. doi: 10.1097/MIB.0000000000000011
26. Ren W, Liu G, Yin J, Tan B, Wu G, Bazer FW, et al. Amino-acid transporters in T-cell activation and differentiation. *Cell Death Dis.* (2017) 8:1–9. doi: 10.1038/cddis.2016.222
27. Langston PK, Shibata M, Horng T. Metabolism supports macrophage activation. *Front Immunol.* (2017) 8:61. doi: 10.3389/fimmu.2017.00061
28. Palego L, Betti L, Rossi A, Giannaccini G. Tryptophan biochemistry: structural, nutritional, metabolic, and medical aspects in humans. *J Amino Acids* (2016) 2016:1–13. doi: 10.1155/2016/8952520
29. Lee JS, Cella M, McDonald KG, Garlanda C, Kennedy GD, Nukaya M, et al. AHR drives the development of gut ILC22 cells and postnatal lymphoid tissues via pathways dependent on and independent of Notch. *Nat Immunol.* (2012) 13:144–52. doi: 10.1038/ni.2187

30. Mezrich JD, Fechner JH, Zhang X, Johnson BP, Burlingham WJ, Bradfield CA. An interaction between Kynurenine and the Aryl Hydrocarbon receptor can generate regulatory T cells. *J Immunol.* (2010) 185:3190–8. doi: 10.4049/jimmunol.0903670
31. Hashimoto T, Perlot T, Rehman A, Trichereau J, Ishiguro H, Paolino M, et al. ACE2 links amino acid malnutrition to microbial ecology and intestinal inflammation. *Nature* (2012) 487:477–81. doi: 10.1038/nature11228
32. Islam J, Sato S, Watanabe K, Watanabe T, Ardiansyah, Hirahara K, et al. Dietary tryptophan alleviates dextran sodium sulfate-induced colitis through aryl hydrocarbon receptor in mice. *J Nutr Biochem.* (2017) 42:43–50. doi: 10.1016/j.jnutbio.2016.12.019
33. Kim CJ, Kovacs-Nolan JA, Yang C, Archbold T, Fan MZ, Mine Y. L-Tryptophan exhibits therapeutic function in a porcine model of dextran sodium sulfate (DSS)-induced colitis. *J Nutr Biochem.* (2010) 21:468–75. doi: 10.1016/j.jnutbio.2009.01.019
34. Bettenworth D, Nowacki TM, Ross M, Kyme P, Schwammbach D, Kerstiens L, et al. Nicotinamide treatment ameliorates the course of experimental colitis mediated by enhanced neutrophil-specific antibacterial clearance. *Mol Nutr Food Res.* (2014) 58:1474–90. doi: 10.1002/mnfr.201300818
35. Lanis JM, Alexeev EE, Curtis VF, Kitzenberg DA, Kao DJ, Battista KD, et al. Tryptophan metabolite activation of the aryl hydrocarbon receptor regulates IL-10 receptor expression on intestinal epithelia. *Mucosal Immunol.* (2017) 10:1133–44. doi: 10.1038/mi.2016.133
36. El-Zaatari M, Bass AJ, Bowlby R, Zhang M, Syu LJ, Yang Y, et al. Indoleamine 2,3-Dioxygenase 1, increased in human gastric pre-neoplasia, promotes inflammation and metaplasia in mice and is associated with type II hypersensitivity/autoimmunity. *Gastroenterology* (2018) 154:140–53.e17. doi: 10.1053/j.gastro.2017.09.002
37. Nikolaus S, Schulte B, Al-Massad N, Thieme F, Schulte DM, Bethge J, et al. Increased Tryptophan metabolism is associated with activity of inflammatory bowel diseases. *Gastroenterology* (2017) 153:1504–16.e2. doi: 10.1053/j.gastro.2017.08.028
38. Gurtner GJ, Newberry RD, Schloemann SR, McDonald KG, Stenson WF. Inhibition of Indoleamine 2,3-Dioxygenase augments trinitrobenzene sulfonic acid colitis in mice. *Gastroenterology* (2003) 125:1762–73. doi: 10.1053/j.gastro.2003.08.031
39. Takamatsu M, Hirata A, Ohtaki H, Hoshi M, Hatano Y, Tomita H, et al. IDO1 plays an immunosuppressive role in 2,4,6-Trinitrobenzene sulfate-induced colitis in mice. *J Immunol.* (2013) 191:3057–64. doi: 10.4049/jimmunol.1203306
40. Jasperson LK, Bucher C, Panoskaltsis-Mortari A, Taylor PA, Mellor AL, Munn DH, et al. Indoleamine 2,3-dioxygenase is a critical regulator of acute graft-versus-host disease lethality. *Blood* (2008) 111:3257–65. doi: 10.1182/blood-2007-06-096081
41. Harrington L, Srikanth CV, Antony R, Rhee SJ, Mellor AL, Hai NS, et al. Deficiency of indoleamine 2,3-dioxygenase enhances commensal-induced antibody responses and protects against Citrobacter rodentium-induced colitis. *Infect Immun.* (2008) 76:3045–53. doi: 10.1128/IAI.00193-08
42. Quintana FJ, Basso AS, Iglesias AH, Korn T, Farez MF, Bettelli E, et al. Control of Treg and TH17 cell differentiation by the aryl hydrocarbon receptor. *Nature* (2008) 453:65–71. doi: 10.1038/nature06880
43. Quiros M, Nishio H, Neumann PA, Siuda D, Brazil JC, Azcutia V, et al. Macrophage-derived IL-10 mediates mucosal repair by epithelial WISP-1 signaling. *J Clin Invest.* (2017) 127:3510–20. doi: 10.1172/JCI 90229
44. Miyajima M, Zhang B, Sugiura Y, Sonomura K, Guerrini MM, Tsutsui Y, et al. Metabolic shift induced by systemic activation of T cells in PD-1-deficient mice perturbs brain monoamines and emotional behavior. *Nat Immunol.* (2017) 18:1342–52. doi: 10.1038/ni.3867
45. Bauer EP. Serotonin in fear conditioning processes. *Behav Brain Res.* (2015) 277:68–77. doi: 10.1016/j.bbr.2014.07.028
46. Selinger CP, Bannaga A. Inflammatory bowel disease and anxiety: links, risks, and challenges faced. *Clin Exp Gastroenterol.* (2015) 8:111–7. doi: 10.2147/CEG.S57982
47. Zelante T, Iannitti RG, Cunha C, DeLuca A, Giovannini G, Pieraccini G, et al. Tryptophan catabolites from microbiota engage aryl hydrocarbon receptor and balance mucosal reactivity via interleukin-22. *Immunity* (2013) 39:372–85. doi: 10.1016/j.immuni.2013.08.003
48. Dudakov JA, Hanash AM, van den Brink MRM. Interleukin-22: immunobiology and pathology. *Annu Rev Immunol.* (2015) 33:747–85. doi: 10.1146/annurev-immunol-032414-112123
49. Lamas B, Richard ML, Leducq V, Pham HP, Michel ML, Da Costa G, et al. CARD9 impacts colitis by altering gut microbiota metabolism of tryptophan into aryl hydrocarbon receptor ligands. *Nat Med.* (2016) 22:598–605. doi: 10.1038/nm.4102
50. Patel VB, Preedy VR, Rajendram R. *L -Arginine in Clinical Nutrition*. New York, NY: Humana Press (2017).
51. Coburn LA, Horst SN, Allaman MM, Brown CT, Williams CS, Hodges ME, et al. L -Arginine availability and metabolism is altered in ulcerative colitis. *Inflamm Bowel Dis.* (2016) 22:1847–58. doi: 10.1097/MIB.0000000000000790
52. Coburn LA, Gong X, Singh K, Asim M, Scull BP, Allaman MM, et al. L-arginine supplementation improves responses to injury and inflammation in dextran sulfate sodium colitis. *PLoS ONE* (2012) 7:e33546. doi: 10.1371/journal.pone.0033546
53. Singh K, Coburn LA, Barry DP, Asim M, Scull BP, Allaman MM, et al. Deletion of cationic amino acid transporter 2 exacerbates dextran sulfate sodium colitis and leads to an IL-17-predominant T cell response. *AJP Gastrointest Liver Physiol.* (2013) 305:G225–40. doi: 10.1152/ajpgi.00091.2013
54. Singh K, Coburn LA, Barry DP, Boucher J-L, Chaturvedi R, Wilson KT. L-arginine uptake by cationic amino acid transporter 2 is essential for colonic epithelial cell restitution. *AJP Gastrointest Liver Physiol.* (2012) 302:G1061–73. doi: 10.1152/ajpgi.00544.2011
55. Geiger R, Rieckmann JC, Wolf T, Basso C, Feng Y, Fuhrer T, et al. L-Arginine modulates T cell metabolism and enhances survival and anti-tumor activity. *Cell* (2016) 167:829–42.e13. doi: 10.1016/j.cell.2016.09.031
56. Fletcher M, Ramirez ME, Sierra RA, Raber P, Thevenot P, Al-Khami AA, et al. L-Arginine depletion blunts antitumor T-cell responses by inducing myeloid-derived suppressor cells. *Cancer Res.* (2015) 75:275–83. doi: 10.1158/0008-5472.CAN-14-1491
57. Rodriguez PC, Quiceno DG, Zabaleta J, Ortiz B, Zea AH, Piazuelo MB, et al. Arginase I production in the tumor microenvironment by mature myeloid cells inhibits T-cell receptor expression and antigen-specific T-cell responses Arginase I production in the tumor microenvironment by mature myeloid cells inhibits T-cell responses. *Cancer Res.* (2004) 64:5839–49. doi: 10.1158/0008-5472.CAN-04-0465
58. Rodriguez PC, Zea AH, DeSalvo J, Culotta KS, Zabaleta J, Quiceno DG, et al. L-Arginine consumption by macrophages modulates the expression of CD3 chain in T lymphocytes. *J Immunol.* (2003) 171:1232–9. doi: 10.4049/jimmunol.171.3.1232
59. Tarasenko TN, Gomez-Rodriguez J, McGuire PJ. Impaired T cell function in argininosuccinate synthetase deficiency. *J Leukoc Biol.* (2015) 97:273–8. doi: 10.1189/jlb.1AB0714-365R
60. Hesse M, Modolell M, La Flamme AC, Schito M, Fuentes JM, Cheever AW, et al. Differential regulation of nitric oxide Synthase-2 and Arginase-1 by Type 1/Type 2 cytokines *in vivo*: granulomatous pathology is shaped by the pattern of L-Arginine metabolism. *J Immunol.* (2001) 167:6533–44. doi: 10.4049/jimmunol.167.11.6533
61. Munder M, Eichmann K, Modolell M. Alternative metabolic states in murine macrophages reflected by the nitric oxide synthase/arginase balance: competitive regulation by CD4+ T cells correlates with Th1/Th2 phenotype. *J Immunol.* (1998) 160:5347–54.
62. MacMicking J, Xie QW, Nathan C. Nitric oxide and macrophage function. *Annu Rev Immunol.* (1997) 15:323–50. doi: 10.1146/annurev.immunol.15.1.323
63. Campbell L, Saville CR, Murray PJ, Cruickshank SM, Hardman MJ. Local arginase 1 activity is required for cutaneous wound healing. *J Invest Dermatol.* (2013) 133:2461–70. doi: 10.1038/jid.2013.164
64. Hankard R, Mauras N, Hammond D, Haymond M, Darmaun D. Is glutamine a "conditionally essential" amino acid in Duchenne muscular dystrophy? *Clin Nutr.* (1999) 18:365–9. doi: 10.1054/clnu.1999.0054
65. Sayles C, Hickerson SC, Bhat RR, Hall J, Garey KW, Trivedi MV. Oral glutamine in preventing treatment-related mucositis in adult patients with cancer: a systematic review. *Nutr Clin Pract.* (2016) 31:171–9. doi: 10.1177/0884533615611857

66. Carr EL, Kelman A, Wu GS, Gopaul R, Senkevitch E, Aghvanyan A, et al. Glutamine uptake and metabolism are coordinately regulated by ERK/MAPK during T lymphocyte activation. *J Immunol.* (2010) 185:1037–44. doi: 10.4049/jimmunol.0903586
67. Wang R, Dillon CP, Shi LZ, Milasta S, Carter R, Finkelstein D, et al. The transcription factor myc controls metabolic reprogramming upon T lymphocyte activation. *Immunity* (2011) 35:871–82. doi: 10.1016/j.immuni.2011.09.021
68. Sido B, Seel C, Hochlehnert A, Breitkreutz R, Droge W. Low intestinal glutamine level and low glutaminase activity in Crohn's disease: a rational for glutamine supplementation? *Dig Dis Sci.* (2006) 51:2170–9. doi: 10.1007/s10620-006-9473-x
69. Shiomi Y, Nishiumi S, Ooi M, Hatano N, Shinohara M, Yoshie T, et al. GCMS-based metabolomic study in mice with colitis induced by dextran sulfate sodium. *Inflamm Bowel Dis.* (2011) 17:2261–74. doi: 10.1002/ibd.21616
70. Vicario M, Amat C, Rivero M, Moretó M, Pelegrí C. Dietary glutamine affects mucosal functions in rats with mild DSS-induced colitis. *J Nutr.* (2007) 137:1931–7. doi: 10.1093/jn/137.8.1931
71. Crespo I, San-Miguel B, Prause C, Marroni N, Cuevas MJ, González-Gallego J, et al. Glutamine treatment attenuates endoplasmic reticulum stress and apoptosis in TNBS-induced colitis. *PLoS ONE* (2012) 7:e50407. doi: 10.1371/journal.pone.0050407
72. Kretzmann NA, Fillmann H, Mauriz JL, Marroni CA, Marroni N, González-Gallego J, et al. Effects of glutamine on proinflammatory gene expression and activation of nuclear factor Kappa B and signal transducers and activators of transcription in TNBS-induced colitis. *Inflamm Bowel Dis.* (2008) 14:1504–13. doi: 10.1002/ibd.20543
73. Tian Y, Wang K, Wang Z, Li N, Ji G. Chemopreventive effect of dietary glutamine on colitis-associated colon tumorigenesis in mice. *Carcinogenesis* (2013) 34:1593–600. doi: 10.1093/carcin/bgt088
74. Suzuki T. Regulation of intestinal epithelial permeability by tight junctions. *Cell Mol Life Sci.* (2013) 70:631–59. doi: 10.1007/s00018-012-1070-x
75. Seth A. L-Glutamine ameliorates acetaldehyde-induced increase in paracellular permeability in Caco-2 cell monolayer. *AJP Gastrointest Liver Physiol.* (2004) 287:G510–7. doi: 10.1152/ajpgi.00058.2004
76. Li N. Glutamine regulates Caco-2 cell tight junction proteins. *AJP Gastrointest Liver Physiol.* (2004) 287:G726–33. doi: 10.1152/ajpgi.00012.2004
77. Li N, Neu J. Glutamine deprivation alters intestinal tight junctions via a PI3-K/Akt mediated pathway in Caco-2 cells 1,2. *J Nutr.* (2009) 139:710–4. doi: 10.3945/jn.108.101485
78. Moore SR, Guedes MM, Costa TB, Vallance J, Maier EA, Betz KJ, et al. Glutamine and alanyl-glutamine promote crypt expansion and mTOR signaling in murine enteroids. *Am J Physiol Liver Physiol.* (2015) 15:831–9. doi: 10.1152/ajpgi.00422.2014
79. Ockenga J, Borchert K, Stüber E, Lochs H, Manns MP, Bischoff SC. Glutamine-enriched total parenteral nutrition in patients with inflammatory bowel disease. *Eur J Clin Nutr.* (2005) 59:1302–9. doi: 10.1038/sj.ejcn.1602243
80. Akobeng AK, Miller V, Stanton J, Elbadri AM, Thomas AG. Double-blind randomized controlled trial of glutamine-enriched polymeric diet in the treatment of active Crohn's disease. *J Pediatr Gastroenterol Nutr.* (2000) 30:78–84. doi: 10.1097/00005176-200001000-00022
81. Wade AM, Tucker HN. Antioxidant characteristics of L-histidine. *J Nutr Biochem.* (1998) 9:308–15. doi: 10.1016/S0955-2863(98)00022-9
82. Son DO, Satsu H, Shimizu M. Histidine inhibits oxidative stress- and TNF-α-induced interleukin-8 secretion in intestinal epithelial cells. *FEBS Lett.* (2005) 579:4671–7. doi: 10.1016/j.febslet.2005.07.038
83. Andou A, Hisamatsu T, Okamoto S, Chinen H, Kamada N, Kobayashi T, et al. Dietary histidine ameliorates murine colitis by inhibition of proinflammatory cytokine production from macrophages. *Gastroenterology* (2009) 136:564–74.e2. doi: 10.1053/j.gastro.2008.09.062
84. Hisamatsu T, Ono N, Imaizumi A, Mori M, Suzuki H, Uo M, et al. Decreased plasma histidine level predicts risk of relapse in patients with ulcerative colitis in remission. *PLoS ONE* (2015) 10:e0140716. doi: 10.1371/journal.pone.0140716
85. Tsune I, Ikejima K, Hirose M, Yoshikawa M, Enomoto N, Takei Y, et al. Dietary glycine prevents chemical-induced experimental colitis in the rat. *Gastroenterology* (2003) 125:775–85. doi: 10.1016/S0016-5085(03)01067-9
86. Johansson MEV, Hansson GC. Immunological aspects of intestinal mucus and mucins. *Nat Rev Immunol.* (2016) 16:639–49. doi: 10.1038/nri.2016.88
87. Johansson MEV, Gustafsson JK, Sjöberg KE, Petersson J, Holm L, Sjövall H, et al. Bacteria penetrate the inner mucus layer before inflammation in the dextran sulfate colitis model. *PLoS ONE* (2010) 5:e12238. doi: 10.1371/journal.pone.0012238
88. Johansson MEV, Gustafsson JK, Holmen-Larsson J, Jabbar KS, Xia L, Xu H, et al. Bacteria penetrate the normally impenetrable inner colon mucus layer in both murine colitis models and patients with ulcerative colitis. *Gut* (2014) 63:281–91. doi: 10.1136/gutjnl-2012-303207
89. Faure M, Moënnoz D, Montigon F, Mettraux C, Breuillé D, Ballèvre O. Dietary threonine restriction specifically reduces intestinal mucin synthesis in rats. *J Nutr.* (2005) 135:486–91. doi: 10.1093/jn/135.3.486
90. Faure M, Mettraux C, Moennoz D, Godin J-P, Vuichoud J, Rochat F, et al. Specific amino acids increase mucin synthesis and microbiota in dextran sulfate sodium-treated rats. *J Nutr.* (2006) 136:1558–64. doi: 10.1093/jn/136.6.1558
91. Pakos-Zebrucka K, Koryga I, Mnich K, Ljujic M, Samali A, Gorman AM. The integrated stress response. *EMBO Rep.* (2016) 17:1374–95. doi: 10.15252/embr.201642195
92. Ravindran R, Loebbermann J, Nakaya HI, Khan N, Ma H, Gama L, et al. The amino acid sensor GCN2 controls gut inflammation by inhibiting inflammasome activation. *Nature* (2016) 531:523–7. doi: 10.1038/nature17186
93. Holscher HD. Dietary fiber and prebiotics and the gastrointestinal microbiota. *Gut Microbes* (2017) 8:172–84. doi: 10.1080/19490976.2017.1290756
94. Thorburn AN, Macia L, Mackay CR. Diet, Metabolites, and "Western-Lifestyle" Inflammatory Diseases. *Immunity* (2014) 40:833–42. doi: 10.1016/j.immuni.2014.05.014
95. Salyers AA, West SE, Vercellotti JR, Wilkins TD. Fermentation of mucins and plant polysaccharides by anaerobic bacteria from the Fermentation of Mucins and Plant Polysaccharides by Anaerobic Bacteria from the Human Colon. *Appl Environ Microbiol.* (1977) 34:529–33.
96. Marsman KE, McBurney MI. Dietary fiber increases oxidative metabolism in colonocytes but not in distal small intestinal enterocytes isolated from rats. *J Nutr.* (1995) 125:273–82.
97. Singh N, Gurav A, Sivaprakasam S, Brady E, Padia R, Shi H, et al. Activation of Gpr109a, receptor for niacin and the commensal metabolite butyrate, suppresses colonic inflammation and carcinogenesis. *Immunity* (2014) 40:128–39. doi: 10.1016/j.immuni.2013.12.007
98. Maslowski KM, Vieira AT, Ng A, Kranich J, Sierro F, Di Yu, et al. Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43. *Nature* (2009) 461:1282–6. doi: 10.1038/nature08530
99. Smith PM, Howitt MR, Panikov N, Michaud M, Gallini CA, Bohlooly-Y M, et al. The microbial metabolites, short-chain fatty acids, regulate colonic treg cell homeostasis. *Science* (2013) 341:569–73. doi: 10.1126/science.1241165
100. Furusawa Y, Obata Y, Fukuda S, Endo TA, Nakato G, Takahashi D, et al. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. *Nature* (2013) 504:446–50. doi: 10.1038/nature12721
101. Kelly CJ, Zheng L, Campbell EL, Saeedi B, Scholz CC, Bayless AJ, et al. Crosstalk between microbiota-derived short-chain fatty acids and intestinal epithelial HIF augments tissue barrier function. *Cell Host Microbe* (2015) 17:662–71. doi: 10.1016/j.chom.2015.03.005
102. Saeedi BJ, Kao DJ, Kitzenberg DA, Dobrinskikh E, Schwisow KD, Masterson JC, et al. HIF-dependent regulation of claudin-1 is central to intestinal epithelial tight junction integrity. *Mol Biol Cell* (2015) 26:2252–62. doi: 10.1091/mbc.E14-07-1194
103. Peng L, Li Z-R, Green RS, Holzman IR, Lin J. Butyrate enhances the intestinal barrier by facilitating tight junction assembly via activation of AMP-activated protein kinase in Caco-2 cell monolayers. *J Nutr.* (2009) 139:1619–25. doi: 10.3945/jn.109.104638
104. Burger-van Paassen N, Vincent A, Puiman PJ, van der Sluis M, Bouma J, Boehm G, et al. The regulation of intestinal mucin MUC2 expression by

short-chain fatty acids: implications for epithelial protection. *Biochem J.* (2009) 420:211–9. doi: 10.1042/BJ20082222
105. Kaiko GE, Ryu SH, Koues OI, Collins PL, Solnica-Krezel L, Pearce EJ, et al. The colonic crypt protects stem cells from microbiota-derived metabolites. *Cell* (2016) 165:1708–20. doi: 10.1016/j.cell.2016.05.018
106. Desai MS, Seekatz AM, Koropatkin NM, Kamada N, Hickey CA, Wolter M, et al. A dietary fiber-deprived gut microbiota degrades the colonic mucus barrier and enhances pathogen susceptibility. *Cell* (2016) 167:1339–53.e21. doi: 10.1016/j.cell.2016.10.043
107. Nagao-Kitamoto H, Kamada N. Host-microbial Cross-talk in inflammatory bowel disease. *Immune Netw.* (2017) 17:1–12. doi: 10.4110/in.2017.17.1.1
108. Machiels K, Joossens M, Sabino J, De Preter V, Arijs I, Eeckhaut V, et al. A decrease of the butyrate-producing species roseburia hominis and faecalibacterium prausnitzii defines dysbiosis in patients with ulcerative colitis. *Gut* (2014) 63:1275–83. doi: 10.1136/gutjnl-2013-304833
109. Wang W, Chen L, Zhou R, Wang X, Song L, Huang S, et al. Increased proportions of Bifidobacterium and the Lactobacillus group and loss of butyrate-producing bacteria in inflammatory bowel disease. *J Clin Microbiol.* (2014) 52:398–406. doi: 10.1128/JCM.01500-13
110. Soergel KH, Lashner BA, Hanauer SB, Vanagunas A, Sellin JH, Weinberg D, et al. Short chain fatty acid rectal irrigation for left-sided ulcerative colitis: a randomised, placebo controlled trial. *Gut* (1997) 40:485–91.
111. Scheppach W, Sommer H, Kirchner T, Paganelli GM, Bartram P, Christl S, et al. Effect of butyrate enemas on the colonic mucosa in distal ulcerative colitis. *Gastroenterology* (1992) 103:51–6. doi: 10.1016/0016-5085(92)91094-K
112. Chapman MA, Grahn MF, Boyle MA, Hutton M, Rogers J, Williams NS. Butyrate oxidation is impaired in the colonic mucosa of sufferers of quiescent ulcerative colitis. *Gut* (1994) 35:73–6. doi: 10.1136/gut.35.1.73
113. De Preter V, Arijs I, Windey K, Vanhove W, Vermeire S, Schuit F, et al. Impaired butyrate oxidation in ulcerative colitis is due to decreased butyrate uptake and a defect in the oxidation pathway. *Inflamm Bowel Dis.* (2012) 18:1127–36. doi: 10.1002/ibd.21894
114. Duffy MM, Regan MC, Ravichandran P, O'Keane C, Harrington MG, Fitzpatrick JM, et al. Mucosal metabolism in ulcerative colitis and Crohn's disease. *Dis Colon Rectum* (1998) 41:1399–405. doi: 10.1007/BF02237056
115. De Preter V, Rutgeerts P, Schuit F, Verbeke K, Arijs I. Impaired expression of genes involved in the butyrate oxidation pathway in crohn's disease patients. *Inflamm Bowel Dis.* (2013) 19:43–4. doi: 10.1002/ibd.22970
116. Thibault R, De Coppet P, Daly K, Bourreille A, Cuff M, Bonnet C, et al. Down-regulation of the monocarboxylate Transporter 1 is involved in butyrate deficiency during intestinal inflammation. *Gastroenterology* (2007) 133:1916–27. doi: 10.1053/j.gastro.2007.08.041
117. Hartog A, Belle FN, Bastiaans J, De Graaff P, Garssen J, Harthoorn LF, et al. A potential role for regulatory T-cells in the amelioration of DSS induced colitis by dietary non-digestible polysaccharides. *J Nutr Biochem.* (2015) 26:227–33. doi: 10.1016/j.jnutbio.2014.10.011
118. Wang H, Shi P, Zuo L, Dong J, Zhao J, Liu Q, et al. Dietary non-digestible polysaccharides ameliorate intestinal epithelial barrier dysfunction in IL-10 knockout mice. *J Crohns Colitis* (2016) 10:1076–86. doi: 10.1093/ecco-jcc/jjw065
119. Silveira ALM, Ferreira AVM, de Oliveira MC, Rachid MA, da Cunha Sousa LF, dos Santos Martins F, et al. Preventive rather than therapeutic treatment with high fiber diet attenuates clinical and inflammatory markers of acute and chronic DSS-induced colitis in mice. *Eur J Nutr.* (2017) 56:179–91. doi: 10.1007/s00394-015-1068-x
120. Wedlake L, Slack N, Andreyev HJN, Whelan K. Fiber in the treatment and maintenance of inflammatory bowel disease: a systematic review of randomized controlled trials. *Inflamm Bowel Dis.* (2014) 20:576–86. doi: 10.1097/01.MIB.0000437984.92565.31
121. Benjamin JL, Hedin CRH, Koutsoumpas A, Ng SC, McCarthy NE, Hart AL, et al. Randomised, double-blind, placebo-controlled trial of fructo-oligosaccharides in active Crohn's disease. *Gut* (2011) 60:923–9. doi: 10.1136/gut.2010.232025
122. Kovatcheva-Datchary P, Nilsson A, Akrami R, Lee YS, De Vadder F, Arora T, et al. Dietary fiber-induced improvement in glucose metabolism is associated with increased abundance of prevotella. *Cell Metab.* (2015) 22:971–82. doi: 10.1016/j.cmet.2015.10.001
123. Halpin SJ, Ford AC. Prevalence of symptoms meeting criteria for irritable bowel syndrome in inflammatory bowel disease: systematic review and meta-analysis. *Am J Gastroenterol.* (2012) 107:1474–82. doi: 10.1038/ajg.2012.260
124. Simrén M, Axelsson J, Gillberg R, Abrahamsson H, Svedlund J, Björnsson ES. Quality of life in inflammatory bowel disease in remission: the impact of IBS-like symptoms and associated psychological factors. *Am J Gastroenterol.* (2002) 97:389–96. doi: 10.1111/j.1572-0241.2002.05475.x
125. Staudacher HM, Whelan K. The low FODMAP diet: recent advances in understanding its mechanisms and efficacy in IBS. *Gut* (2017) 66:1517–27. doi: 10.1136/gutjnl-2017-313750
126. Cox SR, Prince AC, Myers CE, Irving PM, Lindsay JO, Lomer MC, et al. Fermentable Carbohydrates [FODMAPs] exacerbate functional gastrointestinal symptoms in patients with inflammatory bowel disease: a randomised, double-blind, placebo-controlled, cross-over, re-challenge trial. *J Crohns Colitis* (2017)1420–9. doi: 10.1093/ecco-jcc/jjx073
127. Zhan YL, Zhan YA, Dai SX. Is a low FODMAP diet beneficial for patients with inflammatory bowel disease? A meta-analysis and systematic review. *Clin Nutr.* (2017) 37:123–9. doi: 10.1016/j.clnu.2017.05.019
128. Zhou SY, Gillilland M, Wu X, Leelasinjaroen P, Zhang G, Zhou H, et al. FODMAP diet modulates visceral nociception by lipopolysaccharide-mediated intestinal inflammation and barrier dysfunction. *J Clin Invest.* (2018) 128:267–80. doi: 10.1172/JCI92390
129. Kim KA, Gu W, Lee IA, Joh EH, Kim DH. High fat diet-induced gut microbiota exacerbates inflammation and obesity in mice via the TLR4 signaling pathway. *PLoS ONE* (2012) 7:e47713. doi: 10.1371/journal.pone.0047713
130. Gulhane M, Murray L, Lourie R, Tong H, Sheng YH, Wang R, et al. High fat diets induce colonic epithelial cell stress and inflammation that is reversed by IL-22. *Sci Rep.* (2016) 6:1–17. doi: 10.1038/srep28990
131. Cani PD, Bibiloni R, Knauf C, Neyrinck AM, Delzenne NM. Changes in gut microbiota control metabolic diet–induced obesity and diabetes in mice. *Diabetes* (2008) 57:1470–81. doi: 10.2337/db07-1403
132. Martinez-Medina M, Denizot J, Dreux N, Robin F, Billard E, Bonnet R, et al. Western diet induces dysbiosis with increased e coli in CEABAC10 mice, alters host barrier function favouring AIEC colonisation. *Gut* (2014) 63:116–24. doi: 10.1136/gutjnl-2012-304119
133. Hou JK, Abraham B, El-Serag H. Dietary intake and risk of developing inflammatory bowel disease: A systematic review of the literature. *Am J Gastroenterol.* (2011) 106:563–73. doi: 10.1038/ajg.2011.44
134. Shores DR, Binion DG, Freeman BA, Baker PRS. New insights into the role of fatty acids in the pathogenesis and resolution of inflammatory bowel disease. *Inflamm Bowel Dis.* (2011) 17:2192–204. doi: 10.1002/ibd.21560
135. Chen C, Shah YM, Morimura K, Krausz KW, Miyazaki M, Richardson TA, et al. Metabolomics reveals that hepatic Stearoyl-CoA Desaturase 1 downregulation exacerbates inflammation and acute colitis. *Cell Metab.* (2008) 7:135–47. doi: 10.1016/j.cmet.2007.12.003
136. Devkota S, Wang Y, Musch MW, Leone V, Fehlner-Peach H, Nadimpalli A, et al. Dietary-fat-induced taurocholic acid promotes pathobiont expansion and colitis in Il10-/-mice. *Nature* (2012) 487:104–8. doi: 10.1038/nature11225
137. Miyazaki M, Kim YC, Gray-Keller MP, Attie AD, Ntambi JM. The biosynthesis of hepatic cholesterol esters and triglycerides is impaired in mice with a disruption of the gene for stearoyl-CoA desaturase 1. *J Biol Chem.* (2000) 275:30132–8. doi: 10.1074/jbc.M005488200
138. Liu-Wu Y, Hurt-Camejo E, Wiklund O. Lysophosphatidylcholine induces the production of IL-1beta by human monocytes. *Atherosclerosis* (1998) 137:351–7.
139. Fernández-Bañares F, Esteve-Comas M, Mañé J, Navarro E, Bertrán X, Cabré E, et al. Changes in mucosal fatty acid profile in inflammatory bowel disease and in experimental colitis: a common response to bowel inflammation. *Clin Nutr.* (1997) 16:177–83. doi: 10.1016/S0261-5614(97)80003-9
140. Esteve-Comas M, Nunez MC, Fernandez-Banares F, Abad-Lacruz A, Gil A, Cabre E, et al. Abnormal plasma polyunsaturated fatty acid pattern in non-active inflammatory bowel disease. *Gut* (1993) 34:1370–3. doi: 10.1136/gut.34.10.1370
141. Serra-Majem L, Roman B, Estruch R. Scientific evidence of interventions using the Mediterranean diet: a systematic review. *Nutr Rev.* (2006) 64:S27–47. doi: 10.1301/nr.2006.feb.S27

142. De Silva PSA, Luben R, Shrestha SS, Khaw KT, Hart AR. Dietary arachidonic and oleic acid intake in ulcerative colitis etiology: a prospective cohort study using 7-day food diaries. *Eur J Gastroenterol Hepatol.* (2014) 26:11–8. doi: 10.1097/MEG.0b013e328365c372
143. Camuesco D, Gálvez J. Dietary olive oil supplemented with fish oil, rich in EPA and DHA (n-3) polyunsaturated fatty acids, attenuates colonic inflammation in rats with DSS-induced colitis. *J Nutr.* (2005) 135:687–94. doi: 10.1093/jn/135.4.687
144. Morita H, Makanishi K, Dohi T, Yasugi E, Oshima M. Phospholipid turnover in the inflammed intestinal mucosa: arachidonic ccid-rich phosphatidyl/plasmenyl-ethanolamine in the mucosa in inflammatory bowel disease. *J Gastroenterol.* (1999) 34:46–53.
145. Serhan CN, Chiang N. Endogenous pro-resolving and anti-inflammatory lipid mediators: a new pharmacologic genus. *Br J Pharmacol.* (2009) 153:S200–15. doi: 10.1038/sj.bjp.0707489
146. Arita M, Yoshida M, Hong S, Tjonahen E, Glickman JN, Petasis NA, et al. Resolvin E1, an endogenous lipid mediator derived from omega-3 eicosapentaenoic acid, protects against 2,4,6-trinitrobenzene sulfonic acid-induced colitis. *Proc Natl Acad Sci USA.* (2005) 102:7671–6. doi: 10.1073/pnas.0409271102
147. Haas-Stapleton EJ, Lu Y, Hong S, Arita M, Favoreto S, Nigam S, et al. *Candida albicans* modulates host defense by biosynthesizing the pro-resolving mediator resolvin E1. *PLoS ONE* (2007) 2:e1316. doi: 10.1371/journal.pone.0001316
148. Arita M, Bianchini F, Aliberti J, Sher A, Chiang N, Hong S, et al. Stereochemical assignment, antiinflammatory properties, and receptor for the omega-3 lipid mediator resolvin E1. *J Exp Med.* (2005) 201:713–22. doi: 10.1084/jem.20042031
149. Ishida T, Yoshida M, Arita M, Nishitani Y, Nishiumi S, Masuda A, et al. Resolvin E1, an endogenous lipid mediator derived from eicosapentaenoic acid, prevents dextran sulfate sodium-induced colitis. *Inflamm Bowel Dis.* (2010) 16:87–95. doi: 10.1002/ibd.21029
150. Lee JY, Plakidas A, Lee WH, Heikkinen A, Chanmugam P, Bray G, et al. Differential modulation of Toll-like receptors by fatty acids. *J Lipid Res.* (2003) 44:479–86. doi: 10.1194/jlr.M200361-JLR200
151. Novak TE, Babcock TA, Jho DH, Helton WS, Espat NJ. NF-κB inhibition by ω-3 fatty acids modulates LPS-stimulated macrophage TNF-α transcription. *Am J Physiol.* (2003) 284:L84–9. doi: 10.1152/ajplung.00077.2002
152. Simopoulos AP. The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases. *Exp Biol Med.* (2008) 233:674–88. doi: 10.3181/0711-MR-311
153. Jia Q, Ivanov I, Zlatev ZZ, Alaniz RC, Eeks BR, Calla Ay ES, et al. Dietary fish oil and curcumin combine to modulate colonic cytokinetics and gene expression in dextran sodium sulphate-treated mice. *Br J Nutr.* (2011) 106:519–29. doi: 10.1017/S0007114511000390
154. Hassan A, Ibrahim A, Mbodji K, Coëffier M, Ziegler F, Bounoure F, et al. An α-linolenic acid-rich formula reduces oxidative stress and inflammation by regulating NF-κB in rats with TNBS-induced colitis. *J Nutr.* (2010) 140:1714–21. doi: 10.3945/jn.109.119768
155. Matsunaga H, Hokari R, Kurihara C, Okada Y, Takebayashi K, Okudaira K, et al. Omega-3 fatty acids exacerbate DSS-induced colitis through decreased adiponectin in colonic subepithelial myofibroblasts. *Inflamm Bowel Dis.* (2008) 14:1348–57. doi: 10.1002/ibd.20491
156. Hudert CA, Weylandt KH, Lu Y, Wang J, Hong S, Dignass A, et al. Transgenic mice rich in endogenous omega-3 fatty acids are protected from colitis. *Proc Natl Acad Sci USA.* (2006) 103:11276–81. doi: 10.1073/pnas.0601280103
157. Turner D, Shah PS, Steinhart AH, Zlotkin S, Griffiths AM. Maintenance of remission in inflammatory bowel disease using omega-3 fatty acids (fish oil): A systematic review and meta-analyses. *Inflamm Bowel Dis.* (2011) 17:336–45. doi: 10.1002/ibd.21374
158. Turner D, Zlotkin SH, Shah PS, Griffiths AM. Omega 3 fatty acids (fish oil) for maintenance of remission in Crohn ' s disease (Review). *Cochrane Libr.* (2014) CD006320. doi: 10.1002/14651858.CD006320.pub2
159. Fontaine-bisson B, Wolever TMS, Chiasson J, Rabasa-Lhoret R, Maheux P, Josse RG, et al. Genetic polymorphisms of tumor necrosis factor-alpha modify the association between dietary polyunsaturated fatty acids and fasting HDL-concentrations and apo A-1 concentrations. *Am J Clin Nutr.* (2007) 86:768–74. doi: 10.1093/ajcn/86.3.768
160. Paradis A-M, Fontaine-Bisson B, Bossé Y, Robitaille J, Lemieux S, Jacques H, et al. The peroxisome proliferator-activated receptor alpha Leu162Val polymorphism influences the metabolic response to a dietary intervention altering fatty acid proportions in healthy men. *Am J Clin Nutr.* (2005) 81:523–30. doi: 10.1093/ajcn.81.2.523
161. Ferreira P, Cravo M, Guerreiro CS, Tavares L, Santos PM, Brito M. Fat intake interacts with polymorphisms of Caspase9, FasLigand and PPARgamma apoptotic genes in modulating Crohn's disease activity. *Clin Nutr.* (2010) 29:819–23. doi: 10.1016/j.clnu.2010.06.008
162. Ananthakrishnan AN, Khalili H, Song M, Higuchi LM, Lochhead P, Richter JM, et al. Genetic polymorphisms in fatty acid metabolism modify the association between dietary n3. *Inflamm Bowel Dis.* (2017) 23:1898–904. doi: 10.1097/MIB.0000000000001236
163. Zhang YJ, Gan RY, Li S, Zhou Y, Li AN, Xu DP, et al. Antioxidant phytochemicals for the prevention and treatment of chronic diseases. *Molecules* (2015) 20:21138–56. doi: 10.3390/molecules201219753
164. Maheshwari RK, Singh AK, Gaddipati J, Srimal RC. Multiple biological activities of curcumin: a short review. *Life Sci.* (2006) 78:2081–7. doi: 10.1016/j.lfs.2005.12.007
165. Wang J, Ghosh SS, Ghosh S. Curcumin improves intestinal barrier function: modulation of intracellular signaling, and organization of tight junctions. *Am J Physiol.* (2017) 312:438–45. doi: 10.1152/ajpcell.00235.2016
166. Pan MH, Lin-Shiau SY, Lin JK. Comparative studies on the suppression of nitric oxide synthase by curcumin and its hydrogenated metabolites through down-regulation of IκB kinase and NFκB activation in macrophages. *Biochem Pharmacol.* (2000) 60:1665–76. doi: 10.1016/S0006-2952(00)00489-5
167. Yang J, Zhong X, Kim S, Kim D, Kim HS, Lee J, et al. Comparative effects of curcumin and tetrahydrocurcumin on dextran sulfate sodium-induced colitis and inflammatory signaling in mice. *J Cancer Prev.* (2018) 23:18–24. doi: 10.15430/JCP.2018.23.1.18
168. Sugimoto K, Hanai H, Tozawa K, Aoshi T, Uchijima M, Nagata T, et al. Curcumin prevents and ameliorates trinitrobenzene sulfonic acid-induced colitis in mice. *Gastroenterology* (2002) 123:1912–22. doi: 10.1053/gast.2002.37050
169. Manach C. Polyphenols: food sources and bioavailability. *Am J Clin Nutr.* (2004) 79:727–47. doi: 10.1038/nature05488
170. Cardona F, Andrés-Lacueva C, Tulipani S, Tinahones FJ, Queipo-Ortuño MI. Benefits of polyphenols on gut microbiota and implications in human health. *J Nutr Biochem.* (2013) 24:1415–22. doi: 10.1016/j.jnutbio.2013.05.001
171. Hassaninasab A, Hashimoto Y, Tomita-Yokotani K, Kobayashi M. Discovery of the curcumin metabolic pathway involving a unique enzyme in an intestinal microorganism. *Proc Natl Acad Sci USA.* (2011) 108:6615–20. doi: 10.1073/pnas.1016217108
172. Lai CS, Wu JC, Yu SF, Badmaev V, Nagabhushanam K, Ho CT, et al. Tetrahydrocurcumin is more effective than curcumin in preventing azoxymethane-induced colon carcinogenesis. *Mol Nutr Food Res.* (2011) 55:1819–28. doi: 10.1002/mnfr.201100290
173. Lu Y, Zamora-Ros R, Chan S, Cross AJ, Ward H, Jakszyn P, et al. Dietary polyphenols in the aetiology of crohn's disease and ulcerative colitis-A multicenter European Prospective Cohort Study (EPIC). *Inflamm Bowel Dis.* (2017) 23:2072–82. doi: 10.1097/MIB.0000000000001108
174. Hanai H, Iida T, Takeuchi K, Watanabe F, Maruyama Y, Andoh A, et al. Curcumin maintenance therapy for ulcerative colitis: randomized, multicenter, double-blind, placebo-controlled trial. *Clin Gastroenterol Hepatol.* (2006) 4:1502–6. doi: 10.1016/j.cgh.2006.08.008
175. Lang A, Salomon N, Wu JCY, Kopylov U, Lahat A, Har-Noy O, et al. Curcumin in combination with mesalamine induces remission in patients with mild-to-moderate ulcerative colitis in a randomized controlled trial. *Clin Gastroenterol Hepatol.* (2015) 13:1444–9. doi: 10.1016/j.cgh.2015.02.019
176. Roberts CL, Rushworth SL, Richman E, Rhodes JM. Hypothesis: increased consumption of emulsifiers as an explanation for the rising incidence of Crohn's disease. *J Crohns Colitis* (2013) 7:338–41. doi: 10.1016/j.crohns.2013.01.004

177. Chassaing B, Koren O, Goodrich JK, Poole AC, Srinivasan S, Ley RE, et al. Dietary emulsifiers impact the mouse gut microbiota promoting colitis and metabolic syndrome. *Nature* (2015) 519:92–6. doi: 10.1038/nature 14232
178. Viennois E, Merlin D, Gewirtz AT, Chassaing B. Dietary emulsifier-induced low-grade inflammation promotes colon carcinogenesis. *Cancer Res.* (2017) 77:27–40. doi: 10.1158/0008-5472.CAN-16-1359
179. Suez J, Korem T, Zeevi D, Zilberman-Schapira G, Thaiss CA, Maza O, et al. Artificial sweeteners induce glucose intolerance by altering the gut microbiota. *Nature* (2014) 514:181–6. doi: 10.1038/nature 13793
180. Rodriguez-Palacios A, Harding A, Menghini P, Himmelman C, Retuerto M, Nickerson KP, et al. The artificial sweetener splenda promotes gut proteobacteria, dysbiosis, and myeloperoxidase reactivity in crohn's disease-like ileitis. *Inflamm Bowel Dis.* (2018) 24:1005–20. doi: 10.1093/ibd/izy060
181. Sugihara K, Masuda M, Nakao M, Abuduli M, Imi Y, Oda N, et al. Dietary phosphate exacerbates intestinal inflammation in experimental colitis. *J Clin Biochem Nutr.* (2017) 61:91–9. doi: 10.3164/jcbn.16
182. Jowett SL, Seal CJ, Pearce MS, Phillips E, Gregory W, Barton JR, et al. Influence of dietary factors on the clinical course of ulcerative colitis: a prospective cohort study. *Gut* (2004) 53:1479–84. doi: 10.1136/gut.2003.024828
183. Sakamoto N, Kono S, Wakai K, Fukuda Y, Satomi M, Shimoyama T, et al. Dietary risk factors for inflammatory bowel disease. *Inflamm Bowel Dis.* (2005) 11:154–63. doi: 10.1097/00054725-200502000-00009

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REVIEW
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Dietary Habits and Intestinal Immunity: From Food Intake to CD4$^+$ T_H Cells

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Dietary habits have a profound impact on intestinal homeostasis and in general on human health. In Western countries, high intake of calories derived from fried products, butter and processed meat is favored over dietary regimens rich in fruits and vegetables. This type of diet is usually referred to as Western-type diet (WTD) and it has been associated with several metabolic and chronic inflammatory conditions of the gastrointestinal tract. In this review, we describe how WTD promotes intestinal and extra-intestinal inflammation and alters mucosal immunity acting on CD4$^+$ T cells in a microbiota-dependent or –independent fashion, ultimately leading to higher susceptibility to infectious and autoimmune diseases. Moreover, summarizing recent findings, we propose how dietary supplementation with fiber and vitamins could be used as a tool to modulate CD4$^+$ T cell phenotype and function, ameliorating inflammation and restoring mucosal homeostasis.

Keywords: inflammation, mucosal immunity, CD4 T cells, western diet, fat, salt, fiber, microbiota

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INTRODUCTION

In the gastrointestinal (GI) tract, the immune system is constantly under great environmental pressure, continuously facing a wide variety of antigens derived both from intestinal microbiota and from food. Intestinal CD4$^+$ T helper (T_H) cells are key mediators of mucosal immunity, and according to their effector functions, they can be divided into different populations, namely T_H1, T_H2, and T_H17, with the T_H17 cells being relatively abundant within the GI tract (1–4). Here, different bacterial species dictate whether intestinal CD4$^+$ T cells acquire pro- or anti-inflammatory effector phenotypes (5, 6), highlighting the crucial function of the intestinal microbiota in maintaining mucosal homeostasis. Pro-inflammatory responses driven by T_H cells are controlled by different subsets of CD4$^+$ T cells with regulatory capacities, namely T_{reg} and T_R1 cells, key players in promoting and maintaining mucosal tolerance to self- and food-related antigens (7–9). However, when mucosal tolerance fails to limit pro-inflammatory immune responses, this results in intestinal inflammation which can lead to the development of immune-mediated inflammatory diseases (IMIDs) such as inflammatory bowel diseases (IBDs). IBDs are among the leading diseases in Western countries (10, 11) and the observation that T_H17 cells and T_H17-associated cytokines such as IL-17A, IL-17F, and IL-22 are generally enriched in the inflamed mucosa of IBD patients, suggests that T_H17 cells drive intestinal inflammation (12, 13). Interestingly, CD4$^+$ T cells, especially T_H17 cells, are highly susceptible to components of Western-type diet (WTD)

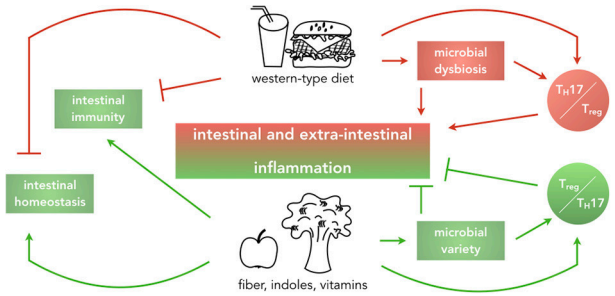

FIGURE 1 | Impact of dietary habits on local and systemic homeostasis and immunity. Graphical abstract summarizing the main findings that this review will discuss. Western-type diets, rich in fat, cholesterol, sugar, and salt are reported to drive intestinal and extra-intestinal inflammation by causing microbial dysbiosis and alteration of the balance of pro- and anti-inflammatory T cells in the intestine, ultimately dampening intestinal immunity and affecting intestinal homeostasis. In contrast, diets enriched in fiber, indoles and vitamins implement beneficial effects on intestinal homeostasis by increasing microbial variety and inducing a regulatory environment.

(14, 15), and WTD has been associated with higher incidence of IBDs (16). Moreover, high intake of calories derived from processed meat, butter and fried products, all components of WTD, have been described to instantly alter the composition of the intestinal microbiota, a phenomenon called dysbiosis, toward a lower *Bacteroidetes* to *Firmicutes* ratio (17–19). Dysbiosis is also commonly found among patients suffering from IMIDs, including IBDs (20–23). However, despite these strong associations, a direct cause/effect link between WTD and development of IBDs has not yet been proven.

In this review, we discuss how changes in dietary habits favoring WTD affect intestinal immunity by altering composition of the intestinal microbiota and phenotype and functions of effector and regulatory $CD4^+$ T cells. Furthermore, we propose that WTD leads both to higher susceptibility to infections and higher incidence of chronic autoimmune diseases, thus exacerbating intestinal and extra-intestinal inflammation. We support the hypothesis that supplementation of diets with defined products of bacterial or dietary origin can ameliorate WTD-induced inflammation, acting on the effector/regulatory T cell axis and, in turn, restoring intestinal homeostasis (**Figure 1**). The findings presented in this review are mostly based on murine experiments and are cross-validated in humans, where possible.

THE INTESTINAL IMMUNE SYSTEM AND THE MICROBIOTA

The intestinal immune system promotes mucosal immunity and maintains tolerance to dietary and microbial antigens, both through its innate and adaptive components located within intestinal epithelia and lamina propria.

In addition to M cells and intraepithelial lymphocytes (IELs), goblet cells, Paneth cells and innate lymphoid type 3 cells (ILC3s) constitute the innate arm of the intestinal immune system. On the other hand, antibody-secreting plasma cells, $CD4^+$ and $CD8^+$ T cells represent the intestinal adaptive immune system. Mucins secreted by goblet cells form the single mucus layer of the small intestine and the two-layered mucus of the colon with the inner layer being impermeable to bacteria (24). ILC3s efficiently contribute to intestinal homeostasis through secretion of IL-17 and IL-22 (25, 26) that instruct Paneth cells to secrete antimicrobial peptides (AMPs) into the intestinal lumen. Although the innate components of the intestinal immune system are fundamental in providing a first line of protection from invading microbes, this review focuses on $CD4^+$ T_H cells given their unique role in orchestrating adaptive immune responses, protecting from infections.

Among the different CD4 T_H cell subsets, T_H17 cells are relatively abundant within the GI tract (27). They are characterized by the expression of the master transcription factor RORγt, the chemokine receptor CCR6 and the transcription factor aryl hydrocarbon receptor (AhR) (28, 29). T_H17 cells secrete the highest amount of IL-17 and IL-22, contributing to protection against fungal and bacterial infections, ultimately maintaining mucosal immunity (1). However, the observation that high levels of IL-17 and IL-22 are found in the inflamed mucosa of patients suffering from IBDs, highlights their dualistic role in limiting or promoting intestinal inflammation (12, 13). Complete blockage of IL-17A failed to ameliorate intestinal inflammation in Crohn's disease, which might be explained by preventing the beneficial actions of IL-17A, such as promotion of AMP production that ultimately protects the host against invading microbes (30). In line with this, it has been also shown that IL17-secreting $TCR\gamma\delta^+$ T cells mediate gut permeability and exert a protective function on epithelial barrier integrity (31). At the same time IL-17A-deficient T cells have been shown to induce a more aggressive disease outcome in a mouse model of transfer colitis (32). Taken together, these findings suggest that the cellular source of IL-17A production might determine the beneficial or detrimental role of the cytokine itself. Therefore, cell-specific targeting of IL-17A could open new therapeutic approaches. Furthermore, it has been described that T_H17 cells are a highly plastic cell population, able to acquire properties typical of other $CD4^+$ T cell subsets (33). Due to their high plasticity, T_H17 cells can be either beneficial or detrimental to the host according to the cytokine profile they exhibit in response to inflammatory stimuli. While IL-12 and IL-23 drive the conversion of T_H17 cells into pro-inflammatory T_H1 cells, inducing acquisition of T-bet and CXCR3 and secretion of IFN-γ (33, 34), exposure to TGF-β and AhR ligands mediate the acquisition of IL-10 secretion from T_H17, thus converting them into anti-inflammatory T_R1^{exTH17} cells (35, 36). Characterized by lack of Foxp3 and expression of the co-inhibitory receptors CD49b and LAG-3 (37), both *bona fide* and T_H17-derived T_R1 cells (i.e., T_R1^{exTH17}) limit expansion of pro-inflammatory T_H17 cells in an IL-10-dependent manner (38). In addition, pathogenicity of T_H17 cells is also controlled by $Foxp3^+$ T_{reg} cells (38), which, similarly to T_R1 and T_H17 cells, are relatively abundant in the small intestine, where most of the dietary products are absorbed (9). In summary, pro- and anti-inflammatory $CD4^+$ T cells co-exist within the GI tract being subject to a highly dynamic microenvironment.

An additional layer of complexity to this tight balance of pro- and anti-inflammatory cells is added by the microbiota, whose composition and abundance vary along the GI tract (39–41). It is increasingly recognized that the intestinal microbiota exerts non-redundant functions in the maintenance of homeostasis of the host, ranging from synthesis of nutrients to protection against invading pathogens and modulation of immune responses (42–44). Indeed, studies on germ-free (GF) mice have underlined a higher susceptibility to viral or bacterial infections of mice deprived of their intestinal microbiota as compared to mice housed under specific pathogen free (SPF) conditions (45, 46). This is probably due to the fact that bacterial species dictate the phenotype of $CD4^+$ T cells. For example, *Bacteroides fragilis* favor differentiation of naïve $CD4^+$ T cells toward IFN-γ-producing T_H1 cells (6), while *Segmented Filamentous Bacteria* (SFB) drives differentiation toward IL-17-secreting T_H17 cells (5). Of note, presence of SFB within the intestinal microbiota prevents the growth of pathogenic *Citrobacter rodentium*, probably due to T_H17 induction, ameliorating colonic inflammation (5). These findings highlight once more the dualistic nature of T_H17 cells in preventing or exacerbating intestinal inflammation.

Composition of the intestinal microbiota and in turn, intestinal $CD4^+$ T cells are therefore key players in promoting mucosal homeostasis. On the one hand, bacterial species are able to shape intestinal immune functions by modulating $CD4^+$ T cell responses. On the other hand, while T_H17 cells mediate immunity to invading microbes, $Foxp3^+$ T_{reg} and T_R1 cells maintain tolerance to self and dietary antigens, preventing, as well, uncontrolled T_H17 cell-mediated immune responses. Failure to suppress uncontrolled $CD4^+$ T_H cell-mediated immune responses may lead to IBDs, as seen in mice lacking critical immunosuppression-associated genes, such as IL-10, which develop spontaneous colitis (47). In agreement, IBDs have been defined by aberrant $CD4^+$ T_H cell responses against the commensal microbiota in genetically susceptible hosts (48). How commensal-specific $CD4^+$ T_H cell responses develop has been reviewed elsewhere (49).

DIETARY HABITS IN WESTERN COUNTRIES

Dietary habits have a profound impact on the lifestyle of individuals. High lipid content in WTD often derived from saturated fatty acids and cholesterol, in addition to excess intake of sugar is linked to higher incidence of colorectal cancer and IMIDs (50–52). Additionally, elevated salt intake and consumption of medium (MCFA) and long chain fatty acids (LCFA), such as lauric and palmitic acids, induce or exacerbate inflammation, acting on the intestinal microbiota, as well as on the innate and adaptive components of the intestinal immune system (53–55).

WTD-favoring dietary habits are also in line with a reduced absorption of vitamins and intake of vegetables and fruits rich in fiber. Dietary fiber consists of non-starch polysaccharides, cellulose, lignin and other plant-derived oligo- or polysaccharides that are not digestible or absorbable in the small intestine (56). It is accepted that diets rich in fiber are beneficial to the host, and dietary regimens favoring consumption of fiber have been associated with a decreased risk of type 2 diabetes (T2D), cardiovascular diseases and intestinal inflammation (57–59). This suggests that fiber can potentially modulate intestinal related and unrelated immune responses. However, how the fiber ameliorates inflammation remains poorly understood. One possible mechanism could reside in its fermentation by bacteria within the colon, which results in the production of short-chain fatty acids (SCFAs) (60, 61). Indeed, acetate, butyrate and propionate, all SCFAs, mediate beneficial effects on the host by engagement of G protein–coupled receptors (GPRs) expressed by a variety of cells, including intestinal $CD4^+$ T cells (62). In addition, recent evidences suggest that the beneficial effects of fiber consumption on the host might reside in the changes it induces in the composition of the intestinal microbiota itself (63).

In short, dietary habits greatly influence human health, modulating function of $CD4^+$ T cells and composition of intestinal microbiota.

WTD INDUCES MUCOSAL INFLAMMATION ALTERING IMMUNITY

Lipids, Cholesterol, and Salt

In this part of the review, we describe the effects that high intake of lipids, cholesterol and salt have on the intestinal immune system, dissecting the complex interplay between adaptive immune cells and the intestinal microbiota. We then summarize recent findings on how WTD-favoring dietary regimens increase susceptibility to chronic autoimmune diseases and infections with commensal bacteria (**Table 1**). Ultimately, we propose how intestinal and extra-intestinal inflammation driven by WTD can be modulated by supplementation of defined bio-products of microbial or dietary origin, which in turn act on $CD4^+$ T cells.

In addition to inducing systemic low-grade chronic inflammation typical of obesity (76, 77), WTD promotes local intestinal inflammation through a variety of mechanisms often linked to alteration of the intestinal microbiota composition (i.e., dysbiosis). **Figure 2** provides a graphical summary.

High intake of fat increases the levels of IL-1β, IL-6, TNF-α, and NF-κB in the colon (64, 65), resulting in higher concentration of lipocalin 2 (Lcn2) in the feces, a biomarker of intestinal inflammation (78). Thinning of the mucus layers of the small intestine and colon (67, 68) and higher gut permeability (66) lead to increased presence of invading Gram-negative bacteria and higher plasma levels of LPS (79, 80), exacerbating local and systemic inflammation. These findings indicate that the high lipid content of WTD affects mucosal homeostasis, inducing thinning of the protective intestinal mucus layers and thus, increasing gut permeability and levels of pro-inflammatory cytokines.

Similarly, high intake of cholesterol increases levels of IL-1β in the small intestine of mice and frequencies of $CD11b^+$ and $CD11c^+$ cells (74). Along the same line, liver X receptor (LXR)-deficient mice ($LXR\alpha\beta^{-/-}$), which lack the receptor for oxysterols (i.e. cholesterol metabolites), fed for 8 or 16 weeks with WTD showed higher titers of antinuclear antibodies (ANA),

TABLE 1 | Table showing how different components of WTD drive cellular and functional phenotypes associated with intestinal and extra-intestinal inflammation.

Components of WTD	Intestinal inflammation	Effects on $CD4^+$ T cells	Effects on gut microbiota	Susceptibility to infection/diseases
High fat	↑ colonic IL-1β, IL-6, TNF-α (64, 65) ↑ gut permeability (66) ↓ mucus layers (67, 68)	↑ Th1 cells (69)	↑ Proteobacteria ↑Firmicutes ↓Bacteroidetes (17–19, 70)	↑ *Bilophila wadsworthia* (69) ↑ invasive *E. coli* (71, 72)
High salt	↑ colitis (54, 55)	↑ Th17 cells (15) ↓ inhibitory Treg cells (73)	↓ *Lactobacillus* spp. (14, 54)	↑ colitis (54, 55) ↑ EAE (15) ↑ GVHD (73)
High cholesterol	↑ small intestine IL-1β, $CD11b^+$ myeloid cells (74)	Not reported	↑ *Bilophila wadsworthia* (69)	↑ ANA ↑ T cell priming ↑ B cell expansion (75)
High LCFA	Not reported	↑ Th1, Th17 cells (53)	↓ *Prevotellaceae* ↓ S24-7 families (53)	↑ EAE (53)

ANA, Anti-Nuclear Antibodies; EAE, Experimental Autoimmune Encephalomyelitis; GVHD, Graft-vs.-Host Disease; LCFA, Long-Chain Fatty Acid.

increased B cell numbers and augmented T_H cell priming, developing a lupus-like autoimmune disease (75). While these findings suggest that metabolism of cholesterol through LXR is crucial for preventing autoimmunity, the effect of excess intake of dietary cholesterol on T_H cells still remains unclear. Indeed, oxysterols have been shown to both favor and inhibit T_H17 cell differentiation via direct binding to RORγt (81) or engagement of LXR (82) respectively, ascribing a context-dependent beneficial or detrimental role to cholesterol. High cholesterol levels might also increase the production of bile acids (BAs) and high BA concentration in the colon, together with BA malabsorption, has been suggested as a possible cause of diarrhea (83), a condition that is commonly present in patients suffering from IBDs. However, the etiology of diarrhea in IBD patients is still under debate and it can be ascribed to a sum of factors, including intestinal inflammation and disruption of barrier integrity, rather than to one single factor.

In addition to direct effects on $CD4^+$ T cells, diets rich in lipids and cholesterol have been shown to drastically alter the composition of the intestinal microbiota. Of note, phenotype and effector functions of intestinal $CD4^+$ T cells are strictly associated with the different bacterial species of the microbiota, and the intestinal microbiota quickly responds to changes in dietary regimens. Indeed, long-term feeding of mice with WTD shifts the composition of intestinal microbiota toward a higher ratio of *Proteobacteria* and *Firmicutes* species over *Bacteroidetes* (19, 70), leading to higher susceptibility to pathobiont infections by invasive *E. coli* (71, 72). Similarly, diets rich in saturated milk-derived fat favor the growth of the pathobiont *Bilophila wadsworthia* (BW) in the colon of IL-$10^{-/-}$ mice, increasing the incidence of spontaneous colitis (69), and SPF mice colonized with BW present higher expression of IL-6 and Serum Amyloid A (SAA), exhibiting systemic inflammation (84). Of note, BW requires bile in its medium to be cultured (85), and diets rich in saturated milk-derived fat increased the amount of taurine conjugated bile acids (TCA) (69), indicating an as-of-yet undefined cross-talk between bile acid composition and intestinal microbiota.

These findings not only show that the intestinal microbiota is highly susceptible to perturbations of dietary regimes, but they also reveal a direct link between WTD, dysbiosis and increased susceptibility to infections and colitis. Further studies are needed to dissect how specific bacterial species act on the different $CD4^+$ T cell populations.

Interestingly, dysbiosis is a common feature of patients suffering from IBDs, which is reflected by lower complexity of microbial species (86) and also exhibit increased frequencies of T_H17 cells and amounts of IL-17 and IL-22 as compared to healthy individuals (12, 13).

It is however still unclear whether WTD first alters the composition of the intestinal microbiota that in turn induces pro-inflammatory T_H17 cells and promotes intestinal inflammation, or vice versa. Moreover, the exact components of WTD able to target microbiota and/or T_H17 cells still remain to be fully identified.

In the last years salt has been given a lot of attention. *Wu et al.* have shown that serum glucocorticoid kinase-1 (SGK1) drives the expression of IL-23R in T_H17 cells via inactivation of Foxo1 (15), and it is known that IL-23R expression on T_H17 cells defines their pathogenicity (87, 88). Interestingly, SGK1 has been shown to regulate salt sensing by different cell types, including epithelial colonic cells (89, 90). In their work, *Wu et al.* showed that mice fed with high salt diet (HSD) for 3 weeks exhibited higher frequencies of lamina propria (LP) T_H17 cells as compared to normal chow-fed mice. Furthermore, HSD-fed mice were more susceptible to experimental autoimmune encephalomyelitis (EAE), showing prominent infiltration of T_H17 in their central nervous system (CNS) (15). A similar phenotype has been described in mice fed with diets rich in lauric acid (53). Along the same line, HSD-fed mice presented increased intestinal inflammation in IL-$10^{-/-}$ mice (55) and increased severity of Dextran Sulfate Sodium (DSS)- and 2,4-Dinitrobenzene Sulfonic Acid (DNBS)-induced colitis (54). These studies not only indicate that dietary habits favoring WTD act locally inducing intestinal inflammation, but they also suggest a link between dietary regimes and extra-intestinal inflammation. Furthermore, recent

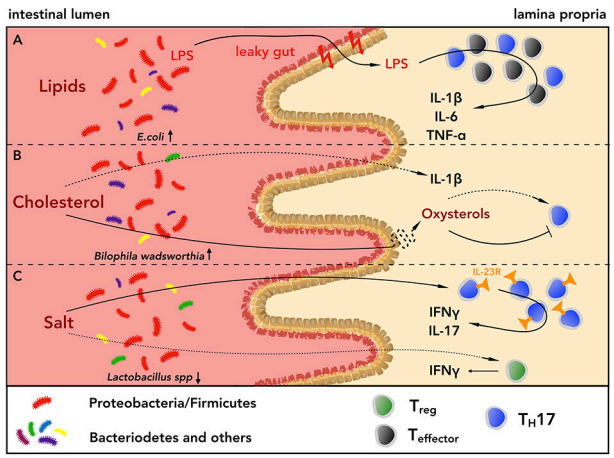

FIGURE 2 | Lipids, cholesterol and salt shape the intestinal CD4$^+$ T cell phenotype in a microbiota-dependent or independent-manner. **(A)** High intake of lipids induces dysbiosis, shifting the composition of the intestinal microbiota toward a higher ratio of *Proteobacteria* and *Firmicutes* to *Bacteroidetes*. This can lead to higher susceptibility to pathobiont infections, e.g. from invasive *E. coli* or *Bilophila wadsworthia* (BW). The mucus layers of both small intestine and colon get thinned, leading to higher gut permeability, which in turn favors the invasion of Gram-negative bacteria, and exacerbates intestinal inflammation. Furthermore, diets rich in saturated fatty acids increase the levels of pro-inflammatory cytokines, such as IL-1β, IL-6, TNF-α, within the gastrointestinal (GI) tract, contributing to the inflammatory state. **(B)** Diets rich in cholesterol alter the composition of bile acids and increase the levels of IL-1β in the small intestine, creating an inflammatory environment, which can lead to higher susceptibility to infections with BW, a pathogen known to require bile acids to outgrow. Cholesterol metabolites can also modulate intestinal inflammation inhibiting or promoting T_H17 cell development through interaction with LXR or RORγt, respectively. **(C)** Within the WTD-driven intestinal inflammation, salt can alter the phenotype of CD4$^+$ T_H17 and T_{reg} cells either directly or indirectly, worsening mucosal homeostasis. Via triggering of serum glucocorticoid kinase-1 (SGK1), salt drives the expression of IL-23R on T_H17 cells, inducing their pathogenicity, and it promotes IFN-γ secretion from T_{reg} cells, attenuating their suppressive capacities. Furthermore, high intake of salt can increase the frequencies of pathogenic T_H17 cells reducing the amount of *Lactobacillus* spp. within the GI tract.

TABLE 2 | Table showing effects that diet supplementation with defined bio-products can have on CD4$^+$ T cell phenotype and on intestinal and extra-intestinal inflammation.

Dietary supplements	Intestinal and extra-intestinal inflammation	Effects on CD4$^+$ T cells
SCFAs	↓ colitis (93) ↓ small intestinal tumors (*K-ras*^{G12Din}) (94)	↑ T_{reg} cells (93, 95) ↑ T_H17 cells (96)
Indoles/AhR ligands	↑ Resolution of inflammation (35)	↑ T_H17 cells (97) ↑ T_R1^{exTH17} cells (35)
Vitamin A	↓ DSS- or TNBS-induced colitis (98, 99)	↓ T_H17 cells ↑ T_{reg} cells (100, 101) ↑ LP T_{reg} cells (7, 102)
Vitamin D	↓ EAE (103) ↓ clinical symptoms in UC patients* (104)	↓ T_H17 cells (103)

AhR, Aryl hydrocarbon Receptor; DSS, Dextran Sulfate Sodium; EAE, Experimental Autoimmune Encephalomyelitis; LP, Lamina Propria; SCFAs, Short-Chain Fatty Acids; TNBS, 2,4,6-Trinitrobenzene Sulfonic Acid; UC, Ulcerative Colitis.
**The study showed no amelioration of inflammation.*

evidence in a pilot human study showed that 14 days high salt challenge increased the number of circulating IL-17A- and TNF-α-secreting T_H17 cells. This was associated with higher blood pressure, which is a risk factor for atherosclerosis (14). High salt consumption has also been described to induce IFN-γ secretion from human T_{reg} cells, inhibiting their suppressive function both *in vitro* and *in vivo* in a SGK1-dependent fashion (73). Taken together, these studies show that salt directly alters phenotype and effector functions of CD4$^+$ T cells in a microbiota-independent manner.

However, salt has also been reported to have a profound impact on the composition of intestinal microbiota, and only indirectly on the effector functions of intestinal T_H cells (91).

Two research groups have independently reported that high salt intake decreases the levels of *Lactobacillus spp*, ultimately favoring inflammation (14, 54). HSD-driven depletion of *Lactobacillus murinus* (*L. murinus*) increased the frequencies of LP T_H17 cells within small intestine and colon and *L. murinus* supplementation ameliorated EAE by reducing numbers of T_H17 cells within the spinal cord of mice fed with HSD (14). Importantly, colonization of GF mice either with SFB alone or SFB and *L. murinus* resulted in high or low frequencies of LP T_H17 cells, suggesting that *L. murinus* presence modulates T_H17 cells. These findings suggest that dietary habits influence T cell phenotype and their effector functions both in a microbiota-dependent and –independent fashion, determining whether they exhibit protective or pathogenic roles in intestinal immunity.

In addition, HSD has been reported to decrease luminal levels of indole-3-lactic acid (ILA) and butyrate (14, 54). Butyrate promotes the expression of Foxp3, stabilizing the LP T_{reg} phenotype, therefore, its reduction induced by HSD can alter intestinal homeostasis (92).

Taken together, favoring increased lipid, cholesterol and salt consumption leads to alterations of the composition of the intestinal microbiota that in turn affect phenotype and effector function of intestinal CD4$^+$ T cells. This can ultimately result in higher susceptibility to both intestinal and extra-intestinal infections and an increased risk of developing chronic autoimmune diseases.

Fiber, Indoles, and Vitamins

In this part of the review, we describe the effects that WTD-associated low contents of fiber, indoles and vitamins have on adaptive components of the intestinal immune system. Then we propose how supplementation of diets with defined bio-products of bacterial and dietary origin can restore the perturbed intestinal homeostasis. These findings are briefly summarized in **Table 2**.

Individuals with low bacterial species diversity have been shown to exhibit higher body mass index (BMI), serum triglyceride, hemoglobin A1c (HbA1c) and C-reactive protein levels as compared to those with higher diversity, indicating

a pivotal role of the intestinal microbiota in maintaining metabolic homeostasis (105, 106). Provision of fiber to the microbial community supports its species diversity (107) and diets low in dietary fiber have been associated with intestinal inflammation (108, 109). The beneficial effects of dietary fiber on mucosal homeostasis are graphically summarized in **Figure 3**. Deprivation of fiber induces proximity of intestinal bacteria to the epithelium by thinning of mucus layers (110), predisposing to pathogenic infections with *Citrobacter rodentium* (63). Along the same line, mice fed with fiber or inulin alone showed enrichment of SCFA-producing bacteria species, limiting *Clostridium difficile* growth, thus highlighting the therapeutic potential of fiber supplementation (111). In addition, *Kim et al.* showed that mice lacking the G-protein coupled receptor GPR43, one of the main receptors for SCFAs in the intestine, exhibited higher susceptibility to pathogenic infections, DSS-induced colitis and Azoxymethane (AOM)/DSS-induced carcinogenesis, all associated with increased frequencies of colonic LP T_H17 and decreased frequencies of T_{reg} cells (112, 113). Similarly, in a transfer-colitis model, SCFA supplementation ameliorated intestinal inflammation, increasing T_{reg} cell population in a GPR43-dependent manner (93). Among SCFAs, butyrate has indeed been shown to increase the generation of extra-thymic T_{reg} cells via promoting acetylation of the Foxp3 promoter and the conserved non-coding sequence 1 (CNS1), an enhancer element within Foxp3 locus (95). Butyrate supplementation protected also mice fed with HFD from developing spontaneous small intestinal tumors in the *K-ras*^{G12Din} model (94). Similarly, acetate administration increased the frequencies of IL-17-producing cells during an active immune response to *Citrobacter rodentium,* resulting in augmented bacterial clearance (96).

Taken together, these findings reveal not only the crucial role of SCFAs as mediators of intestinal immunity and mucosal homeostasis through their direct effect on CD4$^+$ T cells, but they indirectly point out also the importance of the presence of SCFA-producing bacteria species within the intestinal microbiota.

Besides being characterized by a low content of fiber, WTD is also poor in fruits and vegetables, which have been shown to have a positive impact on human health (114). Green vegetables, especially belonging to the genus *Brassica*, contain indoles that are converted to biologically active ligands of AhR (115). AhR is expressed by various intestinal cell types, including IELs, ILC3s and T_H17 cells (116, 117). Lack of AhR impairs expression of AMPs, increases gut permeability in DSS-induced colitis and exacerbates immune activation (115). Furthermore, AhR$^{-/-}$ mice exhibit reduction of IL-22-producing ILC3s, leading to higher fitness of SFB that in turn promotes intestinal T_H17 cells (117, 118). At the same time, the observation that AhR supports T_H17 cell differentiation through interaction with STAT1 (97), suggests that LP T_H17 cell development can be mediated by an AhR dependent mechanism. Interestingly, engagement of AhR via 6-Formylindolo[3,2-b]carbazole (FICZ) in *in vitro* differentiated T_H17 cells induces acquisition of IL-10, favoring their conversion into T_R1 cells. This indicates that AhR ligands could promote resolution of immune responses (35). It is therefore interesting to speculate that diets rich in green vegetables could limit intestinal inflammation of patients suffering from IBDs via AhR-driven conversion of pro-inflammatory T_H17 cells into regulatory T_R1 cells. However, this remains to be proven.

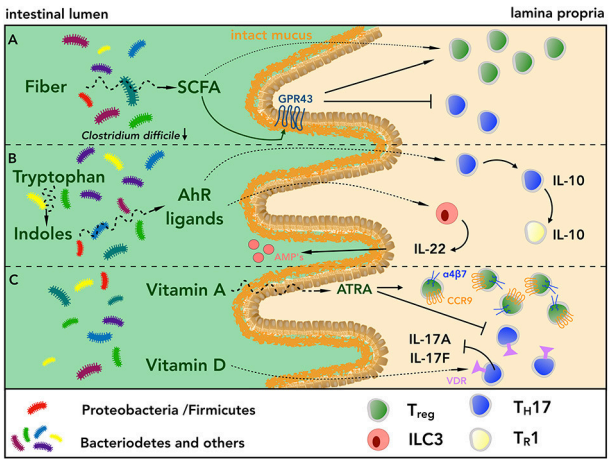

FIGURE 3 | Fiber, indoles and vitamins act in a synergistic fashion promoting intestinal homeostasis. **(A)** Diets rich in fiber promote bacterial species diversity and short chain fatty acid (SCFA)-producing bacteria, which in turn limit pathogen growth, e.g. *Clostridium difficile*, and promote barrier integrity. SCFAs also support intestinal homeostasis upon engagement of GPR43 expressed on intestinal epithelial cells. Furthermore, SCFAs can directly interact with intestinal CD4$^+$ T cells, shifting the balance of T_{reg}/T_H17 cells toward T_{reg} cells, thus inducing a regulatory microenvironment. **(B)** Within the lumen of the GI tract, the conversion of indoles to biologically active ligands of AhR contributes to maintain intestinal homeostasis acting on innate and adaptive immune cells. On the one hand, engagement of AhR by its ligands induces secretion of IL-22 from ILC3s, which in turn promotes the production of antimicrobial peptides (AMPs) from Paneth cells. On the other hand, triggering of AhR axis on T_H17 cells mediates their conversion into IL-10-secreting T_R1 cells, which are in turn able to terminate immune responses. **(C)** The biologically active form all-*trans* retinoic acid (ATRA) of vitamin A induces the expression of the gut homing receptors CCR9 and α4β7 integrin on T_{reg} cells, contributing to create a regulatory microenvironment within the GI tract, which is further augmented by the reduction of intestinal T_H17 cells. Similarly, engagement of VDR by active vitamin D metabolites dampens IL-17 secretion by T_H17 cells.

Differentiation and stability of the T_H17 cell phenotype can also be modulated by vitamins, especially A and D, the contents of which are reduced in WTD. Within the small intestine, dietary vitamin A is converted by CD103$^+$ DCs into the biologically active form all-*trans* retinoic acid (ATRA) (119, 120). The detailed roles of ATRA in shaping intestinal immunity have been reviewed elsewhere (121). Administration of ATRA has been shown to ameliorate intestinal inflammation in mice suffering from DSS- or TNBS-induced colitis (98, 99), likely by shifting the balance T_{reg}/T_H17 in favor of T_{reg} cells (100, 101). Addition of ATRA to TGF-β during T_{reg} cell differentiation has also been shown to augment their capacity to migrate to the LP (7) and their *in vivo* suppressive capacities in a murine model of transfer-colitis (100). Of note, mice fed with vitamin A-deficient diet (VAD) showed a substantial decrease in the number of LP CD4$^+$ T cells within the small intestine, due to the crucial role of vitamin A in mediating the induction of CCR9 and

α4β7 integrin, key gut homing molecules (102). Along the same line, *Tejon et al.* showed that during intestinal inflammation, *in vitro* differentiated T_{reg} cells were able to efficiently convert into T_H17 cells when transferred into VAD-fed mice, suggesting an anti-inflammatory effect of ATRA (122).

On the one hand, inducing a more regulatory environment within the inflamed mucosa of patients suffering from IBDs via vitamin A supplementation could seem tempting. On the other hand, however, translating its effects in the clinics has been shown to be problematic, and as for now there is no evidence showing beneficial effects of vitamin A supplementation for the health of IBD patients.

Similar to vitamin A, vitamin D content is low in WTD and polymorphisms in the vitamin D receptor (VDR) gene have been associated with higher incidence of IBDs (123). Of note, T_H17 cells can be sensitive to vitamin D levels given their expression of VDR. In line with this, high intake of vitamin D has been shown to dampen IL-17A and IL-17F secretion of T_H17 cells, ultimately ameliorating clinical manifestations of EAE (103). Similarly, it has been recently reported that clinical disease activity of patients with active ulcerative colitis (UC) improved after weekly supplementation of cholecalciferol (104). However, no changes in intestinal and systemic inflammation were observed, and other clinical trials involving vitamin D supplementation to patients suffering from IBDs did not show substantial improvement of clinical parameters (124).

Taken together, while these findings question a possible therapeutic role of vitamin supplementation alone in ameliorating intestinal inflammation, they highlight the potential of dietary components in modulating the $CD4^+$ T cell phenotype. Among them, SCFAs and AhR ligands could promote intestinal homeostasis and favor mucosal immunity. Translational studies are however required and will eventually shed the light on their efficacy in the clinics.

CONCLUSIONS AND PERSPECTIVES

Evidences on the impact of biologically active dietary components in modulating mucosal immunity and homeostasis are starting to emerge. Western dietary habits favoring high intake of lipids, cholesterol and salt promote local intestinal and extra-intestinal inflammation shaping phenotype and effector functions of $CD4^+$ T cells in a microbiota-dependent or –independent fashion. This can result in altered intestinal immunity, ultimately leading to higher susceptibility to infections caused by intestinal pathogens and increasing the risk for chronic inflammatory autoimmune diseases. The WTD-induced inflammatory state could, however, be potentially reverted by supplementing diets with food rich in fiber and indoles, which represent a promising therapeutic tool to modulate intestinal homeostasis by acting on the T_H17/T_{reg} cell axis and restoring SCFA-producing bacteria species.

AUTHOR CONTRIBUTIONS

FS and NS wrote the manuscript and prepared tables and figures. EV and SH edited the manuscript. NG supervised and edited the manuscript.

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REFERENCES

1. Korn T, Bettelli E, Oukka M, Kuchroo VK. IL-17 and Th17 cells. *Ann Rev Immunol.* (2009) 27:485–517. doi: 10.1146/annurev.immunol.021908.132710
2. Luckheeram RV, Zhou R, Verma AD, Xia B. $CD4^{(+)}$ T cells: differentiation and functions. *Clin Dev Immunol.* (2012) 2012:925135. doi: 10.1155/2012/925135
3. Mosmann TR, Coffman RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Ann Rev Immunol.* (1989) 7:145–73. doi: 10.1146/annurev.iy.07.040189.001045
4. Romagnani S. Th1 and Th2 in human diseases. *Clin Immunol Immunopathol.* (1996) 80:225–35. doi: 10.1006/clin.1996.0118
5. Ivanov II, Atarashi K, Manel N, Brodie EL, Shima T, Karaoz U, et al. Induction of intestinal Th17 cells by segmented filamentous bacteria. *Cell* (2009) 139:485–98. doi: 10.1016/j.cell.2009.09.033
6. Mazmanian SK, Liu CH, Tzianabos AO, Kasper DL. An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. *Cell* (2005) 122:107–18. doi: 10.1016/j.cell.2005.05.007
7. Cassani B, Villablanca EJ, Quintana FJ, Love PE, Lacy-Hulbert A, Blaner WS, et al. Gut-tropic T cells that express integrin alpha4beta7 and CCR9 are required for induction of oral immune tolerance in mice. *Gastroenterology* (2011) 141:2109–18. doi: 10.1053/j.gastro.2011.09.015
8. Coombes JL, Siddiqui KR, Arancibia-Carcamo CV, Hall J, Sun CM, Belkaid Y, et al. A functionally specialized population of mucosal $CD103^+$ DCs induces $Foxp3^+$ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. *J Exp Med.* (2007) 204:1757–64. doi: 10.1084/jem.20070590
9. Kim KS, Hong SW, Han D, Yi J, Jung J, Yang BG, et al. Dietary antigens limit mucosal immunity by inducing regulatory T cells in the small intestine. *Science* (2016) 351:858–63. doi: 10.1126/science.aac5560
10. El-Gabalawy H, Guenther LC, Bernstein CN. Epidemiology of immune-mediated inflammatory diseases: incidence, prevalence, natural history, and comorbidities. *J Rheumatol Suppl.* (2010) 85:2–10. doi: 10.3899/jrheum.091461
11. Teng MW, Bowman EP, McElwee JJ, Smyth MJ, Casanova JL, Cooper AM, et al. IL-12 and IL-23 cytokines: from discovery to targeted therapies for immune-mediated inflammatory diseases. *Nat Med.* (2015) 21:719–29. doi: 10.1038/nm.3895
12. Fujino S, Andoh A, Bamba S, Ogawa A, Hata K, Araki Y, et al. Increased expression of interleukin 17 in inflammatory bowel disease. *Gut* (2003) 52:65–70. doi: 10.1136/gut.52.1.65
13. Seiderer J, Elben I, Diegelmann J, Glas J, Stallhofer J, Tillack C, et al. Role of the novel Th17 cytokine IL-17F in inflammatory bowel disease (IBD): upregulated colonic IL-17F expression in active Crohn's disease and analysis of the IL17F p.His161Arg polymorphism in IBD. *Inflamm Bowel Dis.* (2008) 14:437–45. doi: 10.1002/ibd.20339
14. Wilck N, Matus MG, Kearney SM, Olesen SW, Forslund K, Bartolomaeus H, et al. Salt-responsive gut commensal modulates TH17 axis and disease. *Nature* (2017) 551:585–9. doi: 10.1038/nature24628

15. Wu C, Yosef N, Thalhamer T, Zhu C, Xiao S, Kishi Y, et al. Induction of pathogenic TH17 cells by inducible salt-sensing kinase SGK1. *Nature* (2013) 496:513–7. doi: 10.1038/nature11984
16. Chapman-Kiddell CA, Davies PS, Gillen L, Radford-Smith GL. Role of diet in the development of inflammatory bowel disease. *Inflamm Bowel Dis.* (2010) 16:137–51. doi: 10.1002/ibd.20968
17. Cani PD, Bibiloni R, Knauf C, Waget A, Neyrinck AM, Delzenne NM, et al. Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. *Diabetes* (2008) 57:1470–81. doi: 10.2337/db07-1403
18. Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* (2006) 444:1027–31. doi: 10.1038/nature05414
19. Zhang C, Zhang M, Pang X, Zhao Y, Wang L, Zhao L. Structural resilience of the gut microbiota in adult mice under high-fat dietary perturbations. *ISME J.* (2012) 6:1848–57. doi: 10.1038/ismej.2012.27
20. Cox AJ, West NP, Cripps AW. Obesity, inflammation, and the gut microbiota. *Lancet Diabetes Endocrinol.* (2015) 3:207–15. doi: 10.1016/S2213-8587(14)70134-2
21. Frank DN, Robertson CE, Hamm CM, Kpadeh Z, Zhang T, Chen H, et al. Disease phenotype and genotype are associated with shifts in intestinal-associated microbiota in inflammatory bowel diseases. *Inflamm Bowel Dis.* (2011) 17:179–84. doi: 10.1002/ibd.21339
22. Maeda Y, Kurakawa T, Umemoto E, Motooka D, Ito Y, Gotoh K, et al. Dysbiosis contributes to arthritis development via activation of autoreactive T cells in the intestine. *Arthritis Rheumatol.* (2016) 68:2646–61. doi: 10.1002/art.39783
23. Ott SJ, Musfeldt M, Wenderoth DF, Hampe J, Brant O, Folsch UR, et al. Reduction in diversity of the colonic mucosa associated bacterial microflora in patients with active inflammatory bowel disease. *Gut* (2004) 53:685–93. doi: 10.1136/gut.2003.025403
24. Pelaseyed T, Bergstrom JH, Gustafsson JK, Ermund A, Birchenough GM, Schutte A, et al. The mucus and mucins of the goblet cells and enterocytes provide the first defense line of the gastrointestinal tract and interact with the immune system. *Immunol Rev.* (2014) 260:8–20. doi: 10.1111/imr.12182
25. Cella M, Fuchs A, Vermi W, Facchetti F, Otero K, Lennerz JK, et al. A human natural killer cell subset provides an innate source of IL-22 for mucosal immunity. *Nature* (2009) 457:722–5. doi: 10.1038/nature07537
26. Takatori H, Kanno Y, Watford WT, Tato CM, Weiss G, Ivanov II, et al. Lymphoid tissue inducer-like cells are an innate source of IL-17 and IL-22. *J Exp Med.* (2009) 206:35–41. doi: 10.1084/jem.20072713
27. Ivanov II, Frutos Rde L, Manel N, Yoshinaga K, Rifkin DB, Sartor RB, et al. Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine. *Cell Host Microbe* (2008) 4:337–49. doi: 10.1016/j.chom.2008.09.009
28. Annunziato F, Cosmi L, Liotta F, Maggi E, Romagnani S. The phenotype of human Th17 cells and their precursors, the cytokines that mediate their differentiation and the role of Th17 cells in inflammation. *Int Immunol.* (2008) 20:1361–8. doi: 10.1093/intimm/dxn106
29. Veldhoen M, Hirota K, Westendorf AM, Buer J, Dumoutier L, Renauld JC, et al. The aryl hydrocarbon receptor links TH17-cell-mediated autoimmunity to environmental toxins. *Nature* (2008) 453:106–9. doi: 10.1038/nature06881
30. Hueber W, Sands BE, Lewitzky S, Vandemeulebroecke M, Reinisch W, Higgins PD, et al. Secukinumab, a human anti-IL-17A monoclonal antibody, for moderate to severe Crohn's disease: unexpected results of a randomised, double-blind placebo-controlled trial. *Gut* (2012) 61:1693–700. doi: 10.1136/gutjnl-2011-301668
31. Lee JS, Tato CM, Joyce-Shaikh B, Gulen MF, Cayatte C, Chen Y, et al. Interleukin-23-Independent IL-17 Production Regulates Intestinal Epithelial Permeability. *Immunity* (2015) 43:727–38. doi: 10.1016/j.immuni.2015.09.003
32. O'Connor WJr, Kamanaka M, Booth CJ, Town T, Nakae S, Iwakura Y, et al. A protective function for interleukin 17A in T cell-mediated intestinal inflammation. *Nat Immunol.* (2009) 10:603–9. doi: 10.1038/ni.1736
33. Annunziato F, Cosmi L, Santarlasci V, Maggi L, Liotta F, Mazzinghi B, et al. Phenotypic and functional features of human Th17 cells. *J Exp Med.* (2007) 204:1849–61. doi: 10.1084/jem.20070663
34. Tanaka K, Martinez GJ, Yan X, Long W, Ichiyama K, Chi X, et al. Regulation of pathogenic T Helper 17 cell differentiation by steroid receptor coactivator-3. *Cell Rep.* (2018) 23:2318–29. doi: 10.1016/j.celrep.2018.04.088
35. Gagliani N, Amezcua Vesely MC, Iseppon A, Brockmann L, Xu H, Palm NW, et al. Th17 cells transdifferentiate into regulatory T cells during resolution of inflammation. *Nature* (2015) 523:221–5. doi: 10.1038/nature14452
36. Heinemann C, Heink S, Petermann F, Vasanthakumar A, Rothhammer V, Doorduijn E, et al. IL-27 and IL-12 oppose pro-inflammatory IL-23 in $CD4^+$ T cells by inducing Blimp1. *Nat Commun.* (2014) 5:3770. doi: 10.1038/ncomms4770
37. Gagliani N, Magnani CF, Huber S, Gianolini ME, Pala M, Licona-Limon P, et al. Coexpression of CD49b and LAG-3 identifies human and mouse T regulatory type 1 cells. *Nat Med.* (2013) 19:739–46. doi: 10.1038/nm.3179
38. Huber S, Gagliani N, Esplugues E, O'Connor WJr, Huber FJ, Chaudhry A, et al. Th17 cells express interleukin-10 receptor and are controlled by Foxp3(-) and $Foxp3^+$ regulatory $CD4^+$ T cells in an interleukin-10-dependent manner. *Immunity* (2011) 34:554–65. doi: 10.1016/j.immuni.2011.01.020
39. Gill SR, Pop M, Deboy RT, Eckburg PB, Turnbaugh PJ, Samuel BS, et al. Metagenomic analysis of the human distal gut microbiome. *Science* (2006) 312:1355–9. doi: 10.1126/science.1124234
40. Li J, Jia H, Cai X, Zhong H, Feng Q, Sunagawa S, et al. An integrated catalog of reference genes in the human gut microbiome. *Nat Biotechnol.* (2014) 32:834–41. doi: 10.1038/nbt.2942
41. Sender R, Fuchs S, Milo R. Revised estimates for the number of human and bacteria cells in the body. *PLoS Biol.* (2016) 14:e1002533. doi: 10.1371/journal.pbio.1002533
42. Backhed F, Ley RE, Sonnenburg JL, Peterson DA, Gordon JI. Host-bacterial mutualism in the human intestine. *Science* (2005) 307:1915–20. doi: 10.1126/science.1104816
43. Belkaid Y, Hand TW. Role of the microbiota in immunity and inflammation. *Cell* (2014) 157:121–41. doi: 10.1016/j.cell.2014.03.011
44. Hill DA, Artis D. Intestinal bacteria and the regulation of immune cell homeostasis. *Ann Rev Immunol.* (2010) 28:623–67. doi: 10.1146/annurev-immunol-030409-101330
45. Inagaki H, Suzuki T, Nomoto K, Yoshikai Y. Increased susceptibility to primary infection with Listeria monocytogenes in germfree mice may be due to lack of accumulation of L-selectin$^+$ $CD44^+$ T cells in sites of inflammation. *Infect Immunity* (1996) 64:3280–7.
46. Zachar Z, Savage DC. Microbial interference and colonization of the murine gastrointestinal tract by Listeria monocytogenes. *Infect Immunity* (1979) 23:168–74.
47. Kuhn R, Lohler J, Rennick D, Rajewsky K, Muller W. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* (1993) 75:263–74. doi: 10.1016/0092-8674(93)80068-P
48. Peloquin JM, Goel G, Villablanca EJ, Xavier RJ. Mechanisms of pediatric inflammatory bowel disease. *Ann Rev Immunol.* (2016) 34:31–64. doi: 10.1146/annurev-immunol-032414-112151
49. Sorini C, Cardoso RF, Gagliani N, Villablanca EJ. Commensal bacteria-specific $CD4^+$ T cell responses in health and disease. *Front Immunol.* (2018) 9:2667. doi: 10.3389/fimmu.2018.02667
50. Amre DK, D'Souza S, Morgan K, Seidman G, Lambrette P, Grimard G, et al. Imbalances in dietary consumption of fatty acids, vegetables, and fruits are associated with risk for Crohn's disease in children. *Am J Gastroenterol.* (2007) 102:2016–25. doi: 10.1111/j.1572-0241.2007.01411.x
51. Loftus EV Jr. (2004). Clinical epidemiology of inflammatory bowel disease: incidence, prevalence, and environmental influences. *Gastroenterology* 126, 1504–1517. doi: 10.1053/j.gastro.2004.01.063
52. Nystrom M, Mutanen M. Diet and epigenetics in colon cancer. *World J Gastroenterol.* (2009) 15:257–63. doi: 10.3748/wjg.15.257
53. Haghikia A, Jorg S, Duscha A, Berg J, Manzel A, Waschbisch A, et al. Dietary fatty acids directly impact central nervous system autoimmunity via the small intestine. *Immunity* (2015) 43:817–29. doi: 10.1016/j.immuni.2015.09.007
54. Miranda PM, De Palma G, Serkis V, Lu J, Louis-Auguste MP, McCarville JL, et al. High salt diet exacerbates colitis in mice by decreasing *Lactobacillus* levels and butyrate production. *Microbiome* (2018) 6:57. doi: 10.1186/s40168-018-0433-4

55. Tubbs AL, Liu B, Rogers TD, Sartor RB, Miao EA. Dietary salt exacerbates experimental colitis. *J Immunol.* (2017) 199, 1051–1059. doi: 10.4049/jimmunol.1700356
56. Harris PJ, Ferguson LR. Dietary fibre: its composition and role in protection against colorectal cancer. *Mutation Res.* (1993) 290:97–110. doi: 10.1016/0027-5107(93)90037-G
57. Hou JK, Abraham B, El-Serag H. Dietary intake and risk of developing inflammatory bowel disease: a systematic review of the literature. *Am J Gastroenterol.* (2011) 106:563–73. doi: 10.1038/ajg.2011.44
58. Meyer KA, Kushi LH, Jacobs DRJr, Slavin J, Sellers TA, Folsom AR. Carbohydrates, dietary fiber, and incident type 2 diabetes in older women. *Am J Clin Nutr.* (2000) 71:921–30. doi: 10.1093/ajcn/71.4.921
59. Wannamethee SG, Whincup PH, Thomas MC, Sattar N. Associations between dietary fiber and inflammation, hepatic function, and risk of type 2 diabetes in older men: potential mechanisms for the benefits of fiber on diabetes risk. *Diabetes Care* (2009) 32:1823–5. doi: 10.2337/dc09-0477
60. Flint HJ, Duncan SH, Scott KP, Louis P. Links between diet, gut microbiota composition and gut metabolism. *Proc Nutr Soc.* (2015) 74:13–22. doi: 10.1017/S0029665114001463
61. Miller TL, Wolin MJ. Pathways of acetate, propionate, and butyrate formation by the human fecal microbial flora. *Appl Environ Microbiol.* (1996) 62:1589–92.
62. Le Poul E, Loison C, Struyf S, Springael JY, Lannoy V, Decobecq ME, et al. Functional characterization of human receptors for short chain fatty acids and their role in polymorphonuclear cell activation. *J Biol Chem.* (2003) 278:25481–9. doi: 10.1074/jbc.M301403200
63. Desai MS, Seekatz AM, Koropatkin NM, Kamada N, Hickey CA, Wolter M, et al. A dietary fiber-deprived gut microbiota degrades the colonic mucus barrier and enhances pathogen susceptibility. *Cell* (2016) 167:1339–53.e1321. doi: 10.1016/j.cell.2016.10.043
64. Kim KA, Gu W, Lee IA, Joh EH, Kim DH. High fat diet-induced gut microbiota exacerbates inflammation and obesity in mice via the TLR4 signaling pathway. *PLoS ONE* (2012) 7:e47713. doi: 10.1371/journal.pone.0047713
65. Liu Z, Brooks RS, Ciappio ED, Kim SJ, Crott JW, Bennett G, et al. Diet-induced obesity elevates colonic TNF-alpha in mice and is accompanied by an activation of Wnt signaling: a mechanism for obesity-associated colorectal cancer. *J Nutr Biochem.* (2012) 23:1207–13. doi: 10.1016/j.jnutbio.2011.07.002
66. Murakami Y, Tanabe S, Suzuki T. High-fat diet-induced intestinal hyperpermeability is associated with increased bile acids in the large intestine of mice. *J Food Sci.* (2016) 81:H216–222. doi: 10.1111/1750-3841.13166
67. Gulhane M, Murray L, Lourie R, Tong H, Sheng YH, Wang R, et al. High fat diets induce colonic epithelial cell stress and inflammation that is reversed by IL-22. *Sci Rep.* (2016) 6:28990. doi: 10.1038/srep28990
68. Schroeder BO, Birchenough GMH, Stahlman M, Arike L, Johansson MEV, Hansson GC, et al. Bifidobacteria or fiber protects against diet-induced microbiota-mediated colonic mucus deterioration. *Cell Host Microbe* (2018) 23:27–40.e27. doi: 10.1016/j.chom.2017.11.004
69. Devkota S, Wang Y, Musch MW, Leone V, Fehlner-Peach H, Nadimpalli A, et al. Dietary-fat-induced taurocholic acid promotes pathobiont expansion and colitis in Il10-/- mice. *Nature* (2012) 487:104–8. doi: 10.1038/nature11225
70. Hildebrandt MA, Hoffmann C, Sherrill-Mix SA, Keilbaugh SA, Hamady M, Chen YY, et al. High-fat diet determines the composition of the murine gut microbiome independently of obesity. *Gastroenterology* (2009) 137:1716–24.e1711–1712. doi: 10.1053/j.gastro.2009.08.042
71. Agus A, Denizot J, Thevenot J, Martinez-Medina M, Massier S, Sauvanet P, et al. Western diet induces a shift in microbiota composition enhancing susceptibility to Adherent-Invasive E. *coli* infection and intestinal inflammation. *Sci Rep.* (2016) 6:19032. doi: 10.1038/srep19032
72. Martinez-Medina M, Denizot J, Dreux N, Robin F, Billard E, Bonnet R, et al. Western diet induces dysbiosis with increased *E coli* in CEABAC10 mice, alters host barrier function favouring AIEC colonisation. *Gut* (2014) 63:116–24. doi: 10.1136/gutjnl-2012-304119
73. Hernandez AL, Kitz A, Wu C, Lowther DE, Rodriguez DM, Vudattu N, et al. Sodium chloride inhibits the suppressive function of FOXP3^{+} regulatory T cells. *J Clin Invest.* (2015) 125:4212–22. doi: 10.1172/JCI81151
74. Progatzky F, Sangha NJ, Yoshida N, McBrien M, Cheung J, Shia A, et al. Dietary cholesterol directly induces acute inflammasome-dependent intestinal inflammation. *Nat Commun.* (2014) 5:5864. doi: 10.1038/ncomms6864
75. Ito A, Hong C, Oka K, Salazar JV, Diehl C, Witztum JL, et al. Cholesterol accumulation in CD11c$^{(+)}$ immune cells is a causal and targetable factor in autoimmune disease. *Immunity* (2016) 45:1311–26. doi: 10.1016/j.immuni.2016.11.008
76. Borst SE, Conover CF. High-fat diet induces increased tissue expression of TNF-alpha. *Life Sci.* (2005) 77:2156–65. doi: 10.1016/j.lfs.2005.03.021
77. Morin CL, Eckel RH, Marcel T, Pagliassotti MJ. High fat diets elevate adipose tissue-derived tumor necrosis factor-alpha activity. *Endocrinology* (1997) 138:4665–71. doi: 10.1210/endo.138.11.5519
78. Chassaing B, Srinivasan G, Delgado MA, Young AN, Gewirtz AT, Vijay-Kumar M. Fecal lipocalin 2, a sensitive and broadly dynamic non-invasive biomarker for intestinal inflammation. *PLoS ONE* (2012) 7:e44328. doi: 10.1371/journal.pone.0044328
79. Cani PD, Amar J, Iglesias MA, Poggi M, Knauf C, Bastelica D, et al. Metabolic endotoxemia initiates obesity and insulin resistance. *Diabetes* (2007) 56:1761–72. doi: 10.2337/db06-1491
80. Pendyala S, Walker JM, Holt PR. A high-fat diet is associated with endotoxemia that originates from the gut. *Gastroenterology* (2012) 142:1100–1101.e1102. doi: 10.1053/j.gastro.2012.01.034
81. Soroosh P, Wu J, Xue X, Song J, Sutton SW, Sablad M, et al. Oxysterols are agonist ligands of RORgammat and drive Th17 cell differentiation. *Proc Natl Acad Sci USA.* (2014) 111:12163–8. doi: 10.1073/pnas.1322807111
82. Cui G, Qin X, Wu L, Zhang Y, Sheng X, Yu Q, et al. Liver X receptor (LXR) mediates negative regulation of mouse and human Th17 differentiation. *J Clin Invest.* (2011) 121:658–70. doi: 10.1172/JCI42974
83. Fromm H, Malavolti M. Bile acid-induced diarrhoea. *Clin Gastroenterol.* (1986) 15:567–82.
84. Feng Z, Long W, Hao B, Ding D, Ma X, Zhao L, et al. A human stool-derived Bilophila wadsworthia strain caused systemic inflammation in specific-pathogen-free mice. *Gut Pathog.* (2017) 9:59. doi: 10.1186/s13099-017-0208-7
85. Baron EJ, Summanen P, Downes J, Roberts MC, Wexler H, Finegold SM. Bilophila wadsworthia, gen. nov and sp nov, a unique gram-negative anaerobic rod recovered from appendicitis specimens and human faeces. *J General Microbiol.* (1989) 135:3405–11.
86. Manichanh C, Rigottier-Gois L, Bonnaud E, Gloux K, Pelletier E, Frangeul L, et al. Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach. *Gut* (2006) 55:205–11. doi: 10.1136/gut.2005.073817
87. Aggarwal S, Ghilardi N, Xie MH, de Sauvage FJ, Gurney AL. Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. *J Biol Chem.* (2003) 278:1910–4. doi: 10.1074/jbc.M207577200
88. Zhou L, Ivanov II, Spolski R, Min R, Shenderov K, Egawa T, et al. IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. *Nat Immunol.* (2007) 8:967–74. doi: 10.1038/ni1488
89. Kuntzsch D, Bergann T, Dames P, Fromm A, Fromm M, Davis RA, et al. The plant-derived glucocorticoid receptor agonist Endiandrin A acts as co-stimulator of colonic epithelial sodium channels (ENaC) via SGK-1 and MAPKs. *PLoS ONE* (2012) 7:e49426. doi: 10.1371/journal.pone.0049426
90. Wulff P, Vallon V, Huang DY, Volkl H, Yu F, Richter K, et al. Impaired renal Na$^{(+)}$ retention in the sgk1-knockout mouse. *J Clin Invest.* (2002) 110:1263–8. doi: 10.1172/JCI0215696
91. Yang Y, Torchinsky MB, Gobert M, Xiong H, Xu M, Linehan JL, et al. Focused specificity of intestinal TH17 cells towards commensal bacterial antigens. *Nature* (2014) 510:152–6. doi: 10.1038/nature13279
92. Furusawa Y, Obata Y, Fukuda S, Endo TA, Nakato G, Takahashi D, et al. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. *Nature* (2013) 504:446–50. doi: 10.1038/nature12721
93. Smith PM, Howitt MR, Panikov N, Michaud M, Gallini CA, Bohlooly YM, et al. The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. *Science* (2013) 341:569–73. doi: 10.1126/science.1241165

94. Schulz MD, Atay C, Heringer J, Romrig FK, Schwitalla S, Aydin B, et al. High-fat-diet-mediated dysbiosis promotes intestinal carcinogenesis independently of obesity. *Nature* (2014) 514:508–12. doi: 10.1038/nature13398
95. Arpaia N, Campbell C, Fan X, Dikiy S, van der Veeken J, deRoos P, et al. Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. *Nature* (2013) 504:451–5. doi: 10.1038/nature12726
96. Park J, Kim M, Kang SG, Jannasch AH, Cooper B, Patterson J, et al. Short-chain fatty acids induce both effector and regulatory T cells by suppression of histone deacetylases and regulation of the mTOR-S6K pathway. *Mucosal Immunol.* (2015) 8:80–93. doi: 10.1038/mi.2014.44
97. Kimura A, Naka T, Nohara K, Fujii-Kuriyama Y, Kishimoto T. Aryl hydrocarbon receptor regulates Stat1 activation and participates in the development of Th17 cells. *Proc Natl Acad Sci USA*. (2008) 105:9721–6. doi: 10.1073/pnas.0804231105
98. Bai A, Lu N, Guo Y, Liu Z, Chen J, Peng Z. All-trans retinoic acid down-regulates inflammatory responses by shifting the Treg/Th17 profile in human ulcerative and murine colitis. *J Leukocyte Biol.* (2009) 86:959–69. doi: 10.1189/jlb.0109006
99. Hong K, Zhang Y, Guo Y, Xie J, Wang J, He X, et al. All-trans retinoic acid attenuates experimental colitis through inhibition of NF-kappaB signaling. *Immunol Lett.* (2014) 162:34–40. doi: 10.1016/j.imlet.2014.06.011
100. Mucida D, Park Y, Kim G, Turovskaya O, Scott I, Kronenberg M, et al. Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid. *Science* (2007) 317:256–60. doi: 10.1126/science.1145697
101. Xiao S, Jin H, Korn T, Liu SM, Oukka M, Lim B, et al. Retinoic acid increases Foxp3^{+} regulatory T cells and inhibits development of Th17 cells by enhancing TGF-beta-driven Smad3 signaling and inhibiting IL-6 and IL-23 receptor expression. *J Immunol.* (2008) 181, 2277–84. doi: 10.4049/jimmunol.181.4.2277
102. Iwata M, Hirakiyama A, Eshima Y, Kagechika H, Kato C, Song SY. Retinoic acid imprints gut-homing specificity on T cells. *Immunity* (2004) 21:527–38. doi: 10.1016/j.immuni.2004.08.011
103. Chang SH, Chung Y, Dong C. Vitamin D suppresses Th17 cytokine production by inducing C/EBP homologous protein (CHOP) expression. *J Biol Chem.* (2010) 285:38751–5. doi: 10.1074/jbc.C110.185777
104. Garg M, Hendy P, Ding JN, Shaw S, Hold G, Hart A. The effect of vitamin D on intestinal inflammation and faecal microbiota in patients with ulcerative colitis. *J Crohn's Colitis* (2018) 12:963–972. doi: 10.1093/ecco-jcc/jjy052
105. Cotillard A, Kennedy SP, Kong LC, Prifti E, Pons N, Le Chatelier E, et al. Dietary intervention impact on gut microbial gene richness. *Nature* (2013) 500:585–8. doi: 10.1038/nature12480
106. Le Chatelier E, Nielsen T, Qin J, Prifti E, Hildebrand F, Falony G, et al. Richness of human gut microbiome correlates with metabolic markers. *Nature* (2013) 500:541–6. doi: 10.1038/nature12506
107. Bourquin LD, Titgemeyer EC, Fahey GCJr. Vegetable fiber fermentation by human fecal bacteria: cell wall polysaccharide disappearance and short-chain fatty acid production during *in vitro* fermentation and water-holding capacity of unfermented residues. *J Nutr.* (1993) 123:860–9. doi: 10.1093/jn/123.5.860
108. Burkitt DP. Epidemiology of large bowel disease: the role of fibre. *Proc Nutr Soc.* (1973) 32:145–9. doi: 10.1079/PNS19730032
109. Rodriguez-Cabezas ME, Galvez J, Camuesco D, Lorente MD, Concha A, Martinez-Augustin O, et al. Intestinal anti-inflammatory activity of dietary fiber (Plantago ovata seeds) in HLA-B27 transgenic rats. *Clin Nutr.* (2003) 22:463–71. doi: 10.1016/S0261-5614(03)00045-1
110. Earle KA, Billings G, Sigal M, Lichtman JS, Hansson GC, Elias JE, et al. Quantitative imaging of gut microbiota spatial organization. *Cell Host Microbe* (2015) 18:478–88. doi: 10.1016/j.chom.2015.09.002
111. Hryckowian AJ, Van Treuren W, Smits SA, Davis NM, Gardner JO, Bouley DM, et al. Microbiota-accessible carbohydrates suppress *Clostridium difficile* infection in a murine model. *Nat Microbiol.* (2018) 3:662–9. doi: 10.1038/s41564-018-0150-6
112. Kim M, Friesen L, Park J, Kim HM, Kim CH. Microbial metabolites, short-chain fatty acids, restrain tissue bacterial load, chronic inflammation, and associated cancer in the colon of mice. *Eur J Immunol.* (2018) 48:1235–47. doi: 10.1002/eji.201747122
113. Kim MH, Kang SG, Park JH, Yanagisawa M, Kim CH. Short-chain fatty acids activate GPR41 and GPR43 on intestinal epithelial cells to promote inflammatory responses in mice. *Gastroenterology* (2013) 145:396–406.e391–e310. doi: 10.1053/j.gastro.2013.04.056
114. D'Souza S, Levy E, Mack D, Israel D, Lambrette P, Ghadirian P, et al. Dietary patterns and risk for Crohn's disease in children. *Inflamm Bowel Dis.* (2008) 14:367–73. doi: 10.1002/ibd.20333
115. Li Y, Innocentin S, Withers DR, Roberts NA, Gallagher AR, Grigorieva EF, et al. Exogenous stimuli maintain intraepithelial lymphocytes via aryl hydrocarbon receptor activation. *Cell* (2011) 147:629–40. doi: 10.1016/j.cell.2011.09.025
116. Nakahama T, Hanieh H, Nguyen NT, Chinen I, Ripley B, Millrine D, et al. Aryl hydrocarbon receptor-mediated induction of the microRNA-132/212 cluster promotes interleukin-17-producing T-helper cell differentiation. *Proc Natl Acad Sci USA*. (2013) 110:11964–9. doi: 10.1073/pnas.1311087110
117. Qiu J, Heller JJ, Guo X, Chen ZM, Fish K, Fu YX, et al. The aryl hydrocarbon receptor regulates gut immunity through modulation of innate lymphoid cells. *Immunity* (2012) 36:92–104. doi: 10.1016/j.immuni.2011.11.011
118. Qiu J, Guo X, Chen ZM, He L, Sonnenberg GF, Artis D, et al. Group 3 innate lymphoid cells inhibit T-cell-mediated intestinal inflammation through aryl hydrocarbon receptor signaling and regulation of microflora. *Immunity* (2013) 39:386–99. doi: 10.1016/j.immuni.2013.08.002
119. Harrison EH. Mechanisms involved in the intestinal absorption of dietary vitamin A and provitamin A carotenoids. *Biochim Biophys Acta* (2012) 1821:70–7. doi: 10.1016/j.bbalip.2011.06.002
120. Villablanca EJ, Wang S, de Calisto J, Gomes DC, Kane MA, Napoli JL, et al. MyD88 and retinoic acid signaling pathways interact to modulate gastrointestinal activities of dendritic cells. *Gastroenterology* (2011) 141:176–85. doi: 10.1053/j.gastro.2011.04.010
121. Czarnewski P, Das S, Parigi SM, Villablanca EJ. Retinoic acid and its role in modulating intestinal innate immunity. *Nutrients* (2017) 9:68. doi: 10.3390/nu9010068
122. Tejon G, Manriquez V, De Calisto J, Flores-Santibanez F, Hidalgo Y, Crisostomo N, et al. Vitamin a impairs the reprogramming of Tregs into IL-17-producing cells during intestinal inflammation. *BioMed Res Int.* (2015) 2015:137893. doi: 10.1155/2015/137893
123. Simmons JD, Mullighan C, Welsh KI, Jewell DP. Vitamin D receptor gene polymorphism: association with Crohn's disease susceptibility. *Gut* (2000) 47:211–4. doi: 10.1136/gut.47.2.211
124. Ardesia M, Ferlazzo G, Fries W. Vitamin D and inflammatory bowel disease. *BioMed Res Int.* (2015) 2015:470805. doi: 10.1155/2015/470805

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Reduced CD27$^-$IgD$^-$ B Cells in Blood and Raised CD27$^-$IgD$^-$ B Cells in Gut-Associated Lymphoid Tissue in Inflammatory Bowel Disease

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The intestinal mucosa in inflammatory bowel disease (IBD) contains increased frequencies of lymphocytes and a disproportionate increase in plasma cells secreting immunoglobulin (Ig)G relative to other isotypes compared to healthy controls. Despite consistent evidence of B lineage cells in the mucosa in IBD, little is known of B cell recruitment to the gut in IBD. Here we analyzed B cells in blood of patients with Crohn's disease (CD) and ulcerative colitis (UC) with a range of disease activities. We analyzed the frequencies of known B cell subsets in blood and observed a consistent reduction in the proportion of CD27$^-$IgD$^-$ B cells expressing all Ig isotypes in the blood in IBD (independent of severity of disease and treatment) compared to healthy controls. Successful treatment of patients with biologic therapies did not change the profile of B cell subsets in blood. By mass cytometry we demonstrated that CD27$^-$IgD$^-$ B cells were proportionately enriched in the gut-associated lymphoid tissue (GALT) in IBD. Since production of TNFα is a feature of IBD relevant to therapies, we sought to determine whether B cells in GALT or the CD27$^-$IgD$^-$ subset in particular could contribute to pathology by secretion of TNFα or IL-10. We found that donor matched GALT and blood B cells are capable of producing TNFα as well as IL-10, but we saw no evidence that CD27$^-$IgD$^-$ B cells from blood expressed more TNFα compared to other subsets. The reduced proportion of CD27$^-$IgD$^-$ B cells in blood and the increased proportion in the gut implies that CD27$^-$IgD$^-$ B cells are recruited from the blood to the gut in IBD. CD27$^-$IgD$^-$ B cells have been implicated in immune responses to intestinal bacteria and recruitment to GALT, and may contribute to the intestinal inflammatory milieu in IBD.

Keywords: memory B cells, inflammatory bowel disease, GALT, mass cytometry, biologics, ustekinumab, infliximab

INTRODUCTION

Inflammatory bowel disease (IBD) encompasses two clinical entities: Crohn's disease (CD) and ulcerative colitis (UC). Both are chronic debilitating diseases characterized by relapsing intestinal inflammation. While the exact etiology of both diseases is still not clearly understood, current literature suggests that an aberrant immune response to the intestinal flora contributes to pathology in genetically susceptible individuals (1–3). The evidence for this is however stronger for CD than for UC (4), which can be associated with autoimmune conditions like primary sclerosing cholangitis (PSC) in a small subgroup of patients (5). CD is a condition hallmarked by granulomatous transmural inflammation that can affect the entire gastrointestinal tract and is frequently complicated by fistulae and strictures. In contrast, UC is predominantly a disease affecting the rectum and distal colon, where it manifests as ulceration limited to the mucosal layer. However, rectum-sparing forms of UC are known to exist and in rare cases the pathology can extend into the terminal ileum (6, 7). Whilst big strides have been made in developing biologic therapies for both diseases, a significant proportion of patients with CD and UC remain treatment refractory, highlighting the unmet need for a better understanding of the mechanisms contributing to the pathogenesis of both diseases (8, 9).

Most immunological research in CD and UC has focused on T lymphocytes and macrophages that infiltrate the mucosa in active disease (10–12). Th1 and Th17 cells have been implicated especially in the pathogenesis of CD, whilst their role in UC is still not fully understood (13, 14). UC is also characterized by the infiltration of neutrophils into the inflamed mucosa, where they are proposed to have a pathogenic role by the maintenance of inflammation (15). However, in both CD and UC, a significant increase in the number of plasma cells in the lamina propria has been observed and linked to disease pathogenesis (16–18).

The gut-associated lymphoid tissue (GALT) comprises the appendix, Peyer's patches in the ileum and isolated lymphoid follicles in the small and large intestine. GALT is the inductive site for the generation of protective immune responses against enteric pathogens as well as the tolerogenic response to commensal species (19). Interestingly, the appendicectomy of the (inflamed) appendix at a young age has been correlated with a reduced risk of developing UC later in life (20). The underlying mechanism of this protective effect is unknown; however, it strongly suggests that an aberrant immune response in the appendix can predispose to UC. In addition, several reports have described appendiceal inflammation in patients with UC affecting only the distal parts of the colon (21, 22). Based on these studies that suggest that plasma cells, B cells and GALT could be implicated in the development of IBD, we sought to study the B cell populations in blood and GALT of CD and UC patients. In addition, we aimed to study the gut-homing capacity of the different populations in blood by investigating the expression of the β7 subunit of α4β7 integrin in active disease vs. remission. We further looked at how the three main biologic treatments for IBD, the anti-TNF treatment infliximab, the anti-α4β7 integrin treatment vedolizumab, and the anti-IL12/IL23 p40 antibody ustekinumab affect peripheral B cell subsets in IBD. While several B cell aberrations in blood and GALT in IBD were observed, we focus this report on the poorly understood $CD27^{-}IgD^{-}$ population that are likely to be unconventional memory B cells (23–27), that we found to be consistently depleted in CD and UC blood, but enriched in GALT.

Abbreviations: IBD, inflammatory bowel disease; UC, ulcerative colitis; CD, Crohn's disease; HC, healthy control; CRP, C-reactive peptide; GALT, gut-associated lymphoid tissue.

MATERIALS AND METHODS

Sample Collection

All recruited IBD patients had a confirmed diagnosis of either CD or UC and took part with informed written consent. The study was approved by the local ethics committee (REC 10/H0704 and REC 15/LO/2127). Blood samples were obtained from patients who attended the biologics infusion clinic or the outpatient IBD clinic for a routine review. Controls for the study of blood cells were healthy donors who were age and gender matched to the patients in each group. Endoscopic samples were taken from IBD patients undergoing routine colonoscopies to assess disease activity, and from patients undergoing polypectomy who had no signs of intestinal inflammation, serving as controls. GALT samples were obtained by targeted biopsies of ileal Peyer's patches and the appendiceal orifice. Mononuclear cells from biopsies were isolated using a collagenase digest as previously described (28). Blood samples were collected in sodium heparin tubes and peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque (GE Healthcare, Amersham, UK) density gradient centrifugation as previously described (28). PBMCs were cryopreserved using fetal bovine serum (FBS)/10% dimethyl sulfoxide (DMSO; both Sigma Aldrich, Gillingham, UK) and stored in the vapor phase of liquid nitrogen until use. An overview of recruited patients is provided in **Supplemental Tables 1**, **2**. For cell sorting experiments blood cones from healthy donors were purchased from NHS Blood and Transplant (Tooting, UK).

Patient Stratification

We included 10 patients receiving infliximab (8 CD and 2 UC; 55% female, median age 41); six patients receiving vedolizumab (1 CD and 5 UC, 50% female, median age 32), and 14 CD patients receiving ustekinumab (50% female, median age 32). Patients received:

- Intravenous (IV) ustekinumab (Stelara®) 6 mg/kg (induction) and 90 mg subcutaneously every 8 weeks from week 8.
- IV infliximab (Remsima®) 5 mg/kg at week 0, 2, and 6 thereafter every 8 weeks.
- IV vedolizumab (Entyvio®) 300 mg at week 0, 2, and 6 and thereafter every 8 weeks.

Blood samples were prospectively collected pre-treatment (week 0), at 6 weeks (infliximab and vedolizumab) or 8 weeks (ustekinumab). Clinical response/remission was assessed at week 14 (infliximab and vedolizumab) or week 16 (ustekinumab).

Clinical remission was defined as Harvey-Bradshaw index (HBI) <5 or partial Mayo score 0–1 at week 14/16 (29, 30). Clinical response was defined as HBI reduction ≥3 or partial Mayo score reduction >/=2 for patients with inactive disease at baseline, or >30% reduction if disease activity index was abnormal (HBI ≥5, partial Mayo ≥2) at week 0. Biological response was defined as a 50% reduction in CRP, if baseline CRP >5 mg/l. Biological remission was defined as CRP <5 mg/l (31).

Multi-Parameter Flow Cytometry

Frozen PBMCs from healthy volunteers and IBD patients were thawed, washed and rested for 45 min in RPMI-1640 containing 10% FBS, 100 U/ml penicillin/ 100 μg/ml streptomycin (all Gibco/Thermo Fisher Scientific, Waltham, MA, USA), with DNase I (0.1 mg/ml; from Roche, Welwyn Garden City, UK). Cells were resuspended in PBS prior to live dead staining with Zombie Aqua (Biolegend, San Diego, CA, USA). Cells were then stained with fluorochrome conjugated monoclonal antibodies to CD19, CD27, CD10, IgA, IgD, IgM, and integrin β7 (**Supplemental Table 3**). Cells were analyzed using a BD Biosciences LSR Fortessa flow cytometer (Wokingham, UK) before determination of B cell populations using the gating strategy shown in **Figure 1**. Further detail and explanation are provided in **Supplemental Figure 1** and **Supplemental Table 4**. IgG was not used in the flow cytometry panel, but preliminary experiments demonstrated that cells lacking IgM, IgA (and IgD for the $CD27^-$ subset) could be reasonably classified as IgG^+, so this method was used to reduce antibodies in the panel. One of five test experiments is illustrated in **Supplemental Figure 2**. Although theoretically IgE expressing B cells could have been included, these are known to be very small in number and are difficult to detect due to the possibility of IgE binding to Fc receptors.

Cell Sorting

Frozen blood cone derived PBMCs were thawed, washed and rested in RPMI-1640 containing 10% FBS, 100 U/ml penicillin/ 100 μg/ml streptomycin, and DNase I (0.1 mg/ml) for 45 min. Cells were stained with DAPI, anti-CD19, CD27, and IgD prior to sorting using a BD Biosciences FACSAria II Cell Sorter. B cells were sorted initially by CD19 expression, with four populations isolated based on CD27 and IgD expression: $CD27^+IgD^-$, $CD27^-IgD^-$, $CD27^+IgD^+$, and $CD27^-IgD^+$ (**Supplemental Figure 3A**).

Analysis of Intracellular Cytokines

Unsorted cell suspensions or B cell populations sorted by FACS were suspended in RPMI-1640 containing 10% FBS, 100 U/ml penicillin/ 100 μg/ml streptomycin and seeded into 96 well plates prior to stimulation with 250 ng/ml ionomycin, 50 ng/ml PMA (both Sigma) and GolgiStop (1:1,000; BD Biosciences) for 4 h. Cells were washed and resuspended with PBS prior to live dead staining with Zombie Aqua. Subsequently, cells were washed and fixed with 2% paraformaldehyde, prior to treatment with permeabilization buffer (both eBioscience/Thermo Fisher Scientific). Cells were stained with anti-TNFα (Mab11, BioLegend, 1:50) and anti-IL10 (JES3-19F1, BioLegend, 1:20) and data acquired using a FACS Canto II instrument (BD Biosciences). See **Supplemental Figure 4** for gating strategies for analysis of cytokine production by whole $CD19^+$ populations from GALT and blood and **Supplemental Figure 3** for sorting $CD27^+IgD^-$, $CD27^-IgD^-$, $CD27^+IgD^+$, and $CD27^-IgD^+$ populations and for analysis of cytokine production by cultured sorted cells.

Mass Cytometry

Cells isolated from healthy control mucosal biopsy samples ($n = 6$) and IBD (UC: $n = 6$ and CD: $n = 1$) were washed and rested in RPMI-1640 10% FBS, 100 U/ml penicillin/100 μg/ml streptomycin and DNase I (0.1 mg/ml) for 30 min. Cell counts were obtained using trypan blue (Sigma) on a Countess II FL cell counter (Thermo Fisher Scientific). From each sample two million live cells were stained with Cell-ID Intercalator-103 Rh (1:500; Fluidigm, San Francisco, CA, USA) for DNA for 15 min. After washing cells were stained with metal-conjugated antibodies (**Supplemental Table 5**) for 30 min, then washed and fixed in 2% paraformaldehyde overnight at 4°C. Cells were pelleted and frozen in FBS/10% DMSO at −80°C for no longer than 14 days. On the day of the mass cytometry run, cells were thawed and washed prior to incubation with 0.3% saponin with Cell-ID Intercalator-Ir for 20 min to permeabilize and live/dead stain, respectively (32). Cells were then washed with PBS followed by two water washes before being run into a Fluidigm Helios mass cytometry instrument.

Data Analysis

Flow cytometry data was analyzed in FlowJo software (FlowJo LCC, Ashland, Oregon, USA) to identify nine B cell populations, as described in **Figure 1**, **Supplemental Figures 1**, **2**, and **Supplemental Table 4**. The designation of cells that do not express IgM or IgA (or IgD for $CD27^-$ cells) as IgG^+ was based on preliminary experiments **Supplemental Figure 2**.

Mass cytometry data was analyzed using cloud-based cytometry platform, Cytobank (Santa Clara, CA USA https://mrc.cytobank.org). Bead-based normalization of the individual mass cytometry data was performed with the Normalizer v0.3 software from Garry P. Nolan Laboratory (downloaded from https://github.com/nolanlab/bead-normalization/releases/tag/v0.3). **Supplemental Figure 5A** shows pre and post-normalization plots. Normalized files were gated as shown in **Supplemental Figures 5B,C** after uploading onto Cytobank to remove doublets by gating on DNA and removing cells with implausible marker combinations. Data was generated by further subset gating in Cytobank.

Statistical Analysis

Statistical testing was performed using GraphPad software (La Jolla, CA, USA). Individual groups were compared using Mann-Whitney U non-parametric test. Multiple groups were compared using Kruskal-Wallis ANOVA. A $p < 0.05$ was considered significant. Identifiers of statistical values derived from Kruskal-Wallis ANOVA are identified with red lines and asterisks in the

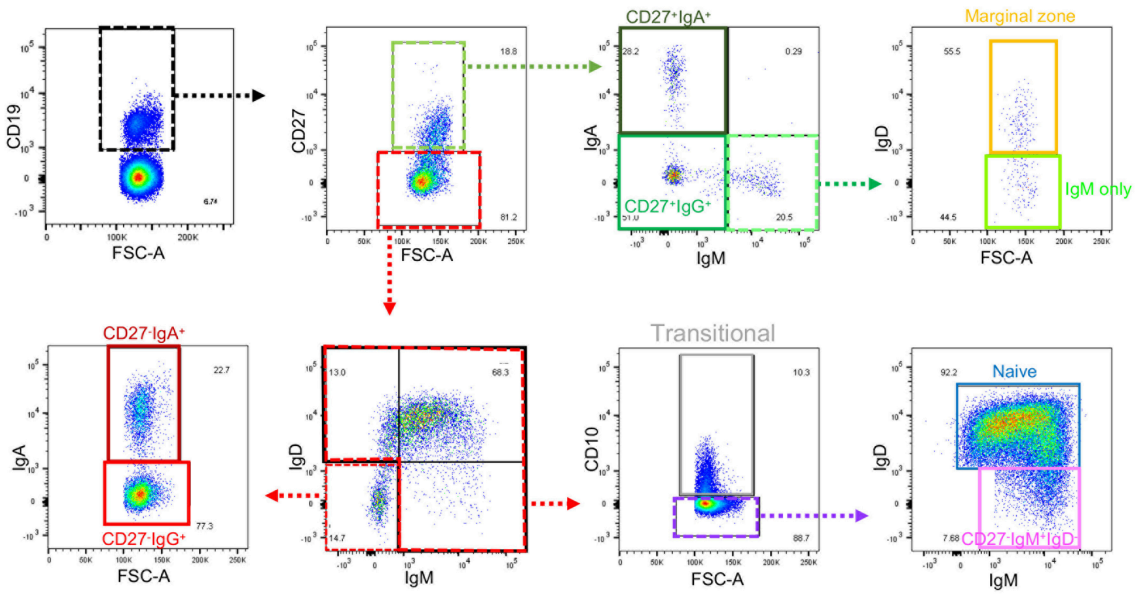

FIGURE 1 | Gating strategy for B cell subsets from blood. Gating strategy to define nine subsets of B cells using the same color code as the gating plan in **Supplemental Table 4**. Further details of gating prior to the selection of CD19$^+$ cells are in **Supplemental Figure 1**. Gates that define intermediate populations are dotted. Gates that define final populations studied are solid.

figures. Identifiers of statistical values derived using the Mann-Whitney *U* test are identified with black lines and asterisks in the figures.

RESULTS

Altered B Cell Subset Frequencies in Peripheral Blood of IBD Patients

In order to assess relative frequencies of the main B cell subsets in peripheral blood of CD and UC patients, we subjected the PBMCs of patients with various disease activities and treatments (see **Supplemental Table 1**) to six color flow cytometry. B cell subsets were gated as described in **Figure 1**, **Supplemental Figure 1** and **Supplemental Table 4**. In UC blood we observed a moderate reduction of transitional B cells (CD27$^-$IgM$^+$IgD$^+$CD10$^+$) and an increase in marginal zone B cells (CD27$^+$IgM$^+$IgD$^+$) compared to controls (**Figure 2**). The frequencies of naïve, CD27$^+$ IgA$^+$, and CD27$^+$IgM$^+$ subsets did not differ between IBD and controls (**Figure 2**). In UC blood, the frequency of CD27$^+$IgG$^+$ was increased compared to both HC and CD (**Figure 2**). Interestingly, the most striking difference compared to controls was the overall reduction of CD27$^-$IgD$^-$ populations expressing IgM, IgA or IgG in the blood of both UC and CD patients (**Figure 2**).

Increased Frequencies of CD27$^-$IgD$^-$ B Cells in IBD GALT

Since the proportion of CD27$^-$IgD$^-$ B cells of total CD19 B cells was reduced in blood of patients with IBD, we asked whether this is also a feature of B cells in GALT of patients with IBD. To answer this specific question, we interrogated a large dataset available in our lab that had been generated by mass cytometric analysis of cells isolated from GALT of healthy individuals and patients with IBD. Following initial normalization and quality control we analyzed the data by manually gating the .fcs files in Cytobank (**Supplemental Figure 5**, **Supplemental Table 4** and **Figure 3**). We initially gated on CD19$^+$ cells and then excluded CD10$^+$ cells since these would include germinal center cells that are not comparable with blood. We then analyzed the frequencies of CD27$^+$IgD$^-$, CD27$^-$IgD$^-$, CD27$^+$IgD$^+$, and CD27$^-$IgD$^+$ B cells and observed an increase in the proportion of CD27$^-$IgD$^-$ cells and a reduction in CD27$^-$IgD$^+$ cells in GALT in IBD. This demonstrated that the CD27$^-$IgD$^-$ B cell subset is not globally depleted in patients with IBD.

Expression of Gut-Homing Marker Beta7 Integrin on CD27$^-$IgD$^-$ B Cells

The enrichment of CD27$^-$IgD$^-$ cells in GALT in IBD, and the reduction of all subsets of this population expressing IgM, IgA, and IgG in blood in IBD, implies that CD27$^-$IgD$^-$ cells may be recruited from the blood to the gut in IBD. Therefore, we went on to investigate the gut-homing capacity of those cells in blood of UC and CD patients by analyzing the expression of α4β7 integrin (by staining the β7 sub-unit). In addition, we were interested in determining whether this might differ in active disease compared to remission state. For the analysis samples were retrospectively stratified into raised C-reactive peptide (CRP >5 mg/l, indicating active inflammation) and normal CRP (CRP <5 mg/l). Samples were also stratified by a global clinical assessment of the patients being having active disease compared to being in remission.

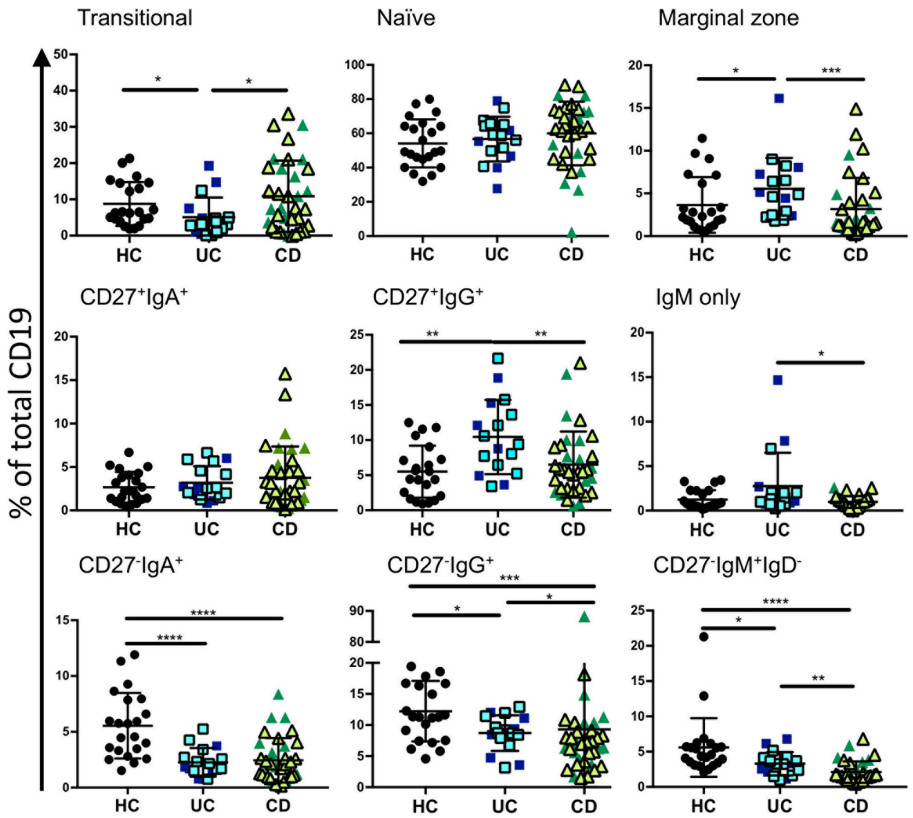

FIGURE 2 | Analysis of B cell subsets in healthy controls and IBD patients. Peripheral blood mononuclear cells from IBD and HC patients were stained using fluorescent antibodies and gated according to the strategy in **Figure 1** and **Supplemental Table 4**. Data is presented as percentage of total CD19 positive cells from each individual HC ($n = 22$), UC patient ($n = 17$), and CD patient ($n = 35$). Patients with UC in are represented with blue symbols. Symbols that are lighter blue with black borders are patients in remission. Patients with CD in are represented with green symbols. Symbols that are lighter green with black borders are patients in remission. Differences between groups were analyzed by Mann-Whitney *U* test where $p < 0.05 = *$, $p < 0.01 = **$, $p < 0.001 = ***$, and $p < 0.0001 = ****$.

The frequency of $CD27^{-}IgA^{+}$ cells was slightly raised in UC patients with CRP >5 compared to CRP<5 and the expression of β7 integrin increased when CRP was elevated. A similar trend was observed for the CD samples but did not reach significance (**Figures 4A,B**). In UC with CRP>5, the frequencies of $CD27^{-}IgG^{+}$ cells and $CD27^{-}IgM^{+}IgD^{-}$ cells showed a non-significant trend toward being raised with increased β7 expression. No differences were seen for those two subsets and β7 in CD with a CRP>5 in terms of % positive cells (**Figure 4B**), however MFI of β7 integrin expression was lower in $CD27^{-}IgM^{+}IgD^{-}$ cells in patients with CD when CRP was >5 compared to <5 (**Supplemental Figure 6**). When patients were stratified clinically into either active disease or in remission, we still observed a bias toward reduced proportion of $CD27^{-}IgD^{-}$ cells. The reduction of $CD27^{-}IgD^{-}$ was significantly reduced in blood from CD patients compared to HC independently of disease activity (**Figure 4C**). For the UC samples we observed a non-significant trend of such a reduction. The expression of β7 integrin on CD27- cells in CD blood as either % positive cells or MFI tended to be raised but values did not reach statistical significance (**Figure 4D** and **Supplemental Figure 6**).

Treatment With Biologics has Little Impact on Blood $CD27^{-}IgD^{-}$ Cells in IBD

Treatment escalation in advanced CD and UC cases involves the use of biologic treatments. The most frequently used are the anti-TNF antibody infliximab and the anti-α4β7 antibody vedolizumab for UC and CD. The latter disease can also be treated with the anti-p40 antibody ustekinumab. Little is known how those antibodies affect blood B cells populations. While we did not anticipate a direct effect of these drugs on B cells, we wondered whether the induction of remission and hence improved gut barrier function could have an impact. $CD27^{-}IgD^{-}$ cells have been shown to be responsive to bacterial antigens (33). Therefore, we hypothesized that mucosal healing during the course of treatment might affect the proportion of $CD27^{-}IgD^{-}$ B cells in the blood stream of IBD patients. Infliximab treatment (sample obtained at week 6; "post") did not change the frequencies of $CD27^{-}IgA^{+}$, $CD27^{-}IgG^{+}$, or $CD27^{-}$ IgM^{+} IgD^{-} populations compared to the corresponding sample taken before treatment commenced (**Figure 5A**). The effect of vedolizumab was also assessed at week 6 ("post") and, interestingly, only the $CD27^{-}IgA^{+}$ population was increased compared to the sample taken before treatment

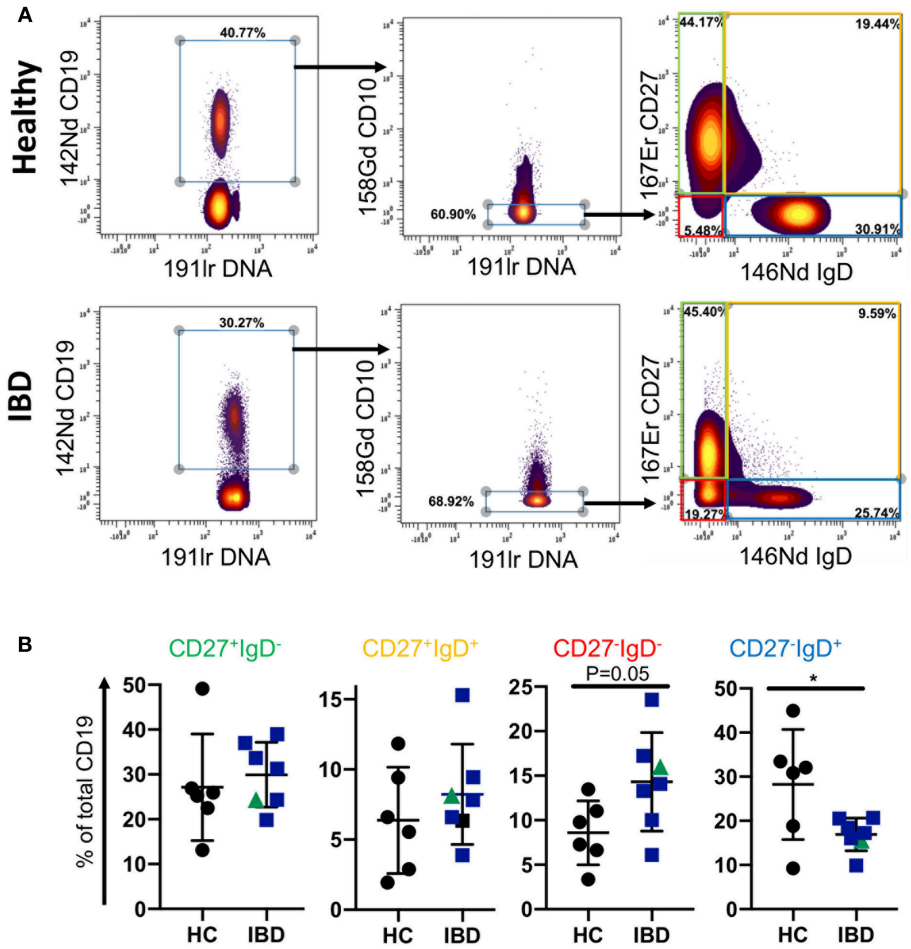

FIGURE 3 | B cell subsets in GALT of healthy control and IBD patients. Data was gated on the Cytobank platform. Following selection of $CD19^+$ cells and elimination of $CD10^+$ cells (since these would include germinal center cells in tissues and therefore not be equivalent to $CD10^+$ subsets in blood), cells were gated according to their expression of CD27 and IgD. Examples of a HC and an IBD sample are illustrated in **(A)**. In **(B)** dotplots represent individually gated data points for HC ($n = 6$), patients with UC ($n = 6$ in blue) and a patient with CD in green, groups together as IBD. Groups were compared by Mann-Whitney *U* test where $p < 0.05 = *$.

("pre"; **Figure 5B**), suggesting that vedolizumab retains this population in the blood by blocking gut homing via α4β7. The $CD27^-IgG^-$, and $CD27^-IgM^+IgD^-$ populations were lower in IBD blood compared to controls but did not change during treatment (**Figure 5B**). Ustekinumab treatment mildly lowered the frequency of $CD27^-IgA^+$ cells (week 16; post, compared to treatment start; pre. **Figure 5C**) but did not affect the frequencies of $CD27^-IgG^+$, or $CD27^-IgM^+IgD^-$ cells (**Figure 5C**).

B Cells in GALT Produce TNFα and IL-10

Since our data show that B cell subsets are represented differently in blood and GALT, we wondered whether those B cells might be capable of producing cytokines such as TNFα and IL-10 (34). We analyzed B cells isolated from blood and two GALT sites: the Peyer's patches in the terminal ileum and colonic follicles in the rectum from the same donors for their production of intracellular TNFα and IL10 (**Supplemental Figure 4**). Compared to the donor-matched PBMCs, the B cells in healthy GALT showed greater tendency to produce TNFα (∼25% compared to 10%). Interestingly, slightly more B cells isolated from colonic follicles produced TNFα than their counterparts isolated from Peyer's patches. B cells in GALT from CD patients also tended to produce more TNFα than the corresponding PBMCs (**Figure 6A**). In both health and CD, PBMCs and matching GALT B cells were able to produce IL-10 (around 20% of all B cells). There was a non-significant trend for more B cells producing IL-10 in blood compared to GALT (**Figure 6B**).

To determine if $CD27^-IgD^-$ B cells might differ in cytokine production and therefore might differ in their potential to drive inflammation compared to other subsets, FACS sorted $CD27^+IgD^-$, $CD27^-IgD^-$, $CD27^+IgD^+$, and $CD27^-IgD^+$ B cells underwent intracellular staining and flow cytometry for intracytoplasmic TNFα and IL-10 **(Supplemental Figure 3)**. $CD27^+IgD^-$, $CD27^-IgD^-$, $CD27^+IgD^+$ populations but fewer $CD27^-IgD^+$ B cells had intracytoplasmic TNFα (**Figure 6C).** In contrast, neither $CD27^+IgD^-$, $CD27^-IgD^-$ nor $CD27^+IgD^+$ cells produced IL10 following stimulation, while between 5 and 20% $CD27^-IgD^+$ cells produced IL-10 (**Figure 6D**).

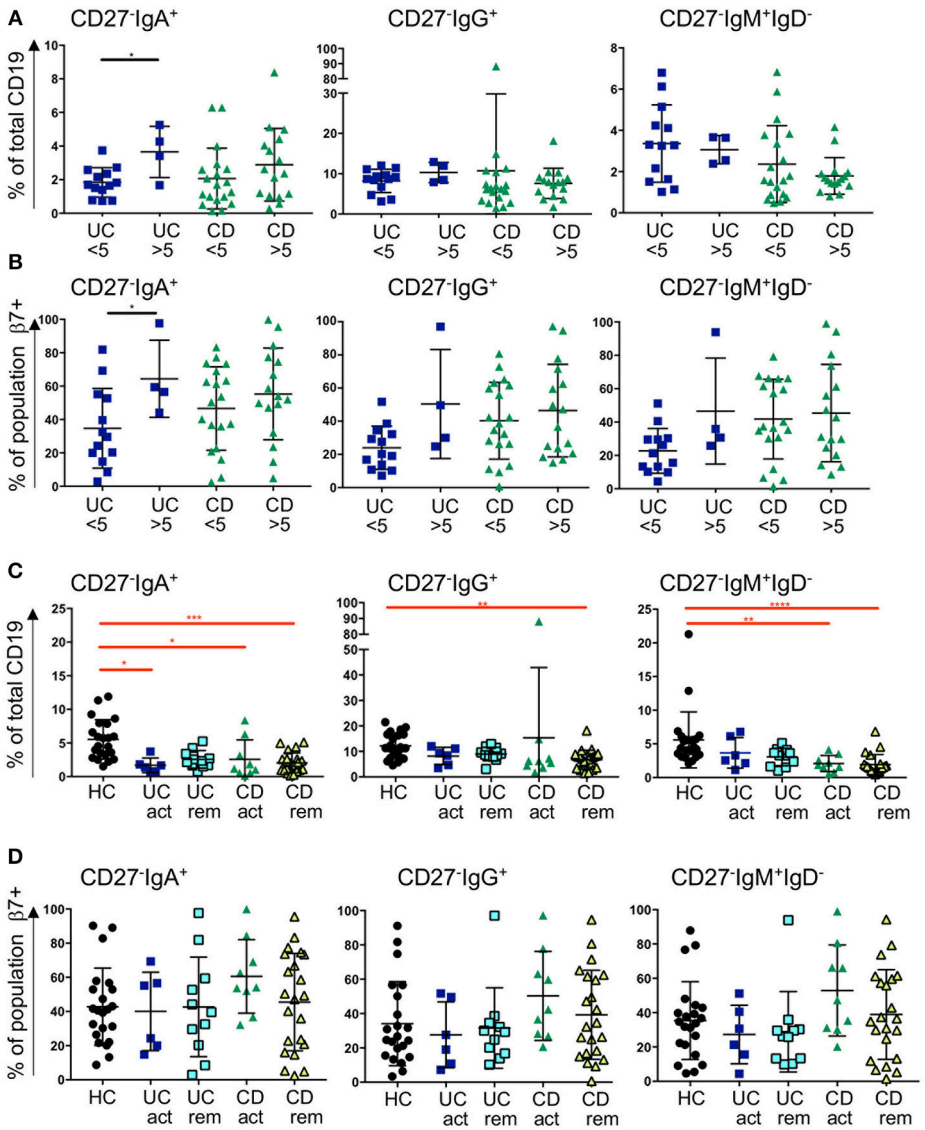

FIGURE 4 | Frequency of blood $CD27^{-}IgD^{-}$ subsets and β7 integrin expression in CD and UC patients stratified according to CRP levels and remission status. Three subsets of $CD27^{-}IgD^{-}$ B cells ($CD27^{-}IgA^{+}$, $CD27^{-}IgG^{+}$, and $CD27^{-}IgM^{+}$) are presented as percentage of CD19 positive cells in **(A)** and in **(B)** the percentage of individual populations expressing of β7 integrin. For **(A,B)**, UC < 5 $n = 13$, UC > 5 $n = 4$, CD < 5 $n = 19$, CD > 5 $n = 16$; for **(C,D)** HC $n = 22$, UC active $n = 6$, UC remission $n = 11$, CD active $n = 9$ and CD remission $n = 22$. Data is analyzed by Mann-Whitney *U* test (black bars and asterisks) or Kruskal-Wallis ANOVA test (red bars and asterisks) where $p < 0.05 = *$, $p < 0.01 = **$, $p < 0.001 = ***$ and $p < 0.0001 = ****$.

DISCUSSION

In this study we aimed to gain insights into the peripheral B cell subsets in IBD and how those subsets might differ between active disease and the state of remission. We observed a consistent proportionate reduction of $CD27^{-}IgD^{-}$ B cells expressing IgM, IgA, and IgG in the blood in IBD and an enrichment of the $CD27^{-}IgD^{-}$-subset in GALT in IBD, consistent with recruitment of $CD27^{-}IgD^{-}$ B cells from the blood to the gut in IBD. Depletion of $CD27^{-}IgD^{-}$ B cells from the blood is seen in patients with both CD and UC even when patients are in remission and when disease activity is low. Although, differences in transitional B cells, marginal zone B cells and $CD27^{+}IgG^{+}$ B cells were observed, mainly between UC and controls, this could be in compensation for the relative fall in the $CD27^{-}IgD^{-}$ subset, as populations were quantified with $CD19^{+}$ cells remaining at 100% and total B cell counts were not determined. We propose that the major feature of the data is the depletion of $CD27^{-}IgD^{-}$ subsets of B cells because this was observed consistently across all isotypes and despite the different gating strategies required for their identification from the blood of patients with UC and CD.

The white blood count in IBD can be altered during a disease flare or lowered by treatments like azathioprine and methotrexate (35). However, we also observed our findings in

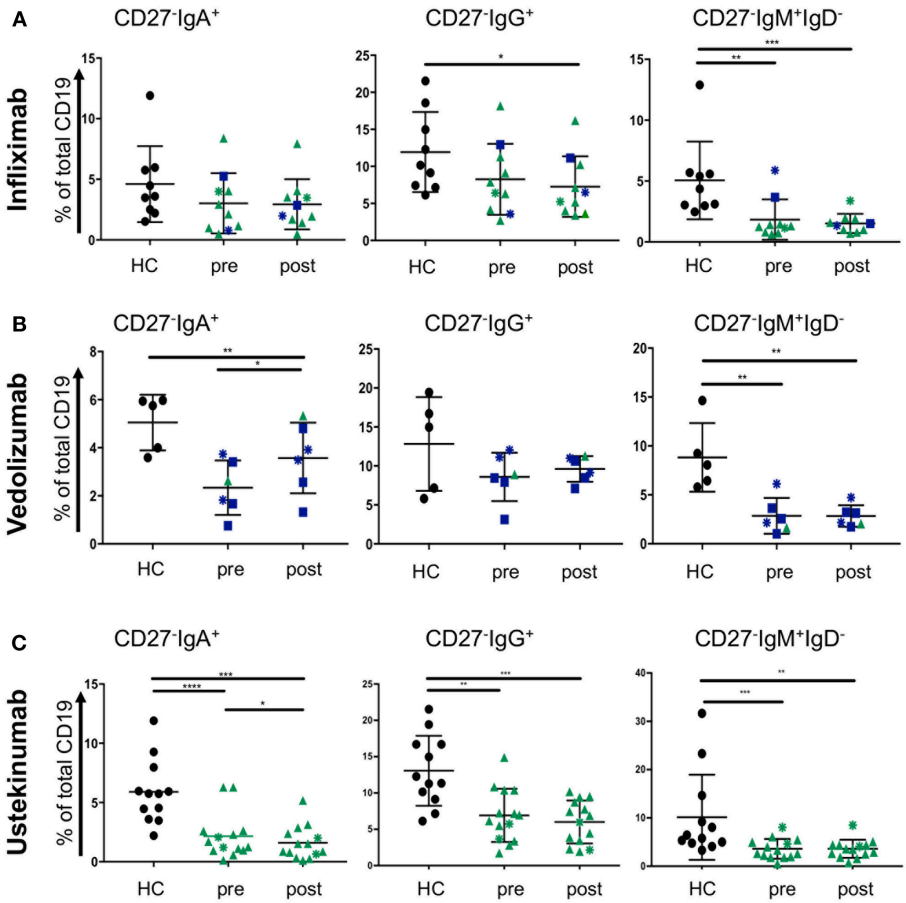

FIGURE 5 | CD27$^-$IgD$^-$ B cells subsets in blood of CD and UC patients treated with biologics. Patients were treated with either **(A)** infliximab ($n = 10$, HC $n = 9$), **(B)** vedolizumab ($n = 6$, HC $n = 5$), or **(C)** ustekinumab ($n = 14$, HC $n = 12$) with a baseline (pre) and a post-treatment (6 weeks for infliximab and vedolizumab, and 8 weeks for ustekinumab) sample obtained. Subsets of CD27$^-$IgD$^-$ cells were compared to age and gender matched healthy controls (Dark blue squares: UC patients responsive to treatment; Dark blue asterisks: UC patients non-responsive to treatment; Green triangles: CD patients responsive to treatment; Green asterisks: CD patients non-responsive to treatment). Data was analyzed by Mann-Whitney *U* tests where $p < 0.05 = *$, $p < 0.01 = **$, $p < 0.001 = ***$ and $p < 0.0001 = ****$.

patients with only mild disease or remission, and most patients were not on treatments that cause lymphopenia, suggesting the observed reduction of CD27$^-$IgD$^-$ subsets is not directly linked to treatment or a flare.

As we were interested in whether peripheral B cell subsets are affected by disease activity in CD and UC, we stratified patients based on their CRP values (CRP >5 mg/l as a marker of ongoing inflammation) and based on clinical scoring by a clinical gastroenterologist. Interestingly, we observed higher frequencies of CD27$^-$IgA$^+$ cells and an increase in β7 integrin on those cells in blood of UC patients with raised CRP levels. This was not significant for UC patients stratified clinically (**Figure 4**). Treatment with the antibody vedolizumab significantly raised the frequency of this subset during treatment (measured at 6 weeks; **Figure 5B**), suggesting that blood CD27$^-$IgA$^+$ B cells are retained in blood by vedolizumab which hinders gut homing by blocking α4β7. Interestingly, treatment with infliximab and ustekinumab did not have much effect on blood frequencies of CD27$^-$IgD$^-$ B cells. We only noticed a mild reduction in CD27$^-$IgA$^+$ cells at 8 weeks of ustekinumab treatment (**Figure 5C**). Whether this finding is of therapeutic relevance remains to be determined.

CD27$^-$IgD$^-$ B cells comprise ∼5% of blood B cells in healthy individuals and are considered to be part of the memory B cell pool because they have mutations in their Ig heavy chain genes consistent with having transited though a germinal center (23–27). However, the role of these cells is not understood. They have been associated with aging and inflammatory diseases including rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and Alzheimer's disease, in which increased frequencies are present in the blood (36–39). Whilst the cause of the increase is not known, this double negative subset has been described as responsive to anti-inflammatory treatments; anti-TNF therapy in RA reduced the cell frequency (40), and conventional treatment in SLE increased it (36). It is therefore very interesting that the change in frequency of CD27$^-$IgD$^-$ B cells in IBD is in the opposite direction to that observed in aging and other disease settings, and that we do not observe changes in response

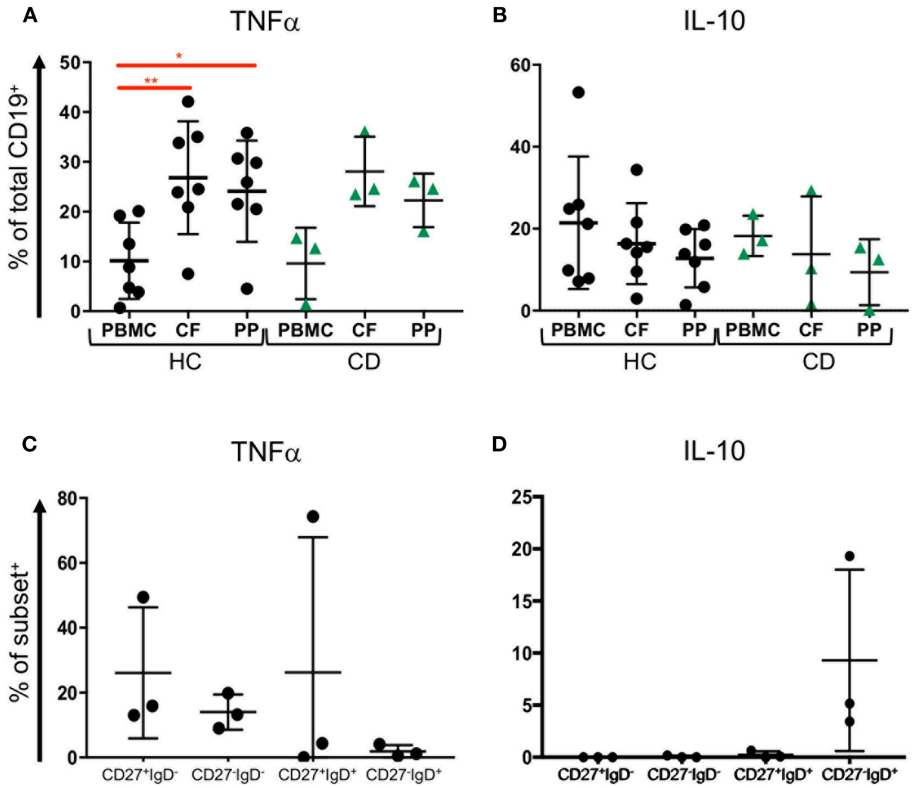

FIGURE 6 | TNFα and IL-10 expression by blood and tissue B cells. Blood (PBMC) and GALT (CF, colonic follicles; PP, Peyer's patches) samples were obtained from HC ($n = 7$) and CD patients ($n = 3$) and isolated $CD19^+$ cells were analyzed for **(A)** TNFα and **(B)** IL-10 expression according to the strategy in **Supplemental Figure 4**. Subsets of B cells were sorted from blood as in **Supplemental Figure 3**, and expression of **(C)** TNFα and **(D)** IL-10 measured. Data was analyzed by Kruskal Wallis ANOVA test, where $p < 0.05 = *$ and $p < 0.01 = **$.

to biologic therapies. As is often the case with studies of inflammatory diseases, we cannot rule out the possibility that medication given at some time may have induced the changes we see. However, other inflammatory diseases may share the same therapeutic strategies, suggesting that the reduced frequency of $CD27^-IgD^-$ B cells in IBD is a feature of the condition rather than its treatment.

It has been suggested previously that $CD27^-$ memory B cells are associated with intestinal B cell responses (25, 33). They are increased in frequency in healthy GALT compared to other lymphoid tissues (41), $CD27^-IgA^+$ memory cells have been shown to have a distinctive repertoire and a bias toward lambda light chain usage. They are polyspecific and able to bind multiple bacterial species in health (33). $CD27^-IgA^+$ memory B cells have also been reported to be generated by T cell independent immune responses since they are present in the blood of patients with deficient CD40/CD40L interactions (25). The increased frequency of CD27-IgD- B cells we observe in GALT in IBD in our study may be due to local proliferation of this subset in response to local challenge rather than selective recruitment from the blood. On the other hand, and in contrast, it has also been suggested that $CD27^-$ memory B cells are part of the normal spectrum of conventional memory B cells since clones of B cells can span the $CD27^+$ and $CD27^-$ subsets (26). In this case, loss or gain of CD27 from members of a clone may reflect time or tissue context rather than lineage or clone specificity.

Our data linking $CD27^-IgD^-$ B cells with intestinal inflammation and their relative depletion from the blood and enhanced frequencies in gut would be consistent with the concept that they are a distinct population with a role in antibacterial immunity. The mucosal barrier is known to be disrupted to varying degrees in IBD so that the local bacterial challenge is likely to be higher (42). UC and CD have markedly different pathogeneses and are impacted by different genetic predispositions and environmental drivers. It is interesting therefore that they share the feature of depletion of $CD27^-$ B cells from the blood. This suggests that recruitment of $CD27^-$ B cells to the gut is more likely a response to an intestinal challenge rather than a feature of the disease process *per se*. This is potentially also the case in quiescent IBD as persistent changes to epithelial barrier function have been described in UC patients who had no mucosal defects (43, 44).

Since our data shows that GALT B cells and blood B cells, including $CD27^-IgD^-$ memory cells, are able to produce TNFα, we suggest that increased recruitment of these cells to the gut might contribute to the inflammatory milieu in IBD.

AUTHOR CONTRIBUTIONS

JS and AV designed the study and wrote the manuscript. CP performed flow cytometric analysis and mass cytometry. NZ, TT, and JaS performed experimental design, mass cytometry, data analysis, and data visualization. MC performed sample preparation and flow cytometry. WG and KC analyzed B cell cytokine production. KK, AV, JeS, and LL provided clinical samples and stratified patients by treatment response. All authors contributed to the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00361/full#supplementary-material

REFERENCES

1. Xavier RJ, Podolsky DK. Unravelling the pathogenesis of inflammatory bowel disease. *Nature*. (2007) 448:427–34. doi: 10.1038/nature06005
2. Murdoch TB, Xu W, Stempak JM, Landers C, Targan SR, Rotter JI, et al. Pattern recognition receptor and autophagy gene variants are associated with development of antimicrobial antibodies in Crohn's disease. *Inflamm Bowel Dis.* (2012) 18:1743–8. doi: 10.1002/ibd.22884
3. de Souza HS, Fiocchi C. Immunopathogenesis of IBD: current state of the art. *Nat Rev Gastroenterol Hepatol.* (2016) 13:13–27. doi: 10.1038/nrgastro.2015.186
4. Andoh A, Imaeda H, Aomatsu T, Inatomi O, Bamba S, Sasaki M, et al. Comparison of the fecal microbiota profiles between ulcerative colitis and Crohn's disease using terminal restriction fragment length polymorphism analysis. *J Gastroenterol.* (2011) 46:479–86. doi: 10.1007/s00535-010-0368-4
5. Khan N, Trivedi C, Shah Y, Cole E, Lewis J, Yang YX. The natural history of newly diagnosed ulcerative colitis in patients with concomitant primary sclerosing cholangitis. *Inflamm Bowel Dis.* (2018) 24, 2062–7. doi: 10.1093/ibd/izy106
6. Abraham C, Cho JH. Inflammatory bowel disease. *N Engl J Med.* (2009) 361:2066–78. doi: 10.1056/NEJMra0804647
7. Horio Y, Uchino M, Bando T, Chohno T, Sasaki H, Hirata A, et al. Rectal-sparing type of ulcerative colitis predicts lack of response to pharmacotherapies. *BMC Surg.* (2017) 17:59. doi: 10.1186/s12893-017-0255-5
8. Danese S, Angelucci E, Malesci A, Caprilli R. Biological agents for ulcerative colitis: hypes and hopes. *Med Res Rev.* (2008) 28:201–18. doi: 10.1002/med.20103
9. Scribano ML. Vedolizumab for inflammatory bowel disease: from randomized controlled trials to real-life evidence. *World J Gastroenterol.* (2018) 24:2457–67. doi: 10.3748/wjg.v24.i23.2457
10. Kobayashi T, Okamoto S, Hisamatsu T, Kamada N, Chinen H, Saito R, et al. IL23 differentially regulates the Th1/Th17 balance in ulcerative colitis and Crohn's disease. *Gut*. (2008) 57:1682–9. doi: 10.1136/gut.2007.135053
11. Caprioli F, Bose F, Rossi RL, Petti L, Vigano C, Ciafardini C, et al. Reduction of CD68+ macrophages and decreased IL-17 expression in intestinal mucosa of patients with inflammatory bowel disease strongly correlate with endoscopic response and mucosal healing following infliximab therapy. *Inflamm Bowel Dis.* (2013) 19:729–39. doi: 10.1097/MIB.0b013e318280292b
12. Bain CC, Mowat AM. Macrophages in intestinal homeostasis and inflammation. *Immunol Rev.* (2014) 260:102–17. doi: 10.1111/imr.12192
13. Rovedatti L, Kudo T, Biancheri P, Sarra M, Knowles CH, Rampton DS, et al. Differential regulation of interleukin 17 and interferon gamma production in inflammatory bowel disease. *Gut*. (2009) 58:1629–36. doi: 10.1136/gut.2009.182170
14. Kamada N, Hisamatsu T, Honda H, Kobayashi T, Chinen H, Takayama T, et al. TL1A produced by lamina propria macrophages induces Th1 and Th17 immune responses in cooperation with IL-23 in patients with Crohn's disease. *Inflamm Bowel Dis.* (2010) 16:568–75. doi: 10.1002/ibd.21124
15. Bennike TB, Carlsen TG, Ellingsen T, Bonderup OK, Glerup H, Bogsted M, et al. Neutrophil extracellular traps in ulcerative colitis: a proteome analysis of intestinal biopsies. *Inflamm Bowel Dis.* (2015) 21:2052–67. doi: 10.1097/MIB.0000000000000460
16. Thoree VC, Golby SJ, Boursier L, Hackett M, Dunn-Walters DK, Sanderson JD, et al. Related IgA1 and IgG producing cells in blood and diseased mucosa in ulcerative colitis. *Gut*. (2002) 51:44–50. doi: 10.1136/gut.51.1.44
17. Uo M, Hisamatsu T, Miyoshi J, Kaito D, Yoneno K, Kitazume MT, et al. Mucosal CXCR4+ IgG plasma cells contribute to the pathogenesis of human ulcerative colitis through FcgammaR-mediated CD14 macrophage activation. *Gut*. (2013) 62:1734–44. doi: 10.1136/gutjnl-2012-303063
18. Virk R, Shinagare S, Lauwers GY, Yajnik V, Stone JH, Deshpande V. Tissue IgG4-positive plasma cells in inflammatory bowel disease: a study of 88 treatment-naive biopsies of inflammatory bowel disease. *Mod Pathol.* (2014) 27:454–9. doi: 10.1038/modpathol.2013.121
19. Boursier L, Gordon JN, Thiagamoorthy S, Edgeworth JD, Spencer J. Human intestinal IgA response is generated in the organized gut-associated lymphoid tissue but not in the lamina propria. *Gastroenterology*. (2005) 128:1879–89. doi: 10.1053/j.gastro.2005.03.047
20. Frisch M, Pedersen BV, Andersson RE. Appendicitis, mesenteric lymphadenitis, and subsequent risk of ulcerative colitis: cohort studies in Sweden and Denmark. *BMJ*. (2009) 338:b716. doi: 10.1136/bmj.b716
21. Naves JE, Lorenzo-Zuniga V, Marin L, Manosa M, Oller B, Moreno V, et al. Long-term outcome of patients with distal ulcerative colitis and inflammation of the appendiceal orifice. *J Gastrointestin Liver Dis.* (2011) 20:355–8. doi: 10.6018/red/45/6
22. Park SH, Loftus EV Jr, Yang SK. Appendiceal skip inflammation and ulcerative colitis. *Dig Dis Sci.* (2014) 59:2050–7. doi: 10.1007/s10620-014-3129-z
23. Fecteau JF, Cote G, Neron S. A new memory CD27-IgG+ B cell population in peripheral blood expressing VH genes with low frequency of somatic mutation. *J Immunol.* (2006) 177:3728–36. doi: 10.4049/jimmunol.177.6.3728
24. Wei C, Anolik J, Cappione A, Zheng B, Pugh-Bernard A, Brooks J, et al. A new population of cells lacking expression of CD27 represents a notable component of the B cell memory compartment in systemic lupus erythematosus. *J Immunol.* (2007) 178:6624–33. doi: 10.4049/jimmunol.178.10.6624
25. Berkowska MA, Driessen GJ, Bikos V, Grosserichter-Wagener C, Stamatopoulos K, Cerutti A, et al. Human memory B cells originate from three distinct germinal center-dependent and -independent maturation pathways. *Blood*. (2011) 118:2150–8. doi: 10.1182/blood-2011-04-345579

26. Wu Y-CB, Kipling D, Dunn-Walters DK. The Relationship between CD27 negative and positive B cell populations in human peripheral blood. *Front Immunol.* (2011) 2:81. doi: 10.3389/fimmu.2011.00081
27. Clavarino G, Delouche N, Vettier C, Laurin D, Pernollet M, Raskovalova T, et al. Novel strategy for phenotypic characterization of human b lymphocytes from precursors to effector cells by flow cytometry. *PLoS ONE*. (2016) 11:e0162209. doi: 10.1371/journal.pone.0162209
28. Vossenkamper A, Hundsrucker C, Page K, van Maurik A, Sanders TJ, Stagg AJ, et al. A CD3-specific antibody reduces cytokine production and alters phosphoprotein profiles in intestinal tissues from patients with inflammatory bowel disease. *Gastroenterology*. (2014) 147:172–83. doi: 10.1053/j.gastro.2014.03.049
29. Harvey RF, Bradshaw JM. A simple index of Crohn's-disease activity. *Lancet*. (1980) 1:514.
30. Schroeder KW, Tremaine WJ, Ilstrup DM. Coated oral 5-aminosalicylic acid therapy for mildly to moderately active ulcerative colitis. A randomized study. *N Engl J Med*. (1987) 317:1625–9. doi: 10.1056/NEJM198712243172603
31. Vermeire S, Van Assche G, Rutgeerts P. The role of C-reactive protein as an inflammatory marker in gastrointestinal diseases. *Nat Clin Pract Gastroenterol Hepatol.* (2005) 2:580. doi: 10.1038/ncpgasthep0359
32. Sumatoh HR, Teng KW, Cheng Y, Newell EW. Optimization of mass cytometry sample cryopreservation after staining. *Cytometry A*. (2017) 91:48–61. doi: 10.1002/cyto.a.23014
33. Berkowska MA, Schickel JN, Grosserichter-Wagener C, de Ridder D, Ng YS, van Dongen JJ, et al. Circulating human CD27-IgA+ memory B cells recognize bacteria with polyreactive Igs. *J Immunol.* (2015) 195:1417–26. doi: 10.4049/jimmunol.1402708
34. Lighaam LC, Unger PA, Vredevoogd DW, Verhoeven D, Vermeulen E, Turksma AW, et al. *In vitro*-induced human IL-10(+) B cells do not show a subset-defining marker signature and plastically co-express IL-10 with pro-inflammatory Cytokines. *Front Immunol.* (2018) 9:1913. doi: 10.3389/fimmu.2018.01913
35. Meijer B, Wilhelm AJ, Mulder CJJ, Bouma G, van Bodegraven AA, de Boer NKH. Pharmacology of thiopurine therapy in inflammatory bowel disease and complete blood cell count outcomes: a 5-year database study. *Ther Drug Monit.* (2017) 39:399–405. doi: 10.1097/FTD.0000000000000414
36. Rodriguez-Bayona B, Ramos-Amaya A, Perez-Venegas JJ, Rodriguez C, Brieva JA. Decreased frequency and activated phenotype of blood CD27 IgD IgM B lymphocytes is a permanent abnormality in systemic lupus erythematosus patients. *Arthritis Res Ther.* (2010) 12:R108. doi: 10.1186/ar3042
37. Bulati M, Buffa S, Martorana A, Candore G, Lio D, Caruso C, et al. Trafficking phenotype and production of granzyme B by double negative B cells (IgG(+)IgD(-)CD27(-)) in the elderly. *Exp Gerontol.* (2014) 54:123–9. doi: 10.1016/j.exger.2013.12.011
38. Bulati M, Buffa S, Martorana A, Gervasi F, Camarda C, Azzarello DM, et al. Double negative (IgG+IgD-CD27-) B cells are increased in a cohort of moderate-severe Alzheimer's disease patients and show a pro-inflammatory trafficking receptor phenotype. *J Alzheimers Dis.* (2015) 44:1241–51. doi: 10.3233/JAD-142412
39. Tony HP, Roll P, Mei HE, Blumner E, Straka A, Gnuegge L, et al. Combination of B cell biomarkers as independent predictors of response in patients with rheumatoid arthritis treated with rituximab. *Clin Exp Rheumatol.* (2015) 33:887–94.
40. Moura RA, Quaresma C, Vieira AR, Goncalves MJ, Polido-Pereira J, Romao VC, et al. B-cell phenotype and IgD-CD27- memory B cells are affected by TNF-inhibitors and tocilizumab treatment in rheumatoid arthritis. *PLoS ONE*. (2017) 12:e0182927. doi: 10.1371/journal.pone.0182927
41. Zhao Y, Uduman M, Siu JHY, Tull TJ, Sanderson JD, Wu YB, et al. Spatiotemporal segregation of human marginal zone and memory B cell populations in lymphoid tissue. *Nat Commun.* (2007) 9:3857. doi: 10.1038/s41467-018-06089-1
42. McDonnell M, Liang Y, Noronha A, Coukos J, Kasper DL, Farraye FA, et al. Systemic Toll-like receptor ligands modify B-cell responses in human inflammatory bowel disease. *Inflamm Bowel Dis.* (2011) 17:298–307. doi: 10.1002/ibd.21424
43. Vivinus-Nebot M, Frin-Mathy G, Bzioueche H, Dainese R, Bernard G, Anty R, et al. Functional bowel symptoms in quiescent inflammatory bowel diseases: role of epithelial barrier disruption and low-grade inflammation. *Gut*. (2014) 63:744–52. doi: 10.1136/gutjnl-2012-304066
44. Dotti I, Mora-Buch R, Ferrer-Picon E, Planell N, Jung P, Masamunt MC, et al. Alterations in the epithelial stem cell compartment could contribute to permanent changes in the mucosa of patients with ulcerative colitis. *Gut*. (2017) 66:2069–79. doi: 10.1136/gutjnl-2016-312609

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REVIEW
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Short Chain Fatty Acids (SCFAs)-Mediated Gut Epithelial and Immune Regulation and Its Relevance for Inflammatory Bowel Diseases

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Ulcerative colitis (UC) and Crohn's disease (CD), collectively known as Inflammatory Bowel Diseases (IBD), are caused by a complex interplay between genetic, immunologic, microbial and environmental factors. Dysbiosis of the gut microbiome is increasingly considered to be causatively related to IBD and is strongly affected by components of a Western life style. Bacteria that ferment fibers and produce short chain fatty acids (SCFAs) are typically reduced in mucosa and feces of patients with IBD, as compared to healthy individuals. SCFAs, such as acetate, propionate and butyrate, are important metabolites in maintaining intestinal homeostasis. Several studies have indeed shown that fecal SCFAs levels are reduced in active IBD. SCFAs are an important fuel for intestinal epithelial cells and are known to strengthen the gut barrier function. Recent findings, however, show that SCFAs, and in particular butyrate, also have important immunomodulatory functions. Absorption of SCFAs is facilitated by substrate transporters like MCT1 and SMCT1 to promote cellular metabolism. Moreover, SCFAs may signal through cell surface G-protein coupled receptors (GPCRs), like GPR41, GPR43, and GPR109A, to activate signaling cascades that control immune functions. Transgenic mouse models support the key role of these GPCRs in controlling intestinal inflammation. Here, we present an overview of microbial SCFAs production and their effects on the intestinal mucosa with specific emphasis on their relevance for IBD. Moreover, we discuss the therapeutic potential of SCFAs for IBD, either applied directly or by stimulating SCFAs-producing bacteria through pre- or probiotic approaches.

Keywords: SCFAs, IBD, immune cells, IECs, intestinal mucosa, dysbiosis

INTRODUCTION

Inflammatory Bowel Diseases (IBD), comprising mainly ulcerative colitis (UC) and Crohn's disease (CD), are characterized by chronic and recurrent inflammation in the gastrointestinal tract. Symptoms such as diarrhea, abdominal cramps, weight loss, fatigue, anemia, and extra-intestinal signs (arthralgia or arthritis among others), have major impact on quality of life. Both disorders are characterized by intermittent active (mild, moderate, or severe) and inactive periods (remission or quiescence). The incidence and prevalence of UC and CD have increased worldwide in the last 50 years, especially in developing/Western countries. IBD is a result of a complex interplay between genetic, immunologic, microbial, and environmental factors, making development of a subtype-specific treatment a challenging task. Thus, increasing efforts are ongoing to develop personalized therapies to induce remission of these diseases and improve the patient's quality of life (1–3).

The gut microbiome has gained increasing attention as a factor that controls intestinal homeostasis in healthy individuals. Various lifestyle and environmental factors, such as hygiene and the use of antibiotics, together with the consumption of a "Western diet" low in fiber and high in fat and sugar are associated with an imbalanced intestinal microbiota, or dysbiosis, which may lead to chronic inflammation and metabolic dysfunction (4, 5). The perturbation of the microbiota can create an inflammatory environment in the gastrointestinal tract, altering intestinal homeostasis (6, 7), as seen in IBD. Innate and adaptive inflammatory cells infiltrating the lamina propria(LP) can produce pro-inflammatory cytokines (such as IFN-γ, IL-17, TNF-α, or IL-1β) exacerbating the inflammatory process, causing epithelial damage and intestinal and extra-intestinal symptoms (3, 8). However, it remains unclear whether dysbiosis is a cause or a consequence of IBD (9).

The intestinal microbiome of a healthy individual is a balanced community of different microorganisms, including bacteria, bacteriophages, viruses, archaea, and fungi (10). The bacterial community participates in maintaining intestinal homeostasis through the "training" of the immune system and inhibiting growth of pathogens and pathobionts (11, 12). Intestinal inflammatory responses are modulated by the gut microbiome. This may go either way, e.g., IL-10 deficient mice show less severe chronic bowel inflammation in germ-free (GF) conditions (13, 14), while acute chemically-induced colitis is exacerbated in GF mice compared to mice with a normal microbiome (15). Also in humans the importance of microbiota in controlling inflammation, for instance when a bowel segment is excluded from the fecal stream leading to diversion colitis/pouchitis (16). Particularly important appear to be bacterial species that feed on non-digestible dietary fibers (DF) and produce metabolites that exert positive effects on the intestinal mucosa; examples being short-chain fatty acids (SCFAs), mainly acetate, propionate, and butyrate. Butyrate is a primary energy source for colonocytes and also maintains intestinal homeostasis through anti-inflammatory actions (17, 18). At the cellular level, SCFAs can have direct or indirect effects on processes such as cell proliferation, differentiation, and gene expression. They may be absorbed by passive diffusion, but uptake by intestinal epithelial cells is greatly enhanced by dedicated transporters, e.g., the monocarboxylate transporter 1 (MCT1; encoded by *SLC16A1*) and the sodium-coupled monocarboxylate transporter 1 (SMCT1; encoded by *SLC5A8*). Moreover, SCFAs act as ligands for G-protein coupled receptors (GPCRs), including GPR109A, GPR43, and GPR41, thereby activating anti-inflammatory signaling cascades (5, 19–24). Importantly, IBD patients not only show reduced levels of dominant SCFAs-producing bacteria (like *Faecalibacterium prausnitzii* and *Roseburia intestinalis*) in intestinal mucosa and feces, but the actual steady state levels of SCFAs herein also appear to be lower compared to healthy controls (25–29).

IBD patients show dysbiosis and loss of microbiome diversity, most prominently in CD patients (28), and the associated alterations in SCFA levels might be restored by new treatment strategies. One method currently evaluated is fecal microbiota transplantation (FMT) obtained from healthy donors, which effectively induces remission in UC (30). However, long-term durability and safety still needs to be established. Other strategies for microbiome restitution are the use of prebiotics or fiber-rich diets combined with probiotics, as SCFAs-producing single microorganism or combinations may alleviate symptoms by improving butyrate levels.

Here, we aim to provide an overview of microbial SCFAs production in the intestine and their effect on intestinal cells and the immune response. Moreover, gut microbiome changes in IBD are reviewed and how they are related to impaired intestinal SCFAs production and associate to cell metabolism and signaling pathways controlling mucosal homeostasis. Finally, the therapeutic potential of SCFAs for IBD will be discussed; either applied directly or through activation of SCFAs-producing bacteria by prebiotic or probiotic approaches.

SHORT CHAIN FATTY ACIDS (SCFAs) BACTERIAL PRODUCTION

Intestinal SCFAs Production

SCFAs are carboxylic acids with aliphatic tails of 1–6 carbons of which acetate (C2), propionate (C3), and butyrate (C4) are the most abundant produced by anaerobic fermentation of dietary fibers (DF) in the intestine. DF were defined in 2009 as "*carbohydrate polymers with three or more monomeric units, which are neither digested nor absorbed in the small intestine of humans*" by the Codex Alimentarius ("Food Code") Commission (CAC), which is part of the Food and Agriculture Organization of the United Nations and the World Health Organization (FAO/WHO) Food Standards Programme (31). From the non-digestible DF, the main substrates for bacterial fermentation and SCFA production are resistant starch (RS), inulin, oat bran, wheat bran, cellulose, Guar gum, and pectin. In particular, RS is an important source for butyrate production (32). Bacteroidetes (gram-negative) and Firmicutes (gram-positive) are the most abundant phyla in the intestine, with members of the Bacteroidetes mainly producing acetate and propionate, while Firmicutes mostly produce butyrate in the human gut (33, 34).

Butyrate and propionate formation in the gut occurs mainly from carbohydrate metabolism in glycolysis, but can also take place from organic acids and amino acids metabolism (34). In addition, acetate is the most abundant SCFA in the gut produced from acetyl-CoA derived from glycolysis and can also be transformed into butyrate by the enzyme butyryl-CoA:acetyl-CoA transferase (**Figure 1**) (35–38).

Quantification of human intestinal SCFAs only provides steady state levels and may not accurately reflect the level of bacterial production as most SCFAs produced in the colonic lumen (90–95%) are absorbed by the gut mucosa (39). Nevertheless, the analysis of SCFAs in fecal samples is used as an approximation of gut levels, since excreted SCFA concentrations are associated with RS enriched diets (substrates of SCFAs-producing bacteria), inferring the relationship between intestinal SCFAs production and fecal levels (40, 41).

SCFAs concentrations (expressed as molality or molarity) have been measured in intestinal tissue and fecal samples from individuals of different ethnicity (42–45). In the human gastrointestinal tract, the highest SCFA concentration is found in colon at a molar ratio of approximately 60:20:20 for acetate:propionate:butyrate (**Table 1**), taken from *post mortem* human subjects (42).

In contrast, the molar ratio of acetate:propionate:butyrate in fecal samples of healthy subjects varies among cohorts, while propionate and butyrate content are similar with an estimated concentration of 20 and 15 mM, respectively (**Table 1**).

SCFAs concentrations were found higher in proximal colon (around 70–140 mM) than distal colon (around 20–70 mM) in pigs, although this varies depending on the intake of DF (43).

Finally, SCFAs levels in other tissues such as liver or blood (**Table 1**) are much lower than in the intestine (42), demonstrating that SCFAs signaling, uptake and/or metabolism mainly occur at the intestinal mucosa. However, detection of extra intestinal levels implies that these metabolites have systemic functions, as established for central nervous system autoimmunity (46).

Main SCFAs Producers

The main butyrate producing-bacteria in the human gut belong to the phylum Firmicutes, in particular *Faecalibacterium prausnitzii* and *Clostridium leptum* of the family *Ruminococcaceae,* and *Eubacterium rectale* and *Roseburia* spp. of the family *Lachnospiraceae* (33, 34). In addition, sugar-and/or lactate-utilizing bacteria produce butyrate from lactate and acetate, such as *Eubacterium hallii* and *Anaerostipes* spp. (33).

Still, the list of butyrate-producing bacteria may be much longer as members of Actinobacteria, Bacteroidetes, Fusobacteria, Proteobacteria, Spirochaetes, and Thermotogae are potential butyrate producers according to the genes they express, including those that encode enzymes that synthesize butyrate, such as butyryl-CoA dehydrogenase, butyryl-CoA transferase and/or butyrate kinase (47). Moreover, apart from butyrate, the production of other SCFAs is mediated by bacteria such as *Bifidobacterium* species (belonging to the Phylum Actinobacteria) that produce acetate and lactate during carbohydrate fermentation (48). Also, the mucin-degrading bacteria *Akkermansia muciniphila* (Phylum *Verrucomicrobia*) produces both propionate and acetate (34, 49).

The main butyrate-producing bacteria are anaerobes, including the Bacteroidetes and Clostridia, and the low O_2 concentrations in the colon create a favorable niche for them. Moreover, butyrate absorbed and metabolized by the epithelium consumes (local) O_2 and thereby stabilizes the hypoxia-inducible factor (HIF, a transcription factor coordinating barrier protection) (50). These data are consistent with studies demonstrating that streptomycin-treated mices how relapse of gastroenteritis by *Salmonella* (51) as well as the expansion of potentially pathogenic *E. coli* (52). The susceptibility due to the depletion of anaerobic bacteria (induced by antibiotics) is associated to a reduction in butyrate levels, thus promoting an aerobic environment and the expansion of aerobic bacteria such as *Salmonella typhimurium* (51, 52). In addition, depletion of butyrate-producing bacteria by antibiotic treatment reduces the intracellular butyrate/PPARγ signaling, increasing iNOS and nitrate levels, favoring Enterobacteriaceae expansion (52).

SCFAs FUNCTIONS IN THE INTESTINAL MUCOSA

In the intestinal mucosa; acetate, propionate and butyrate exert beneficial effects over intestinal epithelial cells (IECs) and immune cells through induction of intracellular or extracellular processes (see **Figure 2** for more details). SCFA may permeate through the cell membrane by passive diffusion (19). However, their absorption is greatly enhanced by two different solute transporters, the proton-coupled monocarboxylate-transporter 1 (MCT1/*SLC16A1*) and the sodium-coupled monocarboxylate-transporter 1 (SMCT1/*SLC5A8*) (20, 21). Alternatively, SCFA may activate signaling pathways via at least 3 different GPCRs: GPR41 (free fatty acid receptor 3; *FFAR3*), GPR43 (free fatty acid receptor 2; *FFAR2*), and GPR109A (hydroxycarboxylic acid receptor 2; *HCAR2*). These receptors are pertussis toxin (PTX)-sensitive, thus coupled to G_{i-o} type G proteins mediate the inhibition of adenylyl cyclase whilst activating AMP-dependent and, to a lesser extent, the phospholipase C (PLC) pathway. In addition, GPR43 mediates G_q protein whilst signaling through the PLC pathway (5, 22–24) (see **Table 2** for transporters and GPCRs tissue and cell expression).The main cellular functions of SCFAs in the intestinal mucosa are described below.

SCFAs and Cell Proliferation

Small intestinal IECs show a reduced proliferative activity and turnover in GF or antibiotic-treated specific pathogen-free (SPF) mice (69). This is reversed, however, when GF or SPF mice treated with Gram-positive commensal bacteria or a mix of SCFA (acetate, propionate and butyrate) (69). These observations demonstrate the role of the commensal microbiota and their products maintaining the intestinal homeostasis and IECs turnover. In line, SCFAs regulate epithelial gene expression involved in energy metabolism (e.g., lipid metabolism), and

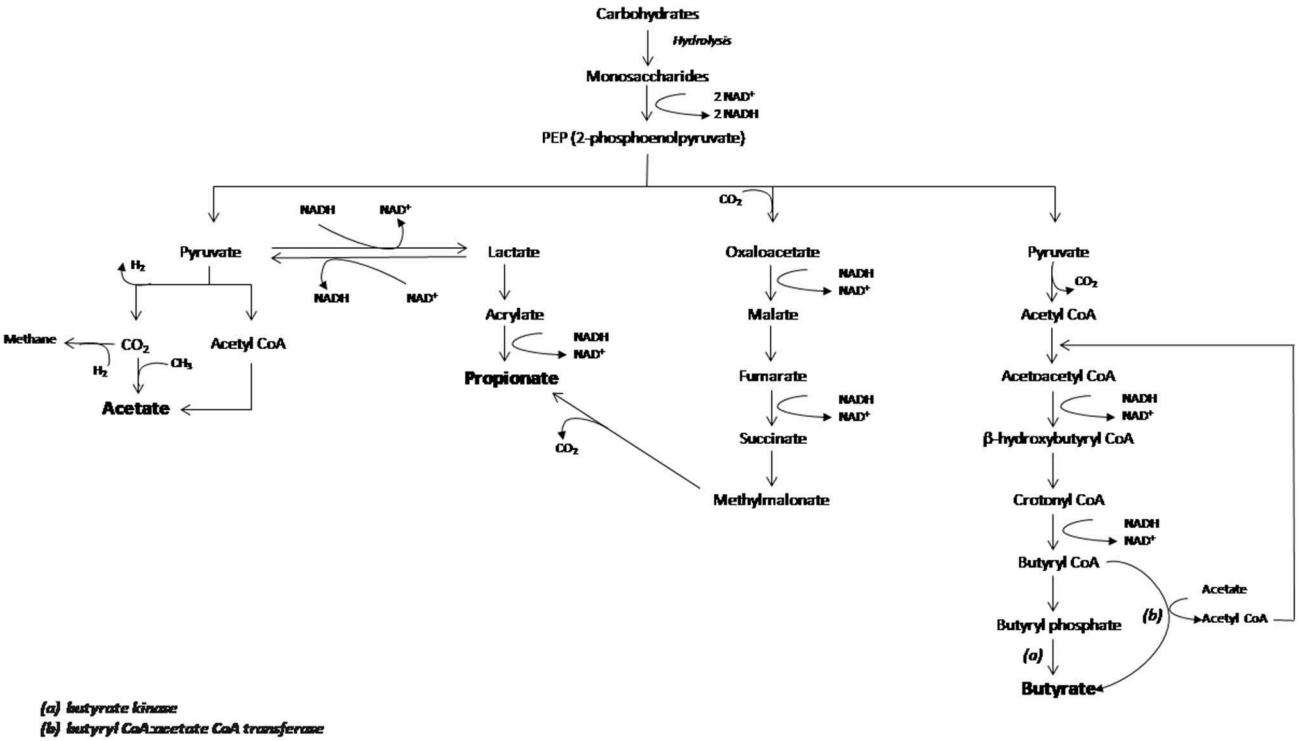

FIGURE 1 | Schematic representation of carboydrates fermentation pathways that lead acetate, propionate and butyrate production. The main enzymes involved in the butyrate production are indicated as (a) butyrate kinase and (b) butyryl CoA:acetate CoA transferase. Figure adapted from den Besten et al. (35).

TABLE 1 | SCFAs concentration in human samples.

	Samples	Concentration	Cohort	References
Total SCFA	Cecum (mmol/kg)	131 ± 9	English	(42)
	Descending colon (mmol/kg)	80 ± 11		
	Ileum (mmol/kg)	13 ± 6		
	Portal blood (mM)	0.375 ± 0.070		
	Liver (mM)	0.148 ± 0.42		
	Peripheral blood (mM)	0.079 ± 0.022		

	Samples	Acetate	Propionate	Butyrate	Cohort	References
SCFAs variants	Colon molar ratio	60	20	20	English	(42)
	Fecal concentration (μmol/g = mmol/kg)	209.7 ± 14.0	93.3 ± 5.3	176.0 ± 16.0	Malaysian	(44)
	Fecal concentration (mM)	87 (58.4–114.9)	21.6 (16.5–27.2)	14.7 (10.3–24.6)	Belgian	(45)
	Fecal concentration (mM)	39.9–56.1	12.8–23.6	12.2–19.0	Japanese, Chinese and Australian	(43)

promote the development of mouse intestinal organoids (69, 70), further reinforcing their role in supporting epithelial cell proliferation.

On the other hand, recently it was shown that butyrate appears to have a different effect on intestinal stem/progenitor cells, inhibiting their proliferation and delaying wound repair through the transcription factor Foxo3 (71). This suggests that SCFAs and particularly butyrate has cell type-specific effects in the intestinal epithelium and may be linked to local SCFA concentrations, where differentiated IECs in the villus are exposed to higher concentrations of microbial metabolites compared to the stem cells in the crypt (71). As part of the maintenance of intestinal homeostasis, and in contrast to the effect on IECs in healthy conditions, SCFAs suppress cancer cell proliferation and induce apoptotic cell death (72). Moreover, SCFAs induce autophagy in colon cancer cell lines (HCT-116, SW480, and HT-29) (73, 74), as a protective response against apoptosis. These observations are interesting in the context of host-microbe interaction in healthy and colorectal cancer (75) though this is beyond the scope of this review.

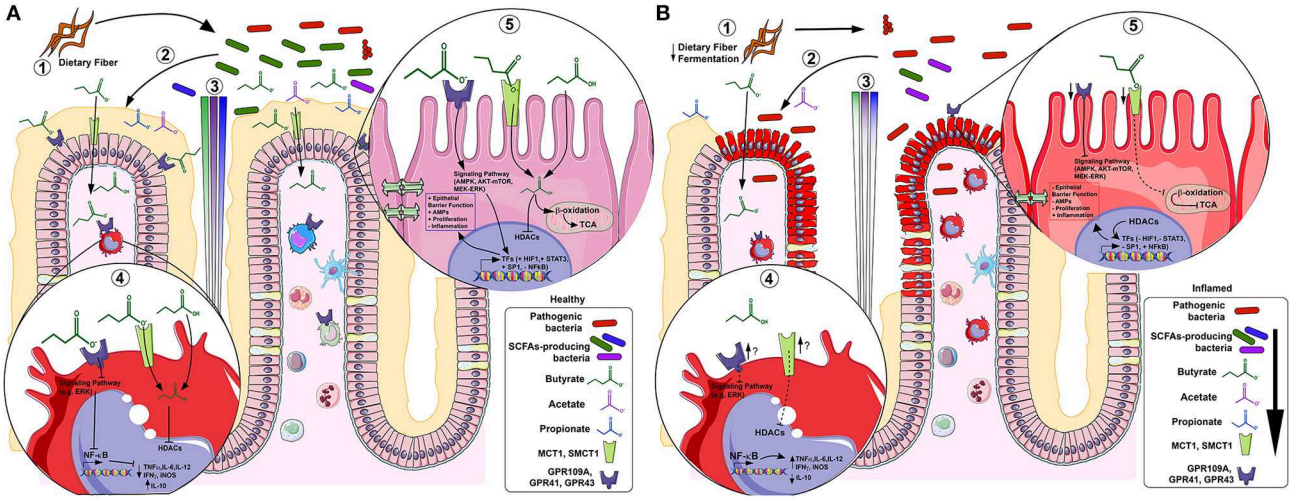

FIGURE 2 | SCFAs in healthy **(A)** and inflamed **(B)** colonic mucosa. In healthy mucosa, (1) bacterial fermentation of dietary fiber (DF) by SCFAs-producing bacteria (e.g., *F. prausnitzii*), increases luminal content of butyrate (green), propionate (blue) and acetate (purple) (2), forming a gradient along the crypt. In lamina propria (LP) macrophages under acute inflammatory stimulus (4), butyrate inhibits histone deacetylases (HDACs) thus; NF-κB-induced pro-inflammatory mediators (e.g. TNF-α, IL-6, IL-12 and iNOS) expression whereas increases anti-inflammatory mediators (e.g., IL-10). In colonocytes (5), butyrate is β-oxidized to Acetyl-CoA and constitutes the main source of energy by entering the TCA cycle. Alternatively, butyrate initiates signaling pathway activation (or repression) by GPCRs and/or directly inhibits HDACs, thus activating (e.g., HIF-1, STAT3 and SP1) or repressing (e.g., NF-κB) transcription factors (TFs), increasing epithelial barrier function, antimicrobial peptides (AMPs) production, cell proliferation and decreasing inflammation. In inflamed mucosa as IBD, (1) a decreased fermentation of DF by low levels of SCFAs-producing bacteria (e.g., *F. prausnitzii*) (2), reduces SCFAs luminal content (3). In LP inflammatory macrophages (4), butyrate-GPCRs activation and -HDACs inhibition are downregulated, thus, there is uncontrolled NF-κB-induced pro-inflammatory mediators' expression (e.g. TNF-α, IL-6, IL-12 and iNOS) and decrease of anti-inflammatory mediators (e.g., IL-10), although it appears that the inflammation increasesthe GPCRs and transporters expression. In inflamed colonocytes (5), butyrate uptake and oxidation are decreased and GPCRs and transporters are also downregulated. This contributes to decreased epithelial barrier integrity, AMPs production, cell proliferation and increased inflammation.

SCFAs and the Epithelial Barrier

The SCFA butyrate promotes the epithelial barrier function, being a main stabilizing mechanism for HIF-1 (as previously mentioned in Main SCFAs producers). Both HIF-1α expression and butyrate levels are reduced in antibiotic-treated or GF mice, but HIF-1α expression is restored after butyrate supplementation (76). Importantly, butyrate induces the barrier function (measured by FITC-dextran flux) in T84 cells, but not in the absence of HIF-1β, demonstrating a crucial role for HIF-1 in maintaining barrier integrity (76). Furthermore, butyrate promotes the epithelial barrier function through induction of genes encoding tight-junctions (TJ) components and protein reassembly through the activation of other transcription factors, including STAT3 and SP1 (**Table 3**). As a result, butyrate maintains and/or increases transepithelial electrical resistance (TEER) in human colonic Caco-2 and T84 cells (77–80), rat small intestine cdx2-IEC cells (81) and small intestine porcine IPEC-J2 cells even when exposed to inflammatory conditions (82). Such effect can also be achieved in Caco-2 cells by supplementing a supernatant of CD microbiota with probiotic *Butyricicoccus pullicaecorum* 25-3^T or a mix of six butyrate-producers when compared to the treatment of CD microbiota-supernatant alone (87). These results reinforce the evidence that the metabolite butyrate restores intestinal barrier function in inflammatory conditions *in vitro* (82), being relevant in the context of IBD, where intestinal epithelial healing is an important therapeutic target. Another important mechanism involved in the epithelial barrier function is the production of antimicrobial peptides (AMPs) by IECs. Recently it was shown that the expression of the AMPs RegIIIγ and β-defensins is strongly impaired in Gpr43 KO mice, while butyrate/Gpr43 activation induced AMP production in *in vitro*, *ex vivo,* and *in vivo* models (88). This indicates that the effects of SCFAs are not only restricted to inter-epithelial junctions, but also involve regulation of epithelium/luminal bacteria interaction through the production of AMPs as first line defense effectors against pathogens.

SCFAs as Energy Source

Butyrate is the main energy source of colonocytes (48), as demonstrated for primary colonocytes from the human ascending and descending colon, which consume more than 70% of oxygen due to butyrate oxidation (89). Interestingly, an energy-deprived state (reflected by decrease of enzymes involved in tricarboxylic acid cycle) leads to lower ATP levels and, ultimately autophagy, observed in GF mice colonocytes. Recolonization of GF mice with butyrate-producing bacteria and butyrate treatment of GF colonocytes *ex-vivo*, increases oxidative phosphorylation and suppresses autophagy to normal levels (17), implying the importance of host-microbe interaction in energy metabolism of colonic epithelium.

Anti-inflammatory Effects of SCFAs

Apart from the physiological functions of SCFAs detailed above, they also exert anti-inflammatory effects in intestinal mucosa

TABLE 2 | SCFAs transporters and receptors.

	Ligands	Tissue or cell expression	Species	References
TRANSPORTERS				
MCT1	**Butyrate**, lactate, pyruvate	Distal colon> proximal colon>ileum>jejunum	H	(53)
		Transverse colon>ascending and descending colon>sigmoid colon	H	(54)
		Cecum>colon>stomach and small intestine	M, R	(55, 56)
		Monocytes, granulocytes and, lymphocytes	H	(57, 58)
		Peritoneal macrophages	M	(59)
SMCT1	**Butyrate > propionate** > lactate >> **acetate**	From terminal ileum to distal colon	M	(55)
		Distal colon>proximal colon and ileum	H, M	(60)
G-PROTEIN COUPLED RECEPTORS				
GPR41	**Propionate**=pentanoate =**butyrate**>**acetate**>formate	Adipose tissue > PBMCs, pancreas, spleen, and placenta	H	(22)
		Monocytes, neutrophils and, monocyte-derived DCs	H	(61, 62)
GPR43	**Acetate**=**propionate**= **butyrate**>pentanoate> hexanoate>formate	Intestinal epithelium	M, H	(6)
		Monocytes, neutrophils and PBMCs and B/T-lymphocytes	H	(22, 61, 63)
		Treg (colon > spleen and MLN) and colonic myeloid cells	H	(64)
GPR109A	D-beta-hydroxybutyrate, **butyrate** and nicotinic acid	Adipose tissue (>lung, adrenal gland, and spleen)	H, M	(65)
		Colon (> ileum, jejunum, and duodenum)	H, M	(66)
		Monocytes, monocyte-derived DCs, DCs (blood, splenic and colonic), macrophages (splenic and colonic), and BMDM	H, M	(62, 67, 68)

PBMCs, peripheral blood mononuclear cells; DCs, dendritic cells; BMDM, bone marrow-derived macrophages; H, human; M, mouse; R, rat.
Main SCFAs (acetate, propionate, and butyrate) are highlighted in bold font.

by histone deacetylases (HDACs) inhibition and activating the GPCRs present in IECs and immune cells (**Table 3**). In IECs models, butyrate suppresses lipopolysaccharide (LPS)-induced NF-κB activation via GPR109A *in vitro* in colonic cell lines and *ex vivo* in mouse colon (66). In addition, the acetate/GPR43 pathway stimulates potassium efflux and hyperpolarization in HT-29 and NMC460 colonic cells leading to NLRP3 inflammasome activation (90). In concordance with these observations, IL-18 is activated in colonic epithelial cells from mice fed on high fiber diet following dextran sulfate sodium (DSS)-colitis (90). These results confirm an important role of GPR109A and GPR43 activation by SCFAs in controlling inflammation and promoting epithelial repair in the colon. Interestingly, butyrate enhances the MCT1 surface expression in the colonic cell line C2BBe1 in a GPR109A-dependent manner (91), suggesting a "cooperative role" between these proteins in mediating butyrate effects.

With respect to innate immune functions, SCFAs induce prostaglandin E_2 release and expression of the anti-inflammatory cytokine IL-10 through PTX-sensitive GPCRs, thereby inhibiting inflammatory responses in human monocytes (61). The molecular mechanism involved in pro-inflammatory mediator suppression (e.g., LPS-induced chemokines and cytokines) by SCFAs has not been completely determined in other human/mouse mononuclear cell models (62, 67, 83). In addition to the anti-inflammatory effects of the microbial metabolism of dietary fibers to SCFAs, it is important to note that such fibers may also modulate the intestinal immune system directly. For instance, DF pectin (with low degree of methyl esterification) blocks the pro-inflammatory Toll-like receptor (TLR) 2-1 pathway in human dendritic cells (DCs) and mouse macrophage cell lines as well as in an ileitis *in vivo* mouse model (92). These results show that DF regulates inflammatory reactions in intestinal immune and epithelial cells not only after being metabolized by gut bacteria.

The inhibition or reversal of the immune cell inflammatory profile (M1-like macrophages toward a M0-like non-polarized) or polarization toward M2-like anti-inflammatory macrophages is a therapeutic target in the context of IBD. In this way, butyrate effects on mouse IL-4-polarized M2 macrophages are contradictory, as it enhances or suppresses *Arg-1* and *Ym1* expression (M2-profile markers) (93, 94). Therefore, clarification is needed of SCFAs effect on macrophage polarization including the evaluation of human *ex vivo* models and other markers that could ensure stronger conclusions.

Also, SCFAs (mainly butyrate) have inhibitory effect over HDACs activity promoting histone acetylation, affecting gene regulation of cell proliferation, differentiation, and inflammatory response, contributing to intestinal homeostasis and cancer protection (67, 95–99). HDACs regulate innate immunity pathways, controlling myeloid cell differentiation and inflammatory response mediated by TLR- and IFN-inducible gene expression (100). Furthermore, the use of HDACs inhibitors

TABLE 3 | Impact of SCFAs on intestinal homeostasis.

Cell type		Model	SCFAs	Effect	Mechanism	References
Epithelial cells	Cell lines	Caco-2	Butyrate 2 mM	↑ TEER, ZO-1, occludin	Activation of AMPK Inhibition of MLCK/MLC2 pathway and phosphorylationof PKCβ2	(77, 78)
			Butyrate 5 mM	↑ TEER, claudin-7, claudin-2	Not determined	(79)
		Caco-2, T84	Butyrate 5 mM Propionate 20 mM	↑ TEER, ↓ Claudin-2	Induction of IL-10RA through STAT3 activation and HDAC inhibition	(80)
		Cdx2-IEC	Butyrate 4 mM	↑ TEER, claudin-1, ZO-1, occludin	Induction of Claudin-1 transcription through SP1	(81)
		IPEC-J2	Butyrate 1 mM	↓ LPS impairment of intestinal barrier ↑ Claudin 3 and claudin 4	Activation of Akt/mTOR signaling	(82)
		CCD841, KM12L4, and HCT116	Butyrate 1 mM	Blockade of LPS-induced NF-κB	Activation of GPR109A	(66)
	Primary cells	Colon culture	Butyrate 0.5 mM	↑ IL-18 mRNA and protein	Activation of GPR109A	(68)
		Mouse small intestine organoids	Acetate, propionate and butyrate 5 mM	↑*Fiaf*, Hdac3, Hdac5 ↓ Gpr43, Pparγ	Not determined	(70)
			Acetate, propionate and butyrate 0.5 mM	↑Promotion of organoids development	Activation of GPR41 or GPR43 and MEK-ERK signaling	(69)
Immune cells	Cell lines	RAW 264.7	Sodium butyrate (NaB), sodium phenylbutyrate (NaPB) and sodium phenylacetate (NaPA) 0.5–1 mM	↑ IL-10 ↓ IFN-γ-induced iNOS, TNF-α, IL-6	Inhibition of NF-κB and ERK signaling pathways	(83)
	Primary cells	Human LP macrophages	Butyrate enemas 100 mM	↓ Inhibition of NF-κB translocation	Not determined	(84)
		Human monocytes	Acetate, propionate and butyrate 0.2–20 mM	↑ PGE_2 ↓ MCP-1, IL-10	Activation of PTX-sensitive GPCRs	(61)
		PBMC	Acetate, propionate and butyrate 0.2–20 mM	↓ LPS-induced TNF-α and IFN-γ	Not determined	
		Human monocyte-derived DCs	Propionate and butyrate 1 mM	↓ LPS-induced chemokines and cytokines (CXCL9-CXCL11), cytokines (IL-6 and IL-12p40)	Not determined	(62)
		Mouse LP macrophages and BMDM	Butyrate 0.1–2 mM	↓ LPS-induced mediators NO, IL-6, IL-12p40	Inhibition of HDACs	(67)
		Mouse DCs	Butyrate 0.125–2 mM	↑ $Foxp3^+CD4^+$ T cells	Inhibition of HDACs	(85)
		Mouse LP macrophages and DCs	Butyrate 0.5 mM	↑ $Foxp3^+CD4^+$ T cells	Activation of GPR109A	(68)
		Mouse T cells	Propionate butyrate 0.1 mM	↑ Foxp3 and IL-10 in naïve $CD4^+$ T cells	Activation of GPR43 and Inhibition of HDACs	(64, 86)

(e.g., valproic acid) reduce disease severity and inhibit colonic proinflammatory cytokines (TNF-α, IFN-γ, and IL-6) in experimental murine colitis (101). These results are promising in regard to the search of alternatives for IBD therapy and support the importance of butyrate as an HDAC inhibitor.

REGULATION OF SCFA TRANSPORTERS AND RECEPTORS IN THE INTESTINAL MUCOSA

Physiological Regulation of Transporters and GPCRs by Ligands

In line with the SCFAs production in the gut, prominent expression of MCT1 and SMCT1 is observed in the colon of humans, mice and rats, while much lower levels are detected in ileum (see **Table 2**). Effective absorption of SCFAs from the gut lumen requires an apical location of MCT1, however, depending on experimental approaches it has also been detected in basolateral membranes of the human colonic epithelium (53–55). SMCT1, on the other hand, has been mainly detected in the apical membranes in proximal and distal colon (55, 60), as well as in the ileal enterocytes (55, 60, 102). Interestingly, GF mice show a decreased expression of SMCT1 in colon and ileum, which is recovered by recolonization of the gut with bacteria (102).

MCT1 is considered to be the primary transporter for butyrate uptake in intestinal epithelial cells and its expression is induced by butyrate and fermentable carbohydrates, as demonstrated in *in vitro, ex vivo, and in vivo* models, as described below.

Butyrate induces *SLC16A1* (encoding MCT1) mRNA expression coinciding with enhanced protein expression in Caco-2 cells and in the apical membrane of human colonic AA/C1 and C2BBe1 cells (91, 103, 104). In addition, the direct effect of butyrate studied in *ex vivo* pig colonic mucosa culture showed an up-regulation of *SLC16A1* mRNA expression (103).

These *in vitro*/*ex vivo* observation are confirmed *in vivo* where gastrointestinal levels of MCT1 are enhanced in rats receiving a pectin-supplemented diet, particularly in the apical membrane of colonic mucosa, and increases the transepithelial flux of butyrate (56, 91). Similar observations were made in pigs, where *SLC16A1* mRNA levels increased in cecal and colonic mucosa after dietary supplementation with RS compared to digestible starch (DS) (41). In contrast, *SLC5A8* mRNA levels (encoding SMCT1) were not affected by RS or DS diet (41), showing a specific regulation of MCT1 expression by RS in comparison to SMCT1. On the other hand, a high-protein diet (with or without fermentable carbohydrates) lead to a reduction in colonic MCT1/*SLC16A1* expression in pigs without affecting the butyrate levels (103). This was accompanied by an induction of *TNF-α*, *IL-8,* and *IFN-γ* mRNA expression, suggesting that the inflammatory environment influences in the expression of the butyrate transporter. Similar as described for SCFA transporters, epithelial GPR43 and GPR109A expression in mouse and human intestinal mucosa is related to the proximity to bacterial metabolite production in the colonic lumen (6, 66, 68), suggesting that the levels of these proteins are controlled by their own substrates. In line, GPR109A protein and gene levels are reduced in the ileum and colon of GF mice compared to conventional mice, recovering their normal levels after bacterial re-colonization (102). Moreover, GPR43 expression is reduced in intestinal mucosa of mice fed a "Western-like diet" high in fat and sugar (6). These observations suggest that a reduction in colonic SCFAs as a result of deficiency in specific bacteria or a high fiber diet leads to down-regulation of these SCFA-sensitive GPCRs. In contrast, intestinal mucosal levels of *FFAR2* and *FFAR3* (encoding GPR43 and GPR41, respectively) were not different in pigs fed either a DS- or a RS-containing diet (41), suggesting that dietary fiber does not regulate the gene expression of its metabolite-sensing receptors in these animals. Future studies need to address whether this is truly a species difference or may be caused by experimental differences.

Taken together, it appears that particularly SCFA transporters in the intestinal mucosa, especially MCT1, are highly regulated by their substrates in healthy/non-inflammatory conditions, while this is less well-established for the SCFA-sensing GPCRs. The effect of inflammatory conditions on these mediators of SCFA uptake and signaling is described next.

Interaction of SCFA Uptake and Signaling With the Intestinal Mucosa in the IBD Context

Among the deregulations detected in the intestinal mucosa of IBD patients, it has been found that the *SLC16A1* gene and MCT1 protein expression is reduced in inflamed mucosa of UC and CD patients (105–107). This may be direct effect of the inflammation or caused by a reduction in butyrate-producing bacteria (see for more details Microbiome changes in CD and UC in relation to SCFAs-producers). In addition, butyrate uptake, and oxidation is inhibited in UC patients compared to healthy individuals (106). Most notably, a significant inverse correlation is observed between butyrate uptake/oxidation and the Mayo endoscopic subscore and Geboes histological score (106). In particular, genes encoding enzymes involved in butyrate metabolism/oxidation (such as *ACSM3, ACADS, ECHS1, HSD17B10, ACAT1, ACAT2*, *ABAT, ALDH1A1, ALDH2, ALDH9A1, EHHADH, HADHA, HMGCL,* and *PDHA1*) are down-regulated in inflamed mucosa of UC patients (105–108), revealing a specific inflammation-driven gene regulation in the intestine. Interestingly, gene expression of *ACSM3, ACADS, ECHS1, HSD17B10* and *ACAT2* (all enzymes involved in butyrate oxidation), but not *SLC16A1,* increased in mucosa of UC patients that responded to infliximab (human anti-TNF-α antibody) therapy (although only *ACSM3* mRNA levels were higher after therapy than in healthy controls) (106). This suggests that butyrate oxidation is impaired by mucosal inflammation and butyrate supplementation alone would be insufficient to regain homeostasis (106). Hence, these results show that inflammation is tightly linked to the inhibition of genes related to SCFAs uptake and metabolism.

The pro-inflammatory cytokine TNF-α inhibits butyrate oxidation in normal colonic mucosa culture (109), reinforcing the role of inflammatory mediators as part of the intestinal SCFA uptake regulation. Similar observations were made *in vitro* in intestinal HT-29 and (IEC)-6 cell lines showing that inflammatory cytokines inhibit butyrate uptake (60), oxidation and MCT1/*SLC16A1* expression (105). Additionally, MCT1 was downregulated in Caco-2 cells and *ex vivo* porcine colonic tissue culture, exposed to TNF-α (103).

Regulation of MCT1 expression has mostly been studied in IECs, although it also modulates immune cell functions (**Table 2**). Interestingly, pro-inflammatory stimuli like lipopolysaccharide (LPS) and TNF-α induce *Slc16a1* mRNA and protein expression in mouse peritoneal and J774.1 macrophages, suggesting inflammatory macrophages are sensitive to butyrate (59), but possible respond differently than intestinal epithelial cells. However, more studies are needed to understand how MCT1 is regulated in inflammatory macrophages and its implications for IBD, as they are innate immune cells exacerbating inflammation in intestinal mucosa.

In CD, GPR43 protein expression was lower in ileum of patients either in acute/active or in the quiescent/remissive phase when compared to control subjects (6), suggesting that CD-specific factors are involved in the downregulation of this SCFA receptor, where inflammation seems not to be a crucial determinant.

Animal models have demonstrated the importance of the SCFA/GPCR pathway in IBD. Acute and chronic DSS-induced colitis leads to higher disease activity and colonic inflammation in *Gpr43* KO mice compared to WT littermates, as characterized by increased histological score, neutrophil infiltration together with TNF-α and IL-17 protein levels in the colonic mucosa (15, 90, 110).

Interestingly, high fiber diet or acetate/GPR43 activation suppresses colonic inflammation through NLRP3 inflammasome or cytokine/mediator regulation in DSS-treated GF and WT mice, but not in *Gpr43* KO mice (15, 90, 110), indicating that GPR43 mediates the anti-inflammatory effects of SCFAs in intestinal mucosa. In the same way, LPS-induced TNF-α secretion in mouse-derived peripheral blood mononuclear cells (PBMCs) was suppressed by acetate and reversed by an anti-GPR43 antibody, confirming that acetate/GPR43 signaling mediates anti-inflammatory effects (110). In support of a role for GPR43 in the prevention of intestinal inflammation, mice treated with a GPR43 agonist appear less susceptible to DSS-induced colitis than WT controls (6).

However, not all GPR43-focussed studies appear to give consistent results. A study by Sina et al. reported that *Gpr43* KO mice actually showed less colonic mucosal damage and inflammatory cell infiltration after acute or chronic DSS exposure compared to WT littermates (111). Future studies need to address whether these apparent contradicting results may be explained by the use of different DSS concentrations, time of treatment and/or transgenic mouse services.

Propionate and butyrate treatment increases the chemotactic migration of *ex vivo*-cultured polymorphonuclear leukocytes (PMN) from WT, but not from *Gpr43* KO mice, suggesting that GPR43 activation is relevant in PMN recruitment (111). These observations support the fact that GPR43 activation by SCFAs is important in mounting prompt immune responses.

Gpr41 and *Gpr43* KO mice show an impaired immune response when exposed to ethanol-induced gut barrier disruption, 2, 4, 6-trinitrobenzene sulfonic-acid (TNBS)-induced colitis, or oral infection with the mucosal pathogen *Citrobacter rodentium*, which is characterized by a decreased neutrophil frequency and expression of inflammatory-associated genes (112). The activation of the acetate/GPCRs pathway accelerates the immune response to *C. rodentium* infection in WT mice demonstrating that IECs mediate the fast immune response dependent on GPR41 and GPR43 activation (112). These observations reveal differences between colonic inflammation models in GPCR KO mice, as acute DSS-induced colitis is characterized by a T_{H1}-T_{H17} immune response and in chronic phase is predominantly T_{H2}-mediated (113). In TNBS-induced colitis the immune response can be T_{H1}, T_{H17}, or T_{H2} depending on the mouse strain (113, 114), with *C. rodentium* infection inducing a T_{H1} immune response (115).

As described for *Gpr43* deficient mice, *Gpr109a* KO mice are more susceptible to chemically-induced colonic inflammation and inflammation-associated colon cancer (68). However, the butyrate/GPR109A pathway activates colonic homeostasis by suppressing inflammation in colonocytes (mediated by IL-18 expression and NLRP3 inflammasome activation) (68, 90) and LP macrophages / DCs by differentiating *naïve* T cells to $Foxp3^+$ Treg cells and IL-10-producing T cells in WT, but not in *Gpr109a* KO mice (68).

Among the multiple factors involved in IBD pathogenesis, the imbalance between Treg and T effector cells has been the subject of considerable attention to improve IBD therapy. Therefore, in addition to Treg induction mediated by butyrate-induced macrophages and DCs (68), propionate also directly stimulates Treg proliferation and function through GPR43 and HDAC inhibition (64). Also, propionate and butyrate induce colonic Treg differentiation from *naïve* $CD4^+$ T cells upregulating *Foxp3* transcription through histone acetylation (85, 86).

In addition, butyrate increases IL-10 production by *ex vivo*-differentiated human Tregs with GPR43-agonists further increasing the suppressive capacity of human Tregs (116), reinforcing the previous evidence of tolerance induction by SCFA in animal models.

As a side note, it is important to mention that depending on the SCFA concentration and cytokine milieu the effects can be 2-fold, either stimulating IL-10-producing T and Treg expansion or T *naïve* differentiation into effector T cells (expressing T-bet transcription factor and IFN-γ; T_{H1}*cells*, or IL-17; T_{H17}) independent of GPR41, GPR43, or SMCT1, but dependent on direct HDAC inhibitor activity (117, 118).

These findings generate new research questions in IBD patient's therapy, such as what is the best formulation of a DF-enriched diet to induce gut immune tolerance? or what is the effect of a high fiber diet or SCFA supplementation on Treg function in IBD patients within an acute or chronic phase? In summary, pharmacokinetic studies, high fiber diet design, and another approach need to be explored to clarify novel therapeutic options for IBD.

Mononuclear cells and neutrophils are innate immune cells mediating the protection against pathogens through recognition and elimination of antigens that cross the epithelial barrier and cytokine/chemokine secretion, thus activating the adaptive immune response. In these cells, expression of SCFA-activated GPCRs is induced by inflammation; thus sensitivity to potential anti-inflammatory actions of SCFAs is increased. Examples are LPS from *E. coli* O55:B5 increases *Gpr109a* mRNA levels in mouse macrophages (119), and also *GPR43* mRNA expression induced by TNF-α, GM-CSF (63) and TLRs (Toll-like receptors) ligands (61, 63) in human monocytes. Moreover, the effect of *E. coli* LPS on *GPR43* mRNA expression in human monocytes may be strain-dependent, as *E. coli* O55:B5 LPS induces *GPR43* mRNA expression (63) whereas *E. coli* O127:B8 LPS does not (61).

Taken together, these studies provide strong evidence for the role of SCFAs/GPCRs (particularly GPR43 and GPR109A), in maintaining colon integrity by inducing mucosa healing and suppressing inflammation. These are relevant therapeutic targets for numerous diseases, but in particular for IBD.

MICROBIOME CHANGES IN CD AND UC IN RELATION TO SCFAs-PRODUCERS

Various changes occur in the intestinal mucosa of IBD patients in active or quiescent status compared to healthy individuals, one being the composition and function of the microbiota, a change often referred to as dysbiosis. In general, dysbiosis in IBD patients is associated with a decrease in the number of SCFAs/butyrate-producing bacteria, in particular members of the phylum Firmicutes. In addition, more specific studies show that a decrease in *F. prausnitzii,* a butyrate producing-bacteria

from the *Clostridium* cluster IV, is a hallmark of active IBD patients, as reviewed previously covering different populations (25, 26, 120–122). Additionally, CD appears to have a more pronounced dysbiosis than UC, with lower diversity, altered composition and an unstable microbial community (28). Thus, CD and UC are being recognized as distinct diseases even at the microbiome level.

At the species level, alterations in other butyrate-producing species have been detected in UC patients, such as *Roseburia intestinalis* and *Roseburia hominis* (25, 45). Alternatively, stool samples of CD patients show an increase of *Ruminococcus gnavus* and decrease of *F. prausnitzii*, *Bifidobacterium adolescentis, Dialister invisus*, an uncharacterized species of Clostridium cluster XIVa, and other SCFAs-producing bacteria (*Blautia faecis, Roseburia inulinivorans, Clostridium lavalense,* and *Bacteroides uniformis*) (27, 29).

The microbiome diversity is affected by geography, ethnicity and lifestyle even in the healthy population (123), which also includes the abundance of SCFAs/butyrate-producing bacteria. However, the "environmental" factors in IBD remain unclear. Reported dysbiosis in IBD patients from different populations might be due to sample size, patient selection criteria or genetic heterogeneity, therefore, further studies are required to clarify differences in microbiome diversity among IBD patients.

As a consequence of the reduction in SCFAs-producers, SCFA levels are often found to be decreased in fecal samples of IBD patients. One study showed that acetate and propionate, but not butyrate, are reduced in fecal samples of UC patients (45). In another study, a reduction of butyrate and propionate in stool samples of IBD patients was found (44). Similarly, a low content of n-butyrate, iso-butyrate and acetate was detected in feces from patients with severe UC. The reduction in SCFAs levels might be related to disease activity, as a higher n-butyrate level was detected in UC patients in remission compared to ones with active disease (25).

THERAPEUTIC APPROACHES OF SCFAs IN IBD AND DIVERSION COLITIS

SCFAs are considered a promising supplementary treatment in the current clinical management of active IBD patients and diversion colitis. Different approaches, including enemas of butyrate and/or mixtures of SCFAs (acetate, propionate, and butyrate) have resulted in diverse clinical outcomes (16, 124, 125).

The direct effects of butyrate or mixtures of SCFAs in enemas showed clinical and histological improvement in active UC patients and diversion colitis (84, 125–127). At the molecular level, butyrate enemas decrease NF-κB nuclear translocation in LP macrophages in tissue sections from distal UC patients (84), as well as in LPS-induced cytokine expression and NF-κB activation in LP mononuclear cells and PBMCs from CD patients (128).

Alternatively, SCFAs enemas (100 ml of 80 mM acetate, 30 mM propionate, and 40 mM butyrate twice a day for 6 weeks) produced clinical remission only in a subset of UC patients (129). Butyrate enemas (60 ml of 100 mM once daily for 20 days) do not affect daily symptoms score, stool consistency and frequency (Bristol scale), and oxidative stress in UC patients in clinical remission, although they have a small effect on inflammation parameters (130). Moreover, no endoscopic or histological changes were observed in diversion colitis patients treated with SCFAs enemas (60 ml of 60 mM acetate, 30mM propionate, and 40 mM butyrate twice a day for 2 weeks) (131).

Inconsistent effects of SCFAs intervention in murine models undergoing colonic inflammation have been reported. For example, SCFAs enemas did not prevent or reduce intestinal damage in TNBS-induced colitis in rats (132), while butyrate reduced colonic mucosal damage and serum inflammatory cytokines (IL-6, TNF-α, and IL-1β) in DSS-treated mice (93). In contrast, butyrate did not revert/prevent DSS-induced intestinal damage in mice exposed to antibiotics (67). Similarly, butyrate was less effective in eliciting an anti-inflammatory response in the TNBS-induced colitis mouse model, vs. an injection of live *F. prausnitzii* or *F. prausnitzii* supernatant, while they both induced IL-10 and decreased IL-12 and TNF-α (133).

Interestingly, oral treatment with the spent medium of a culture of the SCFA-producer *Clostridium butyricum* ("supernatant") decreased DSS-induced colonic mucosal damage (134). These contradictory effects of butyrate or SCFAs might be species-specific or due to the colitis model (DSS vs. TNBS), commensal bacteria depletion, butyrate dosing and route of administration. Still, these results suggest that, by itself, butyrate or SCFAs are probably not as effective as administrating direct live SCFAs-producing bacteria to the mucosa. As mentioned earlier, there needs to be a constant production and delivery of SCFA to the mucosa to have anti-inflammatory effects. Nevertheless, anti-inflammatory effects of SCFAs seem (also) to be directed to immune cells. Therefore, the success of SCFAs in restoring intestinal mucosa homeostasis might be achieved by enriching or recovering SCFAs-producing bacteria through the use of pre- or probiotics.

Use of Prebiotics for SCFAs Production in IBD

The definition of prebiotic is "*a substrate that is selectively utilized by host microorganisms conferring a health benefit*" (135). Typically, these substrates are not digested in the human small bowel, thus promoting selective growth of beneficial bacteria in the colon (136). It is therefore sensible to explore the possible therapeutic role of different supplementary DF as substrates for gut bacteria and SCFA production in order to suppress inflammatory pathways in IBD patients, animal, and *in vitro* models.

A 4- and 12-weeks "intervention" with an oat bran-supplemented diet resulted in an increase of fecal butyrate concentrations and a decrease of abdominal pain or reflux in UC patients (137). Moreover, a double-blind pilot trial demonstrated that oral inulin (oligofructose)-supplementation was well-tolerated by UC patients, with active disease and decreased dyspeptic symptoms and, more importantly, a reduction in fecal calprotectin, as an important marker of intestinal inflammation (138). In contrast, the use of prebiotics has been associated to side effects in CD patients, such as abdominal pain, flatulence,

bloating, and diarrhea (139–141). Consequently, the adherence to this supplementation may be compromised in clinical trials, hindering an objective evaluation of the effect of the prebiotic in IBD patients. It remains to be determined whether the difference in patients' response might be related to the specific pathophysiology of both forms of IBD.

Neutrophils may play a dual role in IBD pathophysiology (142). Over activation of neutrophils may cause excessive tissue damage in UC patients, while defective neutrophil recruitment fails to control microorganism invasion in CD, subsequently leading to uncontrolled inflammation and formation of macrophage-containing granulomas in an attempt to contain the microorganism.

This aspect was addressed in CD patients receiving DF supplementation to their enteral nutrition (143), which resulted in an increase in $GPR43^{+}$ neutrophil infiltration when compared to enteral nutrition alone or patients in remission. Thus, prebiotics may be used to improve intestinal neutrophil recruitment.

In pigs fed an RS-supplemented diet, SCFAs concentrations and abundance of butyrate- (*F. prausnitzii*) or propionate-producing (*Propionibacterium*, *Veillonella, Phascolarctobacterium*) bacteria were increased in the luminal part of cecum and colon, while potentially pathogenic bacteria (*Escherichia coli* and *Pseudomonas* spp.) were decreased (41). Similarly, a high fiber diet protects mice against DSS-induced colitis, increasing protective Bacteroidetes (families *Porphyromonadaceae* and *Rikenellaceae*) and Firmicutes (family *Lachnospiraceae*), compared to a zero fiber diet (90).

Recently, also other prebiotics have been tested to promote intestinal SCFA production, including non-digestible dextrin (DEX), α-cyclodextrin (α-CD), and dextran (DXR) that increased acetate and propionate production in an *in vitro* fecal fermentation model of human colonic microbiota (144). Thus, non-digestible fibers may be a complementary therapy for IBD to increase intestinal butyrate production, especially in UC patients, as supporting evidence in animal and *in vitro* models suggests their benefit in promoting SCFAs-producing bacteria. Nevertheless, well-controlled randomized placebo-controlled trials (RCT) are needed to fine tune a prebiotics supplementation plan to manage gastrointestinal tolerance in IBD patients, especially in CD, before rigorously confirming an actual clinical improvement.

Use of Probiotics for SCFAs Production in IBD

A probiotic is defined as "*live microorganisms that, when administered in adequate amounts, confer a health benefit on the host*" (145). In IBD patients, the potential effect of probiotics in inducing or maintaining remission, showed encouraging benefits mainly in UC, as described below.

Two meta-analyses and systematic reviews of RCT of IBD with probiotics showed that they have significant effects in achieving remission, particularly for VSL#3 (mixture of four strains of *Lactobacillus*, three strains of *Bifidobacterium*, and one strain of *Streptococcus salivarius* subsp. *thermophilus*), being safe and effective in achieving remission in UC patients (146, 147). Moreover, the treatment with the probiotic preparation VSL#3 induced remission, as determined by a decrease in Ulcerative Colitis Disease Activity Index (UCDAI) in 50–53% UC patients with mild to moderately active disease (148, 149). In addition, VSL#3 combined with *Lactobacillus* have a significant effect in achieving clinical response in children with IBD (146). In an alternative approach, UC patients benefitted from a *Lactobacillus* probiotic when combined with prebiotics (146). Similarly, an oral treatment with the non-pathogenic *Escherichia coli* strain Nissle 1917 (EcN) (for 12 months), reduced relapses of UC patients in clinical remission, as compared to the standard treatment with mesalazine (150). Also, *Bifidobacterium infantis* 35,624 supplementation (for 6 weeks) reduced plasma C-reactive protein levels and tended to decrease IL-6 levels in mild to moderately active UC under treatment with mesalazine, compared to placebo-supplemented patients (151).

So far, probiotic treatments have not shown a significant effect in inducing or maintaining remission of active or quiescent CD, or in preventing relapse of CD after surgically-induced remission (146, 147). However, probiotics evaluated in these studies were not butyrate-producing bacteria. Interestingly, a recent proof-of-concept study explored the effect of six butyrate producers (*B. pullicaecorum* 25-3^{T}, *F. prausnitzii, Roseburia hominis, Roseburia inulinivorans, Anaerostipescaccae,* and *Eubacterium hallii*) in an *in vitro* fed batch system that simulates the mucus- and lumen-associated microbiota. A co-culture of these bacteria with fecal microbiota derived from CD patients with active disease showed increased butyrate production and improved epithelial barrier function *in vitro* (87).

These results encourage the exploration of pre- and probiotic therapies for specific SCFAs/butyrate production in restoring intestinal homeostasis and providing resolution and remission in IBD patients. Such approaches may complement alternative strategies to modulate microbiota, such as fecal microbiome transplantation (FMT), which has generated inconsistent results so far. As such, a detailed description of FMT is outside the scope of this review. As promising these results seem, more robust pre-clinical and further RCT studies are still necessary to test safety and efficacy of new SCFAs- or butyrate-producing bacteria (mixtures) with potential to be tested in association with FMT for reconstituting a healthy microbiome.

CONCLUSIONS AND FUTURE PERSPECTIVES

IBD is characterized by gastrointestinal dysbiosis, both in patients and in animal models, which particularly impairs SCFA production, thereby restraining energy supply to colonocytes and local control of mucosal inflammation. UC and CD patients show decreased butyrate-producing bacteria, especially *F. prausnitzii*, and consequently, SCFAs are reduced in feces, as well as butyrate uptake and oxidation, a process dependent on the mucosal inflammatory context. Empirical modulation of the microbiota using prebiotics or probiotics can increase SCFAs-producing bacteria *in vitro* and *in vivo*, enriching microbiome diversity in animal models and UC patients, demonstrating clinical and histological improvement. However, limited evidence exists indicating clinical improvement through theses

therapeutics in CD patients; nevertheless, supplementation with specific probiotics for butyrate formation may still provide new avenues to manage disease activity. The mechanisms involved in IBD pathophysiology are still not resolved, nor how butyrate regulates inflammation, influences metabolism and transcription in colonic mucosa. Future studies are needed to understand how to specifically modulate the microbiota and thus predict possible responses to therapy with personalized strategies in intestinal inflammation.

AUTHOR CONTRIBUTIONS

DPV wrote most of the review. MD and GL contributed to writing and correcting the manuscript. MG, RQ, GD, HH, KF, and MH participated reviewing and critically correcting the manuscript. KF and MH contributed to manuscript structure and supervised the work.

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REFERENCES

1. Ananthakrishnan AN. Epidemiology and risk factors for IBD. *Nat Rev Gastroenterol Hepatol*. (2015) 12:205–17. doi: 10.1038/nrgastro.2015.34
2. Cosnes J, Gowerrousseau C, Seksik P, Cortot A. Epidemiology and natural history of inflammatory bowel diseases. *Gastroenterology*. (2011) 140:1785–94. doi: 10.1053/j.gastro.2011.01.055
3. De Souza HSP, Fiocchi C. Immunopathogenesis of IBD: current state of the art. *Nat Rev Gastroenterol Hepatol*. (2016) 13:13–27. doi: 10.1038/nrgastro.2015.186
4. Sommer F, Bäckhed F. The gut microbiota-masters of host development and physiology. *Nat Rev Microbiol*. (2013) 11:227–38. doi: 10.1038/nrmicro2974
5. Thorburn AN, Macia L, Mackay CR. Diet, metabolites, and "Western-Lifestyle" inflammatory diseases. *Immunity*. (2014) 40:833–842. doi: 10.1016/j.immuni.2014.05.014
6. Agus A, Denizot J, Thévenot J, Martinez-Medina M, Massier S, Sauvanet P, et al. Western diet induces a shift in microbiota composition enhancing susceptibility to Adherent-Invasive E. coli infection and intestinal inflammation. *Sci Rep*. (2016) 6:1–14. doi: 10.1038/srep19032
7. Scott NA, Andrusaite A, Andersen P, Lawson M, Alcon-Giner C, Leclaire C, et al. Antibiotics induce sustained dysregulation of intestinal T cell immunity by perturbing macrophage homeostasis. *Sci Transl Med*. (2018) 10:eaao4755. doi: 10.1126/scitranslmed.aao4755
8. Neurath MF. New targets for mucosal healing and therapy in inflammatory bowel diseases. *Mucosal Immunol*. (2014) 7:6–19. doi: 10.1038/mi.2013.73
9. Nie Y, Lin Q, Luo F. Effects of non-starch polysaccharides on inflammatory bowel disease. *Int J Mol Sci*. (2017) 18:E1372. doi: 10.3390/ijms18071372
10. Koboziev I, Reinoso Webb C, Furr KL, Grisham MB. Role of the enteric microbiota in intestinal homeostasis and inflammation. *Free Radic Biol Med*. (2014) 68:122–33. doi: 10.1016/j.freeradbiomed.2013.11.008
11. Rakoff-Nahoum S, Paglino J, Eslami-varzaneh F, Edberg S, Medzhitov R. Recognition of commensal microflora by toll- like receptors is required for intestinal homeostasis recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. *Cell*. (2015) 118:229–41. doi: 10.1016/j.cell.2004.07.002
12. Kamada N, Chen GY, Inohara N, Núñez G. Control of pathogens and pathobionts by the gut microbiota. *Nat Immunol*. (2013) 14:685–90. doi: 10.1038/ni.2608
13. Madsen KL, Doyle JS, Tavernini MM, Jewell LD, Rennie RP, Fedorak RN. Antibiotic therapy attenuates colitis in interleukin 10 gene-deficient mice. *Gastroenterology*. (2000) 118:1094–105. doi: 10.1016/S0016-5085(00)70362-3
14. Hörmannsperger G, Schaubeck M, Haller D. Intestinal microbiota in animal models of inflammatory diseases. *ILAR J*. (2015) 56:179–91. doi: 10.1093/ilar/ilv019
15. Maslowski KM, Vieira AT, Ng A, Kranich J, Sierro F, Di Yu, et al. Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43. *Nature*. (2009) 461:1282–6. doi: 10.1038/nature08530
16. Tominaga K, Kamimura K, Takahashi K, Yokoyama J, Yamagiwa S, Terai S. Diversion colitis and pouchitis: a mini-review. *World J Gastroenterol*. (2018) 24:1734–47. doi: 10.3748/wjg.v24.i16.1734
17. Donohoe DR, Garge N, Zhang X, Sun W, O'Connell TM, Bunger MK, et al. The microbiome and butyrate regulate energy metabolism and autophagy in the mammalian colon. *Cell Metab*. (2011) 13:517–26. doi: 10.1016/j.cmet.2011.02.018
18. Corrêa-Oliveira R, Fachi JL, Vieira A, Sato FT, Vinolo MAR. Regulation of immune cell function by short-chain fatty acids. *Clin Transl Immunol*. (2016) 5:e73. doi: 10.1038/cti.2016.17
19. Rechkemmer G, von Engelhardt W. Concentration- and pH-dependence of short-chain fatty acid absorption in the proximal and distal colon of guinea pig (*Cavia porcellus*). *Comp Biochem Physiol A Comp Physiol*. (1988) 91:659–63.
20. Ritzhaupt A, Wood IS, Ellis A, Hosie KB, Shirazi-Beechey SP. Identification and characterization of a monocarboxylate transporter (MCT1) in pig and human colon: its potential to transport l -lactate as well as butyrate. *J Physiol*. (1998) 513:719–32. doi: 10.1111/j.1469-7793.1998.719ba.x
21. Miyauchi S, Gopal E, Fei YJ, Ganapathy V. Functional identification of SLC5A8, a tumor suppressor down-regulated in colon cancer, as a Na+-coupled transporter for short-chain fatty acids. *J Biol Chem*. (2004) 279:13293–6. doi: 10.1074/jbc.C400059200
22. Brown AJ, Goldsworthy SM, Barnes AA, Eilert MM, Tcheang L, Daniels D, et al. The orphan G protein-coupled receptors GPR41 and GPR43 are activated by propionate and other short chain carboxylic acids. *J Biol Chem*. (2003) 278:11312–9. doi: 10.1074/jbc.M211609200
23. Le Poul E, Loison C, Struyf S, Springael JY, Lannoy V, Decobecq ME, et al. Functional characterization of human receptors for short chain fatty acids and their role in polymorphonuclear cell activation. *J Biol Chem*. (2003) 278:25481–9. doi: 10.1074/jbc.M301403200
24. Taggart AKP, Kero J, Gan X, Cai TQ, Cheng K, Ippolito M, et al. (D)-β-hydroxybutyrate inhibits adipocyte lipolysis via the nicotinic acid receptor PUMA-G. *J Biol Chem*. (2005) 280:26649–52. doi: 10.1074/jbc.C500213200
25. Kumari R, Ahuja V, Paul J. Fluctuations in butyrate-producing bacteria in ulcerative colitis patients of North India. *World J Gastroenterol*. (2013) 19:3404–14. doi: 10.3748/wjg.v19.i22.3404
26. Wang W, Chen L, Zhou R, Wang X, Song L, Huang S, et al. Increased proportions of *Bifidobacterium* and the *Lactobacillus* group and loss of butyrate-producing bacteria in inflammatory bowel disease. *J Clin Microbiol*. (2014) 52:398–406. doi: 10.1128/JCM.01500-13
27. Joossens M, Huys G, Cnockaert M, De Preter V, Verbeke K, Rutgeerts P, et al. Dysbiosis of the faecal microbiota in patients with Crohn's disease and their unaffected relatives. *Gut*. (2011) 60:631–637. doi: 10.1136/gut.2010.223263
28. Pascal V, Pozuelo M, Borruel N, Casellas F, Campos D, Santiago A, et al. A microbial signature for Crohn's disease. *Gut*. (2017) 66:813–22. doi: 10.1136/gutjnl-2016-313235

29. Takahashi K, Nishida A, Fujimoto T, Fujii M, Shioya M, Imaeda H, et al. Reduced abundance of butyrate-producing bacteria species in the fecal microbial community in crohn's disease. *Digestion*. (2016) 93:59–65. doi: 10.1159/000441768
30. Paramsothy S, Paramsothy R, Rubin DT, Kamm MA, Kaakoush NO, Mitchell HM, et al. Faecal microbiota transplantation for inflammatory bowel disease: a systematic review and meta-analysis. *J Crohns Colitis*. (2017) 11:1180–99. doi: 10.1093/ecco-jcc/jjx063
31. Joint FAO/WHO CODEX Alimentarius Commission. *Guidelines on nutrition labelling CAC/GL 2-1985.* Codex Alimentarius International Food Stand.
32. Champ MMJ. Physiological aspects of resistant starch and *in vivo* measurements. *J AOAC Int*. (2004) 87:749–55.
33. Louis P, Flint HJ. Diversity, metabolism and microbial ecology of butyrate-producing bacteria from the human large intestine. *FEMS Microbiol Lett*. (2009) 294:1–8. doi: 10.1111/j.1574-6968.2009.01514.x
34. Louis P, Flint HJ. Formation of propionate and butyrate by the human colonic microbiota. *Environ Microbiol*. (2017) 19:29–41. doi: 10.1111/1462-2920.13589
35. den Besten G, van Eunen K, Groen AK, Venema K, Reijngoud D-J, Bakker BM. The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *J Lipid Res*. (2013) 54:2325–40. doi: 10.1194/jlr.R036012
36. Miller TL, Wolin MJ. Pathways of acetate, propionate, and butyrate formation by the human fecal microbial flora. *Appl Environ Microbiol*. (1996) 62:1589–92.
37. Duncan SH, Holtrop G, Lobley GE, Calder AG, Stewart CS, Flint HJ. Contribution of acetate to butyrate formation by human faecal bacteria. *Br J Nutr*. (2004) 91:915. doi: 10.1079/BJN20041150
38. Duncan SH, Barcenilla A, Stewart CS, Pryde SE, Flint HJ. Acetate utilization and butyryl coenzyme A (CoA):acetate-CoA transferase in butyrate-producing bacteria from the human large intestine. *Appl Environ Microbiol*. (2002) 68:5186–90. doi: 10.1128/AEM.68.10.5186-5190.2002
39. McNeil NI, Cummings JH, James WP. Short chain fatty acid absorption by the human large intestine. *Gut*. (1978) 19:819–22. doi: 10.1136/gut.19.9.819
40. McOrist AL, Miller RB, Bird AR, Keogh JB, Noakes M, Topping DL, et al. Fecal butyrate levels vary widely among individuals but are usually increased by a diet high in resistant starch. *J Nutr*. (2011) 141:883–9. doi: 10.3945/jn.110.128504
41. Haenen D, Zhang J, Souza da Silva C, Bosch G, van der Meer IM, van Arkel J, et al. A diet high in resistant starch modulates microbiota composition, SCFA concentrations, and gene expression in pig intestine. *J Nutr*. (2013) 143:274–83. doi: 10.3945/jn.112.169672
42. Cummings JH, Pomare EW, Branch WJ, Naylor CP, Macfarlane GT. Short chain fatty acids in human large intestine, portal, hepatic and venous blood. *Gut*. (1987) 28:1221–7. doi: 10.1136/gut.28.10.1221
43. Topping DL, Clifton PM. Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides. *Physiol Rev*. (2001) 81:1031–64. doi: 10.1152/physrev.2001.81.3.1031
44. Huda-Faujan N, Abdulamir AS, Fatimah AB, Anas OM, Shuhaimi M, Yazid AM, et al. The impact of the level of the intestinal short chain fatty acids in inflammatory bowel disease patients versus healthy subjects. *Open Biochem J*. (2010) 4:53–8. doi: 10.2174/1874091X01004010053
45. Machiels K, Joossens M, Sabino J, De Preter V, Arijs I, Eeckhaut V, et al. A decrease of the butyrate-producing species roseburia hominis and faecalibacterium prausnitzii defines dysbiosis in patients with ulcerative colitis. *Gut*. (2014) 63:1275–83. doi: 10.1136/gutjnl-2013-304833
46. Haghikia A, Jörg S, Duscha A, Berg J, Manzel A, Waschbisch A, et al. Dietary fatty acids directly impact central nervous system autoimmunity via the small intestine. *Immunity*. (2015) 43:817–29. doi: 10.1016/j.immuni.2015.09.007
47. Vital M, Howe AC, Tiedje JM. Revealing the bacterial butyrate synthesis pathways by analyzing (Meta)genomic data. *MBio*. (2014) 5:1–11. doi: 10.1128/mBio.00889-14
48. Rivière A, Selak M, Lantin D, Leroy F, De Vuyst L. Bifidobacteria and butyrate-producing colon bacteria: importance and strategies for their stimulation in the human gut. *Front Microbiol*. (2016) 7:979. doi: 10.3389/fmicb.2016.00979
49. Derrien M, Vaughan EE, Plugge CM, de Vos WM. Akkermansia municiphila gen. nov., sp. nov., a human intestinal mucin-degrading bacterium. *Int J Syst Evol Microbiol*. (2004) 54:1469–76. doi: 10.1099/ijs.0.02873-0
50. Kelly CJ, Colgan SP. Breathless in the Gut: implications of luminal O2for microbial pathogenicity. *Cell Host Microbe*. (2016) 19:427–8. doi: 10.1016/j.chom.2016.03.014
51. Rivera-Chávez F, Zhang LF, Faber F, Lopez CA, Byndloss MX, Olsan EE, et al. Depletion of butyrate-producing clostridia from the gut microbiota drives an aerobic luminal expansion of *Salmonella. Cell Host Microbe*. (2016) 19:443–54. doi: 10.1016/j.chom.2016.03.004
52. Byndloss MX, Olsan EE, Rivera-Chávez F, Tiffany CR, Cevallos SA, Lokken KL, et al. Microbiota-activated PPAR-γ signaling inhibits dysbiotic Enterobacteriaceae expansion. *Science*. (2017) 357:570–5. doi: 10.1126/science.aam9949
53. Gill RK. Expression and membrane localization of MCT isoforms along the length of the human intestine. *AJP Cell Physiol*. (2005) 289:C846–52. doi: 10.1152/ajpcell.00112.2005
54. Al-mosauwi H, Ryan E, McGrane A, Riveros-Beltran S, Walpole C, Dempsey E, et al. Differential protein abundance of a basolateral MCT1 transporter in the human gastrointestinal tract. *Cell Biol Int*. (2016) 40:1303–12. doi: 10.1002/cbin.10684
55. Iwanaga T, Takebe K, Kato I, Karaki S-I, Kuwahara A. Cellular expression of monocarboxylate transporters (MCT) in the digestive tract of the mouse, rat, and humans, with special reference to slc5a8. *Biomed Res*. (2006) 27:243–54. doi: 10.2220/biomedres.27.243
56. Kirat D, Kondo K, Shimada R, Kato S. Dietary pectin up-regulates monocaboxylate transporter 1 in the rat gastrointestinal tract. *Exp Physiol*. (2009) 94:422–33. doi: 10.1113/expphysiol.2009.046797
57. Merezhinskaya N, Ogunwuyi SA, Mullick FG, Fishbein WN. Presence and localization of three lactic acid transporters (MCT1,−2, and −4) in separated human granulocytes, lymphocytes, and monocytes. *J Histochem Cytochem*. (2004) 52:1483–93. doi: 10.1369/jhc.4A6306.2004
58. Murray CM, Hutchinson R, Bantick JR, Belfield GP, Benjamin AD, Brazma D, et al. Monocarboxylate transporter Mctl is a target for immunosuppression. *Nat Chem Biol*. (2005) 1:371–6. doi: 10.1038/nchembio744
59. Hahn EL, Halestrap AP, Gamelli RL. Expression of the lactate transporter MCT1 in macrohages. *Shock*. (2000) 13:253–260. doi: 10.1097/00024382-200004000-00001
60. Borthakur A, Anbazhagan AN, Kumar A, Raheja G, Singh V, Ramaswamy K, et al. The probiotic Lactobacillus plantarum counteracts TNF- -induced downregulation of SMCT1 expression and function. *AJP Gastrointest Liver Physiol*. (2010) 299:G928–34. doi: 10.1152/ajpgi.00279.2010
61. Cox MA, Jackson J, Stanton M, Rojas-Triana A, Bober L, Laverty M, et al. Short-chain fatty acids act as antiinflammatory mediators by regulating prostaglandin E2and cytokines. *World J Gastroenterol*. (2009) 15:5549–57. doi: 10.3748/wjg.15.5549
62. Nastasi C, Candela M, Bonefeld CM, Geisler C, Hansen M, Krejsgaard T, et al. The effect of short-chain fatty acids on human monocyte-derived dendritic cells. *Sci Rep*. (2015) 5:1–10. doi: 10.1038/srep16148
63. Ang Z, Er JZ, Ding JL. The short-chain fatty acid receptor GPR43 is transcriptionally regulated by XBP1 in human monocytes. *Sci Rep*. (2015) 5:1–9. doi: 10.1038/srep08134
64. Smith PM, Howitt MR, Panikov N, Michaud M, Gallini CA, Bohlooly-YM, et al. The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. *Science*. (2013) 341:569–73. doi: 10.1126/science.1241165
65. Tunaru S, Kero J, Schaub A, Wufka C, Blaukat A, Pfeffer K, et al. PUMA-G and HM74 are receptors for nicotinic acid and mediate its anti-lipolytic effect. *Nat Med*. (2003) 9:352–5. doi: 10.1038/nm824
66. Thangaraju M, Cresci GA, Liu K, Ananth S, Gnanaprakasam JP, Browning DD, et al. GPR109A is a g-protein–coupled receptor for the bacterial fermentation product butyrate and functions as a tumor suppressor in colon. *Cancer Res*. (2009) 69:2826–32. doi: 10.1158/0008-5472.CAN-08-4466
67. Chang PV, Hao L, Offermanns S, Medzhitov R. The microbial metabolite butyrate regulates intestinal macrophage function via histone deacetylase inhibition. *Proc Natl Acad Sci USA*. (2014) 111:2247–52. doi: 10.1073/pnas.1322269111

68. Singh N, Gurav A, Sivaprakasam S, Brady E, Padia R, Shi H, et al. Activation of Gpr109a, receptor for niacin and the commensal metabolite butyrate, suppresses colonic inflammation and carcinogenesis. *Immunity*. (2014) 40:128–39. doi: 10.1016/j.immuni.2013.12.007
69. Park J, Kotani T, Konno T, Setiawan J, Kitamura Y, Imada S, et al. Promotion of intestinal epithelial cell turnover by commensal bacteria: role of short-chain fatty acids. *PLoS ONE*. (2016) 11:e0156334. doi: 10.1371/journal.pone.0156334
70. Lukovac S, Belzer C, Pellis L, Keijser BJ, de Vos WM, Montijn RC, et al. Differential modulation by *Akkermansia muciniphila* and *Faecalibacterium prausnitzii* of host peripheral lipid metabolism and histone acetylation in mouse gut organoids. *MBio*. (2014) 5:1–10. doi: 10.1128/mBio.01438-14
71. Kaiko GE, Ryu SH, Koues OI, Collins PL, Solnica-Krezel L, Pearce EJ, et al. The colonic crypt protects stem cells from microbiota-derived metabolites. *Cell*. (2016) 165:1708–20. doi: 10.1016/j.cell.2016.05.018
72. Matthews GM, Howarth GS, Butler RN. Short-chain fatty acids induce apoptosis in colon cancer cells associated with changes to intracellular redox state and glucose metabolism. *Chemotherapy*. (2012) 58:102–9. doi: 10.1159/000335672
73. Tang Y, Chen Y, Jiang H, Nie D. Short-chain fatty acids induced autophagy serves as an adaptive strategy for retarding mitochondria-mediated apoptotic cell death. *Cell Death Differ*. (2011) 18:602–18. doi: 10.1038/cdd.2010.117
74. Zhang J, Yi M, Zha L, Chen S, Li Z, Li C, et al. Sodium butyrate induces endoplasmic reticulum stress and autophagy in colorectal cells: implications for apoptosis. *PLoS ONE*. (2016) 11:1–25. doi: 10.1371/journal.pone.0147218
75. Wu X, Wu Y, He L, Wu L, Wang X, Liu Z. Effects of the intestinal microbial metabolite butyrate on the development of colorectal cancer. *J Cancer*. (2018) 9:2510–7. doi: 10.7150/jca.25324
76. Kelly CJ, Zheng L, Campbell EL, Saeedi B, Scholz CC, Bayless AJ, et al. Crosstalk between microbiota-derived short-chain fatty acids and intestinal epithelial HIF augments tissue barrier function. *Cell Host Microbe*. (2015) 17:662–71. doi: 10.1016/j.chom.2015.03.005
77. Peng L, Li Z-R, Green RS, Holzman IR, Lin J. Butyrate enhances the intestinal barrier by facilitating tight junction assembly via activation of AMP-activated protein kinase in Caco-2 cell monolayers. *J Nutr*. (2009) 139:1619–25. doi: 10.3945/jn.109.104638
78. Miao W, Wu X, Wang K, Wang W, Wang Y, Li Z, et al. Sodium butyrate promotes reassembly of tight junctions in Caco-2 monolayers involving inhibition of MLCK/MLC2 pathway and phosphorylation of PKCβ2. *Int J Mol Sci*. (2016) 17:1–12. doi: 10.3390/ijms17101696
79. Valenzano MC, DiGuilio K, Mercado J, Teter M, To J, Ferraro B, et al. Remodeling of tight junctions and enhancement of barrier integrity of the CACO-2 intestinal epithelial cell layer by micronutrients. *PLoS ONE*. (2015) 10:e0133926. doi: 10.1371/journal.pone.0133926
80. Zheng L, Kelly CJ, Battista KD, Schaefer R, Lanis JM, Alexeev EE, et al. Microbial-derived butyrate promotes epithelial barrier function through IL-10 receptor–dependent repression of claudin-2. *J Immunol*. (2017) 199:2976–84. doi: 10.4049/jimmunol.1700105
81. Wang HB, Wang PY, Wang X, Wan YL, Liu YC. Butyrate enhances intestinal epithelial barrier function via up-regulation of tight junction protein claudin-1 transcription. *Dig Dis Sci*. (2012) 57:3126–35. doi: 10.1007/s10620-012-2259-4
82. Yan H, Ajuwon KM. Butyrate modifies intestinal barrier function in IPEC-J2 cells through a selective upregulation of tight junction proteins and activation of the Akt signaling pathway. *PLoS ONE*. (2017) 12:1–20. doi: 10.1371/journal.pone.0179586
83. Park JS, Lee EJ, Lee JC, Kim WK, Kim HS. Anti-inflammatory effects of short chain fatty acids in IFN-γ-stimulated RAW 264.7 murine macrophage cells: involvement of NF-κB and ERK signaling pathways. *Int Immunopharmacol*. (2007) 7:70–77. doi: 10.1016/j.intimp.2006.08.015
84. Lührs H, Gerke T, Müller JG, Melcher R, Schauber J, Boxberger F, et al. Butyrate inhibits NF-κB activation in lamina propria macrophages of patients with ulcerative colitis. *Scand J Gastroenterol*. (2002) 37:458–66. doi: 10.1080/003655202317316105
85. Arpaia N, Campbell C, Fan X, Dikiy S, Van Der Veeken J, Deroos P, et al. Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. *Nature*. (2013) 504:451–5. doi: 10.1038/nature12726
86. Furusawa Y, Obata Y, Fukuda S, Endo TA, Nakato G, Takahashi D, et al. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. *Nature*. (2013) 504:446–50. doi: 10.1038/nature12721
87. Geirnaert A, Calatayud M, Grootaert C, Laukens D, Devriese S, Smagghe G, et al. Butyrate-producing bacteria supplemented *in vitro* to Crohn's disease patient microbiota increased butyrate production and enhanced intestinal epithelial barrier integrity. *Sci Rep*. (2017) 7:11450. doi: 10.1038/s41598-017-11734-8
88. Zhao Y, Chen F, Wu W, Sun M, Bilotta AJ, Yao S, et al. GPR43 mediates microbiota metabolite SCFA regulation of antimicrobial peptide expression in intestinal epithelial cells via activation of mTOR and STAT3. *Mucosal Immunol*. (2018) 1–11: 752–62. doi: 10.1038/mi.2017.118
89. Roediger WEW. Role of anaerobic bacteria in the metabolic welfare of the colonic mucosa in man. *Gut*. (1980) 21:793–8. doi: 10.1136/gut.21.9.793
90. Macia L, Tan J, Vieira AT, Leach K, Stanley D, Luong S, et al. Metabolite-sensing receptors GPR43 and GPR109A facilitate dietary fibre-induced gut homeostasis through regulation of the inflammasome. *Nat Commun*. (2015) 6:1–15. doi: 10.1038/ncomms7734
91. Borthakur A, Priyamvada S, Kumar A, Natarajan AA, Gill RK, Alrefai WA, et al. A novel nutrient sensing mechanism underlies substrate-induced regulation of monocarboxylate transporter-1. *AJP Gastrointest Liver Physiol*. (2012) 303:G1126–33. doi: 10.1152/ajpgi.00308.2012
92. Sahasrabudhe NM, Beukema M, Tian L, Troost B, Scholte J, Bruininx E, et al. Dietary fiber pectin directly blocks toll-like receptor 2-1 and prevents doxorubicin-induced ileitis. *Front Immunol*. (2018) 9:1–19. doi: 10.3389/fimmu.2018.00383
93. Ji J, Shu D, Zheng M, Wang J, Luo C, Wang Y, et al. Microbial metabolite butyrate facilitates M2 macrophage polarization and function. *Sci Rep*. (2016) 6:1–10. doi: 10.1038/srep24838
94. Fernando MR, Saxena A, Reyes J-L, McKay DM. Butyrate enhances antibacterial effects while suppressing other features of alternative activation in IL-4-induced macrophages. *Am J Physiol Gastrointest Liver Physiol*. (2016) 310:G822–31. doi: 10.1152/ajpgi.00440.2015
95. Riggs MG, Whittaker RG, Neumann JR, Ingram VM. n-Butyrate causes histone modification in HeLa and Friend erythroleukaemia cells. *Nature*. (1977) 268:462–4. doi: 10.1038/268462a0
96. Boffa LC, Vidali G, Mann RS, Allfrey VG. Suppression of histone deacetylation *in vivo* and *in vitro* by sodium butyrate. *J Biol Chem*. (1978) 253:3364–6.
97. Vidali G, Boffa LC, Bradbury EM, Allfrey VG. Butyrate suppression of histone deacetylation leads to accumulation of multiacetylated forms of histones H3 and H4 and increased DNase I sensitivity of the associated DNA sequences. *Proc Natl Acad Sci USA*. (1978) 75:2239–43. doi: 10.1073/pnas.75.5.2239
98. Candido EPM, Reeves R, Davie JR. Sodium butyrate inhibits histone deacetylation in cultured cells. *Cell*. (1978) 14:105–13. doi: 10.1016/0092-8674(78)90305-7
99. Davie JR. Inhibition of histone deacetylase activity by butyrate. *J Nutr*. (2003) 133:2485S−93S. doi: 10.1093/jn/133.7.2485S
100. Shakespear MR, Halili MA, Irvine KM, Fairlie DP, Sweet MJ. Histone deacetylases as regulators of inflammation and immunity. *Trends Immunol*. (2011) 32:335–43. doi: 10.1016/j.it.2011.04.001
101. Glauben R, Batra A, Fedke I, Zeitz M, Lehr HA, Leoni F, et al. Histone hyperacetylation is associated with amelioration of experimental colitis in mice. *J Immunol*. (2006) 176:5015–22. doi: 10.4049/jimmunol.176.8.5015
102. Cresci GA, Thangaraju M, Mellinger JD, Liu K, Ganapathy V. Colonic gene expression in conventional and germ-free mice with a focus on the butyrate receptor GPR109A and the butyrate transporter SLC5A8. *J Gastrointest Surg*. (2010) 14:449–61. doi: 10.1007/s11605-009-1045-x
103. Villodre Tudela C, Boudry C, Stumpff F, Aschenbach JR, Vahjen W, Zentek J, et al. Down-regulation of monocarboxylate transporter 1 (MCT1) gene expression in the colon of piglets is linked to bacterial protein fermentation and pro-inflammatory cytokine-mediated signalling. *Br J Nutr*. (2015) 113:610–7. doi: 10.1017/S0007114514004231
104. Cuff MA, Lambert DW, Shirazi-Beechey SP. Substrate-induced regulation of the human colonic monocarboxylate transporter, MCT1. *J Physiol*. (2002) 539:361–71. doi: 10.1113/jphysiol.2001.014241

105. Thibault R, De Coppet P, Daly K, Bourreille A, Cuff M, Bonnet C, et al. Down-regulation of the monocarboxylate transporter 1 is involved in butyrate deficiency during intestinal inflammation. *Gastroenterology*. (2007) 133:1916–27. doi: 10.1053/j.gastro.2007.08.041
106. De Preter V, Arijs I, Windey K, Vanhove W, Vermeire S, Schuit F, et al. Impaired butyrate oxidation in ulcerative colitis is due to decreased butyrate uptake and a defect in the oxidation pathway. *Inflamm Bowel Dis*. (2012) 18:1127–36. doi: 10.1002/ibd.21894
107. Planell N, Lozano JJ, Mora-Buch R, Masamunt MC, Jimeno M, Ordás I, et al. Transcriptional analysis of the intestinal mucosa of patients with ulcerative colitis in remission reveals lasting epithelial cell alterations. *Gut*. (2013) 62:967–76. doi: 10.1136/gutjnl-2012-303333
108. Palmieri O, Creanza TM, Bossa F, Palumbo O, Maglietta R, Ancona N, et al. Genome-wide pathway analysis using gene expression data of colonic mucosa in patients with inflammatory bowel disease. *Inflamm Bowel Dis*. (2015) 21:1260–8. doi: 10.1097/MIB.0000000000000370
109. Nancey S, Moussata D, Graber I, Claudel S, Saurin J-C, Flourié B. Tumor necrosis factor alpha reduces butyrate oxidation *in vitro* in human colonic mucosa: a link from inflammatory process to mucosal damage? *Inflamm Bowel Dis*. (2005) 11:559–66. doi: 10.1097/01.MIB.0000161918.04760.f3
110. Masui R, Sasaki M, Funaki Y, Ogasawara N, Mizuno M, Iida A, et al. G protein-coupled receptor 43 moderates gut inflammation through cytokine regulation from mononuclear cells. *Inflamm Bowel Dis*. (2013) 19:2848–56. doi: 10.1097/01.MIB.0000435444.14860.ea
111. Sina C, Gavrilova O, Forster M, Till A, Derer S, Hildebrand F, et al. G protein-coupled receptor 43 is essential for neutrophil recruitment during intestinal inflammation. *J Immunol*. (2009) 183:7514–22. doi: 10.4049/jimmunol.0900063
112. Kim MH, Kang SG, Park JH, Yanagisawa M, Kim CH. Short-chain fatty acids activate GPR41 and GPR43 on intestinal epithelial cells to promote inflammatory responses in mice. *Gastroenterology*. (2013) 145:396–406.e10. doi: 10.1053/j.gastro.2013.04.056
113. Alex P, Zachos NC, Nguyen T, Gonzales L, Chen TE, Conklin LS, et al. Distinct cytokine patterns identified from multiplex profiles of murine DSS and TNBS-induced colitis. *Inflamm Bowel Dis*. (2009) 15:341–52. doi: 10.1002/ibd.20753
114. Wirtz S, Popp V, Kindermann M, Gerlach K, Weigmann B, Fichtner-Feigl S, et al. Chemically induced mouse models of acute and chronic intestinal inflammation. *Nat Protoc*. (2017) 12:1295–309. doi: 10.1038/nprot.2017.044
115. Higgins LM, Frankel G, Douce G, Dougan G, MacDonald TT. *Citrobacter rodentium* infection in mice elicits a mucosal Th1 cytokine response and lesions similar to those in murine inflammatory bowel disease. *Infect Immun*. (1999) 67:3031–9.
116. Mamontov P, Neiman E, Cao T, Perrigoue J, Friedman J, Das A MJ. Effects of short chain fatty acids and GPR43 stimulation on human Treg function (IRC5P.631). *J Immunol*. (2015) 194:58.14.
117. Park J, Kim M, Kang SG, Jannasch AH, Cooper B, Patterson J, et al. Short-chain fatty acids induce both effector and regulatory T cells by suppression of histone deacetylases and regulation of the mTOR-S6K pathway. *Mucosal Immunol*. (2014) 8:80–93. doi: 10.1038/mi.2014.44
118. Kespohl M, Vachharajani N, Luu M, Harb H, Pautz S, Wolff S, et al. The microbial metabolite butyrate induces expression of Th1-associated factors in cD4+T cells. *Front Immunol*. (2017) 8:1–12. doi: 10.3389/fimmu.2017.01036
119. Feingold KR, Moser A, Shigenaga JK, Grunfeld C. Inflammation stimulates niacin receptor (GPR109A/HCA2) expression in adipose tissue and macrophages. *J Lipid Res*. (2014) 55:2501–8. doi: 10.1194/jlr.M050955
120. Frank DN, St Amand AL, Feldman RA, Boedeker CE, Harpaz N, Pace NR. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proc Natl Am Sci USA*. (2013) 104:13780–5. doi: 10.1073/pnas.0706625104
121. Imhann F, Vila AV, Bonder MJ, Fu J, Gevers D, Visschedijk MC, et al. Interplay of host genetics and gut microbiota underlying the onset and clinical presentation of inflammatory bowel disease. *Gut*. (2016) 67:108–19. doi: 10.1136/gutjnl-2016-312135
122. Prosberg M, Bendtsen F, Vind I, Petersen AM, Gluud LL. The association between the gut microbiota and the inflammatory bowel disease activity: a systematic review and meta-analysis. *Scand J Gastroenterol*. (2016) 51:1407–15. doi: 10.1080/00365521.2016.1216587
123. Gupta VK, Paul S, Dutta C. Geography, ethnicity or subsistence-specific variations in human microbiome composition and diversity. *Front Microbiol*. (2017) 8:1162. doi: 10.3389/fmicb.2017.01162
124. Gill PA, van Zelm MC, Muir JG, Gibson PR. Review article: short chain fatty acids as potential therapeutic agents in human gastrointestinal and inflammatory disorders. *Aliment Pharmacol Ther*. (2018) 48:15–34. doi: 10.1111/apt.14689
125. Hamer HM, Jonkers D, Venema K, Vanhoutvin S, Troost FJ, Brummer RJ. Review article: the role of butyrate on colonic function. *Aliment Pharmacol Ther*. (2008) 27:104–19. doi: 10.1111/j.1365-2036.2007.03562.x
126. Scheppach W, Sommer H, Kirchner T, Paganelli GM, Bartram P, Christl S, et al. Effect of butyrate enemas on the colonic mucosa in distal ulcerative colitis. *Gastroenterology*. (1992) 103:51–56. doi: 10.1016/0016-5085(92)91094-K
127. Harig JM, Soergel KH, Komorowski RA, Wood CM. Treatment of diversion colitis with short-chain-fatty acid irrigation. *N Engl J Med*. (1989) 320:23–8. doi: 10.1056/NEJM198901053200105
128. Segain JP, Raingeard de la Blétière D, Bourreille A, Leray V, Gervois N, Rosales C, et al. Butyrate inhibits inflammatory responses through NFkappaB inhibition: implications for Crohn's disease. *Gut*. (2000) 47:397–403. doi: 10.1136/gut.47.3.397
129. Breuer RI, Soergel KH, Lashner BA, Christ ML, Hanauer SB, Vanagunas A, et al. Short chain fatty acid rectal irrigation for left-sided ulcerative colitis: a randomised, placebo controlled trial. *Gut*. (1997) 40:485–91. doi: 10.1136/gut.40.4.485
130. Hamer HM, Jonkers DMAE, Vanhoutvin SALW, Troost FJ, Rijkers G, de Bruïne A, et al. Effect of *Butyrate enemas* on inflammation and antioxidant status in the colonic mucosa of patients with ulcerative colitis in remission. *Clin Nutr*. (2010) 29:738–44. doi: 10.1016/j.clnu.2010.04.002
131. Guillemot FÇ, Colombel JF, Neut C, Verplanck N, Lecomte M, Romond C, et al. Treatment of diversion colitis by short-chain fatty acids - Prospective and double-blind study. *Dis Colon Rectum*. (1991) 34:861–4. doi: 10.1007/BF02049697
132. Tarrerias AL, Millecamps M, Alloui A, Beaughard C, Kemeny JL, Bourdu S, et al. Short-chain fatty acid enemas fail to decrease colonic hypersensitivity and inflammation in TNBS-induced colonic inflammation in rats. *Pain*. (2002) 100:91–7. doi: 10.1016/S0304-3959(02)00234-8
133. Sokol H, Pigneur B, Watterlot L, Lakhdari O, Bermudez-Humaran LG, Gratadoux J-J, et al. *Faecalibacterium prausnitzii* is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. *Proc Natl Acad Sci USA*. (2008) 105:16731–6. doi: 10.1073/pnas.0804812105
134. Araki Y, Andoh A, Takizawa J, Takizawa W, Fujiyama Y. *Clostridium butyricum*, a probiotic derivative, suppresses dextran sulfate sodium-induced experimental colitis in rats. *Int J Mol Med*. (2004) 13:577–80. doi: 10.3892/ijmm.13.4.577
135. Gibson GR, Hutkins R, Sanders ME, Prescott SL, Reimer RA, Salminen SJ, et al. Expert consensus document: the international scientific association for probiotics and prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. *Nat Rev Gastroenterol Hepatol*. (2017) 14:491–502. doi: 10.1038/nrgastro.2017.75
136. Gibson GR, Probert HM, Van Loo J, Rasrall RA, Roberfroid MB. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *Nutr Res Rev*. (2004) 17:259–75. doi: 10.1079/NRR200479
137. Hallert C, Björck I, Nyman M, Pousette A, Grännö C, Svensson H. Increasing fecal butyrate in ulcerative colitis patients by diet: controlled pilot study. *Inflamm Bowel Dis*. (2003) 9:116–21. doi: 10.1097/00054725-200303000-00005
138. Casellas F, Borruel N, Torrejon A, Varela E, Antolin M, Guarner F, et al. Oral oligofructose-enriched inulin supplementation in acute ulcerative colitis is well tolerated and associated with lowered faecal calprotectin. *Aliment Pharmacol Ther*. (2007) 25:1061–7. doi: 10.1111/j.1365-2036.2007.03288.x
139. Swennen K, Courtin CM, Delcour JA. Non-digestible oligosaccharides with prebiotic properties. *Crit Rev Food Sci Nutr*. (2006) 46:459–71. doi: 10.1080/10408390500215746

140. Looijer–Van Langen MAC, Dieleman LA. Prebiotics in Chronic Intestinal Inflammation. *Inflamm Bowel Dis*. (2009) 15:454–62. doi: 10.1002/ibd.20737
141. Rasmussen HE, Hamaker BR. Prebiotics and Inflammatory Bowel Disease. *Gastroenterol Clin North Am*. (2017) 46:783–95. doi: 10.1016/j.gtc.2017.08.004
142. Wéra O, Lancellotti P, Oury C. The dual role of neutrophils in inflammatory bowel diseases. *J Clin Med*. (2016) 5:118. doi: 10.3390/jcm5120118
143. Zhao M, Zhu W, Gong J, Zuo L, Zhao J, Sun J, et al. Dietary fiber intake is associated with increased colonic mucosal GPR43+ polymorphonuclear infiltration in active Crohn's disease. *Nutrients*. (2015) 7:5327–46. doi: 10.3390/nu7075223
144. Sasaki D, Sasaki K, Ikuta N, Yasuda T, Fukuda I, Kondo A, et al. Low amounts of dietary fibre increase *in vitro* production of short-chain fatty acids without changing human colonic microbiota structure. *Sci Rep*. (2018) 8:1–7. doi: 10.1038/s41598-017-18877-8
145. Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, et al. Expert consensus document: the international scientific association for probiotics and prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat Rev Gastroenterol Hepatol*. (2014) 11:506–14. doi: 10.1038/nrgastro.2014.66
146. Ganji-Arjenaki M, Rafieian-Kopaei M. Probiotics are a good choice in remission of inflammatory bowel diseases: a meta analysis and systematic review. *J Cell Physiol*. (2018) 233:2091–103. doi: 10.1002/jcp.25911
147. Derwa Y, Gracie DJ, Hamlin PJ, Ford AC. Systematic review with meta-analysis: the efficacy of probiotics in inflammatory bowel disease. *Aliment Pharmacol Ther*. (2017) 46:389–400. doi: 10.1111/apt.14203
148. Bibiloni R, Fedorak RN, Tannock GW, Madsen KL, Gionchetti P, Campieri M, et al. VSL#3 probiotic-mixture induces remission in patients with active ulcerative colitis. *Am J Gastroenterol*. (2005) 100:1539–46. doi: 10.1111/j.1572-0241.2005.41794.x
149. Sood A, Midha V, Makharia GK, Ahuja V, Singal D, Goswami P, et al. The probiotic preparation, VSL#3 induces remission in patients with mild-to-moderately active ulcerative colitis. *Clin Gastroenterol Hepatol*. (2009) 7:1202–9.e1. doi: 10.1016/j.cgh.2009.07.016
150. Kruis W, Frič P, Pokrotnieks J, Lukáš M, Fixa B, Kaščák M, et al. Maintaining remission of ulcerative colitis with the probiotic *Escherichia coli* Nissle 1917 is as effective as with standard mesalazine. *Gut*. (2004) 53:1617–23. doi: 10.1136/gut.2003.037747
151. Groeger D, O'Mahony L, Murphy EF, Bourke JF, Dinan TG, Kiely B, et al. *Bifidobacterium infantis* 35624 modulates host inflammatory processes beyond the gut. *Gut Microbes*. (2013) 4:325–339. doi: 10.4161/gmic.25487

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Corrigendum: Short Chain Fatty Acids (SCFAs)-Mediated Gut Epithelial and Immune Regulation and Its Relevance for Inflammatory Bowel Diseases

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Soybean Meal-Induced Intestinal Inflammation in Zebrafish Is T Cell-Dependent and Has a Th17 Cytokine Profile

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Currently, inflammatory bowel disease (IBD) is a serious public health problem on the rise worldwide. In this work, we utilized the zebrafish to introduce a new model of intestinal inflammation triggered by food intake. Taking advantage of the translucency of the larvae and the availability of transgenic zebrafish lines with fluorescently labeled macrophages, neutrophils, or lymphocytes, we studied the behavior of these cell types *in vivo* during the course of inflammation. We established two feeding strategies, the first using fish that were not previously exposed to food (naïve strategy) and the second in which fish were initially exposed to normal food (developed strategy). In both strategies, we analyzed the effect of subsequent intake of a control or a soybean meal diet. Our results showed increased numbers of innate immune cells in the gut in both the naïve or developed protocols. Likewise, macrophages underwent drastic morphological changes after feeding, switching from a small and rounded contour to a larger and dendritic shape. Lymphocytes colonized the intestine as early as 5 days post fertilization and increased in numbers during the inflammatory process. Gene expression analysis indicated that lymphocytes present in the intestine correspond to T helper cells. Interestingly, control diet only induced a regulatory T cell profile in the developed model. On the contrary, soybean meal diet induced a Th17 response both in naïve and developed model. In addition, when feeding was performed in *rag1*-deficient fish, intestinal inflammation was not induced indicating that inflammation induced by soybean meal is T cell-dependent.

Keywords: intestinal inflammation, zebrafish, innate immune, adaptive immunity, Th17 T cells, macrophage, lymphocyte

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INTRODUCTION

Intestinal inflammation (or enteritis) is a feature of several chronic pathologies such as inflammatory bowel disease (IBD) in humans as well as of similar pathologies in fish (1–3). One of the earliest signs of intestinal inflammation is the infiltration of neutrophils into the gut mucosa and the epithelial layer, in addition to polarization of macrophages and dendritic cells toward an inflammatory phenotype (4–6). Enteritis is also characterized by a drastic increase of natural killer cells in the gut as well as activation of mast cells (7). Each of these cell types secretes specific cytokines that trigger several pathways characteristic of enteritis (7). On the other hand, $CD4^+$ T helper (Th) cells are critical for proper immune cell homeostasis and

host defense but are also major contributors to the pathology of autoimmune and inflammatory diseases (8). Depending on the cytokine milieu, different Th subsets can be induced, such as Th1, Th2, Th17, Th22, and Th9, each with specific functional outcomes (9). Likewise, regulatory T cells (Tregs) are essential for the development of tolerance to self and non-self antigens. Activation of Tregs inhibits the inflammatory response to commensal bacteria and is central for mucosal tolerance (10). Conversely, functional defects in Tregs underlie infectious, autoimmune and chronic inflammatory conditions, including IBD (11, 12).

Several models for IBD have been developed in the mouse model. Based on how inflammation is brought about, they can be categorized into four groups: chemical models, genetically engineered models, cell transfer models, and congenic models. Although each of them covers a specific aspect of this pathological condition, none of them encompasses the spontaneous and fluctuating nature of the human disease (13). Thus, in order to extrapolate the experimental findings from mouse studies toward the improvement of knowledge and therapy in IBD pathogenesis in humans, it is necessary to understand the specific advantages and limitations of each model (14, 15). One of the key limitations shared between all models is the difficulty to follow cell behavior and gene function *in vivo*. To overcome this situation, we propose the use of the zebrafish (*Danio rerio*) for studying the contribution of different immune cell types to intestinal inflammation *in vivo*. This teleost fish constitutes a unique tool that allows to combine live imaging of specific fluorescently-labeled cell types with molecular strategies to manipulate gene function to monitor the course of an inflammatory process in real-time and at the whole organism level (16–19). Moreover, the anatomy and architecture of the zebrafish intestine closely resembles the one of mammals, and all main immune cell lineages have been described in this vertebrate model (20–22). Importantly, most chemical-induced intestinal inflammation models used in mice have also been used in zebrafish with similar results (23–25). In order to obtain a more physiological intestinal inflammation model, we established a novel strategy in zebrafish larvae based on the intake of a soybean meal-based diet (26). Using this approach, we have reported that, as early as 2 days after feeding, the number of neutrophils increased in the gut, as well as the mRNA levels of several proinflammatory cytokines such as *il1b* and *cxcl8*. Conversely, no changes in intestinal architecture were detected, suggestive of an early stage in the inflammatory process (26). In this new study, we have compared the behavior of innate cells such as neutrophils, macrophages, and mast cells, in addition to T cells, between two conditions: naïve intestine (not previously exposed to food) and developed intestine (already exposed to regular food). Our findings show that innate immune responses were similarly triggered after fish maintained under both conditions are afterwards exposed to a control or inflammatory diet. T cells, in contrast, responded differently. In the case of naïve intestines, an inflammatory process with increased numbers of helper T cells was induced under both control and inflammatory diet (soybean meal-based diet), the latter with a Th17 profile. Conversely, in developed intestines, the control diet triggered a tolerogenic response with abundant Treg cells, and the inflammatory diet a Th17 profile with decreased presence of Treg cells. When *rag1*$^{-/-}$ fish were fed with inflammatory diet, no increase in neutrophils, or lymphocytes was observed, indicating that T cells are needed to trigger immune responses to soybean meal. These results demonstrate, for the first time, functional adaptive immune response in zebrafish as early as 5 days post-fertilization. They also reveal an evolutionarily conserved response between zebrafish and mammals, supporting the suitability of the zebrafish model to study intestinal inflammation with biomedical purposes.

MATERIALS AND METHODS

Zebrafish Strains and Maintenance

Zebrafish were maintained and bred in our facility according to standard protocols (27). The following strains of fish were used in this study: Tab5, Tg(lck:lck-eGFP) (28), Tg(lysC:DsRed) (29), Tg(mpeg1:Dendra2) (30), and *rag1*$^{-/-}$ mutants (31). All embryos were collected by natural spawning and maintained at 28°C in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4, with methylene blue, equilibrated to pH 7.0) in petri dishes (32). Embryonic and larval ages are expressed in hours post fertilization (hpf) or days post fertilization (dpf).

Feeding Strategies

Two feeding strategies were used; the naïve feeding strategy and the developed feeding strategy (**Supplementary Figure 1A**). The naïve feeding protocol comprised larval feeding from 5 dpf (T0n) to 9 dpf (T4) with a control diet (fishmeal-based diet) or an inflammatory diet (soybean meal-based diet). Diets were prepared as described previously (26). As controls we included non-fed larvae from 9 dpf (T4n). The developed feeding protocol consisted of two parts; the maintenance feeding regime where larvae were fed from 5 to 17 dpf with regular food (gemma micron 300) and the experimental feeding phase where larvae were fed from 18 to 25 dpf with control or inflammatory diet. In all cases larvae were fed *ad libitum* and maintained in 100 ml fish water in a 200 ml tank at 28°C. The last feeding was performed 19 h before processed for the different analyses performed, qPCR, immunohistochemistry or imaging.

Immunohistochemistry

Immunohistochemistry was performed essentially as previously described (33). Briefly, larvae were fixed for 1 h in 4% paraformaldehyde in phosphate-buffered saline (PBS), then rinsed in PBS + 1% Tween 20 (PBS-Tween), dehydrated in 100% methanol and stored at −20°C until use. The following antibodies were used: rabbit anti-GFP (Invitrogen cat: A11122), anti-MPO (Genetext cat: GTX128379) and anti-tryptase (Abcam cat: ab2378). Quantification of neutrophils, mast and lymphoid cells in the intestine was performed in whole-mount larvae; for this purpose, a region in the midgut was defined (red rectangle in **Figure 1**). Thirty larvae per condition were analyzed in three different experiments.

Proliferation Assay

Tg(lck:lck-eGFP) larvae were exposed to the nucleotide analog 5-ethynyl-2′-deoxyuridine (EdU) as was previously described (34)

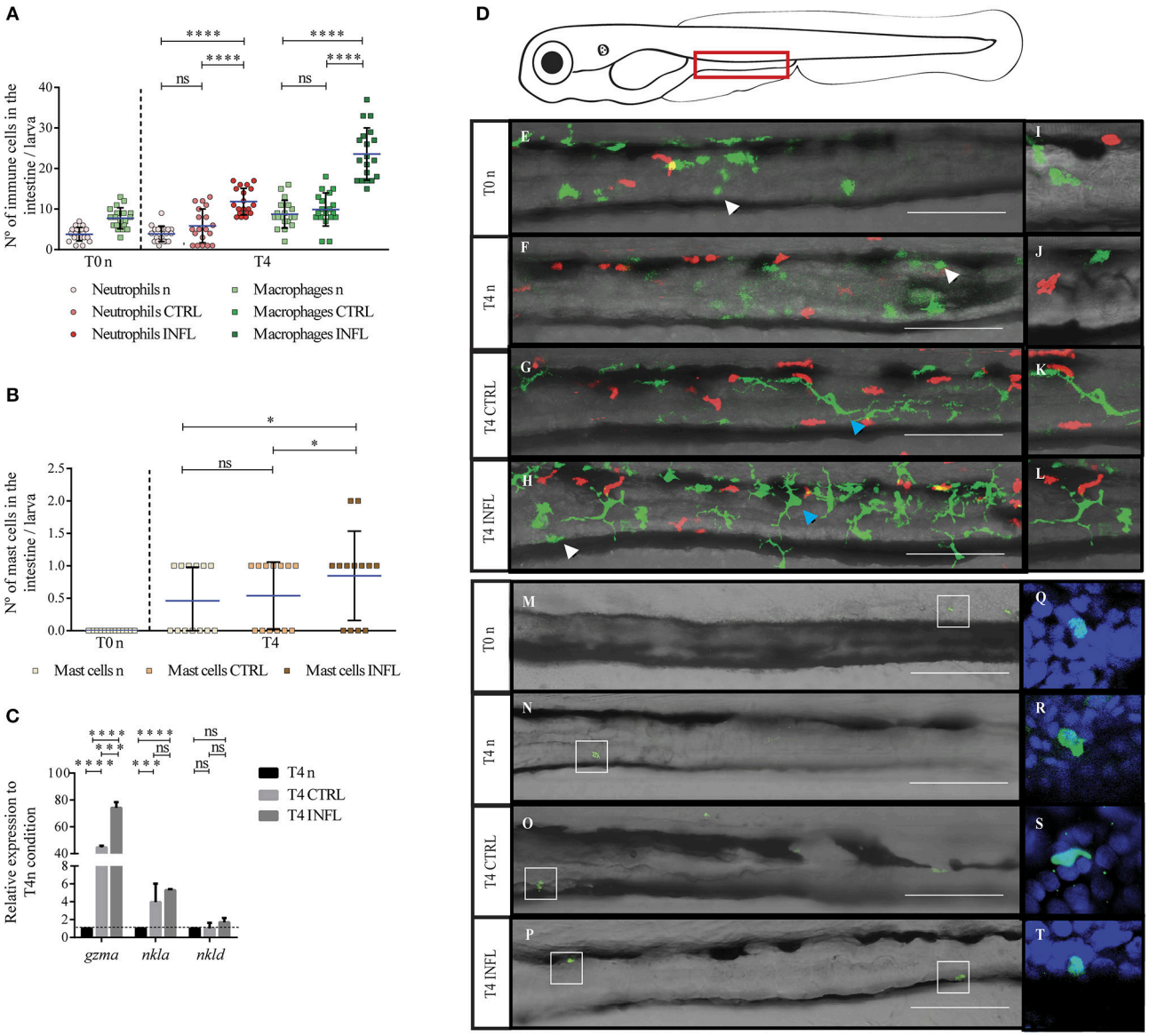

FIGURE 1 | Neutrophils, macrophages, and mast cells increase their presence in the gut during the inflammatory process triggered in the naïve feeding model. The number of neutrophils/macrophages **(A)**, and mast cells **(B)** was quantified under different conditions (n, naïve; CTRL, control; INFL, inflammation) and time points (T0: 5 dpf; T4: 9 dpf). **(C)** Relative mRNA levels of *gzma*, *nkla,* and *nkld* were analyzed. Data were normalized against *rpl13a* and compared to T4 naïve condition (dotted line). **(D)** Scheme of a lateral view of a 9 dpf larva with the zone of the intestine used to quantify delimited by the red rectangle. **(E–H)** Lateral view of the intestine of a Tg(lysC:DsRed)xTg(mpeg1:Dendra2) larva showing fluorescently labeled neutrophils (red) and macrophages (green). **(M–P)** Lateral view of the intestine showing mast cells labeled by anti-tryptase immunohistochemistry. Zoom of macrophages and neutrophils **(I–L)** and mast cells **(Q–T)**. *$p < 0.05$; ***$p < 0.001$; ****$p < 0.0001$. Scale bar 200μm. Experiments were done at least in three biological replicates with 20 individuals per condition.

with some modifications. Larvae were immersed in 100 μg/ml EdU (Invitrogen) with 0.5% DMSO for 16 h and fixed 1 h at RT with 4% PFA in PBS. Larvae were then processed using the Click-iT EdU Imaging Kit (Invitrogen) according to manufacturer's instructions.

Histological Section

Twenty-five dpf larvae were fixed in Bouin's solution for 3 h at RT, washed twice in water and mounted in 3% low melting agarose (Cleaver) for paraffin embedding and sectioning as previously described (26). Sections of 5 μm were obtained and stained with hematoxylin & eosin (Merck.) The quantification of the number of intestinal folds was analyzed as previously described by Hedrera et al. (26).

Confocal Microscopy

For time-lapse and photography, larvae were anesthetized with tricaine 4% (Fluka, cat: 1001011075), mounted in low-melting agarose 1% in E3 and registered under a Leica TCS Sp8 microscope, with Leica application suite X (LAS X software

version 3.1.5) and using an optical zoom of 40 and 60x. The images were analyzed with Image J 1.44, representing at least 70% of each population.

Intestine Dissection and RT-qPCR

Intestines were dissected at different times points according to **Supplementary Figure 1A**. Dissections were performed with entomological pins in RNAasa free conditions. One hundred intestines per condition were directly stored in TRIzol reagent (Invitrogen, cat: 15596-026) for total RNA extraction. Extraction was performed according to the manufacturer's instructions. Synthesis of cDNA was performed with 1 μg RNA and SuperScript II RT (Invitrogen, cat: 100004925), according to manufacturer's instructions and using oligo-dT primers. Real-time PCR was performed following the methodology described previously (35). The mean Ct values from each sample were normalized against the mean Ct value of a reference gene (*rpl13a*, housekeeping gene). The genes analyzed are detailed in **Table 1**. All experiments were performed on at least three biological replicates.

Statistics

Statistical analysis for quantification of cell numbers was performed using unpaired *t*-test or a non-parametric test, the Kruskall–Walls one-way ANOVA test. RT-qPCR analyses were performed using Kruskall–Walls one-way ANOVA test. All analyses were made using Prim 6 (GraphPad Software). The significance level was set to $P < 0.05$.

RESULTS

To analyze mucosal immune responses in intestines of fish exposed for first time to food, we used a well-established inflammation model (26), which consists of 4 days of feeding with soybean meal-based diet, from 5 to 9 dpf (hereafter, naïve model) (**Supplementary Figure 1A**). To address if inflammation induced by this model remits after treatment, we carried out the naïve model and after that, fed larvae with control diet during 4 more days. We then quantified the number of neutrophils present in the intestine. As expected, larvae fed the inflammatory diet had significantly more neutrophils than larvae fed the control diet at 4 days of treatment (9 dpf, T4); 9.5 and 4.3, respectively. Then, 4 days after feeding with the inflammatory diet (T4+4) the number of neutrophils in the intestine of control and inflamed larvae was similar: 4.7 and 5.9, respectively (**Supplementary Figure 1B**). In order to provide a model more comparable to mammalian intestinal inflammatory models, in which the intestine has been exposed to food for a considerable period of time, we established a new intestinal inflammation model, hereafter "developed model." This new strategy comprised a first step of 12 days of feeding with regular food (from 5 to 17 dpf), followed by the second step of 8 days of experimental feeding (from 18 to 25 dpf) (**Supplementary Figure 1A**). In this approach, the gut is first exposed to a regular diet for a few days, allowing the differentiation and functionality of intestinal cell types as well as the colonization of commensal microbes (36). To determine if the inflammation triggered in the developed model is sufficient to induce changes in the morphology of the intestine, we analyzed histological cross sections of the midgut of larvae fed with

TABLE 1 | Sequences of primers used in qPCR.

Gene	Forward primer	Reverse primer	Accession number
rpl13a	TCTGGAGGACTGTAAGAGGTATGC	AGACGCACAATCTTGAGAGCAG	NM_212784
il17a/f1	CATTCGGTGCTGAGGGGG	AGCCGGTATGAATGATCTGC	NM_001020787
il17a/f3	AAGATGTTCTGGTGTGAAGAAGTG	ACCCAAGCTGTCTTTCTTTGAC	NM_001020790
il22	GATGACTGATACAGCACGAAA	CATTGATGCAGCAGGAACCT	NM_001020792
rorca	TCTTTTCCTATCCAACCTCTCTACA	GAGTGGTCTCTTTATGTGAGCGTA	XM_001344013
gzma	GATTTTTGGCTGAGAGGACGG	ACGTCAGAGCAAACTGTCACT	XM_001335130
nkla	GATGACGAATGACGGAGTAAAC	TCTCATTCACAGCCCGGT	NM_001311794
nkld	TGTGATCAGATCGGGTTCCT	AGCACAGATGGTTCTGGCAT	NM_212741
il10	TCACGTCATGAACGAGATCC	CCTCTTGCATTTCACCATATCC	NM_001020785
foxp3a	CTCGGCTCATCTCGCAATCA	CGGTGTCCACAACCCAATCA	NM_001329567
ccr9a	TGCACCATGGTCTACTGGAA	ATAACCCGAAGTGCCTTGTG	NM_001244716
ccl25	ACATCCCAGCCATTGTCTTC	GCTGAAATGAGCCCTCGTAG	XM_002660965
lck	GCCGAAGAAGATCTCGATGGT	TCCCCATGTTTACGTATTTTGTCG	NM_001001596
trac	TCGTTTTCAATGTGCTGGTG	GATGATCTGGAATGGGATGC	NM_001199372
cd4.1	AAGAGTTGAGAAAGCTCCAGTG	CTGGTCTTGCGTCGTCTGTA	NM_001135096
mhczea	GGCTGTTTTTGCCGCTCTG	GTGGACAGGTCTGGATAAAG	NM_001089550
mhc2ab	CTCTGTGGGGAAGTTTGTG	CCAGATCCGAGCATTATGTC	NM_131476
hamp	GCCGTTCCCTTCATACAGCA	CCTGAACAGAACCAGAGGGTC	NM_205583
leap2	TGTGGGTACTAAACCACACGG	GCCCATCCTGCATATTCCTGT	NM_001128777
trim33	GAACCCGAACTCCAGAGCAA	AGCATTAGTAGCACCGCCTC	NM_001002871

control and experimental diets (**Supplementary Figure 1C**). We found that the number of folds present in the intestine of larvae fed the inflammatory diet was significantly lower than the number of folds present in control larvae, 9.12 and 9.78, respectively, (**Supplementary Figure 1D**), indicating that after 8 days of feeding, the inflammation caused by the developed model is detectable at a morphological level.

Neutrophils, Macrophages, Mast Cells, and Natural Killer Cells Respond Similarly to Innocuous and Harmful Food During Naïve Feeding Model

To analyze innate immune responses triggered upon inflammation in the naïve model, we monitored neutrophil, macrophage, and mast cell behavior. We took advantage of the double transgenic line Tg(lysC:DsRed)xTg(mpeg1:Dendra2), in which neutrophils and macrophages are fluorescently labeled in red and green, respectively. To evaluate mast cells, we performed immunofluorescence with an anti-tryptase antibody, which has been shown to label specifically mast cells (37). Using confocal microscopy, we determined cell morphology and the number of infiltrated cells in the intestine at two different time points: T0n (5 dpf, before feeding) and T4 (9 dpf, after feeding) (**Figure 1**). Also, we included a control with larvae without feeding at 9 dpf (T4n). We found that in both unfed conditions, T0n and T4n, there were 4 to 5 neutrophils in the intestine (**Figure 1A**). Later, at T4, we found a similar number of neutrophils. Conversely, inflammatory diet induced an increase to an average of 12 neutrophils per intestine (**Figure 1A**). In the case of macrophages, both naïve conditions (T0n and T4n) displayed a mean of 9 cells in the intestine. Similarly, to neutrophils, the number of macrophages at T4 only increased in larvae fed with the inflammatory diet, with 23 cells per intestine (**Figure 1A**). Remarkably, the number of neutrophils and macrophages did not increase due to larval development, T0 vs. T4n, suggesting that the increase of both types of immune cells is due to the inflammatory response. Finally, we did not observe mast cells in intestine at T0 and T4n and after feeding, in T4, larvae fed with control and inflammatory diet displayed a mean of 1 mast cell per intestine (**Figure 1B**).

To further characterize the participation of other innate immune cells during the naïve model of inflammation, we analyzed the natural killer cell markers Granzyme A (*gzma)*, NK lysin-a (*nkla),* and NK lysin-d (*nkld)* by RT-qPCR (38). We found increased expression of *gzma* and *nkla* in both control and inflammatory diet compared to the naïve situation (T4n), suggesting the participation of NK cells in the response triggered by both the inflammatory and the control diet (**Figure 1C**).

Macrophage Morphology Changes After the Intestine Is Exposed to Food

Cell morphology can change when cell function changes, we thus evaluated if the inflammatory diet induces alterations in the morphology of myeloid cells. To this end, we analyzed the shape of neutrophils, macrophages and mast cells by confocal microscopy before (T0n, T4n) and after feeding (T4). At T0n and T4n, neutrophils displayed a rounded shape that switched to an elongated morphology (**Figures 1E,F,I,J**, red cells), probably due to increased motility. Later, at T4, neutrophil morphology was similar to that observed before feeding, both under control and inflammatory conditions (**Figures 1G,H,K,L**, red cells). Likewise, mast cells displayed a rounded shape at T0 and T4n, which remained unaltered among the different conditions at T4 (**Figures 1M–P,Q–T**). Macrophages, on the other hand, displayed a rounded shape at T0 and T4n (**Figures 1E,F,I,J**, green cells) and a size similar to neutrophils. Conversely, after food intake, either under the control or the inflammatory diet, morphology drastically changed, showing cells with several long protrusions and increased size compared to the naïve situation (**Figures 1G,H,K,L**). Thus, these results suggest that the activation and/or function of macrophages change upon feeding, regardless if the food is innocuous or inflammatory.

Neutrophils, Macrophages, Mast Cells, and Natural Killer Cells Respond Similarly to Food Antigens, Either Innocuous or Inflammatory Upon the Developed Feeding Model

In the case of the developed feeding model, the number of neutrophils, macrophages and mast cells in the gut at T0 was similar to that observed at the same time-point in the naïve feeding protocol, with 5, 9, and 1 cells, respectively, (**Figures 2A,B**). During the inflammatory condition, both neutrophils and macrophages increased in the gut from T0 to T4, with neutrophils remaining constant up to T8 and macrophages maintaining their increment (**Figure 2A**). Meanwhile, the number of mast cells did not vary during the entire period, as observed in the naïve feeding model (**Figure 2B**). Finally, mRNA levels of NK cell markers increased under control and inflammatory conditions, compared to the regular maintenance condition (**Figure 2C**). In summary, these results indicate that if the intestine is exposed to antigens for first time or after continuous exposure to food, the response exerted by innate immune cells is similar.

Lymphoid Cells Colonize the Gut at Early Larval Stages and Are Able to Respond to Inflammatory Stimuli

Currently available literature indicates that zebrafish develop a functional adaptive immune system only after 3 weeks of development (18). Since we observed that the inflammatory diet triggered a strong myeloid cell response at 9 dpf, we decided to investigate if lymphoid cells are present at this developmental stage and if they are able to respond to the different food antigens. To this end, we used the Tg(lck:lck-eGFP) transgenic line with fluorescently labeled lymphoid cells (28). At 5 dpf, before feeding, we found few lymphoid cells (1 or 2) in the gut (**Figures 3A,I**). At this developmental stage, the intestine has a freshly formed lumen (39). Later, at 9 dpf (T4n), the number of lymphoid cells present in the gut of naïve larvae remained unchanged (**Figures 3B,I**). On the other hand, the intake of both diets, control and inflammatory, triggered an increase of lymphoid cells in the

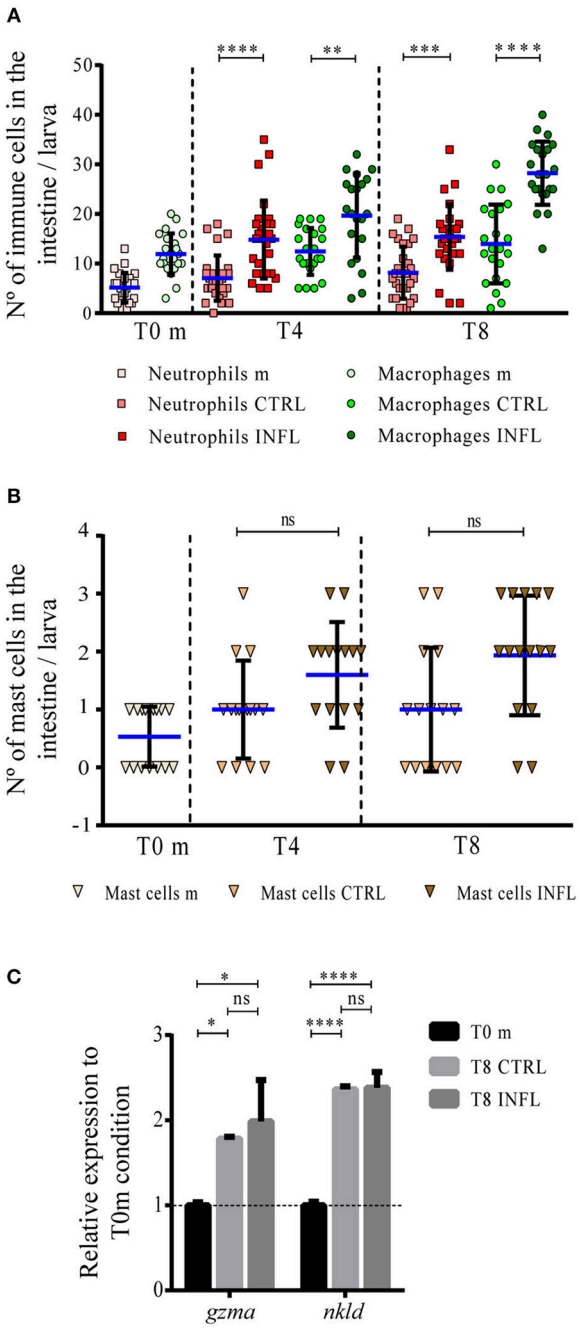

FIGURE 2 | Neutrophils and macrophages increase in number in the gut during the inflammation process triggered in the developed feeding model. The number of neutrophils/macrophages **(A)**, and mast cells **(B)** was quantified under the different conditions (m, maintenance; CTRL, control; INFL, inflammation) and time points (T0m: 18 dpf; T4: 21 dpf; T8: 25 dpf). **(C)** Relative mRNA levels of *gzma* and *nkld* were analyzed. Data were normalized against *rpl13a* and compared to T0 maintenance condition (dotted line). $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$. Experiments were done at least in three biological replicates with 20 individuals per condition.

intestine, 3 and 4 cells, respectively, (**Figures 3C,D,I**). Regarding morphology, we compared lymphoid cell shape between all conditions using confocal microscopy. We found that in both naïve conditions, T0 and T4n, and after feeding either control or inflammatory diet, lck^+ cells displayed a rounded shape with a lower proportion of cytoplasm and a prominent nucleus (**Figures 3E–H**). Interestingly, in the inflammatory condition, we found few lck^+ cells with long protrusions (**Figure 3H**).

To determine if the increase in lck^+ cells observed in larvae fed the inflammatory diet is due to new cells recruitment or due to proliferation, we incubated larvae with thymidine nucleoside (EdU) for 16 h and then immunodetected it. We did not observe any colocalization between lck^+ cells and EdU^+ cells (**Figures 3 J–O**). To corroborate this results we performed immunofluorescence against phosphorylated Histone 3 (H3P) in Tg(lck:lck-eGFP). As in the EdU assay, we did not find any colocalization between $H3P^+$ cells and lck^+ cells (data not shown).

To analyze the behavior of lymphoid cells during the control and the inflammatory condition, we performed time-lapse analysis (**Figures 3P,Q**). We found that GFP^+ cells did not display motility at steady-state, they only moved their protrusions but without displacement (**Figure 3P**, **Supplementary Movie 1**). Interestingly, during inflammation, GFP^+ cells showed substantial and continuous motility along the intestine (**Figure 3Q**, **Supplementary Movie 2**). This behavioral change could be an indicator of presence of T cells in an activated state. Finally, and to determine if lymphoid cell recruitment to the intestine is regulated by similar signaling pathways than in mammals, we quantified mRNA levels of the *ccr9a* receptor and its ligand *ccl25*. Our results showed that in both feeding conditions, *ccl25* mRNA increased upon inflammation (**Figure 3R**), suggesting evolutionary conservation of signaling pathways regulating T cell recruitment to the intestine.

Lymphoid Cells Present in the Intestine Are Helper T Cells With a Th17 Transcriptional Profile

To determine if the lymphoid cells present in the intestine were T cells and if they have a specific Th profile, we evaluated general T cell markers (*lck; trac; cd4.1; cd8*), as well as Treg (*foxp3a* and *il10*), and Th17 (*il17a/f1; il17a/f3; il22; rorca*) markers. We compared the expression levels of all these genes between naïve (or maintenance) condition vs. control or inflammatory diet. In the case of the naïve feeding strategy, we observed an increase in the mRNA levels of *lck* and *trac* in the control and inflammatory conditions compared with the naïve situation. In contrast, there was no difference in the mRNA levels between the control with the inflammatory condition (**Figure 4A**). Also, we evaluated *cd4-1* expression, which strongly increased in the control and inflammatory conditions compared to the naïve situation (**Figure 4A**). It is important to highlight that, although the mRNA levels of *cd8* were analyzed, expression was not detected (data not shown). These results suggest that lymphoid cells increase in fed larvae, regardless of the type of food and that at least part of them are helper T cells. Similarly, in the developed feeding model, *lck, trac,* and *cd4.1* expression increased in control and inflammatory conditions at T8 when compared to T0m (**Figure 4D**). In the

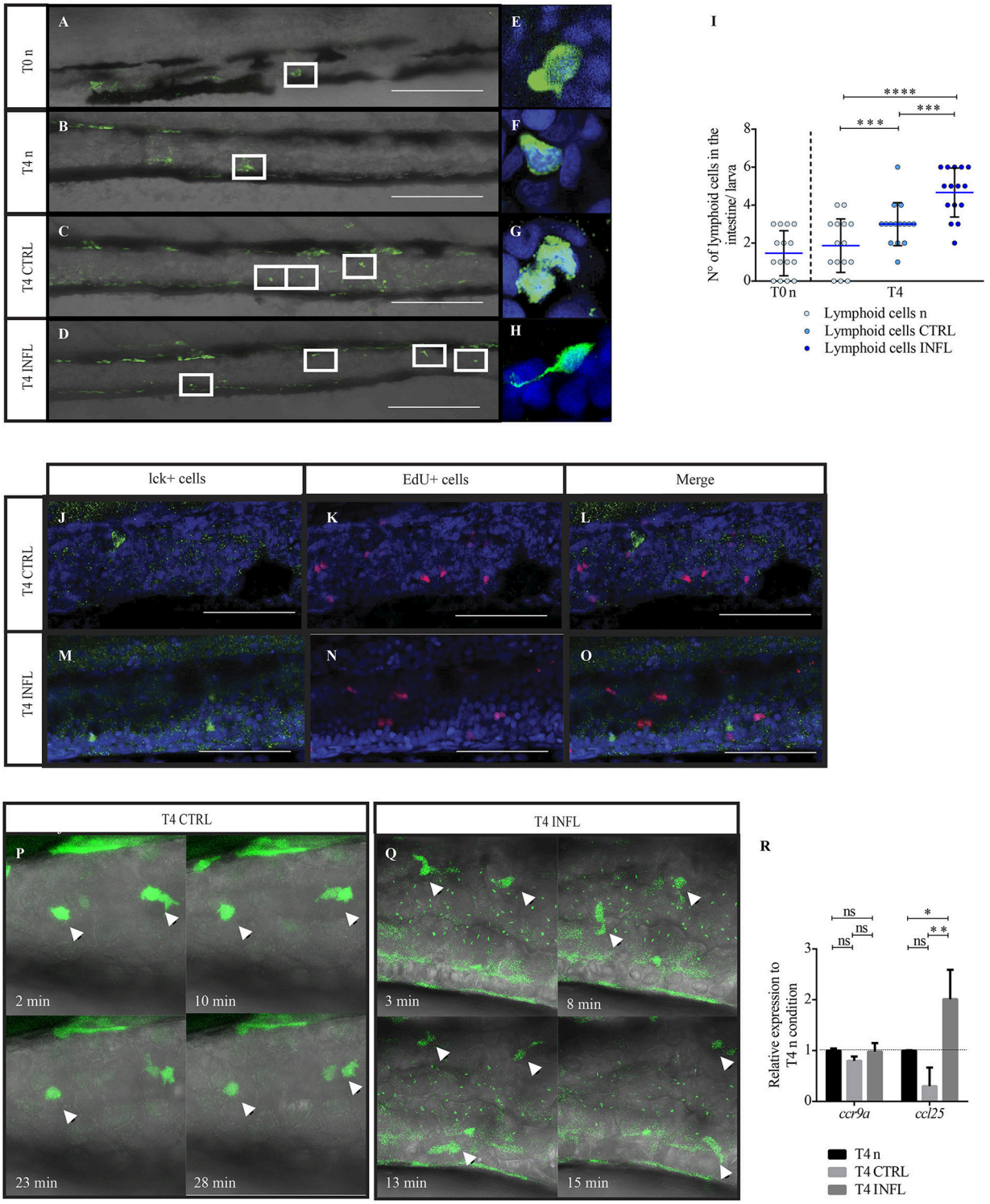

FIGURE 3 | Lymphoid cells are present in the intestine as early as 5 dpf and are able to respond to food antigens. **(A–D)** Lateral view of the intestine from a Tg(lck:lck-eGFP) larva showing fluorescently labelled lymphoid cells (green). **(E–H)** Higher magnification of lymphocytes under the different conditions. **(I)** Quantification of lymphoid cells under the different conditions (n: naive; CTRL: control; INFL: inflammation) and time points (T0: 5 dpf; T4: 9 dpf). **(J–O)** T cells in control **(J)** or inflamed **(M)** intestine do not colocalize with EdU+ cells **(K,L,N,O)**. **(P)** Example of migration of T cells (arrowhead) in a Tg(lck:lck-eGFP) larvae fed with control diet (derived from **Supplementary Movie 1**). **(Q)** Example of the migration of T cells (arrowheads) in a Tg(lck:lck-eGFP) larva fed with inflammatory diet (derived from **Supplementary Movie 2**). **(R)** Relative mRNA levels of ccl25 and ccr9a were analyzed in the intestine of control and inflamed larvae. Data was normalized against rpl13a and compared to naïve or maintenance condition (dotted line). $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p > 0.0001$. Scale bar for **A–D** 200 μm and **J–O** 100 μm. Experiments were done at least in three biological replicates with 20 individuals per condition.

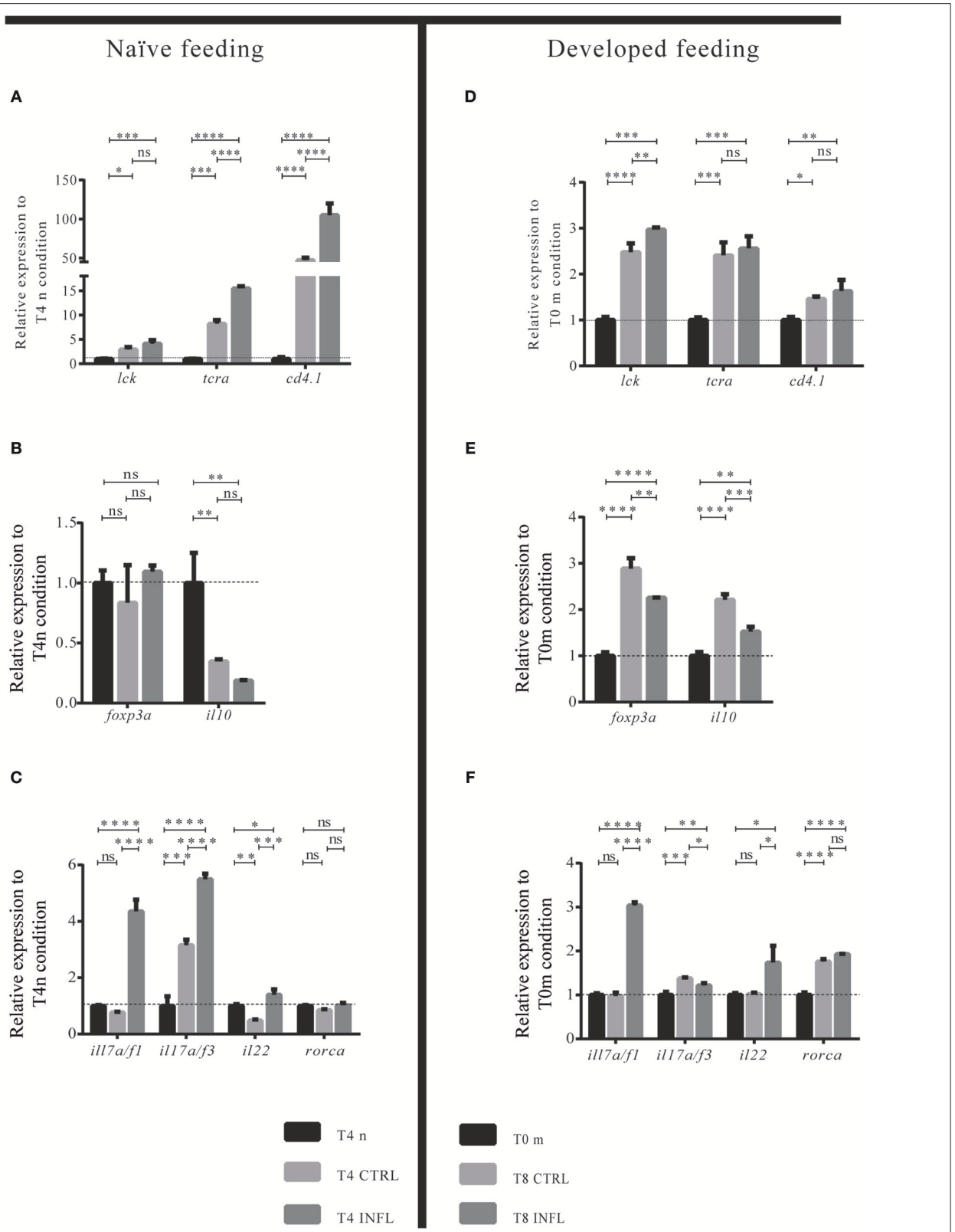

FIGURE 4 | Intestinal lymphoid cells are $CD4^+$ T cells with a Th17-like transcriptional profile. **(A–F)** Relative transcriptional level of lymphoid-associated genes was analyzed. Data was normalized against *rpl13a* and compared to the naïve condition (T0n; dotted line) or to the maintenance condition, T0m. For each condition, 100 guts were analyzed, and three biological replicates were made. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p > 0.0001$.

naïve model, we also evaluated if *mhc1zea*, *mhc2ab*, *hamp, leap2, and trim33* genes changed their mRNA level between control and inflammatory conditions. We found an increase in *mhc1zea* and *mhc2ab* genes in larvae fed the inflammatory diet. On the contrary, none of the antimicrobial peptide markers genes, *hamp, leap2, and trim33,* altered their transcription level (**Supplementary Figure 2**).

To determine the type of Th profile acquired by helper T cells after intake of the control or inflammatory diet, we quantified the mRNA levels of the Th17 markers *il17a/f1*, *il17a/f3*, *il22,* and *rorca,* and the Treg markers *foxp3a* and *il10*. After the naïve feeding model, larvae displayed increased numbers of intestinal T cells, however, they did not show a defined Treg or Th17 profile since only *il17a/f1* was increased (**Figures 4B,C**). In contrast, in the developed feeding model, T cells displayed a Treg profile with an increase in the mRNA levels of *foxp3a* and *il10* (**Figure 4E**). In the case of the inflammatory diet, the mRNA levels of Th17 gene markers were increased while Treg markers decreased compared to control conditions, both in the naïve and developed feeding models (**Figures 4B,C,E,F**). In summary, these results show that in a naïve intestine, helper T cells do not polarize to a specific Th profile in response to innocuous and noxious food antigens, responding similarly in both cases. Conversely, in an intestine already exposed to food, helper T cells selectively detect innocuous or noxious antigens, triggering a tolerogenic or inflammatory response, respectively.

Soybean Meal Based Inflammatory Diet Triggered Intestinal Inflammation in a T Cell-Dependent Manner

To determine if the inflammation triggered by the intake of inflammatory diet was T cell-dependent, we carried out the naïve model in *rag1*$^{-/-}$ larvae, which lack adaptive lymphocytes. First, we quantified number of neutrophils in intestines of larvae without feeding as well as in larvae fed with control and inflammatory diet. We found no significant difference between these three conditions; in naïve larvae, the average of neutrophils per intestine was 2.65 cells; in larvae fed with control diet was 2.95 cells and in larvae fed with the inflammatory diet was 3.2 cells (**Figure 5A**). Likewise, we performed the naïve model in *rag1*$^{-/-}$ Tg(lck:lck-eGFP), and evaluated if the number of lck^{+} cells (innate lymphocytes and/or natural killer cells) increase in the intestine of larvae fed the inflammatory diet compared to control

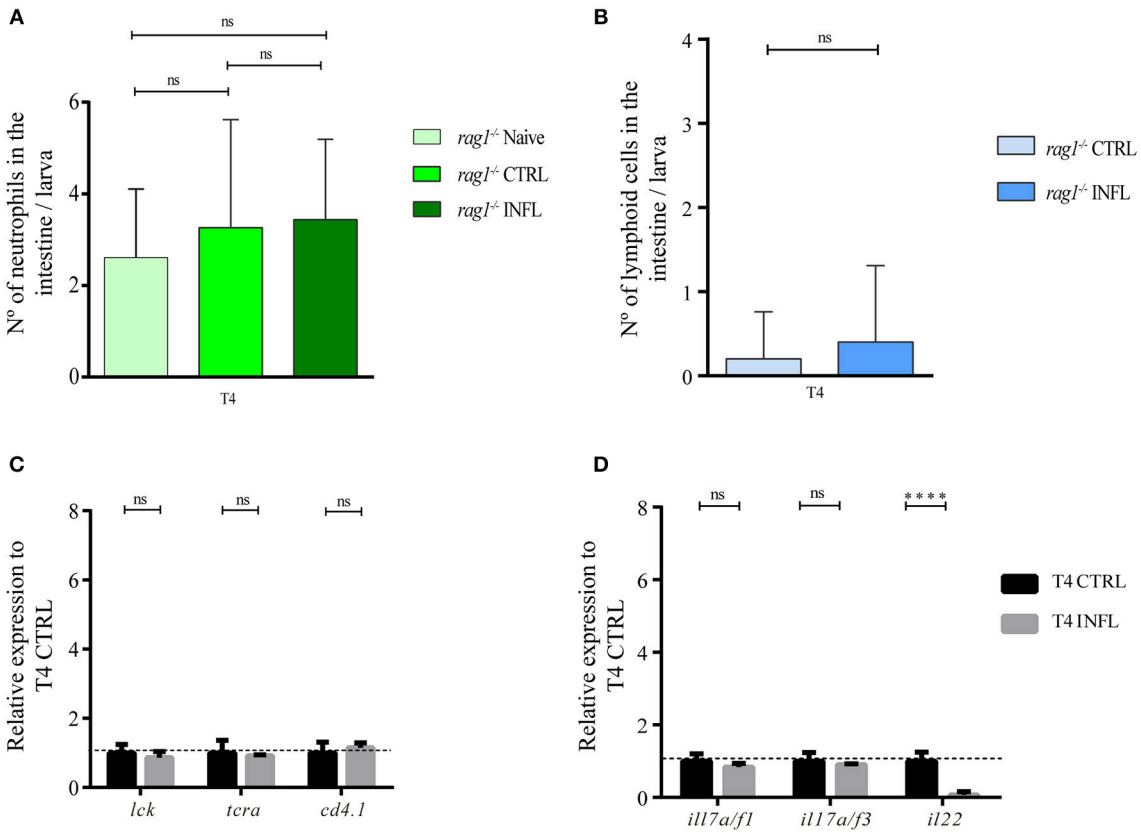

FIGURE 5 | Soybean meal based inflammatory diet triggered intestinal inflammation in a T cell dependent manner. **(A)** Neutrophils quantification in naïve, control and inflamed *rag1*$^{-/-}$ Tg(lck:lck-eGFP) larvae fed according to naïve model. **(B)** Lymphoid cells (lck^{+} cells) quantification in control and inflamed *rag1*$^{-/-}$ Tg(lck:lck-eGFP) larvae fed according to naïve model. **(C–D)** Relative transcriptional level of lymphoid-associated genes were analyzed. Data was normalized against *rpl13a* and compared to the control condition (T4; dotted line). Neutrophils and lymphoid cells quantification were done at least in three biological replicates and with 20 individuals per condition. For each condition in RT-qPCR, 100 guts were analyzed, and three biological replicates were made. ****$p > 0.0001$.

larvae. Our results showed a very low presence of lck^+ cells in intestines in both conditions, with an average of 0.2 lck^+ cells in larvae fed the control diet and 0.4 in larvae fed the inflammatory diet (**Figure 5B**). Regarding mRNA levels of other lymphoid markers, neither *lck, trac,* nor *cd4.1* increased expression level in intestines of larvae fed the inflammatory diet compared to those fed the control diet (**Figure 5C**). In the case of mRNA level of *mhc1zea* and *mhc2ab*, the first decrease their level and the latter increase them (**Supplementary Figure 2**). Likewise, the Th17 makers, *il17/f1* and *il17/f3*, did not change between the control and inflammatory conditions (**Figure 5D**). Altogether, these results indicate that the inflammatory process triggered by the intake of soybean meal-based diet is T cell-dependent.

DISCUSSION

The understanding of biological processes associated with intestinal inflammatory diseases such IBD has historically been a very active research focus due to the high prevalence of these pathologies worldwide. Most of these investigations are based on inflammation models developed in mice which, despite having allowed important advances, are not able to completely encompass the hallmarks of this disease. Thus, in this work we used an intestinal inflammation model established in zebrafish larvae based on the intake of soybean meal in which we monitored *in vivo* the participation of innate and adaptive cells. Specifically, we studied the recruitment of neutrophils, macrophages, mast cells, and T cells to the gut. We show here for the first time that, as early as 5 dpf, T cells are present in the intestine of zebrafish during homeostasis and that they increase in numbers upon soybean meal-induced inflammation. Thus, our results demonstrate that adaptive immune response is already functional at the end of the first week of zebrafish development.

In the case of myeloid cells, we observed a strong increase of macrophages numbers in the intestine in both models of inflammation. A similar phenomenon was observed for neutrophils. On the other hand, we did not detect an increase in the number of mast cells in any of the conditions studied. In humans, disorders such as IBD develop with a large infiltration of neutrophils, macrophages and monocytes to the gut (40, 41). Meanwhile, mast cells undergo activation, leading to a substantial release of mediators such as histamine and proteases (42). Surprisingly, we detected not only an increase in the number of macrophages present in the gut, but also drastic changes in their morphology between conditions where intestines have not yet faced food antigens compared to intestines that have experienced antigen encounter. In the first, all macrophages displayed a rounded shape and, in the latter, a combination of two morphologies was observed: rounded macrophages were accompanied by others with a clear increase in size and presence of long protrusions. Importantly, both macrophage types were observed in the control situation or under inflammatory conditions. Our RT-qPCR analysis indicated that in the control situation of the naïve model, the intestinal response was not tolerogenic but inflammatory. Thus, we speculate that these two morphologies could correspond to M0/homeostatic (round) and M1/pro-inflammatory (with protrusions) states. In mammals, different macrophage populations can be found. In a steady state gut, macrophages are characterized by very high levels of CX3CR1 expression, are avidly phagocytic and $MHCII^{hi,}$ but are resistant to Toll-like receptor stimulation, produce interleukin 10 constitutively, and express CD163 and CD206. Also, these cells have a round morphology (43). On the contrary, during an inflammatory process, macrophages express intermediate levels of CX3CR1, are Toll-like receptor responsive and pro-inflammatory, expressing IL6 and iNOS (43). Another possibility, based on the morphology of both macrophage populations in zebrafish larvae, is that the rounded cells are indeed monocyte and the cells with protrusions correspond to monocyte-derived dendritic cells (DCs). It has been shown that human $CD16^+$ monocytes differentiate into migratory DCs during the inflammatory process (44). Likewise, and using CX3CR1 GFP^+ mice in which monocytes and their daughter cells were tracked by analyzing GFP^+ cells, Qu et al. (45) demonstrated that the $Ly6C^+$ inflammatory monocyte subset gives rise to dendritic cells that migrate to lymph nodes and express Gr1.

Regarding lymphoid cells, we detected transcripts from a very early developmental stage (5 dpf) of *lck*, *trac,* and *cd4.1* genes, suggesting that the lymphoid cells observed in the intestine were helper T cells. Detection of *cd4.1* mRNA at early larval stages has been described before (46). Importantly, we and others (46) did not detect transcription of *cd8*, suggesting absence of $Cd8^+$ T cells at this developmental stage. The *lck* gene has been recently shown to be expressed by zebrafish intestinal innate lymphoid cells (ILCs) (47). Within the ILC family, the ILC3 subset also expresses Th17 cytokines such as Il-22 and Il-17. Furthermore, mouse ILCs populate the intestine at earlier developmental stages than T cells, being crucial in inflammatory responses against enteric viruses in neonatal mice (48). We observed very low numbers of $rag1^{-/-}$ *lck*-$eGFP^+$ cells, thus ILCs, in the intestines of larvae in both control and soybean meal diet fed fish. In addition, we found lack of inflammation upon soybean meal diet in T-cell deficient fish ($rag1^{-/-}$). Altogether, our data suggests that zebrafish ILCs do not play a role in soybean meal-induced inflammation. It remains to be determined the developmental stage at which ILCs populate the intestine of zebrafish as well as their participation in intestinal inflammation.

At the molecular level, it is interesting that in the control condition, the intestine displayed opposite responses in the two models used. In the case of the naïve model, the intestine faced food antigens for the first time, triggering an inflammation with increased expression levels of *il17a/f3*, and more importantly, without a tolerogenic response. Treg cell markers were unaltered and even decreased compared to the situation prior to feeding. Conversely, when the intestine had been previously exposed to food antigens, as in the developed model, we observed polarization of T cells toward a tolerogenic profile showing increased levels of *foxp3* and *il10* transcripts. These results suggest that the intestine must

be educated to develop food tolerance even in the case of an innocuous antigen.

On the other hand, the inflammatory condition, both in the naïve and the developed models, elicited a clear Th17 response, with increased mRNA levels of the markers *il17a/f1*, *il17a/f3,* and *il22,* and decreased levels of the Treg markers *foxp3* and *il10*. Studies in mice and humans indicate that Th17 cells play a major role in the pathogenesis of Crohn's disease and ulcerative colitis (49–51). Furthermore, a correlation between disease severity and levels of IL-17 secreted by peripheral blood mononuclear cells from ulcerative colitis patients has been observed (52).

Regarding the signaling controlling T cell homing to the intestine during inflammation, our results suggest implication of Ccl25. As in mammals (53), we observed a considerable increase in the transcriptional level of *ccl25* in the intestine during inflammation. It remains to be determinate if Ccr9 is the cognate receptor of Ccl25 in zebrafish. Finally, we observed increased motility of lck-eGFP+ cells under inflammatory conditions, suggesting a functional change of these cells. The role of increased motility as well as the signals regulating this phenomenon remain to be explored. Importantly, *in vivo* imaging of intestinal lymphocytes upon different conditions, highlights the utility of the zebrafish model to analyze behavioral changes of different cell types *in situ* and in a non-invasively manner.

An important aspect we did not cover in this work is the participation of the microbiota in the induction of the soybean meal-induced intestinal inflammation. It is widely accepted is that IBD is triggered only under the presence of microbes, thus it remains to be determined whether our inflammation model is dependent on the presence of intestinal microbiota.

In summary, these two new intestinal inflammation models recapitulate many of the hallmarks of IBD observed in mice and humans, offering opposite situations that allow to generate a broad vision of the intestinal inflammatory process. This fact, added to the key advantages offered by the zebrafish model, positions our inflammatory model in a favorable position to offer a complementary alternative to the currently available IBD murine models.

ETHICS STATEMENT

All animals were handled in strict accordance with good animal practice as defined by the European Union guidelines for the handling of laboratory animals and the Bioethics Committee of the Universidad Andres Bello, which approved this study, certificate number 007-2016.

AUTHOR CONTRIBUTIONS

MC, PH, and CF contributed to the conception and design of the study. MC and CS developed the experiments and performed the statistical analysis. CF wrote the first draft of the manuscript. All authors contributed to revising the manuscript, reading, and approving the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00610/full#supplementary-material

Supplementary Figure 1 | (A) Experimental strategy for naïve and developed feeding model. In the case of the naïve feeding model, 5 dpf larvae were fed with control diet (fishmeal-based diet) or inflammatory diet (soybean meal-based diet) during 4 days until 9 dpf. Samples were collected before and after feeding and behavior of neutrophils, macrophages, mast cells, and lymphocytes was analyzed. Also, transcriptional levels of different immune gene markers were evaluated. In the case of the developed feeding model, larvae were fed from 5 to 17 dpf with a commercial diet and then changed to control or inflammatory diet for 8 days (18–25 dpf). Before, at half time and after feeding, samples were collected and analyzed as for the naïve feeding model. **(B)** The amount of neutrophils was quantified in control and inflammatory conditions at T4 and at T4+4. **(C)** Transversal paraffin cross sections of the intestine of larvae fed according to the developed model stained with hematoxylin and eosin. **(D)** Quantification of the number of intestinal folds in control and inflamed larvae fed according the developed model. $*p < 0.05$; $***p < 0.001$.

Supplementary Figure 2 | Relative transcriptional level of *mhc1zea, mhc2ab, hamp, leap2, trim33,* and *il23* genes were analyzed in wild type and *rag1*$^{-/-}$ larvae after performed the naïve model. Data was normalized against *rpl13a* and compared to the control condition. For each condition, 100 guts were analyzed, and three biological replicates was made. $**p < 0.01$; $***p < 0.001$; $****p > 0.0001$.

Supplementary Movie 1 | Time lapse showing lck^{+} cells, lymphocytes, in the intestine of a Tg(lck:lck-eGFP) larva fed the control diet in the naïve model.

Supplementary Movie 2 | Time lapse showing lck^{+} cells, lymphocytes, in the intestine of a Tg(lck:lck-eGFP) larva fed the inflammatory diet in the naïve model.

REFERENCES

1. Sahlmann C, Sutherland BJ, Kortner TM, Koop BF, Krogdahl A, Bakke AM. Early response of gene expression in the distal intestine of Atlantic salmon (Salmo saar L.) during the development of soybean meal induced enteritis. *Fish Shellfish Immunol.* (2013) 34:599–609. doi: 10.1016/j.fsi.2012.11.031
2. Krogdahl Å, Gajardo K, Kortner TM, Penn M, Gu M, Berge GM, et al. Soya saponins induce enteritis in Atlantic Salmon (Salmo salar L.). *J Agric Food Chem*. (2015) 63:3887–902. doi: 10.1021/jf506242t
3. Gu M, Jia Q, Zhang Z, Bai N, Xu X, Xu B. Soya-saponins induce intestinal inflammation and barrier dysfunction in juvenile turbot (*Scophthalmus maximus*). *Fish Shellfish Immunol*. (2018) 77:264–72. doi: 10.1016/j.fsi.2018.04.004
4. Brazil JC, Louis NA, Parkos CA. The role of polymorphonuclear leukocyte trafficking in the perpetuation of inflammation during inflammatory bowel disease. *Inflamm Bowel Dis*. (2013) 19:1556–65. doi: 10.1097/MIB.0b013e318281f54e
5. Kamada N, Hisamatsu T, Okamoto S, Chinen H, Kobayashi T, Sato T, et al. Unique CD14 intestinal macrophages contribute to the pathogenesis

of Crohn disease via IL-23/IFN-γ axis. *J Clin Invest*. (2008) 118:2269–80. doi: 10.1172/JCI34610
6. Hart AL, Al-Hassi HO, Rigby RJ, Bell SJ, Emmanuel AV, Knight SC, et al. Characteristics of intestinal dendritic cells in inflammatory bowel diseases. *Gastroenterology*. (2005) 129:50–65. doi: 10.1053/j.gastro.2005.05.013
7. Geremia A, Biancheri P, Allan P, Corazza GR, Di Sabatino A. Innate and adaptive immunity in inflammatory bowel disease. *Autoimmun Rev*. (2014) 13:3–10. doi: 10.1016/j.autrev.2013.06.004
8. Raphael I, Nalawade S, Eagar TN, Forsthuber TG. T cell subsets and their signature cytokines in autoimmune and inflammatory diseases. *Cytokine*. (2015) 74:5–17. doi: 10.1016/j.cyto.2014.09.011
9. Caza T, Landas S. Functional and phenotypic plasticity of CD4(+) T cell subsets. *Biomed Res Int*. (2015) 2015: 521957. doi: 10.1155/2015/521957
10. Hoeppli RE, MacDonald KG, Levings MK, Cook L. How antigen specificity directs regulatory T-cell function: self, foreign and engineered specificity. *HLA*. (2016) 88:3–13. doi: 10.1111/tan.12822
11. Mayne CG, Williams CB. Induced and natural regulatory T cells in the development of inflammatory bowel disease. *Inflamm Bowel Dis*. (2013) 19:1772–88. doi: 10.1097/MIB.0b013e318281f5a3
12. Harrison OJ, Powrie FM. Regulatory T cells and immune tolerance in the intestine. *Cold Spring Harb Perspect Biol*. (2013) 5:a018341. doi: 10.1101/cshperspect.a018341
13. Bouma G, Strober W. The immunological and genetic basis of inflammatory bowel disease. *Nat Rev Immunol*. (2003) 3:521–33. doi: 10.1038/nri1132
14. Valatas V, Bamias G, Kolios G. Experimental colitis models: insights into the pathogenesis of inflammatory bowel disease and translational issues. *Eur J Pharmacol*. (2015) 759:253–64. doi: 10.1016/j.ejphar.2015.03.017
15. Cominelli F, Arseneau KO, Rodriguez-Palacios A, Pizarro TT. Uncovering pathogenic mechanisms of inflammatory bowel disease using mouse models of Crohn's disease-like ileitis: what is the right model? *Cell Mol Gastroenterol Hepatol*. (2017) 4:19–32. doi: 10.1016/j.jcmgh.2017.02.010
16. Harvie EA, Huttenlocher A. Neutrophils in host defense: new insights from zebrafish. *J Leukoc Biol*. (2015) 98:523–37. doi: 10.1189/jlb.4MR1114-524R
17. Bajoghli B. Evolution and function of chemokine receptors in the immune system of lower vertebrates. *Eur J Immunol*. (2013) 43:1686–92. doi: 10.1002/eji.201343557
18. Novoa B, Figueras A. Zebrafish: model for the study of inflammation and the innate immune response to infectious diseases. *Adv Exp Med Biol*. (2012) 946:253–75. doi: 10.1007/978-1-4614-0106-3_15
19. Meeker ND, Trede NS. Immunology and zebrafish: spawning new models of human disease. *Dev Comp Immunol*. (2008) 32:745–57. doi: 10.1016/j.dci.2007.11.011
20. Renshaw SA, Trede NS. A model 450 million year in the making: zebrafish and vertebrate immunity. *Dis Model Mech*. (2012) 5:38–47. doi: 10.1242/dmm.007138
21. Nguyen-Chi M, Laplace-Builhe B, Travnickova J, Luz-Crawford P, Tejedor G, Phan QT, et al. Identification of polarized macrophage subsets in zebrafish. *Elife*. (2015) 4:e07288. doi: 10.7554/eLife.07288
22. Wallace KN, Akhter S, Smith EM, Lorent K, Pack M. Intestinal growth and differentiation in zebrafish. *Mech Dev*. (2005) 122:157–73. doi: 10.1016/j.mod.2004.10.009
23. Brugman S, Liu KY, Lindenbergh-Kortleve D, Samsom JN, Furuta GT, Renshaw SA, et al. Oxazolone-induced enterocolitis in zebrafish depends on the composition of the intestinal microbiota. *Gastroenterology*. (2009) 137:1757–67. doi: 10.1053/j.gastro.2009.07.069
24. Oehlers SH, Flores MV, Okuda KS, Hall CJ, Crosier KE, Crosier PS. A chemical enterocolitis model in zebrafish larvae that is dependent on microbiota and responsive to pharmacological agents. *Dev Dyn*. (2011) 240:288–98. doi: 10.1002/dvdy.22519
25. Oehlers SH, Flores MV, Hall CJ, Okuda KS, Sison JO, Crosier KE, et al. Chemically induced intestinal damage models in zebrafish larvae. *Zebrafish*. (2013) 10:184–93. doi: 10.1089/zeb.2012.0824
26. Hedrera MI, Galdames JA, Jimenez-Reyes MF, Reyes AE, Avendaño-Herrera R, Romero J, et al. Soybean meal induces intestinal inflammation in zebrafish larvae. *PLoS ONE*. (2013) 8:e69983. doi: 10.1371/journal.pone.0069983
27. Westerfield M. *The Zebrafish Book: A Guide for the Laboratory Use of the Zebrafish (Danio rerio), 2.1*. Eugene: University of Oregon Press (1994).
28. Langenau DM, Ferrando AA, Traver D, Kutok JL, Hezel JP, Kanki JP, et al. *In vivo* tracking of T cell development, ablation, and engraftment in transgenic zebrafish. *Proc Natl Acad Sci USA*. (2004) 101:7369–74. doi: 10.1073/pnas.0402248101
29. Hall C, Flores MV, Storm T, Crosier K, Crosier P. The zebrafish lysozyme C promoter drives myeloid-specific expression in transgenic fish. *BMC Dev Biol*. (2007) 7:42. doi: 10.1186/1471-213X-7-42
30. Harvie EA, Green JM, Neely MN, Huttenlocher A. Innate immune response to Streptococcus iniae infection in zebrafish larvae. *Infect Immun*. (2013) 81:110–21. doi: 10.1128/IAI.00642-12
31. Petrie-Hanson L, Hohn C, Hanson L. Characterization of rag1 mutant zebrafish leukocytes. *BMC Immunol*. (2009) 10:8. doi: 10.1186/1471-2172-10-8
32. Haffter P, Granato M, Brand M, Mullins MC, Hammerschmidt M, et al. The identification of genes with unique and essential functions in the development of the zebrafish, *Danio rerio*. *Development*. (1996) 123:1–36.
33. Feijóo CG, Sarrazin A, Allende ML, Glavic A. (Cysteine-serinerich nuclear protein 1), Axud1/Csrnp1, is essential for cephalic neural progenitor proliferation and survival in zebrafish. *Dev Dynam*. (2009) 238:2034–43. doi: 10.1002/dvdy.22006
34. Neal JT, Peterson TS, Kent ML, Guillemin K. *H. pylori* virulence factor CagA increases intestinal cell proliferation by Wnt pathway activation in a transgenic zebrafish model. *Dis Model Mech*. (2013) 6:802–10. doi: 10.1242/dmm.011163
35. Rawls JF, Samuel BS, Gordon JI. Gnotobiotic zebrafish reveal evolutionarily conserved responses to the gut microbiota. *Proc Natl Acad Sci USA*. (2004) 101:4596–601. doi: 10.1073/pnas.0400706101
36. Brugman S. The zebrafish as a model to study intestinal inflammation. *Dev Comp Immunol*. (2016) 64:82–92. doi: 10.1016/j.dci.2016.02.020
37. Dobson JT, Seibert J, Teh EM, Da'as S, Fraser RB, Paw BH, et al. Carboxypeptidase A5 identifies a novel mast cell lineage in the zebrafish providing new insight into mast cell fate determination. *Blood*. (2008) 112:2969–72. doi: 10.1182/blood-2008-03-145011
38. Yoder JA. Investigating the morphology, function and genetics of cytotoxic cells in bony fish. *Comp Biochem Physiol C Toxicol Pharmacol*. (2004) 138:271–80. doi: 10.1016/j.cca.2004.03.008
39. Bagnat M, Cheung ID, Mostov KE, Stainier DY. Genetic control of single lumen formation in the zebrafish gut. *Nat Cell Biol*. (2007) 9:954–60. doi: 10.1038/ncb1621
40. Wéra O, Lancellotti P, Oury C. The dual role of neutrophils in inflammatory bowel diseases. *J Clin Med*. (2016) 5:E118. doi: 10.3390/jcm5120118
41. Bain CC, Mowat AM. Macrophages in intestinal homeostasis and inflammation. *Immunol Rev*. (2014) 260:102–17. doi: 10.1111/imr.12192
42. Boeckxstaens G. Mast cells and inflammatory bowel disease. *Curr Opin Pharmacol*. (2015) 25:45–9. doi: 10.1016/j.coph.2015.11.005
43. Bain CC, Scott CL, Uronen-Hansson H, Gudjonsson S, Jansson O, Grip O, et al. Resident and pro-inflammatory macrophages in the colon represent alternative context-dependent fates of the same Ly6Chi monocyte precursors. *Mucosal Immunol*. (2013) 6:498–510. doi: 10.1038/mi.2012.89
44. Geissmann F, Jung S, Littman DR. Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity*. (2003) 19:71–82. doi: 10.1016/S1074-7613(03)00174-2
45. Qu C, Edwards EW, Tacke F, Angeli V, Llodra J, Sanchez-Schmitz G, et al. Role of CCR8 and other chemokine pathways in the migration of monocyte-derived dendritic cells to lymph nodes. *J Exp Med*. (2004) 200:1231–41. doi: 10.1084/jem.20032152
46. Tian Y, Xu J, Feng S, He S, Zhao S, Zhu L, et al. The first wave of T lymphopoiesis in zebrafish arises from aorta endothelium independent of hematopoietic stem cells. *J Exp Med*. (2017) 214:3347–60. doi: 10.1084/jem.20170488

47. Hernández PP, Strzelecka PM, Athanasiadis EI, Hall D, Robalo AF, Collins CM, et al. Single-cell transcriptional analysis reveals ILC-like cells in zebrafish. *Sci Immunol*. (2018) 3:eaau5265. doi: 10.1126/sciimmunol.aau5265
48. Hernández PP, Mahlakoiv T, Yang I, Schwierzeck V, Nguyen N, Guendel F, et al. Interferon-λ and interleukin 22 act synergistically for the induction of interferon-stimulated genes and control of rotavirus infection. *Nat Immunol*. (2015) 16:698–707. doi: 10.1038/ni.3180
49. Fouser LA, Wright JF, Dunussi-Joannopoulos K, Collins M. Th17 cytokines and their emerging roles in inflammation and autoimmunity. *Immunol Rev*. (2008) 226:87–102. doi: 10.1111/j.1600-065X.2008.00712.x
50. Gálvez J. Role of Th17 cells in the pathogenesis of human IBD. *ISRN Inflamm*. (2014) 2014:928461. doi: 10.1155/2014/928461
51. Ueno A, Ghosh A, Hung D, Li J, Jijon H. Th17 plasticity and its changes associated with inflammatory bowel disease. *World J Gastroenterol*. (2015) 21:12283–95. doi: 10.3748/wjg.v21.i43.12283
52. Raza A, Shata MT. Letter: pathogenicity of Th17 cells may differ in ulcerative colitis compared with Crohn's disease. *Aliment Pharmacol Ther*. (2012) 36:204. doi: 10.1111/j.1365-2036.2012.05124.x
53. Rivera-Nieves, J., Ho, J, Bamias, G, Ivashkina N, Ley K, Oppermann M, Cominelli F. Antibody blockade of CCL25/CCR9 ameliorates early but not late chronic murine ileitis. *Gastroenterology*. (2006) 131:1518–29. doi: 10.1053/j.gastro.2006.08.031

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REVIEW
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Group 3 ILCs: Peacekeepers or Troublemakers? What's Your Gut Telling You?!

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A complex network of interactions exists between the microbiome, the epithelium, and immune cells that reside along the walls of the gastrointestinal tract. The intestinal immune system has been assigned with the difficult task of discriminating between commensal, harmless bacteria, and invading pathogens that translocate across the epithelial monolayer. Importantly, it is trained to maintain tolerance against commensals, and initiate protective immune responses against pathogens to secure intestinal homeostasis. Breakdown of this fine balance between the host and its intestinal microbiota can lead to intestinal inflammation and subsequently to development of inflammatory bowel disease (IBD). A decade since their discovery, innate lymphoid cells (ILCs) are now recognized as important regulators of intestinal homeostasis. ILC3s have emerged as a critical subset in the gut. They are the most phenotypically diverse ILC population and interact directly with numerous different cell types (haematopoietic and non-haematopoeitic), as well as interface with the bacterial flora. In addition to their contribution to intestinal pathogen immunity, they also mitigate against tissue damage occurring following acute injury, by facilitating tissue repair and regeneration, a key function in the maintenance of intestinal homeostasis. However, in chronic inflammation the tables are turned and ILC3s may acquire a pro-inflammatory phenotype in the gut. Chronic ILC activation can lead to persistent inflammation contributing to IBD and/or colorectal cancer. In this review, we discuss current knowledge of group 3 ILCs and their contributions to intestinal homeostasis and disease leading to novel therapeutic targets and clinical approaches that may inform novel treatment strategies for immune-mediated disorders, including IBD.

Keywords: group 3 innate lymphoid cells, symbiosis, intestinal inflammation, IBD, Crohn's disease, ulcerative colitis

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INTRODUCTION

With more than 10^{13} microorganisms residing in the human gastrointestinal tract (1), our mucosal immune system has excelled in preserving intestinal homeostasis by generating protective immune responses against invading, harmful pathogens whilst maintaining tolerance toward commensals. However, breakdown of this fine balance may lead to excessive immune activation, persistent inflammation and ultimately to the development of inflammatory bowel diseases (IBD). The most common IBD phenotypes, Crohn's disease (CD) and ulcerative colitis (UC) are characterized by

alternating phases of clinical relapse and remission. In CD inflammation can occur in any part of the gastrointestinal tract, whereas in UC inflammation is restricted to the colon (2). Despite the increasing incidence of IBD in the Western world (3), its complex etiology is yet to be fully understood. In general, it is thought that IBD is caused by a dysregulated immune response against the commensal bacterial flora in a genetically predisposed host (4). In accordance with this notion, genome-wide association studies (GWAS) have so far associated single nucleotide polymorphisms (SNPs) in more than 200 genetic loci with IBD susceptibility (5) including genes involved in bacterial recognition, epithelial barrier integrity and immune activation (6) highlighting the importance of microbiota-host interactions and the role of the intestinal immune system, in particular the innate one, in IBD pathogenesis.

Innate Lymphoid Cells—A New Recruit to Mucosal Sentinel Duty

Defense against intestinal pathogens is multifaceted. The intestinal epithelium comprises a physical barrier, which together with the mucus layer and production of anti-microbial peptides provides a containment barrier, which confines microbes in the lumen. Cells of the innate immune compartment residing in the lamina propria are key early warning sentinels detecting invading pathogens through conserved pattern recognition receptors, such as toll-like receptors. Pathogen detecting populations, include cells of the mononuclear phagocyte system, including macrophages and dendritic cells (DCs), which engulf and process microbial antigens and then shape adaptive immune responses by providing initial signals to adaptive lymphocytes to engage potent antigen-specific T and B cell responses. Innate lymphoid cells (ILCs) are recent additions to the innate immune cell family (7–9). They are distributed throughout the human body in lymphoid and non-lymphoid tissues, but are especially enriched at the mucosal barrier surfaces (7), where they directly interact with a number of different cell types; hematopoietic or other (10–12). ILCs have lymphoid-like morphology, but lack any antigen-specific receptors. Arising from a common lymphoid progenitor and similarly to T cells, they can be further subdivided into phenotypically and functionally distinct populations that produce different combinations of effector cytokines to mediate their functions (9–11). Their development depends on different transcription factors, which are also used to help divide ILCs into 3 main groups; group 1 ILCs that includes the well characterized NK cells, as well as the non-cytotoxic ILC1s, group 2 ILCs or ILC2s, and finally group 3 ILCs including ILC3s and lymphoid tissue inducer (LTi) cells. However, recently discovered regulatory ILCs or ILCregs (13) may now represent an additional, distinct ILC family member generating a potential new 4th group of ILCs.

Much of the early work describing ILCs focused on their developmental requirements and capacity for plasticity. An early ILC progenitor (EILCP) rising form a common lymphoid progenitor, which has lost T and B potential, gives rise to NK cells, as well as all ILC lineages (14–16). Downstream of EILCP, Id2 expressing common helper-like ILC precursor (CHILP) gives rise to all ILCs, but not to NK cells (14), whereas all ILCs (except lymphoid tissue inducer cells, LTis), arise from an ILC precursor (ILCP) that expresses both Id2, and PLZF (17) (**Figure 1**). Group 3 ILCs that represent the most diverse and possibly best-characterized ILC populations both in humans and mice (12), require RORγt for their development (18–20), while the transcriptional factor aryl hydrocarbon receptor (AhR) is essential for their maintenance (21, 22). In general, group 3 ILCs, can be further divided into $CCR6^+$ LTi cells that may or may not express CD4, and $CCR6^{low/-}$ ILC3s. Based on whether or not $CCR6^{low/-}$ ILC3s express natural cytotoxicity receptors (NCRs), they are additionally subdivided into NCR^+ and NCR^- ILC3s (**Figure 1**). Upon activation, NCR^- ILC3s and LTi cells produce mostly interleukin (IL)-17A, IL-17F and IL-22, while NCR^+ ILC3s produce mainly IL-22 (20, 23–27). Several independent studies have provided evidence suggesting plasticity within group 3 ILCs (28, 29). NCR^- ILC3s can differentiate into NCR^+ ILC3s in the presence of increasing expression of T-bet (30, 31), or even acquire an ILC1-like phenotype by completely losing RORγt expression (29, 32). Similarly, ILC1-like ILC3s may also differentiate *in vivo* into NCR^- ILC3s in the presence of a RORγt gradient (32, 33) (**Figure 1**). Given the enriched numbers of group 3 ILCs in the gut mucosa and their role in preserving intestinal homeostasis, additional studies are required to clarify whether this profound, finely tuned plasticity within group 3 ILCs is a mechanism to regulate intestinal inflammation, and whether this plasticity may also be extended to recently identified ILCregs (13).

Although group 3 ILCs are primarily known for their role in anti-bacterial immunity (20, 27), they emerge as key effector cells at barrier surfaces. Recent studies describe their involvement in several immune-mediated diseases such as psoriasis (34, 35), multiple sclerosis (36), or cancer (37, 38), improving our understanding of these highly plastic cells in controlling inflammation, while suggesting new ways of therapeutic immune intervention. In this review, we focus on the role of group 3 ILCs in the gut, during intestinal homeostasis and disease.

GROUP 3 ILCS PROMOTE INTESTINAL PEACE

Microbiota-ILC3 Interactions—A Key Partnership for a Finely Tuned Intestinal Immune System

Group 3 ILCs accumulate in the gastrointestinal tract and gut-associated lymphoid tissues in a microbiota-independent manner (21, 39–41). They directly interact with the bacterial flora, as well as with immune and non-hematopoietic cells creating a dynamic network between the host and its resident microbiota that favors symbiosis and preserves intestinal homeostasis (**Figure 2**). An important aspect of this partnership is containment of commensals to the lumen allowing for controlled bacterial sampling by lamina propria mononuclear phagocytes. ILC3s are key regulators of this process. Loss of ILC3s in the intestine leads to diminished IL-22 production (a key cytokine produced by ILC3s), and impaired production of antimicrobial peptides by intestinal epithelial cells, culminating in peripheral dissemination of *Alcaligenes* bacteria and systemic inflammation that could be prevented by exogenous replacement of IL-22

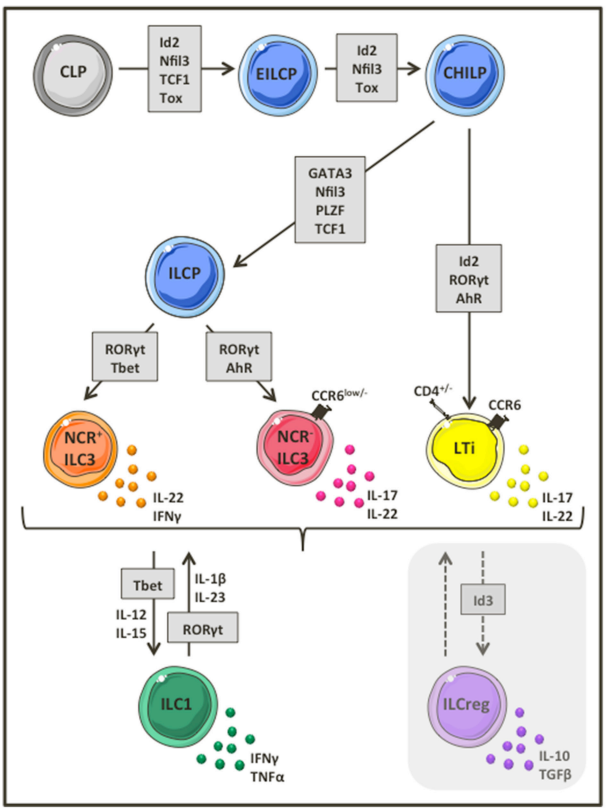

FIGURE 1 | Group 3 ILC development and heterogeneity. Group 3 ILCs represent the most diverse ILC subset, comprising of CCR6$^+$ LTi cells, and ILC3s that can be further divided into NCR$^-$ and NCR$^+$ ILC3s. Group 3 ILCs rise from a common lymphoid progenitor that lost T, B and eventually NK cell potential and depend on the transcriptional factor RORγt for their development. Depending on the environmental stimulus, ILC3s produce a variety of distinct cytokines such as IL-17, IL-22, IFNγ, and GM-CSF to mediate their functions. Interestingly, ILC3s are highly plastic cells and as such NCR$^-$ ILC3s can acquire NCR expression and became NCR$^+$ ILC3s in the presence of a T-bet gradient or loose completely RORγt expression to differentiate into ILC1 like ILCs and vice versa.

(41). ILC3s also play a key role in shaping adaptive immunity. Importantly, Hepworth et al. showed for the first time that loss of RORγt$^+$ ILCs in immunocompetent mice lead to dysregulated adaptive immune responses against commensals, an effect that was not mediated by known ILC3 associated cytokines such as IL-17A, IL-22 and IL-23, but through MHC II:TCR interactions instead (42). In particular, selective deletion of MHC II expression on RORγt$^+$ ILCs resulted in enhanced antigen-specific Th17 responses against bacterial flora, promoting spontaneous intestinal inflammation (42). Others have also reported contribution of ILC3s in the generation of Th17 responses against commensals (43), while antigen presenting capacities through MHC II and expression of co-stimulatory molecules have also been described for splenic ILC3s using *in vitro* systems of T cell priming (44). Although ILC3s can uptake, process and present antigen, they don't express co-stimulatory molecules and as such they can potentially result in T cell energy. Thus, when absent there is uncontrolled T cell activation and exaggerated T cell responses to commensals that would otherwise be regulated. Establishing a balanced, two-way relationship between ILC3s and T cells in the gut, T cells have proven to be crucial for keeping ILC3 numbers and functions in check. Absence of CD4$^+$ T cells resulted in elevated ILC numbers, increased IL-22 production and subsequently enhanced secretion of anti-microbial peptides by epithelial cells (45). Other more complex, dynamic interactions between ILC3s, T cells and the microbiota are important at different development stages of the host. While ILC3s have an important role in influencing bacterial composition at early developmental stages, most likely to prevent unnecessary inflammatory responses, as bacteria burden expands, CD4$^+$ T cells accumulate to establish tolerance using distinct mechanisms compared to their innate counterparts, that may become quiescent (46).

Although IL-22 plays a central role regulating mucosal immunity, the microbiota also play an important role fine-tuning the ILC3/IL-22 axis. Segmented filamentous bacteria (SFB) are commensal bacteria that selectively colonize the terminal ileum of mice, a key inductive site of mucosal immunity. They play a central role in the differentiation of Th17 cells (47, 48), but also regulate innate IL-22 production. Mono-association of germ free mice with SFB results in marked augmentation of IL-22 production by intestinal ILC3s. In this system IL-22 induced production of serum amyloid A proteins 1 and 2 from the intestinal epithelial cells, which in turn played a key role promoting the differentiation of Th17 cells (49). Other commensal bacteria may have an opposing role on IL-22 production, through induction of IL-25 by intestinal epithelial cells, which suppresses IL-22 production by RORγt$^+$ ILCs (50).

The microbiota ILC3 partnership extends its branches to B cells too, the other major adaptive immune cell. In 2008, Tsuji et al. showed that LTi cells are essential for the formation of isolated lymphoid follicles (ILFs) and T cell independent generation of immunoglobulin A (IgA) by B cells in the gut (51). Later that year, a study published in Nature complemented these findings by showing that peptidoglycans from Gram$^-$ bacteria activate LTi cells in the gut, which then induce chemokine production by stromal cells resulting in B cell recruitment and subsequently formation of ILFs (52). Kruglov et al. showed that RORγt$^+$ ILCs mediate both T cell dependent and independent IgA production by B cells through secretion of soluble lymphotoxin α (sLTα3) and membrane-bound lymphotoxin β (LTα1β2), respectively (53). Beneficial interactions have also been described between group 3 ILCs and mononuclear phagocytes (54). Bacterial sampling by intestinal macrophages and DCs induces IL-1β secretion that activates ILC3s, which in turn produce GM-CSF that feeds back to mononuclear phagocytes to produce anti-inflammatory mediators such as IL-10 and retinoic acid eventually leading to Treg expansion and immune tolerance (54). Surprisingly, Ibiza et al. showed that group 3 ILCs also interact with the enteric nervous system in an attempt to maintain intestinal homeostasis (55). Interestingly, it was shown that glial cells in the lamina propria sense bacterial presence and secrete neurotrophic factors that induce IL-22 production by ILC3s through the neuroregulatory receptor RET (55), providing the first evidence

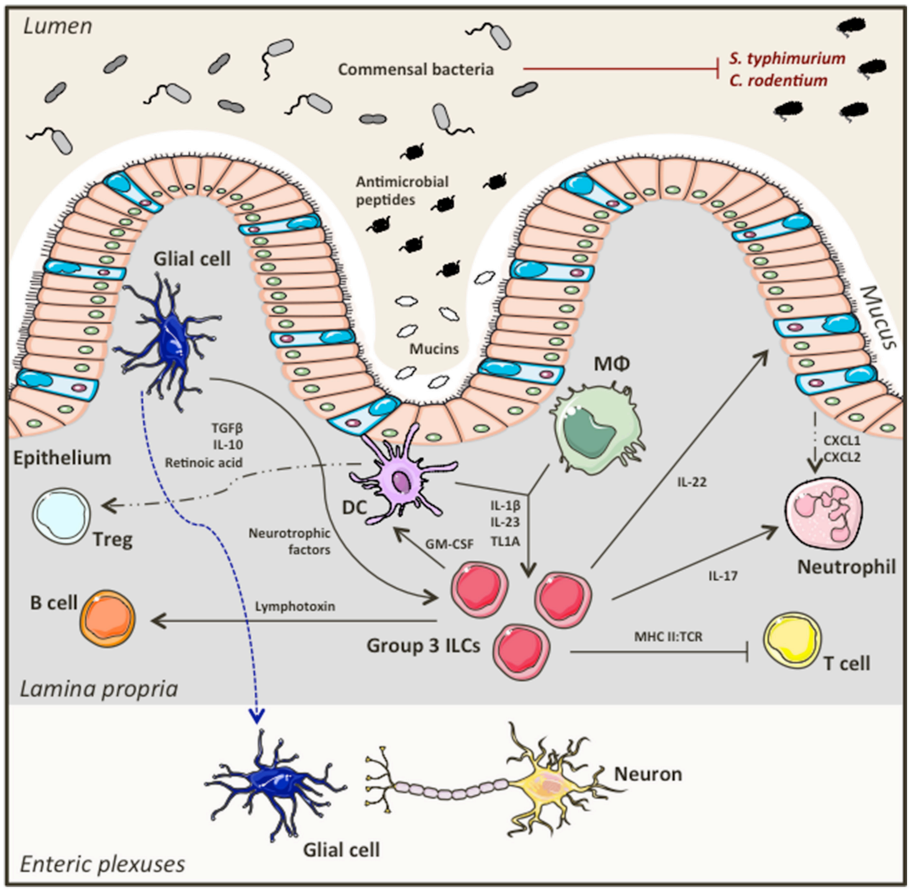

FIGURE 2 | Group 3 ILCs in intestinal homeostasis. Group 3 ILCs including ILC3s and LTi cells directly interact with the commensal bacteria in the lumen, the intestinal epithelium, as well as with other immune cells and neurons in the lamina propria forming a finely tuned network that secures and maintains intestinal homeostasis.

of direct neuron involvement in innate immune regulation in the gut.

ILC3s, Important Soldiers Fighting Foreign Pathogens

In accordance with studies showing how group 3 ILCs directly interact with the bacterial flora while working closely with other hematopoietic and non-immune cells to secure and maintain intestinal homeostasis, ILC3s have also been described as key effector cells in immunity against pathogens. Even prior to acquiring their official name, group 3 ILCs were associated with protection against *Proteobacteria* (20). Loss of NKp46$^+$ RORγt$^+$ IL-22 producing innate immune cells is linked to increased susceptibility to *Citrobacter rodentium* infection, which is a model for enteropathic *E.coli* infection (20) ILC3s were especially important as early producers of IL-22 (56). Lymphotoxin produced by RORγt expressing innate immune cells was necessary to control *C. rodentium* infection as lymphotoxin acted on the intestinal epithelium inducing CXCL1 and CXCL2 chemokine production and subsequently neutrophil recruitment at the early stages of infection (57). Sonnenberg et al. showed that infection with *C. rodentium* induced IL-23 mediated IL-22 production by CD4$^+$ LTi cells, and that this subset of ILC3 was sufficient to promote immunity in immunodeficient hosts (27). More recent studies showed that expression of the G-protein-coupled receptor 183 (GPR183) on LTi cells is not only essential for their migration to cryptopatches and ILFs (58), but also required for ILC3-mediated protection against *C. rodentium* infection (59). Notably, immunity to *Citrobacter* is STAT3 dependent. STAT3 deficiency was associated with impaired IL-22 production, and increased disease severity, which could be rescued with exogenous IL-22 (60). STAT3 expression was only required in ILC3s and T cells to induce protection (60).

One of the mechanisms of IL-22 mediated regulation of the microbiota is through regulation of the glycosylation pattern of epithelial cells. IL-22 and lymphotoxin expressed by ILC3s control the expression of fucosyltransferase 2 (Fut2), which triggers fucosylation of epithelial cells, which in turn can be utilized as a nutrient source by luminal commensals (61). IL-22 mediated fucosylation of the epithelium was dependent on colonization of the GI tract with bacteria, and in a positive circuit, fucosylation promoted colonization with symbiotic bacteria, presumably by providing a favorable nutrient supply. Disruption of this system resulted in loss of host-microbe mutualism and rendered the host susceptible to *Salmonella typhimurium* infection (61). In accordance with these findings, Pickard et al.

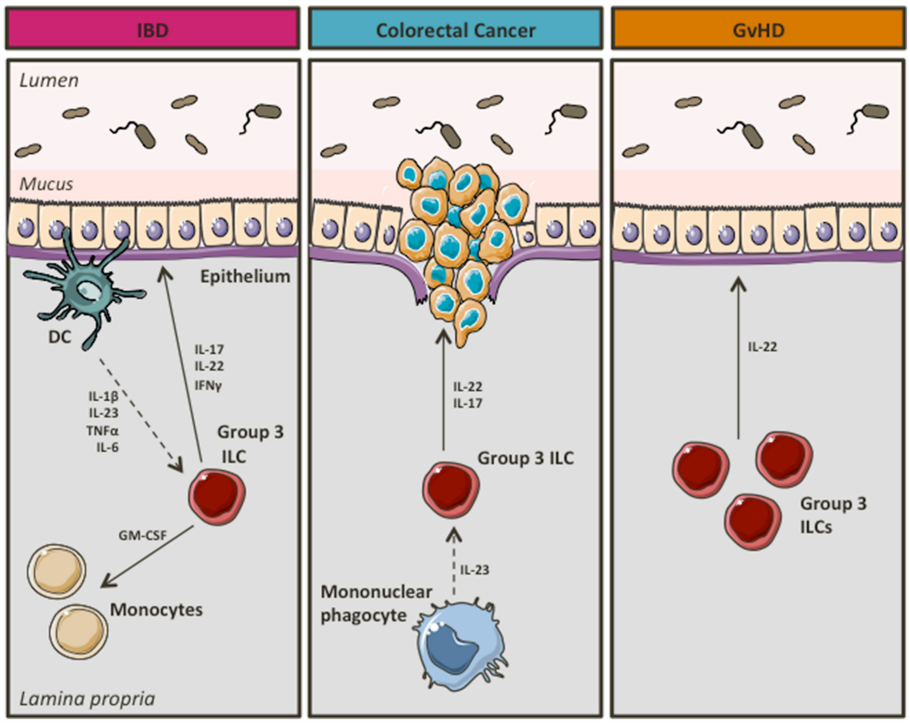

FIGURE 3 | Group 3 ILCs during intestinal disease. In chronic inflammation group 3 ILCs seem to acquire a pro-inflammatory phenotype, thus contributing to the development of IBD and/or colorectal cancer. However, they appear to have a beneficial role in GvHD.

showed that IL-22 induced fucosylation is mediated by ILC3s upon activation with IL-23 produced by DCs during pathogen-induced stress (62). In this setting, rapid fucosylation could also improve tolerance to *C. rodentium* infection (62).

Klose et al. demonstrated that Tbet expressing; IFNγ producing RORγt$^+$ ILCs are key drivers of immunity against *Salmonella* infections (30). Other work supports a role for ILC3s in protective immunity against *E. coli,* or *Klebsiella pneumoniae and Toxoplasma gondi* infection (63, 64). ILC3s also contribute to host resistance to Rotavirus. ILC3 derived IL-22 synergizes with IFNλ to minimize viral replication (65).

These studies point to an important role of group 3 ILCs in promoting symbiosis with commensals and protection against pathogens. ILC3s form a dynamic network of interactions with the microbiome, other immune cells, the intestinal epithelium and surprisingly with neuro-glial cells to secure intestinal homeostasis (**Figure 2**).

GROUP 3 ILCS IN INTESTINAL DISEASE: THE OTHER SIDE OF THE COIN

In a landmark study, Buonocore et al. showed that chronic infection of immunodeficient mice with *Helicobacter hepaticus* resulted in RORγt$^+$ ILC3 mediated gut inflammation. ILC3s produced high levels of IL-17 and IFNγ in response to IL-23, thus contributing to the development of T-cell independent inflammation (26). Similarly, ILC3s were crucial for the development of innate mediated colitis using the anti-CD40 model of IBD (26). In support of a pathogenic role of ILC3s in intestinal inflammation (**Figure 3**), Geremia et al. showed that IL-23 responsive ILC3s were increased in the intestines of patients with CD, where in response to IL-23 they produced high levels of IBD-relevant cytokines such as IL-17 and IL-22 (66). Shedding more light into the mechanisms underlying ILC3-driven intestinal pathology, Pearson et al. showed that ILC3s use GM-CSF to attract inflammatory monocytes on site, whereas mobility of ILC3s in and out of cryptopatches appeared to induce inflammation at non-inflamed sites (67). Recently, Bauché et al. showed that Tregs were able to prevent ILC3-induced colitis (68). In particular, latent activation gene 3 (LAG3) expressing Tregs reduced IL-1β and IL-23 production by CX3CR1$^+$ macrophages resulting in impaired IL-22 production by ILC3s and therefore attenuated disease (68). Moreover, using *Tbx21*$^{-/-}$*Rag2*$^{-/-}$ Ulcerative Colitis (TRUC) mice, which develop spontaneous colitis with clinical features that resemble aspects of human UC (69), Powell et al. showed that IL-23 and TNFα produced by CD103$^-$CD11b$^+$ mononuclear phagocytes activated ILC3s to drive intestinal inflammation through production of IL-17 (70). In particular, NCR$^-$ ILC3s, the major ILC subset present in the colon of TRUC mice, were potent producers of IL-17 and IL-22 in response to IL-1α and IL-23, an effect that was more profound in the presence of IL-6 (71). Interestingly, *in vivo* blockade of IL-6 using neutralizing antibodies significantly attenuated colonic inflammation in TRUC mice (71).

Interestingly, group 3 ILCs have also been associated with a pro-inflammatory, pathogenic role in cancer (**Figure 3**), a feared complication of unopposed inflammation in patients with UC (72, 73). IL-17$^+$IL-22$^+$ ILCs accumulate in colorectal cancer occurring in *Helicobacter hepaticus* associated colorectal cancer. ILC depletion alleviated invasive cancer in this model (74). Importantly, IL-22, but not IL-17, was necessary for cancer maintenance (74). In support of ILC3s having a role in promoting colorectal cancer, Chan et al. showed that ILC3s were key drivers of IL-23 induced tumorigenesis, however in this setting ILC3 actions were mediated by IL-17 (75).

Intestinal inflammation can also occur as a result of graft vs. host disease (GvHD) in recipients of allogenic hematopoietic stem cell transplants (AHSCT) as a treatment of hematopoietic cell disorders including blood cancers (76). Although several studies suggest that ILC3s may contribute to intestinal inflammation promoting the development of IBD or colorectal cancer, in GvHD they might be beneficial (**Figure 3**). Hanash et al. showed that IL-22 production by ILC3s was increased in patients following pretransplant conditioning, whereas IL-22 levels were reduced upon emergence of GvHD (77). Notably, IL-22 deficiency in recipients resulted in significant intestinal inflammation and tissue damage (77). Moreover, Munneke et al. suggested that elevated numbers of NCR$^+$ ILC3s in peripheral blood of leukemia patients following AHSCT were associated with reduced risk of GvHD (78).

CONCLUDING REMARKS

Although they are phenotypically diverse, and exist as multiple different subsets group 3 ILCs are probably the best-characterized ILC lineage, and they appear to play an important role regulating the balance between maintenance and loss of intestinal homeostasis. A decade after their discovery, ILC3s have emerged as important regulators of inflammation at mucosal surfaces, and in the gut in particular ILC3s form a dynamic network of interactions with the microbiome, other immune cells, the intestinal epithelium and enteric neurons to secure intestinal homeostasis. However, ILC3s may also promote inflammation leading to the development of IBD and/or colorectal cancer. Additional work is needed to scrutinize ILC3 biology and their contributions to intestinal disease. Targeting ILCs, their key upstream activating mediators (e.g., IL-23, IL-1β, or IL6), their survival factors (e.g., IL-7), or their effector cytokines (IL-22, IL-17, and IFNγ) hold promise for treating inflammatory diseases such as IBD.

AUTHOR CONTRIBUTIONS

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REFERENCES

1. Gill SR, Pop M, Deboy RT, Eckburg PB, Turnbaugh PJ, Samuel BS, et al. Metagenomic analysis of the human distal gut microbiome. *Science*. (2006) 312:1355–9. doi: 10.1126/science.1124234
2. Baumgart DC, Sandborn WJ. Inflammatory bowel disease: clinical aspects and established and evolving therapies. *Lancet*. (2007) 369:1641–57. doi: 10.1016/S0140-6736(07)60751-X
3. Loftus EV Jr. Clinical epidemiology of inflammatory bowel disease: Incidence, prevalence, and environmental influences. *Gastroenterology*. (2004) 126:1504–17. doi: 10.1053/j.gastro.2004.01.063
4. Abraham C, Cho JH. Inflammatory bowel disease. *N Engl J Med*. (2009) 361:2066–78. doi: 10.1056/NEJMra0804647
5. de Lange KM, Barrett JC. Understanding inflammatory bowel disease via immunogenetics. *J Autoimmun*. (2015) 64:91–100. doi: 10.1016/j.jaut.2015.07.013
6. Jostins L, Ripke S, Weersma RK, Duerr RH, McGovern DP, Hui KY, et al. Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. *Nature*. (2012) 491:119–24. doi: 10.1038/nature11582
7. Spits H, Cupedo T. Innate lymphoid cells: emerging insights in development, lineage relationships, and function. *Annu Rev Immunol*. (2012) 30:647–75. doi: 10.1146/annurev-immunol-020711-075053
8. Sonnenberg GF, Mjosberg J, Spits H, Artis D. SnapShot: innate lymphoid cells. *Immunity*. (2013) 39:622.e1. doi: 10.1016/j.immuni.2013.08.021
9. Artis D, Spits H. The biology of innate lymphoid cells. *Nature*. (2015) 517:293–301. doi: 10.1038/nature14189
10. Spits H, Di Santo JP. The expanding family of innate lymphoid cells: regulators and effectors of immunity and tissue remodeling. *Nat Immunol*. (2011) 12:21–7. doi: 10.1038/ni.1962
11. Eberl G. Development and evolution of RORgammat+ cells in a microbe's world. *Immunol Rev*. (2012) 245:177–88. doi: 10.1111/j.1600-065X.2011.01071.x
12. Spits H, Artis D, Colonna M, Diefenbach A, Di Santo JP, Eberl G, et al. Innate lymphoid cells–a proposal for uniform nomenclature. *Nat Rev Immunol*. (2013) 13:145–9. doi: 10.1038/nri3365
13. Wang S, Xia P, Chen Y, Qu Y, Xiong Z, Ye B, et al. Regulatory innate lymphoid cells control innate intestinal inflammation. *Cell*. (2017) 171:201–16.e18. doi: 10.1016/j.cell.2017.07.027
14. Klose CSN, Flach M, Mohle L, Rogell L, Hoyler T, Ebert K, et al. Differentiation of type 1 ILCs from a common progenitor to all helper-like innate lymphoid cell lineages. *Cell*. (2014) 157:340–56. doi: 10.1016/j.cell.2014.03.030
15. Possot C, Schmutz S, Chea S, Boucontet L, Louise A, Cumano A, et al. Notch signaling is necessary for adult, but not fetal, development of RORgammat(+) innate lymphoid cells. *Nat Immunol*. (2011) 12:949–58. doi: 10.1038/ni.2105
16. Cherrier M, Sawa S, Eberl G. Notch, Id2, and RORgammat sequentially orchestrate the fetal development of lymphoid tissue inducer cells. *J Exp Med*. (2012) 209:729–40. doi: 10.1084/jem.20111594
17. Constantinides MG, McDonald BD, Verhoef PA, Bendelac A. A committed precursor to innate lymphoid cells. *Nature*. (2014) 508:397–401. doi: 10.1038/nature13047
18. Sun Z, Unutmaz D, Zou YR, Sunshine MJ, Pierani A, Brenner-Morton S, et al. Requirement for RORgamma in thymocyte survival and lymphoid organ development. *Science*. (2000) 288:2369–73. doi: 10.1126/science.288.5475.2369
19. Eberl G, Marmon S, Sunshine MJ, Rennert PD, Choi Y, Littman DR. An essential function for the nuclear receptor RORgamma(t) in the generation of fetal lymphoid tissue inducer cells. *Nat Immunol*. (2004) 5:64–73. doi: 10.1038/ni1022
20. Satoh-Takayama N, Vosshenrich CA, Lesjean-Pottier S, Sawa S, Lochner M, Rattis F, et al. Microbial flora drives interleukin 22 production in intestinal

NKp46+ cells that provide innate mucosal immune defense. *Immunity*. (2008) 29:958–70. doi: 10.1016/j.immuni.2008.11.001
21. Lee JS, Cella M, McDonald KG, Garlanda C, Kennedy GD, Nukaya M, et al. AHR drives the development of gut ILC22 cells and postnatal lymphoid tissues via pathways dependent on and independent of Notch. *Nat Immunol*. (2011) 13:144–51. doi: 10.1038/ni.2187
22. Kiss EA, Vonarbourg C, Kopfmann S, Hobeika E, Finke D, Esser C, et al. Natural aryl hydrocarbon receptor ligands control organogenesis of intestinal lymphoid follicles. *Science*. (2011) 334:1561–5. doi: 10.1126/science.1214914
23. Takatori H, Kanno Y, Watford WT, Tato CM, Weiss G, Ivanov II, et al. Lymphoid tissue inducer-like cells are an innate source of IL-17 and IL-22. *J Exp Med*. (2009) 206:35–41. doi: 10.1084/jem.20072713
24. Cupedo T, Crellin NK, Papazian N, Rombouts EJ, Weijer K, Grogan JL, et al. Human fetal lymphoid tissue-inducer cells are interleukin 17-producing precursors to RORC+ CD127+ natural killer-like cells. *Nat Immunol*. (2009) 10:66–74. doi: 10.1038/ni.1668
25. Cella M, Fuchs A, Vermi W, Facchetti F, Otero K, Lennerz JK, et al. A human natural killer cell subset provides an innate source of IL-22 for mucosal immunity. *Nature*. (2009) 457:722–5. doi: 10.1038/nature07537
26. Buonocore S, Ahern PP, Uhlig HH, Ivanov II, Littman DR, Maloy KJ, et al. Innate lymphoid cells drive interleukin-23-dependent innate intestinal pathology. *Nature*. (2010) 464:1371–5. doi: 10.1038/nature08949
27. Sonnenberg GF, Monticelli LA, Elloso MM, Fouser LA, Artis D. CD4(+) lymphoid tissue-inducer cells promote innate immunity in the gut. *Immunity*. (2011) 34:122–34. doi: 10.1016/j.immuni.2010.12.009
28. Cella M, Otero K, Colonna M. Expansion of human NK-22 cells with IL-7, IL-2, and IL-1beta reveals intrinsic functional plasticity. *Proc Natl Acad Sci USA*. (2010) 107:10961–6. doi: 10.1073/pnas.1005641107
29. Vonarbourg C, Mortha A, Bui VL, Hernandez PP, Kiss EA, Hoyler T, et al. Regulated expression of nuclear receptor RORgammat confers distinct functional fates to NK cell receptor-expressing RORgammat(+) innate lymphocytes. *Immunity*. (2010) 33:736–51. doi: 10.1016/j.immuni.2010.10.017
30. Klose CS, Kiss EA, Schwierzeck V, Ebert K, Hoyler T, d'Hargues Y, et al. A T-bet gradient controls the fate and function of CCR6-RORgammat+ innate lymphoid cells. *Nature*. (2013) 494:261–5. doi: 10.1038/nature11813
31. Rankin LC, Groom JR, Chopin M, Herold MJ, Walker JA, Mielke LA, et al. The transcription factor T-bet is essential for the development of NKp46+ innate lymphocytes via the Notch pathway. *Nat Immunol*. (2013) 14:389–95. doi: 10.1038/ni.2545
32. Bernink JH, Krabbendam L, Germar K, de Jong E, Gronke K, Kofoed-Nielsen M, et al. Interleukin-12 and−23 control plasticity of CD127(+) Group 1 and Group 3 innate lymphoid cells in the intestinal lamina propria. *Immunity*. (2015) 43:146–60. doi: 10.1016/j.immuni.2015.06.019
33. Rankin LC, Girard-Madoux MJ, Seillet C, Mielke LA, Kerdiles Y, Fenis A, et al. Complementarity and redundancy of IL-22-producing innate lymphoid cells. *Nat Immunol*. (2016) 17:179–86. doi: 10.1038/ni.3332
34. Pantelyushin S, Haak S, Ingold B, Kulig P, Heppner FL, Navarini AA, et al. Rorgammat+ innate lymphocytes and gammadelta T cells initiate psoriasiform plaque formation in mice. *J Clin Invest*. (2012) 122:2252–6. doi: 10.1172/JCI61862
35. Villanova F, Flutter B, Tosi I, Grys K, Sreeneebus H, Perera GK, et al. Characterization of innate lymphoid cells in human skin and blood demonstrates increase of NKp44+ ILC3 in psoriasis. *J Invest Dermatol*. (2014) 134:984–91. doi: 10.1038/jid.2013.477
36. Perry JS, Han S, Xu Q, Herman ML, Kennedy LB, Csako G, et al. Inhibition of LTi cell development by CD25 blockade is associated with decreased intrathecal inflammation in multiple sclerosis. *Sci Transl Med*. (2012) 4:145ra106. doi: 10.1126/scitranslmed.3004140
37. Langowski JL, Zhang X, Wu L, Mattson JD, Chen T, Smith K, et al. IL-23 promotes tumour incidence and growth. *Nature*. (2006) 442:461–5. doi: 10.1038/nature04808
38. Shields JD, Kourtis IC, Tomei AA, Roberts JM, Swartz MA. Induction of lymphoidlike stroma and immune escape by tumors that express the chemokine CCL21. *Science*. (2010) 328:749–52. doi: 10.1126/science.1185837
39. Monticelli LA, Sonnenberg GF, Abt MC, Alenghat T, Ziegler CG, Doering TA, et al. Innate lymphoid cells promote lung-tissue homeostasis after infection with influenza virus. *Nat Immunol*. (2011) 12:1045–54. doi: 10.1038/ni.2131
40. Sawa S, Cherrier M, Lochner M, Satoh-Takayama N, Fehling HJ, Langa F, et al. Lineage relationship analysis of RORgammat+ innate lymphoid cells. *Science*. (2010) 330:665–9. doi: 10.1126/science.1194597
41. Sonnenberg GF, Monticelli LA, Alenghat T, Fung TC, Hutnick NA, Kunisawa J, et al. Innate lymphoid cells promote anatomical containment of lymphoid-resident commensal bacteria. *Science*. (2012) 336:1321–5. doi: 10.1126/science.1222551
42. Hepworth MR, Monticelli LA, Fung TC, Ziegler CG, Grunberg S, Sinha R, et al. Innate lymphoid cells regulate CD4+ T-cell responses to intestinal commensal bacteria. *Nature*. (2013) 498:113–7. doi: 10.1038/nature12240
43. Goto Y, Panea C, Nakato G, Cebula A, Lee C, Diez MG, et al. Segmented filamentous bacteria antigens presented by intestinal dendritic cells drive mucosal Th17 cell differentiation. *Immunity*. (2014) 40:594–607. doi: 10.1016/j.immuni.2014.03.005
44. von Burg N, Chappaz S, Baerenwaldt A, Horvath E, Bose Dasgupta S, Ashok D, et al. Activated group 3 innate lymphoid cells promote T-cell-mediated immune responses. *Proc Natl Acad Sci USA*. (2014) 111:12835–40. doi: 10.1073/pnas.1406908111
45. Korn LL, Thomas HL, Hubbeling HG, Spencer SP, Sinha R, Simkins HM, et al. Conventional CD4+ T cells regulate IL-22-producing intestinal innate lymphoid cells. *Mucosal Immunol*. (2014) 7:1045–57. doi: 10.1038/mi.2013.121
46. Mao K, Baptista AP, Tamoutounour S, Zhuang L, Bouladoux N, Martins AJ, et al. Innate and adaptive lymphocytes sequentially shape the gut microbiota and lipid metabolism. *Nature*. (2018) 554:255–9. doi: 10.1038/nature25437
47. Ivanov II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ, et al. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell*. (2006) 126:1121–33. doi: 10.1016/j.cell.2006.07.035
48. Zhou L, Ivanov II, Spolski R, Min R, Shenderov K, Egawa T, et al. IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. *Nat Immunol*. (2007) 8:967–74. doi: 10.1038/ni1488
49. Sano T, Huang W, Hall JA, Yang Y, Chen A, Gavzy SJ, et al. An IL-23R/IL-22 circuit regulates epithelial serum amyloid A to promote local effector Th17 responses. *Cell*. (2016) 164:324. doi: 10.1016/j.cell.2015.12.047
50. Sawa S, Lochner M, Satoh-Takayama N, Dulauroy S, Berard M, Kleinschek M, et al. RORgammat+ innate lymphoid cells regulate intestinal homeostasis by integrating negative signals from the symbiotic microbiota. *Nat Immunol*. (2011) 12:320–6. doi: 10.1038/ni.2002
51. Tsuji M, Suzuki K, Kitamura H, Maruya M, Kinoshita K, Ivanov II, et al. Requirement for lymphoid tissue-inducer cells in isolated follicle formation and T cell-independent immunoglobulin A generation in the gut. *Immunity*. (2008) 29:261–71. doi: 10.1016/j.immuni.2008.05.014
52. Bouskra D, Brezillon C, Berard M, Werts C, Varona R, Boneca IG, et al. Lymphoid tissue genesis induced by commensals through NOD1 regulates intestinal homeostasis. *Nature*. (2008) 456:507–10. doi: 10.1038/nature07450
53. Kruglov AA, Grivennikov SI, Kuprash DV, Winsauer C, Prepens S, Seleznik GM, et al. Nonredundant function of soluble LTalpha3 produced by innate lymphoid cells in intestinal homeostasis. *Science*. (2013) 342:1243–6. doi: 10.1126/science.1243364
54. Mortha A, Chudnovskiy A, Hashimoto D, Bogunovic M, Spencer SP, Belkaid Y, et al. Microbiota-dependent crosstalk between macrophages and ILC3 promotes intestinal homeostasis. *Science*. (2014) 343:1249288. doi: 10.1126/science.1249288
55. Ibiza S, Garcia-Cassani B, Ribeiro H, Carvalho T, Almeida L, Marques R, et al. Glial-cell-derived neuroregulators control type 3 innate lymphoid cells and gut defence. *Nature*. (2016) 535:440–3. doi: 10.1038/nature18644
56. Zheng Y, Valdez PA, Danilenko DM, Hu Y, Sa SM, Gong Q, et al. Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. *Nat Med*. (2008) 14:282–9. doi: 10.1038/nm1720
57. Wang Y, Koroleva EP, Kruglov AA, Kuprash DV, Nedospasov SA, Fu YX, et al. Lymphotoxin beta receptor signaling in intestinal epithelial cells orchestrates innate immune responses against mucosal bacterial infection. *Immunity*. (2010) 32:403–13. doi: 10.1016/j.immuni.2010.02.011

58. Emgard J, Kammoun H, Garcia-Cassani B, Chesne J, Parigi SM, Jacob JM, et al. Oxysterol sensing through the receptor GPR183 promotes the lymphoid-tissue-inducing function of innate lymphoid cells and colonic inflammation. *Immunity*. (2018) 48:120–32.e8. doi: 10.1016/j.immuni.2017.11.020
59. Chu C, Moriyama S, Li Z, Zhou L, Flamar AL, Klose CSN, et al. Anti-microbial functions of Group 3 innate lymphoid cells in gut-associated lymphoid tissues are regulated by G-protein-coupled receptor 183. *Cell Rep*. (2018) 23:3750–8. doi: 10.1016/j.celrep.2018.05.099
60. Guo X, Qiu J, Tu T, Yang X, Deng L, Anders RA, et al. Induction of innate lymphoid cell-derived interleukin-22 by the transcription factor STAT3 mediates protection against intestinal infection. *Immunity*. (2014) 40:25–39. doi: 10.1016/j.immuni.2013.10.021
61. Goto Y, Obata T, Kunisawa J, Sato S, Ivanov II, Lamichhane A, et al. Innate lymphoid cells regulate intestinal epithelial cell glycosylation. *Science*. (2014) 345:1254009. doi: 10.1126/science.1254009
62. Pickard JM, Maurice CF, Kinnebrew MA, Abt MC, Schenten D, Golovkina TV, et al. Rapid fucosylation of intestinal epithelium sustains host-commensal symbiosis in sickness. *Nature*. (2014) 514:638–41. doi: 10.1038/nature13823
63. Deshmukh HS, Liu Y, Menkiti OR, Mei J, Dai N, O'Leary CE, et al. The microbiota regulates neutrophil homeostasis and host resistance to *Escherichia coli* K1 sepsis in neonatal mice. *Nat Med*. (2014) 20:524–30. doi: 10.1038/nm.3542
64. Wagage S, Harms Pritchard G, Dawson L, Buza EL, Sonnenberg GF, Hunter CA. The Group 3 innate lymphoid cell defect in aryl hydrocarbon receptor deficient mice is associated with T cell hyperactivation during intestinal infection. *PLoS ONE*. (2015) 10:e0128335. doi: 10.1371/journal.pone.0128335
65. Hernandez PP, Mahlakoiv T, Yang I, Schwierzeck V, Nguyen N, Guendel F, et al. Interferon-lambda and interleukin 22 act synergistically for the induction of interferon-stimulated genes and control of rotavirus infection. *Nat Immunol*. (2015) 16:698–707. doi: 10.1038/ni.3180
66. Geremia A, Arancibia-Carcamo CV, Fleming MP, Rust N, Singh B, Mortensen NJ, et al. IL-23-responsive innate lymphoid cells are increased in inflammatory bowel disease. *J Exp Med*. (2011) 208:1127–33. doi: 10.1084/jem.20101712
67. Pearson C, Thornton EE, McKenzie B, Schaupp AL, Huskens N, Griseri T, et al. ILC3 GM-CSF production and mobilisation orchestrate acute intestinal inflammation. *Elife*. (2016) 5:e10066. doi: 10.7554/eLife.10066
68. Bauche D, Joyce-Shaikh B, Jain R, Grein J, Ku KS, Blumenschein WM, et al. LAG3(+) regulatory T cells restrain interleukin-23-producing CX3CR1(+) gut-resident macrophages during Group 3 innate lymphoid cell-driven colitis. *Immunity*. (2018) 49:342–52.e5. doi: 10.1016/j.immuni.2018.07.007
69. Garrett WS, Lord GM, Punit S, Lugo-Villarino G, Mazmanian SK, Ito S, et al. Communicable ulcerative colitis induced by T-bet deficiency in the innate immune system. *Cell*. (2007) 131:33–45. doi: 10.1016/j.cell.2007.08.017
70. Powell N, Walker AW, Stolarczyk E, Canavan JB, Gokmen MR, Marks E, et al. The transcription factor T-bet regulates intestinal inflammation mediated by interleukin-7 receptor+ innate lymphoid cells. *Immunity*. (2012) 37:674–84. doi: 10.1016/j.immuni.2012.09.008
71. Powell N, Lo JW, Biancheri P, Vossenkamper A, Pantazi E, Walker AW, et al. Interleukin 6 increases production of cytokines by colonic innate lymphoid cells in mice and patients with chronic intestinal inflammation. *Gastroenterology*. (2015) 149:456–67.e15. doi: 10.1053/j.gastro.2015.04.017
72. Eaden JA, Abrams KR, Mayberry JF. The risk of colorectal cancer in ulcerative colitis: a meta-analysis. *Gut*. (2001) 48:526–35. doi: 10.1136/gut.48.4.526
73. Rutter MD, Saunders BP, Wilkinson KH, Rumbles S, Schofield G, Kamm MA, et al. Thirty-year analysis of a colonoscopic surveillance program for neoplasia in ulcerative colitis. *Gastroenterology*. (2006) 130:1030–8. doi: 10.1053/j.gastro.2005.12.035
74. Kirchberger S, Royston DJ, Boulard O, Thornton E, Franchini F, Szabady RL, et al. Innate lymphoid cells sustain colon cancer through production of interleukin-22 in a mouse model. *J Exp Med*. (2013) 210:917–31. doi: 10.1084/jem.20122308
75. Chan IH, Jain R, Tessmer MS, Gorman D, Mangadu R, Sathe M, et al. Interleukin-23 is sufficient to induce rapid de novo gut tumorigenesis, independent of carcinogens, through activation of innate lymphoid cells. *Mucosal Immunol*. (2014) 7:842–56. doi: 10.1038/mi.2013.101
76. Ferrara JL, Levine JE, Reddy P, Holler E. Graft-versus-host disease. *Lancet*. (2009) 373:1550–61. doi: 10.1016/S0140-6736(09)60237-3
77. Hanash AM, Dudakov JA, Hua G, O'Connor MH, Young LF, Singer NV, et al. Interleukin-22 protects intestinal stem cells from immune-mediated tissue damage and regulates sensitivity to graft versus host disease. *Immunity*. (2012) 37:339–50. doi: 10.1016/j.immuni.2012.05.028
78. Munneke JM, Bjorklund AT, Mjosberg JM, Garming-Legert K, Bernink JH, Blom B, et al. Activated innate lymphoid cells are associated with a reduced susceptibility to graft-versus-host disease. *Blood*. (2014) 124:812–21. doi: 10.1182/blood-2013-11-536888

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MINI REVIEW
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Contribution of Non-immune Cells to Activation and Modulation of the Intestinal Inflammation

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The mucosal immune system constitutes a physical and dynamic barrier against foreign antigens and pathogens and exerts control mechanisms to maintain intestinal tolerance to the microbiota and food antigens. Chronic alterations of the intestinal homeostasis predispose to inflammatory diseases of the gastrointestinal tract, such as Inflammatory Bowel Diseases (IBD). There is growing evidence that the frequency and severity of these diseases are increasing worldwide, which may be probably due to changes in environmental factors. Several stromal and immune cells are involved in this delicate equilibrium that dictates homeostasis. In this review we aimed to summarize the role of epithelial cells and fibroblasts in the induction of mucosal inflammation in the context of IBD. It has been extensively described that environmental factors are key players in this process, and the microbiome of the gastrointestinal tract is currently being intensively investigated due to its profound impact the immune response. Recent findings have demonstrated the interplay between dietary and environmental components, the gut microbiome, and immune cells. "Western" dietary patterns, such as high caloric diets, and pollution can induce alterations in the gut microbiome that in turn affect the intestinal and systemic homeostasis. Here we summarize current knowledge on the influence of dietary components and air particulate matters on gut microbiome composition, and the impact on stromal and immune cells, with a particular focus on promoting local inflammation.

Keywords: gut inflammation, inflammatory bowel disease, intestinal epithelial cells, intestinal fibroblasts, immune cell activation/modulation, intestinal microbiota

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GUT INFLAMMATION

Inflammation is a central component of innate immunity, comprising the physiopathological response to infection or tissue damage. As a local response to cellular injury, it is initiated when tissue-resident cells of the innate immune system detect the damaging insult and alarm resident cells and circulating neutrophils. These cells migrate to the inflamed tissue, promote recruitment of inflammatory monocytes and potentiate the pro-inflammatory environment, allowing to deal with the harmful agent (1). Hence, the acute inflammatory response is marked by increased blood flow, capillary dilatation, leukocyte infiltration, and the localized production of chemical mediators, which serves to initiate the elimination of toxic agents and the repair of the damaged tissue. Hence, the acute inflammatory response is a physiological process committed to control an offending stimulus.

The intestinal mucosa has evolved as a well-structured barrier against physical, chemical, and microbial insults. The epithelial layer, mucus, antimicrobial peptides, secreted immunoglobulin A, and innate and adaptive immune cells, together help to establish a beneficial environment to tolerate the diverse community of microbes of the microbiota and food antigens. The intestinal mucosal surface constitutes the major interface between the internal tissues and a potentially hostile outer environment. To deal with this universe of antigenic components, intestinal homeostasis has been evolutionary developed through a constant crosstalk between metabolites and microbes of the microbiota, intestinal stromal cells, and the mucosal immune system. Perturbations of the homeostatic state can result in severe inflammatory conditions in the gut that may lead to tissue damage. Therefore, intestinal inflammation is a double-edge sword that should be tightly regulated. Although it is an essential component for immunosurveillance and host defense, chronic inflammatory processes may promote pathology such as inflammatory bowel diseases (IBD) (2), irritable bowel syndrome (IBS) (3), diverticular disease (4), food allergy (5), celiac disease (4), etc.

In this homeostatic scenario, the controlled and physiological inflammation of the gut promotes a barrier permeability that allows the penetration of luminal antigens to the underlying mucosal tissue. In chronic inflammatory disorders, such as IBD, microbial components of the microbiota are translocated through the damaged mucosal barrier, and trigger and maintain a sustained inflammatory response, as it is represented in **Figure 1**. Epithelial cells, dendritic cells (DCs), macrophages, and innate lymphocytes (ILCs), which sense the presence of microbes or an altered tissue environment, are activated and promote the induction of the adaptive immune response. As it is summarized in **Figure 1**, the pro-inflammatory cytokines secreted by innate cells and activated T cells are key to amplify and perpetuate mucosal inflammation. Therefore, the mucosal immune system is responsible for the induction of the inflammatory process, while tissue damage results from continuous activation and differentiation of local cells, such as myofibroblasts, that release noxious mediators (2, 6).

NON-IMMUNE CELLS INVOLVED IN GUT INFLAMMATION

Epithelial Cells

The alteration of the intestinal epithelial barrier permeability leads to local inflammation. The fact that mice expressing dominant negative N-cadherin adherent junction protein suffer from spontaneous inflammatory bowel disease, clearly highlights the fundamental role of barrier integrity in the development of colitis and, probably, IBD (7). Many cytokines that are increased in IBD favor gut permeability, such as TNF-α. Indeed, impaired barrier function has been described for Crohn's disease (CD) and ulcerative colitis (UC) patients and it is a marker for predicting the course of these relapsing diseases (8, 9). Intestinal pro-inflammatory cytokines have been documented to modify the expression of different tight junction proteins. Claudins showed deregulation and differential expression in human active IBD (8, 9). However, studies in mice null for claudins and other tight junction proteins were controversial, demonstrating that other factors may contribute to gut permeability (10). Overall, these reported evidences highlight the relevance of epithelial cells in intestinal inflammation. Amongst these, Paneth cells secrete antimicrobial peptides that modulate the gut microbial composition (11), and it has been recently described that metabolites produced by luminal microbes control the secretion of these peptides (12). In other words, there is a mutual monitoring of the microbiota composition and the mucosal immune system. Defects in defensin production have been found in patients with CD and NOD-2 mutations (13). Taking into account the role of the microbiome in intestinal homeostasis, these findings should not be underestimated. Recently, a study involving Paneth cell deficient mice showed that they develop dysbiosis and visceral hypersensitivity (14). Other relevant epithelial cell type are Goblet cells, responsible for secretion of mucus and sampling of luminal antigens (15, 16). These cells showed a protective role in gut inflammation since MUC2-null mice developed spontaneous colitis (17), and patients with UC showed polymorphism in MUC2 in the Dutch population (18). Goblet cell loss and decreased mucous levels are commonly observed in UC patients, with endoplasmic reticulum stress and accumulation of MUC2 precursors (17, 19). In addition, micro-fold cells, or M cells, found in the follicle-associated epithelium of the gut, have a key function in the immunosurveillance of the luminal content (16, 20, 21). There is no report showing M cell dysfunction in colitis or any other inflammatory condition. Enteroendocrine cells, which are also sensors of gut luminal content, were found to be altered in mouse models of colitis and in patients with active IBD (22).

The most abundant cells in the epithelial compartment are the absorptive cells, which not only constitute a physical barrier against luminal antigens, but mediate the crosstalk between intestinal microbiome and the host immune system, mainly through innate immune receptors. Conserved molecular patterns are mainly recognized by Toll-like receptors (TLRs) and Nod-like receptors (NLR), among others, which are expressed along the intestinal tract (23–26). Healthy human small intestine expresses TLR-2 and−4 proteins, whereas high levels of TLR-5 are found in the colon. These receptors show a polarized distribution, being located in the basolateral membranes, ensuring that commensal bacteria do not trigger an inflammatory response in homeostasis (27). These particular expression patterns of innate receptors are modified in an inflammatory setting. IBD patients have increased levels of TLR4 expression, and lower level of TLR2 and TLR5 in epithelial cells, while TLR4 was shown to be expressed in the apical surface of epithelial cells (28). Current studies have demonstrated the importance of TLR1 in the prevention of gut inflammation (29). In addition to NOD-2 mutations, abnormal mucosal NLRP3 activity has been reported in IBD and in experimental colitis; GWAS studies revealed polymorphisms in these receptors (30–33).

Intestinal epithelial cells (IECs) also contribute to intestinal homeostasis through interaction with microbiota and secretion of TGF-β and IL-10 (34, 35). Although IECs crosstalk with T

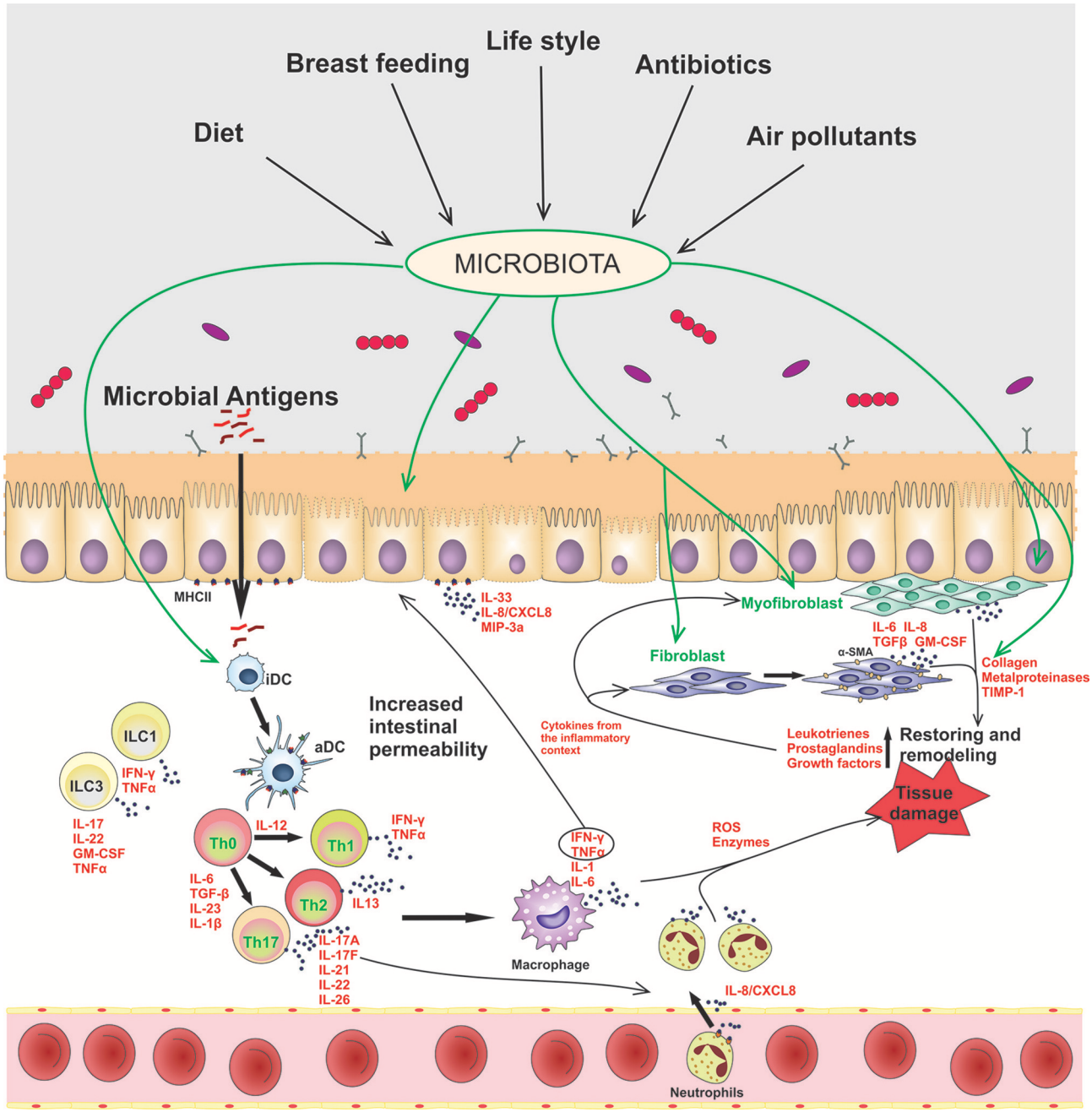

FIGURE 1 | Contribution of epithelial cells and intestinal fibroblasts to the induction and modulation of mucosal inflammation in IBD. In this disorder the intestinal barrier permeability is impaired, allowing the passage of luminal antigens into the lamina propria. The exposure of stromal and immune cells to the luminal content induces cell activation, inflammatory soluble-mediators release (IFN-γ, TNF-α, IL-1β, IL-6, IL-17A), cell-crosstalk (represented with arrows) and neutrophil recruitment to the inflamed tissue. Several environmental factors can modulate the microbiota composition and the activation of stromal and immune cells in the gut. iDC, innactivated dendritic cells; aDC, activated dendritic cells; ILCs, innate lymphoid cells; Th, T helper cells.

cells through cell-cell interactions (36), the role of these cells as antigen presenting cells *in vivo* is controversial (37). They express class 2 MHC, but no co-stimulatory molecules, though they do express the ICOS-L; of note, variants in this gene have been related to the IBD early onset clinical entity (38). Colonic epithelial cell isolated from active IBD patients have shown to secrete the neutrophil-attracting IL-8/CXCL8 (39, 40) and IL-33 (41–44), which contribute to inflammation (**Figure 1**). IL-1β, produced through activation of the inflammasome, and IL-17 are also secreted (45, 46), contributing to Th1 and Th17

responses in IBD. IL-21R expression is up-regulated in IECs of IBD patients, which leads to increased CCL20 synthesis, a T cell and DC chemo attractant (47, 48). IL-22, IL-31, and IL-33 have been also described to be augmented in IBD, which leads to deregulation of IEC proliferation and migration functions, whereas they stimulate IL-1, TNF-α, IL-6, and IL-8 secretion (49–51). Cytokines generated by innate and adaptive responses, on the other hand, interact with IECs, activating them and altering barrier permeability. It is well known that TNF-α induces IECs apoptosis (52) and that IFN-γ alters epithelial permeability by affecting tight junctions and bacterial translocation (53), as represented in **Figure 1**. All these factors contribute to immune cell activation and cytokine secretion which, in the context of IBD, favor an inflammation and damage perpetuation.

In conclusion, IECs are relevant cell sources of pro-inflammatory and regulatory mediators, which should be tightly controlled to achieve intestinal homeostasis.

Fibroblasts

Intestinal myofibroblasts are localized beneath the epithelial compartment, particularly around the crypts of the small intestine and the colon, and participate in the repair process. Fibroblasts, which are involved in formation of the extracellular matrix (ECM), differentiate into contractile myofibroblasts, which are also involved in the inflammatory response to injury through the secretion of cytokines (54–56). Furthermore, the origin of myofibroblasts in chronic inflammation is not clear, and they may have an epithelial origin through an epithelial-mesenchymal transition (57).

Myofibroblast activation is associated with tissue injury and inflammation (**Figure 1**). They have the ability to migrate to the injured sites, where they contract the wound area and produce extracellular matrix components that restore and remodel the damaged mucosal tissue. However, persistent myofibroblast activation in an inflammatory environment may promote an irreversible damage of the affected tissue with fibrosis and cell proliferation in the submucosa (58). Cells can secrete a number of soluble cytokines (IL-6), chemokines (IL-8, MCP-1), growth factors (TGF-β, GM-CSF), collagen, metalloproteinases (MMP-1, MMP-3, MMP-9, MMP-12), and MMPs' inhibitor (TIMP-1), that attract other cells to perpetuate the inflammation. Depending on the balance of MMPs and TIMP-1, and collagen deposition, it will be the consequence of tissue remodeling in IBD, thus resulting in fibrosis, stricture formation or ulceration (59–62). It is unknown why in some cases the intestinal inflammation induces penetrating damage with perforation and fistulae, and with increased risk of colorectal cancer in UC patients, whereas in CD patients the long-term complications include abscesses, granuloma, strictures, obstruction, fibrosis, and stenosis. Overall, the control of myofibroblast differentiation is critical to prevent or reverse complications in CD and UC patients.

INFLAMMATION IS MODULATED BY DIFFERENT FACTORS

It has been previously described that the immune system is regulated at different points for homeostasis, while aberrant or inappropriate regulation may result in failure to protect the body from pathogens or any injury.

Environmental factors influence the incidence and development of IBD in many ways that are not fully understood, with a higher incidence in developed countries and urban populations, compared to rural areas and underdeveloped regions of the world. It has been shown that young migrants from low incidence countries have a similar incidence of IBD compared to non-immigrants, highlighting the importance of the environment in these diseases (63). Several studies discuss associations of different dietary components in gut inflammation and IBD. High meat/fat (64) consumption has been linked to a higher risk of IBD onset, and studies in animal models correlate this finding with the high heme intake (65). Also, trans- and poly-unsaturated long-chain fatty acids have been related to the disease (66, 67), while unsaturated fatty acids seem to exert a preventive role (67). Emulsifiers, which are commonly found in processed foods, have a pro-inflammatory effect in the gut (68). On the other hand, dietary fiber, especially from fruits, has been associated to a lower IBD incidence (69). Also, vitamin consumption is thought to be beneficial (70). These results should now be reevaluated after the recently published study in which the effect of different dietary ingredients was addressed in the context of IBD, using experimental colitis models in pathogen-free and germ-free mice in order to identify specific triggers (71).

More recently, the microbiota has been targeted as a critical player in establishing and sustaining the tight equilibrium of the immune system that is constantly exposed to a myriad of antigens in the mucosal surfaces. The intestinal microbiota is key for maintaining intestinal homeostasis, and it may be involved in inflammatory disorders when the composition and diversity is modified, which is called dysbiosis. In all mucosa an associated microbiota has been described and it is known that the gut microbiota might be the most complex and dynamic one. It is established during the intrauterine life and modified after birth. Several factors have been described as contributing to condition the composition of the microbiota throughout life. There is a link between diet, gut composition and gut metabolism which undoubtedly impact on the gastrointestinal health. There is growing evidence that a dysbiotic intestinal microbiota is associated with immune and non-immune disorders. However, it is debatable whether dysbiosis is a cause or a consequence of the inflammatory process (72, 73). Commonly, dysbiosis implies a change from a diverse anaerobe community, rich in *Firmicutes* and *Bacteroidetes* to a lower diversity community with enrichment of facultative anaerobes including *Proteobacteria* and *Bacilli*, although it depends on the pathology (74). There is broad consensus that pathology-associated dysbiosis is accompanied with a restriction in the diversity of species (75).

Microbiota composition may be affected by different external factors: diet (fiber, calories, etc.), urbanization, use of antibiotics, age, mode of birth, exposure to air pollutants, etc. (76–78). A westernized diet with high red meat-high fat and processed carbohydrates content, is associated with a loss in gut microbial diversity, with an increase of pathogenic adherent-invasive *E. coli* (AIEC), as it has been reported in IBD patients (79). It has been demonstrated that a high-fat/high-sugar diet leads to dysbiosis

with increased *Bacteroides* spp and *Ruminococcus torques* in mice (79). On the other hand, digestible fibers, which are fermented by bacteria in the gut, generate beneficial, anti-inflammatory short-chain fatty acids (SCFA). Modern diet components such as artificial sweeteners and emulsifiers are being subjected to investigation as they are suspected to induce dysbiosis (80–82). Human and murine studies have demonstrated that the use of antibiotics generates variations in gut microbiota mostly according to the type and the period of time used (78). Antibiotics directed to anaerobes, such as vancomicin, seem to have a more severe impact on gut microbiota composition (76). The contamination of food with particulate matter, as occurs in a contaminated environment, has shown to impair intestinal permeability and to generate inflammation, altering microbial gut composition (77, 83).

There are several evidences that highlight the relevance of the microbiota in IBD: germ-free animals do not develop experimental colitis (84), and antibiotic therapy has been successful in the treatment of CD, while it has given promising results in some forms of UC (84), etc. Dysbiosis has been well characterized in CD, with a decrease in *Clostridiales* and an increase in *Enterobacteriales* (85), while a diminished, but less defined, microbial diversity has been described for UC (85). Emerging insights on intestinal dysbiosis during immune and non-immune disorders has attracted the attention to target the microbiota composition as a novel therapeutic approach to control intestinal and extra-intestinal inflammation. However, it should be considered that the microbiota-derived metabolites are the true messengers that control the development, differentiation, and activity of the immune system associated to the local, and also, distant mucosa. Microbiota transfer strategies, or fecal microbiota transplant (FMT), have been used in Chinese ancestral medicine for centuries and have proven to be useful for *Clostridium difficile* recurrent infections (86). Several clinical trials have demonstrated that FMT restores gut microbial diversity, diminishing disbiosis and controlling mucosal inflammation. More recently, FMT has been explored in IBD (87). Most clinical trials and randomized controlled studies have been made in UC, with promising results when they were performed early in the disease course (88). Results in CD have been variable and rigorous randomized controlled trials are needed (89). More studies are mandatory to confirm the beneficial effect of this therapy.

Another therapeutic approach aimed to modify the gut microbiome has been the use of probiotics. Numerous studies using animal models (90–93) and *in vitro* approaches (94–96) provide evidence on the beneficial use of probiotics in colitis. Results depend on the specific strain of probiotics used, and vary with the experimental model used. In particular, *Bifidobacterium* and *Lactobacillus* are the most widely used probiotic bacteria (97), although strains of *E. coli* (98–101), *Propionibacterium* (102–105), *Bacillus* (106–108), and *Saccharomyces* (109, 110) are amongst those mostly studied.

Different mechanisms of action have been described for probiotics, including binding to IECs and thus preventing the binding of pathogenic microorganisms (109, 110), acidification of the lumen of the colon by nutrient fermentation, production of SCFA like acetate, propionate, and butyric acid as a source of energy, but also with immune modulating properties and anti-inflammatory effects (109, 110), as enhancement of epithelial barrier integrity (97), etc. Mack et al. (111) described an increased production of MUC2 and MUC3 secretion by epithelial cells stimulated with *Lactobacillus plantarum* and *Lactobacillus rhamnosus* in HT-29 intestinal cell line, while Anderson et al. described that *L. plantarum* MB452 increased the trans-epithelial electrical resistance of Caco-2 cells monolayer through the induction of tight junction proteins (112). Recently, components of *L. amylovorus* DSM 16698 cell wall demonstrated to have protective effects toward *E. coli* induced damage on Caco-2/TC7 cells, protecting membrane leakage and reducing the phosphorylation of the p65 component of the NF-κB intracellular signaling pathway (113). The inhibition of NF-κB activation has also been described for other *Lactobacilli* strains and for *Bifidobacterium* (114–116). The increased expression of tight junction proteins is linked to an activation of the ERK and p38 MAPK signaling cascades, via Toll like receptors (114). Probiotics have also been described for preventing IECs apoptosis by reducing oxidative stress, as in the case of *L. amylovorous* DSM16698 (117), and to inhibit apoptosis through the activation of the epidermal growth factor receptor by the release of a soluble protein known as p40 by *L. rhamnosus* GG (114, 118). In addition, probiotics act by diminishing pro-inflammatory responses and contributing to tolerogenic responses, modulating TLR-2 and TLR-4 signaling (114), and driving DCs to a suppressive phenotype, which further promote the generation of Tregs (119, 120).

It has been demonstrated in the inflamed gut, that the mucosal damage not only affects the epithelial compartment, but also colonic myofibroblasts located beneath the epithelium. It has been reported that the exposure to colonic microbiota products promotes cell activation through TLR (121). Beswick and col. demonstrated that isolated myofibroblasts from normal human colonic mucosa respond to TLR4 stimulation by LPS with the induction of PD-L1, which mediates the suppression of activated $CD4^+$ $PD\text{-}1^+$ T cell response and inhibition of IFN-γ secretion *in vitro*. Authors also showed that *in vivo* up-regulation of PD-L1 in colonic myofibroblast is MyD88-dependent in colitis (122). This provides evidence that colonic myofibroblasts might help to maintain the equilibrium between tolerance and immunity to protect the colonic mucosa against inflammatory responses toward the microbiota. In agreement with these findings, Scheibe and col. reported that IL-36R ligands released upon mucosal damage activate $IL\text{-}36R^+$ colonic fibroblasts via MyD88, thereby inducing expression of chemokines, granulocyte-macrophage colony-stimulating factor and IL-6. These mediators induce the migration and recruitment of leukocytes and neutrophils to the inflamed colon and contribute to control mucosal healing (123).

On the other hand, dysbiosis and specific bacterial taxa correlated with fibrostenosis in a CD cohort study (32), although there are no studies showing the contribution of the microbiome to fibrosis and fibroblasts modulation in the gut. It has recently been published the first evidence that fibroblast activation and the intestinal fibrosis require specific microbial cues provided by the mouse microbiota. Authors reported that intestinal

fibrosis is microbiota-dependent, by giving gavages of feces from germ-free mice, pathogen-free feces or healthy human donor feces, in TL1A-overexpressing mice. In addition, they found that the microbial composition affects fibroblast differentiation into myofibroblasts. Furthermore, they identified several candidates that correlated directly with the fibrosis degree in mice (123, 124). Although the key role of TL1A in inducing fibrostenosis is known (125, 126), the interplay between microbiota, TL1A and fibroblasts is novel (124).

Given that there are a number of chronic inflammatory disorders with fibrotic evolution, such as rheumatoid arthritis, cirrhosis, IBD, and pulmonary fibrosis, there is great effort to develop therapies that could control or reverse fibrosis. Particularly, intestinal fibrosis has been exclusively associated to IBD, mostly CD. The use of probiotics is becoming increasingly important for the prevention and treatment of gastrointestinal conditions. In a mouse model of DSS-induced colitis, administration of *Lactobacillus acidophilus* ameliorated collagen mucosal deposition and improved intestinal fibrosis. Indeed, *L. acidophilus* treated mice showed decreased α-SMA and collagen I expression levels, compared to untreated mice (127). Alternatively to probiotics, a role for Vitamin D in modulating the immune system and the integrity of intestinal epithelium and gut microbioma has also been proposed. In this sense, vitamin D supplementation improves IBD patient's condition (128) and regarding intestinal fibrosis, it was demonstrated that vitamin D exerted protective effects on colonic fibrosis caused by TNBS-induced chronic colitis, through direct inhibition of TGFβ-1/Smad3 pathway and up/regulation of vitamin D receptor in sub-epithelial myofibroblasts (129). More recently, the overexpression of fibroblasts activation protein (FAP), an inducible surface glycoprotein, has been associated with fibrosis in strictured CD patients. *In vitro* assays with anti-FAP treatment have shown promising results in controlling ECM deposition (130). Overall, despite the widely described contribution of fibroblasts to intestinal inflammation, the control of fibrosis in chronic intestinal inflammatory disorders still remains a big challenge for therapeutic purposes.

CONCLUSIONS

As the intestinal mucosa surface constitutes the major surface of the body which is in direct contact with the outer environment, intestinal immune homeostasis must be accurately regulated. The interplay between commensal microbiota, intestinal stromal cells, and the mucosal immune system components should guarantee the intestinal homeostasis to avoid a sustained inflammation that could induce tissue damage. However, several factors can lead to inflammation through homeostasis breakdown. **Figure 1** summarizes the main points that have been reviewed here. We have described what it is known so far about the role of epithelial cells and intestinal fibroblasts in the induction and modulation of mucosal inflammation in IBD. In this chronic inflammatory disorders the intestinal barrier permeability is compromised and the selective passage of luminal antigens into the lamina propria is altered, triggering cell activation, and inflammation. A plethora of evidences demonstrate the impact of dietary and environmental factors on the gut microbioma and on the modulation of the intestinal immunity. Notwithstanding the efforts made to find alternatives to conventional anti-inflammatory treatments (steroids, antibiotics, immunosuppressive drugs and biologics) by modulation of non-immune cells response, no current evidences arise that support the replacement of conventional therapies. Regarding probiotics, for instance, it has recently been demonstrated that the use of probiotics to achieve effective mucosal protection should be personalized according to the individual affection (131–133).

In conclusion, in this review we summarized the most recent findings in animal models and cohort studies, that show the contribution of epithelial cells and fibroblasts to gut inflammation with the influence of different environmental and dietary factors. Considering that the frequency and severity of IBD are increasing worldwide, changes on environmental factors and dietary habits should not be underestimated. Based on these observations, and those regarding the modulation of the intestinal microbioma and mucosal immune cells, it has been prompted to develop novel therapeutic interventions to prevent, control or reverse gut inflammation.

AUTHOR CONTRIBUTIONS

RC, CIM, KEC, and GHD conceptualized the review. RC and CIM provided an initial draft of the manuscript and KEC provided the figure. RC, CIM, and GHD performed the final editions.

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REFERENCES

1. Mantovani A, Cassatella MA, Costantini C, Jaillon S. Neutrophils in the activation and regulation of innate and adaptive immunity. *Nat Rev Immunol.* (2011) 11:519–31. doi: 10.1038/nri3024
2. Baumgart DC, Carding SR. Inflammatory bowel disease: cause and immunobiology. *Lancet*. (2007) 369:1627–40. doi: 10.1016/S0140-6736(07)60750-8
3. Choung RS, Locke GR. Epidemiology of IBS. *Gastroenterol Clin North Am*. (2011) 40:1–10. doi: 10.1016/j.gtc.2010.12.006
4. Strate LL, Modi R, Cohen E, Spiegel BMR. Diverticular disease as a chronic illness: evolving epidemiologic and clinical insights. *Am J Gastroenterol*. (2012) 107:1486–93. doi: 10.1038/ajg.2012.194
5. Tordesillas L, Berin MC, Sampson HA. Immunology of food allergy. *Immunity*. (2017) 47:32–50. doi: 10.1016/j.immuni.2017.07.004

6. MacDonald TT, Monteleone I, Fantini MC, Monteleone G. Regulation of homeostasis and inflammation in the intestine. *Gastroenterology*. (2011) 140:1768–75. doi: 10.1053/j.gastro.2011.02.047
7. Hermiston ML, Gordon JI. Inflammatory bowel disease and adenomas in mice expressing a dominant negative N-cadherin. *Science*. (1995) 270:1203–7. doi: 10.1126/science.270.5239.1203
8. Zeissig S, Burgel N, Gunzel D, Richter J, Mankertz J, Wahnschaffe U, et al. Changes in expression and distribution of claudin 2, 5 and 8 lead to discontinuous tight junctions and barrier dysfunction in active Crohn's disease. *Gut*. (2007) 56:61–72. doi: 10.1136/gut.2006.094375
9. Martini E, Krug SM, Siegmund B, Neurath MF, Becker C. Mend your fences. *Cell Mol Gastroenterol Hepatol*. (2017) 4:33–46. doi: 10.1016/j.jcmgh.2017.03.007
10. Ahmad R, Sorrell MF, Batra SK, Dhawan P, Singh AB. Gut permeability and mucosal inflammation: bad, good or context dependent. *Mucosal Immunol*. (2017) 10:307–17. doi: 10.1038/mi.2016.128
11. Salzman NH, Hung K, Haribhai D, Chu H, Karlsson-Sjöberg J, Amir E, et al. Enteric defensins are essential regulators of intestinal microbial ecology. *Nat Immunol*. (2010) 11:76–83. doi: 10.1038/ni.1825
12. Schoenborn AA, von Furstenberg RJ, Valsaraj S, Hussain FS, Stein M, Shanahan MT, et al. The enteric microbiota regulates jejunal Paneth cell number and function without impacting intestinal stem cells. *Gut Microbes*. (2018) 8:1–32. doi: 10.1080/19490976.2018.1474321
13. Gassler N. Paneth cells in intestinal physiology and pathophysiology. *World J Gastrointest Pathophysiol*. (2017) 8:150–60. doi: 10.4291/wjgp.v8.i4.150
14. Riba A, Olier M, Lacroix-Lamandé S, Lencina C, Bacquié V, Harkat C, et al. Paneth cell defects induce microbiota dysbiosis in mice and promote visceral hypersensitivity. *Gastroenterology*. (2017) 153:1594–1606.e2. doi: 10.1053/j.gastro.2017.08.044
15. McGuckin MA, Lindén SK, Sutton P, Florin TH. Mucin dynamics and enteric pathogens. *Nat Rev Microbiol*. (2011) 9:265–78. doi: 10.1038/nrmicro2538
16. Knoop KA, McDonald KG, McCrate S, McDole JR, Newberry RD. Microbial sensing by goblet cells controls immune surveillance of luminal antigens in the colon. *Mucosal Immunol*. (2015) 8:198–210. doi: 10.1038/mi.2014.58
17. Heazlewood CK, Cook MC, Eri R, Price GR, Tauro SB, Taupin D, et al. Aberrant mucin assembly in mice causes endoplasmic reticulum stress and spontaneous inflammation resembling ulcerative colitis. Schreiber S, editor. *PLoS Med*. (2008) 5:e54. doi: 10.1371/journal.pmed.0050054
18. Visschedijk MC, Alberts R, Mucha S, Deelen P, de Jong DJ, Pierik M, et al. Pooled resequencing of 122 ulcerative colitis genes in a large dutch cohort suggests population-specific associations of rare variants in MUC2. Prokunina-Olsson L, editor. *PLoS ONE*. (2016) 11:e0159609. doi: 10.1371/journal.pone.0159609
19. Gersemann M, Becker S, Kübler I, Koslowski M, Wang G, Herrlinger KR, et al. Differences in goblet cell differentiation between Crohn's disease and ulcerative colitis. *Differentiation*. (2009) 77:84–94. doi: 10.1016/j.diff.2008.09.008
20. Tahoun A, Mahajan S, Paxton E, Malterer G, Donaldson DS, Wang D, et al. Salmonella transforms follicle-associated epithelial cells into M cells to promote intestinal invasion. *Cell Host Microbe*. (2012) 12:645–56. doi: 10.1016/j.chom.2012.10.009
21. Ohno H, Kanaya T, Williams IR. M cell differentiation: distinct lineage or phenotypic transition? Salmonella provides answers. *Cell Host Microbe*. (2012) 12:607–9. doi: 10.1016/j.chom.2012.11.003
22. Harrison E, Lal S, McLaughlin JT. Enteroendocrine cells in gastrointestinal pathophysiology.Curr Opin Pharmacol. (2013) 13(6):941-5. doi: 10.1016/j.coph.2013.09.012
23. Claes A-K, Zhou JY, Philpott DJ. NOD-like receptors: guardians of intestinal mucosal barriers. *Physiology*. (2015) 30:241–50. doi: 10.1152/physiol.00025.2014
24. Hug H, Mohajeri MH, La Fata G. Toll-like receptors: regulators of the immune response in the human gut. *Nutrients*. (2018) 10:203. doi: 10.3390/nu10020203
25. Latorre E, Layunta E, Grasa L, Pardo J, García S, Alcalde AI, et al. Toll-like receptors 2 and 4 modulate intestinal IL-10 differently in ileum and colon. *United Eur Gastroenterol J*. (2018) 6:446–53. doi: 10.1177/2050640617727180
26. Abreu MT. Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function. *Nat Rev Immunol*. (2010) 10:131–44. doi: 10.1038/nri2707
27. Yu S, Gao N. Compartmentalizing intestinal epithelial cell toll-like receptors for immune surveillance. *Cell Mol Life Sci*. (2015) 72:3343–53. doi: 10.1007/s00018-015-1931-1
28. Cario E. Toll-like receptors in inflammatory bowel diseases: a decade later. *Inflamm Bowel Dis*. (2010) 16:1583–97. doi: 10.1002/ibd.21282
29. Kamdar K, Johnson AMF, Chac D, Myers K, Kulur V, Truevillian K, et al. Innate recognition of the microbiota by TLR1 promotes epithelial homeostasis and prevents chronic inflammation. *J Immunol*. (2018) 201:230–42. doi: 10.4049/jimmunol.1701216
30. Hugot JP. CARD15/NOD2 mutations in Crohn's disease. *Ann N Y Acad Sci*. (2006) 1072:9–18. doi: 10.1196/annals.1326.011
31. Cummings JRF, Cooney RM, Clarke G, Beckly J, Geremia A, Pathan S, et al. The genetics of NOD-like receptors in Crohn's disease. *Tissue Antigens*. (2010) 76:48–56. doi: 10.1111/j.1399-0039.2010.01470.x
32. Kugathasan S, Denson LA, Walters TD, Kim M-O, Marigorta UM, Schirmer M, et al. Prediction of complicated disease course for children newly diagnosed with Crohn's disease: a multicentre inception cohort study. *Lancet*. (2017) 389:1710–8. doi: 10.1016/S0140-6736(17)30317-3
33. Hanaei S, Sadr M, Rezaei A, Shahkarami S, Ebrahimi Daryani N, Bidoki AZ, et al. Association of NLRP3 single nucleotide polymorphisms with ulcerative colitis: a case-control study. *Clin Res Hepatol Gastroenterol*. (2018) 42:269–75. doi: 10.1016/j.clinre.2017.09.003
34. Martin-Gallausiaux C, Béguet-Crespel F, Marinelli L, Jamet A, Ledue F, Blottière HM, et al. Butyrate produced by gut commensal bacteria activates TGF-beta1 expression through the transcription factor SP1 in human intestinal epithelial cells. *Sci Rep*. (2018) 8:9742. doi: 10.1038/s41598-018-28048-y
35. Fonseca-Camarillo G, Yamamoto-Furusho JK. Immunoregulatory pathways involved in inflammatory bowel disease. *Inflamm Bowel Dis*. (2015) 21:2188–93. doi: 10.1097/MIB.0000000000000477
36. Cruickshank SM, McVay LD, Baumgart DC, Felsburg PJ, Carding SR. Colonic epithelial cell mediated suppression of CD4 T cell activation. *Gut*. (2004) 53:678–84. doi: 10.1136/gut.2003.029967
37. Nakazawa A, Dotan I, Brimnes J, Allez M, Shao L, Tsushima F, et al. The expression and function of costimulatory molecules B7H and B7-H1 on colonic epithelial cells. *Gastroenterology*. (2004) 126:1347–57. doi: 10.1053/j.gastro.2004.02.004
38. Imielinski M, Baldassano RN, Griffiths A, Russell RK, Annese V, Dubinsky M, et al. Common variants at five new loci associated with early-onset inflammatory bowel disease. *Nat Genet*. (2009) 41:1335–40. doi: 10.1038/ng.489
39. Izutani R, Loh EY, Reinecker H-C, Ohno Y, Fusunyan RD, Lichtenstein GR, et al. Increased expression of interleukin-8 mRNA in ulcerative colitis and Crohn's disease mucosa and epithelial cells. *Inflamm Bowel Dis*. (1995) 1:37–47.
40. Struyf S, Gouwy M, Dillen C, Proost P, Opdenakker G, Van Damme J. Chemokines synergize in the recruitment of circulating neutrophils into inflamed tissue. *Eur J Immunol*. (2005) 35:1583–91. doi: 10.1002/eji.200425753
41. Beltrán CJ, Núñez LE, Díaz-Jiménez D, Farfan N, Candia E, Heine C, et al. Characterization of the novel ST2/IL-33 system in patients with inflammatory bowel disease. *Inflamm Bowel Dis*. (2010) 16:1097–107. doi: 10.1002/ibd.21175
42. Di Salvo E, Ventura-Spagnolo E, Casciaro M, Navarra M, Gangemi S. IL-33/IL-31 axis: a potential inflammatory pathway. *Mediators Inflamm*. (2018) 2018:3858032. doi: 10.1155/2018/3858032
43. Travers J, Rochman M, Caldwell JM, Besse JA, Miracle CE, Rothenberg ME. IL-33 is induced in undifferentiated, non-dividing esophageal epithelial cells in eosinophilic esophagitis. *Sci Rep*. (2017) 7:17563. doi: 10.1038/s41598-017-17541-5
44. De la Fuente M, MacDonald TT, Hermoso MA. The IL-33/ST2 axis: role in health and disease. *Cytokine Growth Factor Rev*. (2015) 26:615–23. doi: 10.1016/j.cytogfr.2015.07.017
45. Lazaridis L-D, Pistiki A, Giamarellos-Bourboulis EJ, Georgitsi M, Damoraki G, Polymeros D, et al. Activation of NLRP3 inflammasome in inflammatory

bowel disease: differences between Crohn's disease and ulcerative colitis. *Dig Dis Sci*. (2017) 62:2348–56. doi: 10.1007/s10620-017-4609-8
46. Friedrich M, Diegelmann J, Schauber J, Auernhammer CJ, Brand S. Intestinal neuroendocrine cells and goblet cells are mediators of IL-17A-amplified epithelial IL-17C production in human inflammatory bowel disease. *Mucosal Immunol*. (2015) 8:943–58. doi: 10.1038/mi.2014.124
47. Henderson P, van Limbergen JE, Schwarze J, Wilson DC. Function of the intestinal epithelium and its dysregulation in inflammatory bowel disease. *Inflamm Bowel Dis*. (2011) 17:382–95. doi: 10.1002/ibd.21379
48. Caruso R, Fina D, Peluso I, Stolfi C, Fantini MC, Gioia V, et al. A functional role for interleukin-21 in promoting the synthesis of the T-cell chemoattractant, MIP-3alpha, by gut epithelial cells. *Gastroenterology*. (2007) 132:166–75. doi: 10.1053/j.gastro.2006.09.053
49. Mizoguchi A, Yano A, Himuro H, Ezaki Y, Sadanaga T, Mizoguchi E. Clinical importance of IL-22 cascade in IBD. *J Gastroenterol*. (2018) 53:465–74. doi: 10.1007/s00535-017-1401-7
50. Andoh A, Yagi Y, Shioya M, Nishida A, Tsujikawa T, Fujiyama Y. Mucosal cytokine network in inflammatory bowel disease. *World J Gastroenterol*. (2008) 14:5154–61. doi: 10.3748/wjg.14.5154
51. Netea MG, Azam T, Ferwerda G, Girardin SE, Walsh M, Park J-S, et al. IL-32 synergizes with nucleotide oligomerization domain (NOD) 1 and NOD2 ligands for IL-1beta and IL-6 production through a caspase 1-dependent mechanism. *Proc Natl Acad Sci USA*. (2005) 102:16309–14. doi: 10.1073/pnas.0508237102
52. Watson AJM, Hughes KR. TNF-α-induced intestinal epithelial cell shedding: implications for intestinal barrier function. *Ann N Y Acad Sci*. (2012) 1258:1–8. doi: 10.1111/j.1749-6632.2012.06523.x
53. Smyth D, McKay CM, Gulbransen BD, Phan VC, Wang A, McKay DM. Interferon-gamma signals via an ERK1/2-ARF6 pathway to promote bacterial internalization by gut epithelia. *Cell Microbiol*. (2012) 14:1257–70. doi: 10.1111/j.1462-5822.2012.01796.x
54. Mifflin RC, Pinchuk IV, Saada JI, Powell DW. Intestinal myofibroblasts: targets for stem cell therapy. *Am J Physiol Liver Physiol*. (2011) 300:G684–96. doi: 10.1152/ajpgi.00474.2010
55. Pinchuk IV, Mifflin RC, Saada JI, Powell DW. Intestinal mesenchymal cells. *Curr Gastroenterol Rep*. (2010) 12:310–8. doi: 10.1007/s11894-010-0135-y
56. Valentich JD, Powell DW. Intestinal subepithelial myofibroblasts and mucosal immunophysiology. *Curr Opin Gastroenterol*. (1994) 10:645–51. doi: 10.1097/00001574-199411000-00013
57. Willis BC, duBois RM, Borok Z. Epithelial origin of myofibroblasts during fibrosis in the lung. *Proc Am Thorac Soc*. (2006) 3:377–82. doi: 10.1513/pats.200601-004TK
58. Chen W, Lu C, Hirota C, Iacucci M, Ghosh S, Gui X. Smooth muscle hyperplasia/hypertrophy is the most prominent histological change in Crohn's fibrostenosing bowel strictures: a semiquantitative analysis by using a novel histological grading scheme. *J Crohn's Colitis*. (2017) 11:92–104. doi: 10.1093/ecco-jcc/jjw126
59. Lawrance IC, Maxwell L, Doe W. Altered response of intestinal mucosal fibroblasts to profibrogenic cytokines in inflammatory bowel disease. *Inflamm Bowel Dis*. (2001) 7:226–36. doi: 10.1097/00054725-200108000-00008
60. Bamba S, Andoh A, Yasui H, Araki Y, Bamba T, Fujiyama Y. Matrix metalloproteinase-3 secretion from human colonic subepithelial myofibroblasts: role of interleukin-17. *J Gastroenterol*. (2003) 38:548–54. doi: 10.1007/s00535-002-1101-8
61. Di Sabatino A, Jackson CL, Pickard KM, Buckley M, Rovedatti L, Leakey NAB, et al. Transforming growth factor beta signalling and matrix metalloproteinases in the mucosa overlying Crohn's disease strictures. *Gut*. (2009) 58:777–89. doi: 10.1136/gut.2008.149096
62. Biancheri P, Pender SL, Ammoscato F, Giuffrida P, Sampietro G, Ardizzone S, et al. The role of interleukin 17 in Crohn's disease-associated intestinal fibrosis. *Fibrogenesis Tissue Repair*. (2013) 6:13. doi: 10.1186/1755-1536-6-13
63. Benchimol EI, Mack DR, Guttmann A, Nguyen GC, To T, Mojaverian N, et al. Inflammatory bowel disease in immigrants to Canada and their children: a population-based cohort study. *Am J Gastroenterol*. (2015) 110:553–63. doi: 10.1038/ajg.2015.52
64. Niewiadomski O, Studd C, Wilson J, Williams J, Hair C, Knight R, et al. Influence of food and lifestyle on the risk of developing inflammatory bowel disease. *Intern Med J*. (2016) 46:669–76. doi: 10.1111/imj.13094
65. Constante M, Fragoso G, Calvé A, Samba-Mondonga M, Santos MM. Dietary heme induces gut dysbiosis, aggravates colitis, and potentiates the development of adenomas in mice. *Front Microbiol*. (2017) 8:1809. doi: 10.3389/fmicb.2017.01809
66. Ananthakrishnan AN, Khalili H, Konijeti GG, Higuchi LM, de Silva P, Fuchs CS, et al. Long-term intake of dietary fat and risk of ulcerative colitis and Crohn's disease. *Gut*. (2014) 63:776–84. doi: 10.1136/gutjnl-2013-305304
67. IBD in EPIC Study Investigators, Tjonneland A, Overvad K, Bergmann MM, Nagel G, Linseisen J, et al. Linoleic acid, a dietary n-6 polyunsaturated fatty acid, and the aetiology of ulcerative colitis: a nested case-control study within a European prospective cohort study. *Gut*. (2009) 58:1606–11. doi: 10.1136/gut.2008.169078
68. Viennois E, Merlin D, Gewirtz AT, Chassaing B. Dietary emulsifier–induced low-grade inflammation promotes colon carcinogenesis. *Cancer Res*. (2017) 77:27–40. doi: 10.1158/0008-5472.CAN-16-1359
69. Amre DK, D'Souza S, Morgan K, Seidman G, Lambrette P, Grimard G, et al. Imbalances in dietary consumption of fatty acids, vegetables, and fruits are associated with risk for Crohn's disease in children. *Am J Gastroenterol*. (2007) 102:2016–25. doi: 10.1111/j.1572-0241.2007.01411.x
70. Reif S, Klein I, Lubin F, Farbstein M, Hallak A, Gilat T. Pre-illness dietary factors in inflammatory bowel disease. *Gut*. (1997) 40:754–60. doi: 10.1136/gut.40.6.754
71. Llewellyn SR, Britton GJ, Contijoch EJ, Vennaro OH, Mortha A, Colombel J-F, et al. Interactions between diet and the intestinal microbiota alter intestinal permeability and colitis severity in mice. *Gastroenterology*. (2018) 154:1037–1046.e2
72. Lee N, Kim W-U. Microbiota in T-cell homeostasis and inflammatory diseases. *Exp Mol Med*. (2017) 49:e340. doi: 10.1038/emm.2017.36
73. Lynch SV, Pedersen O. The human intestinal microbiome in health and disease. Phimister EG, editor. *N Engl J Med*. (2016) 375:2369–79. doi: 10.1056/NEJMra1600266
74. Kriss M, Hazleton KZ, Nusbacher NM, Martin CG, Lozupone CA. Low diversity gut microbiota dysbiosis: drivers, functional implications and recovery. *Curr Opin Microbiol*. (2018) 44:34–40. doi: 10.1016/j.mib.2018.07.003
75. Brown K, DeCoffe D, Molcan E, Gibson DL. Diet-induced dysbiosis of the intestinal microbiota and the effects on immunity and disease. *Nutrients*. (2012) 4:1095–119. doi: 10.3390/nu4081095
76. Vrieze A, Out C, Fuentes S, Jonker L, Reuling I, Kootte RS, et al. Impact of oral vancomycin on gut microbiota, bile acid metabolism, and insulin sensitivity. *J Hepatol*. (2014) 60:824–31. doi: 10.1016/j.jhep.2013.11.034
77. Salim SY, Kaplan GG, Madsen KL. Air pollution effects on the gut microbiota. *Gut Microbes*. (2014) 5:215–9. doi: 10.4161/gmic.27251
78. Dudek-Wicher RK, Junka A, Bartoszewicz M. The influence of antibiotics and dietary components on gut microbiota. *Gastroenterol Rev*. (2018) 13:85–92. doi: 10.5114/pg.2018.76005
79. Agus A, Denizot J, Thévenot J, Martinez-Medina M, Massier S, Sauvanet P, et al. Western diet induces a shift in microbiota composition enhancing susceptibility to Adherent-Invasive *E. coli* infection and intestinal inflammation. *Sci Rep*. (2016) 6:19032. doi: 10.1038/srep19032
80. Pearlman M, Obert J, Casey L. The association between artificial sweeteners and obesity. *Curr Gastroenterol Rep*. (2017) 19:64. doi: 10.1007/s11894-017-0602-9
81. Chi L, Bian X, Gao B, Tu P, Lai Y, Ru H, et al. Effects of the artificial sweetener neotame on the gut microbiome and fecal metabolites in mice. *Molecules*. (2018) 23:367. doi: 10.3390/molecules23020367
82. Nickerson KP, McDonald C. Crohn's disease-associated adherent-invasive *Escherichia coli* adhesion is enhanced by exposure to the ubiquitous dietary polysaccharide maltodextrin. Mizoguchi E, editor. *PLoS ONE*. (2012) 7:e52132. doi: 10.1371/journal.pone.0052132
83. Kish L, Hotte N, Kaplan GG, Vincent R, Tso R, Gänzle M, et al. Environmental particulate matter induces murine intestinal inflammatory responses and alters the gut microbiome. *PLoS ONE*. (2013) 8:e62220. doi: 10.1371/journal.pone.0062220

84. Onderdonk AB, Hermos JA, Bartlett JG. The role of the intestinal microflora in experimental colitis. *Am J Clin Nutr*. (1977) 30:1819–25. doi: 10.1093/ajcn/30.11.1819
85. Pascal V, Pozuelo M, Borruel N, Casellas F, Campos D, Santiago A, et al. A microbial signature for Crohn's disease. *Gut*. (2017) 66:813–22. doi: 10.1136/gutjnl-2016-313235
86. Kim P, Gadani A, Abdul-Baki H, Mitre R, Mitre M. Fecal microbiota transplantation in recurrent Clostridium difficile infection: a retrospective single-center chart review. *JGH Open*. (2018) 3:4–9. doi: 10.1002/jgh3.12093
87. Chen T, Zhou Q, Zhang D, Jiang F, Wu J, Zhou J-Y, et al. Effect of faecal microbiota transplantation for treatment of clostridium difficile infection in patients with inflammatory bowel disease: a systematic review and meta-analysis of cohort studies. *J Crohn's Colitis*. (2018) 12:710–7. doi: 10.1093/ecco-jcc/jjy031
88. Allegretti J, Eysenbach LM, El-Nachef N, Fischer M, Kelly C, Kassam Z. The current landscape and lessons from fecal microbiota transplantation for inflammatory bowel disease: past, present, and future. *Inflamm Bowel Dis*. (2017) 23:1710–7. doi: 10.1097/MIB.0000000000001247
89. Baktash A, Terveer EM, Zwittink RD, Hornung BVH, Corver J, Kuijper EJ, et al. Mechanistic insights in the success of fecal microbiota transplants for the treatment of clostridium difficile infections. *Front Microbiol*. (2018) 9:1242. doi: 10.3389/fmicb.2018.01242
90. Madsen KL, Doyle JS, Jewell LD, Tavernini MM, Fedorak RN. *Lactobacillus* species prevents colitis in interleukin 10 gene-deficient mice. *Gastroenterology*. (1999) 116:1107–14. doi: 10.1016/S0016-5085(99)70013-2
91. Hvas CL, Bendix M, Dige A, Dahlerup JF, Agnholt J. Current, experimental, and future treatments in inflammatory bowel disease: a clinical review. *Immunopharmacol Immunotoxicol*. (2018) 10:1–15. doi: 10.1080/08923973.2018.1469144
92. Kim DH, Kim S, Lee JH, Kim JH, Che X, Ma HW, et al. *Lactobacillus acidophilus* suppresses intestinal inflammation by inhibiting endoplasmic reticulum stress. *J Gastroenterol Hepatol*. (2018) 34:178–85. doi: 10.1111/jgh.14362
93. Je I-G, Lee D-G, Jeong D-G, Hong D, Yoon J-M, Moon JS, et al. The probiotic, ID-JPL934, attenuates dextran sulfate sodium-induced colitis in mice through inhibition of proinflammatory cytokines expression. *J Med Food*. (2018) 23:jmf.2017.4152. doi: 10.1089/jmf.2017.4152
94. Nishitani Y, Tanoue T, Yamada K, Ishida T, Yoshida M, Azuma T, et al. *Lactococcus lactis* subsp. cremoris FC alleviates symptoms of colitis induced by dextran sulfate sodium in mice. *Int Immunopharmacol*. (2009) 9:1444–51. doi: 10.1016/j.intimp.2009.08.018
95. Borthakur A, Bhattacharyya S, Kumar A, Anbazhagan AN, Tobacman JK, Dudeja PK. *Lactobacillus acidophilus* alleviates platelet-activating factor-induced inflammatory responses in human intestinal epithelial cells. Dahiya R, editor. *PLoS ONE*. (2013) 8:e75664. doi: 10.1371/journal.pone.0075664
96. Mattar AF, Drongowski RA, Coran AG, Harmon CM. Effect of probiotics on enterocyte bacterial translocation *in vitro*. *Pediatr Surg Int*. (2001) 17:265–8. doi: 10.1007/s003830100591
97. Plaza-Diaz J, Gomez-Llorente C, Fontana L, Gil A. Modulation of immunity and inflammatory gene expression in the gut, in inflammatory diseases of the gut and in the liver by probiotics. *World J Gastroenterol*. (2014) 20:15632. doi: 10.3748/wjg.v20.i42.15632
98. Rodríguez-Nogales A, Algieri F, Garrido-Mesa J, Vezza T, Utrilla MP, Chueca N, et al. The administration of *Escherichia coli* nissle 1917 ameliorates development of DSS-induced colitis in mice. *Front Pharmacol*. (2018) 9:468. doi: 10.3389/fphar.2018.00468
99. Lodinová-Žádniková R, Sonnenborn U. Effect of preventive administration of a nonpathogenic *Escherichia coli* strain on the colonization of the intestine with microbial pathogens in newborn infants. *Neonatology*. (1997) 71:224–32.
100. Barbaro MR, Fuschi D, Cremon C, Carapelle M, Dino P, Marcellini MM, et al. *Escherichia coli* Nissle 1917 restores epithelial permeability alterations induced by irritable bowel syndrome mediators. *Neurogastroenterol Motil*. (2018) 30:e13388. doi: 10.1111/nmo.13388
101. Secher T, Maillet I, Mackowiak C, Le Bérichel J, Philippeau A, Panek C, et al. The probiotic strain *Escherichia coli* Nissle 1917 prevents papain-induced respiratory barrier injury and severe allergic inflammation in mice. *Sci Rep*. (2018) 8:11245. doi: 10.1038/s41598-018-29689-9
102. Fukumoto S, Toshimitsu T, Matsuoka S, Maruyama A, Oh-oka K, Takamura T, et al. Identification of a probiotic bacteria-derived activator of the aryl hydrocarbon receptor that inhibits colitis. *Immunol Cell Biol*. (2014) 92:460–5. doi: 10.1038/icb.2014.2
103. Le Maréchal C, Peton V, Plé C, Vroland C, Jardin J, Briard-Bion V, et al. Surface proteins of *Propionibacterium freudenreichii* are involved in its anti-inflammatory properties. *J Proteomics*. (2015) 113:447–61. doi: 10.1016/j.jprot.2014.07.018
104. Barouei J, Moussavi M, Hodgson DM. Effect of maternal probiotic intervention on HPA axis, immunity and gut microbiota in a rat model of irritable bowel syndrome. Heimesaat MM, editor. *PLoS ONE*. (2012) 7:e46051. doi: 10.1371/journal.pone.0046051
105. Okada Y, Tsuzuki Y, Narimatsu K, Sato H, Ueda T, Hozumi H, et al. 1,4-Dihydroxy-2-naphthoic acid from *Propionibacterium freudenreichii* reduces inflammation in interleukin-10-deficient mice with colitis by suppressing macrophage-derived proinflammatory cytokines. *J Leukoc Biol*. (2013) 94:473–80. doi: 10.1189/jlb.0212104
106. De Cupere F, Deprez P, Demeulenaere D, Muylle E. Evaluation of the effect of 3 probiotics on experimental *Escherichia coli* enterotoxaemia in weaned piglets. *Zentralbl Veterinarmed B*. (1992) 39:277–84. doi: 10.1111/j.1439-0450.1992.tb01169.x
107. Sudha MR, Jayanthi N, Aasin M, Dhanashri RD, Anirudh T. Efficacy of *Bacillus* coagulans unique IS2 in treatment of irritable bowel syndrome in children: a double blind, randomised placebo controlled study. *Benef Microbes*. (2018) 9:563–72. doi: 10.3920/BM2017.0129
108. Yang Y, Jing Y, Yang J, Yang Q. Effects of intranasal administration with Bacillusï¿½subtilis on immune cells in the nasal mucosa and tonsils of piglets. *Exp Ther Med*. (2018) 15:5189–98. doi: 10.3892/etm.2018.6093
109. Sivananthan K, Petersen AM. Review of *Saccharomyces boulardii* as a treatment option in IBD. *Immunopharmacol Immunotoxicol*. (2018) 17:1–11. doi: 10.1080/08923973.2018.1469143
110. Roussel C, Sivignon A, de Vallée A, Garrait G, Denis S, Tsilia V, et al. Anti-infectious properties of the probiotic *Saccharomyces cerevisiae* CNCM I-3856 on enterotoxigenic *E. coli* (ETEC) strain H10407. *Appl Microbiol Biotechnol*. (2018) 102:6175–89. doi: 10.1007/s00253-018-9053-y
111. Mack DR, Michail S, Wei S, McDougall L, Hollingsworth MA. Probiotics inhibit enteropathogenic *E. coli* adherence *in vitro* by inducing intestinal mucin gene expression. *Am J Physiol*. (1999) 276(4 Pt 1):G941–50.
112. Anderson RC, Cookson AL, McNabb WC, Park Z, McCann MJ, Kelly WJ, et al. Lactobacillus plantarum MB452 enhances the function of the intestinal barrier by increasing the expression levels of genes involved in tight junction formation. *BMC Microbiol*. (2010) 10:316. doi: 10.1186/1471-2180-10-316
113. Dai C, Zhao DH, Jiang M. VSL#3 probiotics regulate the intestinal epithelial barrier *in vivo* and *in vitro* via the p38 and ERK signaling pathways. *Int J Mol Med*. (2012) 29:202–8. . doi: 10.3892/ijmm.2011.839
114. Llewellyn A, Foey A. Probiotic modulation of innate cell pathogen sensing and signaling events. *Nutrients*. (2017) 9:E1156. doi: 10.3390/nu9101156
115. Kim SW, Kim HM, Yang KM, Kim S-A, Kim S-K, An MJ, et al. Bifidobacterium lactis inhibits NF-kappaB in intestinal epithelial cells and prevents acute colitis and colitis-associated colon cancer in mice. *Inflamm Bowel Dis*. (2010) 16:1514–25. doi: 10.1002/ibd.21262
116. Tomosada Y, Villena J, Murata K, Chiba E, Shimazu T, Aso H, et al. Immunoregulatory effect of bifidobacteria strains in porcine intestinal epithelial cells through modulation of ubiquitin-editing enzyme A20 expression. *PLoS ONE*. (2013) 8:e59259. doi: 10.1371/journal.pone.0059259
117. Finamore A, Ambra R, Nobili F, Garaguso I, Raguzzini A, Serafini M. Redox role of *Lactobacillus casei* shirota against the cellular damage induced by 2,2′-Azobis (2-Amidinopropane) dihydrochloride-induced oxidative and inflammatory stress in enterocytes-like epithelial cells. *Front Immunol*. (2018) 9:1131. doi: 10.3389/fimmu.2018.01131
118. Yan F, Liu L, Dempsey PJ, Tsai Y-H, Raines EW, Wilson CL, et al. A *Lactobacillus rhamnosus* GG-derived soluble protein, p40, stimulates ligand release from intestinal epithelial cells to transactivate epidermal growth factor receptor. *J Biol Chem*. (2013) 288:30742–51. doi: 10.1074/jbc.M113.492397
119. Coombes JL, Siddiqui KRR, Arancibia-Cárcamo CV, Hall J, Sun C-M, Belkaid Y, et al. A functionally specialized population of mucosal CD103 + DCs

induces Foxp3 + regulatory T cells via a TGF-β- and retinoic acid–dependent mechanism. *J Exp Med*. (2007) 204:1757–64. doi: 10.1084/jem.20070590
120. Iliev ID, Spadoni I, Mileti E, Matteoli G, Sonzogni A, Sampietro GM, et al. Human intestinal epithelial cells promote the differentiation of tolerogenic dendritic cells. *Gut*. (2009) 58:1481–9. doi: 10.1136/gut.2008.175166
121. Otte J-M, Rosenberg IM, Podolsky DK. Intestinal myofibroblasts in innate immune responses of the intestine. *Gastroenterology*. (2003) 124:1866–78. doi: 10.1016/S0016-5085(03)00403-7
122. Beswick EJ, Johnson JR, Saada JI, Humen M, House J, Dann S, et al. TLR4 activation enhances the PD-L1-mediated tolerogenic capacity of colonic CD90+ stromal cells. *J Immunol*. (2014) 193:2218–29. doi: 10.4049/jimmunol.1203441
123. Scheibe K, Backert I, Wirtz S, Hueber A, Schett G, Vieth M, et al. IL-36R signalling activates intestinal epithelial cells and fibroblasts and promotes mucosal healing *in vivo*. *Gut*. (2016) 66:823–38. doi: 10.1136/gutjnl-2015-310374
124. Jacob N, Jacobs JP, Kumagai K, Ha CWY, Kanazawa Y, Lagishetty V, et al. Inflammation-independent TL1A-mediated intestinal fibrosis is dependent on the gut microbiome. *Mucosal Immunol*. (2018) 11:1466–76. doi: 10.1038/s41385-018-0055-y
125. Shih DQ, Zheng L, Zhang X, Zhang H, Kanazawa Y, Ichikawa R, et al. Inhibition of a novel fibrogenic factor Tl1a reverses established colonic fibrosis. *Mucosal Immunol*. (2014) 7:1492–503. doi: 10.1038/mi.2014.37
126. Barrett R, Zhang X, Koon HW, Vu M, Chang J-Y, Yeager N, et al. Constitutive TL1A expression under colitogenic conditions modulates the severity and location of gut mucosal inflammation and induces fibrostenosis. *Am J Pathol*. (2012) 180:636–49. doi: 10.1016/j.ajpath.2011.10.026
127. Park JS, Choi JW, Jhun J, Kwon JY, Lee BI, Yang CW, et al. Lactobacillus acidophilus improves intestinal inflammation in an acute colitis mouse model by regulation of th17 and treg cell balance and fibrosis development. *J Med Food*. (2018) 21:215–24. doi: 10.1089/jmf.2017.3990
128. Gubatan J, Moss AC. Vitamin D in inflammatory bowel disease: more than just a supplement. *Curr Opin Gastroenterol*. (2018) 34:217–25. doi: 10.1097/MOG.0000000000000449
129. Tao Q, Wang B, Zheng Y, Jiang X, Pan Z, Ren J. Vitamin D prevents the intestinal fibrosis via induction of vitamin D receptor and inhibition of transforming growth factor-beta1/Smad3 pathway. *Dig Dis Sci*. (2015) 60:868–75. doi: 10.1007/s10620-014-3398-6
130. Truffi M, Sorrentino L, Monieri M, Fociani P, Mazzucchelli S, Bonzini M, et al. Inhibition of fibroblast activation protein restores a balanced extracellular matrix and reduces fibrosis in Crohn's disease strictures *ex vivo*. *Inflamm Bowel Dis*. (2018) 24:332–45. doi: 10.1093/ibd/izx008
131. Suez J, Zmora N, Zilberman-Schapira G, Mor U, Dori-Bachash M, Bashiardes S, et al. Post-antibiotic gut mucosal microbiome reconstitution is impaired by probiotics and improved by autologous FMT. *Cell*. (2018) 174:1406–23.e16. doi: 10.1016/j.cell.2018.08.047
132. Zmora N, Zilberman-Schapira G, Suez J, Mor U, Dori-Bachash M, Bashiardes S, et al. Personalized gut mucosal colonization resistance to empiric probiotics is associated with unique host and microbiome features. *Cell*. (2018) 174:1388–405.e21. doi: 10.1016/j.cell.2018.08.041
133. Cho JA, Chinnapen DJF. Targeting friend and foe: emerging therapeutics in the age of gut microbiome and disease. *J Microbiol*. (2018) 56:183–8. doi: 10.1007/s12275-018-8037-z

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