LOSS OF EPITHELIAL BARRIER INTEGRITY IN INFLAMMATORY DISEASES: CELLULAR MEDIATORS AND THERAPEUTIC TARGETS

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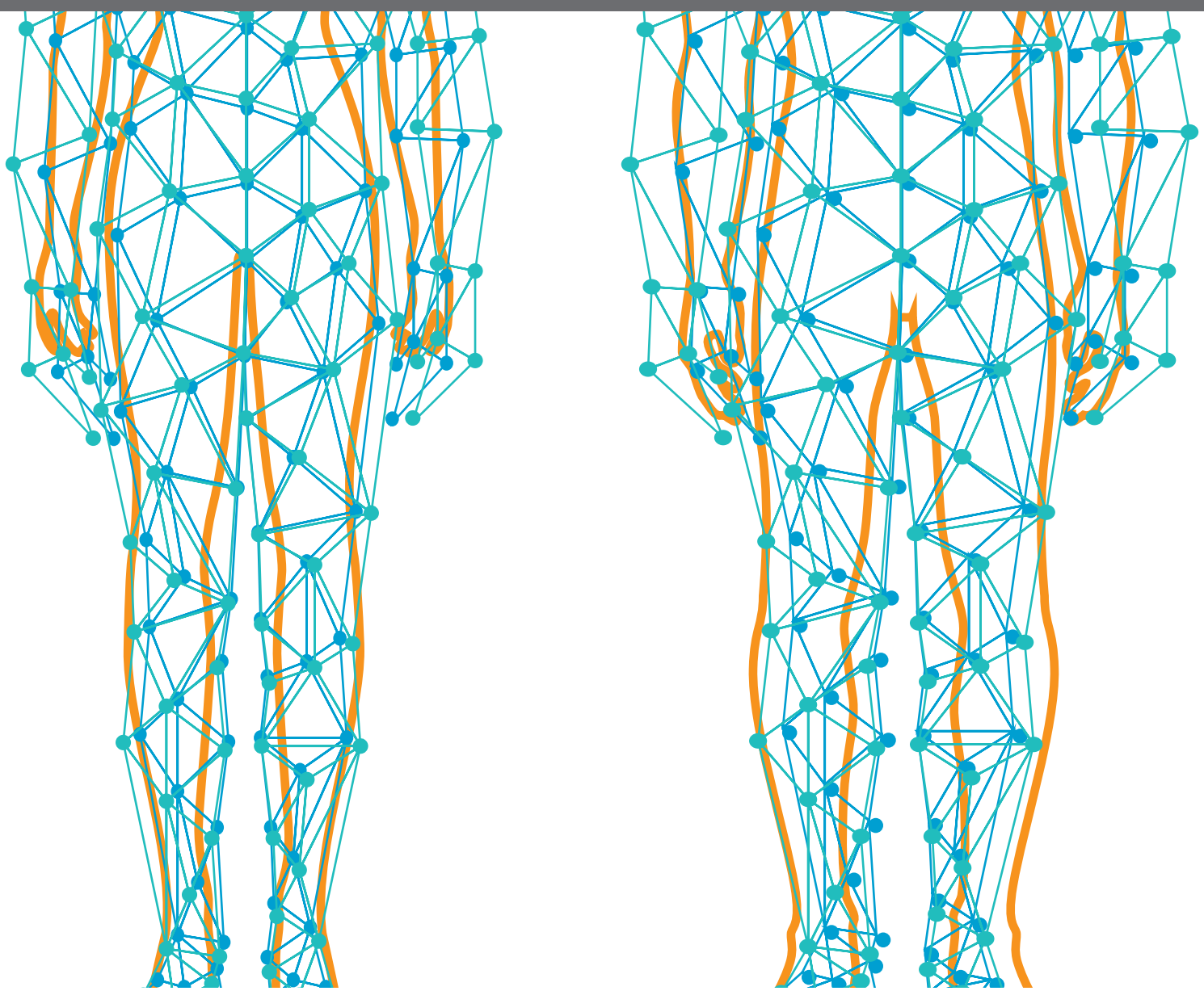

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LOSS OF EPITHELIAL BARRIER INTEGRITY IN INFLAMMATORY DISEASES: CELLULAR MEDIATORS AND THERAPEUTIC TARGETS

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Editorial: Loss of Epithelial Barrier Integrity in Inflammatory Diseases: Cellular Mediators and Therapeutic Targets

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Editorial on the Research Topic

Loss of Epithelial Barrier Integrity in Inflammatory Diseases: Cellular Mediators and Therapeutic Targets

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IMPAIRED INTESTINAL BARRIER INTEGRITY IN IBD

About 0.75% of the inhabitants of the industrialized countries of the Western world were affected by one of the two main entities of inflammatory bowel diseases (IBD), Crohn's disease or ulcerative colitis, in 2020 (1). This means that a significant part of the general population is affected by chronically remitting gastroenterological symptoms, such as bloody diarrhea, abdominal pain, and anemia. Importantly, besides its high incidence, IBD mainly affects the younger population, who are often significantly hindered in their social and professional lives by the disease. Despite the successful clinical establishment of new hallmark therapies during the last three decades, about 40% of IBD patients do not satisfactorily respond to current treatment strategies or suffer a secondary loss of response (2). Together, this promotes a scientific and clinical interest in identifying innovative therapeutic targets (3), and in this course, there is a steadily growing improvement in knowledge regarding the exact molecular and cellular processes of IBD pathogenesis. Although in the past, many pioneering studies focused on intestinal immune cells (4–6), as their overwhelmingly enhanced activation status and cytokine release were long assumed as predominant drivers of IBD, nowadays, there is growing awareness that the entire mucosal barrier and its function decisively determines the development and resolution of chronic intestinal inflammation (7–9). Addressing this aspect in our Research Topic, we present a diverse collection of original and review articles focusing on the pathological dysregulation and clinical relevance of the mucosal barrier integrity and its cellular key players in IBD.

Besides absorptive and secretory intestinal epithelial cells (IECs) forming the tightly closed epithelial monolayer, a broad spectrum of locally accumulating immune cells including also innate

lymphoid cells as a rather newly described immune cell population (reviewed by Schulz-Kuhnt et al.), as well as non-hematopoietic cells, such as endothelial cells, enteric neurons, adipocytes, and fibroblasts, contribute to the protection of the intestinal lamina propria against potentially invading luminal pathogens or foreign antigens (9–16). Furthermore, numerous intestinal blood vessels are located in direct proximity to the epithelial layer and are influenced by local inflammatory processes (e.g., IFNγ-induced disruption of endothelial barrier integrity) (17), while, *vice versa*, the inflammation-triggered increased blood flow supports recruitment of pro-inflammatory immune cells from the circulation (comprehensively summarized by Stürzl et al.). In addition, the enteric nervous system represents another factor of impact on intestinal homeostasis that has been neglected in the past and whose potential contribution in intestinal inflammation and impaired gut barrier is discussed by Drobny et al. While we are constantly getting better understanding of how the dysregulated function of individual cell populations in the gut contributes to the loss of intestinal homeostasis and the initiation or maintenance of chronic inflammatory processes, it is also becoming increasingly clear that the real challenge is deciphering the communication between the different components of this cellular network and to identify central molecular switches driving the loss of mucosal barrier integrity, as well as the counteracting process of mucosal wound healing in IBD (summarized by Sommer et al.). For sure, immune cell-derived cytokines represent central signaling molecules within this and a study by Delbue et al. provides new mechanistic insights into IL-22-mediated effects on epithelial integrity and wound healing. Moreover, implying even higher complexity, the interplay between different cellular compartments of the gut may further be influenced by external factors derived from the lumen, as very well-established for intestinal microbiota (an overview is provided by Jergens et al.) and of clear relevance also for defined nutritional components (18, 19). For example, Yeung et al. observed that reduced uptake of vitamin D resulted in impaired mucosal barrier properties.

IMPLICATIONS FOR IBD THERAPY

Most of the clinically applied strategies in IBD therapy, including classic immunosuppressive drugs (e.g., azathioprine and 6-mercaptopurine), but also more specific approaches like anti-cytokine antibodies (e.g., anti-TNF therapy and IL-12/IL-23-neutralizing ustekinumab) and anti-adhesion therapy (e.g., vedolizumab) primarily target the pathologically increased activation and/or accumulation of pro-inflammatory immune cells in the intestinal mucosa (3, 6). Lately, the maintenance and restoration of the intestinal barrier function and mucosal healing emerged as relatively new therapeutic goal in the clinical management of IBD (8, 20) and this also resulted in a better consideration of the epithelial-protective effects of established therapeutics. For example, the recognized capacity of anti-TNF therapy to restore the pathologically increased rate of apoptotic IECs and the subsequent loss of epithelial resistance in IBD patients (21), as well as the counteracting influence of azathioprine and 5-aminosalicylic acid on the inflammation-triggered downregulation and rearrangement of junctional proteins in *in vitro* cultured IECs and intestinal organoids (22). In addition, defined molecular mediators and intracellular signaling pathways involved in the maintenance of the epithelial tightness (e.g., STAT6, angulin-1, leptin, RhoA, and IL13Rα2) (23–26), in IEC survival (e.g., Caspase-8) (27), in the production of antimicrobial peptides (e.g., human β-defensin 2) (28) and in wound healing (e.g., STAT1, STAT3) (29, 30) have also been suggested as innovative therapeutic targets. As exemplarily demonstrated by Gerbeth et al. summarizing the multiple effects of histone deacetylase inhibitors on gut homeostasis, it will in general be essential to always consider the above outlined cellular complexity of the protective mucosal barrier and carefully validate the role of potential innovative target structures for the entire panel of involved cell types. Moreover, the fact that pathological conditions significantly differ dependent on the phase of disease (nicely described by Semin et al.) and its site of manifestation (emphasized by the study of Stolzer et al. describing a different impact of STAT1 signaling on IEC cell death in the context of inflammation in ileum and colon, and by comparative transcriptomic results reported by Gonzalez Acera et al.), makes it important to also develop diagnostic strategies allowing a careful clinical and molecular characterization of the individual disease status prior to the selection and initiation of therapy. In this context, Bojarski et al. provide a valuable overview of innovative advanced gastrointestinal endoscopic technologies and their potential future contribution in paving the way for personalized medicine in IBD.

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

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MINI REVIEW
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Intestinal Mucosal Wound Healing and Barrier Integrity in IBD–Crosstalk and Trafficking of Cellular Players

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The intestinal epithelial barrier is carrying out two major functions: restricting the entry of potentially harmful substances while on the other hand allowing the selective passage of nutrients. Thus, an intact epithelial barrier is vital to preserve the integrity of the host and to prevent development of disease. Vice versa, an impaired intestinal epithelial barrier function is a hallmark in the development and perpetuation of inflammatory bowel disease (IBD). Besides a multitude of genetic, molecular and cellular alterations predisposing for or driving barrier dysintegrity in IBD, the appearance of intestinal mucosal wounds is a characteristic event of intestinal inflammation apparently inducing breakdown of the intestinal epithelial barrier. Upon injury, the intestinal mucosa undergoes a wound healing process counteracting this breakdown, which is controlled by complex mechanisms such as epithelial restitution, proliferation and differentiation, but also immune cells like macrophages, granulocytes and lymphocytes. Consequently, the repair of mucosal wounds is dependent on a series of events including coordinated trafficking of immune cells to dedicated sites and complex interactions among the cellular players and other mediators involved. Therefore, a better understanding of the crosstalk between epithelial and immune cells as well as cell trafficking during intestinal wound repair is necessary for the development of improved future therapies. In this review, we summarize current concepts on intestinal mucosal wound healing introducing the main cellular mediators and their interplay as well as their trafficking characteristics, before finally discussing the clinical relevance and translational approaches to therapeutically target this process in a clinical setting.

Keywords: wound healing, intestinal epithelial cells, mucosal healing, IBD, intestinal epithelial barrier function

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INTRODUCTION

The intestinal mucosa forms a tight barrier with two opposing functions. While it is selectively permeable allowing the absorption of nutrients, it also separates the host from luminal toxins, antigens and microbes that potentially promote disease [reviewed in (1)]. Upon mucosal damage, the epithelial barrier gets leaky facilitating the translocation and therefore excessive exposure of deeper layers of the mucosa to intestinal microbial antigens. This may lead to the recruitment of immune cells releasing different cytokines and may result in disturbed homeostasis [further

reviewed in (2, 3)]. Therefore, the regulation of the epithelial barrier function is essential to maintain mucosal homeostasis.

A variety of factors may potentially contribute to mucosal damage, including environmental factors, medication, diet, the host microbiota, infections like HIV as well as genetic factors such as polymorphisms in the CDH1 gene encoding E-Cadherin, which is associated with increased risk to develop ulcerative colitis (UC) [reviewed in (4, 5)]. In general, the pathogenesis of several chronic inflammatory diseases including the inflammatory bowel diseases (IBD) UC and Crohn's disease (CD) is associated with a dysfunctional intestinal epithelial barrier as well as insufficient and delayed mucosal wound healing (6–9). Particularly, wound repair as a pre-requisite to re-establish the mucosal epithelial barrier and intestinal homeostasis is crucial for efficient resolution of inflammation. Hence, mucosal healing (MH) is an increasingly acknowledged goal in IBD therapy in order to achieve and maintain long-term remission. However, mucosal repair and wound healing are complex processes coordinated by the dynamic crosstalk of different cellular players including epithelial cells and infiltrating immune cells as well as their mediators [reviewed in (10)] that are still incompletely understood. A better understanding of these interactions might therefore help to develop tissue-specific approaches to promote wound healing and to treat intestinal inflammation.

In the following paragraphs, we will review the current concepts of intestinal mucosal wound healing, shedding light on the contribution of infiltrating immune cells and their interaction with epithelial cells. Finally, we highlight the clinical relevance of MH and translational approaches to therapeutically target this process.

INTESTINAL EPITHELIAL WOUND HEALING

Intestinal epithelial wound healing is a complex process modulated by various regulatory peptides, including growth factors (GF), and cytokines. Three different phases can be distinguished: Restitution, proliferation, and differentiation and maturation. However, *in vivo*, these processes merge into each other and overlap [reviewed in (11)].

First, epithelial cells surrounding the wound migrate rapidly into the denuded area, form pseudopodia-like structures, re-organize themselves in order to extend into the wound and then re-differentiate after closing the wound defect. This process is termed epithelial restitution and occurs within minutes to hours [reviewed in (12)]. Interestingly, restitution is independent of cell proliferation and one of the most important stimulators of intestinal epithelial cell (IEC) restitution is transforming growth factor β (TGF-β) (13–15). Within the intestinal mucosa, TGF-β is produced by different cell types including epithelial cells, stromal cells, regulatory T cells (T_{regs}), dendritic cells (DC) and macrophages [reviewed in (16)]. Once TGF-β is activated, it enhances restitution by upregulating the expression of matrix metalloproteinase-1 (MMP-1), MMP-10 and a set of genes, including Slc28a2, Tubb2a, and Cpe that are preferentially expressed in fetal IECs (17, 18). Furthermore, mediators, such as vascular endothelial growth factor (VEGF), which are released from the inflamed mucosa, are involved in epithelial cell migration in a TGF-β-dependent manner (19). In addition, it was shown that amino acids like histidine and arginine play an important role in TGF-β-mediated IEC restitution probably via interaction with Smad signaling (20). Furthermore, Lopetuso et al. (21) showed that during acute resolving colitis, IL-33/ST2 promote epithelial repair and restitution by inducing miR-320. It had earlier been demonstrated that miR-320 is decreased in the context of intestinal inflammation, suggesting that this might lead to an inherent defect of epithelial repair (22). Recently, Desmocollin-2 (Dsc2), a desmosomal cadherin exclusively expressed on IECs, was identified as a further key contributor to IEC migration and restitution *in vivo* (23).

In order to increase the number of cells able to resurface the wound area, proliferation is necessary and occurs within hours or days [reviewed in (12)]. This phase is pre-dominantly promoted by various GFs, such as epidermal growth factor (EGF), keratinocyte growth factor (KGF), and fibroblast growth factor (FGF) (24–27), as well as different cytokines including IL-28, which was shown to control proliferation of IECs by activating STAT1 (28), and IL-22, which induces STAT3 signaling, an important regulator of immune homeostasis and mucosal wound healing in the gut (29). Moreover, TLR2 was shown to suppress apoptosis of IECs *in vivo* by selectively regulating trefoil factor 3 (TFF) expression and controlling intestinal epithelial wound repair by modulating epithelial connexin-43 (30, 31).

Finally, differentiation and maturation is needed to re-establish and maintain the mucosal barrier function. Under normal conditions, $Lgr5^+$ intestinal stem cells (ISCs), which are located at the base of the crypts, differentiate into short-lived proliferating transit-amplifying progenitors, which further differentiate into absorptive (enterocyte) and secretory progenitors under the control of Wnt/Notch signaling [reviewed in (32, 33)]. Secretory precursors then develop into enteroendocrine cells in a Neurog3-dependent manner or into Goblet or Paneth cells following activation of Atoh1 also known as Math1. Later on, the different cell types acquire their lineage-specific expression of transcription factors (TFs), such as Sox9 for Paneth cells and Klf4 for Goblet cells (34–36). It is also worth mentioning, that there are two distinct ISC populations: Crypt base columnar (CBC) cells, which are actively proliferating and reserve intestinal stem cells (rISC) that are quiescent stem cells until activated upon injury. In line with this, Gonzalez et al. (37) showed that $Hopx^+$ cells (rISC) are resistant to injury and are the likely source of epithelial renewal following prolonged ischemic injury (37).

Furthermore, host-microbiota interactions may substantially affect proliferation of epithelial cells and are implicated in intestinal barrier function. E.g., short chain fatty acids (SCFAs) produced by commensal bacteria promote proliferation and differentiation of cells along the crypt-villus axis and, thus, contribute to epithelial restitution (38). Moreover, they are also directly implicated in upholding epithelial integrity to counteract tissue damage (39). In addition to these direct effects on epithelial cells, SCFAs also profoundly impact on the differentiation of

mucosal T cells and induce T_{regs} (40), which are involved in mucosal wound healing as described below. Further details on this emerging field are reviewed elsewhere [reviewed in (41, 42)].

Another important cellular mechanism that should be considered in the context of intestinal epithelial wound healing is epithelial-mesenchymal transition (EMT). During this process epithelial cells lose some of their epithelial characteristics, such as polarity and adhesiveness and acquire migratory functions and properties of mesenchymal cells. This transformation is characterized by the interplay of different mediators like TFs, RNAs, and TGF-β family proteins [reviewed in (43)]. In IBD patients, Leeb et al. (44) reported a reduced migratory ability of fibroblasts, which are normally essential in wound contraction during the initial phase of wound healing (44, 45). Based on these findings it is conceivable that epithelial cells are forced to undergo EMT in order to compensate fibroblast dysfunction and to rapidly restore the intestinal barrier function, which, in turn, might predispose for CD-associated fistulae formation (46).

CONTRIBUTION OF VARIOUS IMMUNE CELL TYPES IN INTESTINAL REPAIR AND THEIR INTERACTION WITH EPITHELIAL CELLS

Lymphocytes and Innate Lymphoid Cells

Cytokines and other mediators secreted by different T cell subsets play essential roles in wound healing (see **Figure 1**). Diverse injury models in mice (including models focusing on other organs than the gut, for which evidence is limited) show that depletion of T_{regs} during different phases of wound healing leads to a worse clinical outcome suggesting that they play an important role in the regulation of wound healing probably by counteracting pro-inflammatory stimuli (47–52). Nosbaum et al. (53) showed that T_{regs} in cutaneous wounds attenuated Interferon-γ (IFN-γ) production and reduced the accumulation of pro-inflammatory macrophages. Their elimination resulted in delayed wound re-epithelialization and wound closure. IFN-γ had previously been shown to affect epithelial intercellular junctions and to attenuate intestinal epithelial wound closure by inhibiting epithelial cell migration in a β1 integrin-dependent mechanism (54, 55). Nosbaum et al. (53) were also able to show that, mechanistically, T_{regs} induced the expression of EGFR early after wounding, and lineage-specific deletion of EGFR in T_{regs} resulted in a reduced accumulation and activation as well as increased accumulation of pro-inflammatory macrophages. Furthermore, there is evidence that FGF2 produced by T_{regs} together with IL-17 is involved in gene regulation to repair damaged cutaneous and intestinal epithelium (53, 56). Moreover, $CD4^+CD25^+Foxp3^+$ T_{regs} isolated from peripheral blood of healthy individuals were reported to induce a phenotypical switch of human monocytes/macrophages to wound healing macrophages (57). Following IL-33 release from damaged epithelia, the GF amphiregulin is another mediator produced by T_{regs}, which is involved in limiting inflammation and promoting epithelial repair (47, 58).

Other important cell types involved in intestinal mucosal wound healing are T helper cells (T_H) and innate lymphoid cells (ILCs). IL-22 is produced by T_H17 and T_H22 cells as well as by group 3 ILCs (ILC3) at mucosal surfaces and is a key mediator of this process [reviewed in (59)]. By activating STAT3, IL-22 can not only accelerate proliferation of IECs, but also induce the expression of mucus-associated molecules and the restitution of mucus-producing cells (29, 60). Specifically, IL-22 produced by ILC3s after intestinal injury has been shown to activate intestinal stem cells to promote regeneration (61). Upstream, upon tissue damage, IL-23 may be released leading to the production of IL-22 by ILC3s (62). In line with this, mice deficient for IL-36γ, a potent inducer of IL-23, showed reduced levels of IL-22 and failed to recover from acute intestinal damage. This impaired recovery could be rescued by exogenous IL-23 application (63).

ILC1 show a similar cytokine expression pattern as T_H1 cells and mainly exhibit their function by secreting tumor necrosis factor α (TNF-α) and IFN-γ to recruit and activate other inflammatory cells (64). As mentioned above, IFN-γ is also involved in the regulation of epithelial barrier integrity (54, 55). Thus, it is not surprising that depletion of intraepithelial ILC1s was associated with reduced proximal colon inflammation in a mouse model of colitis (65).

By contrast, ILC2s produce T_H2-cell-associated cytokines including IL-4, IL-5, IL-9, and IL-13 [reviewed in (66, 67)]. Upon stimulation by IL-33 and similar to T_{regs}, ILC2s produce amphiregulin, which was shown to promote intestinal epithelial cell regeneration in dextran sodium sulfate (DSS)-treated mice (58).

Furthermore, γδ T cells need to be considered when talking about intestinal wound healing as they are the major source of KGF in the mucosa. KGF released from intraepithelial γδ T cells is important for maintaining intestinal epithelial cell proliferation and villus growth, for promoting the repair of epithelial lesions and is also involved in epithelial cell differentiation (68). It was shown that mice lacking γδ T cells have increased susceptibility to DSS-induced colitis and reduced ability to repair damaged epithelia (69). In line with this, Chen et al. (70) found that intraepithelial γδ T cells preserve the integrity of damaged epithelial surfaces by localized delivery of KGF (70, 71).

Neutrophils

Neutrophils play a crucial role in the first line of defense against microbes. Their antimicrobial machineries include the formation of neutrophil extracellular traps called "NETs" (72) and the elimination of invading microbes through phagocytosis, degranulation and production of reactive oxygen species (ROS) [reviewed in (73)]. These mechanisms are essential for wound healing by on the one hand preventing infection through pathogen translocation, and on the other hand by mediating the early so-called inflammatory phase of wound healing. The recruitment of murine neutrophils to the site of cutaneous injury begins 4 h after the initial injury and peaks after 18 h (74). Depletion of neutrophils in damaged mucosa was shown to lead to a severer colitis as well as impaired recovery and restoration of epithelial integrity (75–77). Furthermore,

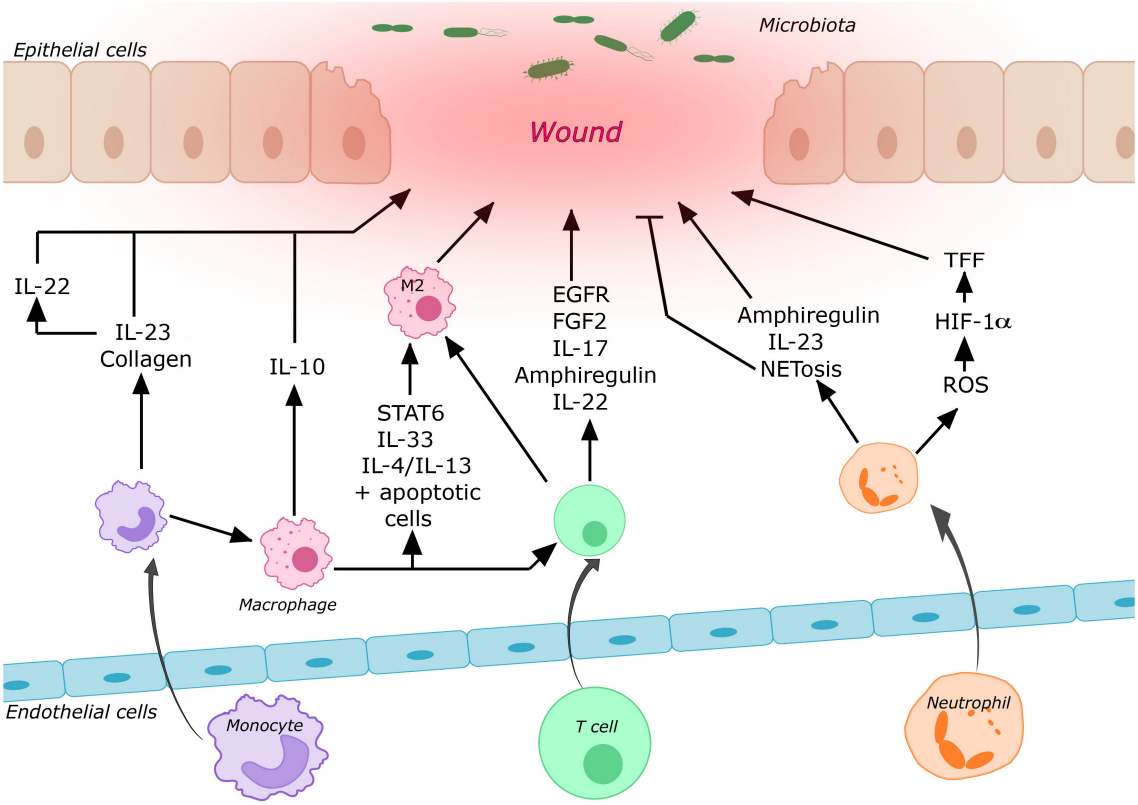

FIGURE 1 | Contribution of some of the most important immune cells to intestinal wound healing. Circulating immune cells are recruited to the wound area by cell trafficking processes. After entering the tissue these cells may undergo differentiation processes and secrete various mediators, which promote or repress mucosal wound healing (for details cf. main text).

it was shown that neutrophils enhance the production of amphiregulin by IECs promoting epithelial barrier function and tissue repair (75). Another mechanism contributing to the wound healing properties of neutrophils is their ability to generate a hypoxic microenvironment within the wounded tissue by producing ROS, which in turn leads to the stabilization of HIF-1α in the intestinal mucosa (78). HIF-1α was shown to enhance the epithelial expression of TFF3, which has a barrier-protective function (79). In addition, HIF-1α as a TF promotes the upregulation of genes involved in wound healing including adhesion proteins, different GFs and extracellular matrix components [reviewed in (80)]. Moreover, neutrophils produce IL-22 and IL-23, which are both essential mediators of wound healing as mentioned above (77, 81, 82).

However, neutrophils may also have a negative impact on wound healing. For instance, it was shown that counteracting the alarmin HMGB1 leads to reduced NET formation resulting in improved wound healing and inhibition of NETosis improves wound healing in diabetic mice (83). Furthermore, the accumulation of double strand breaks in the mucosa induced by neutrophils led to impaired wound healing and genomic instability (84). In summary, the effects of neutrophil in this process can be seen as a double-edged sword.

Monocytes and Macrophages

Circulating monocytes are rapidly recruited to sites of tissue damage or infection, where they further differentiate into inflammatory M1-like macrophages or wound healing M2-like macrophages. Although this classification has been used to explain many experimental observations, it is meanwhile regarded as oversimplification (85).

While the level of CD16 and CD14 expression can be used to differentiate three different monocyte subsets in humans, they are divided into two subpopulations based on their surface expression of Ly6C and/or CX3CR1 in mice (86, 87). Ly6C^{hi} monocytes were shown to be more dominant in the early inflammatory phase exhibiting phagocytic and inflammatory functions, whereas Ly6C^{low} monocytes dominate the later phase displaying anti-inflammatory properties and promoting healing (88). The supportive role of macrophages for barrier function was shown by their ability to increase transepithelial electric resistance and cell height of enteroid monolayers (89). Depletion of macrophages in different mouse models led to severely altered wound morphology, delayed re-epithelialization, reduced collagen deposition, impaired angiogenesis, and decreased cell proliferation in the healing wounds (90, 91). Due to their heterogeneity, macrophages play essential roles in all phases of wound repair. More specifically, depletion after the

inflammatory phase increased injury and delayed regeneration while depletion in the early inflammatory phase significantly reduced the formation of vascularized granulation tissue, impaired epithelialization, but also resulted in reduced scar formation in kidneys and skin (92, 93). As mentioned above, IL-23 is an important mediator of wound healing and macrophages were identified as a major source of this cytokine (94). Furthermore, the release of IL-10 by macrophages leads to endothelial cell proliferation and activation of epithelial pro-proliferative pathways in the intestine (95). Interestingly, monocytes and macrophages express virtually all known collagen and collagen-related mRNAs, which is essential for the remodeling phase of wound healing (96). Macrophages also have an impact on other immune cells, e.g., by inducing the differentiation of $Foxp3^+$ T_{regs} in the lamina propria (97).

The polarization of macrophages to a wound healing phenotype is essential for repair processes and is regulated by different mediators. Blockade of IL-1β was shown to prime the generation of M2-like macrophages in diabetic mice and IL-33 significantly enhanced intestinal wound healing by promoting the M2 phenotype (98, 99). Moreover, STAT6-mediated M2 polarization promoted repair in 2,4,6-trinitrobenzenesulfonic acid (TNBS) treated mice through activation of the Wnt signaling pathway (100). In addition, IL-4 or IL-13 in combination with apoptotic cells are capable of activating wound healing macrophages. In the absence of apoptotic signals, the proliferation of tissue-resident macrophages, the induction of anti-inflammatory and tissue repair genes are impaired after induction of colitis (101). Recently, Fpr2/3, which is expressed by epithelial cells was shown to regulate the migration of monocytes to sites of mucosal injury, and CX3CR1 was important for the accumulation of macrophages in the wound (102).

However, monocytes and macrophages may also have negative effects on the epithelial barrier. Mononuclear phagocytes interact with IECs by E-Cadherin leading to dysregulated epithelial cell differentiation and intestinal inflammation by disrupting mucosal homeostasis (103, 104). In line with this, a combination of paracrine and hetero-cellular communication between IECs and macrophages was suggested to play a pivotal role in regulating epithelial cell function and dysregulation of intestinal epithelial barrier (105). Sablet et al. demonstrated that inflammatory monocytes contribute to the loss of intestinal barrier function during cryptosporidiosis by producing TNF-α and IL-1β (106).

Taken together, macrophages are crucially involved in many aspects of intestinal wound healing. Depending on their polarization and the phase of wound healing, they may either promote wound closure or predispose for dysregulation of MH.

CELL TRAFFICKING IN THE CONTEXT OF INTESTINAL MUCOSAL WOUND HEALING

As all of the immune cells discussed in the scope of this review are circulating cells or descendants from such cells, there is an obvious need of trafficking for these effectors to reach the site of insult. Thus, cell trafficking should be considered as an integral part of wound healing processes and will shortly be reviewed here.

Described in greater detail elsewhere, cell trafficking describes all processes that are involved in the localization of cells and therefore comprises cellular influx to, retention in and egress from effector tissues [as reviewed in (3, 107)]. Influx from the circulation is regulated by a tightly controlled multistep adhesion cascade. As a prerequisite for transmigration through the endothelium, interaction of selectins and their respective ligands on endothelial cells recruit circulating cells to the vessel walls of high endothelial venules (HEVs) leading to rolling and reduced velocity (108). This slow-down increases the availability of circulating cells to chemotactic stimuli, especially to chemokines, thereby enabling chemokine-induced conformational changes of heterodimeric integrins. Activated integrins are able to firmly bind to endothelial cell adhesion molecules, leading to the arrest of circulating cells on the vessel wall and subsequent para- or intracellular transmigration and target tissue invasion (109).

With regard to gut homing, the α4β7 integrin-mucosal vascular addressin cell adhesion molecule 1 (MAdCAM-1) axis was identified as important due to the virtually exclusive expression of MAdCAM-1 on HEVs of the intestinal tract (110). The relevance of this axis in intestinal wound healing was recently demonstrated, as anti-α4β7 antibody treatment of mice in a colon wound model led to impaired intestinal wound closure, most likely due to reduced homing of non-classical monocytes (NCMs) and a reduction of NCM-derived wound healing macrophages (111). Further, gut specificity in trafficking processes may be provided by the exclusive expression of chemokines in the intestine, as for instance of the CCR9 ligand CCL25 and the GPR15 ligand in the small and large intestine, respectively (112, 113). Their participation in cell recruitment to intestinal wounds has not been studied so far and needs to be further elucidated. Interestingly, both α4β7 and CCR9 are induced on gut-homing T cells through retinoic acid (RA) produced by dendritic cells in the gut-associated lymphoid tissue. With regard to ILCs, it has been shown that this is the case only for ILC1s and ILC3s, while α4β7 expression on ILC2s occurs independent of RA and is already induced in the bone marrow (114). In connection with the above-mentioned roles of ILCs in wound healing, it is tempting to speculate that this might lead to continuous gut homing of amphiregulin-secreting ILC2s promoting homeostasis, while ILC3 recruitment might be regulated by the level of inflammation present. However, it is difficult to envision the consequences for wound healing, since ILC3s not only promote mucosal repair through IL-22, but may also promote inflammation and, thus, secondary tissue injury (115).

Retention of homed cells within the target tissues is either controlled indirectly by the regulation of egress signal receptors or by direct anchoring to tissue structures. A key example of indirect retention is the interaction of CD69 with sphingosine 1-phosophate receptor-1 (S1PR1), leading to degradation of the latter and inhibition of extravasation along the S1P gradient into the bloodstream (116, 117). Further extravasation signals might be provided by the interaction of CCR7 and CCL19 or

CCL21, facilitating the recruitment of receptor-bearing cells to the lymphatic system (118, 119). Direct anchoring of recruited cells can be provided by the interaction of integrins with cell adhesion molecules in the tissue. E.g., αE-integrin (CD103) dimerizes with β7-integrin and mediates tissue retention through interaction with E-Cadherin (120, 121). Although the retention of cells in the wound area or their recirculation to the blood will certainly be of relevance in the spatiotemporal orchestration of the wound healing process, since it might lead to the accumulation or reduction of repair-promoting or -impeding cell populations, these mechanisms have not been specifically investigated in this context in the gut. However, and interestingly, there is evidence from skin models, that tissue resident memory T cells (T_{RM} cells), which play important roles in the pathogenesis of IBD (122), promote epithelial wound healing (123, 124).

It is also worth mentioning that mucosal cytokine profiles differ between CD and UC. While UC is dominated by T_H2-associated cytokines like IL-5, IL-13, IL-9, and IL-4 (125–129), CD is marked by cytokines, such as IFN-γ and IL-2, associated with a T_H1 phenotype (125, 130). T_H17 cells and cytokines seem to be involved in both entities (131). At the same time, macroscopic differences in ulcerations between CD and UC exist (132) and inflammation patterns between CD and UC differ with immune cell infiltration restricted to the mucosa of the colon in UC, but being transmural and potentially occurring in the whole gastrointestinal tract in CD [reviewed in (133)]. This strongly suggests that different homing mechanisms apply for immune cells in CD and UC that might also impact wound healing. Interestingly, differences in the expression of gut homing markers on different T cell subsets and differential usage of gut homing pathways in ileal CD as compared to colonic UC have been observed [reviewed in (134, 135)]. However, further dedicated studies are needed to explore this assumption in depth.

Taken together, the implications of immune trafficking for intestinal wound healing are obvious. Particularly, they need to be considered in a therapeutic context, especially when trafficking mechanisms are directly manipulated by antibodies. This also highlights the need for further investigation of the trafficking mechanisms participating in intestinal wound healing.

CLINICAL RELEVANCE OF MUCOSAL HEALING AND THERAPEUTIC APPROACHES

Mucosal healing (MH), a term coined by Truelove and Witts in 1955 (136), is nowadays considered an important study endpoint and increasingly important treatment goal in IBD. Several clinical trials showed the importance and improved clinical outcomes after achieving MH, defined as absent or low signs of mucosal injury on endoscopy (137–141). In UC and CD, it is associated with long-term remission and reduced need for surgery (142, 143). On tissue level and mechanistically, it is obvious that wound healing and restitution of the intestinal epithelial barrier function are major steps in achieving MH. Consequently, the promotion of wound healing has been suggested as a potential therapeutic tool (144). Calprotectin is a soluble protein in the cytosol of neutrophils and known to be elevated in both the intestinal mucosa and feces of IBD patients (145). Several studies have shown a correlation between low fecal calprotectin (FC) concentration and histological remission as well as MH in UC and CD patients. Therefore, low calprotectin levels might be an early predictor of therapeutic success in terms of MH (146, 147).

One experimental approach to achieve wound healing that was addressed by several studies, but not in the gut, was the promotion of recruitment and polarization of monocytes and wound healing macrophages (148, 149). Maruyama and colleagues (150) showed that upon injection of IL-1β-activated macrophages in mice, the production of VEGF-C was increased and cutaneous wound healing improved. Interestingly, one mechanism of action of corticosteroids is M1 macrophage suppression in response to LPS stimulation, which involves the miR-155 (151). Moreover, neutrophils as cellular mediators can be targeted. In the context of peritonitis, Norling et al. (152) showed that nanoparticles containing aspirin-triggered resolvin D1 or a lipoxin A4 analog reduced polymorphonuclear cell influx and enhanced wound healing. As different GFs like EGF, VEGF, and KGF mediate epithelial repair, they might also be interesting candidates [reviewed in (153)]. Another promising therapeutic approach is targeting IL-22, which is considered to promote epithelial integrity via STAT3. Consequently, an IL-22 IgG4 Fc fusion protein (UTTR1147A) is currently tested in patients with moderate-to-severe UC and CD (ClinicalTrials.gov Identifier: NCT03558152, NCT03650413).

In addition to these experimental concepts, several current IBD treatments were shown to have a protective or regenerative effect on the damaged epithelium and to promote MH [reviewed in (154)]. Aminosalicylates not only affect intestinal inflammation via various signaling pathways such as NF-κB, but also directly stimulate epithelial wound healing by enhancing epithelial cell restitution and proliferation (155–157). Anti-TNF-α antibodies such as infliximab and adalimumab are able to induce and maintain MH (144, 158–160) by restricting the inflammatory infiltrate and T cell proliferation within the lamina propria and by downregulating the expression of metalloproteinases and pro-inflammatory molecules (161). For infliximab, a single nucleotide polymorphism in the TRAP1 gene has been described to be associated with MH in CD patients (162). Moreover, anti-TNF-α antibodies support regenerative processes by reducing inflammation, restoring gut barrier function, mucosal secretion and by activating fibroblasts (163). In addition, it has been suggested that these antibodies mediate Fc region-dependent induction of wound healing macrophages. It was shown that infliximab as well as adalimumab can induce wound healing macrophages *in vitro* and *in vivo* (164, 165). Similarly, ustekinumab, a monoclonal antibody directed against IL-12 and IL-23, successfully induced MH in CD patients (166).

As the JAK/STAT pathway seems to play an important role in the interaction of lymphocytes and IECs through a variety of cytokines, it is not surprising that tofacitinib, a JAK inhibitor routinely used in UC treatment, is able to induce and maintain

MH (167). Lechner et al. (168) recently demonstrated that tofacitinib specifically reduces pro-inflammatory cytokines that are produced by lamina propria T cells and affects their homing potential by suppressing the surface integrin expression on T cells. However, in an experimental model of intestinal mucosal wounding, high concentrations of tofacitinib rather prolonged wound healing (168), an observation that requires further translational studies to reconcile it with the clinical outcomes.

Another important class of IBD therapeutics are anti-trafficking agents [reviewed in (3)]. Vedolizumab, a humanized monoclonal anti-α4β7 antibody, inhibits the binding and subsequent migration of lymphocytes into the gut (169). The GEMINI I trial showed that significantly more UC patients treated with vedolizumab than with placebo achieved MH (140, 170). However, mechanistic data explaining the impact of vedolizumab on trafficking of cells implicated in wound healing in inflammation are so far missing. Thus, it is not clear, whether this is a direct effect or secondarily resulting from reduced inflammation and associated changes in the balance of cells promoting and counteracting mucosal repair. In seeming contrast to data on MH as a study endpoint assessing control of inflammation, several (but not all) studies reported that patients treated with vedolizumab are more vulnerable to post-operative complications (171–176). A potential explanation might be that, according to a recent study from our group, blocking α4β7 impaired gut homing of NCMs, which was associated with delayed wound healing and reduced perilesional presence of wound healing macrophages (111). It is important to mention that this is not necessarily contradicting the mentioned MH data, since this study exclusively addressed exogenous tissue injury in the absence of inflammation and it is likely that ongoing inflammation will substantially modulate trafficking, communication and signaling pathways.

Collectively, almost all available therapies for the treatment of IBD have demonstrated their potential to induce MH, although it is not clear to what extent this is a result from direct impact on wound healing processes or a secondary effect of the reduction of inflammation. Thus, further mechanistic data and additional efforts to directly promote wound healing and barrier integrity in the context of IBD are necessary.

CONCLUDING REMARKS

Intestinal mucosal wound repair are key steps for achieving and maintaining MH, which is associated with beneficial clinical outcomes. However, the interplay as well as the trafficking characteristics of the most important cellular mediators like lymphocytes, neutrophils and monocytes/macrophages are not sufficiently characterized. Further research is necessary in order to better understand the contribution of cell trafficking to mucosal wound repair and to base targeted therapeutic approaches on this process.

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Histone Deacetylases in the Inflamed Intestinal Epithelium—Promises of New Therapeutic Strategies

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The intestinal epithelium is a complex, dynamic barrier that separates luminal contents from the immune compartment while mediating nutrient absorption and controlled passage of antigens to convey oral tolerance. A compromised epithelial barrier often leads to inflammation because immune cells in the lamina propria come into direct contact with luminal antigens. Defects in epithelial cell function were also shown to be involved in the etiology of inflammatory bowel diseases. These are severe, chronically relapsing inflammatory conditions of the gastrointestinal tract that also increase the risk of developing colorectal cancer. Despite major efforts of the scientific community, the precise causes and drivers of these conditions still remain largely obscured impeding the development of a permanent cure. Current therapeutic approaches mostly focus on alleviating symptoms by targeting immune cell signaling. The protein family of histone deacetylases (HDACs) has gained increasing attention over the last years, as HDAC inhibitors were shown to be potent tumor cell suppressors and also alleviate morbid inflammatory responses. Recent research continuously identifies new roles for specific HDACs suggesting that HDACs influence the cell signaling network from many different angles. This makes HDACs very interesting targets for therapeutic approaches but predicting effects after system manipulations can be difficult. In this review, we want to provide a comprehensive overview of current knowledge about the individual roles of HDACs in the intestinal epithelium to evaluate their therapeutic potential for inflammatory conditions of the gut.

Keywords: histone deacetylase, HDAC, inflammatory bowel disease, intestinal epithelium, HDAC inhibitor, inflammation

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INTRODUCTION

The intestinal epithelium is a highly dynamic tissue whose functional integrity is indispensable for proper gut homeostasis. Lining the inner walls of the gastrointestinal tract, it establishes the first line of defense from potential pathogens contained in ingested material but simultaneously allows controlled passage of nutrients and selected antigens. Functional defects of the intestinal epithelium can lead to severe dysregulations of gut homeostasis and are a hallmark of many chronic gastrointestinal conditions such as inflammatory bowel diseases (IBD) (1). IBD, with Crohn's disease (CD) and ulcerative colitis (UC) being the most frequent forms,

is characterized by chronically relapsing, exaggerated inflammation that involves drastic alterations in the microbiome and epithelial barrier function (2, 3). Despite rising incidence rates worldwide and extensive research, the precise etiology and drivers of IBD are still not clear with only few therapeutic options and no permanent cure available (4).

The promise of a new therapeutic approach arose when we could show that SAHA (Vorinostat), an inhibitor of histone deacetylases (HDACs), has the potential to alleviate intestinal inflammation in an IBD mouse model (5). Previously, HDAC inhibitors were mainly appreciated for their anticancer activity (6). At the time, most small molecules used in these studies were pan-HDAC inhibitors, meaning they inhibit all members of the classical HDAC family (7). In the mammalian genome, this protein family comprises 11 HDACs, that, are subdivided into three classes depending on their structure, enzymatic function, subcellular localization, and expression patterns (**Table 1**) (22). The eponymous function of epigenetic control via histone deacetylation is mainly implemented by class I HDACs while members of the other classes have either mainly non-histone targets or display a strongly reduced catalytic activity in their deacetylation domain and are considered to function rather *via* sequestering their targets than deacetylating them (22). HDACs are a phylogenetically very old protein family and are deeply rooted into the cellular signaling network. Therapeutic strategies that base on inhibiting all members of this family could therefore bear a certain disruptive potential, which might not be directly evident.

The environment of the intestinal mucosa adds an additional level of complexity to this issue, as different cell types are involved whose signaling network might rely on different HDACs with different functions. Many studies have looked into the role of HDACs in immune cells as the obvious mediators of inflammation (23). As increasing evidence over the last years also ascribed crucial immune regulatory functions to the intestinal epithelium, many recent studies also reported on the role of HDACs in the intestinal epithelium during inflammation. Here, we want to condense their results to provide a bigger picture about currently known inflammation-associated signaling pathways that involve HDAC signaling in intestinal epithelial cells (IECs) to help improve our understanding of the effects of HDAC inhibitor treatment on the intestinal epithelium. Additionally, to explore possibilities of a more targeted treatment, we outline the current knowledge about the roles of specific single HDACs in this context.

Pan-HDAC Inhibition

In the gut, HDAC inhibition is a naturally occurring mechanism that constitutes an integral part of homeostasis. Short chain fatty acids (SCFAs), such as butyrate, propionate, and acetate, produced by various bacterial communities of the microbiome mostly *via* anaerobic fermentation of dietary fibers, act as natural HDAC inhibitors (24, 25). In particular, besides serving as energy source for colonocytes, butyrate elicits a wide array of beneficial effects for gut homeostasis including suppression of pathological inflammation (26).

Considerable advances in understanding the importance of HDACs for inflammatory response pathways in IECs have been made by investigating the anti-inflammatory properties of butyrate and other SCFAs. The influence of butyrate on cell signaling can often be traced back to its ability to act as an HDAC inhibitor. For example, butyrate was found to support barrier function by increasing expression of IL-10 receptor α subunit (IL-10RA) via activation of STAT3 in human colon-derived cell lines Caco-2 and T84. In turn, IL-10RA increases tightness of the epithelial barrier by mediating downregulation of the pore-forming claudin-2. This mechanism depends on HDAC activity, as it can be reproduced by other pan-HDAC inhibitors, such as Trichostatin A (TSA) (27). Similarly, using cell lines and enteroids from mouse and human, the conductive effects of butyrate on the production of retinoic acid, an important immune regulator, could be ascribed to HDAC inhibition in IECs (17).

HDACs are most likely also the main mediators for conveying the effects of butyrate and propionate on nuclear factor kappa light-chain-enhancer of activated B-cells (NF-κB) signaling in response to Toll-like receptor (TLR) or TNFα stimulation. In cell culture models of human colon IECs, HDAC inhibition by butyrate and propionate increase TNFα and decrease IL-8 and MCP-1 expression in response to TLR5 stimulation (28). By contrast, during steady state, phenyl butyrate increases IL-8 and IL-18 production as well as TLR2-dependent expression of host defense peptides pEP2C, pBD-1, and pBD-3 in porcine IECs (29). HDAC inhibition by TSA dramatically increases the production of antimicrobial peptides, such as β-defensins, upon bacterial challenge in cell lines and organoids of human colon epithelium (30). TSA induces phosphorylation of the IκB kinase complex, which in turn phosphorylates inhibitor of NF-κB alpha (IκBα) and serine 10 of histone H3 activating NF-κB signaling and expression of target genes, respectively (30). Silencing of TLR2 or TLR4 increases overall HDAC activity and considerably mitigates the effects of phenyl butyrate on host defense peptide expression (29). Interestingly, TLR2 and TLR4 are two of the main receptors for recognizing extracellular high-mobility group box 1 (HMGB1), which plays an important role in the pathogenesis of IBD and whose secretion is also controlled by HDAC activity (31, 32). HMGB1 is typically localized in the nucleus but can be released into the extracellular space upon stress or tissue damage acting as a damage-associated molecular pattern (DAMP) that induces pro-inflammatory responses by binding its receptors (33). In a study investigating the anti-inflammatory effects of flavonoid isoliquirtigenin using HT-29 cells (human colon IECs), isoliquirtigenin prevented HMGB1 acetylation, leading to subsequent cytosolic translocation and secretion, by increasing HDAC activity (32). While HDACs appear to be negative regulators of TLR2 and TLR4, signal transduction of the intracellular virus-sensing receptor TLR3 heavily depends on HDAC activity. The HDAC inhibitor SAHA causes strong downregulation of TLR3 supposedly through upregulation of interferon response factor 8, which suppresses TLR3 transcription (34). Consequently, SAHA-treated IECs do not react to TLR3 stimulants with upregulation of TLR3-responsive target genes, such as IL-6, TNFα, and IFNβ, or

TABLE 1 | Superfamily of Zn2+-dependent histone deacetylases (HDACs) of the mammalian genome with subcellular localization and reported role in intestinal epithelial cells (IECs) during inflammation.

	HDAC	Subcellular localization	Reported role in IECs during inflammation	References
Class I	HDAC1	Nuclear	Negative regulator of STAT signaling, NF-κB signaling and acute phase response; involved in IL-1β-dependent cytokine production; positive regulator of the p38/MAPK pathway; downregulation of tight-junction proteins	(8–11)
	HDAC2	Nuclear	Positive regulator of inflammatory response and serotonin transporter; negative regulator of STAT signaling and expression of antibacterial lectins	(10, 12, 13)
	HDAC3	Nuclear and cytoplasmic	Negative regulator of retinoic acid metabolism and NF-κB signaling, deacetylation of p65; downregulation of tight-junction proteins; crosstalk with microbiome; activation of intraepithelial lymphocytes during infection	(11, 14–17)
	HDAC8	Nuclear and cytoplasmic	NA	
Class IIa	HDAC4	Nuclear and cytoplasmic	Invovled in acute phase response, interacts with C/EBPδ	(18)
	HDAC5	Nuclear and cytoplasmic	NA	
	HDAC7	Nuclear and cytoplasmic	NA	
	HDAC9	Nuclear and cytoplasmic	NA	
Class IIb	HDAC6	Nuclear and cytoplasmic	Positive regulator of NF-κB signaling; downregulation of tight-junction proteins	(19, 20)
	HDAC10	Nuclear and cytoplasmic	NA	
Class IV	HDAC11	Nuclear and cytoplasmic	Downregulation of tight-junction proteins in response to LPS	(21)

phosphorylation and activation of NF-κB and MAP kinases ERK and JNK (34). In contrast, antiviral defense mechanisms involving IFN-responsive gene induction in response to type III interferons are significantly increased in murine IECs when HDAC activity is hampered (35). The differential influence of HDAC activity on TLR signaling demonstrates the complexity by which HDACs affect certain cellular responses.

We showed recently that the pan-HDAC inhibitors SAHA and ITF2357 (Givinostat) protect the epithelial barrier integrity from TNFα-induced disruption by upregulating expression of tight junction proteins occludin and claudin-1 while downregulating claudin-2 in *in vitro* monolayer models of T84 and CMT93 (murine IECs) cells. HDAC inhibition further supports wound healing by upregulation of IL-8 and TGFβ during inflammation in cell lines and primary murine enteroids. Oral administration of ITF2357 significantly improves regeneration after acute DSS-colitis and alleviates symptoms of inflammation in mice (36). Another recent study linked the beneficial effects of HDAC inhibition in the intestinal epithelium during inflammation to expression changes of the IL-12 cytokine family (37). The heterodimeric members of this protein family can convey inflammatory or anti-inflammatory effects depending on their subunits and play important roles in intestinal inflammation (38). One of these subunits, Epstein-Barr virus-induced gene 3 (EBI3), becomes highly upregulated in human colon epithelial cells when TNFα treatment is combined with HDAC inhibition *via* Trichostatin A (TSA). Considering expression levels of other IL-12 subunits and activated signaling pathways in the cell, the authors suggest that the anti-inflammatory properties of HDAC inhibition in the intestinal epithelium are mainly conveyed through increased formation of the anti-inflammatory IL-35, which is also upregulated in acute phases of ulcerative colitis (37, 39). Strikingly, the anti-inflammatory effects of HDAC inhibition in a DSS-colitis mouse model are completely abolished and even reversed into exacerbation of the disease phenotype when *Ebi3* is silenced indicating a crucial role of EBI3 in mediating the beneficial effects of HDAC inhibition in intestinal inflammation (37).

Research with SCFAs and chemical pan-HDAC inhibitors demonstrates that HDACs play an important role in multiple inflammation-associated pathways in the intestinal epithelium. Yet, potentially distinct roles of single HDACs can only be inferred to a very limited degree from this data. However, considering HDACs as therapeutic targets, pan-inhibition might be neither a necessary nor the safest option. Targeting only single HDACs is likely more efficient and limits undesired off-target effects. The field of HDAC research is still relatively young and the tissue specific expression patterns and functions make their study even more challenging. Nevertheless, many recent studies provided new insights into the functions of single HDACs in the IECs.

Class I HDACs

Class I HDACs are the most intensively studied group of this protein family and are often considered the "true" HDACs since they exert epigenetic control through deacetylation activity toward histones. In rat IEC-6 cells, the class I HDAC HDAC1 was shown to control global acetylation levels (40). Alterations in activity of class I HDACs often lead to profound, global changes of histone acetylation patterns and associated gene expression. In active sites of UC or CD, IECs exhibit significantly decreased levels of histone H3 acetylation compared to healthy controls suggesting increased HDAC activity (11, 41). Indeed, HDAC activity also increases measurably in the inflamed colonic epithelium of mice treated with DSS (41). Paradoxically, most HDAC transcripts, including the class I HDACs HDAC2,

HDAC3, and HDAC8, are downregulated in the epithelium of active IBD patients (36). HDAC1 mRNA levels do not change during inflammation indicating a special role (36).

Indeed, HDAC1 was reported to be an important regulator of inflammatory responses in IECs but also to be involved in certain aspects of homeostasis. Silencing of *HDAC1* impairs cell proliferation and alters cell morphology of rat IECs (8). These effects are most likely an indirect consequence of metabolic reprogramming including downregulation of homeostatic processes and upregulation of survival pathways (40). The cells produce less ATP but are more resistant to nutrient deficiency and oxidative stress (40). In terms of inflammation, HDAC1 depletion causes prolonged activity of the acute phase response and NF-κB signaling by retention of phosphorylated C/EBPβ and phosphorylated p65 in the nucleus upon IL-1β stimulation (8). Interestingly, HDAC1 silencing causes elevated levels of certain inflammatory cytokines in response to IL-1β, such as Cx3cl1, Timp1, and Cxcl2, while others are decreased, such as Cxcl5 and β-NGF (8). *In vitro*, HDAC1 becomes upregulated in human IECs when stimulated with IL-4, IL-5, IL-13, MCP-1, or TNFα, all being activators of the p38/MAPK pathway (9).

HDAC2 is in many ways closely associated to HDAC1 signaling. Certain DNA-binding multiprotein complexes, such as Sin3A, NuRD, or CoREST, require incorporation of HDAC1 and HDAC2 as heterodimer to exert their biological activity and HDAC2 protein levels increase after *Hdac1* silencing suggesting some form of substitution (8, 42). Epithelial HDAC1 and HDAC2 are of critical importance for intestinal homeostasis as simultaneous deletion of both genes in IECs of adult mice leads to profound dysregulations across multiple cell signaling pathways (43). This involves altered tissue architecture caused by an increased proliferative and migratory activity of IECs, differentiation defects affecting especially secretory lineages leading to decreased numbers of goblet cells and Paneth cells, and increased expression of inflammation-associated genes inflicting weight loss and colon shortening (43). Mechanistically, these effects were traced back to changes in the expression levels of certain key regulators. Increased expression of Cyclin D and targets of the mTOR pathway affect cell proliferation and division while elevated levels of activated Notch shift cell fate determination from a secretory to an absorptive phenotype (43). In addition, IEC-specific *Hdac1/2* deletion decreases expression levels of tight junction protein claudin-3 thereby weakening the intestinal barrier and leading to activation of inflammatory regulators, such as Stat3 (43). Combined with reduced microbial protection due to decreased secretion of mucus and antimicrobial products from a diminished number of secretory cells, the tissue exhibits a phenotype of basal chronic inflammation with increased immune cell infiltration (43). Accordingly, IEC-specific *Hdac1/2* knockout mice suffer considerably aggravated symptoms when subjected to DSS-induced colitis (12). Interestingly, IEC-specific deletion of *Hdac2* alone appears to protect mutant mice from DSS colitis as they lose less weight and retain a higher epithelial barrier integrity compared to wild type mice (12). Immune programs are strongly downregulated in these mice while antibacterial lectins, such as Reg3b and Reg3g are strongly increased (12). Silencing of *HDAC2* in Caco-2 cells additionally decreases expression of the transporter of serotonin, whose expression is commonly dysregulated in inflammatory bowel disease (13). Comprehensive analysis of murine intestinal organoids with a *Hdac1* or *Hdac2* deletion suggests that HDAC2 influences the intestinal immune response and regulation of the intestinal barrier function through its involvement in xenobiotic signaling and the aryl hydrocarbon receptor-mediated response to endogenous and exogenous ligands (10). STAT signaling is increased after *Hdac1* or *Hdac2* knockout suggesting them as negative regulators for this pathway (10).

HDAC3 is important for a variety of epithelial cell functions particularly concerning cross-talk with the microbiome. Mice with an IEC-specific *Hdac3* knockout are more susceptible to DSS-induced inflammation and intestinal damage (14). This phenotype may in part be caused by increased activation of NF-κB. HDAC3 was previously shown to restrict NF-κB activity by deacetylating p65 promoting its nuclear export and binding to IκBα (15). Therefore, HDAC3 might be the main mediator for the reported activating effect of phenyl butyrate on NF-κB signaling (29). IEC-specific *Hdac3* knockout mice also display loss of Paneth cells, impaired IEC function, decreased expression of antimicrobial peptides, and altered composition of commensal bacteria (14). Interestingly, this phenotype can be rescued by transferring the animals to germ-free conditions suggesting that HDAC3 is necessary for integrating signals from the microbiome during homeostasis (14). IEC-intrinsic HDAC3 has also been shown to regulate activation of IFNγ-producing intraepithelial lymphocytes by inducing IL-18 expression in the epithelium upon bacterial infection (16).

The class I HDACs HDAC1, HDAC2, and HDAC3 are evidently involved in the regulation of the inflammatory response in IECs. However, describing a precise mechanism of action is still challenging. The available data raise the possibility that the anti-inflammatory properties of pan-HDAC inhibitors are mostly mediated through inhibition of class I HDACs. Inhibition of additional members of the HDAC family might not add to the desired result unnecessarily increasing the risk of off-target effects. For example, the effects of butyrate on STAT signaling and retinoic acid metabolism (see above) might mainly be due to decreased HDAC1 and HDAC2 activity as silencing either is sufficient to reproduce this effect (10, 17). Indeed, symptoms of intestinal inflammation in a DSS-colitis model can also be alleviated with more specific inhibitors, such as MS-275 (Entinostat) that inhibits mainly HDAC1 and HDAC3 activity (11, 44). Inflammation-induced reduction of acetylation, activation of NF-κB, and downregulation of tight-junction proteins zonula occludens 1 (ZO-1) and occludin are all reversed by MS-275 treatment (11). The enhancing effect of pan-HDAC inhibition on IFN-responsive gene induction in response to type III interferons in murine IECs can also be reproduced by inhibiting HDAC1 and HDAC3 alone *via* MS-275 (35). Class I HDACs could drive inflammation by controlling expression of certain key regulators, such as the vitamin D receptor (11), but also by deacetylating proteins of the inflammation signaling chain, such as p65, thereby affecting their activity (15).

Class II HDACs

HDACs of class II are further subdivided into class IIa, containing HDAC4, HDAC5, HDAC7, and HDAC9, and class IIb, containing HDAC6 and HDAC10. Class IIa HDACs influence gene expression by interacting with various transcription factors mostly suppressing their activity. Conserved residues in the protein sequence of class IIa HDACs can be phosphorylated triggering nuclear export (22). To date, the roles of most class II HDACs in the intestinal epithelium are only scarcely investigated. Class IIa HDACs were described as crucial components of protein kinase D1 (PKD1)-dependent mitogenic signaling (45). HDAC4 might play a role in the acute phase response during inflammation as it interacts with C/EBPδ, a key regulator of haptoglobin expression, in cultured IEC models (18). Epithelial HDAC7 was found to be positively associated with development of colorectal cancer (46).

HDAC6 represents a very interesting therapeutic target in intestinal inflammation, as it was recently shown to be important for NF-κB signaling. In a human colonic cell line, the HDAC6-specific inhibitor CKD-506 blocks phosphorylation of IκBα, suppresses IL-8 secretion, and inhibits DNA binding of the NF-κB complex (20). In mouse models of experimental colitis, oral administration of CKD-506 significantly improves symptoms of intestinal inflammation (20). A similarly beneficial effect of HDAC6 inhibition has been found in the context of reperfusion damage of the intestine after hemorrhagic shock (HS). Inhibition of HDAC6 *via* Tubastatin-A prevents loss of tight junction proteins claudin-3 and ZO-1 and attenuates injury-induced tissue alterations, such as villous blunting, epithelial necrosis, and immune cell infiltration in a murine HS model (19).

Class IV HDAC

The only class IV HDAC, HDAC11, has been suggested to play a role in LPS-induced downregulation of tight-junction proteins and subsequent loss of barrier integrity. In human intestinal epithelial cells, Vitamin D protects LPS-induced loss of barrier integrity by upregulation of its receptor, which sequesters HDAC11 and prevents its recruitment to the DNA (21). Chromatin immunoprecipitation revealed ZO-1, claudin-5, and occludin as targets of HDAC11, which binds to their promoters and impairs gene transcription in response to LPS stimulation (21).

CONCLUDING REMARKS

An increasing number of independent studies show that HDACs influence inflammation and barrier function in the intestinal epithelium. Therefore, epithelial HDACs definitely represent promising therapeutic targets that could help to control inflammation and protect barrier integrity in diseases like IBD. HDACs are involved in many inflammatory signaling pathways *via* direct interaction with key regulators or influencing their gene expression. Although many mechanisms of action have already been identified for single HDACs, drawing a comprehensive picture still proves challenging. Especially class I HDACs affect a large number of cellular responses due to their epigenetic activity and extensive effects on gene expression. Data on the role of class II and IV HDACs in IECs is still very limited but they could represent more specific therapeutic targets since they do not affect global histone acetylation levels to the same extend as class I HDACs. This, however, remains to be clarified by future studies.

A major weak point of current HDAC research is that many studies that report a certain role for HDACs in IECs rarely focus on single HDACs or even have the function of HDACs as a primary study goal. HDACs often appear as a side note, a secondary finding that happened to be connected to the initial point of interest. Further elaborations on the precise underlying modes of action that integrate specific HDACs into the signaling pathway under investigation are often missing. However, the fact that HDACs appear in so many different contexts, especially with a focus on inflammation, shows the enormous potential that lies within detailed knowledge of their individual roles. Future studies, which aim directly at deciphering the role of specific HDACs in distinct cell types, are necessary to build on current knowledge and enable novel therapeutic strategies for IBD and other inflammatory diseases by precise modulation of HDAC activity.

AUTHOR CONTRIBUTIONS

LG and RG organized the review structure. LG performed the bibliographic research and wrote the manuscript. Both authors were involved in editing the paper and had final approval of the submitted version.

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REVIEW
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Innate Lymphoid Cells as Regulators of Epithelial Integrity: Therapeutic Implications for Inflammatory Bowel Diseases

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The occurrence of epithelial defects in the gut relevantly contributes to the pathogenesis of inflammatory bowel diseases (IBD), whereby the impairment of intestinal epithelial barrier integrity seems to represent a primary trigger as well as a disease amplifying consequence of the chronic inflammatory process. Besides epithelial cell intrinsic factors, accumulated and overwhelmingly activated immune cells and their secretome have been identified as critical modulators of the pathologically altered intestinal epithelial cell (IEC) function in IBD. In this context, over the last 10 years increasing levels of attention have been paid to the group of innate lymphoid cells (ILCs). This is in particular due to a preferential location of these rather newly described innate immune cells in close proximity to mucosal barriers, their profound capacity to secrete effector cytokines and their numerical and functional alteration under chronic inflammatory conditions. Aiming on a comprehensive and updated summary of our current understanding of the bidirectional mucosal crosstalk between ILCs and IECs, this review article will in particular focus on the potential capacity of gut infiltrating type-1, type-2, and type-3 helper ILCs (ILC1s, ILC2s, and ILC3s, respectively) to impact on the survival, differentiation, and barrier function of IECs. Based on data acquired in IBD patients or in experimental models of colitis, we will discuss whether the different ILC subgroups could serve as potential therapeutic targets for maintenance of epithelial integrity and/or mucosal healing in IBD.

Keywords: innate lymphoid cells, intestinal epithelium, inflammatory bowel diseases, cytokines, ILC plasticity, colitis

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INTRODUCTION

Worldwide, approximately seven million patients are diagnosed with inflammatory bowel diseases (IBD) (1), a chronically remitting inflammatory disease of the gastrointestinal tract. Both entities of IBD, Crohn's disease (CD) and ulcerative colitis (UC), commonly lead to severe intestinal symptoms like chronic diarrhea, abdominal pain, rectal bleeding, and anemia, thus significantly limiting the physical fitness, work ability, and overall quality of life of the affected and often young patients (2–4). Due to a complex and multifactorial pathogenesis, which is crucially influenced by

genetic, environmental, microflora-related, and immunological components, there still exists no causal therapy for IBD (5, 6). Instead, established treatment strategies focus on the control of clinical symptoms, mainly by either inhibiting the overwhelming accumulation and activation of intestinal immune cells or by promoting mucosal healing (7, 8). During the last two decades, the spectrum of available therapeutic regimens underwent a significant shift from a predominance of rather unspecific immunosuppressive drugs, like glucocorticoids, thiopurines, and methotrexate, toward an increasing and often preferential use of biological agents, like anti-TNF antibodies, the anti-IL-12/IL-23 antibody ustekinumab or anti-adhesion strategies, which in principle allow to specifically interfere with disease-driving signaling cascades (8). However, still approximately 40% of IBD patients do not show a satisfactory primary response even to these optimized therapeutic regimens or develop a secondary loss of response (5, 9), emphasizing the urgent need to further fine-tune the definition of therapeutic target structures, and/or to better take into account the full spectrum of interacting cellular players involved in the pathogenesis of IBD. In this context, it will be of particular importance to better elucidate the complex crosstalk between intestinal epithelial cells (IECs) and subepithelial innate immune cells, which together form an early and tightly regulated line of host defense against invading luminal pathogens. Consequently, achieving improved insights into the capacity of lamina propria innate immune cells to interfere with the maintenance of epithelial integrity or the resolution of epithelial defects might subsequently pave the way for therapeutic approaches that pursue a two-pronged strategy: Epithelial restoration and suppression of overwhelming immune activation.

Within the mucosal immune cell compartment, the group of innate lymphoid cells (ILCs) can be characterized by their typical localization in direct spatial proximity to the epithelial surface (10, 11) and by their exceptional capability to initiate an early and rapid response to invading pathogens and epithelial damage (12–14). Although resembling T cells morphologically and in several functional aspects, ILCs are classic representatives of the innate immune system and can thus be distinguished by the lack of rearranged antigen-specific receptor expression and their ability to achieve a full activation status independent from the antigen-presentation and -recognition machinery. This predisposes ILCs for promoting and regulating early defense mechanisms (13). In analogy to T helper (Th) cells and mainly based on their cytokine and transcription factor profile, mature helper ILCs can be categorized into type-1 (ILC1s), type-2 (ILC2s), and type-3 ILCs (ILC3s) (15). Accordingly, ILC1s are associated with type-1 immune responses classically directed against intracellular pathogens and tumor cells, functionally depend on the transcription factor T-bet and preferentially secrete the pro-inflammatory cytokines IFN-γ and TNF-α (16, 17). Besides helper ILC1s, NK cells represent another ILC1 subset, which is characterized by a potent cytotoxic effector function (16) and whose impact on the maintenance of mucosal homoeostasis has been reviewed elsewhere (18). As an important cellular source of the effector cytokines IL-4, IL-5, IL-9, and IL-13 and, hereby, as an integral cellular component of mucosal type-2 immune responses, ILC2s are crucially involved in the immunological control of parasitic worm infections and in allergic diseases. Like in Th2 cells, GATA-3 and RORα represent important signature transcription factors in ILC2s (19–21). Finally, ILC3s depend on the transcription factor RORγt and can be subdivided into lymphoid tissue inducer (LTi) cells, which play a significant role during lymphoid organogenesis, as well as NCR^+ and NCR^- ILC3s as relevant producers of IL-17A, IL-22, and GM-CSF at mucosal sites, appearing accordingly in many functional aspects like innate counterparts of Th17 cells (22, 23). An important functional attribute, which is common to all local helper ILC pools in mucosal organs, is their early activation in response to pathological tissue damage and their subsequent capacity to support, amplify, and modulate local adaptive immune responses (24, 25). While this sequential cascade of primary or externally triggered epithelial damage, subsequent ILC activation via epithelial cell-released factors and, finally, the ILC-mediated modulation of adaptive immune responses has drawn a lot of scientific attention during the last decade (15, 24, 26, 27), the opposite direction, meaning the ILC-to-epithelium communication, often seemed to fade a bit into the background. However, several studies described a significant influence of the ILC compartment on tissue regeneration, resolution of inflammatory tissue damage, and mucosal barrier integrity (28–32), although several details of the underlying mechanisms and the particular contribution of the different types of epithelial cells still remain incompletely understood. Due to the implicated high significance of the bidirectional crosstalk between locally accumulating ILCs and epithelial cells for maintenance of mucosal homeostasis in the gastrointestinal tract and its potential therapeutic targetability in IBD, we will here summarize the insights in the capacity of helper ILCs to influence the fate of different epithelial cell types in the gut under physiological and inflammatory conditions and discuss potentially resulting therapeutic perspectives.

INTESTINAL ILCS IN HEALTHY AND CHRONIC INFLAMMATORY CONDITIONS

The distinct distribution of the three classical helper ILC subtypes observed at different anatomical sites upon homeostasis implies a specific function of each local subset in non-diseased tissues (**Figure 1**). In the human gastrointestinal tract $NKp44^+$ ILC3s represent the main helper ILC population in the caecum, ileum, and colon, while ILC1s predominantly populate the upper gastrointestinal tract, including esophagus, stomach, and duodenum (33, 34). In contrast, ILC2s constitute only a small ILC population throughout the whole healthy intestine (33–36). However, when differentiating between lamina propria and intraepithelial ILCs more precisely, a distinct ILC2 population could be observed in the intraepithelial compartment of the colon together with a large ILC1 pool and only a minor fraction of ILC3s (35), suggesting a distinct function of each helper ILC subset in the gut under homeostatic conditions. In addition to the three classical helper ILC subsets, regulatory ILCs (ILCregs), which resemble regulatory T cells in several key

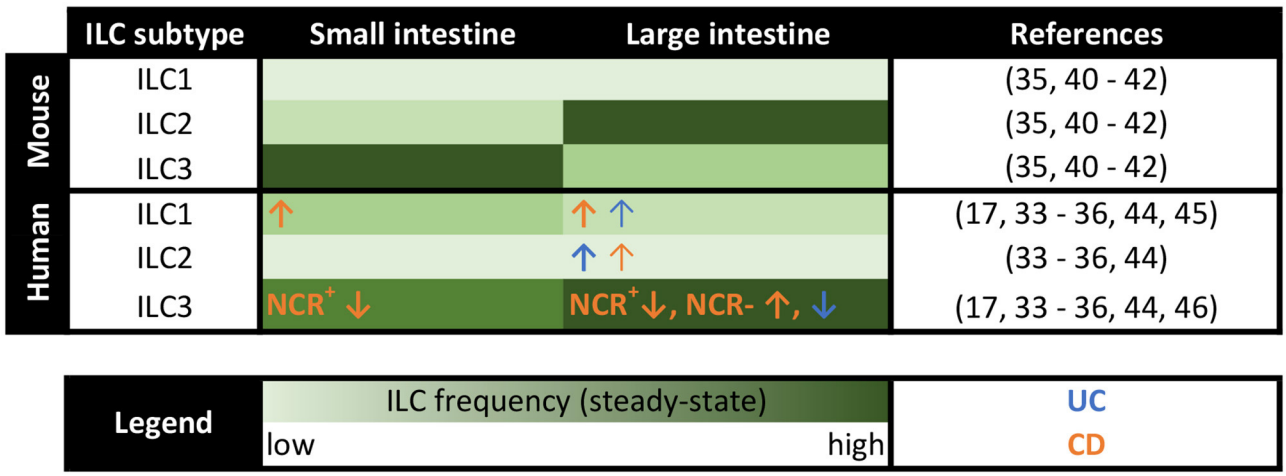

FIGURE 1 | Distribution of murine and human ILCs along the intestine in steady-state and IBD patients. Heatmap summarizing the distribution of the classical helper ILC subsets in the lamina propria of the small and large intestine in both mice and humans. The frequency of ILCs is color-coded with the light green color indicating low frequencies and the dark green color representing high ILC percentages. Changes in local ILC frequencies in patients with ulcerative colitis (UC) and Crohn's disease (CD) are indicated by blue and orange arrows, respectively.

features, were suggested to exist in both human and murine intestines. ILCregs were initially defined by their constitutive expression of IL-10 and were found to primarily accumulate in the lamina propria of the small intestine (37). Their existence, however, is still a matter of controversial discussion, given the inability of Bando and colleagues to detect ILCregs in the murine gut based on the expression of IL-10 in their study. Furthermore, they could not identify a helper ILC subset distinct from ILC1s, ILC2s, and ILC3s using mice from different breeding facilities in both steady-state and under inflammatory conditions (38). Instead, the authors described ILC2s as inducible source of intestinal IL-10 production (38), which is in line with the findings from other groups (39), raising the idea of IL-10-producing ILC2s rather than the existence of a distinct ILCreg subset.

Importantly, the murine intestine is populated by differently distributed ILC classes compared to human intestines (**Figure 1**). Unlike in humans, ILC2s make up a clear intestinal cell population in naive mice, which even outnumbers ILC3s or ILC1s in the lamina propria of the large intestine (35, 40). In the intraepithelial compartment and the small intestine, in contrast, murine ILC3s turned out as dominant ILC subtype (35, 41, 42). In total, murine ILCs were specifically enriched in the lamina propria of the large intestine (35). Keeping the species-specific differences in mind is important when assessing the translational relevance of results obtained in the murine organism. Additionally, the use of immunodeficient $Rag1^{-/-}$ mice and, thus, the absence of functional adaptive immune cells in many murine *in vivo* ILC studies might bias the obtained results (13), emphasizing the need for confirmatory human studies. Nevertheless, the ability of highly controlled breeding, housing, and the availability of elegant genetic knockout mouse models, makes murine studies in the field of intestinal ILCs inevitable. The intensified consideration of humanized mouse models, in which the function of primary human ILCs can be analyzed under experimentally defined *in vivo* conditions, might even allow for better transferability of acquired data to the clinical context of human diseases (43).

Significant alterations in local ILC pools were observed in inflamed areas in IBD patients compared to unaffected control tissue (**Figure 1**), indicating a functional role of ILCs in chronic inflammation of the gut. While $NKp44^{+}$ ILC3s constitute the dominant helper ILC population in the lower gastrointestinal tract in homeostasis (33, 34), their frequency was markedly reduced at sites of active inflammation in patients suffering from IBD, including both UC and CD (17, 34, 44). This ILC3 decrease further correlated with severe disease cases (34), highly suggesting a regulatory or protective function of ILC3s in intestinal inflammation. Contrary to $NKp44^{+}$ ILC3s, the percentage of ILC1s, ILC2s, and $NKp44^{-}$ ILC3 was found to be increased in IBD patients (34, 44–46). Especially in CD patients an enhanced percentage of intestinal ILC1s has been described in multiple studies (17, 34, 44) and was obviously associated with an advanced disease severity (34). Regarding the underlying mechanism for the accumulation of ILC1s in the inflamed intestine of CD patients, transdifferentiation of other ILC subtypes into ILC1s was suggested to take place in the IL-12-enriched microenvironment of the inflamed gut of CD patients (25). In *in vitro* experiments, ILC2s, ILC3s as well as c-$Kit^{+}NKp44^{-}$ immature ILCs were described to transdifferentiate into IFN-γ-secreting ILC1-like cells in the presence of IL-12 (17, 36, 47–50). And indeed, an increased local secretion of IL-12 was reported in CD patients (51, 52). Moreover, the biological relevance of this *in vitro* induced ILC3-to-ILC1 transition could be reinforced by an inverse link of ILC3 and ILC1 frequencies in the inflamed mucosa of CD patients (17, 34, 44) and the presence of an ILC subgroup

harboring both ILC3 and ILC1 characteristics in human ileal LPMCs (53). Similarly, IL-13^{+}IFN-γ^{+} ex-ILC2s were detected in the intestine of CD patients (48), hinting at ILC2-to-ILC1 transitions *in vivo*. In contrast to patients with CD, the intestinal tissue of UC patients was associated with an accumulation of NKp44^{-} ILC3s, which correlated with severe illness (34), making ILC1s and NKp44^{-} ILC3s specifically important in CD and UC, respectively. Although the scientific debate on the existence of ILCregs is still ongoing (37, 38), experimental models of innate colitis revealed an enhanced frequency of IL-10-producing ILCs, which the authors defined as ILCregs, upon intestinal inflammation, temporally following an increase of ILC3s and ILC1s. Accordingly, the transfer of ILCregs into *Rag1*$^{-/-}$*Il10*$^{-/-}$ mice resulted in reduced signs of innate colitis, thus claiming the importance of ILCreg expansion for the resolution of intestinal inflammation (37). Focusing on the intraepithelial compartment, intraepithelial ILC1s were described to be increased in CD patients (54), which, however, could not be confirmed in a later study (34), making further research on intraepithelial ILCs necessary, especially since their prime location in direct proximity to the intestinal epithelium might predispose them for impacting on the integrity of the epithelial layer. Given that altered ILC frequencies were predominantly observed at inflamed intestinal sites but were absent in non-inflamed areas (34), suggests an active role of intestinal ILCs in inflammatory processes but argues against a primary and disease-predisposing alteration of the ILC compartment in IBD patients.

Based on these numerical ILC alterations observed in the inflamed gut of patients suffering from IBD, the next section will discuss our current knowledge on the functional role of local ILCs in intestinal inflammation with a particular focus on their capacity to interact and regulate IEC functions.

ILC–IEC INTERACTIONS

Enterocytes represent the most frequent cell type in the intestinal epithelium and as such build the fundament for a tight barrier between gut lumen and tissue. This is achieved by tight junctions, which connect the enterocytes to form a robust but selectively permeable wall, allowing a targeted paracellular transport. Together with the transcellular transport through enterocytes, this enables them to absorb nutrients and antigens from the gut lumen in a highly controlled fashion (55), while simultaneously forming an effective first line of defense for pathogens. When disrupted, however, overwhelming invasion of pathogens can cause severe inflammatory immune responses within the intestinal mucosa (56), making a tightly controlled regulation inevitable.

Several studies have described a crucial involvement of ILC3s in the regulation of epithelial integrity in the gut, which has been attributed mainly to their capacity to secrete the effector cytokine IL-22 (57–60). Based on the well-known protective functions of IL-22 in IBD (61) and the fact that ILC3s are thought to be the main producers of IL-22 in the homeostatic and inflamed murine gut (58, 60), the favorable functions described for IL-22 on IECs might be largely assigned to ILC3s. Indeed, ILC3-derived IL-22 could be demonstrated to play a protective role on dextran sulfate sodium (DSS)-induced tissue disruption (57–60). DSS-induced colitis represents a widely used model for UC, that is initiated by the chemical disruption of the intestinal epithelium, resulting in colonic inflammation (62), and thereby makes up an ideal model system to study the influence of ILCs on gut barrier integrity. Whereas, under homeostatic conditions ILC3 activity is largely repressed by IL-25 and adaptive immune responses, this repression is downregulated upon DSS-induced tissue disruption and inflammation, allowing the secretion of the protective cytokine IL-22 by intestinal ILC3s (60). In IBD patients an increased IL-22 expression was observed exclusively in ILC3s rather than T cells (63), fortifying the relevance of ILC3-derived IL-22 on gut barrier integrity in humans (**Figure 2**). Interestingly, the murine norovirus has been shown to take advantage of this ILC3-IL22-IEC-axis in order to protect IECs against tissue disruption (64). Besides the beneficial effect of ILC3s on the barrier function of the intestinal epithelium, they were additionally described to directly foster the glycosylation of eptihelial cells via their effector cytokines IL-22 and lymphotoxin (32) (**Figure 2**). In general, the colonic glycocalyx functions as additional barrier for pathogens, but also enables communication and adherence of specific bacteria (65). In mice, ILC3s were detected to mediate epithelial fucosylation via the induction of the responsible enzyme fucosyltransferase 2, which turned out to be important for host protection against *Salmonella typhimurium* infection (32).

In IBD patients, decreased frequencies of NKp44^{+} ILC3s were detected at inflamed intestinal sites compared to samples from non-inflamed IBD and non-IBD subjects (17, 34) which was significantly associated with an increased endoscopic disease severity score in both CD and UC patients (34). Since NKp44^{+} ILC3s represent the main producers of IL-22 in the adult intestine (66), the lack of the protective IL-22 effect on the epithelial barrier in IBD patients might at least partially explain the gut barrier disruption. However, in another study, increased IL-22 expression levels have been observed in colonic tissue samples derived from CD and UC patients which could be shown to result from NKp44^{+} ILC3s (63). Interestingly, the presence of fecal microbiota clearly triggered IL-22 production in human LPMCs (63) and potential differences in the composition of the gut microbiota might thus explain, at least partly, the controversy of published data on the intestinal IL-22 levels in IBD patients. Based on findings acquired in innate experimental colitis models (e.g., a model of anti-CD40-induced colitis), ILC3-derived IL-22 could be demonstrated to even have a pathogenic effect (67), indicating a double-edged role of ILC3-derived IL-22 in IBD (**Figure 2**) that might depend on the local micromilieu and microbiota.

Although less abundant in the gut compared to ILC3s (34), ILC2s can significantly contribute to the preservation and restoration of the intestinal integrity as well (**Figure 2**). Findings in other organs with a barrier function, like the lung and skin, support this idea. ILC2s could for example be shown to facilitate wound healing of the skin (68), and ILC2-derived

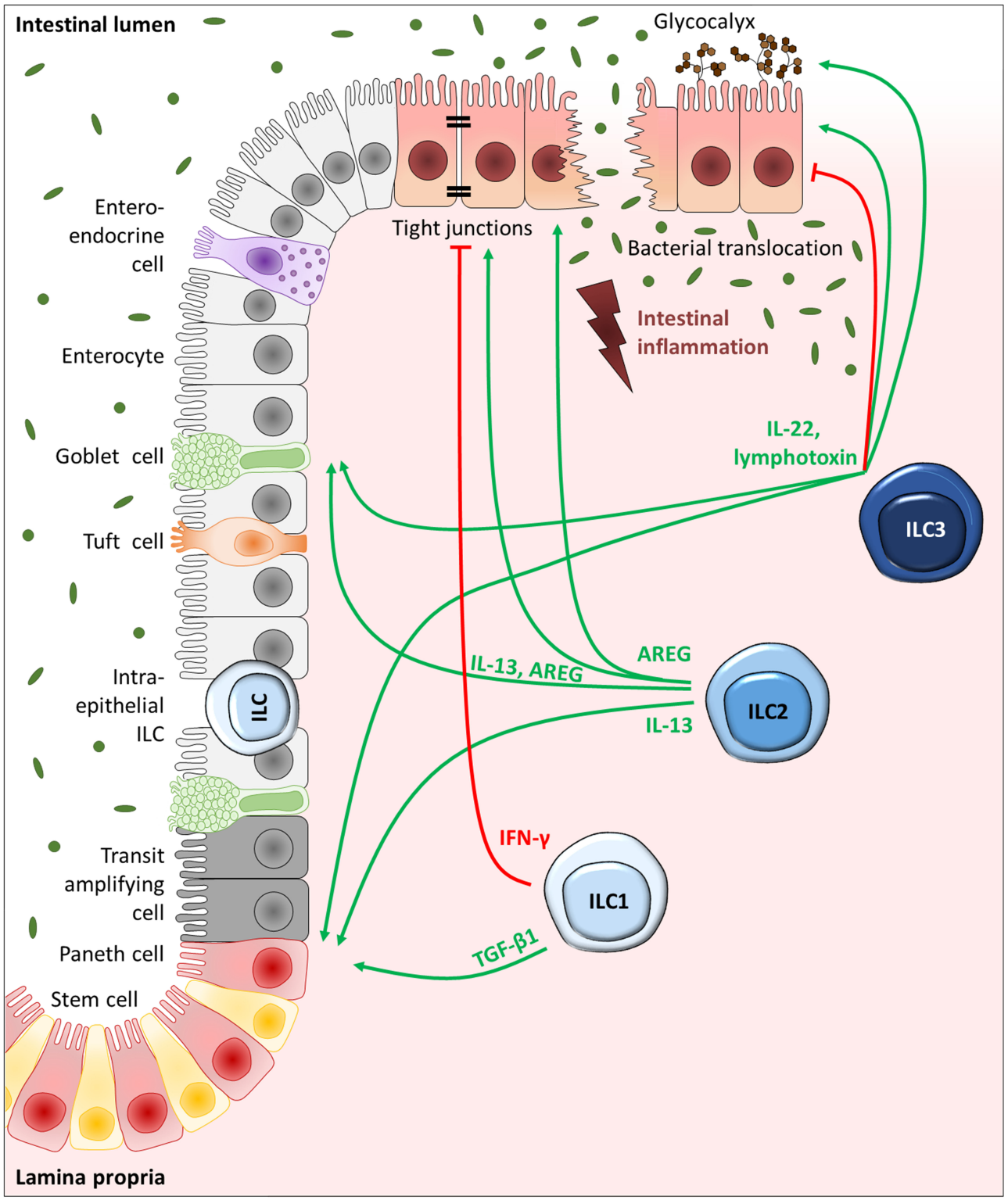

FIGURE 2 | ILC-driven regulation of IECs in intestinal inflammation. Schematic depiction of the intestinal epithelium, consisting of goblet cells, tuft cells, enteroendocrine cells, and M cells dispersed throughout the enterocytes as well as transit-amplifying progenitor cells, paneth cells, and stem cells localized toward the crypt bottom. ILC1s, ILC2s, and ILC3s reside in the mucosa in close proximity to the epithelium or can be directly positioned in between IECs as intraepithelial ILCs, giving them prime positions to interact with IECs. While IECs are important activators of ILCs via the release of selective alarmins, ILCs can in return control the

(Continued)

FIGURE 2 | different IEC subtypes via the release of effector cytokines. With the secretion of IL-22 and lymphotoxin, ILC3s can drive stem and progenitor cell proliferation and differentiation. ILC3s can additionally drive mucus production by goblet cells and promote fucosylation of enterocytes. The effect of ILC3-derived IL-22, however, is largely dependent on the microenvironment. ILC2-driven IEC regulation is mainly based on their ability to release IL-13 and AREG, which can trigger stem and progenitor cells, goblet cells as well as robust tight junctions interconnecting enterocytes. Moreover, ILC1-derived TGF-β1, although not a classical type-1 cytokine, can drive stem cell proliferation and differentiation, while IFN-γ secreting ILC1s can weaken the epithelial stability.

amphiregulin (AREG) was suggested as effective player in the epithelial restoration of influenza virus-infected lungs (30). Indeed, first data acquired in the gut could underpin this, showing a tissue-protective role of ILC2s in a mouse model of acute DSS colitis via the secretion of the epidermal growth factor AREG (69). Here the authors suggest a circuit of damaged epithelial cells, releasing the alarmin IL-33 and thereby activating AREG production in ILC2s, which ameliorates DSS-induced tissue disruption, likely via the upregulation of Claudin-1 and Mucin 2 (Muc2) (see section Goblet cells—ILCs for further details on *Muc2*). Claudin-1 represents a well-known tight junction protein (70), whose increased expression favors tightly connected enterocytes forming an efficient barrier in the gut. The observation of a protective function of ILC2-derived AREG was further confirmed in a mouse model of acute gastrointestinal graft-vs.-host disease, in which the intravenous injection of ILC2s could significantly reduce intestinal leakiness in an AREG-dependent manner (71). In contrast to these results, one research group observed detrimental effects of both murine and human ILC2s on the tight junctions of bronchial epithelial cells and epidermal keratinocytes via the secretion of IL-13 (72, 73). These opposing results might indicate the presence of polyfunctional ILC2s (69) which can adapt to local requirement based on the specific microenvironment to gain either protective or destructive functions on epithelial barriers. In the inflamed gut, however, current knowledge indicates a beneficial effect of ILC2s on the intestinal barrier integrity (69, 71). This was additionally indirectly supported by the finding, that the increased intestinal permeability observed in $Itk^{-/-}$ mice compared to wildtype mice upon acute DSS colitis was associated with a gut-specific reduction of ILC2s. Injection of IL-2 complexes, however, could both, restore gut ILC2 frequencies and diminish disease severity (74). In IBD patients an increased ILC2 frequency was observed at inflamed intestinal sites (34), suggesting a certain clinical relevance. Just recently, a regulatory subset of IL-10 producing ILC2s was described to have a protective effect in the context of grass-pollen allergy and lung inflammation (75, 76). This was not only mediated by their ability to dampen disease-driving type-2 immune responses (75, 76), but additionally relied on the restoration of the epithelial barrier (75). Whether this regulatory ILC subset is relevant in the inflamed gut as well, has to be determined in future studies, though. First hints, however, imply the potential formation of IL-10 producing ILCs in the inflamed gut mucosa: The transdifferentiation of ILC2s into IL-10^{+} ILC2s or ILCregs interestingly turned out to depend on the vitamin A metabolite retinoic acid (75, 77), elevated levels of which have been reported in patients with active UC (78).

Likewise, ILC1s turned out to accumulate in the inflamed ileum of CD patients (17), pointing toward a functional role in IBD-associated tissue disruption as well. In line with this, the frequency of intraepithelial ILC1s was shown to be increased in the small intestine of CD patients (54). Based on their prime position in immediate proximity to IECs, it can be assumed that intraepithelial ILC1s might play a special role in the regulation of the intestinal epithelial barrier. Further evidence derived from a mouse model of experimental colitis, showing a marked IFN-γ production by intraepithelial ILC1s and to a lesser extent also by plastic ILC3s. Deletion of intraepithelial ILC1s with an anti-NK1.1 antibody was associated with reduced epithelial disruption and a diminished accumulation of inflammatory cells (54), proposing a negative influence of ILC1s on colitis-associated barrier destruction. In the context of celiac disease, increased percentages of intraepithelial $NKp44^{-}$ cytotoxic ILC1s have been reported in the human small intestine, which significantly correlated with an increased IFN-γ production as well as an increased disease severity and epithelial breakdown (79). In addition, enhanced levels of ILC1- and ILC3-derived IFN-γ and TNF-α upon simian immunodeficiency virus infection in rhesus macaques associated with an increased loss of colonic tight junctions, resulting in epithelial instability and increased microbial translocation (80), supporting a detrimental functional role of ILC1s on the maintenance of an intact epithelial gut barrier (**Figure 2**). A clear proof of ILC1s directly interacting with IECs, however, is still lacking to date, making further research interesting.

Next to enterocytes, the intestinal epithelium consists of highly specialized cell types, including goblet cells, tuft cells, stem cells, paneth cells, enteroendocrine cells, microfold cells, and cup cells (81, 82). While each cell type contributes to the integrity of the epithelium by its unique function, the following section takes a closer look on the direct impact of ILCs on the differentiation and functions of these cell types.

Goblet Cells—ILCs

Goblet cells represent the secretory cell type of the intestinal epithelium specialized for the production and secretion of mucus components. By the release of mucus, goblet cells form a protective layer over the gut epithelium and thereby relevantly contribute to the intestinal barrier function and the maintenance of mucosal homeostasis (83).

Most prominently, ILC2s could be shown to regulate mucin responses by goblet cells and to thereby protect mice from colonic inflammation. First evidence came from a study by Monticelli and colleagues: Using an acute model of DSS-induced colitis, they could show AREG-derived from IL-33-activated ILC2s to be sufficient for the induction of goblet cell hyperplasia and expression of the mucin *Muc2*. Since this goblet cell activation was accompanied by a decreased overall disease severity of DSS-treated mice (69), this indicates that the protective effect of ILC2s on experimental colitis could be partially mediated

by AREG-induced mucus production by goblet cells. This might additionally be supported by ILC2-derived IL-13, which was shown to promote goblet cell differentiation. Applying a coculture system of mesenteric lymph node cells and enteroids, representing a stem cell-derived 3D model system of the small intestinal epithelium, a direct effect of IL-33-induced IL-13 on goblet cell differentiation has been indicated by increased *Muc2* and *Atoh1* expression levels. In this setting, ILC2s were identified as primary source of IL-13 upon IL-33 induction (84), pointing toward a combined action of the ILC2 effector cytokines AREG and IL-13 on goblet cell activation and thus epithelium protection (**Figure 2**). While a direct effect of IL-13 on goblet cells was demonstrated *in vitro*, no direct impact of AREG could be detected (84), indicating ILC2s to activate goblet cells via both direct and indirect mechanisms. Regarding reported variations in the strength and quality of the impact of ILC2-derived IL-13 and AREG on goblet cell activation (85), it might be important to take into account the potential influence of the local micromilieu, mirrored in different experimental model systems. Another study even expanded these local findings on distal mucosal sites (84). In the context of experimental helminth infection of the gut, it was demonstrated that ILC2s activated by gastrointestinal infection with the helminth *Trichinella spiralis*, do not only activate local goblet cell hyperplasia (86), but could also induce expression of the mucin *Muc5* in the uninfected lung. This phenomenon has been discussed as potential priming mechanism to protect distal sites from secondary helminth infections (84) and strongly implied a systemic relevance of ILC2-triggered goblet cell activation.

During intestinal *Listeria monocytogenes* infection, ILC3s appeared as important inducers of goblet cell differentiation and function, enabling efficient bacteria control. Making use of an *in vitro* coculture transwell assay, the necessity of a direct interaction of lymphotoxin-secreting RORγt$^+$ cells and lymphotoxin-β receptor (LTβR) expressing IECs for efficient *Muc2* induction was shown, with ILC3s being suggested as lymphotoxin-expressing interaction partner (87). Though the role of direct ILC3-goblet cell interactions have not been described in IBD yet, a prominent function of IL-22 has been confirmed in the inflamed gut (**Figure 2**). Using a mouse model of Th2-mediated colitis, IL-22 gene delivery could induce expression of mucus components and goblet cells in a STAT3-dependent manner, which resulted in increased mucus production and thus less severe colitis (88).

In case of *Salmonella typhimurium* infection, IFN-γ-production by an intermediate ILC subtype characterized by NKp46, T-bet, and RORγt expression was claimed to be critical for successful mucus secretion by goblet cells (89). Whether this interaction might play a role in IBD as well, has to be clarified in future studies.

Collectively, ILC2- and ILC3-derived effector cytokines as well as the type-1 cytokine IFN-γ have been shown to strongly impact on goblet cell-driven mucus production and secretion. This allows ILCs to directly regulate the production and composition of the protective mucus layer and to thereby effectively shield IECs from intestinal pathogens.

Stem and Progenitor Cells—ILCs

The impressive capacity of the intestinal epithelium to renew itself every 3–7 days is driven by a rare cell population positioned at the crypt bottom of the ileum and colon: the intestinal stem cells. Intestinal stem cells can give rise to enterocytes, goblet cells, enteroendocrine cells, and tuft cells throughout life, while simultaneously renewing themselves. Therefore, dividing stem cells partially differentiate into highly proliferative transit-amplifying cells, which successively form terminally differentiated IEC types upon upward migration (90). With their ability to interact with and orchestrate intestinal stem and progenitor cells, ILCs are able to greatly influence overall epithelial functionality despite their rareness, once again demonstrating the direct link between innate immune responses and epithelial restoration.

Most prominently, a positive role of the ILC3-associated cytokine IL-22 on intestinal stem cells has been repeatedly shown (31, 91–95). While a regulatory role of ILC3s on the intestinal epithelium has been proposed shortly after their identification as unique cell population (60), their influence on intestinal stem cells was described only 1 year later. In the context of allogeneic hematopoietic transplantation, Hanash and colleagues identified IL-22 derived from radio-resistant host ILCs in the gut as critical protective factor against epithelial damage during graft-vs.-host disease. With the development of graft-vs.-host reactions, IL-22-secreting ILC frequencies markedly decreased, which was associated with a reduction of IL-22R-expressing intestinal stem cells. Indeed, *Il-22*$^{-/-}$ transplant recipient mice showed an increased loss of intestinal stem cells compared to wildtype mice, which was accompanied by severe disruption of the epithelial barrier integrity. Based on these findings, the authors suggested intestinal ILCs to maintain stem and progenitor cells during tissue damage via the secretion of IL-22, while Paneth cells were proposed to be responsible for basal stem cell maintenance (91). Using an *ex vivo* organoid culture system, they could later translate the importance of IL-22 on stem cell-driven epithelial regeneration to the human system. Moreover, they deciphered the underlying mechanism, showing the dependency of IL-22-mediated intestinal Lgr5$^+$ stem cell preservation and resulting organoid growth on STAT3 signaling, while the classical signaling pathways involved in intestinal stem cell maintenance, including Wnt/β-catenin and Notch signaling, were not affected by IL-22. A gene set enrichment analysis of the intestinal stem cell gene signature in STAT3-deficient and -sufficient mice with DSS colitis, reinforced these results under *in vivo* tissue-destructive conditions. Furthermore, the capacity of IL-22 to induce epithelial regeneration appeared to be restricted to intestinal stem and progenitor cells, since the inability of IL-22 to affect Paneth cells could be demonstrated in organoid culture experiments (92). A far-reaching importance of IL-22-secreting ILC3s on stem cell-driven intestinal barrier maintenance by the interaction with crypt stem cells could be further established in a setting of chemotherapy-induced small intestinal tissue disruption (31). Beyond that, ILC3-derived IL-22 turned out to contribute to the initiation of the DNA damage response in intestinal stem cells, preventing them from the

acquisition of potential mutations that might cause intestinal cancer development. This was mediated via aryl hydrocarbon receptor (AhR) signaling in ILC3s as well as γδ T cells in response to genotoxic stress triggered by dietary compounds (93). A recent study differentiated more explicitly between the maintenance and proliferation of intestinal stem cells, showing that upon acute damage of the small intestine by methotrexate, ILC3-derived IL-22 was primarily relevant in protecting and preserving stem cells in a STAT3-dependent manner, while ILC3-driven, but IL-22-independent amplification of the Hippo-Yap1 pathway in stem cells turned out to mediate crypt cell proliferation in a SFK-dependent fashion. The authors thus proposed that ILC3s might not only interact with $Lgr5^+$ stem cells for their maintenance, but might also directly or indirectly trigger proliferation of damage-linked progenitor cells to restore an intact epithelial barrier after tissue disruption (94). The exact mechanisms through which ILC3s drive epithelial restoration independently of IL-22, however, still need to be evaluated. Somehow in contrast, another study proposed a role of high IL-22 concentrations primarily for the amplification of transit-amplifying progenitor cells rather than intestinal stem cells based on findings in an *in vitro* ileal organoid culture model. There the authors observed a negative effect of 500 pmol/l IL-22 on organoid survival, whereas remaining organoids showed an increase in size, which was suggested to results from highly proliferating transit-amplifying progenitor cells. The principle idea for analyzing IL-22 concentrations as high as 500 pmol/l was derived from a computational modeling of the local ILC3-secreted IL-22 concentration in the stem cell niche (95). To date, however, absolute IL-22 concentrations in the microenvironment of intestinal stem cells have not been experimentally confirmed yet. Thus, more research is necessary to determine the functional role of ILC3-derived IL-22 on distinct stem and progenitor cell subsets under defined inflammatory conditions. Taken together, multiple studies were able to identify ILC3s as key players in preserving and rebuilding an intact epithelial barrier in the gut after tissue damage (**Figure 2**). This can be mediated by a direct interaction of IL-22-secreting ILC3s and IL-22R-expressing intestinal stem cells and by ILC3-driven activation of Yap1 signaling in stem cells. ILC3s are obviously able to rapidly rebuild an efficient gut barrier upon various kinds of tissue disruptions and, in addition, can prevent intestinal cancer development originating from DNA damage in stem cells.

Besides a fundamental role of ILC3s on intestinal stem and progenitor cells, ILC2s were suggested to interact with intestinal progenitor cells as well. In helminth infection models with *Nippostrongylus brasiliensis* and *Heligmosomoides polygyrus*, they have been demonstrated to induce goblet and tuft cell differentiation via the secretion of IL-13 (96). In general, the latter represent a chemosensory IEC subset with striking similarities to our taste buds. Thus, tuft cells are suggested to "taste" luminal signals unable to cross the intestinal barrier to trigger a specific response in the intestinal tissue (97), though their exact function has long been undetermined and is still insufficiently clarified. However, a pivotal role of intestinal tuft cells has been suggested during helminth infections, during which their rare number literally explodes (96, 98). Since tuft cells turned out to be the major source of IL-25 in the intestinal epithelium, the functional relevance of ILC2-driven tuft cell hyperplasia upon helminth infection was suggested to lie in the activation of ILC2s themselves via the secretion of IL-25, in order to mount an efficient anti-helminth immune response. Indeed, in systemic and epithelium-specific *Il-25* knockout mice helminth infections were only inefficiently cleared (96, 98). Similarly, $Trpm5^{-/-}$ mice, which are unable to transduce taste signals in tuft cells, are characterized by an increased worm burden after helminth infection compared to wildtype mice. This could be restored upon intraperitoneal injection of IL-25 (98). Thus, a regulatory circuit was suggested with IL-25-secreting tuft cells stimulating IL-13 release by local ILC2s, which in turn triggers tuft cell proliferation from progenitor cells via a positive feedback mechanism, finally resulting in successful worm clearance (96). Next to this pathologic context, this circuit was suggested to be important even under homeostatic conditions, showing in uninfected $Il\text{-}25^{-/-}$ and $Il\text{-}4ra^{-/-}$ mice that constitutive IL-25 secretion by tuft cells as well as ILC2-derived IL-13 were important to maintain intestinal tuft cell numbers in naive mice (96). Collectively, intestinal tuft cells can be positively regulated by ILC2-derived IL-13 (**Figure 2**), leading to tuft cell expansion via activating crypt progenitor cells and thus directly regulating anti-helminth responses, raising the question whether this tuft cell-ILC2 circuit might play a role in IBD as well. Indeed, decreased tuft cell counts were recently described in CD patients at inflamed ileal tissue sites (99). Moreover, using a model of acute DSS colitis, a beneficial role of $Dclk^+$ tuft cells on intestinal barrier integrity was observed (100). In line with this, reduced IL-25 levels were found in the inflamed gut mucosa of IBD patients with active disease, which correlated with an increased disease severity (101), suggesting the loss of intestinal tuft cells and their IL-25 secretion as disease-driving factor in IBD. In the murine ileum, tuft cells could be reconstituted together with local ILC2 frequencies and classical type-2 cytokines by the administration of the microbiota-derived metabolite succinate, finally resolving ileal inflammation (99). This implies an *in vivo* relevance of the ILC2-driven tuft cell regulation upon intestinal inflammation in CD patients and might additionally explain the recently described protective role of helminth infections in the development of IBD (99).

Also for ILC1s a regulatory role on intestinal stem cells has been described lately (**Figure 2**). Though not representing a classical ILC1-associated cytokine, ILC1-derived TGF-β1 was able to specifically induce the expression of the variant 6 of the stem cell marker CD44 (*Cd44v6*) in the intestinal epithelium. This resulted in enhanced crypt budding of small intestinal organoids *in vitro* via p38γ-induced proliferation. Having observed an enhanced expression of *CD44v6* in enlarged intestinal crypts in the inflamed tissue of IBD patients as well (45), a positive regulatory role of ILC1s on epithelial expansion upon inflammation might be of significance *in vivo*.

Collectively, ILC1s, ILC2s, and ILC3s are described to have a beneficial impact on epithelial restoration and growth upon inflammation, resulting from direct interactions with intestinal crypt stem and progenitor cells.

Further IEC Subtypes—ILCs

Next to enterocytes, goblet, progenitor and stem cells as well as tuft cells, the IEC fraction additionally consists of enteroendocrine, paneth, and M cells, each of these subtypes contributing to the diverse functions of the intestinal epithelium. Enteroendocrine and paneth cells for instance are known for the secretion of hormones and antimicrobial peptides, respectively, while M cells can transport antigens from the gut lumen into the intestinal tissue and thus serve the intestinal immune system (81).

To date, no interaction of ILCs with these IEC subtypes has been shown but might be implicated by the presence of potential interaction sites between IECs and ILCs. In case of enterochromaffine cells for example, which constitute a subset of enteroendocrine cells and are critical for the production of serotonin, expression of the IL-13 receptor α1-chain could be demonstrated (102), implicating a potential signal induction in enterochromaffine cells by ILC2-derived IL-13. Enteroendocrine cells might in addition be positively regulated by IL-22 released from ILC3s. As suggested in a mouse model of *Citrobacer rodentium* infection, secretion of antimicrobial peptides, primarily of the Reg family, from epithelial cells turned out to depend on IL-22 production from IL-23 responsive cells of the innate immune system (103), providing a direct link between ILC3s and the bacterial defense mechanisms of the intestinal epithelium. Moreover, the localization of paneth cells at the crypt bottom exposes them to an environment that was suggested by computational modeling to be characterized by particularly high ILC3-derived IL-22 levels (95). Together with the recently shown importance of IL-22 signaling for paneth cell differentiation and effector functions (104), this predisposes ILC3s as potential regulators of paneth cells. Future research will help to clarify the biological relevance of these suggested ILC-IEC interactions in IBD.

ILC–IEC INTERACTIONS AS THERAPEUTIC TARGET

Given the preferential accumulation of ILCs at mucosal surfaces in close proximity to the epithelium (10, 11) as well as their rapid and early activation as part of the innate immune system (12–14), makes them ideal interaction partners of IECs. Moreover, the ILC-driven regulation of IECs is of particular importance as the integrity of the epithelial barrier is critical to preserve a stable and efficient control of bacterial translocation, which might otherwise trigger the initiation of mucosal inflammation. Based on our current knowledge, ILC3s represent the main helper ILC population in the healthy human gut (33, 34) (**Figure 1**) with an incredible ability to secrete large amounts of the effector cytokine IL-22 (58, 60). Together with the release of lymphotoxin, ILC3-derived IL-22 was ascribed a predominantly protective effect on enterocytes, goblet cells and even crypt stem and progenitor cells (57–60, 87, 91, 92). Therefore, the observed decrease of intestinal ILC3s (17, 34, 44) in the inflamed mucosa of IBD patients might contribute to local inflammatory responses in IBD. However, the effect of ILC3-derived IL-22 on IECs appeared to highly depend on the surrounding micromilieu (67) and thus has to be treated with care. With an increasing number of intestinal ILC1s in CD (17, 44), their TGF-β1-mediated beneficial effect on crypt stem cells (45) might contribute to the resolution of intestinal inflammation by promoting the reconstruction of an intact epithelial barrier. In contrast, via the secretion of IFN-γ, ILC1s were suggested to additionally have a negative influence on tight junctions (54, 79, 80), implying functional subgroups of ILC1s. Despite their overall low number in the healthy and inflamed intestine, ILC2s could be demonstrated to favor mucosal healing and disease control on the level of enterocytes, goblet cells, tuft cells and crypt stem cells by secreting AREG and IL-13 (69, 71, 84). Overall, the described ILC-IEC interactions prove once again, that a rare cell population like ILCs can have an extensive impact on disease induction, progression, and resolution irrespective of their small cell number (**Figure 2**), making the interference with ILC-driven IEC regulation an interesting new therapeutic option. Targeting ILCs appears to be particularly elegant regarding the ability of intestinal ILCs to regulate both, epithelial integrity as well as mucosal immune responses. However, this also makes clear that the development of ILC-modulating therapeutic strategies will require a careful fine-tuning to prevent accidental ILC-mediated immune cell activation upon boosting protective ILC-IEC interactions. Notably, no fully ILC-specific target structures exist these days, which is mainly due to the shared key features of ILCs and Th cells. This makes it currently impossible to clearly differentiate between ILC- and T cell-targeting therapeutic approaches. A broad overview of ILC-related therapeutic strategies was given by Goldberg and colleagues, postulating the following points of potential attack: targeting ILC effector functions by therapeutically modulating their activity, intracellular signaling, and effector cytokine production or targeting local ILC numbers by interfering with their survival and trafficking (105). To date, most of the postulated therapies involving ILC targeting aim at the inhibition of pro-inflammatory ILC functions. However, these strategies need to be critically revised, since inhibition of total ILC functions might not only favor resolution of inflammation but might additionally abolish their mainly positive influence on the intestinal epithelium upon inflammation. Thus, novel therapeutic approaches might focus on the controlled stimulation of specific and defined aspects of ILC functions, rather than mediating a broad or complete blockade of ILC activation. The following section will therefore focus on potential therapeutic options, which might allow to directly and specifically interfere with defined ILC-IEC interactions.

Given the great ability of ILC3-derived IL-22 to preserve the intestinal epithelium (57–60) and the fact that the IL-22 receptor (IL-22R) is absent on hematopoietic cells (106), ILC3-derived IL-22 appears as an attractive therapeutic target, since this might enable to support the protective effect of IL-22 on epithelial cells without triggering pro-inflammatory immune responses in parallel. Accordingly, different strategies to boost IL-22-mediated epithelial regeneration and preservation have been discussed. On the one hand, IL-22 can be supplemented directly and, on the other hand, therapeutic activation of endogenous IL-22 production and signaling can be targeted. In the latter

category, a site-specific *Il-22* gene delivery system was suggested and proven to improve signs of intestinal inflammation in a preclinical mouse model of Th2-driven colitis (88). However, there is still plenty of safety issues to be addressed carefully until this local gene-delivery strategy might be translated to the human system. Alternatively, therapeutic activation of the IL-22 pathway might represent an interesting strategy. To date, most promising approaches involve AhR-mediated IL-22 induction, which is of functional relevance in ILC3s as well (107, 108). Activation of the AhR pathway can be achieved, for instance, by dietary compounds (107) or microbiota-driven metabolization of tryptophan to the AhR ligand indole-3-aldehyde (109, 110), the latter of which was demonstrated to augment intestinal inflammation in murine models (109, 110). Similarly, indigo naturalis, which has been traditionally used in Chinese medicine, turned out to mediate its protective effect via AhR-mediated IL-22 production in ILC3s as well, resulting in reduced disease severity in several murine models of colitis (111). Moreover, especially a short-term treatment with indigo naturalis has been identified as effective treatment in patients with UC (112), whereas 8 weeks of administration cannot yet be fully excluded to potentially be associated with severe adverse events (113). Importantly, reduced AhR activation was observed in fecal samples from IBD compared to healthy subjects (110). Therefore, together with other AhR ligand candidates, including FICZ (114) and ABX464 (115), tryptophan and indigo naturalis might be promising therapeutic strategies in IBD patients by activating endogenous IL-22 production from intestinal ILC3s in order to protect and restore the intestinal epithelium. Since, however, AhR activation can trigger multiple signaling pathways dependent on its ligand (116), potential therapeutic interference with AhR signaling would have to be carefully controlled and monitored. Another strategy to target IL-22 signaling might involve blocking of the soluble IL-22 receptor IL-22 binding protein (IL-22BP), which functions as endogenous inhibitor of IL-22 by capturing IL-22 with high affinity and thus suppressing IL-22 signaling through its membrane-bound receptor (117). A beneficial effect of blocking IL-22BP in intestinal inflammation has been implicated by experimental colitis models, showing a detrimental role of local IL-22BP. Local gene delivery of *IL-22bp*, for instance, hindered IL-22-mediated goblet cell proliferation in a mouse model of acute DSS colitis (88). Moreover, $CD4^+$ T cell-derived IL-22BP could be shown as driver of intestinal inflammation in the model of adaptive transfer colitis using *Il-22bp*-sufficient and -deficient donor T cells (118). The other way around, IL-22bp-deficient rats recovered significantly faster from first signs of colitis upon DSS treatment compared to wildtype control animals, which could be traced back to the protective effects of efficient IL-22 signaling on the epithelial barrier (119). Since inflamed areas in IBD patients are characterized by an increased expression of *IL-22BP* (118, 119), blocking IL-22BP might potentially have therapeutic potential in humans as well. Alternatively, recombinant human IL-22 fusion proteins, like F-652 or UTTR1147A, allow direct IL-22 supplementation with the advantage of increased IL-22 stability and thus extension of the IL-22-induced beneficial effects. In case of UTTR1147A, efficient STAT3 activation via the IL-22 receptor could be demonstrated *in vitro*, resulting in protective *in vivo* effects in a murine colitis model (120). Moreover, the therapeutic efficacy of F-652 has been shown in a mouse model of graft-vs.-host disease (92) and its safety profile has even been validated successfully in a clinical trial (121), paving the way for further clinical development. Irrespective of the afore mentioned strategies, fine-balancing of any therapeutic interference with ILC3-driven IL-22 secretion and individual patient selection will be of critical importance, since next to its protective effects on IECs, a detrimental potential of IL-22 on the epithelial barrier has been observed in selected mouse models, as for instance in the anti-CD40-induced innate colitis model (67). Likewise, uncontrolled IL-22 was suggested to favor formation of colitis-associated colon cancer (122), allowing IL-22 modulations only in a highly controlled fashion.

Boosting the ILC2-progenitor cell-tuft cell circuit might represent another innovative, yet currently still hypothetical therapeutic option in IBD patients. Indeed, data from a murine study indicated that succinate supplementation resulted in the amelioration of intestinal disease, which was attributed to an activation of the tuft cell-ILC2 circuit (99). Even though expansion of the helminth-sensing tuft cells by defined microbiota-derived metabolites has not directly been targeted in IBD patients yet, helminth infections themselves have been discussed to prevent intestinal inflammation, though to date with controversial outcomes (123–126). Thus, it appears promising to pursue strategies that aim on a more specific therapeutic activation of the tuft cell-ILC2 circuit by specific microbial metabolites, like succinate.

Hypothetically, a direct transfer of *ex vivo* expanded and specifically activated human ILCs might represent another future therapeutic strategy, aiming at the restoration of a functional epithelial barrier. Motivated by the first promising results achieved by the adoptive transfer of regulatory T cells (127, 128) with a phase I clinical trial currently running in UC patients (NCT04691232) (129), the transfer of other protective immune cell populations might also be of advantage for IBD patients. While there already exist established protocols for the *ex vivo* expansion and differentiation of human ILCs (48, 130, 131), a targeted *ex vivo* generation of specific ILC subsets with distinct effector functions is certainly still an unsolved challenge that needs to be addressed as a first step on the way to a potential clinical translation. However, pursuing this idea, the therapeutic use of *ex vivo* expanded primarily AREG-producing ILC2s might, for instance, be desirable, based on their beneficial effect observed in a mouse model of intestinal inflammation (69). Nevertheless, many more questions need to be answered, until adoptive ILC transfer might be further discussed as therapeutic strategy in IBD. For example, it will be important to define the *in vivo* stability of the intended ILC phenotype, the capacity of transferred ILCs to accumulate at the site of epithelial tissue damage as well as their potential risk to drive inflammation rather than epithelial healing under *in vivo* conditions.

In summary, the here described insights into the capacity of helper ILCs to impact on the fate of IECs clearly point to these innate mucosal immune cells as key regulators of the gut barrier integrity and as an interesting and still largely unexplored research topic with great therapeutic potential in the clinical context of 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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Reprogramming Intestinal Epithelial Cell Polarity by Interleukin-22

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Background: Interleukin-22 (IL-22) impacts the integrity of intestinal epithelia and has been associated with the development of colitis-associated cancer and inflammatory bowel diseases (IBD). Previous data suggest that IL-22 protects the mucosal barrier and promotes wound healing and barrier defect. We hypothesized, that IL-22 modulates cell polarity of intestinal epithelial cells (IECs) acting on tight junction assembly. The aim of the study was to investigate IL-22-dependent mechanisms in the reprogramming of intestinal epithelia.

Methods: IECs were exposed to IL-22 at various concentrations. IECs in Matrigel® were grown to 3-dimensional cysts in the presence or absence of IL-22 and morphology and expression of polarity proteins were analyzed by confocal microscopy. Epithelial cell barrier (TER and sandwich assay) and TJ assembly analysis (calcium-switch assay) were performed. TJ and cell polarity protein expression were assessed by western blotting and confocal microscopy. Cell migration and invasion assays were performed. Induction of epithelial-mesenchymal transition (EMT) was assessed by RT-qPCR analysis and western blotting. Signaling pathway analyses were performed by phosphoblotting and functional assays after blocking STAT3 and ERK signaling pathways. Using the toxoplasma-model of terminal ileitis, IL-22-knock-out mice were compared to wild-type littermates, analyzed for barrier function using one-path-impedance-analysis and macromolecular flux (H3-mannitol, Ussing-chambers).

Results: IECs exhibited a barrier defect after IL-22 exposure. TJ protein distribution and expression were severely impaired. Delayed recovery in the calcium-switch assay was observed suggesting a defect in TJ assembly. Analyzing the 3D-cyst model, IL-22 induced multi-lumen and aberrant cysts, and altered the localization of cell polarity proteins. Cell migration and invasion was caused by IL-22 as well as induction of EMT. Interestingly, only inhibition of the MAPK pathway, rescued the TJal barrier defect, while

blocking STAT3 was relevant for cell survival. In addition, ileal mucosa of IL-22 deficient mice was protected from the barrier defect seen in Toxoplasma gondii-induced ileitis in wild type mice shown by significantly higher Re values and correspondingly lower macromolecule fluxes.

Conclusion: IL-22 impairs intestinal epithelial cell barrier by inducing EMT, causing defects in epithelial cell polarity and increasing cell motility and cell invasion. IL-22 modulates TJ protein expression and mediates tight junctional (TJal) barrier defects via ERK pathway.

Keywords: intestinal epithelial cells, barrier function, cell polarity, IL-22, tight junctions, MAPK, stat3

INTRODUCTION

Interleukin-22 (IL-22) is a member of the interleukin-10 cytokine family that is primarily acting on epithelial cells, which is secondary to the expressional restriction of the IL-22-receptor-1 chain to epithelia (1–3). IL-22 is upregulated in inflammatory bowel diseases (IBD) as Crohn's disease but also in coeliac disease (4–6). Although there are many reports on IL-22-mediated effects on epithelial cells, the overall outcomes described so far appear to be very heterogeneous. Dependent on the cell type involved or the type of inflammatory trigger, IL-22 was reported to result either in protection of epithelia/wound healing or to induce epithelial damage (1, 7). Specifically, IL-22 protected from colitis in infectious or chronic inflammatory models or induced ileitis in the *Toxoplasma gondii* model (8–12). However, previous analyses of epithelial cell responses to IL-22 are mostly limited to wound closure assays or examination of epithelial proliferation and apoptosis (7).

Epithelial polarity describes a cellular program ensuring proper localization of distinct polarity-relevant molecular constituents (i.e., phospholipids and proteins) to the apical or the basolateral epithelial compartments as well as the coordinated assembly of intercellular junctional structures, including tight junctions (TJ) and adherens junctions (13). It is regulated through a complex network of proteins that are strongly conserved throughout evolution and is described to be dysregulated in inflammation and carcinogenesis (14). However, it is unknown how epithelial polarity and barrier function are regulated during chronic inflammation of the gut. Previously, dysregulation of the polarity protein Pard3 was found in celiac disease and was connected to celiac epithelial barrier defects (15).

Furthermore, the proinflammatory cytokines, such as TNF-α and interferon-γ, have been shown to disturb regular lumen formation in intestinal epithelial cysts as well as alter the epithelial polarity and barrier function (16). In this context, activation of the IL-22 receptor triggers signaling via various pathways including STAT3, AKT and MAPK/ERK, that are crucial for cell survival, proliferation, barrier integrity and establishment of cell polarity (17–20). Therefore, we hypothesized that IL-22 exposure directly modulates the epithelial apical complex and also the establishment of cell polarity, thereby regulating the barrier function.

In this study, we show that IL-22 impairs the intestinal cell barrier integrity by inducing a complex reprogramming of intestinal epithelial cell functions. Within this regulation, IL-22 induces EMT, modulates TJ, and polarity protein expression and mediates TJal barrier defects via ERK- but not STAT3- or AKT-pathway.

MATERIALS AND METHODS

Cell Culture and TER Measurement

Caco-2, HT29/B6, and T84 were maintained in Minimum Essential Medium Eagle's (MEM, Gibco/Thermo Fisher, Waltham, MA, USA), RPMI-1640 and DMEM/Ham's F-12 (Corning, Wiesbaden, Germany) supplemented with 10% fetal bovine serum (FBS, Gibco/Thermo Fisher), 1% penicillin and streptomycin (Corning), respectively. IECs were seeded on PCF filters (0.4 μm; 0.6 cm^2, Merck Millipore, Darmstadt, Germany) and grown to confluence for 7, 10 and 12 to 14 days in culture at 37°C in a 5% CO_2 environment, respectively. IL-22 (Biolegend, San Diego, CA, USA) was added to both, the apical and basolateral compartments of transwell filters for times indicated and transepithelial resistance (TER) was measured using chopstick electrodes. The IEC filters were basolaterally exposed to additional proinflammatory cytokines as TNF-α (1,000 U/ml); IFN-γ (100 U/ml); IL-13 (10 ng/ml); TGF-β1 (20 ng/ml). These cytokines were from Prepotech (Hamburg, Germany).

Impedance Spectroscopy

The experiment was performed as previously described (21). In brief, an electric circuit model was used describing the epithelial properties: Epithelial resistance (R_{epi}) consists of two parallel resistors, transcellular resistance (R_{trans}) being further divided into resistors and capacitors, and the paracellular resistance reflecting the TJ formed resistance (R_{para}). R_{epi} is in series to the subepithelial resistance (R_{sub}), the latter caused by the filter support. IECs were grown on filter support and were mounted into a modified Ussing chamber setup and after application of alternating current (35 $\mu A/cm^2$, frequency range 1.3 Hz to 65 kHz), voltage changes were detected by phase-sensitive amplifiers (402 frequency response analyzer, Beran Instruments, Glen Allen, VA, USA; 1,286 electrochemical interface; Solartron Schlumberger, Atlanta, GA, USA) and the resulting complex

impedance values were calculated and plotted in a Nyquist diagram, which allowed to evaluate R_{sub} and R_{epi} (One-path impedance spectroscopy). R_{trans} and R_{para} (Two-path impedance spectroscopy) were determined from experiments in which the impedance spectra and fluxes of a paracellular marker substance, fluorescein, were obtained before and after chelating extracellular Ca^{2+} with EGTA. This caused TJs to open and to increase paracellular flux inversely proportional to R_{epi} changes.

Sandwich Assay

The sandwich assay was done as previously described (22) and was performed at RT with cells growing on transwells (0.6 cm^2, 0.4 μm pore size). IECs were washed in FBS-free medium and were incubated basolaterally with avidin (15 μM, 10 min). After washing with PBS^+, cells were exposed to 140 μl of biotinylated dextran-3000-TexasRed (10 μM, 10 min, MolProbes) from the apical side. Cells were then fixed (2% PFA, 30 min, RT) and mounted for confocal microscopy.

Calcium Switch Experiment

Experiments were done as previously described (23). Seven days after seeding them on PCF filters (0.4 μm), T84 cells were switched to a low calcium medium (DMEM calcium-free, Gibco) supplemented with 5% of FBS and 1% penicillin and streptomycin (Corning). To disrupt cell adhesion, cells were kept 16 h in low calcium medium after 4 times PBS washing in the presence or absence of IL-22 (10 ng/ml). Then, filters were mounted to Ussing chambers, where TER was monitored in 10 s-intervals throughout the experiment. After 30 min of equilibration, calcium chloride was added to both chamber sides at a final concentration of 1.6 mM for 6 h.

Immunostaining and Confocal Laser Scanning Microscopy

Epithelial cell layers were washed 3× with PBS, then fixed with PFA 4% pH 7,5 and kept in 4°C with PBS for maximally 7 days prior to immunostaining. Cells were washed and stained following the protocol published previously (13) using the following primary antibodies: ZO-1 (1:100; BD Biosciences), JAM-A (1:100; Thermo Fisher). The secondary antibodies used were Alexa Fluor 488 goat anti-mouse or rabbit IgG, and Alexa Fluor 594 goat anti-mouse or rabbit IgG (1:500; Thermo Fisher). To determine occludin expression and cellular distribution, an occludin mouse monoclonal antibody (OC-3F10) was used as an Alexa Fluor® 594 Conjugate (Thermo Fisher). Nuclei were stained using DAPI (4′,6-Diamidin-2-phenylindol, conc. 1:2000). Immunofluorescence staining was analyzed by confocal laser scanning microscopy (LSM 780, Carl Zeiss, Jena).

Migration and Invasion Assay

HT29/B6 cells were kept on at 37°C in a 5% CO_2 environment until reach confluence. Subsequently, a defined scratch (diameter 100 μm) was introduced to filter-grown HT29/B6 cells and kept with medium with 1% of fetal bovine serum (Gibco) to avoid cell proliferation. Cells were exposed to IL-22 (10 ng/ml) and migration was evaluated by measuring the distance at 24 and 48 h after scratching. To perform invasion assay, Matrigel® was diluted (1 mg/ml), placed 100 μl into upper chamber of 24-well transwell and incubated at 37°C for 4–5 h. Subsequently, 2×10^5 CaCo-2 cells in 100 μl plus 100 μl of media (MEM Eagle Medium, Gibco + 1% penicillin and streptomycin, Corning) without FBS were placed into the transwell chamber with Matrigel®; cells were treated with or without IL-22 (100 ng/ml). In transwell lower chamber was added 600 μl of culture media and then, incubated for 24 h. The transwell chamber was removed and cells presented in the lower chamber were washed 2× with PBS^+, fixed (PFA 2%) at room temperature for 30 min and stained with DAPI (1:2000 for 30 min). Number of colonies were counted and analyzed by confocal laser scanning microscopy (LSM 780, Carl Zeiss, Jena).

Culturing 3D-Cysts, Immunostaining

For seeding CaCo-2 cells in Matrigel®, all materials were kept at 4°C under the cell culture bench. 1×10^4 cells CaCo-2 cells were embedded in 150 μl of fluidic Matrigel® (Corning, Wiesbaden, Germany) prior to homogeneously seeding them to Lab-tek slides (Thermo Fisher). To allow the Matrigel® to consolidate, Lab-teks were incubated at 37°C for 30 min. Subsequently, 500 μl of Eagle's MEM, supplemented with 10% FBS (both Gibco/Thermo Fisher) was added. 3D-cysts evolved within 3 to 5 days (37°C, 5% CO_2). Lab-teks were incubated at 4°C (PBS^+) until immunostaining was performed. For immunostaining, cells were washed with PBS^+ and then incubated with prewarmed collagenase (Sigma, Darmstadt, Germany; 8–10 min, 37°C), washed again and fixed using PFA (4%, pH 7.5) for 30 min at RT. Extensive PBS^+ washes, then permeabilization/blocking using PBL-solution (0.7% fish skin gelatin and 0.025% saponin, in PBS^+; 2 h, RT), followed by PBS-washes and quenching using 75 mM NH_4Cl and 20 mM glycine in PBS^+ (10 min, RT). Now, one wash using PBL and incubation with first antibody (PAR3 1:100; Sigma-Aldrich/ DLG1 1:100; Santa Cruz Biotechnology) in PBL was performed in a wet chamber overnight at 4°C. On the next day, samples were extensively washed using PBS^+ at RT. Then incubation with the secondary antibody (in PBL, wet chamber, overnight, 4°C; Alexa594-Fab-fragment donkey anti-rabbit IgG, 1:200; Alexa488-Fab-fragment donkey anti-mouse IgG, 1:200, additionally phalloidin-Atto647, 1:200). Alternatively, antibody stainings with fluorescently tagged first antibodies were performed using a less complex protocol with overnight incubation of PFA-fixed 3D-cysts at 4°C with E-cadherin antibody (1:100; Alexa Fluor647-conjugate, BD Biosciences, San Jose, CA, USA) and DY-594-phalloidin (1:100; Dyomics, Jena, Germany) to stain actin. Nuclei were stained using DAPI (4′,6-Diamidin-2-phenylindol, 1:2000) for 1.5 h at RT. Microscopy was performed using a confocal laser scanning microscopy (LSM 780, Carl Zeiss, Jena).

Treatment With Inhibitors

To inhibit STAT3 phosphorylation, different inhibitors were used. Stattic and STAT3 Inhibitor IV (S31-201) are cell-permeable molecules that inhibit by selectively binding the STAT3-SH2 domain impairing STAT3 activation, dimerization and nuclear translocation (24–26). Furthermore, it was used a cell-permeable peptide analog, which is also a selective blocker

of STAT3 activation (27). As an indirect inhibitor, WP1066 was used that blocks STAT3 phosphorylation by binding to JAK2, a kinase upstream of STAT3 (28, 29). To inhibit the MAPK signaling, the inhibitor U0196 was used. It acts as a selective inhibitor of MEK1 and MEK2 preventing activation of MAP kinases p42 and p44 (ERK1/2) (30). Specifically, after 7 days in culture, HT29/B6 cells growing on transwell filters were exposed to the aforementioned STAT3 and MAPK inhibitors (**Supplementary Table 1**) for 2 h. Subsequently, IL-22 (10 ng/ml) was added for either 1 h after which cells were lysed, or for a maximum of 72 h for measuring TER (48 72 h) and cells were lysate to perform Western blotting experiments.

RT-qPCR

Total RNA was extracted using the *mir*Vana™ mRNA Isolation Kit (Thermo Fisher) according to the manufacturer's instructions. To quantify the extracted RNA, NanoDrop 1000 (Thermo Fisher Waltham, MA, USA) was used. 800 to 1,000 ng of total RNA was applied to synthetize cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems/Thermo Fisher) according to the manufacturer's instructions. Real time-qPCR reactions were performed using 1 μl of cDNA template, 1 μl of the desired probe, 10 μL of RT-qPCR Master Mix (Applied Biosystems/Thermo Fisher) and nuclease-free water to a final volume of 20 μl. Comparative CT reactions were performed in triplicates using the StepOnePlus™ instrument (Applied Biosystems/Thermo Fisher). Calculations for gene expression changes were performed using the $2^{-\Delta\Delta CT}$ method. The human probes used were all from Applied Biosystems/Thermo Fisher and were *SNAI1* (Hs00195591_m1), *SNAI2* (Hs00161904_m1), *MMP −2* (Hs01548727_m1), *−7* (Hs01042796_m1), and *−9* (Hs00957562_m1). *ACTB* (Hs01060665_g1) was used as control of the reaction amplification.

Western Blotting

For protein quantification, epithelial cells were washed twice with ice-cold PBS^+. Protein extraction was done using ice-cold lysis buffer (150 mM NaCl, 10 mM Tris buffer pH of 7.5, 0.5% Triton X-100, and 1% SDS). A volume of 10 ml lysis buffer was supplemented with one Complete Protease Inhibitor Cocktail tablet; Roche AG, Basel, Switzerland). Cells were scraped from the filters, incubated for 60 min on ice, and vortexed every 10 min. The supernatant was collected after centrifugation (30 min, 15,000 g, 4°C). To determine the protein content, Pierce BCA assay (Thermo Fisher, Waltham, MA, USA) was performed according to the product instructions using a Tecan plate reader (Tecan GmbH, Maennedorf, Switzerland) at an absorbance of 562 nm. Protein samples (20 μg) were mixed with 5× Laemmli buffer and loaded on premade SDS polyacrylamide gels (Bio-Rad, Feldkirchen, Germany). After electrophoretic separation, proteins were transferred to a PVDF membrane (Thermo Fisher) using the Trans-Blot system (Bio-Rad) at 25 V for 7 to 10 min and membranes were blocked for 2 h at RT with 1% PVP-40 (Polyvinylpyrrolidone; Sigma, Darmstadt, Germany) in TBST/0.05% Tween-20 buffer. Primary antibodies (**Supplementary Table 2**) were incubated overnight at 4°C. A peroxidase-conjugated secondary antibody was incubated (2 h, RT). Detection of proteins on the membrane was performed using SuperSignal West Pico Plus Stable Peroxide Solution (Thermo Fisher). Luminescent signals were detected with the Fusion FX7 imaging system (Vilber Lourmat Deutschland GmbH, Eberhardzell, Germany).

Mice

Female WT and IL-$22^{-/-}$ (on a C57BL/6 background), and NMRI mice were 8 to 12 weeks of age and bred and maintained in the Forschungsinstitut für Experimentelle Medizin (Charité—University Medicine, Berlin). Clinical conditions and body weights were determined daily, and all experiments were conducted according to the German animal protection laws. Animal protocols were approved by the Landesamt für Gesundheit und Soziales (Lageso, Berlin; TVV-No G0258/04).

Toxoplasma gondii-Induced Ileitis *In vivo* Murine Model

Cysts of the *T. gondii* ME49 strain were obtained from brains of NMRI mice infected with 10 cysts for 2–3 months. Mice were infected with 100 cysts in 0.3 ml of PBS by gavage. All animal experiments were conducted according to the German animal protection laws. Histological scores and parasite loads were determined in formalin-fixed and paraffin-embedded tissue sections taken from the terminal ileum as described previously (31).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism software (GraphPad Software, La Jolla, CA) by the non-parametric Mann Whitney U test. All data are expressed as mean values ± standard error of the mean (SEM). $p < 0.05$ was considered significant.

RESULTS

IL-22 Impairs Paracellular Intestinal Epithelial Barrier Integrity

To investigate the role of IL-22 on barrier integrity, intestinal epithelial cells (IECs) seeded on transwell filters were exposed to IL-22 (apical and basolateral compartment). A stable epithelial barrier was established in CaCo-2 cells on day 10, in HT-29/B6 cells on day 7 and in T84 cells on day 14. Subsequently, apical and basolateral cell surfaces were exposed to IL-22. Transepithelial electrical resistance (TER) was monitored throughout the experiment (**Figure 1**). IL-22 induced a significant decrease in TER in a dose-dependent (**Figure 1A**) and time dependent manner (**Figures 1B,C)** with reductions in TER as much as 60% of control level at 10 and 100 ng/ml of IL-22 (72 h exposure). Furthermore, IL-22-induced TER decrease was similar to that after 48 hours of exposure to other proinflammatory cytokines (**Figure 1D**). Interestingly, the IL-22-induced barrier leak also allowed the passage of macromolecules like TMR-dextran3000

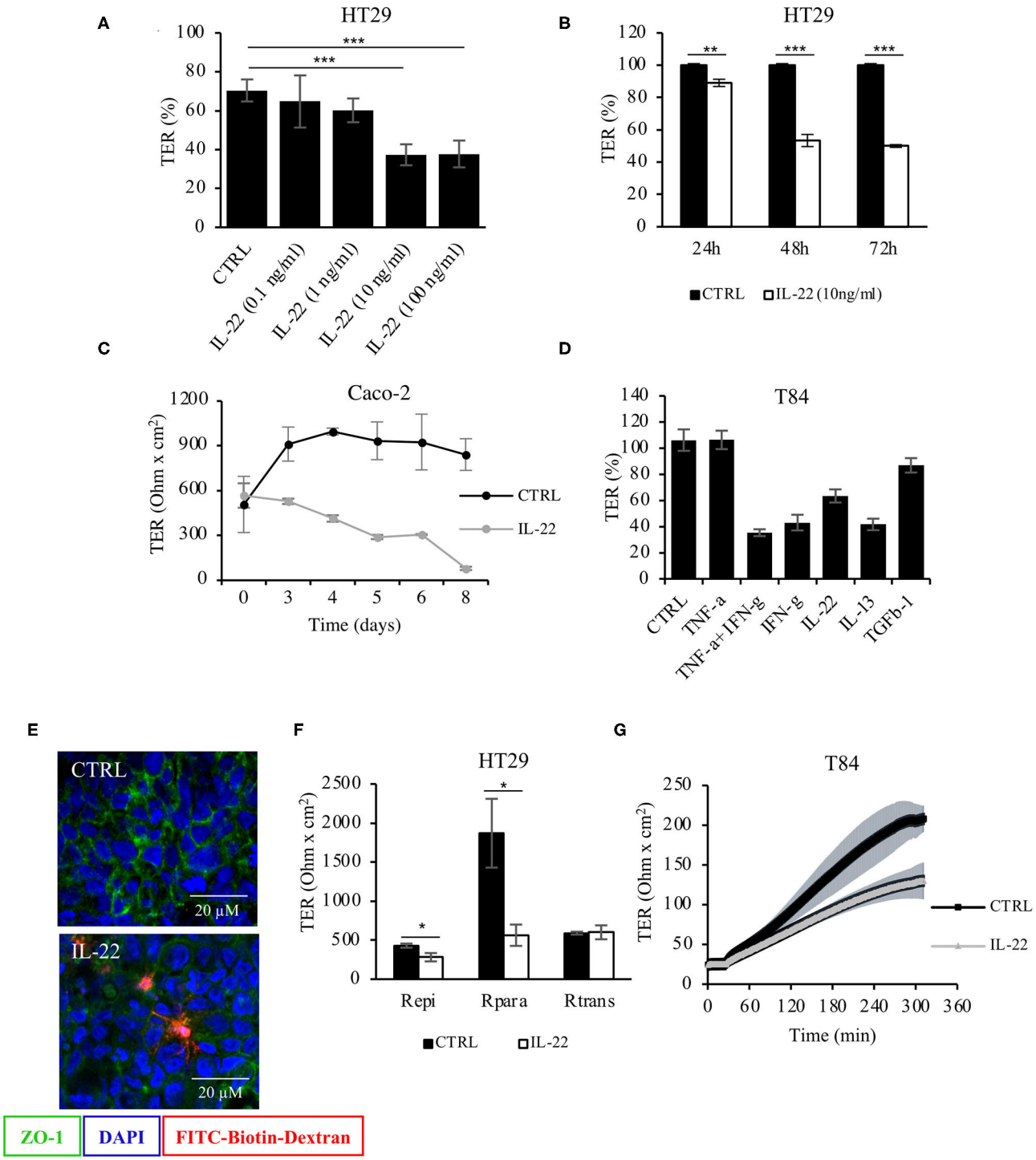

FIGURE 1 | Barrier integrity is affected by IL-22. **(A)** Transepithelial resistance (TER) was determined in HT-29/B6 cells grown on transwell filters. Cells were exposed to IL-22 at different concentrations (0.1, 1, 10, and 100 ng/ml). TERs after 72 h of IL-22 exposure are shown $n = 25$. Mann–Whitney *U* test; $^{***}p < 0.001$. **(B)** TER time course in HT29/B6 cells exposed to IL-22 (10 ng/ml); $n = 32$. Mann–Whitney *U* test; $^{**}p < 0.01$; $^{***}p < 0.001$. **(C)** TER measured in CaCo-2 cells exposed to IL-22 (10 ng/ml) for a longer time course (up to 8 days); $n = 3$. **(D)** Comparative analysis of TER in T84 cells (grown on transwell filters) after a 48 h-exposure to various cytokines (TNFα: 1,000 U/ml, IFNγ: 100 U/ml, IL-22: 10 ng/ml; IL-13: 10 ng/ml; TGF-b1: 20 ng/ml); $n = 8$. **(E)** Sandwich assay revealing transepithelial passage of macromolecules, specifically TexasRed-dextran3000 (red fluorescence) in control and IL-22-treated CaCo-2 cells. E-cadherin, green; nuclei, blue; $n = 3$. **(F)** Two-path impedance analysis: HT-29/B6 cells grown on transwell filters were exposed to IL-22 (10 ng/ml) for 48 h. After mounting filters to Ussing chambers paracellular and transcellular components of TER were determined by two-path impedance; $n = 6$. Mann Whitney *U* test; $^{*}p < 0.05$ **(G)** Calcium switch experiment: T84 cells growing on transwell filters were exposed to IL-22 (10 ng/ml, 48 h) and mounted to Ussing chambers, where TER was monitored in 10 s-intervals throughout the experiment. Transepithelial resistance was measured every 60 min for 6 h; $n = 3$. Mann–Whitney *U*-test; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

as shown by the sandwich assay (**Figure 1E**). Furthermore, a 2-path-impedance analysis showed that the barrier leak occurred exclusively paracellular (**Figure 1F**). In addition, we observed an IL-22-associated delay of TER-recovery after switching the media from calcium-free to normal calcium concentrations in CaCo-2 cell layers in filter transwells that had been mounted to Ussing chambers. This finding is frequently found with a disturbed TJ assembly (**Figure 1G**). Altogether, these results show that IL-22 impairs the paracellular barrier function of IECs and promotes an increased permeability of small ions (measured by TER) and macromolecules (as measured by the sandwich assay).

IL-22 Induces Defective Epithelial Polarity

Next, CaCo-2 cells seeded in Matrigel® were allowed to evolve to 3-dimensional cysts. Formation of cyst lumen was analyzed as this is known to reflect the integrity of the polarization process. Cells were then immunostained and analyzed by confocal laser scanning microscopy. Untreated CaCo-2 3D cysts most often exhibited a single lumen, lined with a single epithelial layer with the apical cell surface pointing to the lumen and the basolateral surface pointing to the Matrigel®-containing matrix. In untreated cysts, phalloidin staining showed a strongly stained subapical network of actin fibers, while the basolateral membrane was E-cadherin as well as β-catenin-positive as expected in polarized IECs (**Figure 2A**, **Supplementary Figure 1**). Interestingly, exposing cysts to IL-22 resulted in an increase of cysts with multiple lumens and a consecutive decrease of hollow cysts, i.e. cysts displaying a single, "ball-shaped" lumen (**Figures 2B,C**). In this regard, IL-22-treated cysts frequently revealed dystopic lumen formation, e.g., in between neighboring IECs of the single cell lining of the cysts (**Figure 2A**, **Supplementary Figures 1E, 2C**). Nevertheless, the number of cysts with mitotic spindles was not significantly changed upon IL-22 treatment (**Figure 2D**). Furthermore, we immunostained key cell polarity proteins, including Par-3, that has been described to orchestrate the assembly of apical junctions in epithelial cells and was thus expected to localize to TJs in polarized IECs, ZO-1 as a protein localizing to TJs, Ezrin as a component of the apical membrane and Dlg-1, demarcating the basolateral membrane. In general, we confirmed the expected protein localizations in established cysts 5 days after seeding (**Figures 3A–C**). Par-3 was localized to the most apical part of the lateral cell membrane in control cysts revealing the same localization as ZO-1 (**Figure 3A**, arrows; **Supplementary Figures 2A,B**). Ezrin was associated with the apical membrane and Dlg1 was restricted to the basolateral membrane. In contrast to that, in IL-22-treated cysts Par-3 was dislocated as it was found diffusely along the entire lateral membrane and also in intracellular vesicles (**Figure 3D**). Furthermore, membranous Dlg-1 staining was reduced compared to controls and was shifted to an intracellular compartment (**Figure 3D**). Ezrin staining was focally enriched at the basal membrane (instead of the apical membrane, **Figure 3E**, arrows), suggesting opposite polarization. In other cysts it demarcated aberrant lumens (**Figure 3F**, arrows). Taken together, these results suggest that IL-22 impairs intestinal epithelial polarity and lumen formation.

IL-22 Increases Cell Motility, Cell Invasion, and Induces EMT

As we had observed IEC polarity defects after IL-22 exposure, we next asked, whether IL-22 might also impact migratory and invasive properties of IECs. Thus, we carried out a CaCo-2 wound healing assay by performing uniform scratches into a single CaCo-2 layer that stably expressed Actin-GFP and monitored live by confocal LSM. Exposure to IL-22 (10 ng/ml) resulted in a statistically significant increased IEC migration, thereby nearly doubling IEC migratory speed (**Figures 4A,B**). Similarly, IL-22 had the capacity to induce invasion of cells in a combined Matrigel®/filter-based assay. After IL-22 exposure, the number of invaded colonies was ∼3-fold higher compared to control cells (**Figure 4C**). To us, these results appeared to be plausible findings in the context of epithelial-to-mesenchymal transition (EMT). Thus, the following experiments were designed to assess whether IL-22 induces EMT in IECs. Firstly, levels for proteins that are regulated within the EMT process, specifically E-cadherin and matrix metalloprotease-7 (MMP7), were quantified by western blotting in the course of exposing IECs to IL-22. While E-cadherin levels declined starting between 4 and 8 h of IL-22 exposure continuously, MMP7 expression peaked 24 h after IL-22 addition (**Figures 4D,E**). To further support the hypothesis that an EMT program is induced by IL-22, mRNA levels of classical EMT transcription factors, *SNAI1* (Snail) and *SNAI2* (Slug), were assessed after exposing the cells for 3 and 24 h to IL-22 (10 and 100 ng/ml). IL-22 significantly increased *SNAI1* and *SNAI2* gene expression at 24 h even higher than at 3 h of IL-22 exposure at both IL-22 concentrations (**Figures 4F,G**). In addition, MMP7-RNA levels were strongly upregulated after 3 and 24 h of IL-22 exposure (**Figure 4H**), in accordance to our previous data showed on protein levels by western blotting. In summary, these data indicate that IL-22 induces an EMT-like cell program, which might contribute to migratory as well as invasive properties of IL-22-treated IECs.

IL-22 Modulates Tight Junction Protein Expression

As we had shown that IL-22 induces a paracellular barrier defect in IECs and modulates the expression of genes that regulate junctional proteins, we next investigated the impact of IL-22 on expression and subcellular localization of TJ proteins. In a first step, we monitored the expression of various TJal claudins in the course of IL-22-exposure by western blotting (**Figure 5A**). As early as 4 h after exposing the cells to IL-22, claudin-1, a barrier-forming claudin, decreased on the protein level, whereas protein levels of the pore-forming claudin-2 and claudin-4, which was previously linked to EMT were increased (**Figure 5A**). Using confocal LSM we moreover found, that the PDZ-containing TJ-associated protein ZO-1 as well as the junctional adhesion molecule-A (JAM-A) were reduced in their junctional expression (**Figure 5B**). Similarly, TJal localization of occludin was shifted

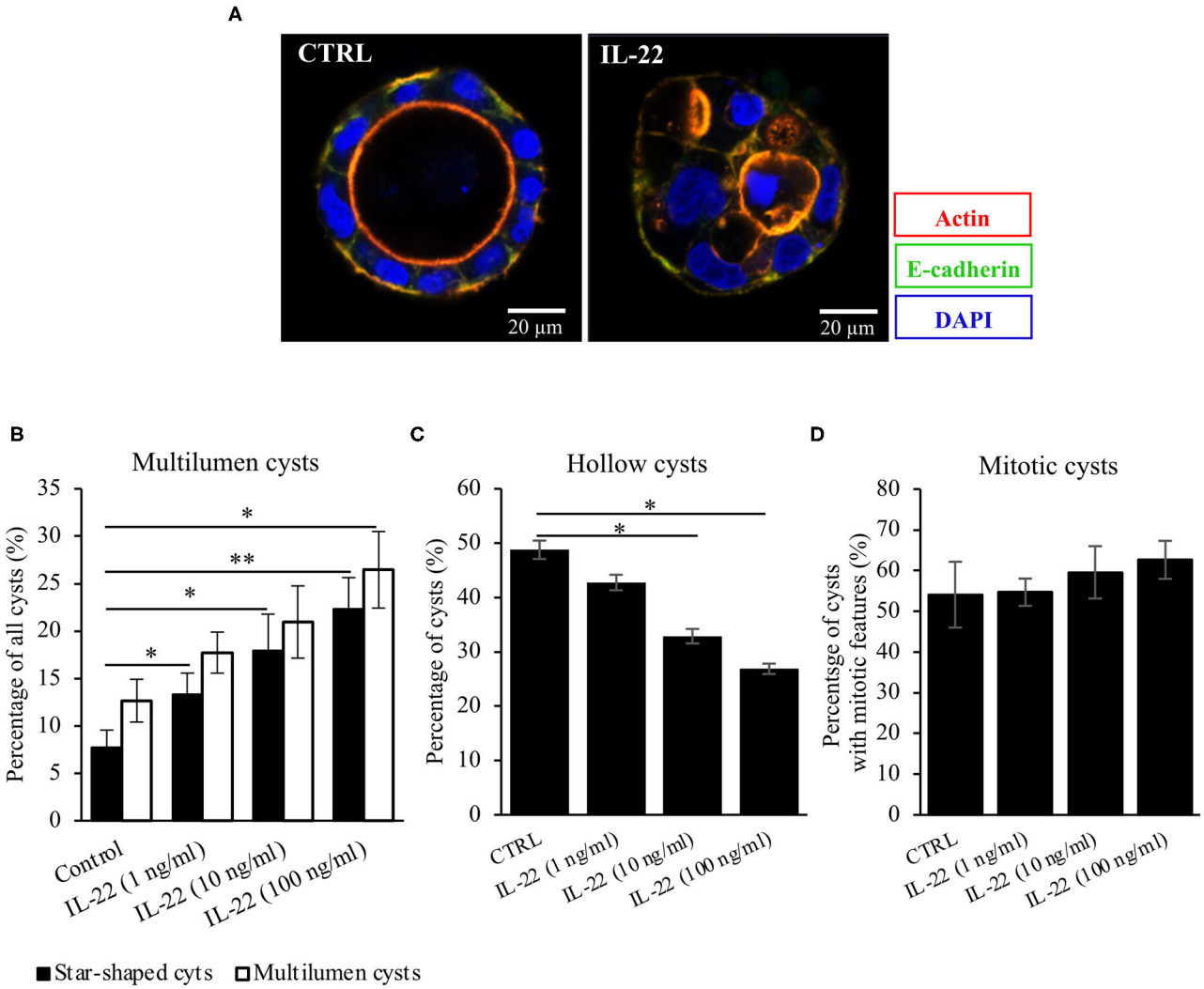

FIGURE 2 | IL-22 exposure causes atypical cysts formation. **(A)** CaCo-2 cells were seeded in Matrigel® and grown for 5–7 days to form 3D cysts. Subsequently, they were fixed and immunostained. Blue, nuclei; red, actin; green, E-cadherin. Representative images $n = 6$. **(B–D)** Quantification of the 3D-cyst experiments: CaCo-2 cysts growing in Matrigel® were analyzed by confocal LSM. Multilumen, hollow cysts and cysts revealing mitoses were microscopically quantified; $n = 6$. Mann–Whitney *U*-test; $^{*}p < 0.05$; $^{**}p < 0.01$.

to an intracellular and a lateral membrane localization in the 2D transwell and the 3D cyst model, respectively (**Figures 5C,D**).

IL-22 Mediates TJal Barrier Defects via ERK Pathway

Next, we aimed to dissect the intracellular signaling pathways after activation of the IL-22 receptor in IECs. All three IEC cell lines involved in this study equally expressed the two IL-22 receptor subunits (IL-22Ra1 and IL-10Rb), but did not express the endogenous IL-22 antagonist, the IL-22 binding protein, IL-22BP (**Supplementary Figure 3**). As shown in various previous studies, upon IL-22 receptor activation the STAT3 pathway as well as the MAPK/ERK pathway were activated. However, this occurred non-simultaneously (STAT3 at 15 min, ERK between 30 min and 4 h, **Figure 6A**). Interestingly, we did not detect any phosphorylation of AKT in our model system. Since activation of STAT3 signaling was previously reported to play a role in epithelial protection, we next determined the effect of various strategies to inhibit STAT3 signaling on STAT3 activation and epithelial barrier function (**Figures 6B,C**). While the STAT3 inhibitor WP1066 reduced the pSTAT3 signal, it also reduced total STAT3 levels which was explained by a strong induction of programmed cell death (as revealed by cleaved caspase-3, **Figure 6B**). Consecutively, STAT3 inhibition did not rescue the IL-22-induced barrier defect (**Figure 6C**). In line with this finding other STAT3 inhibitors were not capable of reversing epithelial barrier defects by IL-22 (**Supplementary Figure 4**). On the other hand, inhibition of ERK/MAPK was successful regarding signaling as well as rescue of barrier function (**Figures 7A–D**). Using the MEK inhibitor U0126, we achieved

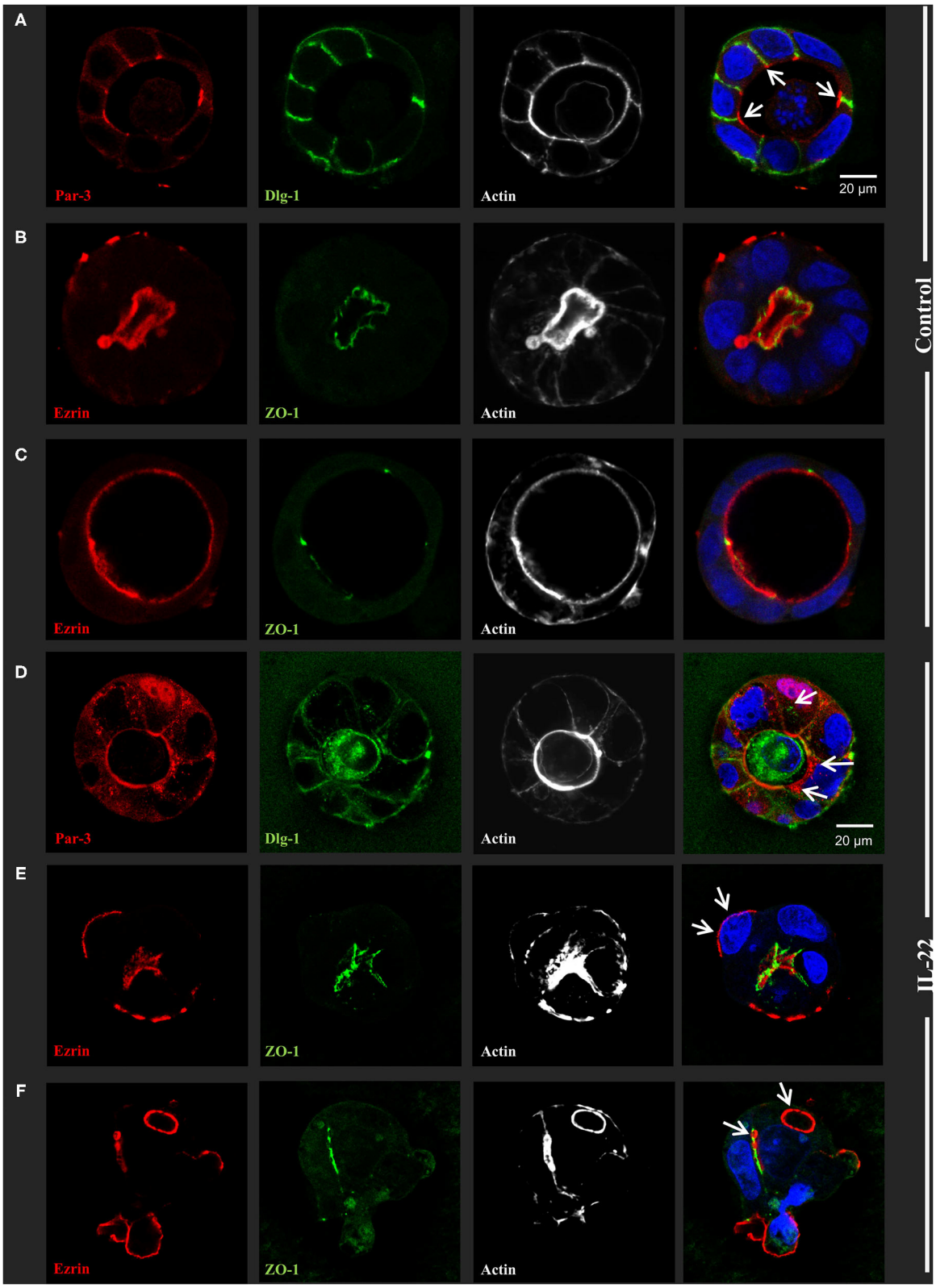

FIGURE 3 | IL-22 induces development of dyspolar 3dimensional cysts. CaCo-2 cells were seeded in Matrigel and 3D-cysts were allowed to develop within 5–7 days. Then cysts were fixed and immunostained according to the Methods section. **(A–C)** control 3D-cysts. **(D–F)** cysts treated with IL-22 (10 ng/ml) starting at the day after seeding. Proteins detected by immunostaining are depicted in each image. The composite image (right column) additionally includes staining for nuclei using DAPI. Structures identified by arrows are explained in the text of the Results chapter; $n = 4$ independent experiments.

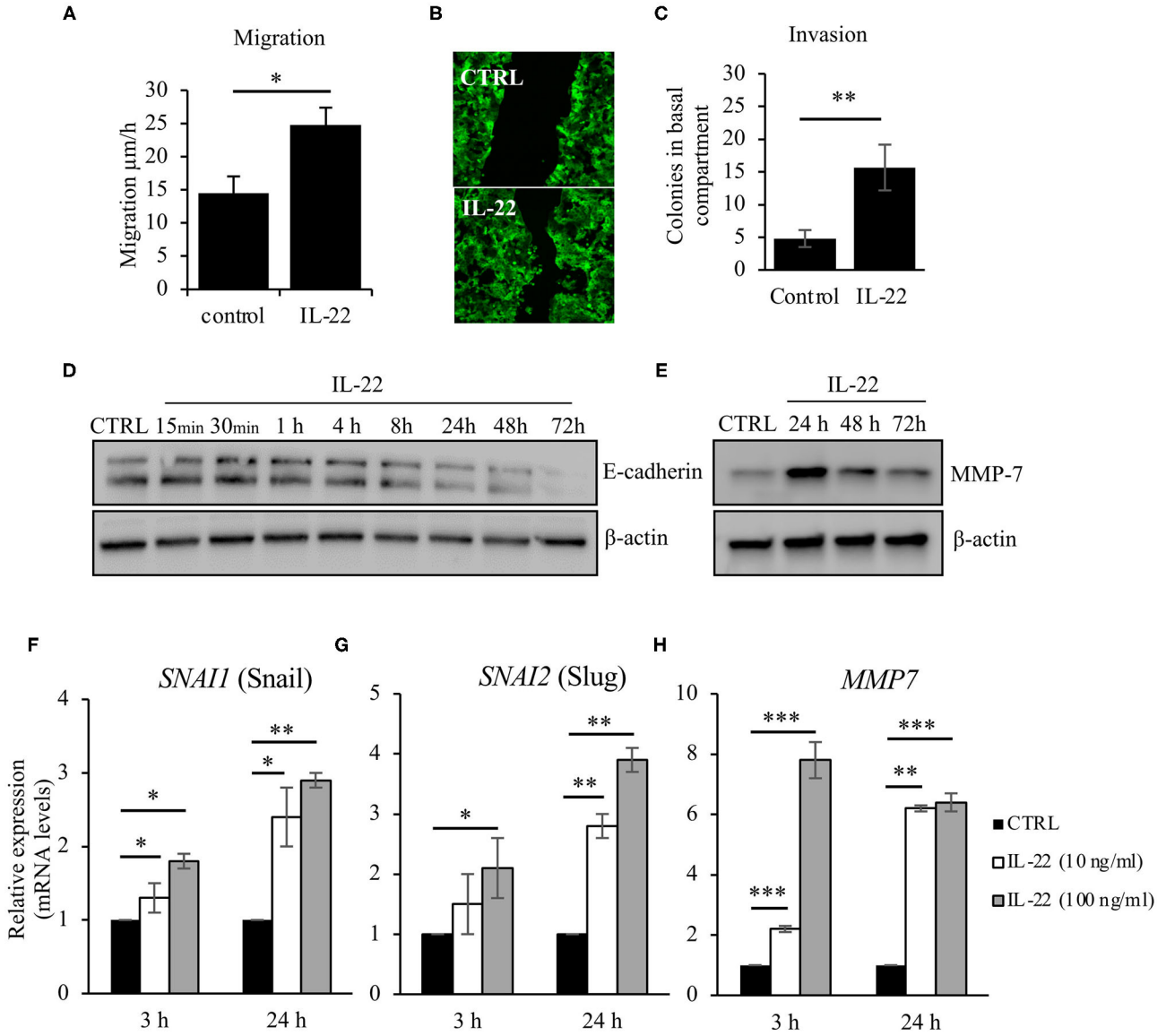

FIGURE 4 | IL-22 increases cell motility and induces EMT on IECs. **(A–C)** HT29/B6 cells exposed to IL-22 (10 ng/ml) growing on transwell filters were scratched (diameter 100 µm). Migration was evaluated by measuring the remaining scratch width at 24 and 48 h after scratching by fluorescence microscopy; $n = 3$ **(B)** For invasion, CaCo-2 cells were seeded on Matrigel®-coated filters and then exposed to IL-22 (100 ng/ml). The invasion of CaCo-2 cells through the Matrigel®-coated filter was quantified by counting colonies that formed on the basal side of the filter $n = 3$. **(D,E)** CaCo-2 cells were exposed to IL-22 (10 ng/ml) and lysed. E-cadherin and MMP-7 protein levels were investigated through western blotting. Representative Western blots of three and two independent experiments, respectively. **(F–H)** CaCo-2 cells were exposed to IL-22 (10 or 100 ng/ml) for 3 and 24 h. RNA was extracted and RT-qPCR was performed to quantify expression of Snail (SNAI1), Slug (SNAI2), and MMP-7. Expression levels were calculated using the $2^{-\Delta\Delta CT}$ method. Mann–Whitney *U*-test; $n = 3$; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

close to total inhibition of ERK phosphorylation, thereby rescuing the IL-22-induced TER-reduction (**Figures 7A,B**). In accordance with the signaling study, IL-22-induced dislocation of occludin was reversed by MAPK inhibition (**Figure 7C**). Similarly, reduction of E-cadherin and claudin-1 protein levels as well as increases in claudin-2,−4 and MMP7 were normalized by U0126 treatment (**Figure 7D**). Altogether, our results indicate that MAPK/ERK signaling is central in mediating IL-22-dependent barrier and EMT signaling in IECs.

IL-22 Induces Barrier Defect in a Mouse Model of Terminal Ileitis

In addition to the IEC *in vitro* experiments, barrier function was examined using the *Toxoplasma gondii* (*T. gondii*) mouse model of terminal ileitis in mice lacking IL-22 (8). *T. gondii* has previously been described to induce IL18 expression in IECs in an IL22-dependent manner. However, the detailed consequences on the structural and functional intestinal barrier mediated by the presence or absence of IL22 have not been investigated. In

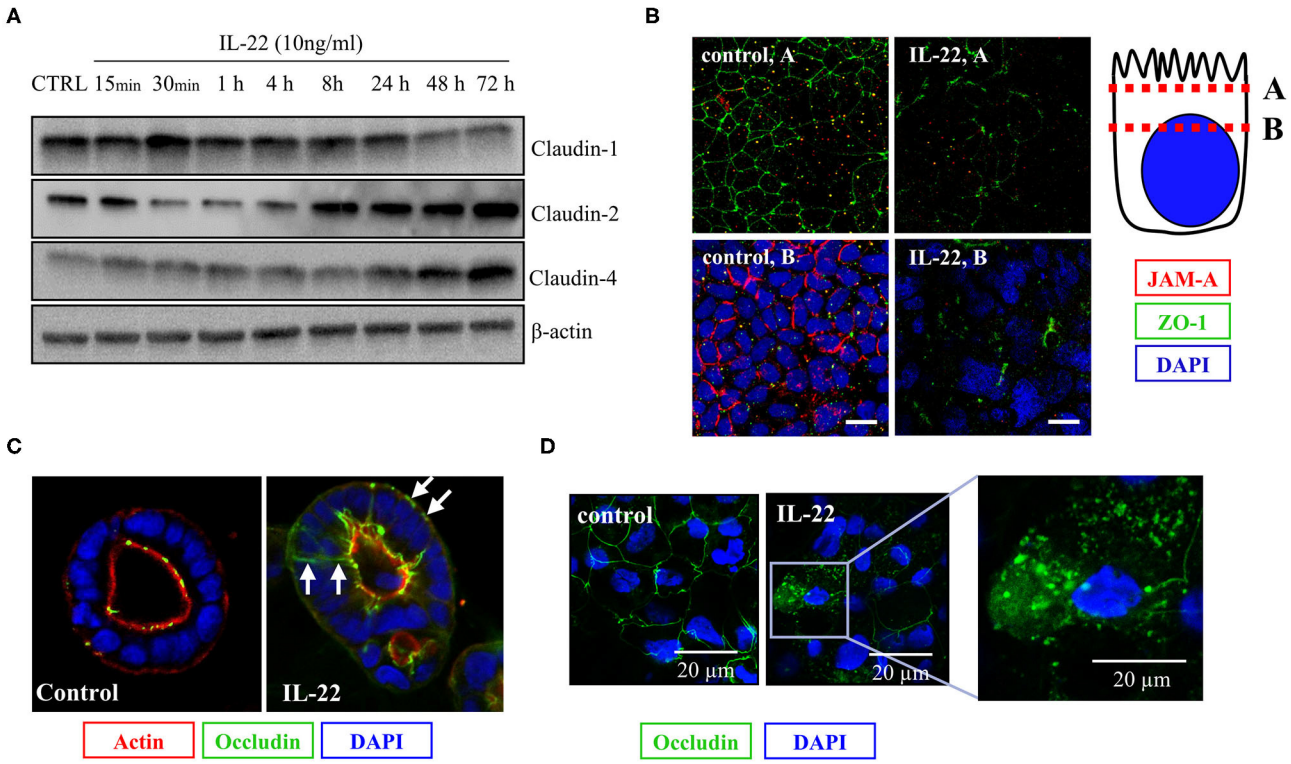

FIGURE 5 | IL-22 affects tight junction proteins. **(A)** Expression of TJ proteins: IECs were treated with IL-22 (10 ng/ml) for the indicated times. Subsequently, cells were lysed. TJ protein levels were determined by Western blotting as explained in the Methods section. Representative blots of three independent experiments. **(B–D)** Confocal LSM after immunostaining of CaCo-2 cells. **(B)** Reduction of JAM-A junctional levels by IL-22. Red, JAM-A; green, ZO-1; blue, nuclei $n = 3$. IECs cartoon represents the confocal microscopy analysis **(C,D)** Dislocation of occludin by IL-22. Green, occludin; red, actin; blue, nuclei; $n = 3$.

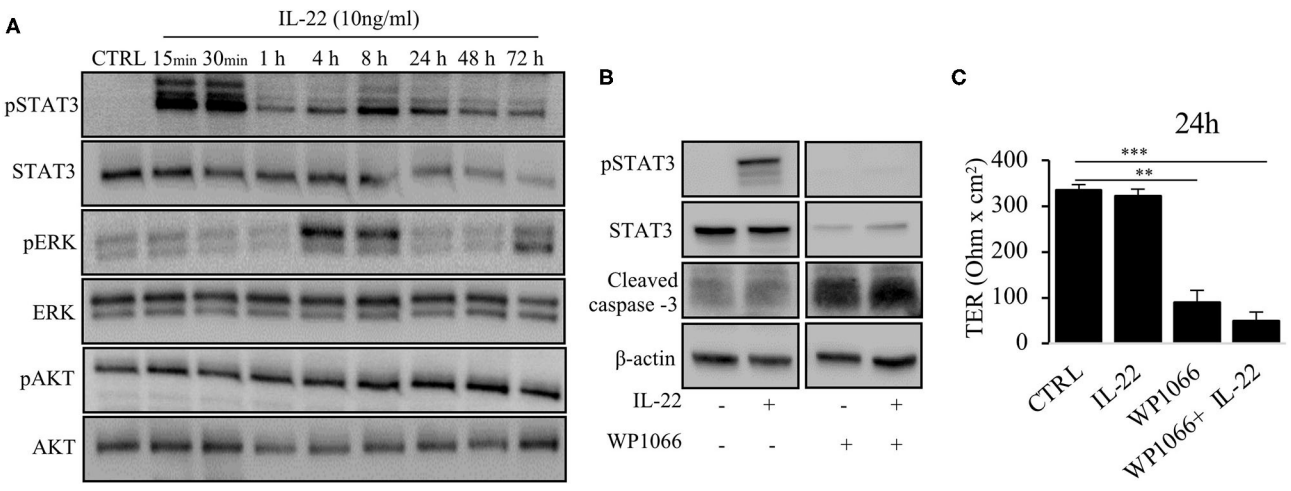

FIGURE 6 | IL-22 induces STAT3 and ERK phosphorylation. **(A)** HT-29/B6 cells were exposed to IL-22 (10 ng/ml) for the indicated times. Subsequently, cells were lysed and protein levels of STAT3, ERK and AKT and their phospho-levels were investigated by Western blotting. Representative blots of three independent experiments are shown. **(B)** HT-29/B6 cells were incubated in the presence of the STAT3 inhibitor WP1066 (50 µM) for 2 h and IL-22 (10 ng/ml) for 1 h as indicated. Western blotting of cell lysates was performed to quantify protein levels of STAT3 total, phospho-STAT3 (pSTAT3), cleaved caspase-3, and b-actin as loading control. **(C)** TER was determined after 24 h of IL-22 exposure of HT-29/B6 cells growing on transwell filters treated with IL-22 (10 ng/ml), WP1066 (50 µM) as indicated. Mann–Whitney *U* test; $n = 3$; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

line with previously published data, *T. gondii* induced a severe terminal ileitis in C57Bl/6 mice after seven days of infection as seen in H&E stainings of formalin-fixed, paraffin-embedded sections (**Figure 8A**). Mucosae from the terminal ileum of IL-22 deficient and wild type control mice were mounted to Ussing chambers and analyzed by one-path impedance spectroscopy to

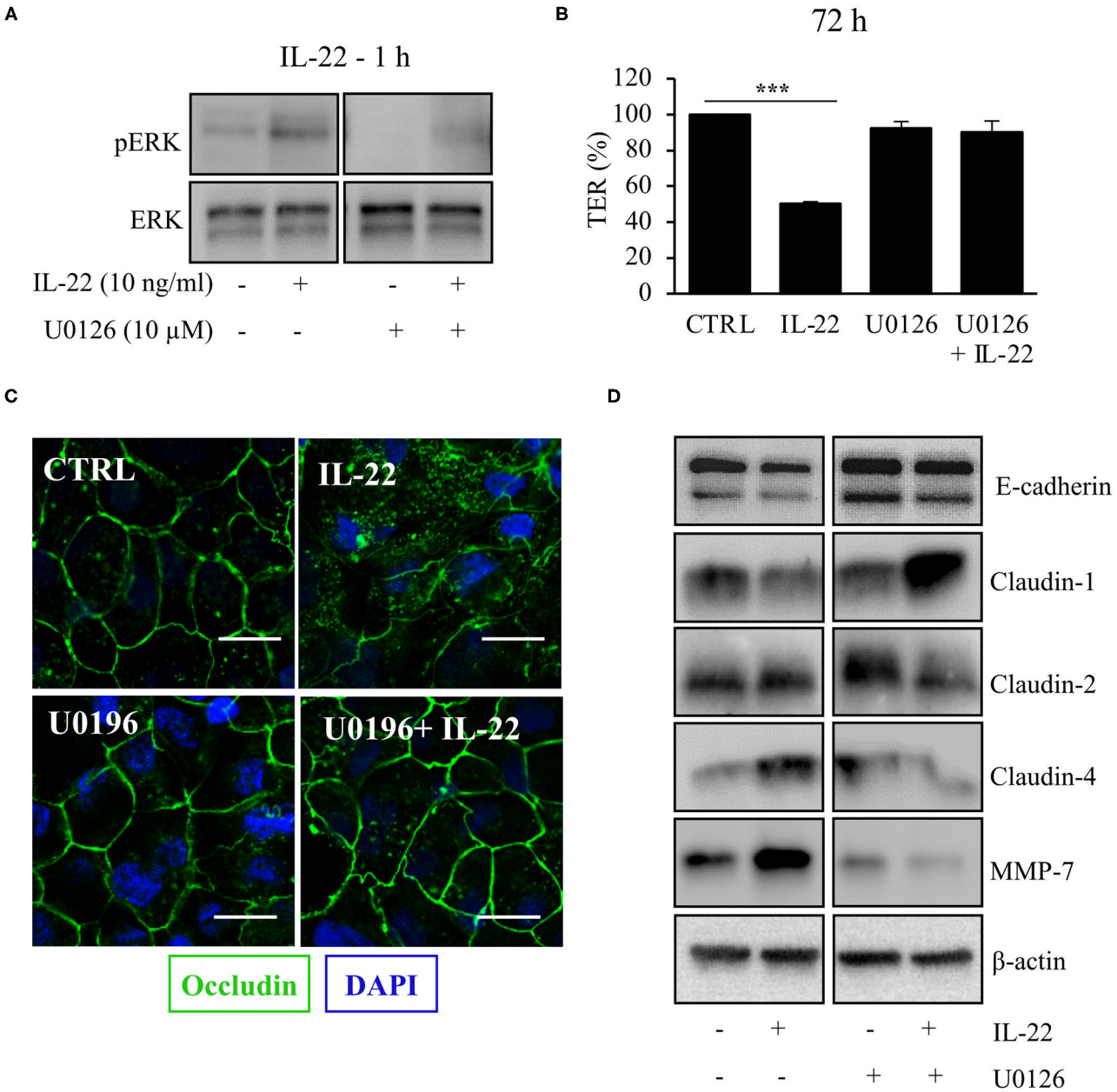

FIGURE 7 | MAPK/ERK signaling pathway is pivotal for IL-22 barrier signaling in IECs. HT-29/B6 cells were exposed to U0196 (2 h) in the presence or absence of IL-22 (1 h). **(A)** Protein levels of ERK and phospho-ERK were assessed by Western blotting. Representative blots of three independent experiments are shown. **(B)** TER of HT-29/B6 cells were measured after treatment with U0126 and IL-22 (10 ng/ml), as depicted; $n = 9$, $^{***}p < 0.001$. **(C)** Confocal LSM after immunostaining of occludin was performed. Green, occludin; blue, nuclei. Scale bar: 20 µm. Representative images of three independent experiments. **(D)** Western blotting was performed to quantify protein levels of E-cadherin, claudin-1,−2,−4, and MMP-7. Representative blots of two independent experiments are shown; $^{***}p < 0.001$.

examine not only the total intestinal wall resistance, but also its epithelial portion [R^e, (15), **Figure 8B**]. As expected, the terminal ileal mucosa of *T. gondii*-infected wild type mice displayed a significant defect of the epithelial barrier and an increase in macromolecular permeability when compared to wildtype mice (3H-mannitol, **Figures 8B,C**). Interestingly, in line with our cell culture findings, mice lacking IL-22 expression were protected from this barrier defect and exhibited a significantly higher R^e and a statistically non-significant tendency toward a higher mannitol permeability (**Figures 8B,C**). In summary, these results give *in vivo* and *ex vivo* evidence showing that IL-22 plays an important role in the intestinal barrier function.

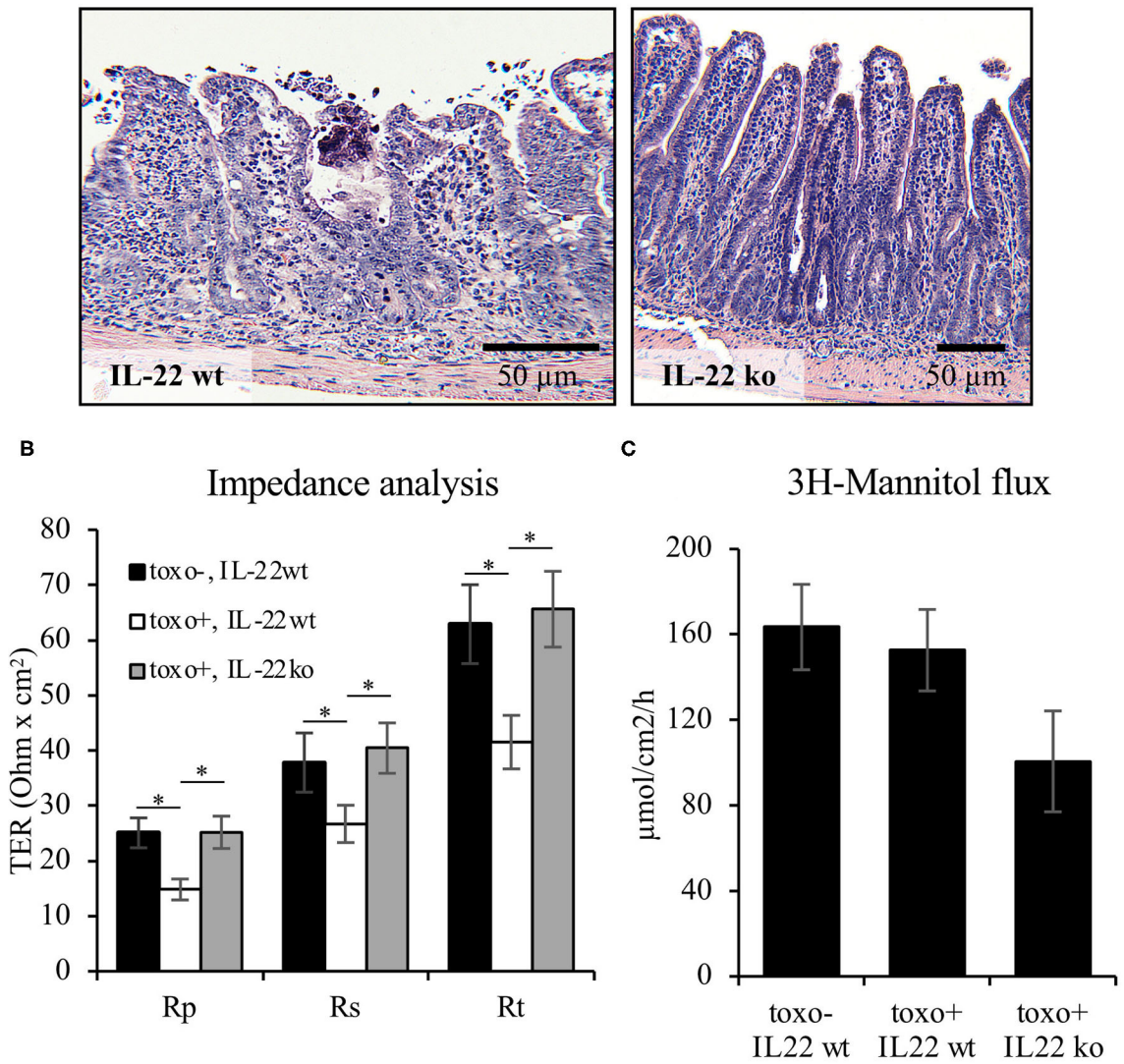

FIGURE 8 | IL-22 induces an epithelial barrier defect in murine mucosa. For induction of a murine *T. gondii* terminal ileitis, IL-22-deficient and wildtype control mice were orally infected with T. gondii cysts. The terminal ileum was explanted at day 7. **(A)** Histological examinations were done in formalin-fixed and paraffin-embedded tissue following H&E staining. **(B,C)** Explanted mucosae from the murine terminal ileum were mounted to Ussing chambers and analyzed by one-path impedance spectroscopy to examine total intestinal wall resistance (R^t), the epithelial resistance (R^e) and the subepithelial resistance [R^s, **(B)**]. Macromolecular permeability was determined by measuring the flux of 3H-mannitol **(C)**; $n = 9$, $^*p < 0.05$.

DISCUSSION

IL-22 has a central role in type 3 mucosal immunity, which is directed against extracellular bacteria and fungi. IL-22 is tonically secreted by ILC3 cells sedentary to the gut mucosa and, additionally, "on demand" by T_H17 cells (10, 32). The fact, that the IL-22 receptor is exclusively expressed on non-hematopoietic epithelial and stromal cells, has prompted the idea of an ILC3-IL-22-IEC axis (32). Current understanding implies that IL-22 is contributing to type 3 immunity by (*ex vivo*) the production of antimicrobial peptides (AMPs) including β-defensins, the C-type lectins RegIIIβ and RegIIIγ, lipocalin-2, and calprotectin as well as amplifying IEC turnover to disturb colonization of the gut epithelial lining by bacteria (10, 12, 33–35). Furthermore, it has been described that IL-22 supports "epithelial integrity," which however, is incompletely analyzed so far, since studies have mostly focused on AMP expression and wound healing assays and missed out classical barrier function as well as analysis of the apical junctional complex (7, 17). Thus, our study aimed to functionally analyze the IL-22-exposed epithelial barrier and

to characterize IL-22-altered epithelial polarity as this might be fundamental to orchestrating barrier function (36, 37).

Our first finding—IL-22 inducing a profound reduction of IEC transepithelial resistance corresponding to a relevant increase in small ion flux—came unexpected as it is common knowledge that IL-22 rather stabilizes but destroys epithelial barrier function (7, 17). Since it was unexpected, we validated this finding in three different intestinal epithelial cell lines, established its dose- and time-dependence and presented evidence that these findings correspond to the IL-22-related epithelial barrier function in murine intestines as *ex vivo* one-path impedance analyses of mucosae from IL-22 knock-out mice revealed an IL-22-dependent epithelial barrier defect. Importantly this defect (i) is clearly localized to the paracellular junction as evaluated by two-path impedance analysis and (ii) is not limited to small ion flux as shown by sandwich assay studies and by a tendency to higher mannitol fluxes in the Ussing studies on murine intestinal epithelia. Nevertheless, two studies, specifically those by Tsai et al. (38) and by Wang et al. (39) are in full accordance to our findings, since both uncovered an IL-22- and claudin-2-dependent mechanism for triggering a leak-flux diarrhea in the murine *Citrobacter rodentium* and an epithelial barrier defect for small solutes in the CaCo-2BBE model.

After having confirmed that the previously described wound healing potential of IL-22 holds also true in our model, we questioned, whether IL-22 might reprogram epithelia in a way that would explain likewise the induction of transient increases in solute permeability and the potential to support the healing of mucosal wounds. Our working hypothesis was that this would be compatible with epithelial-to-mesenchymal transition (EMT). Hence, we established an IL-22-induced expression of transcription factors (Snail, Slug) characteristic for EMT, as well as decreased expression of epithelial markers (E-cadherin) as well as induction of a protein that points to a reorganization of mucosal architecture and allows for epithelial invasion (MMP7). IL-22's potential to induce EMT can be compared to that of IL13 as this T_H2 cytokine had been previously shown to induce EMT in a similar fashion (40). However, if an EMT-like program orchestrates the reorganization of TJs aiming to release junctional tightness and thereby facilitating IEC migration into a wound, epithelial polarity is likely altered beforehand (41). Thus, we investigated the status of epithelial polarity after exposing intestinal epithelial cysts with IL-22. Indeed, IL-22 significantly disturbed epithelial polarity including the establishment of a single lumen in Caco-2-cysts as well as the dislocation of polarity complex proteins that (like Par3) are pivotal to the assembly of primordial apical junctions. Accordingly, TER monitoring of IL-22-exposed IECs after calcium switch provided functional evidence for a defective assembly of TJs.

In terms of intracellular signaling the study was in contradiction to a number of previous studies as it did not confirm the prominent role of JAK/STAT signaling, especially STAT3, in our model system (7, 17, 38, 39). In fact, STAT3 was activated by IL-22 exposure but could only be related to survival signaling and not to TJal and polarity reprogramming. Instead, we found a mostly unprecedented function for IL-22-induced activation of MAPK, since inhibitor studies revealed evidence for ERK signaling to be causative for signaling to TJs. Our experiences, that epithelial cell death occurs as soon as STAT3 is inhibited goes in line with results from several previous publications (17, 42). One should emphasize the limitation that our study was performed using cell lines and that a consecutive study using primary cells, e.g., a 2D organoid model, might help to solve this controversy.

Moreover, our data suggest that the potential of IL-22 to reprogram epithelia in order to close mucosal wounds comes with the expense of acquiring dyspolar epithelia and to induce cellular features as MMP7 expression and actin filaments-driven invadopodes that finally contribute to epithelial invasiveness. This goes in line with data on murine colitis-associated cancer, which, however, reveal some complexity (42). On the one hand, IL-22-knock-out mice develop a higher tumor burden, which was related to a substantially increased inflammatory activity after colitis induction by dextrane sulfate sodium. On the other hand, mice, in which the endogenous IL-22 opponent, IL-22-BP, was knocked out also developed more tumors, which was interpreted as a long-term effect of the increased IL-22-availability and secondary to that prolonged epithelial proliferation during the recovery phase of inflammation (43, 44).

In summary, we have used model systems including three IEC lines, functional as well as subcellular structural experimental setups and a murine terminal ileitis model to describe the epithelial response to the T_H17 cytokine IL-22. From these data we propose that IL-22 induces an EMT-like program that induces intestinal epithelia to reduce their epithelial-specific polarity. Secondary to that, a loosening of intercellular junctions occurs, that allows IECs to migrate into wounds but also to become more invasive. It is so-far speculative, that the latter process might contribute to colitis-associated carcinogenesis once the IL-22:IL-22BP ratio becomes too high. Interestingly, we found rather MAPK/ERK to be responsible for these actions than the JAK/STAT pathway.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Landesamt für Gesundheit und Soziales (Lageso, Berlin; TVV-No G0258/04).

AUTHOR CONTRIBUTIONS

DD: plan and carry out the signaling studies and barrier experiments, confocal LSM, and writing the paper. LL: plan and carry out the 3D cyst studies and barrier studies, and confocal LSM. DC-S: carry out the EMT studies and western blotting. VD: western blotting of STAT3 inhibitor studies. SK: plan and carry out 2-path impedance analysis and revision of manuscript. JR: design of the 3D cyst assay and the sandwich assay. CH:

immunostaining and cell culture. KW and RS: planning and carrying out of IL22 and IL22 receptor RT-PCR. SM: paper writing and statistics. MM and MH: establish and generate the terminal ileitis mouse model. BS: experimental strategy and writing the paper. MS: defining the experimental strategy, barrier studies on mouse mucosa, 3D cyst assays, immunostaining and confocal LSM, and writing the paper. All authors contributed to the article and approved the submitted version.

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The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.656047/full#supplementary-material

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An IFN-STAT Axis Augments Tissue Damage and Inflammation in a Mouse Model of Crohn's Disease

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Blocking interferon-function by therapeutic intervention of the JAK-STAT-axis is a novel promising treatment option for inflammatory bowel disease (IBD). Although JAK inhibitors have proven efficacy in patients with active ulcerative colitis (UC), they failed to induce clinical remission in patients with Crohn's disease (CD). This finding strongly implicates a differential contribution of JAK signaling in both entities. Here, we dissected the contribution of different STAT members downstream of JAK to inflammation and barrier dysfunction in a mouse model of Crohn's disease like ileitis and colitis (*Casp8*$^{\Delta IEC}$ mice). Deletion of STAT1 in *Casp8*$^{\Delta IEC}$ mice was associated with reduced cell death and a partial rescue of Paneth cell function in the small intestine. Likewise, organoids derived from the small intestine of these mice were less sensitive to cell death triggered by IBD-key cytokines such as TNFα or IFNs. Further functional *in vitro* and *in vivo* analyses revealed the impairment of MLKL-mediated necrosis as a result of deficient STAT1 function, which was in turn associated with improved cell survival. However, a decrease in inflammatory cell death was still associated with mild inflammation in the small intestine. The impact of STAT1 signaling on gastrointestinal inflammation dependent on the localization of inflammation, as STAT1 is essential for intestinal epithelial cell death regulation in the small intestine, whereas it is not the key factor for intestinal epithelial cell death in the context of colitis. Of note, additional deletion of STAT2 was not sufficient to restore Paneth cell function but strongly ameliorated ileitis. In summary, we provide here compelling molecular evidence that STAT1 and STAT2, both contribute to intestinal homeostasis, but have non-redundant functions. Our results further demonstrate that STATs individually affect the distinct pathophysiology of inflammation in the ileum and colon, respectively, which might explain the diverse outcome of JAK inhibitors on inflammatory bowel diseases.

Keywords: inflammatory bowel disease, STAT signaling, inflammation, cell death, Paneth cells

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INTRODUCTION

Based on similarity in their structure and function as well as sharing of downstream signaling pathways, interferons (IFNs) are grouped into three families: Type I with IFNα, IFNβ, and several minor subtypes; Type II with IFNγ and Type III with IFNλs. Their expression can be induced in response to diverse viral and bacterial stimuli in an autocrine or paracrine fashion (1–3). Ligation

of IFNs to their corresponding receptors activates the Janus kinase (JAK)-signaling transducer and activator of transcription (STAT) signaling pathway. Activated JAKs induces STAT dimer formation, homo- or heterodimer depending on the context, and subsequently expression of different target genes involved in various biological processes (2, 4, 5). The canonical JAK-STAT signaling includes STAT1-STAT2 heterodimers, with associated complex formation, in response to type-I and type-III interferons and STAT1-homodimers following IFNγ ligation. However, non-canonical JAK-STAT signaling in response to all types of IFNs can provoke homodimer formation (all STATs 1–6) as well as different combinations of heterodimers with other members. Beside this, type-I IFNs can also induce gene activation independently of STATs. This manifold setting, with various dimer and complex formations, enables the modulation of gene transcription of several hundred different genes by IFNs (2–6). Accordingly, IFNs are currently considered as key cytokines in autoimmune diseases such as inflammatory bowel disease (IBD), rheumatoid arthritis or autoimmune hepatitis (7–12). Inflammatory bowel diseases are prototypic immune-mediated inflammatory diseases with a globally increasing prevalence, affecting the gastrointestinal tract. IBD includes the major forms ulcerative colitis and Crohn's disease. The main differences of these both forms is the localization of inflammation: While ulcerative colitis only affects the colon, Crohn's disease can cause inflammatory lesions along the whole gastrointestinal tract predominantly in the terminal ileum. In this context, it has just recently been shown that IFNλ is a key factor for small intestinal inflammation that can trigger mucosal inflammation by influencing host cell death pathways in the context of IBD (13). Crohn's diseases patients displayed increased IFNλ serum and tissue levels associated with severe inflammation, and increased cell death accompanied by a dramatic reduction of Paneth cell numbers in the small intestine. The same study depicted that interferons were able to alter *Caspase-8* as well as *Mlkl* gene expression in intestinal epithelial cells (IECs) to induce apoptosis and necroptosis (13). In contrast to this, IFNλ promotes tissue regeneration and mucosal healing in the colonic tissue, highlighting a specific regulation of downstream signaling mechanisms depending on the cellular and spatial context (14).

Caspase-8 is a central cell death regulator that is involved in various cellular processes. Translational studies have demonstrated that humans with Caspase-8 mutations display an early onset IBD associated with massive epithelial cell death and severe inflammation (15, 16). In line with these studies it has been previously shown that mice lacking this central cell death regulator ($Casp8^{\Delta IEC}$) in intestinal epithelial cells spontaneously develop intestinal inflammation with histomorphological alterations similar to the classical features of Crohn's disease. Accordingly, $Casp8^{\Delta IEC}$ mice mimic several important characteristics including Paneth cell depletion accompanied by microbial dysbiosis, the culprit of inflammation in the ileum, as well as the immune cell signature (Th1 driven) association with elevated IFN levels (17–19). Inflammation in these mice is primarily located in the distal part of the small intestine (ileum), and depending on the microbial composition can vary in extent and localization to cause colitis or extensive enteritis (17, 18). Colonic inflammation is dependent on TNFα signaling, whereas TNFα deficiency is able to ameliorate colonic inflammation, but is not sufficient to prevent Paneth cell loss or enteritis in the small intestine (19, 20). Hence, intestinal epithelial cell death seems to have shared general mechanism but requires a tissue specific regulation.

Emerging evidence have indicated IFNs and their immune-modulatory function, as well as their impact on cell death mechanisms, as important factors in the pathogenesis of IBD and as a central point for therapeutic intervention (13, 21). Current strategies focus on blocking the JAK-STAT signaling downstream of IFNs. Tofacitinib, a small molecule JAK inhibitor, can attenuate disease activity in patients with active ulcerative colitis accompanied by improved clinical response and mucosal healing (22, 23). While these data are promising, tofacitinib was insufficient to induce a clinical benefit for patients with Crohn's disease (24–26). Phase II trials reported biological activity of tofacitinib in Crohn's disease patients, but without a significant clinical benefit (24–26) and even indicated disease worsening (25). In line with this, recent data derived from preclinical studies, demonstrated that intestinal inflammation in CD and UC models displayed different disease mechanisms associated to IFN-coordinated cell death (13, 14). In contrast to the broad JAK inhibitor tofacitinib (JAK1 and JAK3 inhibitor), more selective inhibitors like filgotinib and upadacitinib (JAK1 inhibitor) seem to have more therapeutic benefit in both diseases but clinical trials are currently ongoing. For Crohn's disease patients, filgotinib reduced fecal calprotectin and C-reactive protein levels, and was associated with mucosal healing and clinical remission (27). However, broad JAK inhibitors such as tofacitinib not only block IFN-signaling. Depending on the cellular context, various cytokines can activate the JAK-STAT signaling pathway, e.g., IL-6, IL-10, which can result in the activation of various STAT pathways (28). Clinical trials using either specific or broad JAK-inhibitors, reported differences in clinical benefit and treatment success which might be explained with the differential role and relevance of the underlying signaling cascade depending on JAK-STAT pathway. Accordingly, detailed mechanisms are still missing and further knowledge is required.

Here we provide molecular evidence that STAT1 and STAT2 both contribute to intestinal inflammation but have non-redundant functions. Of note, the impact of STAT1 signaling on intestinal inflammation seems to be strongly dependent on the localization of inflammation as STAT1 is involved in cell death regulation in the small intestine associated with Paneth cell death, whereas it is not the key factor for epithelial death in the context of colitis.

MATERIALS AND METHODS

Mice

$Casp8^{\Delta IEC}$ (17), $Casp8^{\Delta IEC}$x$Mlkl^{-/-}$ (13), $Stat1^{-/-}$ (29), $Stat2^{-/-}$ (30) mice were described earlier. $Casp8^{\Delta IEC}$x$Stat1^{-/-}$ were generated by crossing $Casp8^{\Delta IEC}$ mice to $Stat1^{-/-}$ mice, $Casp8^{\Delta IEC}$x$Stat1^{+/-}$x$Stat2^{-/-}$ and $Casp8^{\Delta IEC}$x$Stat1^{-/-}$x$Stat2^{-/-}$ mice were generated by crossing $Casp8^{\Delta IEC}$ mice to $Stat1^{-/-}$x$Stat2^{-/-}$ mice. As controls we used

littermates or C57BL/6 mice. At the end of the experiments, mice were sacrificed by cervical dislocation. Mice were routinely screened for pathogens according to FELASA guidelines. Animal procedures were approved by the Institutional Animal Care and Use Committee of the Regierung von Unterfranken and conducted by qualified personnel.

DSS-Colitis

Experimental colitis was induced in mice by the administration of 2% dextran sodium sulfate (DSS) in the drinking water for 5 days. The development of colitis was monitored with a high-resolution video mini-endoscopic system. Endoscopic scores for intestinal inflammation were assigned based on the criteria described for the assessment of the murine endoscopic index of colitis severity, which scores the following parameters: translucency, granularity, fibrin, vascularity, and stool consistency, as previously described (31).

Organ Collection and Storage

Tissue for histology and immunohistochemistry were collected and fixed in 4.5% PFA. Tissue was embedded in paraffin in a water-free procedure and stored at room temperature for further analysis. Samples for RNA and protein analyses were instantly frozen in liquid nitrogen and stored at −80 °C until further use.

Histology and Immunohistochemistry

Histopathological analyses were performed on formalin-fixed paraffin-embedded tissue cross sections after Mayer's haematoxylin and eosin (H&E) staining or a combined staining with periodic acid–Schiff reaction and alcian blue (PAS). Immunofluorescence of tissue sections was performed using Streptavidin Protein DyLight (Thermo). Primary antibodies (for detailed information see **Supplementary Table 1**) were incubated overnight. Nuclei were counterstained with Hoechst 33,342 (Invitrogen). Cell death (TUNEL) was analyzed using the *In-Situ* Cell Death Detection Kit (Roche). Images were obtained using the microscope LEICA DMI 4000B together with the LEICA DFC360 FX or LEICA DFC420 C camera or the microscope Leica DMi1 with the corresponding imaging software.

Gene Expression Analysis

Total RNA was extracted from intestinal tissue using the peqGOLD Total RNA Kit or Total peqGOLD Microspin Total RNA Kit for organoids (peqLab/VWR). cDNA was synthesized by reverse transcription using the SCRIPT cDNA Synthesis Kit (Jena Bioscience) and analyzed by real-time qPCR using SYBRGreen reagent (Roche), the LightCycler 480 (Roche) and specific QuantiTect Primer Assays (Qiagen) (for detailed information see **Supplementary Table 2**). Experimental values were normalized to levels of the housekeeping gene *hypoxanthine guanine phosphoribosyl transferase* (*Hprt*) or *Glyceraldehyde 3-phosphate dehydrogenase* (*Gapdh*). For fold change calculation, the average mean of the relative expression of control mice were set as 1.

Organoid Culture

Small intestinal organoids were isolated from the mouse small intestine and cultured with ENR medium (organoid medium with epidermal growth factor/Noggin/R-spondin) for a minimum of 7 days according to Sato et al. (32). Organoid growth was monitored by light microscopy. Organoids were stimulated with TNFα (25 ng/ml) and IFNβ (100 ng/ml) and stained with Propidium Iodide Staining Solution (BD Pharming). Images were obtained using the microscope LEICA DMI 4,000 B together with the LEICA DFC360 FX or LEICA DFC420 C camera or the microscope Leica DMi1 with the corresponding imaging software.

Statistical Analyses

Comparisons of two groups were performed using an unpaired two-tailed *t*-test. Comparisons among multiple groups were performed using ANOVA followed by multiple comparison and statistical significance was accepted with $p < 0.05$ (NS $p \geq 0.05$; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$). Statistical calculations were performed using GraphPad Prism 8 (GraphPad Software).

RESULTS

STAT1 signaling attenuates MLKL mediated necroptosis in the ileum and partially restores Paneth cell function.

Previously, we have demonstrated that IFNs can trigger programmed necrosis by regulating *Mlkl* gene transcription via activation of the transcription factor STAT1 in hepatocytes (33). Furthermore, STAT1 alters the gene expression of *Caspase-8* and *Mlkl* in the small intestine and interferon-induced cell death seems to plays a crucial role during Crohn's disease like ileitis (13). To study the impact of STAT1 on gastrointestinal inflammation with features of Crohn's disease, we deleted this transcription factor in the *Caspase-8* mouse model ($Casp8^{\Delta IEC}$). Generation of double deficient mice strain ($Casp8^{\Delta IEC}$x$Stat1^{-/-}$) was not trivial, as both genes are closely located on chromosome 1 (*Caspase-8*: 1 C1.3; 1 29.19 cM, *Stat1*: 1 C1.1; 1 26.81 cM), suggesting that STAT1 might be relevant upstream of Caspase-8.

$Casp8^{\Delta IEC}$ mice are characterized by MLKL mediated epithelial necroptosis, Paneth cell depletion and ileitis (17). In line with these observations, gene deletion of *Mlkl* in $Casp8^{\Delta IEC}$ mice ($Casp8^{\Delta IEC}$x$Mlkl^{-/-}$ mice) was sufficient to block Paneth cell death and inflammation (17) (**Supplementary Figure 1**). These data clearly demonstrate that indeed Caspase-8 is a negative regulator of MLKL mediated cell death in the intestinal epithelium. In line with our previous observation, mice lacking Caspase-8 displayed rare to no Paneth cells as visualized by PAS staining or specific Lysozyme detection (**Figure 1A**). However, deletion of *Stat1* in $Casp8^{\Delta IEC}$ mice partially restored Paneth cell numbers as visualized by quantification of crypts containing $Lysozyme^{+}$ cells (**Figure 1B**). Accordingly, restriction of IFN-STAT1 pathway increase the ratio from around 5 % Paneth cells in $Casp8^{\Delta IEC}$ mice up to 25 % in $Casp8^{\Delta IEC}$x$Stat1^{-/-}$ mice (**Figures 1A,B**). This could be confirmed by quantification of Lysozyme production by Western Blot (**Figure 1C**). Previously,

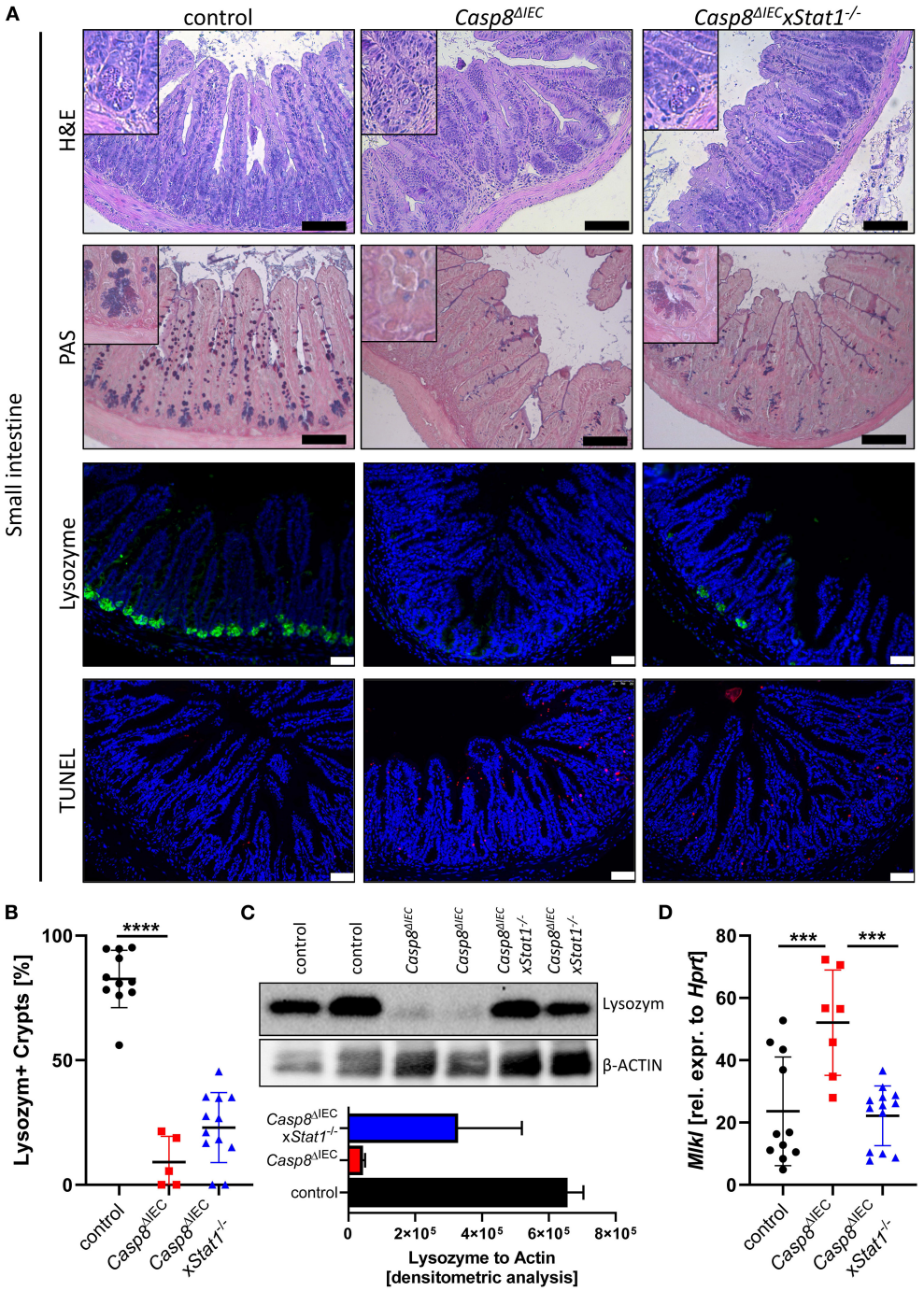

FIGURE 1 | STAT1 signaling attenuates necroptosis and partially restores Paneth cell depletion. **(A)** Representative images of small intestinal tissue cross sections of wild type control, *Casp8*$^{\Delta IEC}$, and *Casp8*$^{\Delta IEC}$xStat1$^{-/-}$ mice stained with standard H&E, PAS (scale bar: 100 μm) or immunohistochemically stained (scale bar: 50 μm) with antibody against Lysozyme (green) or stained with TUNEL assay (red). Nuclei were counterstained with Hoechst 33,342 (blue). **(B)** Quantification of Lysozyme positive crypts. **(C)** Western blot analysis Lysozyme in murine ileal tissue. β-Actin was used as loading control. Densitometry analysis for quantification ($n = 2$). Error bars i1ndicate +SD. **(D)** Gene transcription analysis of *Mlkl* mRNA expression in small intestinal tissue. *Hprt* was used as housekeeping gene. Error bars indicate +/−SD Statistical analyses: One-way ANOVA with Tukey's multiple comparisons test; NS $p \geq 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

we have demonstrated that Paneth cell depletion in a Caspase-8 proficient context is associated with IFN-induced cell death and STAT1 mediated transcriptional control of *Mlkl* gene expression (13). In line with these previous data, STAT1 deletion in *Casp8*$^{\Delta IEC}$ mice was associated with decreased *Mlkl* gene expression and a reduced number of TUNEL (terminal

deoxynucleotidyl transferase dUTP nick end labeling) positive dying cells (**Figures 1A,D**). However, Paneth cell function could not be restored to normal. Furthermore, staining of the Leukocyte Common Antigen (LCA, CD45), displayed higher leucocyte infiltration, especially at the crypt button in *Casp8*$^{\Delta IEC}$ mice but also in *Casp8*$^{\Delta IEC}$x*Stat1*$^{-/-}$ mice compared to control mice (**Supplementary Figure 2A**). Hence, STAT1 seems to modulate cell death and not inflammation.

To further investigate the molecular mechanism underlying epithelial cell death, we took advantage of organoids derived from the small intestine of control, *Stat1*$^{-/-}$, *Casp8*$^{\Delta IEC}$ and *Casp8*$^{\Delta IEC}$x*Stat1*$^{-/-}$ double deficient mice. Surprisingly, in contrast to the reduced number of Paneth cells observed *in vivo*, organoids derived from small intestinal tissue of *Casp8*$^{\Delta IEC}$ and *Casp8*$^{\Delta IEC}$x*Stat1*$^{-/-}$ mice displayed Paneth cells in an equal number and morphology as in control organoids (**Figure 2A**, marked by asterisk). In addition, expression of the Paneth cell marker Lysozyme (Lyz) was similar between all organoid cultures (**Figure 2A**). Interestingly, in contrast to our *in vivo* data, *Mlkl* expression was not elevated in any analyzed group, suggesting that activation of *Mlkl* gene transcription via STAT1 requires IFNs derived from non-epithelial cells or that factors, triggering autocrine IFN production by intestinal epithelial cells, are missing *in vitro*.

In vivo, cell death in intestinal epithelial cells can be induced by TNFα or IFNs (13, 17, 34). Interferons are able to induce the expression of *Mlkl,* whereas TNFα act as cell death trigger (13). To simplify the complex *in vivo* situation, we stimulated organoids with IFNβ, an interferon that can induce canonical and non-canonical STAT pathways (4), alone and in combination with TNFα. In line with previous studies, intestinal organoids lacking only *Caspase-8* were highly susceptible in response to TNFα and INFβ (**Figure 2B**). Additional deletion of *Stat1* was sufficient to block cell death with enhanced viability compared to control organoids (**Figure 2B**). These results highlight that beside factors that activate cell death, also the expression of cell death mediators such as MLKL, are relevant for cell death induction. However, when organoids were stimulated with TNFα in combination with IFNβ, to mimic a more complex *in vivo* situation, also double deficient organoids started to die, suggesting that the synergistic action of these two cytokines can trigger alternative cell death pathways which are independent of Caspase-8 or MLKL.

In summary these data suggest, that STAT1 is a key transcription factor for *Mlkl* gene expression in intestinal epithelial cells that orchestrate cell death in response to either TNF or IFNs.

STAT1 Does Not Modulate Cell Death During Experimental Colitis

Emerging evidence suggests that enterocyte death in the small and the large intestine is mediated by distinct molecular mechanism. Therefore, in a next set of experiments, we aimed to delineate the impact of STAT1 signaling during inflammation-induced cell death in the colon (**Figure 3**). As previously demonstrated, *Casp8*$^{\Delta IEC}$ mice only develop colonic inflammation under specific microbial settings or in response to experimental colitis (18). Accordingly, histological analysis of caecal and colonic tissue revealed no signs of inflammation or cell death in all analyzed mouse strains under steady state conditions (control, *Casp8*$^{\Delta IEC}$, and *Casp8*$^{\Delta IEC}$x*Stat1*$^{-/-}$ mice) (**Supplementary Figure 2**). Hence, we decided to trigger colonic inflammation by administration of low-dose DSS in the drinking water. Surprisingly, in sharp contrast to our *in vitro* observation and *in vivo* data derived from the small intestine, STAT1 was not essential to orchestrate necrotic cell death during chemically induced colitis (**Figure 3**). *Casp8*$^{\Delta IEC}$ as well as *Casp8*$^{\Delta IEC}$x*Stat1*$^{-/-}$ mice displayed body weight loss and increased inflammation after DSS administration (**Figure 3**). Interestingly, body weight loss was most pronounced in *Casp8*$^{\Delta IEC}$x*Stat1*$^{-/-}$ mice (**Figure 3A**), while endoscopic analysis and scoring revealed that both groups (*Casp8*$^{\Delta IEC}$ as well as *Casp8*$^{\Delta IEC}$x*Stat1*$^{-/-}$ mice) developed equal colonic inflammation (**Figures 3A,B**). Furthermore, both genotypes display severe tissue destruction, increased cell death and loss of epithelial integrity as demonstrated by H&E- and E-Cadherin staining (**Figure 3B**). In contrast to the minor contribution of *Stat1* to inflammation induced cell death in the colon, deletion of *Mlkl* was sufficient to rescue *Casp8*$^{\Delta IEC}$ mice from massive epithelial cell death and associated tissue destruction. Accordingly, *Casp8*$^{\Delta IEC}$x*Mlkl*$^{-/-}$ mice displayed a similar disease activity and tissue damage compared to wild type littermates (**Figure 3**). These results suggest that STAT1 acts upstream of MLKL during small intestinal inflammation, but seems to have differential regulatory functions in the large intestine during DSS-induced colitis.

STAT2 Does Not Prevent Paneth Cell Death

IFNs are able to signal through various combination of STATs in response to canonical and non-canonical JAK-STAT signaling (4). To investigate if an additional pathway, mediated by STAT2, might influence cell death in the intestinal epithelium, we generated *Caspase-8* and *Stat2* double deficient mice (*Casp8*$^{\Delta IEC}$x *Stat1*$^{+/-}$*Stat2*$^{-/-}$ mice) as well as triple knockout animals lacking both STAT1 and STAT2 members (*Casp8*$^{\Delta IEC}$x *Stat1*$^{-/-}$x*Stat2*$^{-/-}$). Similar to organoids derived from *Casp8*$^{\Delta IEC}$x*Stat1*$^{-/-}$ mice, Paneth cell numbers and expression of antimicrobial peptides was not influenced by additional deletion of *Stat2* in *Casp8*$^{\Delta IEC}$ mice *in vitro* (**Supplementary Figure 3**). However, in contrast to *Casp8*$^{\Delta IEC}$x*Stat1*$^{-/-}$ mice, *Stat2* deficiency did not improve Paneth cell viability *in vivo* as we observed only rare numbers of these secretory cells at the crypt bottom (**Figure 4A**). These histological features were supported by quantification of Lysozyme by Western Blot, which revealed similar levels between *Casp8*$^{\Delta IEC}$ and double deficient mice (*Casp8*$^{\Delta IEC}$x *Stat1*$^{+/-}$*Stat2*$^{-/-}$ mice) (**Figure 4B**). In line with these data, we identified high numbers of TUNEL positive cell along the crypt-villus axis of *Stat2* deficient *Casp8*$^{\Delta IEC}$ mice (**Supplementary Figure 4A**). In contrast to this, *Mlkl* gene expression was downregulated in all groups lacking a single STAT member or both compared to *Casp8*$^{\Delta IEC}$ mice

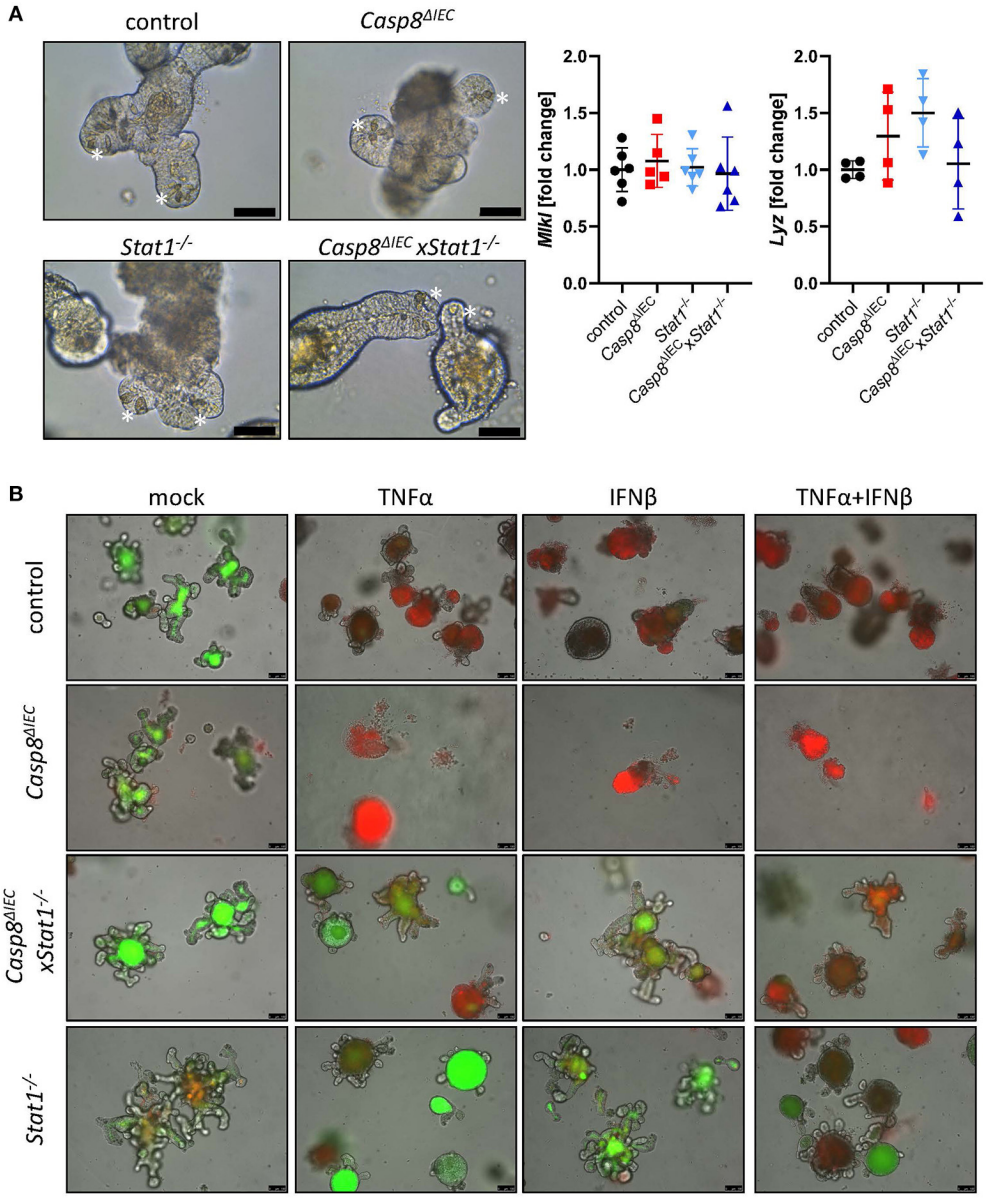

FIGURE 2 | STAT1 coordinates interferon induced cell death *in vitro*. **(A)** Representative pictures of organoids derived from of control, *Casp8*$^{\Delta IEC}$, Stat1$^{-/-}$ and *Casp8*$^{\Delta IEC}$xStat1$^{-/-}$ mice. (Scale bar: 50 μm, Paneth cells marked with asterisks). Gene transcription analysis of organoid mRNA expression. *Gapdh* was used as housekeeping gene. Gene expression levels are shown as fold changes. Error bars indicate +/-SD. **(B)** Organoids derived from of control, *Casp8*$^{\Delta IEC}$, Stat1$^{-/-}$ and *Casp8*$^{\Delta IEC}$xStat1$^{-/-}$ mice. Stimulated with TNFα (25 ng/ml) and IFNβ (100 ng/ml) were stained with propidium iodide (red, dead cells) and live cells visualized by autofluorescence (green; scale bar: 100 μm).

(**Supplementary Figure 4B**), suggesting that alternative cell death pathways are activated.

STAT2 Signaling Contributes to Small Intestinal Inflammation

While our data suggested a neglectable function of STAT2 in coordinating cell death in this mouse model of ileitis, we surprisingly observed that STAT2 influences small intestinal inflammation. Accordingly, as previously described *Casp8*$^{\Delta IEC}$ mice display severe inflammation including bowel wall thickening and an increased cellularity in the lamina propria of the terminal ileum. By contrast, deletion of STAT2 in these mice decreased these histo-morphological features (**Figure 4A**). In line with these results, gene expression of *S100a9* as well as *Nos2*, both associated with intestinal inflammation and upregulated in *Casp8*$^{\Delta IEC}$ mice, were downregulated in *Casp8*$^{\Delta IEC}$x *Stat1*$^{-/-}$*Stat2*$^{-/-}$ mice triple deficient mice and reduced in *Casp8*$^{\Delta IEC}$x *Stat1*$^{+/-}$*Stat2*$^{-/-}$ double deficient mice (**Figure 4C,**

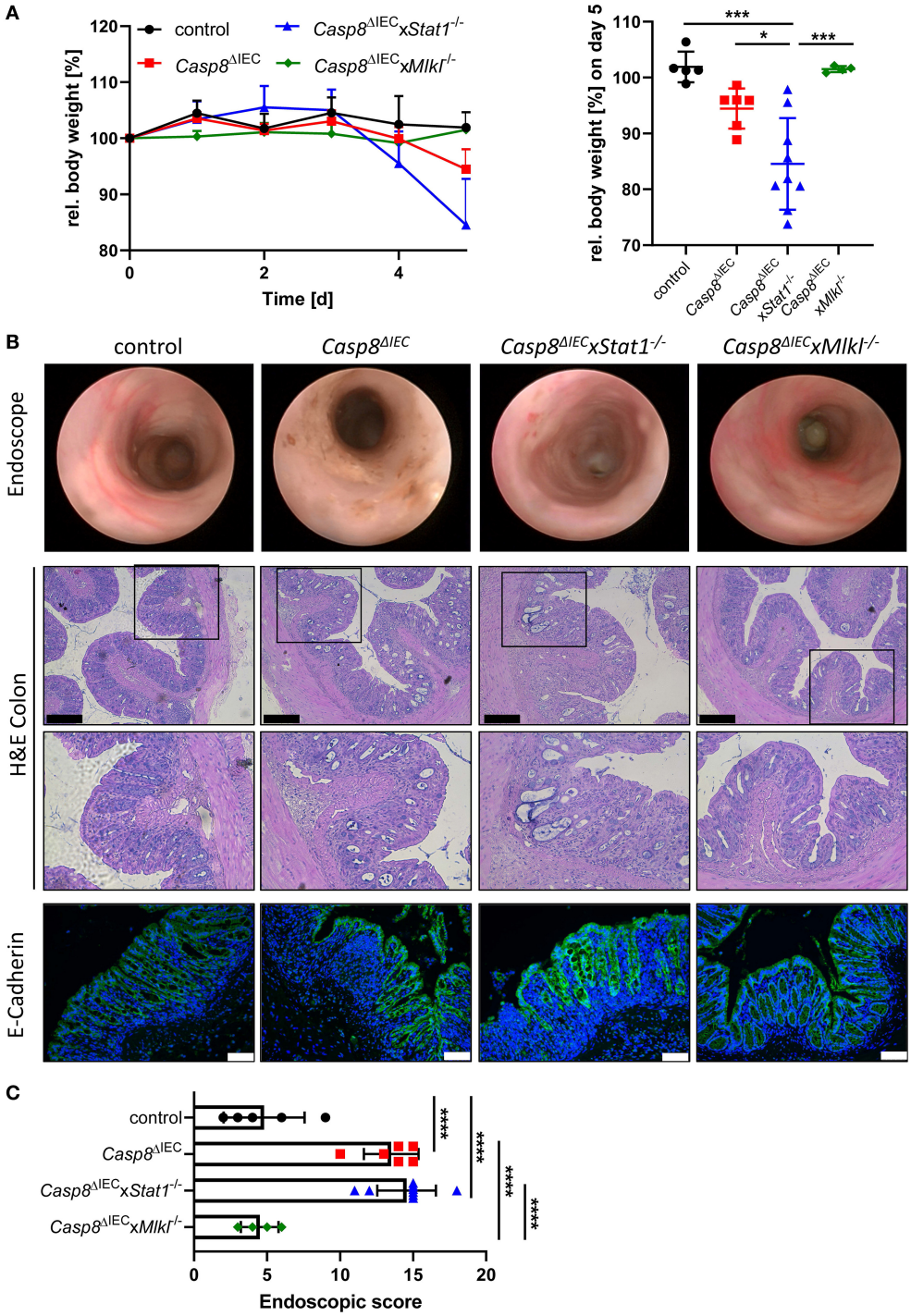

FIGURE 3 | Deletion of STAT1 does not ameliorate inflammation of *Casp8*$^{\Delta IEC}$ mice during DSS colitis. **(A)** Relative body weight of control, *Casp8*$^{\Delta IEC}$, *Casp8*$^{\Delta IEC}$xStat1$^{-/-}$, *Casp8*$^{\Delta IEC}$x*Mlkl*$^{-/-}$ mice after administration of 2% DSS in drinking water. Mice were sacrificed at day 5. **(B)** Representative endoscopic pictures at day 5. Representative images of colonic cross sections stained with H&E (scale bar: 200 μm) or immunohistochemically stained (scale bar: 75 μm) with antibody against E-Cahderin (green). Nuclei were counterstained with Hoechst 33,342 (blue). **(C)** Endoscopic score. Error bars indicate +/-SD. Statistical analyses: One-way ANOVA with Tukey's multiple comparisons test; NS $p \geq 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Pooled data of two individual experiments.

Supplementary Figure 4C). Moreover, *Irf1*, a downstream target of IFNγ-STAT1 signaling was significantly reduced in *Casp8*$^{\Delta IEC}$x *Stat1*$^{+/-}$*Stat2*$^{-/-}$ mice (**Figure 4C**).

In summary, we provide molecular evidence that STAT2 alone or in combination with STAT1 contributes to small intestinal inflammation. Our results further demonstrate that

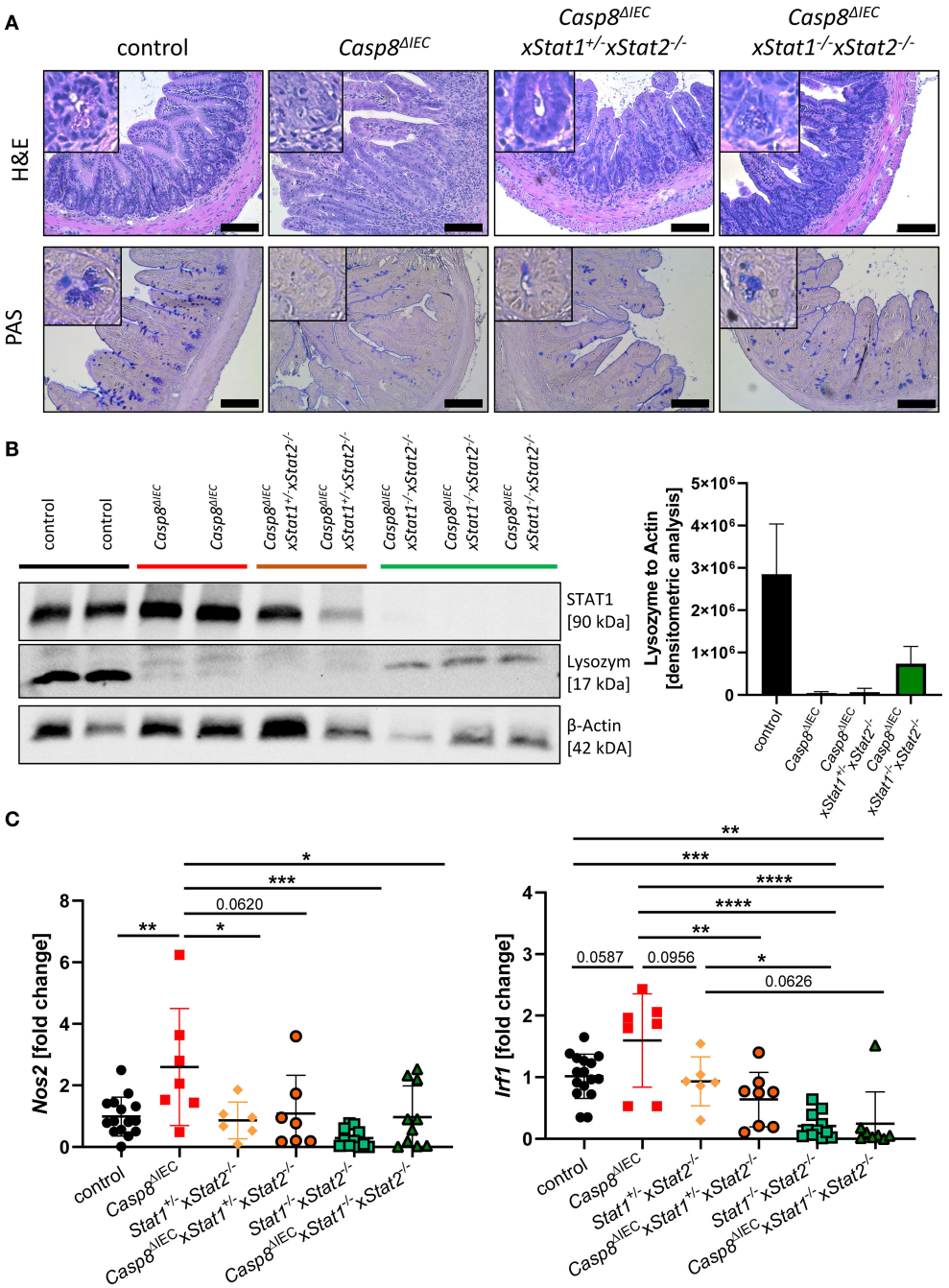

FIGURE 4 | STAT2 signaling fails to restore Paneth cell viability but alters inflammation. **(A)** Representative images of small intestinal tissue cross sections of control, *Casp8*$^{\Delta IEC}$, *Casp8*$^{\Delta IEC}$x*Stat1*$^{+/-}$*Stat2*$^{-/-}$ and *Casp8*$^{\Delta IEC}$x*Stat1*$^{-/-}$*Stat2*$^{-/-}$ mice stained with H&E and PAS (scale bar: 100 μm). **(B)** Western blot analysis and normalization of ileal tissue with antibodies against STAT1 and Lysozyme. β-Actin was used as loading control. Densitometry analysis for quantification ($n \geq 2$). Error bars indicate +SD. **(C)** Gene transcription analysis of *Mlkl* mRNA expression in the small intestine. *Gapdh* was used as housekeeping gene. Gene expression levels are shown as fold changes. Error bars indicate +/-SD. Statistical analyses: One-way ANOVA with Tukey's multiple comparisons test; NS $p \geq 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

STATs individually affect the distinct pathophysiology of IBD in the ileum and colon, respectively, which might explain the diverse outcome of JAK inhibitors in IBD patients with different localization of the inflammation site.

DISCUSSION

The pathogenic mechanisms involved in IBD contain a complex network of several key factors including immune cells, cytokines

and the intestinal epithelial barrier. Breakdown of the intestinal epithelial barrier, caused by massive cell death or missing mucosal healing is a crucial step during IBD pathology, which consequently also influences immune responses and microbial composition by missing antimicrobial defense (35). Moreover, clinical research and studies in experimental disease models have delineated the ambivalent role of IFNs and STAT1 in orchestrating epithelial cell homeostasis including induction of death as a key aspect of chronic inflammation as well as conducting mucosal healing during colonic inflammation (13, 14). Accordingly, blocking IFN signaling is a promising novel therapy for patients suffering from IBD. However, the underlying molecular mechanism and targeted cells are still controversially discussed.

Here, we provided novel evidence that STAT1 and STAT2 might independently influence intestinal inflammation in a highly spatial-dependent process. Blocking STAT1 signaling by genetic deletion of this transcription factor in *Casp8*$^{\Delta IEC}$ mice, was sufficient to partially rescue Paneth cell depletion and to reduce cell death frequency the intestinal epithelium (**Figure 1**). These data are in line with the observation that the IFNλ-STAT1 signaling axis is a key factor in small intestinal inflammation (13). Accordingly, two groups independently demonstrated that IFNs can either directly, or indirectly through IL-22, trigger non-apoptotic cell death (13, 36). Moreover, in a translational approach it has been described that IFNλ might support ileal inflammation by mediating necrotic Paneth cell death coordinated by STAT1 and MLKL (13). Here, we uncovered the fact that STAT1 was not able to fully restore Paneth cell viability *in vivo,* suggesting that further factors, triggering cell death, or additional pathways are present in the context of CD manifestations like ileitis. *In vitro* experiments using organoids, demonstrated that epithelial cells lacking Caspase-8 and STAT1 were protected from TNF or IFN induced toxicity, while single knock-out organoids displayed excessive cell death. These data demonstrate that both factors are sufficient to trigger cell death, but that *in vivo* additional factors are present that might activate Paneth cell necroptosis. Interestingly a recent paper identified the Z-DNA-binding protein 1 (ZBP1) as potential novel player in the pathogenesis of intestinal inflammation. ZBP1, also known as DAI, was initially identified to induce IFN-mediated MLKL-dependent necroptosis in the context of viral infection (37, 38). However, recent studies in mice and humans, further unveiled its contribution to gastrointestinal inflammation (19, 39). Interestingly, genome instability in IBD patients could trigger ZBP1 activation associated with necroptosis. Murine genomic instability, mimicking the human situation, was associated with ZBP1 activation, MLKL-mediated necroptosis and followed disruption of the epithelial barrier (39).

Beside their impact on cell death regulation, IFNs are primarily known for their immune-modulatory function. Accordingly, while STAT1 was sufficient to block TNFα or IFN triggered cell death *in vitro* and partially rescued Paneth cell death *in vivo*, it was surprisingly not involved in inflammation in the small intestine. Moreover, we identified that STAT1 signaling might be associated with tissue injury processes in the colon as *Casp8*$^{\Delta IEC}$x *Stat1*$^{-/-}$ mice exhibited severe tissue injury and inflammation in response to experimental colitis. These data are in line with a previous publication by Chiriac et al., highlighting that the activation of IFNλ-STAT1 signaling specifically in the IECs is responsible for mucosal healing and epithelial regeneration during colitis (14). Our results highlights differential mechanisms and upstream regulatory components underlying cell death pathways in the small and large intestine. In sharp contrast to these results, deletion of STAT2 in *Casp8*$^{\Delta IEC}$ mice, was associated with mucosal healing and reduction of disease activity in the small intestine, while Paneth cell homeostasis was not influenced by STAT2. STAT2 is linked to type I interferon signaling and only little is known about its role during IBD (28). In humans, downregulation of STAT2 gene expression has been observed in LPMCs (lamina propria mononuclear cells) derived from IBD patients (28, 40). Further studies are required to address the role of STAT2 in the context of human and murine intestinal inflammation.

Our findings on the differential role of STAT signaling molecules in the context on ileitis and colitis are in line with previous studies, supporting the concept that the pathogenic mechanism underlying ileal and colonic Crohn's disease are distinct and thus require individual therapies. Our data now provide further mechanistic insights in the role and contribution of the JAK-STAT signaling in the intestinal tract, which is currently in clinical focus. Accordingly, blocking JAK-STAT signaling is a promising therapeutic intervention, but current studies uncovered differential therapeutic success between ulcerative colitis and Crohn's disease (22–26). The broad JAK inhibitor tofacitinib (JAK1 and JAK3 inhibitor) showed promising results for patients with ulcerative colitis but not for Crohn's disease. Beside this, more selective inhibitors like filgotinib and upadacitinib (JAK1 inhibitors) seem to have more therapeutic benefit in both diseases (27). In this context, a recent study investigated the impact of both, selective and broad JAK-inhibitors, on ileitis and uncovered that blocking JAK-STAT signaling inhibited Paneth cell dysfunction and inflammation *in vitro* and *in vivo* (13). In line with these previous results, our data also suggest a major contribution of STAT signaling to small intestinal inflammation but not colitis. The fact that STAT signaling influences homeostasis of the intestinal epithelium, as a key component in the pathogenesis of inflammation, in a highly regional manner, indicates that further studies are required to fully define the contribution of STAT1 and STAT2 to inflammatory processes in the small and large intestine.

In summary, we provide molecular evidence that STAT1 and STAT2 both contribute to intestinal inflammation but have differential functions. Our results demonstrate that STAT1 coordinates cell death in the ileum but not during experimental colitis. Furthermore, STAT2 was able to modulate mucosal inflammation, independent of STAT1. Thus, our data provide further evidence for a differential pathological mechanism responsible for ileal and colonic inflammation.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of the Regierung von Unterfranken and conducted by qualified personnel.

AUTHOR CONTRIBUTIONS

IS, MN, and CG: designed the research. IS and AD: performed the experiments. MC: supplied material. IS, MN, and CG: analyzed the data and wrote the paper. All authors contributed to the article and approved 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/fmed.2021.644244/full#supplementary-material

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Interplay Between Microbiota, Toll-Like Receptors and Cytokines for the Maintenance of Epithelial Barrier Integrity

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The intestinal tract is densely populated by microbiota consisting of various commensal microorganisms that are instrumental for the healthy state of the living organism. Such commensals generate various molecules that can be recognized by the Toll-like receptors of the immune system leading to the inflammation marked by strong upregulation of various proinflammatory cytokines, such as TNF, IL-6, and IL-1β. To prevent excessive inflammation, a single layer of constantly renewing, highly proliferating epithelial cells (IEC) provides proper segregation of such microorganisms from the body cavities. There are various triggers which facilitate the disturbance of the epithelial barrier which often leads to inflammation. However, the nature and duration of the stress may determine the state of the epithelial cells and their responses to cytokines. Here we discuss the role of the microbiota-TLR-cytokine axis in the maintenance of the epithelial tissue integrity. In particular, we highlight discrepancies in the function of TLR and cytokines in IEC barrier during acute or chronic inflammation and we suggest that intervention strategies should be applied based on the type of inflammation.

Keywords: TNF, TLR4, cytokine, intestinal barrier, inflammation

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INTRODUCTION

The intestinal barrier represents a complex system of epithelial cells, Paneth cells, goblet cells, infiltrating immune cells, mucus, immunoglobulin A (IgA) antibodies and antimicrobial peptides (1). Underneath the epithelial cells multiple immune cell subsets are localized, which contribute to the maintenance of the border between the host and the microbiota. Disturbance of this barrier by extrinsic and intrinsic factors may result in the influx of various bacterial products inside of the host body leading to chronic inflammatory reactions. Such stimuli include dietary components, commensal microflora or invading pathobionts from the environmental side. Moreover, genetic variability of the host and adaptive immune response toward these stimuli may also influence barrier integrity.

The main component of the intestinal barrier is a layer of epithelial cells that forms the very first physical border between the host organism and its external surroundings, which could be potentially detrimental for the host cells. These epithelial cells are tightly connected with each

other to ensure proper control of the molecules that enter the body from the intestinal fluids. The junctional complex of intestinal epithelial cells is composed of the three main different types of connections—tight junction (TJ), adherence junction and desmosome. Tight junctions between epithelial cells are facilitated by a set of proteins [Claudin, ZO1, Occludin, F-actin, Myosin, Myosin light chain kinase (MLCK)], which form together an apical junctional complex in order to seal the paracellular space between epithelial cells. There are two additional zones of cell-to-cell contacts beneath TJ named "Adherence junction" and "Desmosome." They consist of E-cadherin, α-catenin 1, β-catenin, catenin- δ1 and desmoglein, desmocollin, desmoplakin, respectively (2). Together they provide cell-to-cell and cell-to-matrix connections and create a paracellular space. Normal gut permeability facilitates paracellular transport of nutrients, water and essential solutes. Disruption of such TJ may result in the penetration of various molecules and microorganisms, leading to inflammation.

The whole spectrum of cell types within the gut epithelium develops from the epithelial stem cells located at the base of the crypts. Stem cells give rise to distinct cell types of the intestinal epithelium: absorptive cells (enterocytes) and secretory cells (goblet, Paneth, enteroendocrine, and tuft cells). Fate decision toward the absorptive phenotype is critically dependent on the NOTCH pathway (3). Genetic and pharmacological manipulation of NOTCH signaling also revealed its crucial role in the maintenance of the epithelial stem cell niche (4–6). Apart from NOTCH, wingless and Int-1 (WNT) signaling plays an essential role in epithelial stem cell functions influencing functioning of different transcription factors including Ascl2, sox9, Lgr5 (7–9).

REGULATION OF THE EPITHELIAL CELL FUNCTIONS DURING HOMEOSTASIS

In steady state, a delicate balance is maintained between bacterial composition, the mucosal immune system and the intact epithelial barrier. Commensal microbiota is transported in a highly controlled manner to be recognized by the immune system in the gut-associated lymphoid tissues (2). Due to the non-pathogenic nature of such microorganisms, the immune system responds with the production of non-inflammatory cytokines, such as TGF-β1, IL-10 and cytokines which are important for the IEC barrier, like IL-22 (**Figure 1**). Both mutation of IL-10 pathway in humans and the genetic ablation of *Il10* resulted in development of intestinal inflammation demonstrating a crucial role for IL-10 in the tolerance maintenance and barrier integrity (10). Although $Il10^{-/-}$ mice are not defective in mucin production, but have its defective loose quality that makes mice suffer from spontaneous colitis (11). Similarly, TGF-β1 directly modulates TJ protein expression (12, 13), significantly decreasing JNK-pathway activation and protects cells from TNF-mediated downregulation of occludin and ZO-1 (14). IL-22 controls not only the expression of TJ proteins (15), but also the expression of various antimicrobial proteins. IL-22 deficient animals exhibited defects in IEC barrier (15) and failed to repair IEC functions in multiple inflammatory models linked to the disruption of the IEC barrier. IL-22 was further reported as a necessary cytokine for TJ formation and mucin production (16). Patients with HIV infections have decreased IL-22 levels and concomitantly impaired IEC barrier and increased bacterial translocation (16). Interestingly, the natural antagonist of IL-22 (IL-22BP; IL-22Ra2) which regulates the biological actions of IL-22 was found to be expressed by various immune cells (17). Recent data suggested that type III innate lymphoid cells (ILC3) instruct a special subset of dendritic cells in the isolated lymphoid follicles to produce IL-22BP via lymphotoxin ($LT\alpha_1\beta_2$)–lymphotoxin β receptor (LTβR) interaction (18), revealing a novel mechanism of the epithelial barrier control in steady state and during inflammation.

Commensal microbiota produces multiple "non-self" ligands and IECs recognize such molecules and tune their transcriptional program to keep the barrier tight. There are several families of receptors sensing various microbial products: Toll-like receptors (TLR), NOD-like receptors (NLR), RIG-like receptors (RLR), and others (19). TLRs are widely expressed on the epithelial cells in the small and large intestine and their expression is tightly regulated in order to ensure the proper innate immune recognition. Mostly, TLRs are expressed among the whole IEC lineage: absorptive enterocytes (20, 21), stem cells (22), enteroendocrine cells (23), goblet cells (24, 25), Paneth cells (26–28), and micro-fold cells (29, 30). The distribution pattern of TLR expression on epithelial cells varies among the intestinal tract. Price et al. recently provided an elegant analysis of TLRs expression in the large and small intestine of mice (27). It was shown that TLR2, TLR5, and TLR9 are more restricted to the small intestine when TLR2, TLR4, and TLR5 are upregulated in the colonic epithelial cells. In addition, TLR signaling is controlled by the polarized expression on the cell surface. For instance, TLR2 and TLR4 are expressed at low levels on basolateral sides of IEC in the small intestine, while TLR5 is expressed mainly on basolateral sides of the colon (31). Furthermore, apical TLR9 recognition of CpG oligonucleotides prevents NFkB translocation into the nucleus and limits inflammatory response.

The tuning of the immune responses via IEC-derived TLRs is achieved by several mechanisms. Epithelial cells modulate TLR receptor-ligand interactions by the downregulation of the receptor expression (32) or by translocating receptors from apical to basolateral sides or to lysosomes (33–36) to avoid excessive sensing of bacterial products. Indeed, overexpression of *Tlr4* on epithelial cells resulted in the overactivation of TLR4 pathway in IECs that lead to the increased production of IgA by plasma B cells (37). This loop potentially demonstrates a regulatory mechanism where IgA antibodies after being induced neutralize excessive bacteria-TLR4 interaction (20). Next, expression of molecules downstream of TLRs is modulated in IEC via various posttranslational modifications like glycosylation, phosphorylation, and ubiquitination (38, 39). Finally, IECs were reported to bind and modify immunogenic parts of MAMPs in order to diminish ligands property to induce signals (40, 41).

Apart from this, TLRs are involved in crypt dynamics control. For instance the depletion of MyD88 or TLR2 was

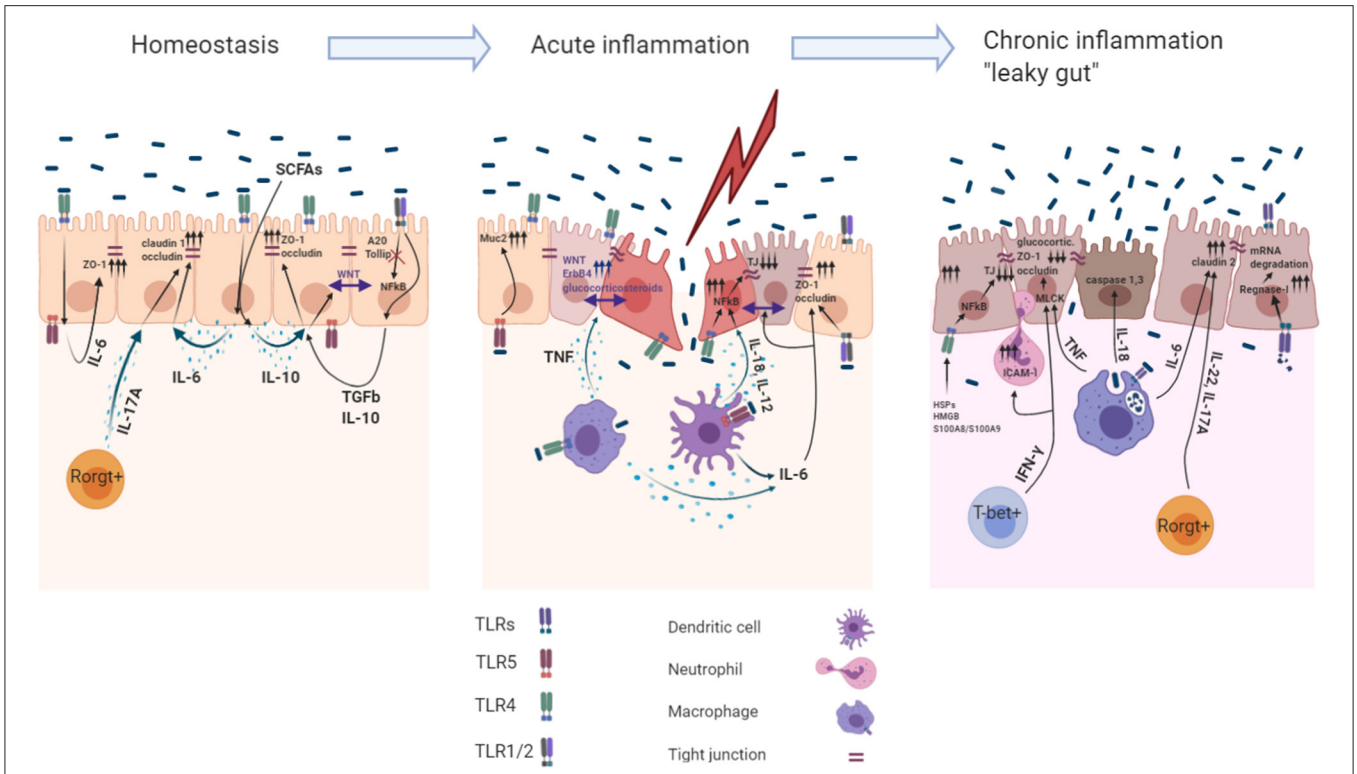

FIGURE 1 | Role of the TLR induced cytokines in acute and chronic intestinal inflammation. The intestinal mucosa is separated from the body immune environment by a single layer of the intestinal epithelial cells (IECs) that provides a physical and functional barrier. Beneath the IECs immune cells reside in the *lamina propria*, maintaining the intestinal tissue at the hyporesponsive state. Intestinal immune homeostasis: Constant recognition of microbiota by TLR4 and TLR1/2 leads to IL- 6, IL−10, TGF-β1 production within IECs**.** Autocrine recognition of these cytokines maintains IEC barrier integrity by promoting expression of the TJ proteins (ZO-1, claudin-1, occludin). Moreover, action of the IL-10 induce Wnt signaling within IECs, which maintains their proliferation. A20 and Tollip are the main inhibitors of the TLR1/2 signaling facilitating the avoidance of undesired response toward microbiota. Rorgt+ cells during homeostasis produce IL-17A to maintain constant production of claudin-1 and occluding within IECs. Acute inflammation: Sensing microbiota within the *lamina propria* induces production of pro-inflammatory cytokines and cytoprotective factors via NFkB dependent mechanism. Basolateral TLR5 mediated recognition of bacteria leads to MUC2 production in IECs. IL-6, TNF production by M?s and DCs during acute inflammation enables barrier repair program within IECs. TNF induced production of the glucocorticoids and ErbB4 receptor tyrosine kinase in IECs induce tissue repair functions and resolve late stages of the acute inflammation. Pro-inflammatory cytokines IL-18, IL-12 involved in IEC barrier dysfunction by downregulating TJ proteins (ZO-1, occludin). TLR1/2 basolateral recognition of bacteria promotes ZO-1/occluding expression in IECs. Chronic inflammation: Chronic TNF sensing by IECs reduces their ability to migrate toward crypts villi, modulates MLCK which decreases claudin-1, ZO-1, and ZO-2 expression and decreases glucocorticoids synthesis, which is indispensable for later inflammation resolution. IFN-γ activates the expression of ICAM-1 which resulted in increased IEC barrier permeability caused by neutrophil migration into subepithelial layers and paracellular space via modulation of MLCK. TLR4 dependent recognition of the HSPs, HMGB1 and S100A8/S100A9 by IECs leads to downregulation of the expression of ZO-1.

associated with an abrogation of trefoil factor 3 (TFF3) expression, which is required for goblet cells maturation (24). Furthermore, TLR4 was shown to mediate NOTCH expression implying that TLRs may interfere with processes of stemness and differentiation in the stem cell niche (1). However, the role of TLR4 on stem cell differentiation remains controversial (42, 43). Deletion of TLR1 or TLR5 in mice induced the loss of the mucus layer integrity via impaired MUC2 production in goblet cells (1). Moreover, ablation of the TLR recognition by MyD88 deletion abrogated the production of antimicrobial peptides RegIIIβ and RegIIIγ by goblet cells in mice (20). Thus, sensing of bacterial products via TLRs modulates mucus layer permeability that limits the direct interaction of commensals with the epithelium and induction of spontaneous inflammation (33, 44).

Altogether, IEC barrier exerts multiple strategies to avoid activation of inflammatory pathways in normal conditions via cytokine production and regulation of TLR signaling to maintain its integrity.

REGULATION OF THE EPITHELIAL CELL FUNCTIONS DURING ACUTE INFLAMMATION

Disruption of the cell-to-cell contacts at the epithelial layer leads to increased bacterial products penetration, which triggers inflammatory immune responses. The nature of the damage may further define the type of immune response and subsequent immune reactions driving IEC repair (**Figure 1**). Epithelial

barrier disruption may be induced by acute stimuli, such as ingestion of toxic substances (oxazolone, dextran sodium sulfate etc.) (45), by physical force or by the invasion of various pathogens, such as *Clostridium difficles, Citrobacter rodentium, Salmonella enterica* etc. These acute stimuli result in IEC layer erosion, the influx of commensal bacteria and activation of the innate arm of the immune system (46), while the chronic reduction of the barrier leads to the mobilization of both arms of the immune system as well as the genomic instability of epithelial cells (47). In case of the acute damage of the epithelium caused by pathogens, the immune system should eliminate the causing agent or pathogen, while ensuring the proper restoration of the barrier. Thus, the gut immune system is determined to restore the barrier functions in both acute and chronic settings, but triggers are different and, thereby, advocate for different intervention strategies.

Eliciting a protective immune response is required for the successful restoration of the barrier during bacteria-induced colitis. Here TLR-proinflammatory cytokine module is instrumental for the clearance of the inflammatory triggers and it is also involved in further tissue repair processes. Indeed, there are multiple examples of protective functions of TLR receptors in this setting. For instance, TLR1 is found to be crucial for the protection during acute intestinal inflammation induced by *Yersinia enterocolitica* in mice and the maintenance of the increased IEC barrier permeability (48). TLR5 was reported to limit intestinal colonization with vancomycin-resistant Enterococcus (VRE) by the induction of RegIIIγ expression (49) and IEC-derived TLR5 mediates production of IL-6 and IL-12 by $CD11c^{+}$ in response to Salmonella enterica infection (50). The significance of TLR/MyD88 signaling pathway for the recovery of IECs was also shown during acute colitis induced by *Helicobacter hepaticus* or *Citrobacter rodentium* (51). Furthermore, $Myd88^{-/-}$, $Tlr1^{-/-}$, $Tlr2^{-/-}$ mice were characterized by the early loss of tight junctions and diminished transepithelial resistance during acute intestinal inflammation (52).

Apart from IEC barrier disruption by pathogens, there is a significant amount of the research directed toward the dissection of the pathways which are crucial for IEC barrier restoration during injury caused by chemical agents, such as DSS, oxazolone and others. Herein the inflammation is caused by the influx of commensal microbiota in the intestinal tissue. Thus, TLR signaling pathways and pro-inflammatory cytokines facilitate the inflammation that is needed for the clearance of the bacteria and may possess protective functions. Consistently, seminal work from Medzhitov's lab showed the crucial role of TLR4/MyD88 signaling for the maintenance of the intestinal homeostasis and barrier repair during acute DSS colitis in microbiota dependent manner (53). Activation of TLR4 signaling pathways was crucial for the clearance of commensal bacteria by infiltrating innate immune cells (54). In contrast, several other studies highlighted the pathogenic function of TLR4 signaling in DSS colitis. In particular, an increase of *E. Coli* in the microbiota was associated with less severe colitis in TLR4 deficient mice (55). LPS, main TLR4 agonist, also may induce epithelial damage *in vitro* and *in vivo* via excessive phosphorylation of the focal actin kinase (FAK) in TLR4/MyD88 dependent pathway in epithelial cells (56). Using an ileal cell line, LPS was further reported to be instrumental in the induction of paracellular permeability via ZO-1 and occludin downregulation via TLR4 (57). Interestingly, LPS serotypes differentially affect inflammatory cytokines expression *in vitro*. Among others, LPS from *S. marcescens* has the most pronounced effect on the reduction of transepithelial electrical resistance. That correlated with an increase in NFkB activation, IL-8 production as well as TNF (58). Furthermore, *E. coli* LPS, but not LPS from *B. dorei*, influenced the incidence of autoimmune diabetes in non-obese diabetic mice and correlated with the development of autoimmunity in humans (59). Therefore, the role of TLR4 during acute IEC disruption is determined by the microbiota composition and therapeutic strategies targeting TLR4 should be considered given the prevalence of various microorganisms and pathogens in individual contexts.

TLR signaling mediates the production of multiple pro-inflammatory cytokines, among them TNF, IL-6, and IL-1β. TNF a cytokine with pleiotropic functions in the body is of particular significance in this context. On the one hand, TNF is crucial for the host defense against intracellular pathogens (60) but on the other hand it drives multiple autoimmune pathologies associated with a reduction of the epithelial barrier, such as inflammatory bowel disease (IBD), ankylosing spondylitis and rheumatoid arthritis. Importantly, anti-TNF therapy is highly effective in the treatment aforementioned autoimmune pathologies (61). Despite the tremendous success of the TNF blockade, a significant proportion of patients do not respond to this type of biological interventions further highlighting the heterogeneity of given autoimmune conditions and pleiotropy of TNF itself. It is worth mentioning that TNF exerts its functions via two receptors, TNFR1 and TNFR2 (62) inducing distinct transcriptional programs. TNF plays a protective role during acute colitis induced by DSS, as TNF deficient mice and anti-TNF therapy in wild type mice during colitis resulted in severe inflammation (63). Short acute IEC exposure to TNF induced glucocorticoid synthesis and, thereby, ameliorated the late stages of DSS colitis (64). Furthermore, TNFR1 mediated protective functions, while TNFR2 was deleterious upon acute disruption of epithelium (65). Apart from the induction of anti-inflammatory mediators that are crucial for the barrier restoration, TNF also contributes to the restoration of the epithelial barrier via modulation of Wnt (66). TNF administration during acute DSS colitis promoted the intestinal cell survival and restitution via elevating expression the ErbB4 receptor tyrosine kinase (67). In addition, another study conducted on the IL-10 deficient mice colitis model suggested that the binding of TNF by TNFR1 and following Il1b upregulation is essential for the early defensive response within colonic epithelial cells (68, 69). Kuhn et al. showed that *Bacteroidales spp*. induced IL-6 secretion by IECs in a MyD88-dependent manner, while $Il6^{-/-}$ mice were more susceptible to *Citrobacter rodentium* infection and had a thinner mucus layer, as well as decreased claudin-1 expression (70). Finally, IL-6 also activated NOTCH dependent program of IEC barrier restoration during acute DSS colitis (71).

Thus, proinflammatory cytokines exert its protective functions during acute barrier injury to facilitate efficient clearance of invading microorganisms.

REGULATION OF THE EPITHELIAL CELL FUNCTIONS DURING CHRONIC INFLAMMATION

Various extrinsic factors, such as the environment, particular diet, and exposure to hazardous chemicals, may result in the chronic elevation of pro-inflammatory cytokines and the reduction of the gut permeability for a long period of time (**Figure 1**). The state of an increased gut permeability and the perturbation of local immunity in the gut is called "leaky gut." This phenomenon has been described not only in IBD patients, but also in many metabolic and autoimmune disorders. "Leaky gut" syndrome is characterized by an impaired mucin synthesis, a decreased expression of junctional proteins and epithelial cell death. Importantly, increased permeability of the epithelium is often found before the development of clinical symptoms (72).

Taking into account the fundamentally different nature of IEC barrier reduction during acute and chronic stress, it is plausible that TLR and cytokines may have distinct, and even opposing functions depending on the duration of inflammation. Consistently, deep analysis of the mutational landscape from inflamed IBD tissue and corresponding non-inflamed parts revealed mutations in several genes, such as *NFKBIZ*, *ZC3H12A (Regnase-1)* and *PIGR*. Interestingly, Regnase-1 is activated in response to TLR stimulation and degrades mRNA of many downstream immune signaling genes (47), including PIGR (73), NFKBIZ (74), and members of the IL-17 pathway (75). Furthermore, DNA methylation patterns and transcriptional program in IECs differed between healthy and IBD patients (76). Chronic exposure of IECs to TNF exclusively affected their migration from the crypt to the villus (77). In addition, chronic inflammation modeled by long-term culture of colonic organoids in the presence of TLR agonists and pro-inflammatory cytokines resulted in chronic NFkB activation and the transformation of epithelial cells. Finally, organoid cultures from IBD patients showed an inflammatory phenotype with decreased size and budding capacity and inverted polarization (78). Altogether, these data suggested that chronic inflammation might transform the genetic program and the functions of IECs and their ability to maintain the epithelial barrier.

Chronic subclinical inflammation is characterized by an increase in cytokine production and in release of endogenous TLR4 ligands. In particular, high mobility group box 1 (HMGB1) protein, the heat shock proteins and calcium binding protein A8 and A9 (S100A8/S100A9) (79) are released during an inflammation and chronic conditions, like metabolic disorders (80). Their binding to TLR4 leads to the secretion of the pro-inflammatory cytokines IL-1β, TNF, IL-6, IL-17A, IL-18, and IL-12 in the intestine (31, 81). Furthermore, TLR4 activation within the gut epithelium is associated with the activation of myosin light chain kinase (MLCK), which reduces the tight junction of IEC barrier and may lead to the development of "leaky gut" (82–84).

As mentioned earlier, increased gut permeability may be induced by extrinsic factors, like diet, environmental factors but also by intrinsic factors, such as elevated levels of pro-inflammatory cytokines (85, 86). In particular, TNF, IL-6 and IFN-γ are associated with the epithelial barrier impairment and increased gut permeability (31, 87–89). These cytokines once produced chronically may significantly reduce IEC barrier. So IFN-γ was found to modulate the expression of the neutrophil adhesion molecule ICAM-1, which resulted in increased permeability and the migration of neutrophils into the subepithelial layers and paracellular space (90). Apart from this, IFN-γ enhanced Th1 immune responses and also increased CD14 and TLR4 expression, as well as LPS uptake by IECs (86). For instance, IL-6 increased permeability-promoting tight junction protein (claudin-2) in colonic cell culture via activation of c-Jun N-terminal kinase (JNK) pathway (91). IEC stimulation with TNF lead to the upregulation of the MLCK, phosphorylation of myosin II light chain (MLC) and the subsequent decrease in barrier integrity. Furthermore, TNF induced the loss of ZO-1 and occludin expression and decreased trans-epithelial electrical resistance (92). In immune-mediated colitis model, it was further shown that TNFR2 pathway, but not TNFR1 signaling, increases MLCK expression resulting in tight junction dysregulation, barrier loss, and more severe disease (93). Chronic exposure to TNF, in contrast to acute stimuli, actually decreased glucocorticosteroid production and perpetuated inflammation (94). Given multiple effects of TNF on the intestinal biology, it is predicted that anti-TNF therapy restores the intestinal barrier in many autoimmune diseases (95). It has been shown in several reports that anti-TNF therapy directly influenced tight junction protein expression (96), while others showed the restoration of EC survival rate (97). *In vitro* experiments also indicated that sera from IBD patients directly regulates ZO-1 and occludin expression in IECs via TNF. Moreover, TNF was further shown to downregulate claudin-1, claudin-2, claudin-4, and occludin expression in IECs layer (95). Interestingly, IL-6 promoted crypt organoid proliferation stem cell numbers (98). Furthermore, anti-IL-6 therapy in IBD patients ameliorated the disease, but increased the risk of developing GI abscesses and perforation (99), suggesting that IL-6 contribute to inflammatory processes, but also may maintain epithelial barrier. Thus, upon chronic inflammatory stimuli epithelial cells modify their transcriptional program, expression patterns of receptors and, thereby, may respond differently toward pro-inflammatory cytokines.

CONCLUSIONS

IEC barrier integrity is maintained not only by a complex system of tight junction proteins and strict compartment-dependent distribution of TLRs on apical and basolateral sides of IECs but also by a network of immune cells that mediate cell proliferation and epithelial permeability via cytokines. In a healthy state IECs exhibit multiple mechanisms that dampen TLR-dependent recognition of the microbiota. During acute injury of IEC barrier by chemical agents or pathogens the TLR-TNF axis is triggered toward the clearance of the pro-inflammatory stimuli and further drives IEC layer restoration via activation of the glucocorticosteroid synthesis, WNT pathway and ErbB4 kinase. In contrast to acute damage, chronic inflammation induces genetic instability, changes of methylome,

transcriptome and the polarity of TLRs expression in IECs. This results in their modified response toward TLR agonists and TNF. Thus, the character and duration of inflammation should be considered for the modeling of studies aiming to dissect the mechanisms of IEC barrier integrity during various injury.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

IS, MB, JN, IG, and AK analyzed the literature and studies and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Comparative Transcriptomics of IBD Patients Indicates Induction of Type 2 Immunity Irrespective of the Disease Ideotype

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Inflammatory cytokines initiate and sustain the perpetuation of processes leading to chronic inflammatory conditions such as inflammatory bowel diseases (IBD). The nature of the trigger causing an inflammatory reaction decides whether type 1, type 17, or type 2 immune responses, typically characterized by the respective T- helper cell subsets, come into effect. In the intestine, Type 2 responses have been linked with mucosal healing and resolution upon an immune challenge involving parasitic infections. However, type 2 cytokines are frequently elevated in certain types of IBD in particular ulcerative colitis (UC) leading to the assumption that Th2 cells might critically support the pathogenesis of UC raising the question of whether such elevated type 2 responses in IBD are beneficial or detrimental. In line with this, previous studies showed that suppression of IL-13 and other type 2 related molecules in murine models could improve the outcomes of intestinal inflammation. However, therapeutic attempts of neutralizing IL-13 in ulcerative colitis patients have yielded no benefits. Thus, a better understanding of the role of type 2 cytokines in regulating intestinal inflammation is required. Here, we took a comparative transcriptomic approach to address how Th2 responses evolve in different mouse models of colitis and human IBD datasets. Our data show that type 2 immune-related transcripts are induced in the inflamed gut of IBD patients in both Crohn's disease and UC and across widely used mouse models of IBD. Collectively our data implicate that the presence of a type 2 signature rather defines a distinct state of intestinal inflammation than a disease-specific pathomechanism.

Keywords: IBD, type 2 immunity, intestine, chronic inflammation, ulcerative colitis

INTRODUCTION

Immune responses that detect and eliminate multicellular metazoan parasites and allergic reactions have evolved fundamentally differently than those that tackle other immune challenges. The tissue destruction caused by such parasitic infections and allergic reactions, signals an "eliminate and repair" type of innate immune response, characterized by the emergence of the type 2 helper T

cells (Th2) (1). The "type 2" nomenclature was initially coined after Th2 lymphocytes, thought of as being the sole cellular sources capable of producing the core type 2 cytokines interleukin (IL)-4, IL-5, and IL-13 (2). However, key discoveries in the field of innate immunity revealed the presence of new cellular sources of type 2 cytokines (3). For e.g., the type 2 innate lymphoid cells, which also express the polarizing transcription factor GATA3 are rich sources of IL-4 and express IL-5 and IL-13 (4). The effector cells of type 2 responses are epithelia, myeloid cells and granulocytes including dendritic cells, macrophages, neutrophils, basophils, eosinophils, and mast cells and the various secretory and absorptive lineages of epithelial cells. Multiple, differentially polarized subsets of T cells are involved in disease pathogenesis in inflammatory conditions, for e.g., Th1, Th2, Th9, and Th17 (5, 6).

It has been speculated that a breakdown in type 2-mediated processes is the underlying cause of the failure of tissue repair and healing in chronic debilitating conditions affecting mucosal tissues (1). However, our understanding of whether this hypothesis holds true is limited. Studies on chronic diseases affecting the gut such as inflammatory bowel diseases (IBD) show a dichotomous relationship between type 2 responses and the two predominant types of IBD, namely Crohn's disease (CD) and ulcerative colitis (UC). Both CD and UC are chronic mucosal inflammatory diseases, an elevated expression of type 2 cytokines may be detected in the tissues and serum of UC, but not of CD patients, however, this concept has recently been challenged (7, 8). Therefore, elevation in type 2 cytokines does not establish a causal connection between UC and type 2 immune responses and crucial questions on when and why such an elevation may be observed remain poorly addressed. Administration of neutralizing antibodies against IL-13 failed to induce any clinical response in UC patients, which begs the question whether defective signaling via respective receptors could cause a compensatory elevation in the expression of the type 2 cytokines in UC (9–11). Indeed some of the well-documented downstream effects of type 2 cytokines such as increased smooth muscle proliferation and cholinergic activation, and elevated mucin production are not uniquely seen to occur in UC, indicating that there is, at least, a partial functional blockade in the mediation of type 2 responses despite the elevation in their levels. By contrast, some studies indicate that elevated type 2 responses in CD patients during parasite infections can in fact prove therapeutically beneficial (12). It is therefore complicated to dissect how type 2 cytokines influence IBD.

Strong type 2 responses are elicited in mucosal tissues during allergic conditions or helminth infections. Therefore, the most common mouse models to study type 2 immune responses are based on infecting mice with parasitic helminths. Some of the most common nematode worms used to provoke mucosal type 2 responses in mice are *Nippostrongylus brasiliensis* and *Heligmosomoides polygyrus* (13). Another mouse model, which is reported to show a predominant type 2 response, is the oxazolone colitis model (14, 15). Other common mouse models of colitis such as the chemically induced dextran sulfate sodium (DSS) colitis and tri-nitrobenzidium sulfate (TNBS) colitis are described to have a more mixed Th 1, Th 17 response (16). Whereas, the chronic DSS colitis model is described to involve more of a type 2 immune response (16). One of the most commonly employed models to study the contribution of T cells to the pathogenicity of colitis is the transfer colitis mouse model, which is more of a type 1 model (17). Although these generalized distinctions hold out, there has also been evidence that indicates the contrary. Therefore, the dogmatic view of UC as a predominantly type 2 and CD a type 1 immune disease has been challenged. This is important given that both therapeutic and diagnostic attempts in IBD have evolved around our understanding of the immune homeostasis.

In order to improve our understanding of the type 2 response in IBD, we present here a comparative transcriptomic analysis of various mouse models and human cohorts of IBD using a well-defined list of markers of type 2 immune responses. Our data show that an elevation in type 2 responses is a generalized phenomenon seen intestinal inflammation and identifies a core network of type 2 associated genes that is shared between preclinical models and patients of IBD.

MATERIALS AND METHODS

Mouse Models of Colitis

The C57Bl6 strain has been widely used in colitis research and was purchased from Charles river laboratories GmbH (Sulzfeld, Germany). All mice included in this study were of both genders and aged between 8 and 18 weeks. Acute and chronic DSS, Oxazolone, and T-cell transfer colitis were induced as previously described (18, 19). At the end of the colitis induction period, animals were euthanized and tissues were harvested for further analysis. The housing, animal care and experimentation was performed according to the institutional guidelines that were preapproved by the ethics commission of Lower Franconia and Rhineland.

RNA Extraction and mRNA Sequencing

The RNA from the tissue samples were harvested using the peqGold total tissue RNA kit (peqlab GmbH) according to the manufacturer's instructions. The RNA degradation, contamination and quantification were checked using a combination of 1% agarose gels checked using the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA), the Nanodrop (thermofischer), the Qbit (Thermo) and the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

For library preparation for transcriptome, sequencing a total amount of 1 μg RNA per sample was used as input material per sample. Sequencing libraries were generated using NEBNext® UltraTM RNA Library Prep Kit for Illumina® (NEB, USA) following manufacturer's recommendations and index codes were added to attribute sequences to each sample. Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried

out using divalent cations under elevated temperature in NEBNext First Strand Synthesis Reaction Buffer (5X). First strand cDNA was synthesized using random hexamer primer and M-MuLV Reverse Transcriptase (RNase H-). Second strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. After adenylation of 3' ends of DNA fragments, NEBNext Adaptor with hairpin loop structure were ligated to prepare for hybridization. In order to select cDNA fragments of preferentially 150–200 bp in length, the library fragments were purified with AMPure XP system (Beckman Coulter, Beverly, USA). Then 3 μl USER Enzyme (NEB, USA) was used with size-selected, adaptorligated cDNA at 37°C for 15 min followed by 5 min at 95°C before PCR. Then PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. At last, PCR products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system.

The clustering of the index-coded samples was performed on a cBot Cluster Generation System using PE Cluster Kit cBot-HS (Illumina) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina platform and paired-end reads were generated. Raw data (raw reads) of FASTQ format were firstly processed through in-house scripts. In this step, clean data (clean reads) were obtained by removing reads containing adapter and poly-N sequences and reads with low quality from raw data. At the same time, Q20, Q30, and GC content of the clean data were calculated. All the downstream analyses were based on the clean data with high quality. Reference genome and gene model annotation files were downloaded from NCBI, UCSC and Ensembl directly. Paired-end clean reads were mapped to the reference mouse genome (GRCm38.p6) using STAR software (20) (2.7.0d).

Meta-Analyses of Publicly Available Datasets

Mouse Models for Helminth Infection

Transcriptomic data from the duodenal samples of mice infected with the parasite *H. polygyrus* that has been previously published was obtained from NCBI's Gene Expression Omnibus (GEO) https://www.ncbi.nlm.nih.gov/gds (GSE102789). Raw RNAseq data was mapped against the mouse genome and processed and analyzed using the same procedures and tools described above for the mouse colitis models.

Human Inflammatory Bowel Disease Cohorts

Transcriptomic data from the respective intestinal tissues from IBD patients from the previously published studies were obtained from –

a) NCBI's Gene Expression Omnibus (GEO) https://www.ncbi.nlm.nih.gov/gds and from
b) EBI's Array Express https://www.ebi.ac.uk/arrayexpress/.

The specifics of the cohorts and the respective accession numbers are provided here:

Cohort	Accession	No. of Samples	Tissue	References
WashU	E-MTAB-5783	36 CD, 32 Control	Ileum	(21)
RISK_I	GSE57945	202 CD, 60 UC, 39 Control	Ileum	(22)
PSC	E-MTAB-7915	10 UC, 10 PSC, 10 Control	Colon	(23)
PROTECT	GSE109142	206 UC, 20 Control	Rectum	(24)
RISK_R	GSE117993	92 CD, 43 UC, 55 Control	Rectum	(24)

CD, Crohn's disease; UC, ulcerative colitis; PSC, primary sclerosing cholangitis.

Where applicable, microarray data and its annotation was downloaded and processed using the limma R package [3.42.2] (25). Raw RNAseq data was mapped against the human genome [GRCh38.p13], processed and analyzed using the same procedures and tools described above for the mouse colitis models.

Network extension for detection of pathways and physical interactors for a subset type 2 identifier genes that were commonly regulated between mouse and human samples was determined using the freely available tool Genemania for Cytoscape 3.7 (26, 27).

Statistical Analysis of Differential Gene Expression

FeatureCounts (v2.0.1) was used to count the read numbers mapped of each gene included in the Ensembl database 21. Then the median of ratios of each gene were calculated, based on the sequencing depth and the RNA composition. Although the Reads Per Kilobase of exon model per Million (RPKM) is currently one of the most commonly used methods for normalization of RNA seq data, the median of ratios used by DESeq2 is more accurate for the differential expression analysis22.

Differential expression analysis between two conditions/groups (three biological replicates per condition) was performed using DESeq2 (v 1.26.0) R package22. DESeq2 provides statistical routines for determining differential expression in digital gene expression data using a model based on the negative binomial distribution22. The resulting *P*-values were adjusted using the Benjamini and Hochberg's approach for controlling the False Discovery Rate (FDR). Genes with an adjusted $P < 0.05$ found by DESeq2 were assigned as differentially expressed.

TABLE 1 | Identifier gene set for type 2 immunity.

Symbol	Full name	Description	References
Il33	Interleukin 33	Cytokine that drives production of Th2 associated cytokines. Expressed in a wide range of cells	(29)
Il25	Interleukin 25	Cytokine that drives production of Th2 associated cytokines. Expressed in a wide range of cells	(2)
Il4	Interleukin 4	Cytokine that induces differentiation of naive Th cells to Th2	(30)
Il5	Interleukin 5	Cytokine that stimulates IgA secretion and activates eosinophils	(31)
Il9	Interleukin 9	Cytokine that regulates hematopoietic cells activating several STAT signaling pathways, stimulating cell proliferation, and preventing apoptosis	(32, 33)
Il10	Interleukin 10	Anti-inflammatory cytokine that down regulates the expression of Th1 cytokines	(34, 35)
Il13	Interleukin 13	Cytokine with similar effects in immune cells than those caused by IL-4	(36, 37)
Il31	Interleukin 31	Pruritic cytokine expressed by Th2 cells in response to stimulation with type 2 cytokines	(38, 39)
TLSP	Thymic stromal lymphoproietin	Cytokine that plays a role on activating Dendritic Cells	(40)
AREG	Amphiregulin	Epidermal growth factor secreted by ILC2 after tissue damage.	(41, 42)
GATA3	GATA binding protein 3	Transcription factor that stimulates the producion of IL-4, IL-5 and IL-13.	(43)
MAF	Avian musculoaponeurotic fibrosarcoma oncogene	Transcription factor that regulates the expression of IL-4 and attenuates type 1 response.	(44)
STAT6	Signal transducer and activator of transcription 6	Transcription factor that acts as the intracellular effector of IL-4 in Th2 cells	(45)
CCL1	Chemokine (C-C motif) ligand 1	Chemokine that acts as a chemoattractant for multiple immune cells.	(46, 47)
CCL8	Chemokine (C-C motif) ligand 8	Chemokine that acts as a chemoattractant for multiple immune cells.	(48)
CCL17	Chemokine (C-C motif) ligand 17	Chemokine that induces chemotaxis in T cells.	(49)
CCL22	Chemokine (C-C motif) ligand 22	Chemokine that induces chemotaxis in T cells.	(49)
IL1RL1	Interleukin 1 Receptor like protein 1	Receptor for IL-33	(50)
PTGDR2	Prostaglandin D2 receptor 2, Interleukin 52 (CRTH2)	Prostaglandin receptor that mediates in the chemotaxis of Th2 cells	(51)
IL17RB	Interleukin 17 Receptor B	Receptor for IL25	(52)
CRLF2	Cytokine receptor-like factor 2	Receptor for TSLP	(53)
CCR4	C-C chemokine receptor type 4	Receptor for multiple chemokines, including CCL17 and CCL22.	(54)
CCR8	C-C chemokine receptor 8	Receptor for CCL1	(55)
IL4R	Interleukin 4 Receptor subunit alpha	Receptor for IL-4 and IL-13, forms a complex with IL-13RA1	(56)
IL13RA1	Interleukin 13 Receptor alpha 1	Receptor for IL-4 and IL-13, forms a complex with IL-4R	(57)
IL31RA	Interleukin 31 receptor A	Receptor for IL-31	(58)
IL9R	Interleukin 9 Receptor	Receptor for IL-9	(59)
DENND1B	DENN domain-containing protein 1A	Protein containing a DENN domain, which interacts with Rab family GTPases	(60)
ITK	IL-2-inducible T-cell kinase	Tyrosine kinase that plays a role on the differentiation and proliferation of Th2 cells	(61)
ARG1	Arginase 1	Metabolic enzyme and marker of activated ILCs	(62)
ARG2	Arginase 2	Paralog of ARG1 and marker of activated T cells	(63)
ECM1	Extracellular matrix protein 1	Extracellular protein that contributes in the manteinance of the epithelium	(64)
PRKCZ	Protein kinase C zeta	Protein kinase C that regulates differentiation of T cells	(65)
RETNLA	Resistin like molecule alpha (FIZZ)	Molecule increased in inflammatory and allergic responses	(66)
RETNLB	Resistin like molecule beta	Molecule increased in inflammatory and allergic responses	(67)
CHIL3	Chitinase-like protein 3	Pseudo chitinase expressed in IECs and macrophages in inflammation	(68)
MUC5AC	Mucin 5AC	Gene involved in the production of mucus	(69)
MRC1	Mannose receptor C-type 1	Protein expressed by intestinal macrophages	(70)

RESULTS

Selection of an Identifier Gene Set Representing Type 2 Responses

To generate a discovery gene set for type 2 immunity that would then be used to query transcriptomic datasets, we surveyed gene sets in the molecular signatures database (MSigDB, https://www.gsea-msigdb.org/gsea/msigdb) (28). The MSigDB database serves as a standard resource for gene sets used in gene set enrichment analyses. A list of all MSigDB gene sets used for this analysis is available as **Supplemental Table 1**. Surprisingly, an integrated list of the 15 gene sets that we investigated, failed to show the necessary attributes of a canonical type 2 signature. Therefore, based on reliable literature resources, we compiled a list of 39 genes, which are known to be classical markers for type 2 immune responses (**Table 1**). This list includes genes categorized into five major groups: cytokines, transcription factors, chemokines, receptors, and other genes, which served as a molecular identifier to interrogate transcriptomic datasets and associate a specific type 2 signature (**Table 1**).

The genes included in the cytokines group are involved in multiple types of signaling for the activation of the type 2 response. These include the classical type 2 cytokines *Il4*, *Il5*, *IL9,* and *IL13* that are involved in the activation of the effector cells (30–33, 36, 37). The cytokines *Il33*, *Il25*, and *TLSP* are primarily produced by parenchymal cells in response to damage, thereby rapidly activating type 2 ILCs (ILC2s) and other immune cells in an early activation stage (2, 29, 40). The ILC2s and Treg cells secrete AREG in order to help repair the damaged epithelium (41, 42). Finally, IL10 is produced by macrophages and lymphocytes, and has an anti-inflammatory function, in order to maintain intestinal homeostasis (34, 35).

Among the key transcription factors (TF) that regulates type 2 polarization and expression of multiple cytokines is *GATA3* (43). Another key regulatory TF assigned to type 2 responses is MAF, which controls the production of *Il4*, and attenuates type 1 signaling (44). The *IL4* signaling is crucial in dictating type 2 responses and its actions are mediated by the activation of the TF *STAT6*, which regulates a plethora of type 2 related effects including polarization and recruitment of Th2 cells (45).

Among the chemokines that represent the type 2 signature were *CCL1* and *CCL8* that promote migration and activation of ILC2s and Treg cells. *CCL17* and *CCL22* have similar effects, dendritic cells (DC) produce both and they interact with T helper cells (46–49).

Key cytokine receptors, which influence the type 2 polarization and function are *IL4R1* (lymphocytes, ILC, fibroblasts, and epithelium), *IL9R* (eosinophils, mast cells, ILC2s), *IL13RA1* (ILC, granulocytes, epithelial cells, fibroblasts), and *IL31RA* (monocytes, epithelium) (56, 57, 59). Other cytokine receptors which influence type 2 behaviors include the IL-33 receptor subunit *IL1RL1* (hematopoietic), which has been seen involved in allergic responses and *IL17RB* (ILC2s, monocyte, Tuft cells), which is receptor of *IL25* (50, 52). The receptors for type 2-related chemokines are also included in this group, with the genes *CCR4* (Th2) and *CCR8* (ILCs, T cells) (54, 55). *PTGDR2* is a prostaglandin D receptor expressed in Th2 cells, mediating allergic responses (51). The receptor of *TSLP*, *CRLF2* (DC, hematopoietic) is also a key component determining the activation and initiation of Th2 responses (40, 53).

Finally, we identified 12 additional genes that are known to control type 2 responses, but do not fall into any of the above categories. Among these were *DENND1B* which is involved in the down-modulation of the T cell receptor, and its absence, malfunction or delay has been associated with asthma and allergic response (60). The Tec family tyrosine kinase *ITK*, also included in this group, is required for the production of type 2 cytokines and the differentiation of ILC2s and T cells (61). *ARG1* and *ARG2* are paralogues, both encoding for the metabolic enzyme arginase, which metabolizes L-arginine, and has been identified as a marker of ILC2s and alternatively activated macrophages; and an upstream regulator of these metabolic genes is *ECM1* (62–64). *PRKCZ* encodes an atypical protein kinase C, which is involved in immune surveillance (65). Next, we included *RETNLA* and *RETNLB* that encode proteins of the Resistin family that are generally elevated upon type 2 immune activation via the actions of IL-13 (66, 67). During nematode driven intestinal type 2 responses, the upregulation of the chitinase *CHIL3* is detectable (68). This enzyme controls the degradation of chitin, and is produced and released by intestinal epithelial cells promoting host cell survival and proliferation. Various scenarios of type 2 activation in the intestine have shown that *MUC5AC* is induced in the epithelium and is responsible for the elevated mucus production, characteristic of these models (69). MRC1 is a receptor induced by the type 2 cytokine IL4 and can bind high-mannose structures on parasite walls, aiding their neutralization and engulfment (70).

Transcriptomic Comparison Across Multiple Mouse Models of IBD Reveals a Non-discriminatory Regulation of Type 2 Response

In an initial attempt to find discernable patterns of the involvement of type 2 immune responses in different mouse models of IBD, we screened colonic transcriptomes against the type 2 immune response identifier gene set. The mouse models included for this were a time course of dextran sulfate sodium (DSS) colitis representing mild inflammation at day 3, high inflammation at day 8, moderate recovery at day 12 and full recovery from colitis at day 19 of the experimental protocol, characterized by distinct changes in body weights during and after DSS challenge (**Figures 1A,B**). Apart from these 4 stages of DSS colitis we also included samples of acute DSS colitis, chronic DSS colitis, Oxazolone colitis, and adoptive T-cell transfer colitis, where inflammation was ascertained by histochemical staining (**Figures 1C,D**). Colonic transcriptomes from unchallenged Rag1 knockout mice and unchallenged C57BL/6 mice were used as control datasets for the T-cell transfer colitis and the rest of the mouse models, respectively. Our exploration yielded 25 genes representing 64% of the identifier gene set which met all requisite criteria for technical thresholds across all the mouse model datasets (**Figures 1C,D**). Intriguingly, while a type 2 immune signature was evident

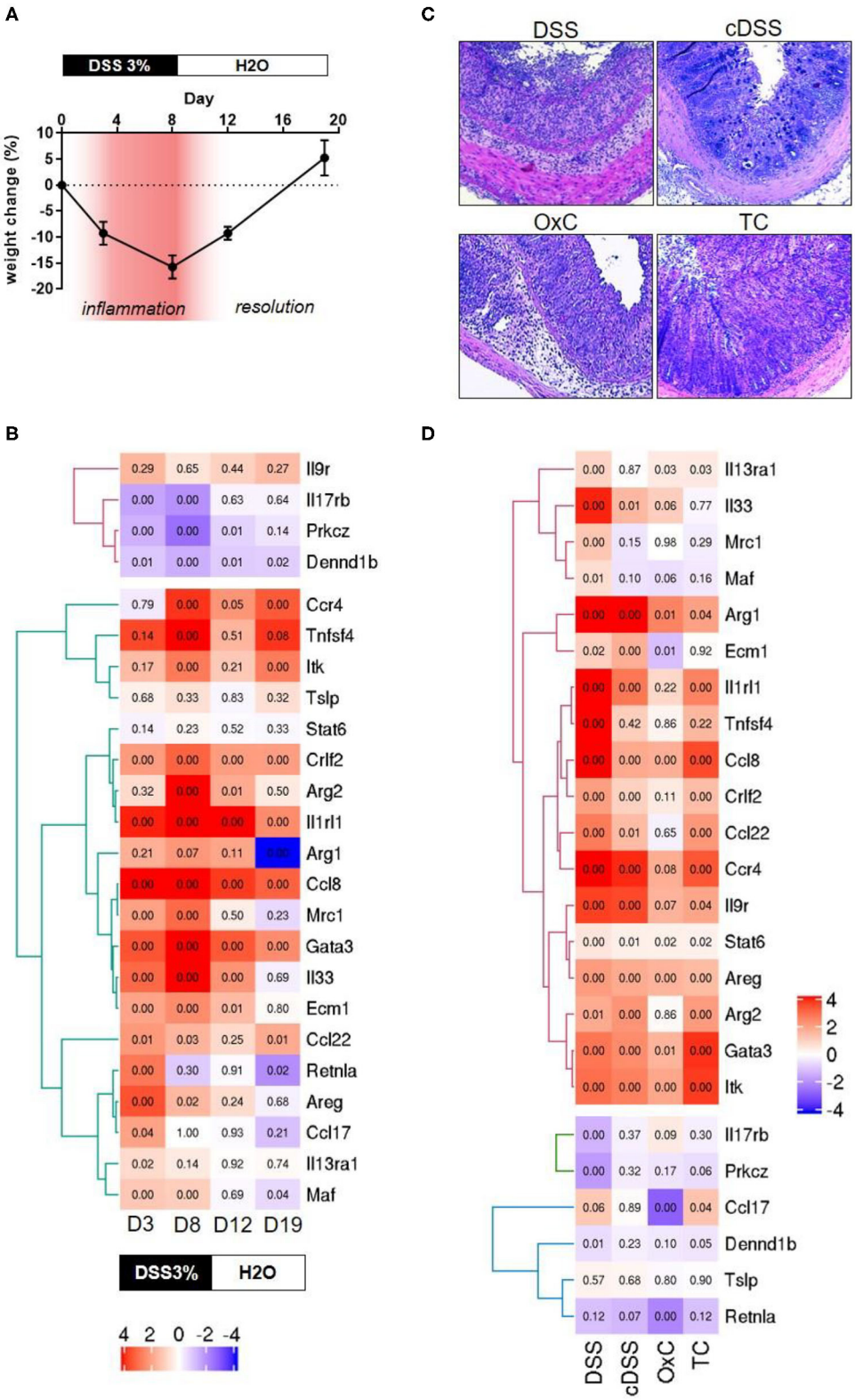

FIGURE 1 | Transcriptomic analyses of type 2 identifiers in multiple mouse models of colitis. **(A)** percent change in body weight from baseline and DSS treatment paradigm. Time points representing inflammation are represented in red **(B)** Heatmap depicting the normalized expression of the indicated genes from four different time-points of dextran sulfate sodium (DSS) induced colitis D3 = day 3, D8 = day 8, D12 = day 12, and D19 = day 19. The text boxes below represent the treatment

(Continued)

FIGURE 1 | type and duration. Hierarchical clustering shows clear segregation of two distinct groups of genes. **(C)** Representative photomicrographs of H&E-stained tissue sections from the indicated colitis mouse models. DSS, acute dextran sulfate sodium colitis; cDSS, chronic dextran sulfate sodium colitis; OxC, Oxazolone induced colitis; TC, T-cell adoptive transfer colitis. **(D)** Heatmap showing normalized expression levels of the indicated genes the type of regimen used to induce colitis is depicted below. Numbers in each quadrant represent the *p*-value and the legend denotes the fold changes in expression ratios.

throughout the course of DSS-induced colitis, we failed to identify a clear type 2 discriminatory signature in specific mouse models and observed that surprisingly, DSS colitis had the most dynamic Th2 response followed by T-cell transfer colitis and lastly oxazolone colitis (**Figure 1D**). Oxazolone colitis showed the mildest regulatory shift to a type 2 response with only 5 genes of the identifier gene set being significantly regulated. These included *Itk*, *Ccl17*, *Retnla*, *Areg*, and *Ccl8* (**Figure 1D**). Interestingly, oxazolone colitis was the only dataset in which *Ccl17* was significantly regulated. There was an overall tendency toward upregulation of most of the transcripts that we assessed barring 3 transcripts, *Dennd1b*, *Prkcz,* and *Il17rb* which showed an overall downregulation and clustered together across all the datasets that we tested (**Figure 1D**). The protein products of these three genes functionally cooperate downstream of IL-17 signaling to modulate cellular stress response (71). Among all the genes and across all the datasets, the expression of *Tslp* showed the least significant variation in expression making it the only gene in the identifier that failed to show significant expression changes in at least one of the datasets (**Figures 1C,D**). The gene that showed high similarity across all the datasets was *Ccl8*, which was significantly upregulated in all the tested datasets (**Figures 1C,D**).

Several genes such as *Gata3*, *Itk*, *Ccl8*, *Ccl22*, *Ccr4*, *Crlf2*, *Il9r*, *Il1rl1*, and *Arg2* are regulated concordantly in T-cell transfer colitis, acute DSS colitis and the time point representing high inflammation in the DSS time course (**Figures 1C,D**). Finally, some genes tended to show a greater proportion of upregulation only in the DSS colitis models. These included *Il33*, *Ecm1*, *Arg1* and *Mrc1,* and *Maf* (**Figures 1C,D**). No gene was exclusively upregulated in the Transfer or Oxazolone colitis models. Thus, our analysis fails to identify a discriminatory type 2 signature in mouse models of IBD, which are known to have distinct immune phenotypes (14–17).

Publicly Available Transcriptomic Datasets Show a Non-discriminatory Regulation of Type 2 Identifier Gene Set Between Different IBD Subtypes

Classically, UC has been classified as having a dominant type 2 immune signature. This view has always been controversial and there is evidence in support of and against this view (72, 73). To address whether the type 2 gene set identifier may discriminate between human IBD subtypes, we screened five publicly available transcriptomic datasets representing 22 distinct comparisons against respective control samples from intestinal biopsies of human IBD patients. The datasets represented ileum (**Figure 2A**) as well as the colon and rectum (**Figure 2B**) at various degrees of inflammation from CD and UC patients of both genders. After applying the appropriate abundance thresholds our analysis yielded 22 genes representing 56% of the identifier gene set that were analyzed across all the cohorts (**Figures 2A,B**). A comparison of the overall magnitude of the most upregulated genes, showed that the rectal tissues of UC patients, on an average, had 11 out of the 22 genes upregulated (**Figure 2B**). These included *ARG2*, *MAF*, *EVM1*, *MRC1*, *CCL22*, *GATA3*, *IL33*, *ITK*, *CCR4*, and *IL1RL1*. However, their regulatory behavior was non-discriminatory when comparing disease subset or tissue of origin because several of them were also upregulated in CD patients and in inflamed ileal tissues (**Figures 2A,B**). The least informative genes vis à vis their significance across all the datasets were *TNFSF4*, *DENND1B*, *STAT6*, and *TSLP* with significant changes in expression detectable in just 1, 3, 3, and 4 comparisons, respectively (**Figures 2A,B**). Whereas, genes *IL1RL1*, *IL33*, *ECM1*, and *PRKCZ* were the most informative with significance reached in 15, 12, 12, 12, and 12 comparisons, respectively (**Figures 2A,B**).

In the analysis of the more severe cases of disease across all the cohorts, the gene *IL1RL1* showed the most significant upregulation in all tissues including ileum, colon and rectum (**Figures 2A,B**). Remarkably, the upregulation in *CCL22* and *GATA3* was restricted to the colonic and rectal samples with no regulation detected in any of the ileal samples irrespective of IBD subtype and gender (**Figures 2A,B**). Among these, CCL22 afforded the most discriminatory power against ileal tissues, with none of the ileal datasets showing changes in regulation and only the rectal and colonic datasets showing an upregulation. We also detected a discordant expression pattern for two genes *ARG2* and *MAF* between the ileum and the rectum with an overall tendency of being downregulated in the inflamed ilea and upregulated in the inflamed rectum (**Figures 2A,B**). Among the comparisons involving inflamed vs. control ileal tissues, the genes *CCL8*, *ARG1* and *AREG* were upregulated in patients of both genders with endoscopic evidence of macroinflammation with deep ulcers (**Figures 2A,B**).

A generalized trend toward downregulation was observed for a cluster of 3 genes that included *IL13RA1*, *PRKCZ*, and *DENND1B* (**Figures 2A,B**). Among this cluster, *IL13RA1* and *PRKCZ* were significantly downregulated across all the cohorts representing rectal tissues, whereas in ileal cohorts, significance was only reached for the most inflamed ileal tissues with evidence of macroinflammation and deep ulceration (**Figures 2A,B**).

Comparative Transcriptomics Reveals Conserved Regulation of a Type 2-Associated Module Shared Between Human IBD and Mouse Models of IBD

By comparing the genes that showed significant regulation (up or down) in the mouse models and the human cohorts, we identified two clusters of genes the regulatory behavior of which was conserved. For both, up as well as down regulated genes

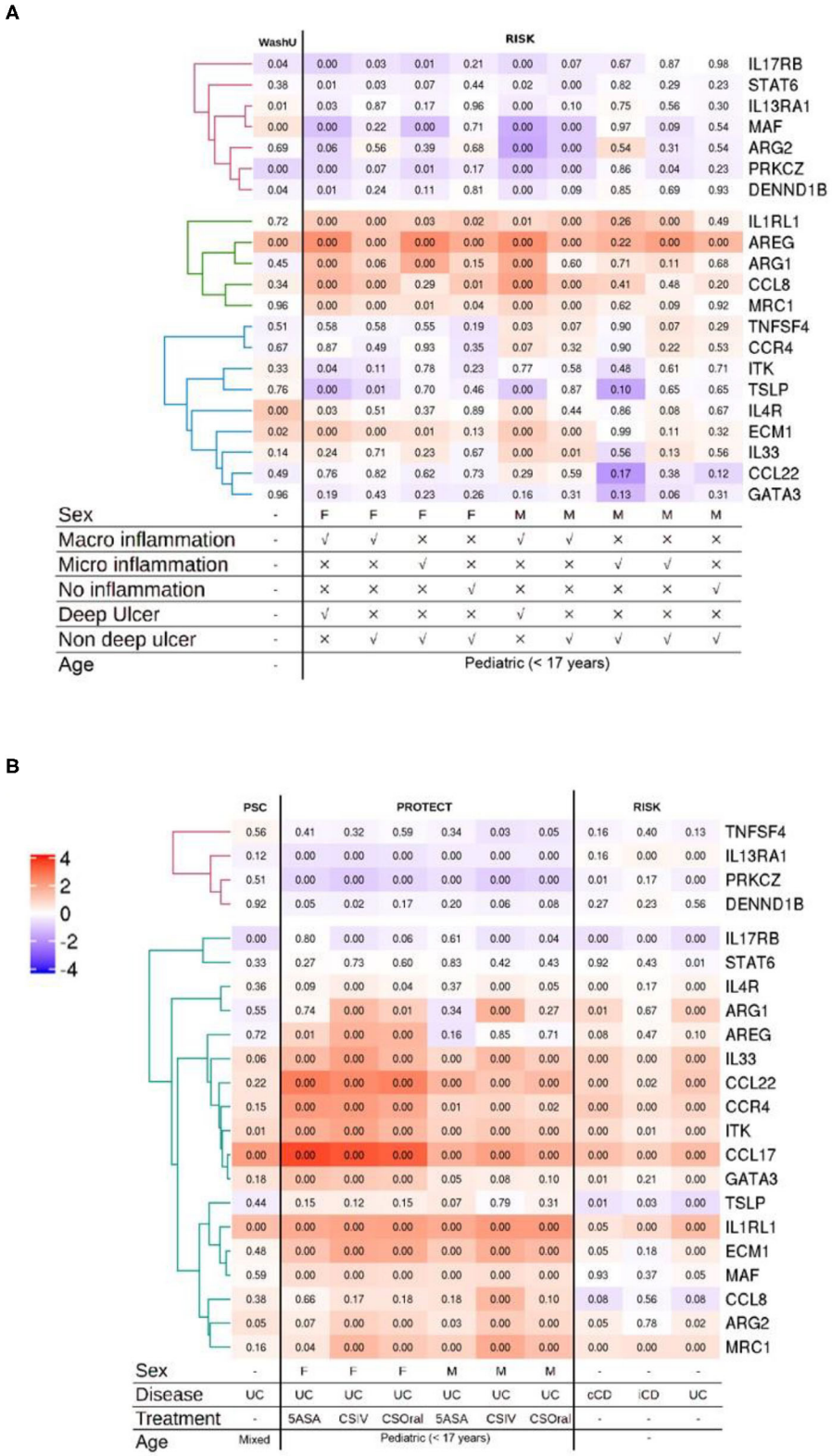

FIGURE 2 | Analysis of multiple IBD cohorts using the type 2 identifier gene set. **(A)** Heatmap of the normalized ileal gene expression profiles from the indicated cohorts of CD patients. Where available, gender, inflammation type and ulceration status are depicted below. **(B)** Heatmap of the normalized gene expression profiles of the indicated genes in the colonic and rectal tissues from IBD patients. Where available, gender, disease diagnosis (UC, Ulcerative Colitis; iCD, ileal Crohn's disease; and cCD, colonic Crohn's disease) and treatments (5-ASA, 5-aminosalicylic acid; CSIV, Cyclosporin intravenous; and CSOral, Cyclosporin oral) are indicated below. Numbers in each quadrant represent the *p*-value and the legend denotes the fold changes in expression ratios.

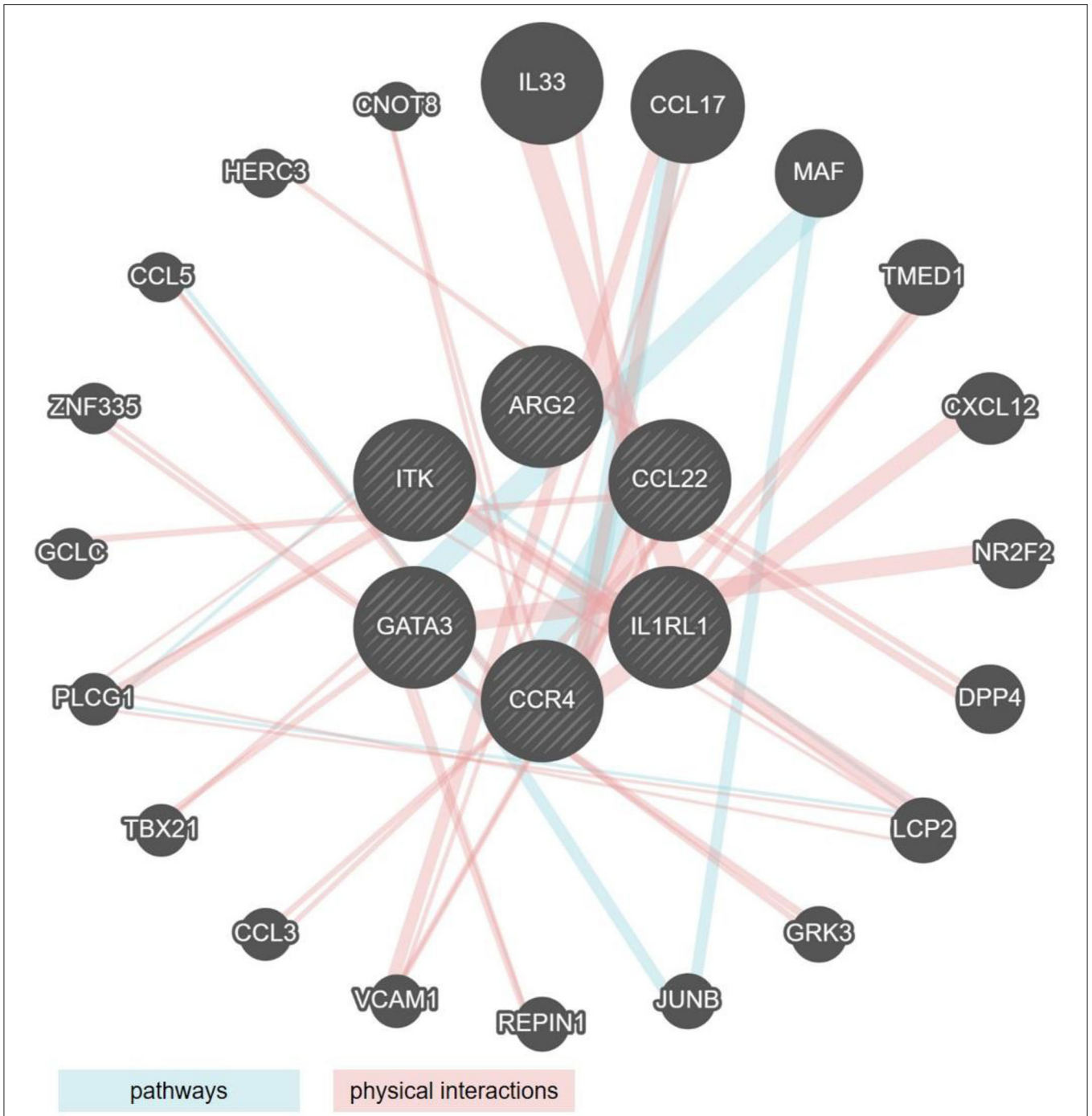

FIGURE 3 | Network analysis of the six type 2 associated genes similarly regulated in human IBD and mouse models of colitis. Radial interaction map of type 2 associated marker genes common to both human IBD and mouse models of colitis (the six central circles, shaded gray). Each non-shaded gray circle represents a predicted pathway interactor (aqua lines) or physical interactor (mauve lines) of the six core factors. Size of each circle represents the rank generated by an automatically selected weighting method in Cytoscape.

that were commonly regulated, the greatest overlap was observed between the mouse models showing acute inflammation and human cohorts that were highly inflamed. The seven genes encoding for *ITK, GATA3, CCL22, IL1RL1, CCR4,* and *ARG2* were upregulated in DSS and T-cell transfer colitis, and in the human colon and rectum cohorts (**Figures 1C,D**, **2A,B**). An analysis of pathways and physical interactors of these genes yielded an extended network (**Figure 3**). Some of the members

of this extended network such as *IL33, CCL17,* and *MAF* were among our type 2 identifier gene set; the others are downstream components, which may participate in regulating type 2 immune response (**Figure 3**).

Interestingly, and contrary to our expectation, except for *ITK*, the regulatory behavior of none of these seven shared genes was significantly altered in the Oxazolone colitis mouse model, a model previously associated with Th2-driven pathology (**Figure 1D**). We also identified another module of genes, including *MRC1*, *ARG2, ECM1*, *MAF*, and *IL33* that was upregulated in the DSS models as well as in the human rectum cohorts with some degree of overlap with the most inflamed of the ileal cohorts (**Figures 1C,D**, **2A,B**). Among these, the upregulation of only *ARG2* and *MAF* was truly restricted solely to the rectal datasets (**Figures 1C,D**, **2A,B**). Among the DSS mouse models, *Maf* expression was restricted to the mild, high and acute inflammation stages, returned to normal levels during the resolution stage, and remained unaltered in the chronic DSS model (**Figures 1C,D**).

Interestingly, a cognate behavior for the regulation of *STAT6* and *TSLP* was identified among the mouse models and human cohorts that showed a lack of any significant regulation (**Figures 1C,D**, **2A,B**). The upregulation of *AREG* was observed across all the mouse models except for controls and recovery cohorts and was shared predominantly among in the human ileal but not colonic and rectal ones (**Figures 1C,D**, **2A,B**). Interestingly, the downregulation of the genes *PRKCZ* and *DENND1B* was common among the mouse models and the human cohorts (**Figures 1C,D**, **2A,B**).

DISCUSSION

Research into the cytokine biology of IBD has nurtured the dogmatic view that different IBD subtypes are characterized by distinct immunotypes (6). This classical view has been repeatedly challenged and recent evidence calls for a revision of this point of view. Although several studies have identified an upregulation in type 2 markers in ulcerative colitis, there is no conclusive evidence that a diagnostic discrimination of UC vs. CD can be attained by measuring the markers of type 1 and type 2 immune responses (8, 74, 75). Thus, the type of mucosal immune response on its own cannot explain the differences in the clinical pathogenesis of CD vs. UC. Preclinical mouse models, which resemble these classical human IBD immunotypes, have been widely employed for gaining a better understanding of disease biology as well as for drug discovery purposes. However, due to a lack of consensus on whether certain archetypal immune responses are associated with a certain mouse model that reflects a specific human IBD subtype, we took advantage of a comparative transcriptomic approach. To our knowledge, such an approach has not been applied so far in gaining an understanding of type 2 immunity in preclinical and clinical IBD.

Classically, colitis induced using the adoptive transfer of $CD4^{+}CD45RB^{high}$ T cells into Rag 1, 2 knockout mice that lack T and B cells has been considered to be Th1 -mediated (76). More recently, both Th1 and Th17 cells were broadly implicated in colitogenic disease mechanisms in this model. Another commonly used mouse model for IBD, DSS colitis, is also considered to be predominantly a Th1/Th17 type of colitis, although colitis can develop even in the absence of T cells (77). Conversely, the models of chronic DSS colitis and Oxazolone colitis are considered to be Th2-driven forms of colitis (78). Interestingly, our analysis showed a significant overlap in the expression of cognate type 2 gene set in the DSS as well as the T cell transfer colitis models, which as stated earlier are classically considered type 1-driven models. Interestingly, and contrary to our expectation, chronic DSS colitis and Oxazolone colitis showed the least regulation of the type 2 identifier gene set. Using a single cell sequencing approach, Kiner et al. recently also failed to identify classical T helper cell subsets and could not define distinct Th1, Th17, or Th2 restricted clusters but rather identified phenotypically flexible clusters that depended on the overarching microbial milieu (79). Notably, the lack of Th stereotypes in the gut mucosa was not only observed at steady state, but also when mice were infected with different pathogens including the metazoan parasite *H. polygurus* and *N. brasiliensi* (79). In line with this, we analyzed two transcriptomic datasets, one from the lung and the other from the small intestine of mice that were infected with the helminth parasites *Nippostrongylus brasiliensis* and *Heligmosomoides polygyrus,* respectively (80, 81). Similar to the findings of Kiner et al., we also failed to identify a significant proportion of canonical type 2 response signatures, which were shared between the two tissues.

One technical caveat of transcriptome sequencing methods used in our study is the cutoff for minimum sequence length that guarantees a good sequence read. In our method, this cutoff was 150 base pairs for a paired end sequencing, which inadvertently causes a loss of short length transcripts, which we were unable to include in our analysis. Nonetheless, our analysis contributes to the growing body of evidence that points to a reassessment of the classical immune subtyping in IBD.

In addition, our data reveal a conserved group of type 2 associated genes, which are regulated similarly in the commonly used mouse models of IBD and in human IBD. The analyses presented in our work shows that most of the classical markers of type 2 immune response do not behave in a presumed categorical pattern. We also show that the conception that in preclinical studies of IBD, specific types of immune responses can be modeled using specific mouse models needs to be revised.

DATA AVAILABILITY STATEMENT

The data for the mRNA sequencing datasets generated in this study for the time course of experimental DSS colitis are available via the array express service of EMBL-EBI through the accession number E-MTAB-9850. All other datasets analyzed in this study along with their accession numbers, references, and sources for the data can be found in the materials and methods section of this article.

ETHICS STATEMENT

The animal study was reviewed and approved by The ethics commission of Lower Franconia and Rhineland.

AUTHOR CONTRIBUTIONS

LD: experimentation and analysis. MG, JP, SW, and CB: study concept, design, literature search, experimentation, analysis, interpretation of data, and critical revision of the manuscript. BS: critical revision of the manuscript. All authors contributed to the article and approved 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/fmed.2021.664045/full#supplementary-material

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frontiers in Medicine

MINI REVIEW
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Innovative Diagnostic Endoscopy in Inflammatory Bowel Diseases: From High-Definition to Molecular Endoscopy

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High-definition endoscopy is one essential step in the initial diagnosis of inflammatory bowel disease (IBD) characterizing the extent and severity of inflammation, as well as discriminating ulcerative colitis (UC) from Crohn's disease (CD). Following general recommendations and national guidelines, individual risk stratification should define the appropriate surveillance strategy, biopsy protocol and frequency of endoscopies. Beside high-definition videoendoscopy the application of dyes applied via a spraying catheter is of additional diagnostic value with a higher detection rate of intraepithelial neoplasia (IEN). Virtual chromoendoscopy techniques (NBI, FICE, I-scan, BLI) should not be recommended as a single surveillance strategy in IBD, although newer data suggest a higher comparability to dye-based chromoendoscopy than previously assumed. First results of oral methylene blue formulation are promising for improving the acceptance rate of classical chromoendoscopy. Confocal laser endomicroscopy (CLE) is still an experimental but highly innovative endoscopic procedure with the potential to contribute to the detection of dysplastic lesions. Molecular endoscopy in IBD has taken application of CLE to a higher level and allows topical application of labeled probes, mainly antibodies, against specific target structures expressed in the tissue to predict response or failure to biological therapies. First pre-clinical and *in vivo* data from label-free multiphoton microscopy (MPM) are now available to characterize mucosal and submucosal inflammation on endoscopy in more detail. These new techniques now have opened the door to individualized and highly specific molecular imaging in IBD in the future and pave the path to personalized medicine approaches. The quality of evidence was stated according to the Oxford Center of evidence-based medicine (March 2009). For this review a Medline search up to January 2021 was performed using the words "inflammatory bowel disease," "ulcerative colitis," "crohn's disease," "chromoendoscopy," "high-definition endoscopy," "confocal laser endomicroscopy," "confocal laser microscopy," "molecular imaging," "multiphoton microscopy."

Keywords: high-definition endoscopy, confocal laser microscopy, chromoendoscopy, molecular endoscopy, multiphoton microscopy

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INTRODUCTION

Gastrointestinal endoscopy plays a crucial role in patients with inflammatory bowel disease (IBD; Crohn's disease CD; ulcerative colitis UC). The initial diagnosis, the determination of disease activity and surveillance are the key steps of rational disease management and include primarily an endoscopic approach to visualize and characterize the extent and severity of mucosal inflammation and to take targeted biopsies of inflamed and non-inflamed tissue areas. High-resolution or high-definition endoscopy should be the gold standard when examining IBD patients. In surveillance colonoscopy, a combination of high-definition endoscopy with classical dye-based chromoendoscopy (e.g., indigocarmine solution 0.1–0.5%) is of additional value to detect flat polypoid neoplastic mucosal lesions and discriminate these areas from colitis-associated pseudopolyps or other benign lesions (level 1, grade of recommendation A). The exclusion or detection of intraepithelial neoplasia (IEN) is the aim of all surveillance colonoscopies in IBD to reduce the risk of malignant transformation to colorectal cancer. Although many study data showed different results this risk seems to be lower than previously assumed (1) and is in the range of 1–7% after 10 and 30 years of UC, respectively (2), (level 2, grade B). In patients with CD the risk of developing colorectal cancer is lower than in UC, but still heightened with an incidence rate of 2% after 30 years (2), (level 2, grade B). The detection rate of IEN can be further improved by using *in-vivo* histology techniques. Confocal laser endomicroscopy (CLE) was introduced in 2006 and gave exclusive insight into the gastrointestinal tract on a cellular and subcellular level in a variety of gastrointestinal diseases. Initially, there were two independent *in-vivo* histology systems available on the market, the endoscope-based CLE (eCLE) by Pentax, Tokyo, Japan, and a probe-based CLE (pCLE) by Mauna Kea Technologies, Paris, France. A couple of years ago the technical support for eCLE was permanently discontinued and research activities with that specific system were restricted to a very small number of research centers with active running systems. In IBD, CLE was used for characterization and classification of inflammatory activity and mucosal healing in active disease as well as for the detection of IEN during surveillance. For example, a combination of chromoendoscopy with CLE can detect 5-fold higher rates of IEN compared with random biopsy protocols (3), (level 4 grade C). After evaluation of CLE as a unique tool for the characterization of normal, inflamed and pre-malignant or malignant intestinal mucosa, some research groups focused on the analysis of the intestinal barrier function for predicting clinical relapse (4), (level 3, grade C). Mucosal healing can predict response to therapy or, vice versa, ongoing mucosal or submucosal inflammation may indicate treatment failure. Kiesslich et al. published a study investigating epithelial barrier function by CLE and described leakage of fluorescein due to epithelial gaps during cell shedding (5), (level 3, grade C). Based on these and other data, highly specific fluorescein-labeled probes binding to their molecular targets on the surface of the gastrointestinal epithelium established a fascinating new era of molecular imaging studies. Molecular endoscopy allows a more specific and individual treatment by predicting the response to anti-inflammatory therapy (6), (level 3, grade C). Recently label-free multiphoton microscopy based on endogenous autofluorescence visualized mucosal inflammation in human biopsies of CD patients (7, 8).

HIGH-DEFINITION ENDOSCOPY AND CHROMOENDOSCOPY IN IBD

Lower optical resolution of previous endoscope generations and random biopsy protocols in all patients were central elements in the surveillance of IBD during the first decade of this century. The lower image quality might be one reason for the increased rate of colorectal cancers described earlier in UC patients (1). High-definition endoscopes have an average diameter of 9–13 mm, a field of view between 140 and 170°, an optical resolution up to 2 million pixels and a 4-way angulation and the newest generation of endoscopes is mostly equipped with bright LEDs (9). Over the last 10 years a more specific and, moreover, individual endoscopic strategy was implemented in national IBD guidelines focusing on defined risk factors. In Germany, surveillance colonoscopy in UC starts 6–8 years after initial diagnosis and should be performed between each year in high-risk patients and every 4 year in patients with low-risk conditions (10), (level 1, grade A). Recently patients with primary sclerosing cholangitis (PSC), a tubular colon and those with a history of neoplasia were identified as having a higher risk for developing colorectal cancer and in these patients targeted and additional random biopsies were recommended during chromoendoscopy (11), (level 1, grade A). For classical chromoendoscopy in the colon, either indigo carmine as a contrast enhanced dye or methylene blue as an absorptive dye can be used, for both agents a 0.1–0.5% working solution is recommended and should be applied with slight pressure via a spraying catheter to the mucosal surface to ensure optimal distribution throughout the entire colon. An adequate withdrawal time and sufficient bowel preparation (Boston Preparation Scale $\geq$ 6) is mandatory for an optimal view of the complete colonic mucosal surface. Huge efforts were made to investigate if virtual chromoendoscopy techniques (NBI, FICE, I-scan) are able to replace classical dye-based chromoendoscopy. NBI can characterize histological inflammation by the determination of mucosal vascular pattern (12), (level 4, grade C). This was recently confirmed and prediction of mucosal proliferation can be helpful in the diagnosis of IEN (13), (level 4, grade C). However, the inconsistent results of various studies currently do not justify the application of virtual chromoendoscopy as a single surveillance strategy (14, 15), (level 3, grade D). Studies favoring virtual chromoendoscopy found that the examination time and the technical efforts were significantly lower and therefore more user-friendly compared to the application of classical dyes via spraying catheter (16), (level 1, grade A). A new meta-analysis identified 11 randomized-controlled trials with a total of 1328 patients and concluded that virtual chromoendoscopy is as good as high-definition endoscopy with dye-based chromoendoscopy (17). This indicates that probably in a couple of years both techniques can be applied equally

depending on the local expertise of the respective endoscopy unit. As we already know from our daily practice, the acceptance rate of classical chromoendoscopy among physicians is low. Therefore, a promising future perspective for any screening colonoscopy might be the pre-interventional intake of oral chromoendoscopy dye. Recently, the results of a phase 3 trial found an increase in the adenoma detection rate of 8.5% when peroral methylene blue tablets (MMX®) were administered together with bowel preparation (18), (level 1, grade A). Further studies will evaluate oral chromoendoscopy in patients with the need for recurring endoscopic surveillance colonoscopies.

CONFOCAL LASER ENDOMICROSCOPY (eCLE, pCLE)

Today we can look back on 15 years of confocal laser endomicroscopy (CLE). This exciting technique was originally designed to allow virtual histology on a cellular and subcellular level with the potential to at least partially replace classical histology. The procedure, however, is time-consuming, technically challenging and intravenous applied fluorescein is necessary for each procedure to generate high-resolution images. This and the fact that no reimbursement was provided by health care authorities restricted the running CLE systems to large research units in University centers. The acquisition of targeted biopsies became reality and a large number of clinical studies investigating a variety of gastrointestinal diseases were published between 2005 and 2012. Most of these studies characterized pre-malignant or inflammatory lesions in Barrett's esophagus (19), gastric cancer (20), celiac disease (21), IBD (22), graft-vs. host disease (23) or adenomatous polyps (24) in the upper and lower gastrointestinal tract (level 3, grade C). A fascinating overview of different cellular and subcellular pathologies was provided and after an initial characterization period the next level of CLE research was reached by explaining functional dynamic changes within the intestinal mucosa. The identification of epithelial gaps during cell shedding and the increase in gaps after stimulation with tumor necrosis factor (TNF) alpha caused loss of barrier function and integrity (5), (level 3, grade C). In IBD patients in clinical remission, increased cell shedding with fluorescein leakage was observed and associated with subsequent relapse 12 months after initial CLE (22) indicating that CLE is able to relapse or can define a stable disease when the barrier function is intact (level C, grade C). These observations were in accordance with electrophysiological measurements in human biopsies of patients with CD as described earlier. After anti-TNF treatment the upregulation of epithelial apoptotic cells in active disease restored to normal and barrier dysfunction completely recovered (25). *In vivo* histology was also able to contribute to the diagnosis and detection of IEN during surveillance colonoscopy. For CLE a meta-analysis revealed a pooled sensitivity and specificity of 91 and 97% for the differentiation of neoplastic from non-neoplastic lesions (26). Data of chromoendoscopy-guided CLE showed inconsistent results (level 4, grade C). Whereas, some studies describe a higher detection rate of IEN (3) in UC patients, other working groups did not observe a benefit over chromoendoscopy alone (27). However, the general use of this approach for surveillance cannot be recommended. Ongoing study activities with eCLE were hampered by the missing combination of the initial confocal microscope device with a newer high-definition endoscope technology due to several, unfortunately also economic reasons. Currently there is only pCLE available on the market and although there are technical and optical differences between the two systems, the usefulness of pCLE in predicting postoperative recurrence in patients with CD was shown recently (28), (level 4, grade C). Now there is a possibility to apply pCLE with nearly any commercial endoscope independent of the manufacturer. For further characterization of intestinal barrier function in IBD *in vivo* by pCLE, reliable and reproducible diagnostic criteria should be defined. The quantification of gaps, fluorescein leakage and cell shedding (5, 29) (level 3–4, grade C) are encouraging first candidates for the measurement of barrier function *in vivo* and may act as main criteria. Crypt tortuosity, distortion of crypt openings and decreased crypt density were additional observations in UC patients (30) and could potentially act as minor criteria (level 3, grade C). A number of CLE-based rating systems and scores have been published so far taking into account the degree of inflammation and the prediction of relapse (31). For the assessment of clinical outcomes or the determination of relapse rates in IBD patients under immunosuppressive therapy further research is necessary. The number of research projects investigating CLE in IBD is currently decreasing. One reason for this may be the introduction of emerging artificial intelligence systems (32) on the market, which will be part of future detection of IEN. However, for the determination of disease activity to predict relapse or therapy response in IBD there is an ongoing need for further CLE evaluation.

MOLECULAR IMAGING

Fluorescence endoscopy (33), near-infrared fluorescence endoscopy (34) and autofluorescence endoscopy were often subsumed under molecular imaging devices. These techniques can be combined with virtual chromoendoscopy (35). However, these technologies were rather classical "red flag" technologies than real molecular imaging techniques. The years of research of the newer *in-vivo* histology techniques deliver the basis for a more detailed analysis of the underlying molecular pathways. More specific and distinct molecular imaging in advanced gastrointestinal endoscopy is the real-time visualization and binding of labeled-molecules to targeted structures on the surface of epithelial cells and the detection of this conjunction by *in vivo* histology. Probes usable for molecular imaging could be labeled antibodies, peptides, enzymes, affibodies or lectins, respectively (36). Molecular imaging is far away from widespread clinical use. However, it potentially allows a highly-individualized and specific characterization of mucosal inflammatory diseases in the future. *In vivo* studies with labeled antibodies imply a long-lasting and extensive process of approval and fulfillment of strict requirements before the use in humans is approved by regulatory authorities. The first *in*

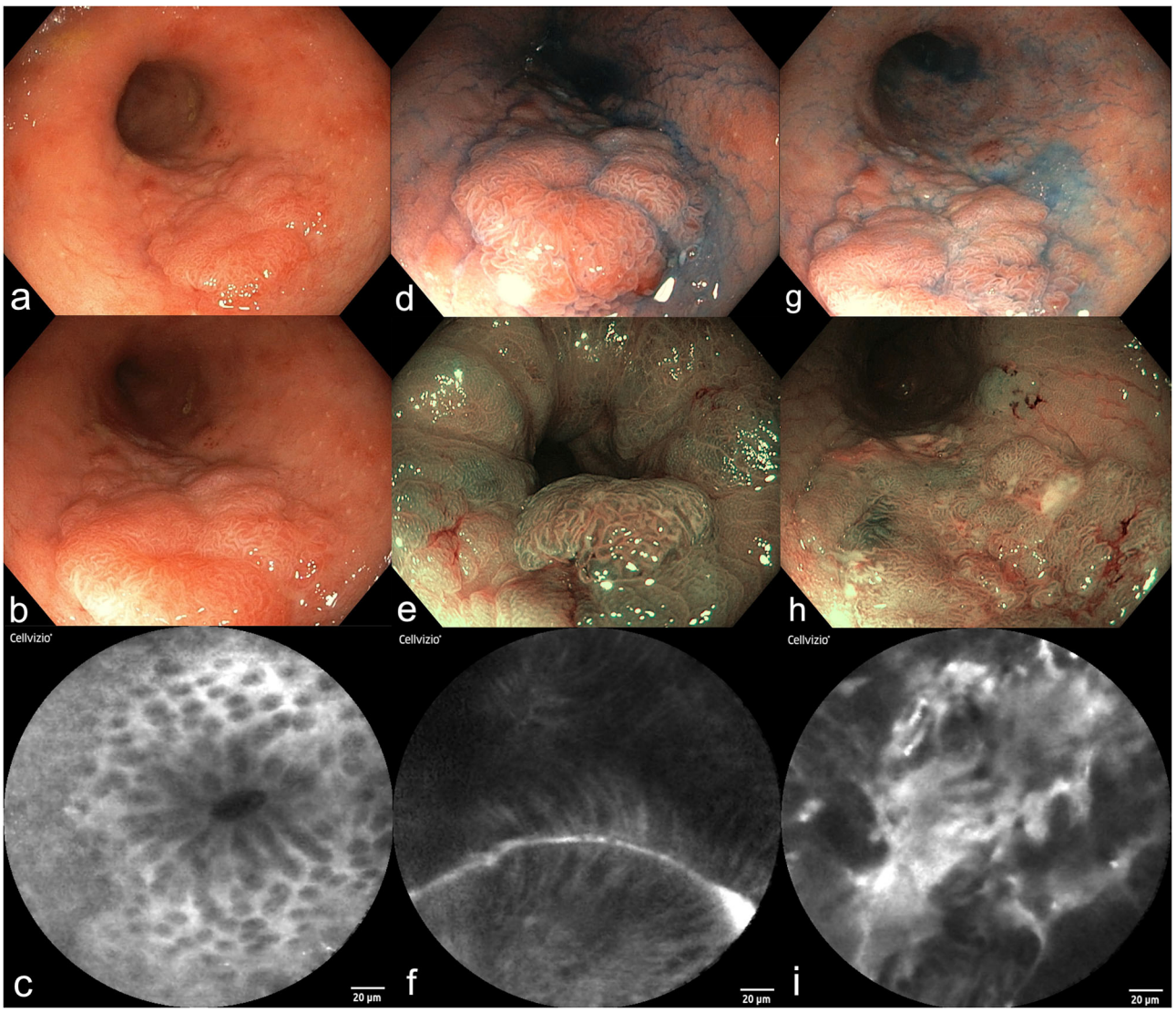

FIGURE 1 | Surveillance colonoscopy in a female patient (64y) with a history of UC for 15 years **(a–i)**. **(a,b)** High-resolution video endoscopy shows a flat polypoid lesion in the sigmoid colon, size 4 × 2 cm, Paris Classification IIa+c. **(c)** Probe-based confocal laser endomicroscopy of the surrounding mucosa revealed mild inflammation and normal crypts. **(d–f)** Dye-based chromoendoscopy with indigocarmine **(d)**, narrow-band imaging [NBI, **(e)**], and pCLE **(f)** of the distal border of the polyp. A tubular structure and distorted mucosal epithelial cells become visible. **(g–i)** Dye-based chromoendoscopy with indigocarmine **(g)**, NBI **(h)**, and pCLE **(i)** of the proximal part of the polyp. **(i)** Shows high-grade intraepithelial neoplasia. Final histology of this lesion after proctocolectomy revealed well-differentiated intramucosal cancer without invasion.

vivo application of fluorescein-labeled heptapeptides during a colonoscopy detecting colonic dysplasia was in 2008 (37). Two years later, targeting of epidermal growth factor receptor (EGFR) in colorectal cancer allowed the discrimination of neoplastic and non-neoplastic tissue areas in living animals and human tissue samples (38). One underlying signaling pathway identified a link between inflammation and tumorigenesis and was described in colitis-associated cancer (39). After demonstrating the feasibility and safety of molecular imaging in pre-malignant or malignant disease *in vivo*, further research focused on inflammatory disease with the goal to predict therapy response or relapse. The first molecular target of interest in IBD was TNF. A landmark study detecting the binding of membrane-bound TNF (mTNF) by a fluorescent-labeled adalimumab anti-TNF antibody showed that high numbers of mTNF-positive cells correlated with higher short-term response rates to treatment with the TNF-neutralizing antibody adalimumab. Patients with high numbers of mTNF-expressing cells demonstrated a higher probability of clinical response than patients with low numbers of mTNF+ cells (92 vs. 15%). The sensitivity, specificity and accuracy for the prediction of therapeutic responses were 92, 85, and 88%, respectively. Positive and negative predictive values were 85 and 92% (40). Recently, first data presented the detection of mucosal α4β7 integrin *ex vivo* with a fluorescent labeled anti-adhesion

antibody vedolizumab in CD (41). In the clinical management of IBD patients, early prediction of response or failure of a planned therapy would be of utmost clinical importance. Consequently, a prompt adjustment of planned immunosuppressive therapy would be possible (42).

MULTIPHOTON MICROSCOPY

Multiphoton microscopy (MPM) is one of the emerging innovative imaging technologies with the potential to visualize intestinal epithelial cells under normal and inflamed conditions without the addition of exogenous fluorescent dyes (43). The first data with MPM as a promising imaging technology in IBD revealed a clear discrimination of epithelial and immune cells and the amount of extracellular matrix (7). This label-free imaging of intestinal cellular and subcellular structures based on autofluorescence and second harmonic generation signals has therefore some advantages compared to CLE and was further developed for *in vivo* use. Recently, the first experiments in normal and inflamed murine colonic mucosa in a dextran-sulfate sodium-induced colitis model showed feasibility and a gradually deformation of the crypt architecture depending on the activity of the colitis (8). A future perspective would be the combination of MPM with a high-definition endoscope to enable the use during routine gastrointestinal endoscopy without the requirement of any exogenous labeling.

FUTURE PERSPECTIVES

On the way to an individualized endoscopic approach, a large number of technical improvements are nowadays available for patients with IBD. These include mainly high-definition endoscopy with nearly comparable efficiency compared to dye-based and virtual chromoendoscopy techniques. If upcoming clinical studies with oral intake of methylene blue prior to surveillance colonoscopy become available and confirm the additional benefit, the reservations against classical dye spraying would finally come to an end. Although CLE as the most widely used *in vivo* histology method brought extensive insight and understanding of gastrointestinal mucosal pathology, its widespread use in routine endoscopy is hampered by the lack of reimbursement and additional examination time (**Figure 1**). However, CLE opened the field for molecular endoscopy allowing specific targeting of surface molecules. The prediction of therapeutic response followed by prompt adjustment of targeted therapeutic strategies improve clinical decisions in complex IBD courses. MPM is an emerging new technology and the first data are now available showing *in vivo* use in an animal model. Label-free high-resolution endomicroscopy would be the logical consequence and a perfect long-term perspective for the use in patients with 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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REVIEW
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Molecular Communication Between Neuronal Networks and Intestinal Epithelial Cells in Gut Inflammation and Parkinson's Disease

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Intestinal symptoms, such as nausea, vomiting, and constipation, are common in Parkinson's disease patients. These clinical signs normally appear years before the diagnosis of the neurodegenerative disease, preceding the occurrence of motor manifestations. Moreover, it is postulated that Parkinson's disease might originate in the gut, due to a response against the intestinal microbiota leading to alterations in alpha-synuclein in the intestinal autonomic nervous system. Transmission of this protein to the central nervous system is mediated potentially via the vagus nerve. Thus, deposition of aggregated alpha-synuclein in the gastrointestinal tract has been suggested as a potential prodromal diagnostic marker for Parkinson's disease. Interestingly, hallmarks of chronic intestinal inflammation in inflammatory bowel disease, such as dysbiosis and increased intestinal permeability, are also observed in Parkinson's disease patients. Additionally, alpha-synuclein accumulations were detected in the gut of Crohn's disease patients. Despite a solid association between neurodegenerative diseases and gut inflammation, it is not clear whether intestinal alterations represent cause or consequence of neuroinflammation in the central nervous system. In this review, we summarize the bidirectional communication between the brain and the gut in the context of Parkinson's disease and intestinal dysfunction/inflammation as present in inflammatory bowel disease. Further, we focus on the contribution of intestinal epithelium, the communication between intestinal epithelial cells, microbiota, immune and neuronal cells, as well as mechanisms causing alterations of epithelial integrity.

Keywords: Parkinson's disease, gut-brain axis, enteroendocrine cells, alpha-synuclein, intestinal inflammation, inflammatory bowel diseases

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INTRODUCTION

Parkinson's Disease

Parkinson's disease (PD) is the second most common neurological disorder characterized by movement disabilities (1), but also by non-motor symptoms, including gastrointestinal dysfunction that often appears years before diagnosis of disease (2, 3). A neuropathological hallmark of PD is the aggregation of the synaptic protein alpha-synuclein (aSyn) within the central nervous system (CNS), leading to degeneration of dopaminergic neurons within the *substantia nigra pars compacta* (SNpc) of the midbrain (1). Moreover, research suggests that inflammatory responses within the CNS contribute to PD pathology. Hence, glial cell reactions and T cell infiltration result in increased levels of inflammatory cytokines within the CNS and are currently recognized as prominent features of PD (4, 5).

Intestinal Dysfunction and Inflammation Within PD

Interestingly, recent data indicate that intestinal inflammation contributes to the pathogenesis of PD (6), and increasing numbers of studies imply that PD may start in the gastrointestinal system years before any motor symptoms develop (7–9). An acute and chronic intestinal inflammation is a prominent feature of Inflammatory bowel disease (IBD) comprising the diseases Ulcerative colitis (UC) and Crohn's disease (CD). While UC mainly affects the colon and rectum, CD injures the entire GI tract (10). IBD is understood to be a result of gut microbiota dysbiosis and mucosal immune dysregulation (11). Also, intestinal inflammation in IBD is associated with intestinal epithelial cell (IEC) alterations and maintaining epithelial homeostasis helps in protecting against inflammation (12). Remarkably, PD and IBD share overlapping genetic factors found within a recent genome-wide-association study (GWAS) (13). The leucin-rich repeat kinase 2 (*LRRK2*) gene appears to be the most susceptibility-factor for both diseases (14, 15). Interestingly, *LRRK2* is one of the genes most commonly associated with familial and sporadic PD (16). Recent studies show, that patients with IBD have a higher risk of developing PD as compared to non-IBD individuals (17, 18). It is well-established that IBD is characterized by chronic pro-inflammatory immune activity (11), which is now suggested to be a fundamental element of neurodegenerative disorders as well (5, 6). Furthermore, animal studies demonstrate that gut inflammation, similar to IBD, induces loss of dopaminergic neurons (19, 20). Additionally, chronic GI inflammation is likely to induce anxiety-like behavior and alter CNS biochemistry in mice (21). Interestingly, CD patients have been shown to accumulate aSyn in the gut (22).

Moreover, aSyn and its aggregated forms were also found in the enteric nervous system (ENS) of PD patients and symptoms outside the CNS were described including GI impairments (2, 23, 24). This gave rise to the hypothesis that PD pathology can spread from the gut to the brain and vice versa (23, 25, 26). This hypothesis is supported by recent animal studies, which recapitulated the transmission of aSyn pathology via the vagal nerve, connecting the central with the peripheral nervous system (27, 28). In this context, the discovery of aSyn expression in enteroendocrine cells (EECs) within the intestinal epithelium suggests these cells as sensors of luminal signals triggering the gut-neural circuit behind aSyn alteration (29, 30). This signal is then transmitted to the CNS, potentially via the vagus nerve. Thus, deposition of aggregated aSyn in the GI tract has been inferred as a potential diagnostic marker for prodromal PD.

This review focuses on overlapping disease pathologies and the molecular communication between the brain and the gut in the context of PD and gut inflammation, as present in IBD (**Figure 1**). We emphasis on the contribution of neurodegeneration and neuroinflammation in PD, gut-brain spreading of PD pathology, intestinal epithelium and the communication between IECs, microbiota and immune cells (**Figure 2**). Moreover, we discuss the mechanisms causing alterations of epithelial integrity and gastrointestinal (GI) dysfunction in PD.

NEUROPATHOLOGY IN PD

Motor and Non-motor Manifestations of PD

PD is clinically characterized by classical motor symptoms including muscular rigidity, bradykinesia, rest tremor, and postural instability (1). Among several putative factors that may contribute to PD pathology, the most crucial indication of PD is the degeneration of neurons in the CNS. The loss of dopaminergic neurons within the SNpc is the most predominant feature during disease progression (31) and leads to excessive dopamine depletion within the basal ganglia, which results in the above mentioned parkinsonian motor characteristics (1). The administration of the amino acid precursor of dopamine, L-DOPA (L-3,4-dihydroxy-L-phenylalanine), has shown to be the most effective symptomatic treatment. However, if the motor symptoms occur in PD patients the continuous loss of neurons is already inexorable (32). Interestingly, PD manifests already >20 years before the motoric problems occur. This premotor or prodromal period of disease is defined by e.g., constipation, olfactory dysfunction, sleep disorder, cognitive impairment, autonomic dysfunction, pain and fatigue (1, 3). Altogether, this leads to the assumption that PD is a complex, multisystem disorder with both neurologic and systemic non-motor manifestations.

Lewy Body Pathology in the CNS

A neuropathological hallmark of PD is the formation of intracellular amyloid inclusions in neuronal bodies and neurites, known as Lewy bodies (LB) and Lewy neuritis (LN), respectively, consisting of aggregated aSyn (33). The appearance of these aSyn-carrying inclusions in patients is also collectively known as synucleinopathies, referring to PD, dementia with Lewy bodies (DLB) and multiple system atrophy (MSA) (33–35).

Physiologically, aSyn is natively unfolded and soluble with an amphipathic N-terminus, a hydrophobic central domain known as non-amyloid-β component (NAC) region, and an acidic C-terminus (36). Under pathological conditions, aSyn aggregates have been shown to exert cell toxic properties (37, 38). The aggregation mechanisms, by which soluble aSyn changes its structure to oligomers and ultimately to insoluble β-sheet rich

fibrils, are still under debate (39–41). However, several factors have been described to induce structural changes of monomeric aSyn, involving the interactions with specific lipids (42, 43) and membranes (44). Further, posttranslational modifications such as phosphorylation (45), nitration and oxidation (46), ubiquitination (47), and sumoylation (48) have been shown to accelerate aSyn pathology. Multiple copies (e.g., duplications and triplications) (49, 50) and missense mutations (e.g., A53T, A30P, E46K, and H50Q) (51–53) of the gene encoding for aSyn (*SNCA*) foster protein aggregation. In addition, the cellular microenvironment of aSyn has been reported to play a role in aSyn conformation and solubility. For instance, aSyn aggregation behavior differs between neutral pH7.4 (e.g., cytosol) and acidic pH5 (e.g., lysosome) and aSyn purified from lysosomes was able to seed aggregation in a concentration-dependent manner (54, 55). Interestingly, dysfunction in lysosomal pathways have been linked to PD (56, 57). For degradation, aSyn is processed within lysosomes by specialized lysosomal enzymes (cathepsins) (58–60). Hence, deficiency within these lysosomal enzymes, important for lysosomal function and aSyn degradation, lead to its aggregation and pathology (61–64). Targeting lysosomal enzymes by boosting their activity has become a promising therapeutic approach, which might lower aSyn burden within neuronal cells and thus decrease the risk of pathological aSyn aggregation and neurotoxicity (65, 66). In a nutshell, intracellular accumulation of aSyn, due to inefficient clearance mechanisms, might drive further aggregation of the protein (67). In this regard, it was shown that toxic aSyn species can be released to the periphery from stressed and/or dying neurons and are subsequently taken up by surrounding cells, leading to spreading of pathology (68–70). Especially, aggregation intermediates, such as aSyn oligomers, exhibit highly cell toxic properties (43, 71, 72).

Neuroinflammation in PD

In recent years, evidence evolved that aSyn and inflammatory processes are extraordinarily connected. In that sense, chronic neuroinflammation is another characteristic indicator of PD pathophysiology and is considered to promote the progression of dopaminergic cell death (73, 74). In general, neuroinflammation is defined as the immune response of cells within the brain and plays an important role in maintenance of nervous tissue homeostasis (4). On the one hand, a moderate inflammation can protect neurons from damage (75); on the other hand, inflammatory factors do also affect neurons directly and convey neurodegeneration. In addition, neuronal cell death induces inflammatory mechanisms, and contributes to a vicious cycle of inflammation and progressive loss of neurons in the brain (76). The neuroinflammatory response is mediated by resident immune cells (microglia and astrocytes), which release cytokines and chemokines (4, 77).

Many neuroinflammatory circumstances at *post-mortem* stage have also been identified on a molecular basis in PD. For example, numerous proinflammatory cytokines and factors such as tumor necrosis factor (TNF)-α, β2-microglobulin, epidermal growth factor (EGF), transforming growth factor α (TGFα), TGFβ1, and interleukin (IL)-1β, IL-6, and IL-2 were found in the striatum of PD patients (78). Furthermore, TNF-α, IL-1β, and interferon (IF)-γ were also detected in the SNpc of PD patients (79). Interestingly, dopaminergic neurons express the receptors of these cytokines (80), that might explain the vulnerability of DA neurons to inflammatory processes inside the brain. In addition, increased levels of proinflammatory mediators, such as IL-1β, IL-2, TNF-α, and IL-6 are present in the serum and the cerebrospinal fluid (CSF) of PD patients (81–84). These results suggest the direct migration of immune cells from the periphery (blood stream) to the brain (or vice versa) during neurodegenerative process.

Microglia, the resident macrophages in the brain, and astrocytes, the most abundant glial subtype in the CNS, are considered to drive the inflammatory response in PD (85). Of relevance, microglia initiate the innate immune response in the brain, therefore representing key players upon inflammatory stimulus (86, 87). Under pathological conditions, activated microglia release proinflammatory cytokines and reactive oxygen species (ROS), which affect dopaminergic neuron viability (73, 88). Reactive microglia were found in various brain regions (89, 90) including the SNpc of PD patients (91). Besides microglia activation, reactive astrogliosis contributes to PD pathogenesis and progression (85). Astroglial cells secret the glial cell-line derived neurotrophic factor (GDNF), which promotes survival of dopaminergic neurons (92), and regulates the permeability of the blood-brain-barrier (BBB) (93, 94). Interestingly, in this regard the BBB is found to be defective in PD patients (95–97). The mechanism of an altered BBB function is still elusive, however, the increased levels of proinflammatory cytokines IL-6, IL-1β, and TNF-α have been associated with a disruption of trans-endothelial electrical resistance, indicating an increased BBB permeability (98). Recently, a study showed that aSyn-mediated release of proinflammatory cytokines and chemokines by pericytes induces disruption of BBB (99). Further, accumulations of aSyn in astrocytes are found in *post-mortem* analysis of PD patients (100). Reactive astrocytes manifest with PD progression by increased proinflammatory cytokine secretion such as IL-1β, TNF-α, and IFN-γ (101–103). A recent study indicates the close interplay between microglia and astrocytes showing induction of neurotoxic A1 astrocytes by microglial secretion of IL-1α, TNF-α and complement component 1q (C1q) (104). In this regard, it was shown that pathological aSyn inoculation *in vitro* and *in vivo* induces microglia to secrete cytokines and chemokines followed by astrocyte A1 activation that caused neuronal cell death in culture and neurodegeneration in mice (105).

Moreover, is has been reported that aSyn itself has an important role in the initiation and maintenance of inflammation in PD. Recent reports have suggested that aSyn acts as a damage-associated molecular pattern (DAMP), capable of modulating inflammatory cytokine production in microglia and inducing intracellular signaling cascades (106, 107). It has been demonstrated that extracellular oligomeric aSyn is a putative activator of toll-like receptor (TLR) 2 and promotes microglia-mediated inflammatory cytokine and ROS production (108, 109). The exact contribution of different aSyn conformations to TLR activation is currently unclear, however, there are strong indications that the activation seems to be conformation dependent. Specifically, TLR4 appears to be involved in the

uptake of fibrillary aSyn (110). While the presence of monomeric aSyn seems to enhance phagocytic function, aggregated forms seem to inhibit this process (111).

Furthermore, lymphocyte infiltration might also play a role in inflammation processes inside the brain of PD patients. It was reported that cytotoxic T lymphocytes (CD8+) as well as CD4+ T helper (Th) cells were more abundant in the brains of PD patients compared to healthy individuals (112). In this regard, T cells, in particular Th17 cells, were increased in number in PD brain and blood. Furthermore, Th17 cells induced cell death in co-cultures autologous induced pluripotent stem cells (iPSC)-derived neurons from PD patients (113). Interestingly, aSyn is able to activate helper and cytotoxic T cell responses in PD patients, which suggests a possible role of autoimmune inflammation in PD (114).

Taken together, these current data implicate that PD is an extraordinary complex disease with many pathophysiological processes driving disease progression. It becomes evident, that PD is rather a systemic disorder with a variety of pathological facets than 'just' neurological degeneration.

GUT-BRAIN AXIS IN PD

Approximately 80% of PD patients suffer from GI manifestations (115), including constipation, which seems to be an important risk factor for PD (116). As mentioned above, intestinal symptoms may precede motor manifestations by several years, suggesting that PD might originate in the gut. This is in line with the estimation that 90% of idiopathic PD cases are due to oral ingestion of substances causing cell toxicity (oxidative stress, mitochondrial dysfunction), such as herbicides and pesticides (117). According to the hypothesis that PD originates from the gut (118), aggregates of aSyn were detected in the intestine of PD samples (119). Despite the evidence of gut to brain communication in the context of PD, there are still open questions regarding (A) the exact localization in the gut where PD might originate, (B) the dissemination pathways within the gut and to the brain, (C) the declutching event of proteinopathy in the gut, and (D) the role of the intestinal microbiota, as well as microbiota-epithelial-immune communication. All this lacking information is indispensable in order to develop potential PD diagnosis strategies based on GI premotor symptoms.

aSyn in the Gut and Its Propagation to the CNS

As already mentioned, LBs and different aSyn conformers were observed in variety of organs despite the brain. aSyn was reported to be present in the spinal cord and the peripheral nervous system (PNS) including the paravertebral sympathetic ganglia, vagus nerve, the GI tract and among others (120, 121). Indeed, phosphorylated aSyn, a pathological form of aSyn, has been detected in the GI tract up to 20 years before onset of PD motor symptoms (9). Also, Braak and colleagues hypothesized that synucleinopathy begins in the anterior olfactory nucleus and the dorsal motor nucleus of the vagus nerve (DMV) (dual-hit theory) (8, 23), favoring the idea that PD pathology invades the brain via retrograde axonal transport (25, 26). Braak and colleagues even suggest that a pathogen, a pathogen-derived component or other exposures are entering the nervous system through axons of the myenteric (Auerbach's) plexus and/or the submucosal (Meissner's) plexus via postganglionic neurons and may trigger aSyn conformation to aggregates and fibrils (8, 122). Thus, the microbiota has been suggested as a key player, since local immune activation can lead to systemic inflammation affecting the BBB, finally causing neuroinflammation and neurodegeneration (123) (**Figure 2**). Although it is still not clear whether microbiota changes are cause or consequence, dysbiosis is considered as a risk factors for PD development.

Detection of aSyn in the Gastrointestinal Tract

In the context of the ENS, aSyn was first identified in the esophagus and the colon (124), but it is still not clear where the deposition under pathological conditions initiates. Current literature demonstrates that aSyn can also be detected in salivary glands (125), pharyngeal sensory nerves (126), the esophagus (120), the stomach and the small intestine (127), the colon (123), and the appendix (128). Colonic aSyn has been detected even in premotor PD (119, 129). These observations postulate detection of intestinal aSyn as a diagnostic tool in PD, even in early phases of the disease. However, inconsistencies in the detection of aSyn conformers imply the need of alternative and more accurate methods for its detection (granular staining in the lamina propia, perivascular/vascular wall mucosa staining, lacy-granular pattern in the submucosa, or epithelial cell nuclear staining, 2D/3D electrophoresis) (24).

Monomeric aSyn expressed in gut neurons can be released in form of free protein or exosomes, which can be taken up by neighboring neurons via endocytosis (130). Most commonly, aSyn is transported directly from neuron to neuron (131, 132), which requires close cellular contacts and intact synaptic connections (133). In the gut, this is possible via the connection between submucosal/myenteric neurons to the preganglional vagal nerves, which allows aSyn propagation (134). Proteinopathy within the GI innervation might be due to a neurotropic pathogen/agent, which initiates Lewy pathology in the gut (8). Therefore, a connection between the ENS and the mentioned agent is necessary, since neurons/nerves do not reach the intestinal lumen. An attractive candidate in this context would be the intestinal epithelium, which is in direct contact with luminal content, and therefore acts as a physical and immunological barrier in the gut. On the other hand, disturbances of intestinal sealing in chronic intestinal inflammation leading to leaky gut might allow direct contact of the initiating factor and the ENS (**Figure 2**) (135–137).

Propagation of aSyn Between the Gut and the CNS

The next important question is how aSyn propagates from the ENS to the CNS. The connection between the ENS and the CNS, so called gut-brain axis, permits a mutual effect from the ENS to the CNS, and vice versa. This communication mainly occurs via the sympathetic system and the vagus nerve of the autonomic nervous system, and the spinal cord. Four levels of

control have been defined (138): (1) ENS, including myenteric and submucosal ganglia, and enteroglial cells; (2) prevertebral ganglia (visceral reflex responses); (3) spinal tract the through tractus solitaires in the brain stem and the dorsal motor nucleus of the vagus nerve; and (4) cortical and basal ganglia neurons. Healthy individuals maintain intestinal functions, and patients with neurodegenerative disease suffer from GI problems, not only PD, but also Alzheimer's disease, transmissible spongiform encephalopathies, or amyotrophic lateral sclerosis; while GI disorders leads to CNS-related symptoms. The connection between the vagus nerve and the luminal content has been suggested to be mediated via EECs (139), which might produce metabolites acting on the vagus nerve, transmitting information from the nutrients toward the brain, in a glutamatergic neurotransmission (140) (**Figure 2**).

The vagus nerve, one of the largest nerves connecting the gut and brain, is considered to be the direct link between these two organs (141). Recent data from rodent models could evaluate a direct propagation of aSyn pathology from the gut to the brain via the vagal nerve (28, 142–144) (see section Animal Models of PD and GI Symptoms). Moreover, there has also been also research in alternative hypothesis of a brain-to-gut spread of aSyn pathology, showing that a vector-mediated overexpression of aSyn in the midbrain lead to accumulations of aSyn in enteric nerves and stomach walls (145). Further, a more recent study presents that a nigral overexpression of aSyn exerts significant alteration on the ENS followed by changes in the microbiome (146). Subsequently, loss of neuronal plexus and activation of glial cells in the gut impact on intestinal permeability, barrier function, inflammation, and GI motor functions. Taken together, this data suggests a bidirectional potential of aSyn to move both anterogradely and retrogradely within neurons (**Figure 2**). If the vagus nerve is the main route of bidirectional aSyn transmission, vagotomy could be protective against developing PD. Studies questioning whether a vagotomy leads to a reduced risk to develop PD could not find a strong association (147, 148). Only when the cases of a full truncal vagatomy were restricted >20 years after surgery a decreased risk for subsequent PD was observed (149). Overall, many studies support the idea of aSyn gut to brain and inversely brain to gut spread, however, there are still clinical studies missing that investigate the start and/or early development of PD progression, respectively.

Another way of aSyn transmission from the gut to the brain and vice-versa is thought to be possible through extracellular vesicles called exosomes, which are found in the blood serum and CSF of PD patients (**Figure 2**) (150–152). In fact, it was shown that exosomes derived from PD patients incorporate oligomeric aSyn and spread oligomerization of aSyn in a dose-dependent manner (130, 153, 154). An alternative gut-brain communication via the circulation has been also suggested in primates, where the damage of the CNS could be observed upon intestinal injection of aSyn without affecting the vagus nerve, but elevated aSyn levels in the circulation (155). Overall, this indicates that exosomes may function as intracellular cargo distributing aSyn pathology throughout the body (**Figure 2**).

Enteric Nervous System (ENS)

Being the largest and most complex part of the peripheral nervous system (PNS), the ENS controls crucial functions within the gastrointestinal tract, such as peristalsis, substance transport, or local blood supplies. The ENS innervates the whole GI tract, from the mouth to the rectum, including the salivary glands. Neuron networks in the gut wall formed ganglia, which are interconnected by dense fiber bundles. The nerve plexuses are organized in myenteric and submucosal plexuses, which are, in turn, interconnected. The myenteric plexus is localized between the longitudinal and circular muscle layers throughout the GI tract, and controls smooth muscle activity and motility. The submucosal plexus is located mainly in the small and large intestine, also in the stomach, but not in the esophagus (156).

ENS-mediated control of the GI function is independent from the CNS; therefore, the ENS allows complete sensory-motor reflexes, based on the existence of primary afferent neurons, interneurons and motor neurons. However, apart from this intrinsic innervation within the ENS, the gut is also innervated via the sympathetic and parasympathetic nervous system. More than 100 million entities from 20 different neuron subtypes (depending on the expression of neuropeptides) coexist with enteroglial cells (EGCs) in the ENS. EGCs express glial fibrillary acidic protein (GFAP), vimentin and S-100, but also receptors for cytokines, neuropeptides and neurotrophins, and therefore, have a dual function on the ENS. As astrocyte-like cells they also contribute to the function of the intestinal immune system. Moreover, ECGs participate in the structure of the ENS and contribute to the maintenance of mucosal barrier and tissue homeostasis (157). Interestingly, EGCs also serve as a communication tool between IECs and the ENS (158). Among ENS neurons, dopaminergic neurons are present in both plexus (159), and are more frequent in the proximal part of the GI tract; although the association between the loss of dopaminergic neurons and PD has been only demonstrated in the colon (160).

Microbiota

Seeing it as a super-organism, the human body is not only composed of human cells but also numerous microorganisms colonizing at mucosal surfaces, allowing various important body's functions such as maturation, education of host immune responses, protection against pathogen proliferation, and induction of responses to specific drugs. The human gut-microbiome carries millions of microorganisms and indeed, has been defined as the most complex ecosystem ever. It contributes greatly to intestinal immune function as a consequence of the continuous contact with gut lumen commensals and potentially harmful agents. A symbiotic relationship between the human body and these microorganisms permits the digestion of nutrients and pathogen colonization resistance. Thus, the intestinal microbiota modulates several functions of the gastrointestinal tract, such as permeability (161), mucosal immune function, motility (162), sensory nerve function and ENS activity (163). Interestingly, it is also associated with brain functions (164), such as response to stress (165), emotions (138), pain, digestive behavior (166), and brain biochemistry (167).

The balance between the human body and the microbiota (eubiosis) is challenged by several external factors, such as antibiotic treatment, various diseases, highly processed foods or lack of sleep. This can lead to microbiota alterations or dysbiosis, which in most cases is shown by variations in the composition and reduced diversity between different species. Dysbiosis has been associated with several pathological situations, including IBD (168) and PD (169) (**Figure 1**); although in many cases it is not clear whether its alteration represents cause or consequence of the subjacent pathology. The most accepted hypothesis for the pathogenesis of IBD claims that chronic intestinal inflammation occurs as an exacerbated immune response against components of the microbiota in genetically predisposed individuals. The first hint pointing to the association between the intestinal microbiota and IBD came from animal studies showing that experimental inflammation in a number of well-established animal models was abolished in germ-free mice (170). In addition, inflammation could be challenged upon colonization with caecal bacteria, while specific species were able to protect upon recolonization. Despite numerous efforts in order to identify a single specie capable of triggering chronic intestinal inflammation (171), nowadays IBD is considered as a polymicrobial disease, where dysregulation in the composition of the microbiota affects several species. In addition to activation of signals upon detection of the microbiota or derived-antigens, another important aspect is the release of metabolites derived from the microbiota. This has been identified using next-generation sequencing, metagenomics and metabolomics, allowing the description of the microbiome and its potential alterations (172, 173).

Based on the relevance of the intestinal microbiota, its modulation in order to restore eubiosis, appears as an attractive strategy for therapy purposes. In this context, fecal transplantation implies the transfer of microbiota from healthy donors to IBD patients. Fecal microbiota transplantation (FMT) has been tested in various pathological conditions, such as IBD, diabetes type 2 and even neurodegenerative disease. A recent study demonstrates the efficacy of this strategy in an experimental colitis model induced by adoptive transfer of naïve T cells, since transfer of healthy vs. IBD patient fecal content permits restoration of T cell responses (decreased Th17/Th2); and increased Treg/IFNγ and ameliorates thereby colitis (174). Despite limitations based on the donor testing, the limited duration of the treatment and the potential alterations upon antibiotic treatment, FMT it is approved for the treatment of other intestinal conditions, such as *Clostridium difficile* infections (175, 176). In addition to fecal transplantation, a recent review collects other therapy strategies based on the modulation of the microbiota via direct or indirect mechanisms, such as enteral nutrition; pre-, pro-, and post-biotics; inhibition of Adherent-invasive *Escherichia coli* (AIEC) adhesion and tungstate treatment (168). All these strategies to restore eubiosis are potentially valuable in diverse pathologies coursing with dysbiosis.

Microbiota in PD

Compared to GI homeostasis, more surprising is the association between microbiota and brain function, and the fact that the intestinal flora modulates immune, endocrine, and neuroendocrine maturation in nervous system sprouting. Colonization of the human gut upon birth is important for neonatal brain development, since it allows the synthesis of vitamins and fatty acids, regulation of BDNF (Brain-derived neurotrophic factor), synaptophysin and PSD-95 (177). Experimentally, sterile mice elicit decreased expression of BDNF in the cerebral cortex and hippocampus, and they show signs of anxiety and less activity performance (178); while another study shows that recolonization with healthy flora permitted production of different neurotransmitters (NTs) and the abolition of anxiety symptoms (179). An additional important aspect to be considered is the ability of the microbiota to directly produce inhibitory NT (GABA) or regulate their synthesis by the host (180, 181). Moreover, GABA signaling system (GAD and $GABA_AR$) was detected in IECs and $GABA_AR$ stimulation played important role in regulating intestinal fluid secretion in rat (182). On the other hand, preventing the reuptake of NTs (for example, inhibiting 5-HT reuptake by fluoxetine) can regulate colonization in the gut (183). In addition, important to mention here is the production of short-chain fatty acids (SCFA) as microbiota-derived factors, which can affect the CNS thank to their passaging through the BBB via specific transporters. SCFAs in the brain regulated microglia homeostasis (184), have impact on G-protein coupled receptors (GPCRs) (185, 186) and maintain to the GPR41-mediated SNS activity (187). According to an association between brain function/development and colonization of the intestinal tract, the microbiota impacts then on social behavior, sleep cycle, mood disorders, and neurodegenerative disease including Alzheimer's disease and PD (188).

In the context of the gut-brain axis, components of the microbiota and its metabolites can act directly on neurons at the ENS, or signal through IECs (**Figure 2**) (189). Nowadays, several pieces of evidence demonstrate a correlation between dysbiosis and prodromal signs in PD (190–192). Importantly, changes affecting *Firmicutes*, *Prevotella*, *Helicobater pylori* (193), *Bacteroides,* or *Bifidobacterium* (194) as well as the imbalance between pro- and anti-inflammatory species, and the increased release of LPS should be mentioned (195). Based on a recent Metabolome wide association studies (MWAS) (196), the dysbiosis in PD patients is characterized by: increase of opportunistic pathogens (*Porphyromonas*, *Corynebacterium*, *Prevotella*, *Porphyromonas*, and *Corynebacterium*); reduction of SCFA-producing bacteria (*Oscillospira*, *Lachnospiraceae*_UCG-04, *Lachnospiraceae*_ND3007_group, *Agathobacter*, *Butyricicoccus*, *Blautia*, *Faecalibacterium*, *Lachnospira*, *Fusicatenibacter*, *Roseburia*); and elevated carbohydrate-metabolizing probiotics becoming immunogenic (*Lactobacillus* or *Biffidoacerium*). An independent meta-analysis of 223 PD vs. 137 control patients from America and Europe suggests elevation of *Akkermansia*, *Catabacter genera,* and *Akkermansiaceae* family together with reduction of general *Roseburia* and *Faecalibacterium* (197). Beyond alterations of the microbiota composition, related metabolic changes have also been observed in PD patients, such as reduced carbohydrates fermentation, butyrate synthesis,

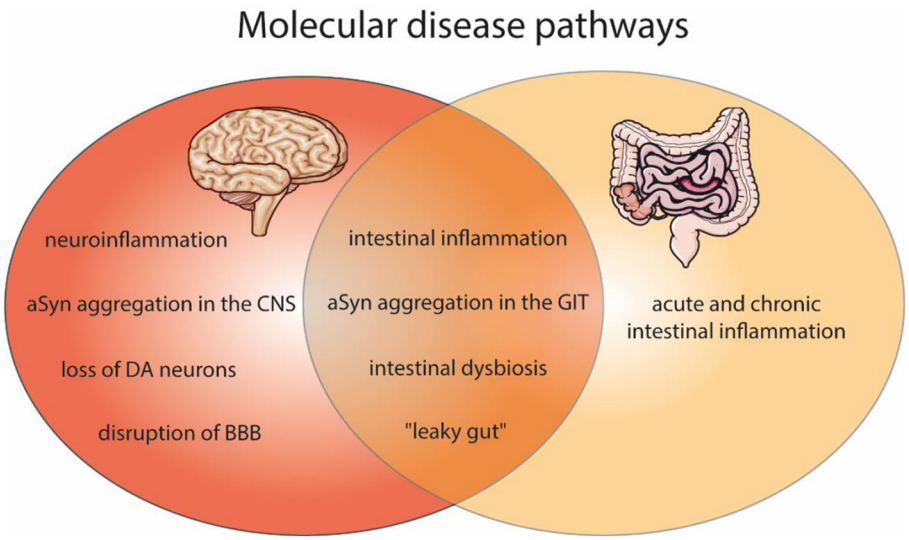

FIGURE 1 | Shared molecular disease pathways of the brain and gut pathologies, as found in PD and IBD. Affected molecular features within PD (red) and gut inflammation (light orange) as well as in both disease (dark orange). Molecular pathways of PD include neuroinflammation, aSyn aggregation in the central nervous system (CNS), dopaminergic neurons (DA) degeneration, and the disruption of blood-brain barrier (BBB). In IBD an acute and chronic intestinal inflammation is described. Both diseases can comprise intestinal inflammation, aSyn aggregation in the gastrointestinal tract (GIT), intestinal dysbiosis and a "leaky gut." The figure contains modified components of Servier Medical Art, licensed under the Creative Commons Attribution 3.0 Unported License (CC BY 3.0) https://smart.servier.com.

increased proteolytic fermentation, and amino acid metabolism (198). Interestingly, some of these metabolites play crucial roles for nervous system-related intestinal functions; for instance, SCFA contribute to 5-HT release and colon motility, proving again the gut-brain connection (199).

Interestingly, many of the PD-related microbiota alterations can also be linked to dysbiosis in IBD. *Akkermansia muciniphila* is a well-known actor in the context of IBD, since it can degrade the mucus layer and thereby impair the barrier function (200), which might favor the contact between the luminal content and the ENS. On the other hand, *Roseburia* and *Faecalibacterium* (197) possess an anti-inflammatory effect in IBD, due to their ability to produce SCFAs (201, 202); while decreased *Prevotellaceae* is associated with alterations of intestinal permeability via a similar mechanism (203). On its part, accumulation of *Enterobacteriaceae* leads to increased levels of LPS, explaining its correlation with disease progression and motor symptoms. Increased LPS levels can contribute to GI alterations by several mechanisms, such as causing epithelial leakage (204), inducing the production of cytokines and inflammation. Moreover, it can pass through the BBB (205), triggering direct destruction of the substantia nigra (206). Based on the neuroprotective effect of SCFA and ghrelin, reduced *Lactobacillaceae* can also affect intestinal inflammation, correlating with disease severity (207). Jointly, overlaps between dysbiosis profiles in IBD and PD might contribute to the associated barrier function alterations.

Changes in the gut microbiota composition might lead to aSyn accumulation in the gut, originating oxidative stress and mucosal inflammation. However, it is not clear whether changes in the microbiota composition, PD associated symptoms (constipation) or PD pharmacological treatment are a consequence of aSyn proteinopathy. Supporting a causative role of microbiota and/or microbiota-derived factors, a recent study shows induction of motor symptoms in mice upon fecal transplantation from human PD patients, due to aSyn pathology and neuroinflammation engendered by microbiota metabolites, such as SCFA. Furthermore, aSyn overexpressing mice (under the Thy1-promoter) show less motor symptoms in germ-free conditions, as well as upon antibiotic-treatment; while colonization with healthy or, in particular, PD patient-derived microbiota, lead to worsening of motor symptoms (192).

Beyond commensal bacteria, also pathogens in the lumen interact with the ENS, mostly via non-neuronal cells, such as EECs within the intestinal epithelium. On the other hand, local gut infections can impact on affective state and emotional responsiveness. This communication occurs via toxins promoting secretion and therefore, diarrhea, as observed in the case of *Vibrio cholera*, *Clostriiodes difficile*; or toxins promoting emesis, including *Staphyloccoccus aureus* or *Bacillus cereus*. However, not only bacteria, also viruses and parasites demonstrate an interplay with the ENS and CNS. The viremic hit hypothesis defends that PD occurs upon Influenza and HSV1 infections (dual-hit theory), leading to the aSyn aggregation in peripheral nervous tissues, and subsequently propagation to the brain (208–210). Interestingly, HIV targets the ENS, since it activates glial cells, which can then be propagated to the CNS. Furthermore, HIV Tat peptide can synergize with LPS by interfering with TLR4, inducing the release of cytokines, and promoting the proinflamamtory effect of LPS (211). ENS infection by HSV-1 leads to macrophage recruitment, releasing ROS and causing ENS neuroplasticity and destruction

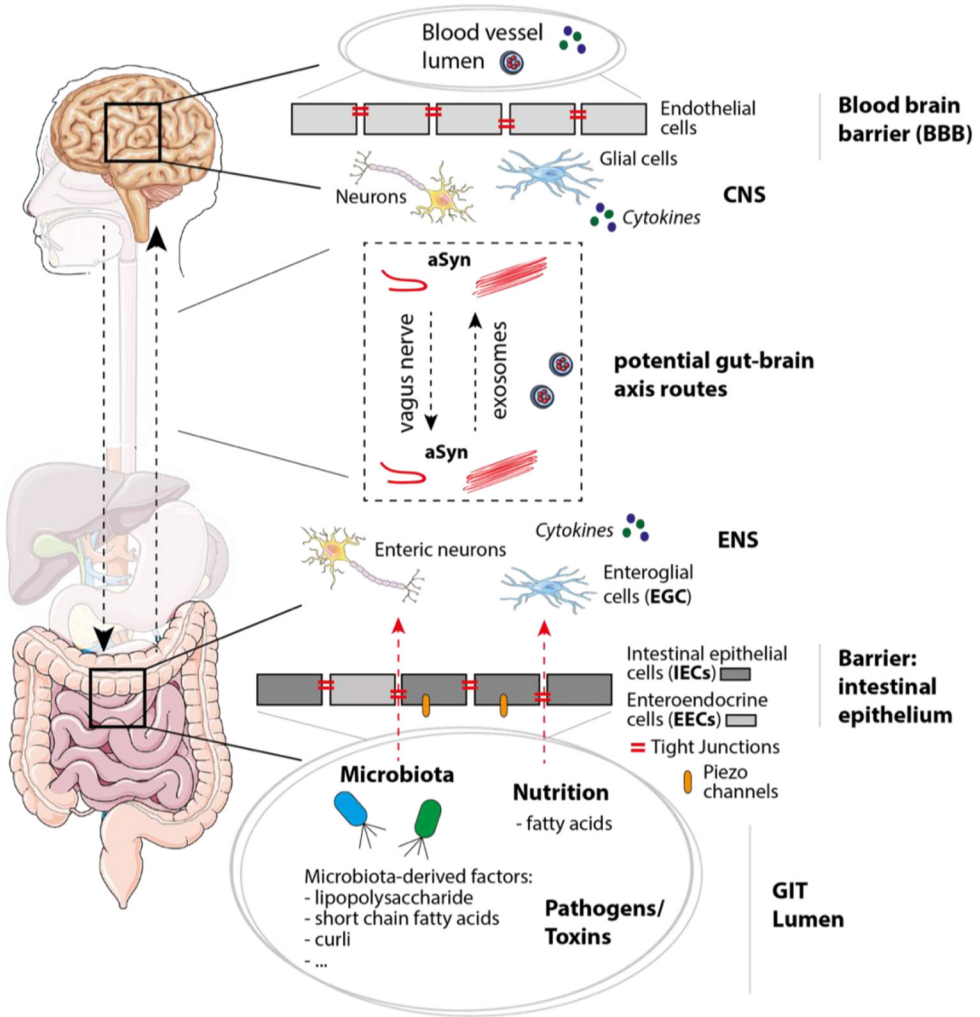

FIGURE 2 | Mechanisms, molecules, and cell types involved in PD/IBD pathology and gut-brain communication. In the gastrointestinal tract (GIT) the intestinal epithelium functions as barrier and separates the GIT lumen from the surrounding enteric nervous system (ENS), which not only contains enteric neurons, but also enteroglial cells. Within the GIT lumen microbiota and microbiota-derived factors like polysaccharides, short chain fatty acids, and curli (bacterial amyloid protein) can be found, but also nutritions, possible pathogens and toxins. When integrity of the intestinal epithelium consisting of epithelial cells including enteroendocrine cells (IECs and EECs) is disturbed, molecules within the GIT lumen get in contact with cells of the ENS. Tight junctions and Piezo channels have been shown to play an important role in mechanosensation, peristalsis, and intestinal barrier function. Within the brain, the blood-brain barrier (BBB) separates the CNS (including neurons and glial cells) from the blood vessel lumen. Potential gut-brain axis routes on which aSyn and molecules like cytokines could be transferred are for instance the vagus nerve or exosomes [via the blood stream or cerebrospinal fluid (CSF)]. The figure contains modified components of Servier Medical Art, licensed under the Creative Commons Attribution 3.0 Unported License (CC BY 3.0) https://smart.servier.com.

of enteric ganglia as well as GI dysmotility (212). Finally, parasites modulate 5-HT secretion, the release of enzymes degrading NTs, such as acetylcholinesterases (*Anisakis* or *Schistosome*), and NT secretion, while they are tightly linked to the immune system function (213).

Intestinal Epithelium and "Leaky Gut"

The intestine is in charge of nutrition and water/ion absorption, but represents also a fundamental immunological organ, harboring the most extended immune cell population in the body. On its part, the intestinal epithelium together with the attached mucus constitute a physical and immunological barrier segregating the environment (intestinal lumen) and the human body. The gut epithelium consists of a monolayer of columnar epithelial cells allowing trans- and para-cellular transport required for nutrition, however, simultaneously impairing the invasion of potentially harmful pathogens. Thus, sealing of the epithelium has to be tightly maintained, in order to prevent transmucosal passage of microbiota-derived factors, which can then get in contact with the plethora of immune cells present in the sub-epithelial space. This is achieved via intercellular junctions (tight junctions, adherens junctions, and desmosomes) (214), as well as a tight regulation of cell architecture and polarity, mostly regulated by the function of the actin-myosin cytoskeleton

(12, 215). Together, the intestinal epithelium accounts for the intestinal barrier function, which has been critically involved in the pathogenesis of intestinal disorders, such as chronic intestinal inflammation (including IBD) (216). This barrier function is challenged during the renewal or turnover of the epithelial layer. $Lgr5^+$ stem cells located at the crypt bottom proliferate and give rise to pluripotent daughter cells located in the transient-amplifying area, which, in turn, differentiate into five IEC subtypes [enterocytes, goblet cells, paneth cells, EECs, and tuft cells (217)]. All differentiated IECs, except paneth cells which remain at the crypt, migrate upwards to the villus tip (small intestine) or the crypt surface (colon), where aged cells will be extruded to the lumen (cell shedding) and finally die. Temporary leakage occurring at the villus tip is tightly regulated by rearrangement of tight junctions and the so-called zipper effect of neighboring cells (218, 219), which allows resealing of the epithelium.

As mentioned above, the maintenance of epithelial integrity plays a fundamental role to keep tissue homeostasis in the gut, and therefore, avoid inflammation (220). Loss of epithelial sealing and leakage of the intestinal layer has been associated with chronic inflammatory disorders, such as IBD (221). Indeed, some observations claim that epithelial-intrinsic alterations can play a causative role in the disease. For instance, increased intestinal permeability appears in non-diagnosed relatives of IBD patients (222), and precedes flares in patients with an IBD diagnosis (223), suggesting that epithelial leakage heads the activation of the inflammatory response. Moreover, based on immune-epithelial communication in the gut, epithelial architecture and function can also be modified due to the effect of pro-inflammatory mediators present in the gut mucosa upon activation of an immune response, such as immune-cell derived cytokines (TNF, IL-6, IL-1β, IL-13, etc.). These cytokines affect mainly tight junction assembly (224), activation of different cell death pathways or cell shedding (225), as well as IEC damage (226). Altogether, via epithelial intrinsic and extrinsic mechanisms, epithelial barrier function is challenged in the context of IBD, and this correlates with pathogenesis of the disease. A proof of this association are recently introduced epithelial restoration therapy strategies, which indeed show promising results in the context of IBD pharmacological management (227, 228).

Beyond being a pure physical fence against components present in the lumen, the intestinal epithelium displays innate immune responses based on the expression of pattern-recognition receptors (PRRs), allowing them to recognize pathogen-associated molecular patterns (PAMPs) from diverse microorganisms in the lumen, amplify the initial immune response, and finally prime the adaptive immune system. PRRs also recognize endogenous molecules produced in stress conditions, so called DAMPs. Membrane-bound [TLRs and C-type lectin receptors (CLRs)] and cytoplasmic Nucleotide-binding oligomerization domain-like receptors or NOD-like receptors (NLRs), retinoic acid-inducible gene-I-like receptor (RLRs), absent-in-melanoma 2 (AIM2)-like receptors, and cyclic GMP-AMP synthase (cGAS) receptors act together in order to detect pathogens in multiple cellular compartments. Although TLRs are the best characterized PRRs, they are not unique in the context of IECs and IBD; others relevant receptors comprise CLRs (229) and NLRs (NOD2) (230, 231).

Focusing on the well-studied TLRs, deficiency of TLR2 is associated with aggravated colitis in DSS-treated mice (232) and multidrug resistance colitis (233). Similarly, poly(I:C)-mediated TLR3 activation protects epithelial barrier function and ameliorate DSS-induced colitis (234, 235). In contrast, several strategies based on TLR4 blocking show promising results in the context of epithelial restoration in IBD (not in the case of necrotizing enterocolitis), while constitutively activated TLR4 predisposes for DSS-colitis and colitis-induced neoplasia (236–238). Mechanistically, this is based on impaired NF-kB-mediated cytokine production and migration of epithelial cells. Although TLR5 was identified as one of the first IBD loci and its deletion triggers spontaneous colitis (239), controversial results regarding flagellin-mediated activation implies the need of future studies in this context (240). Another important candidate is TLR7, since its activation leads to production of antimicrobial peptides (AMPs) and protects against DSS (241) or TNBS colitis. Interestingly, as already mentioned in section Neuroinflammation in PD, recent studies suggest aSyn as a DAMP-activating TLRs on the surface of microglia (108, 109). This opens the hypothesis of a TLR-mediated recognition of aSyn in the gut, even via specific stimulation of intestinal epithelium or IEC subtypes. As mentioned above, it is important to consider potential specificity of TLR activation based on the conformation of the different aSyn aggregates in this context (242).

Intestinal Permeability in PD

PD pathogenesis is associated with "leaky gut" (**Figure 1**) and increased intestinal permeability (243), correlating with aSyn and LPS levels in the mucosa (190). Elevated intestinal permeability in turn promotes subsequent inflammation, and therefore, aSyn accumulation and aggregation in the ENS (192). In fact, increased expression of pro-inflammatory cytokines and glial markers, also in the gut, positively correlated with disease progression and severity. Mechanistically, recent studies have suggested that PD patients (123) and animal models of PD show altered expression and distribution of tight junction proteins, such as ZO-1, E-cadherin (244), and claudin-1 (245).

aSyn and Intestinal Epithelial Cells

Beyond the association between PD and decreased expression of tight junction proteins within the intestinal epithelium (123), the current knowledge about a potential interaction between aSyn and the intestinal epithelium is still scarce. The fact that EECs express aSyn make them attractive candidate players in this context [see chapter Enteroendocrine Cells (EECs)]. aSyn can be transmitted in a prion-like manner from epithelial cells to enteric neurons (30). Enteric glia is a crucial communication tool between the intestinal epithelium and the ENS. Thus, intestinal pathological conditions associated with alterations of epithelial permeability might trigger alterations of the EGCs as the declutching event for a local immune response and neuroinflammation affecting the ENS (see chapter Neuroinflammation in PD). In order to get in contact with IECs, aSyn should translocate across the mucus barrier protecting the

monolayer of epithelial cells. A recent study has shown that, despite mucoadhesive properties, aSyn penetrates the mucus by inducing rearrangement of the mucin matrix (246). Other studies suggest that rather than aSyn itself, other phenomena associated with alterations of the microbiota, such as increased levels of LPS are responsible for epithelial alterations, including redistribution of ZO1 and E-cadherin (244). Enteric biofilms are produced by bacteria in the gut in order to promote their survival, and can in turn, activate local immune response, since some of their components act as DAMPs activating TLRs, for instance. Curli-containing biofilms in several experimental infection models caused alterations of the epithelial layer; the mechanism behind includes the fibrillization of aSyn (247). Undoubtedly, further research on the impact of aSyn on IECs, as well as other mechanisms explaining epithelial alterations in the context of PD pathogenesis are required.

The use of brain organoids derived from PD patient iPSCs has been extended in the last years. Recently, technical development in the field, such as co-culture of neuronal cells with astrocytes (248) and the use of assembloids have permitted modeling of cellular crosstalk between different areas of the brain (249). However, controversial opinions about the ability of these *in vitro* models to mimic complexity of the human brain still exist. In the context of PD, midbrain organoids containing dopamine-related neurons, astrocytes and oligodendrocytes (250) have demonstrated to recapitulate pathological hallmarks upon appropriate conditions (e.g., LRRK2 mutations), such as neurotoxic damage, endosomal phosphorylated aSyn, and increased mitophagy (27). Future advances regarding midbrain organoids may be the inclusion of other cell types, such as microglia, which enables to study the relevance of innate immunity in PD. Therefore, two strategies have been proposed: on one hand, the development of brain organoids including microglia (251); and on the other hand, exogenously add iPSC-derived microglia to brain organoids (252). Moreover, in order to model the BBB and the potential immune cell trafficking, neurovascular communication has been developed and implemented via organ-on-chip technology (endothelial-like cells, astrocytes, and neurons) (253).

The relevance of the gut-brain axis in PD opens the path for exploiting intestinal organoids as *in vitro* models of PD. Described in 2009, intestinal organoids or enteroids are 3D structures developed from intestinal stem cells cultures allowing the intricate differentiation of IECs, and mimicking the complex architecture of the intestinal epithelium (254). Although extremely useful in the context of of epithelial-intrinsic phenomenon, two aspects of intestinal organoids limit their use in studies dealing with microbial-epithelial communication. On one hand, the apical side of the polarized epithelium is projected toward the inside of the organoid (lumen) and makes microbial stimulation highly challenging; and on the other hand, culture conditions with high oxygen concentrations are not optimal for the growth of a vast majority of anaerobic intestinal microbiota. Moreover, some limitations also accounted in the case of co-culture settings with immune cells, for instance, the lack of nutrient support and mechanical constrains to immune cells mediated by blood flow and circulation. Thus, organ on a chip cultures mimicking the inter-organ communication and allowing the interaction with the microbiota as well, appear as suitable alternative. Highly challenging tissue engineering approaches combined with transplantation into mice have tried to implement *in vitro* systems including the ENS to co-cultures of intestinal organoids and smooth muscle cells; however, these strategies have not been successful until now, based on the lack of maturity of neuronal cells (255). More advances have been achieved in the context of immune-epithelial and microbiota-epithelial communication in organoid cultures. Addition of macrophages affected epithelial barrier function and maturity (256); while neutrophils in combination with pathological bacteria cause loss of epithelial integrity (257) and epithelial development and/or maturation is promoted by TNF-producing CD4+ T cells (258).

A step further in the field of PD research will be the combination of gut and brain organoids. Recent advances have focused on "patient-on-chip" models, such as the combination of separately developed multiorgan organoids (259); or the use of gut organ-chip models fluidically coupled to vascular endothelium lined channels (260), such as MINERVA (MIcroboita-Gut-BraiN EngineeRed platform to eVAluate intestinal microflora impact on brain functionality) (261). Experimental setups based on intestinal organoids and multiorgan organoids might provide important knowledge of the communication between the gut and the brain.

Enteroendocrine Cells (EECs)

Considered sensory cells within the secretory lineage of IECs, EECs represent the largest source of hormones in the body and play vital roles in many physiological processes like appetite control, sensing of gut microbiota, GI immunity, motility, barrier function, insulin and growth hormone secretion (262). Upon sensing of nutrients, EECs produce neuropeptides and hormones to the basal space. In the gut epithelium, enterochromaffin cells (ECs)—a subtype of EECs, react to mechanical forces during gut peristalsis by secreting 5-HT, accounting for 95% of body 5-HT (263). For decades, 5-HT is known as an important neurotransmitter signaling molecule, holding a key role in gut motility, secretion and pain sensation. Many studies indeed showed the link between abnormal regulation of 5-HT and GI disorders, such as IBD and irritable bowel syndrome (IBS) as well as in many CNS disorders (264, 265), suggesting a significant role of 5-HT in gut-brain-gut communication. Recently, EECs have been proposed as an alternative source for Notch ligands, supporting the stem cell population in Paneth-deficient mice (266). Therefore, it is predictable that many gut dysfunction diseases, including IBD, are associated with EECs alterations.

EECs possess a tightly organized apical brush border, and basal membrane projections (neuropods) allowing the intercellular communication with nerves and neurons (267). Interestingly, EECs show a certain overlapping expression profile with neuronal cells, such as neurotrophin receptors, pre- and post-synaptic proteins including aSyn, neurofilaments mimicking axons and their functions (neuropods), and dopamine synthesis machinery (268). Indeed, EECs not only synapse with enteric nerves (29) but also establish a direct contact with enteric glia (269). Thus, EECs can serve as a connection between the

intestinal lumen and the ENS, and represents a key population in the context of gut-brain axis in neurodegenerative diseases (267). Besides direct cellular contact, EECs communicate with the ENS via the release of NT and hormones; or even act as the entry pathway for pathogens, which can then act on neurons in the gut. Most importantly, based on their neuron-like features, they can serve as niche for proteinopathy upon luminal signals, which is further supported by the expression of aSyn from these cells (30). Hence, the question arises, whether EECs may be the starting point or declutching event for aSyn pathology in the gut, which is then further transmitted to the CNS.

The fact that EECs express aSyn opens the path for the study of proteinopathy specifically in these cells. An important aspect in this context is the exposure of EECs to the lumen, which make them accessible via endoscopy, as a future early diagnostic tool of premotor PD (270). Interestingly, different TLRs (TLR1, 2, and 4) are expressed in EEC cell lines (271); while TLR4, -5, and -9 ligands induced secretion of EECs hormones in mice (272). On the other hand, *Bacteroides thetaiotaomicron* contributes to neurogenic colon activity via a TLR2- and EEC-dependent mechanism (273). Interestingly, TLR overstimulation has also been suggested in PD pathology (274). Another mechanism by which EECs contribute to the barrier function might be mediated by the expression of SCFA receptors, such as FFAR2 and FFAR3 (275–277).

Interestingly, qualitative and quantitative alterations of EECs have been associated with GI dysfunctions also observed in PD, such as constipation or alterations of transit times. Rotavirus infection courses with EEC-mediated 5-HT secretion, which activates the ENS and the extrinsic vagal afferent to the brain causing nausea, vomiting, and diarrhea (278). In contrast, increased 5-HT secretion protects intestinal barrier function due to the production of neutropic factors (279). Similar EECs-5-HT-dependent mechanisms operate also in diarrhea upon viral infections, such as Adenovirus infection (280) and even COVID-19 patients (281).

Mechanosensations in the Gut

Mechanosensation is vital for proper function of electrically excitable organs, those constantly exposed to and/or generating mechanical forces (heart, bladder, and GI). Physiologically, all cells in the gut epithelial layer are mechanosensitive, they need to sense the static forces (e.g., stretching, crowding) to adjust cell numbers and maintain epithelial integrity. Among them, so-called mechanosensitive cells, develop specific ion channels to sense acute mechanical forces (e.g., pressure from luminal food content); these cells are important to maintain gut functions like food digestion and peristalsis. Beyond peristalsis, mechanical issues are also crucial for maintenance of epithelial architecture. It is well-known that stem cell proliferation is important to maintain tissue homeostasis and avoid pathological conditions. Interestingly, in *Drosophila*, the strict regulation of stem cells is indeed associated with food digestion via gut epithelial stretching. Changes in mechanical properties upon ingestion (gut distension), lead to the decrease of misshapen (a Hippo pathway regulator) membrane association and phosphorylation, which then stimulates stem cell activity and contributes to control intestine adaptive growth (282). During epithelial turnover, aged or damaged cells are shed into the lumen in order to leave space for newly generated cells. This process must be tightly governed to maintain epithelial integrity, and therefore requires intercellular sensing communication between shedding and neighboring cells to finally extrude the dying cell. In general, little is known about biochemical pathways governing sensing and responses to mechanical forces.

Although several membrane ion channels have been revealed as important players in this context, the recently identified Piezo channels show their notable roles in many cellular mechanosensitive processes, from light-touch sensing, controlling red blood cell volume to muscular shear stress (283). In *Drosophila* midgut, the unique Piezo isoform is expressed in low division precursor cells differentiating into EECs. Adult Piezo mutant fly showed decreased number of EECs compared to WT fly. Moreover, Piezo overexpression or increasing Ca^{2+} level in fly intestinal stem cells induced both cell proliferation and EEC differentiation (284). In zebrafish, Piezo1 ion channel is reported to participate in live cell extrusion (285) and cell division (286), in response to crowding and stretching, respectively. Disturbing cell extrusion via Piezo1 channel lead to formation of cell masses, which hypothetically can lead to tumorigenesis. Gudipaty et al. have proposed a model on how Piezo1 acts as a regulator of epithelial cell number by shifting its localization between nuclear envelope and cytoplasm/plasma membrane in order to control cell division and extrusion (286). Altogether, these studies suggested that investigating Piezo-mediated mechanosensations will give us insights into intracellular pathways regulating cell numbers and epithelial integrity, and therefore, be relevant in the context of intestinal inflammation and tumorigenesis.

Peristalsis

Peristalsis, or the impulsion of food based on muscle contraction and relaxation, is regulated by sensation of mechanical forces, but the molecular mechanism behind remains elusive. Generally, peristaltic waves in small intestine consist of weak and infrequent contractions around the bolus, while they continuous and gradually increased toward the anus in the colon. Under specific circumstances, for example diarrhea, an intense and powerful peristaltic wave is triggered in the whole small intestine, which quickly relieves mucosa irritation or unusual gut distension. In the small intestine, peristalsis helps driving food against intestinal wall for nutrient absorption and persistently push it toward the large intestine. In the large intestine, peristalsis is important for feces elimination and mechanical removal of gas and bacteria. At a cellular level, when food particles are formed, EECs are stimulated to secrete 5-HT, while mechanosensory neurons in circular and longitudinal muscles are activated to declutch gut motility (287).

The muscle contraction depends on signals received from ENS or CNS, such as substance P, neuropeptide Y or inhibitory neurotransmitters including nitric oxide (NO) and vasoactive intestinal polypeptide (VIP) (288). How the excitatory and inhibitory motor neurons are activated is still a controversy, however, a population of sensory neurons in the distal colon of

guinea-pig are believed to be stretch-sensitive rather than muscle tone or contraction sensitive (289).

In order to respond to mechanical stimulations, the intestinal tract contains various mechanosensitive cell types carrying membrane mechanically gated ion channels such as ECs within the epithelial layer, smooth muscles, interstitial cells of Cajal or different types of sensory neurons in the lamina propria. They sense and respond to mechanical changes in different ways; for instance, by 5-HT secretion in the case of ECs. Even though the molecular mechanism behind mechanically induced 5-HT-release in ECs is unknown, recent evidence revealed that Piezo2 ion channel is specifically expressed in human and mouse 5-HT positive ECs, and Piezo2 activation by mechanical forces is necessary for 5-HT release and mucosal secretion (290). Another study suggests that Piezo2 is selectively expressed in a large number of NeuroD1+ cells—a subset of EC cell, and mechanical stimulation of NeuroD1+ cells leads to Piezo2-dependent, but not Piezo1-dependent Ca^{2+} increase inducing 5-HT production (291). Paradoxically, a newly published study showed that 5-HT release is crucially regulated upon detection of bacterial derived single-stranded RNA by Piezo1 channel in the gut epithelium, indicative of a new potential pathway for gut and bone disorder therapies. Even though the function of the Piezo family in EECs is not clear, Piezo1 was found to regulate gut peristalsis positively *in vivo* and the lack of Piezo1 in epithelial caused whole gut transit time delay (292). Considering mentioned evidences, Piezo1 and Piezo2 channels in gut epithelium could be possible key elements to uncover the mechanism behind EECs-related mechanisms operating behind constipation and altering transit time in PD (**Figure 2**). This knowledge might even elucidate the phenomena explaining misfolded aSyn-EECs and reveal the initiation of PD origin.

Constipation and PD

The abnormal defecation and reduced peristalsis can lead to constipation. Physically, constipation occurs when there is a decrease of bowel movement frequency, due to primary (idiopathic or functional) or secondary reasons (diet or medication). Approximately 52.48% PD patients experience constipation (293), making it the most common and distressing PD gastrointestinal symptoms (**Figure 1**). Indeed, a study with 551.324 volunteers in Taiwan showed that participants with mild to severe constipation symptoms tended to develop PD within 5.5 years and the constipation severity correlated with the risk of having PD (294).

Targeting the Gut for PD Treatment

Current pharmacological treatment for PD patients is based on the principle of escalating DA brain concentration, by (1) increasing/replacing DA levels; or (2) impairing its degradation. Since DA does not cross the BBB, the most commonly used drug is based on the action of Carbidopa/levodopa, a precursor of DA, which crosses the BBB and is believed to convert to DA in the brain. Other available medicines include DA agonists, monoamine oxidase type B (MAO B) inhibitors, catechol-O-methyltransferase (COMT) inhibitors, anticholinergics, Amantadine or Creatine (295). Pharmacological treatment can be also combined with surgery (deep brain stimulation) (296), gene therapy (297), immunotherapy (e.g., antibodies against aSyn) (298), or cell transplantation (299). However, none of the available therapeutic options is actually curative, nor able to stop disease progression (300, 301). Together, the need of alternative therapy strategies in PD is patent, which opens avenues for the identification of innovative strategies.

Considering gut-brain axis in the context of PD, nowadays it is suggested that PD can be, not only diagnosed based on GI manifestations, but even treated "from the gut." This principle has been also exploited in the context of innovative strategies for levopoda therapy (302). For instance, currently used duopa therapy is based on the application of gels enabling the release of carbidopa/levodopa directly in the gut, allowing slow absorption and, therefore, impairing motion fluctuations and movement disorders. Tightly linked to intestinal function and microbiota, increasing attention has been paid to PD clinical management based on the diet, especially dietary fat. However, conflicting results do not permit drawing conclusive remarks in this context (303, 304); except for the fact that polyunsaturated fatty acid consumption has been associated with lower risk of PD (305). In accordance with the role of the microbiota in the pathogenesis of PD, several strategies modulating the microbiota demonstrated the potential in the context of PD. Antibiotics treatment ameliorate signs of PD, such as IL-1β and, TNF-α at the CNS and dopamine neuron loss (306, 307). Both pre- and pro-biotics have an effect on aSyn proteinopathy. Thus, butyrate activates aSyn autophagy and promotes barrier function of the intestinal epithelium (308). On the other hand, *Bifidobacterium* and *Lactobacillus* are able to reverse PD and PD-related constipation (309); while *Lactobacillus* promotes production of L-DOPA from L-tyrosine (310). The use of probiotics has been found to be beneficial in PD patients (311, 312) and experimental PD models (313, 314). Regarding fecal transplantation, there are controversial results; it is suggested that FMT not only improves GI symptoms (constipation) but also neuroinflammation in PD patients (315, 316); however, safety and efficacy are not clear. Experimentally, FMT lead to further decreased of *Lachnospiraceae* and *Ruminococcaceae*, and worsening of dyskinesia (191); while FMT from PD patients lead to worsening on motor symptoms in a PD model (192), but motor impairment was also observed in normal mice. FMT can impair TLR4 activation, improve gut dysbiosis, reduce activation of microglia, change NT secretion and the destruction of the substantia nigra (315). FMT can also ameliorate comorbidity in PD patients related to the GI tract, including ulcerative colitis (317).

ANIMAL MODELS OF PD AND GI SYMPTOMS

As mentioned above, the interplay between neurological and GI symptoms in PD is also nicely demonstrated in animal models. Thus, here we provide a summary of currently used experimental *in vivo* models of Parkinsonism, and the occurrence

of GI pathological features, as well as aSyn propagation mechanisms supported by experimental observations using corresponding models.

Classical PD models are based on toxin-induced motor manifestations. Intragastric injection of rotenone causes Parkinsonism in mice, without increased systemic rotenone levels (133). The presence of aSyn in the GI tract or ENS depends on factors such as the administration route, dose or length of exposure. Thus, chronic exposure of rotenone involves non-motor GI symptoms (318), however, did not delay gastric emptying (319). Interestingly, rotenone toxicity is associated with changes in the microbiota composition, such as decreased *Bifidobacterium* and increased *Rikenellaceae* or *Allobaculum* (320); while severity of the symptoms is associated with decreased *Lactobacillus* and increased reactivity to LPS (245). In addition, cell toxicity is induced by the prodrug MPTP and the neurotoxin MPP+, causing dopaminergic neurons/tyrosine hydroxylase + (TH) neuron destruction in the brain and in the colonic ENS (321, 322). However, controversial data exists about the outcome of this GI affectation, and it is not clear if this is associated with increased intestinal motility (323) or constipation (322, 324). Strikingly, recent publications support a MPTP-mediated intestinal immune response, which might be provoked by activation of monocytes (325). Furthermore, direct brain injection of 6-OHDA induces PD-like constipation, delayed gastric emptying, and enteric inflammation (326, 327). Last but not least, paraquat injection into rats also demonstrated the relevance of the gut-brain axis, since it evokes reduced gastric motility tone and increased aSyn immunoreactivity in the DMV, which is blocked upon vagotomy (328, 329).

Most experimental genetic models are based on the induced expression of aSyn or mutations on the gene encoding for aSyn. These models recapitulate aSyn aggregation, similar to PD patients, however, require more time for pathological manifestations. In accordance with the gut-brain axis hypothesis, these models confirm that GI dysfunction and non-motor symptoms might represent early pathological features. The most commonly used model is the Thy1-aSyn overexpression model, which presents GI manifestations, delayed colon transit time and defecation accompanied by aSyn accumulation in colonic myenteric plexus (330, 331). CNS pathology in Thy1-aSyn mice is reduced upon microbial depletion, while FMT from PD causes worsening of the phenotype (192). However, a recent study claims that levels of LPS rather than microbiota alterations in Thy1-aSyn mice are responsible for colon intestinal permeability dysfunction and early motor manifestations (244). Other genetic models taking advantage of mutations on the aSyn gene, show aSyn accumulation in the olfactory bulb, myenteric plexus and adrenal neurons (aSyn-A53T) or accumulation of phospho-aSyn, slower transit time, abnormal stool and neuroinflammation at the ENS (PrP-A53T-aSyn) (332, 333). Even unique GI affectations, without motor dysfunction can be observed (BAC-A53T-aSyn) (334).

Mutations in *PINK1* and *PARK2* are associated with PD and activation of immune responses via modulation of mitophagy/autophagy (335). Interestingly, immune response in the context of *PINK1* knockout mice is regulated by the microbiota, since colonization with bacteria leads to T cell mediated destruction of dopaminergic neurons in the periphery and the brain (336). On the other hand, the MitoPark model represents a noticeable example of experimental recapitulation of GI dysfunction and dysbiosis in PD Non-motor symptoms in this model include decreased motility and gradual progression of colon transit times, reduced fecal water content and activation of glial cells in the myenteric plexus. Disease progression in this model goes along with loss of TH+ neurons, reduction of central and intestinal DA levels, as well as changes in the microbiota composition (337).

As mentioned above, another important aspect within the gut-brain axis concept is the propagation route for aSyn. Thus, researchers in the field have concentrated on the development of experimental models based on the injection of aSyn. Therefore, pathological aSyn can be isolated from post-mortem human tissue; or recombinant aSyn preformed fibrils (PFF) are experimentally prepared. It has been demonstrated that the injection of patient-derived pathological aSyn directly into the gut leads to deposition of aSyn in myenteric neurons and intestinal inflammation in A53T transgenic mice (338). Intragastric aSyn can be transmitted to the brain in rats (142). Moreover, the injection of recombinant PFF in the olfactory bulb in WT mice caused the spread of aSyn to distant areas of the brain (339). While spreading of aSyn occurred only in aSyn transgenic mice upon injection into gastric wall and not in WT mice (340). Inoculation of PFFs in the duodenum of mice led to GI deficits and physiological changes of the ENS in addition to changes of aSyn histopathology in the midbrain and subsequent motor defects in elder, but not in young mice (28).

CONCLUSION

As outlined in our review, the disease mechanisms of PD are complex and exhibit a variety of pathological facets. GI manifestations are the most significant symptoms in the prodromal phase of PD (115), suggesting the direct communication of gut and brain. Recent studies have shown that pathogenic aSyn found within the GI system are able to spread and reach the CNS (28, 142, 339). In addition, the role of constipation in PD seems to support the hypothesis that the pathological pathway of PD spreads from the intestine to the brain. Besides, EECs were found to express aSyn and link directly to aSyn-containing nerves, creating neural circuit between the gut and nervous system. This raised an interesting hypothesis that the root of PD might start from misfolded aSyn in EECs, which is transmitted to the nervous system (30). Moreover, constipation is the most troublesome PD-gastrointestinal symptom and likely regulated by abnormal gut peristalsis (293). Accordingly, investigating the roles of EEC-mechanosensitive ion channels, which indeed was proven to be associated to peristalsis, could explain the reason why aSyn in ECs is misfolded, and reveal the mechanism behind PD origin.

Correspondingly, gut inflammation is a main pathological feature occurring in PD and IBD. Inflammatory processes and aSyn pathology appear to be extraordinarily linked to each other. In connection with inflammation, aSyn and its aggregated forms seem to mediate inflammatory responses by TLR activation (108, 109). This indicates the possibility of TLR-mediated release of proinflammatory cytokines in the gut by specific stimulation of IEC. Furthermore, IECs appear as key factor in inflammatory response, as they create a protective barrier against luminal antigens and microbes, helping to preserve gut homeostasis. IEC alterations, for example cytoskeletal rearrangement (12) or cell-to-cell adherens junction reorganization (341) could disturb the epithelial integrity and lead to intestinal permeability as seen in CD patients (342). In addition, PD pathogenesis is also associated with an increased intestinal permeability (243) along with impaired BBB function (97), promoting bidirectional inflammation cascades between the gut and the brain.

Many different routes for transmission between neuronal networks and intestinal cells are described to propagate aSyn pathology. Of interest, extracellular exosomes found in blood and CSF of PD patients have been described to spread pathology (151). Moreover, the vagus nerve is considered to be the most important bidirectional connection between these two organs (141). However, within this context, clinical studies investigating the origin of PD progression are still elusive.

Lastly, it is interesting that dysbiosis is a common feature in PD and IBD (168, 169). In order to affect ENS-specific pathways and spreading to the CNS, a connection between the GI lumen and the neurons/enteroglia is necessary. The intestinal epithelium is in direct contact with luminal content and therefore, acts as a physical and immunological barrier in the gut. Hence, a disturbance of the intestinal sealing allows direct contact of pathological factors and cells of the ENS. Interestingly, IBD (221) as well as PD (243) patients can suffer from intestinal inflammation concomitantly exhibiting a leaky gut. The disturbance of intestinal barrier function has been suggested to promote aSyn aggregation in the ENS, which is further able to spread to the CNS (30, 304), along the so-called gut-brain axis.

In recent years, numerous studies have been addressing the role of the gut-brain axis in neurodegenerative disorders, like PD. However, there are still open questions regarding the understanding about its impact in disease progression and regulation. Further studies and comparisons of disease mechanisms of PD and IBD, as presented in this review, might help to connect missing dots and shed light into the role of aSyn aggregation within the intestine as well as intestinal inflammation in PD. A detailed comprehension of the mechanisms and regulation of the gut-brain axis is essential to establish novel disease biomarkers, clinical read-outs and identify novel targets for (early) treatment strategies.

AUTHOR CONTRIBUTIONS

AD, PN, FZ, and RL-P wrote the manuscript. MN read the manuscript and contributed to the finalized version. All authors approved the final version of the manuscript.

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GLOSSARY

6-OHDA, 6-Hydroxydopamine; AIEC, Adherent invasive Escherichia coli; AMPs, Antimicrobial peptides; aSyn, alpha-Synuclein; BBB, Blood-brain barrier; BDNF, Brain-derived neurotrophic factor; CD, Crohn's disease; cGAS, cyclic GMP-AMP synthase; CLR, C-type lectin receptor; CNS, Central nervous system; COMT, Catechol-O-methyltransferase; CSF, Cerebrospinal fluid; CXCL, Chemokine C-X-X- motif ligand; DA, Dopamine; DAMP, Damage-associated molecular pattern; DLB, Dementia with Lewy Bodies; DMV, Dorsal motor nucleus of the vagus nerve; DSS, Dextran sodium sulfate; EC, Enterochromaffin cells; EEC, Enteroendocrine cells; EGC, Enteroglial cells; EGF, Epidermal growth factor; ENS, Enteric nervous system; FFAR, Free fatty acid receptor; FMT, Fecal Microbiota Transplantation; GABA, γ-Amicobutyric acid; GDNF, Glial cell-line derived neurotrophic factor; GFAP, Glial fibrillary acidic protein; GI, gastrointestinal; GMP-AMP, Guanosin monophosphate-adenosine monophosphate; GPCRS, G-Protein coupled receptors; GWAS, Genome-Wide-Association studies; HSV1, Herpes simplex virus type 1; IBD, Inflammatory bowel disease; IEC, Intestinal epithelial cells; IF, Interferon; IL, Interleukin; iPSC, Induced pluripotent stem cells; LB, Lewy Bodies; L-DOPA, L-3,4-dihydroxy-L-phenylalanine; LN, Lewy neuritis; LRRK2, Leucin-rich repeat kinase 2; LP, Lamina propria; LPS, Lipopolysaccharide; MAO-B, Monoamine oxidase type B; MINERVA, MIcroboita-gut-braiN EngineeRed platform to eVAluate intestinal microflora impact on brain functionality; MPP, 1-Methyl-4-phenylpyridinium; MPTP, 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridin; MSA, Multiple system atrophy; MWAS, Metagenome-wide association studies; NAC, Non-amyloid-β component; NF-kB, Nuclear factor "kappa-light-chain-enhancer" of activated B-cells; NLR, NOD-like receptor; NO, Nitric oxide; NOD, Nucleotide-binding oligomerization domain; NT, Neurotransmitter; PAMPs, Pathogen-associated molecular patterns; PD, Parkinson's disease; PFF, Preformed fibrils; PNS, Peripheral nervous system; PRRs, Pattern-recognition receptors; PSD-95, postsynaptic density protein 95; RLR, Retinoic acid-inducible gene-like receptor; SCFA, Short-chain fatty acids; SNpc, Substantia nigra pars compacta; TGFα, Transforming growth factor α; TH, Tyrosine hydroxylase; Th, T helper cells; TLR, Toll-like receptor; TNBS, Trinitrobenzene sulfate; TNF, Tumor necrosis factor; UC, Ulcerative colitis; VIP, Vasoactive intestinal polypeptide; ZO, Zonula occludens.

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REVIEW
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Angiocrine Regulation of Epithelial Barrier Integrity in Inflammatory Bowel Disease

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Inflammatory bowel disease describes chronic inflammatory disorders. The incidence of the disease is rising. A major step in disease development is the breakdown of the epithelial cell barrier. Numerous blood vessels are directly located underneath this barrier. Diseased tissues are heavily vascularized and blood vessels significantly contribute to disease progression. The gut-vascular barrier (GVB) is an additional barrier controlling the entry of substances into the portal circulation and to the liver after passing the first epithelial barrier. The presence of the GVB rises the question, whether the vascular and endothelial barriers may communicate bi-directionally in the regulation of selective barrier permeability. Communication from epithelial to endothelial cells is well-accepted. In contrast, little is known on the respective backwards communication. Only recently, perfusion-independent angiocrine functions of endothelial cells were recognized in a way that endothelial cells release specific soluble factors that may directly act on the epithelial barrier. This review discusses the putative involvement of angiocrine inter-barrier communication in the pathogenesis of IBD.

Keywords: endothelial, angiocrine, barrier, inflammatory bowel disease, inflammation, angiogenesis, epithelial

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CLINICAL PRESENTATION AND EPIDEMIOLOGY OF INFLAMMATORY BOWEL DISEASE

Inflammatory bowel disease (IBD) includes inflammatory diseases of the colon and small intestine with Crohn's disease and ulcerative colitis being the major clinical presentations (1). Crohn's disease affects the small intestine and large intestine, as well as the mouth, esophagus, stomach and the anus, whereas ulcerative colitis primarily affects the colon and the rectum (2). Crohn's disease and ulcerative colitis are different diseases, but commonly present with any of the following symptoms: abdominal pain, diarrhea, rectal bleeding, severe internal cramps/muscle spasms in the region of the pelvis and weight loss. In addition, anemia is a common extra-intestinal complication of IBD.

IBD is classically considered as a disease of Westernized countries but has started to rise worldwide in the beginning of the twenty first century (3). The rise is population-dependent and categorized into four different epidemiological stages: first, the *Emergence Stage* with sporadic cases of IBD observed in developing countries, second, the *Acceleration in Incidence Stage* with rising incidence and relatively low prevalence in newly industrialized countries, third, the *Compounding Prevalence Stage* with stable incidence and steeply rising prevalence in countries of the Western world, and forth, the *Prevalence Equilibrium Stage*, which represents the opposing forces between

an aging IBD population and the incidence of IBD. In Germany at present 620,085 persons are suffering from IBD with a predicted rise up to 815,200 patients in 2030. In the U.S. presently 2,489,362 patients are registered and a rise up to 3,544,480 is expected within the next 10 years (4).

IBD is characterized by a chronically relapsing intestinal inflammation that is thought to result from an exaggerated immune response to the commensal microbiota. However, the specific molecular mechanisms driving IBD pathogenesis are still unclear. Many different putative susceptibility genes for IBD are reported but all of these are associated with only low risk and differ in different countries of the world. At present, it is commonly accepted, that cytokines, such as, tumor necrosis factor (TNF), interleukin (IL)-10, transforming growth factor (TGF)-β, IL-6, IL-12, IL-13, IL-17, IL-21, IL-23, interferon (IFN)-γ and C-X-C motif chemokine ligand (CXCL)10, are drivers of the excessive immune response, leading to leukocyte infiltration and mucosal damage. In addition, there is agreement that IBD pathogenesis is closely associated with a loss of intestinal epithelial barrier functions associated with bacterial translocation, likely representing an initiating or early event in the disease (5–10).

Recently, it became evident that the intestinal barrier involves two sequential physical barriers. The first being the epithelial barrier consisting of a single cell layer of epithelial cells and a mucus layer which physically separates the microbiota in the gut lumen and epithelial cells (11). Directly below the epithelial barrier an additional barrier was identified, the gut-vascular barrier (GVB) controlling the entry of substances into the portal circulation and their access to the liver after passage of the first epithelial barrier (12, 13). The discriminative control of nutrient uptake and tight sealing towards potentially pathological microorganisms requires a profound regulation of the barrier permeability.

STRUCTURE AND FUNCTION OF THE EPITHELIAL BARRIER IN IBD

The epithelial barrier allows the co-existence of commensal microbiota and mucosal immune cells in the gut. It consists of a physical barrier established by the epithelial cells situated on a basement membrane. Collagen type IV and laminins are the predominant components of the basement membrane (14). The basement membrane is subject of continuous remodeling. Increased remodeling was observed under inflammatory conditions in association with decreased barrier functions (14). At the cellular level barrier functions are established by (i) densely packed microvilli on the apical side of intestinal epithelial cells termed the brush border (15), (ii) tight cell-cell interactions between the epithelial cells, (iii) the cellular resistance to bacterial transcytosis (16), and (iv) specialized epithelial cells, such as mucus-producing goblet cells and anti-microbial peptide secreting Paneth cells (12). Altogether, the epithelium exerts manifold functions, establishing a physical barrier against pathogen invasion and also performing innate immune functions and nutrient uptake (17). Thereby, the preservation of the epithelial integrity is a major aspect in order to preserve homeostasis and to avoid the progress of inflammation in mucosal tissues (18) [for review see: Lopez-Posadas et al. (11)].

At the molecular level the intercellular barrier of the intestinal epithelium is established by apical junction complexes comprised of tight and adherens junctions. Adherens junctions consist of cadherins and nectins and are mainly important for the cell-cell-adhesion (19, 20). Tight junctions are multiprotein-complexes consisting of several transmembrane proteins: tight junction associated MARVEL proteins (TAMP) like occludin, marvelD3 and tricellulin, junctional adhesion molecules (JAM), angulins and the family of claudins, which has in mammalia 27 members that either possess barrier- or channel-forming properties affecting the overall permeability characteristics of the epithelia [for review see Günzel and Fromm (21)]. Adherens junctions as well as tight junctions establish zipper-like structures, sealing the paracellular space within the epithelial cell layer (22). These intercellular junctions are connected to the actin cytoskeleton via cytoplasmic adaptors, such as zonula occludens proteins, and catenins supporting the mechanical strength of the junctions (23–25). Cell activation with molecules that induce permeability causes actin reorganization into stress fibers. This is associated with increasing traction forces, which lead to the detachment of adherens junctions from the cytoskeleton followed by the formation of gaps between adjacent cells (26, 27). Further mechanisms such as the removal of cell-cell interaction molecules from the cell surface by internalization and/or by proteolytic cleavage can regulate the intestinal barrier permeability (11, 28).

The epithelium is constantly renewed without an effect on its tightness. Within this process stem cells at the crypt bottom proliferate and differentiate into the different intestinal epithelial cell subtypes with specialized biological functions (29). Subsequently, most of the differentiated epithelial cells migrate upwards to the villus tip, where aged cells die and are shed into the lumen (30, 31). The tightness of the epithelial layer is maintained by the intercellular junctions during this process (23). During cell shedding, epithelial integrity is maintained in cytoskeleton and membrane trafficking-dependent processes regulating the redistribution of junctional proteins along lateral membranes (32, 33).

Increased epithelial tight junction permeability is a hallmark in the gut of IBD patients (34–38). It is believed that the disruption of intercellular junctions and cytoskeleton rearrangements in the context of infection or inflammation lead to a breakdown of epithelial integrity (39–41). Although a correlation between epithelial barrier permeability and disease activity has been observed in patients with Crohn's disease, the cause of this barrier collapse is still a matter of controversy (42, 43). Experimental animal studies demonstrated that a deficiency of single tight junction proteins is not sufficient to cause pathology due to compensatory mechanisms (44, 45) with the exception of claudin-15 (46). However, agreement exists that inflammation-derived soluble mediators such as IL-6 (47), IL-13 (48, 49), TNF (50), and IFN-γ (51, 52) affect tight junctions and may increase intestinal permeability in experimental colitis models and IBD (53–55). These observations suggested that the epithelial barrier breakdown occurs as a consequence of

proinflammatory cytokine stimulation. In contrast, recent studies in IBD patients demonstrated that an increase of epithelial permeability precedes flares of inflammatory bowel pointing towards a causative role of epithelial barrier breakdown in the development of intestinal inflammation (35, 56–58). The latter is supported by reports that a decrease of epithelial permeability by application of vitamin D (59, 60), probiotics (61–63), IL-22-triggered mucus production (64), butyrate (65, 66), or an anti-TNF antibody caused clinical amelioration of chronic colitis (67, 68). Moreover, alternative portals for gut leakiness such as brush border functions and intestinal bacterial endocytosis by epithelial cells have to be considered and may play important pathogenic roles providing putative targets for therapy of inflammatory bowel disease (15). Altogether, these results suggest that the epithelial barrier function is important and its maintenance can counteract the development of inflammatory bowel disease.

THE IMPACT OF BLOOD VESSELS ON IBD PATHOGENESIS

Capillaries are located in close proximity to the intestinal epithelial cell barrier (**Figure 1A**). Blood vessels in adult tissues evolve through sprouting from preexisting vessels, a process termed angiogenesis (69). Angiogenic activity correlates with disease severity in IBD suggesting that blood vessels may contribute to pathogenesis (70–73). Moreover, elevated levels of angiogenic growth factors including vascular endothelial growth factor (VEGF)-A and basic fibroblast growth factor (bFGF), that synergize in angiogenesis activation, have been detected in the inflamed mucosa and in the blood during active IBD (74, 75). However, experimental colitis models provided conflicting results on the contribution of angiogenesis to disease activity. Neutralization of VEGF-A resulted in a decreased vessel density and improvement of the disease in dextran sulfate sodium (DSS)–induced and 2,4,6-trinitrobenzenesulfonic acid (TNBS)–induced colitis (73, 76). In contrast, reduced angiogenic activity induced by deficiency of placental growth factor failed to ameliorate colitis in the same experimental models (77). These results indicated that besides vessel density additional parameters such as vessel quality are of relevance in IBD pathogenesis. In fact, newly formed vessels in IBD tissues are strongly disorganized and leaky as evident by associated edema (78).

The difficulties in determining the precise role of blood vessel function in IBD may be due to the fact that the intestinal endothelial cells are both, targets and regulators of inflammation (78). In this framework, IBD-associated inflammatory cytokines such as TNF-α, IL-1β and IFN-γ can activate endothelial cells by inducing the expression of adhesion molecules for leukocytes such as E-selectin, intercellular adhesion molecule (ICAM)-1 or vascular cell adhesion molecule (VCAM)-1 (79). Macrophages are important drivers of IBD and are characteristically expressing high amounts of TNF-α and IL-1β, which may amplify the extravasation of these cells being responsible for the high numbers of macrophages present in IBD tissues (80). In addition, inflammation is associated with increased angiogenesis supporting immune cell recruitment by increase of blood flow and endothelial surface (81). As mentioned above the intestinal endothelium also establishes an additional barrier in the gut, the GVB (12, 13). The GVB constitutes a semipermeable barrier between the blood stream and the interstitium regulating the transport of nutrients, tissue fluid homeostasis and the transmigration of immune cells but is non-permissive to bacterial penetration (13, 28, 78, 82). The latter is in agreement with

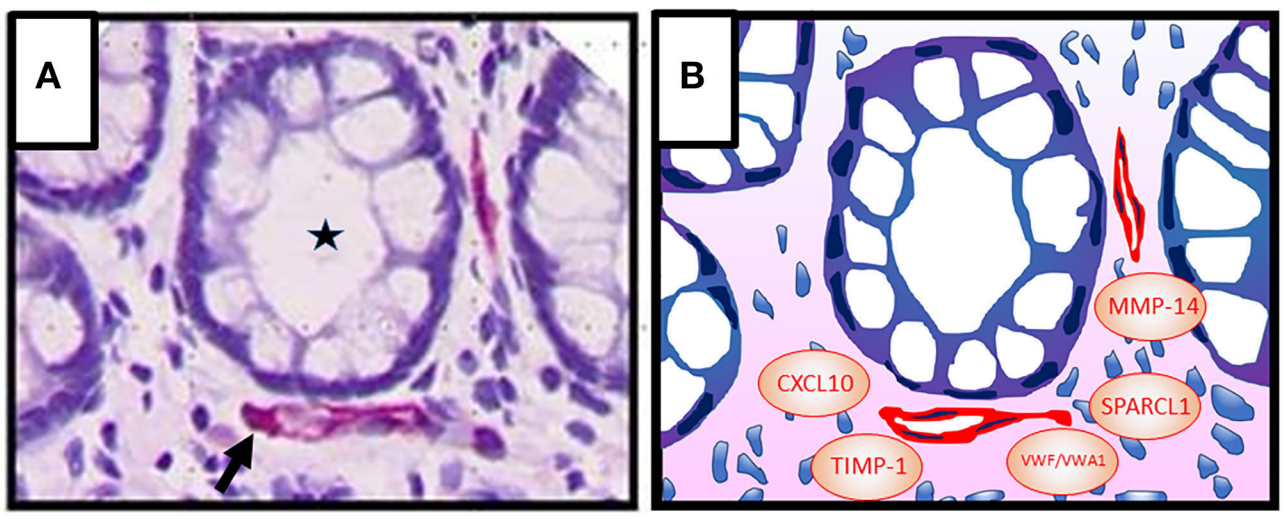

FIGURE 1 | (A) Colonic crypt (intestinal gland, asterisk) with vessels (red, arrow) in the lamina propria. Epithelial cells (1st barrier) and endothelial cells (2nd barrier) are directly adjacent, indicating active inter-barrier communication. Vascular endothelial cells were stained immunohistochemically using an anti-CD31 antibody. Cell nuclei (blue) were stained by haematoxylin. **(B)** Graphic presentation of **(A)** indicating possible factors that may be involved in angiocrine regulation of epithelial barrier functions in IBD [von Willebrand factor A domain containing 1 (VWA1), von Willebrand factor (VWF), matrix metalloproteinase (MMP)-14, tissue inhibitor of metalloproteinases (TIMP)-1, C-X-C motif chemokine ligand (CXCL) 10, secreted protein, acidic and rich in cysteine–like 1 (SPARCL1)].

the observation that bacterial lipopolysaccharides (LPS) in low concentrations are stabilizing the vascular barrier (83). In contrast, high concentrations of LPS (>10 μg/ml) inhibit endothelial cell migration, down-regulate intercellular junction molecules and increase the permeability of the vascular barrier (83). Paracellular (i.e., in between the cells) or transcellular (i.e., across the cells) routes are available to cross the endothelial cell monolayer. Transcellular exchange is accomplished via either solute transporters, or transcytosis via vesicular carriers (e.g., caveolae), or pore-like subcellular structures (i.e., fenestrae and transendothelial channels) (84, 85). The paracellular route is controlled by adherens junctions and tight junction proteins similar as in the epithelial barrier. In intestinal endothelial cells, tight junctions are composed mainly of occludin, junctional adhesion molecule (JAM)-A, zonula occludens (ZO)-1, and cingulin (13). Claudin-3, -5, and -12 from the claudin family are known to be mainly expressed in endothelia (86, 87).

Adherens junctions are formed by vascular endothelial (VE)-cadherin and β-catenin (13). Of note, the same cytokines regulating immune cell extravasation can also deregulate adherens and tight junction formation in endothelial cells supporting translocation of bacteria thereby further amplifying the inflammatory process [for review see: Lopez-Posadas et al. (11)].

The impact of the GVB in intestinal inflammation is substantiated by mouse models of acute and chronic DSS-colitis. In these models intestinal vessel perfusion remained constant during colitis whereas vessel permeability strongly increased (5). Using experimental animal models with an endothelial cell specific knockout of the interferon-γ-receptor 2 (IFNγR2) it was shown that the IBD-associated cytokine IFN-γ induces a breakdown of the vascular barrier based on the disruption of the adherens junction protein VE-cadherin and this was significantly increasing DSS-induced experimental colitis. Importantly, the disease-associated vascular barrier dysfunction could be confirmed in human IBD patients indicating the clinical relevance of the findings. Imatinib (brand name Gleevec) is a kinase inhibitor acting against Abelson tyrosine kinase BCR–ABL, the KIT and PDGF receptors and is used for therapy of chronic myeloid leukemia (CML), gastrointestinal stromal tumors (GIST) and several other malignancies (88). Interestingly, treatment with imatinib restored adherens junctions, inhibited vascular permeability, and significantly reduced colonic inflammation in experimental colitis. Altogether, these results highlighted the pathogenic impact of inflammation–associated vascular barrier defects in IBD and opens new avenues for vascular-directed treatment of the disease (81).

The detection of an additional intestinal barrier rises the question whether the epithelial and the vascular barriers may communicate in prevention or progression of the disease. Epithelial to endothelial cell communication is commonly accepted. For example, the nutrient composition of the chyme (partially digested food) and not simply gut distension modulates blood flow. Specialized subsets of intestinal epithelial cells transport nutrients through the epithelial monolayer into the lamina propria from where they are transported through the fenestrated blood endothelium to be distributed systemically (89, 90). Moreover, in response to pathogen invasion or loss of barrier integrity, both intestinal epithelial cells and tissue-resident leukocytes secrete cytokines, chemokines, reactive oxygen species, and lipid mediators that activate endothelial cells to modulate the number and structure of vessels and to promote immune cell extravasation. For example, intestinal epithelial cells in IBD were shown to secret the chemokines CXCL8/IL-8 and CCL20 (91, 92), both of which can activate angiogenesis (93, 94). In addition, these cells secrete the cytokine TNF-α (91), which regulates vessel remodeling and by directly acting on endothelial cells may inhibit angiogenesis (95, 96). In addition, vascular permeability is increased by inflammatory mediators released from epithelial cells fostering both, inter- and trans-cellular diapedesis (90, 97).

ANGIOCRINE FUNCTIONS OF BLOOD VESSELS IN ORGAN DEVELOPMENT AND DISEASES

The endothelium is not a passive response organ for nutrient supply, tissue entry of immune cells, and metabolite removal, but actively regulates the tissue microenvironment in organ development and diseases as indicated by novel results. These perfusion-independent functions of endothelial cells were recognized in experimental tumor models in mice for the first time, where the inhibition of angiogenesis in certain instances did not abrogate tumor growth but instead enhanced tumor invasiveness (98). Based on this the hypothesis arose that endothelial cells release specific soluble factors that may directly regulate tumor growth in a perfusion-independent manner. This respective mechanism was termed as "angiocrine" regulation of tumorigenesis (98).

Subsequent studies confirmed that endothelial cells may activate tumorigenesis by secreted factors (98, 99). For example, angiocrine factors were reported to stimulate growth and migration of lymphoma tumor cells (100), to maintain stem cell like properties in colorectal carcinoma and glioblastoma cells (101–103), to inhibit anoikis in head and neck cancer stem cells (104) and, to activate proliferation, survival and epithelial to mesenchymal transition of lung carcinoma cells (103) [for review see: Lee et al. (105)].

Vice versa, it was noted that endothelial cells can also suppress cancer growth through angiocrine signaling. In this framework contact-dependent interactions between the endothelial cell surface receptor duffy antigen/receptor for chemokines and the carcinoma cell surface receptor kang ai-1 were shown to suppress metastasis (106). In addition, in breast cancer endothelial cell-released slit homolog 2 protein (Slit 2), perlecan and additional as yet unknown factors were reported to inhibit proliferation, invasion and pro-tumorigenic signaling of the cancer cells (107, 108). In addition, thrombospondin is regarded as a putative anti-angiogenic factor secreted from endothelial cells (98). Angiocrine factors also exert key functions in physiologic condition such as kidney development (109, 110), liver bud (111) and pancreatic bud formation (112), in neuronal development (113),

lung regeneration (114), osteogenesis (115) and hematopoiesis (113).

Of note, a specific impact of angiocrine signaling on epithelial barrier functions was observed in retina development (116). Endothelial cells secrete factors that remodel the retinal pigment epithelium (RPE) basement membrane and integrin receptors sense these changes by triggering GTPase signals that modulate RPE tight junctions and enhance RPE barrier function (116). Similar parenchymal cell barrier regulatory mechanisms may be active in other organs.

Altogether, angiocrine factors are involved in tumorigenic, homeostatic, regenerative and morphogenetic processes in a paracrine or juxtacrine manner. The term "angiocrine" factors meanwhile includes secreted and membrane-bound inhibitory or stimulatory growth factors, trophogens, chemokines, cytokines, extracellular matrix components, exosomes and other cellular products (117). The angiocrine profile of endothelial cells can differ between tissues, reflecting the diversity of cell types found adjacent to endothelial cells in organs (113, 117).

THE IMPACT OF ANGIOCRINE SIGNALING ON EPITHELIAL BARRIER FUNCTION IN IBD

Angiocrine functions in IBD have not been investigated extensively as yet, despite the manifold effects of angiocrine signaling on epithelial cell functions in cancer, organ development and tissue regeneration. However, first results indicating angiocrine activities in the colon have emerged. For example, endothelial cells release jagged 1, generated by proteolytic activity of ADAM metallopeptidase domain 17 (ADAM17) activating Notch in human colorectal cancer cells and thereby promoting a cancer stem cell phenotype and chemo-resistance (103, 118). Moreover, it was shown that selectively endothelial cells isolated from colorectal carcinomas with a prognostically favorable Th-1-like immune environment released the matricellular protein *secreted protein, acidic and rich in cysteine–like 1 (SPARCL1)*, which autocrinely and paracrinely inhibited angiogenesis and proliferation of different cancer cell lines (119, 120). The latter indicated that angiocrine activities in the colon may trigger the course of diseases in a microenvironment–dependent manner. A recent single cell RNAseq approach of intestinal cells and subsequent bioinformatics interaction analyses supported the molecular interaction between endothelial cells and epithelial cells in the colon (121).

Specific support for angiocrine functions in IBD was obtained from a recent report on an increased susceptibility for acute and chronic DSS-induced colonic inflammation in mice lacking the angiocrinely active SPARCL1 protein (122). SPARCL1 is almost exclusively expressed in vascular cells in the colon (119, 123, 124). In SPARCL1 (Sc1) KO animals colonic inflammation and colon vessel permeability were significantly increased and colon length was shorter as compared to wildtype animals. Exaggerated inflammation in Sc1 KO animals was further supported by an increased detection of fibrosis and the presence of tertiary lymphoid structures similar to the human chronic disease. Altogether, these results indicated that intestinal angiocrine functions may establish a chemical barrier affecting both, epithelial and endothelial cell barrier functions in IBD (122).

In a next step, we applied a meta-analysis to further investigate whether angiocrine signaling may impact barrier functions. To this goal, an *in silico* secretome screening against the human proteome was performed using the VerSeDa database [Vertebrate Secretome Database (125)]. Transcripts with a prediction cut-off value > 0.8 (SignalP 4.1, TargetP 1.1, SecretomeP) were considered as secreted proteins. The resulting 1,050 genes (1,959 proteins; 1,959 gene transcripts) were used for a functional gene and phenotype annotation using the Ensembl BioMart database (http://www.ensembl.org/index.html). Next, candidates were selected based on data mining (inflammatory, angiocrine, epithel, extracellular, endothelial, barrier, cytokine, bowel, secreted). Subsequently, the resulting 257 genes were mapped to profiles from human endothelial cells of different origin, including human umbilical vein endothelial cells (HUVEC) exposed to shear stress (126), under LPS-stimulation (127), overexpressing γ-interferon-inducible protein (IFI) 16 (128) and unstimulated (129), as well as endothelial cells from brain, lung, heart (130) and colorectal carcinoma (119, 131). This analysis identified in total 28 genes (**Table 1**). Six of these may be of specific interest as candidates of angiocrine barrier effects in IBD (**Figure 1B**). This includes components of the von Willebrand factor domain superfamily (VWA1, VWF) and tissue inhibitor of metalloproteinases (TIMP)-1, which were retrieved from three different studies, respectively. vWF is a classical endothelial cell marker protein, that promotes adhesion of platelets to the sites of vascular injury by forming a molecular bridge between sub-endothelial collagen matrix and the platelet-surface receptor complex (132). Its impact on the epithelial barrier warrants further investigation. TIMP-1 is an inhibitor of the matrix metalloproteinases (MMPs). It is able to promote cell proliferation in a wide range of cell types, has an anti-apoptotic function and can modulate the vascular barrier (133, 134). TIMP-1 may impact the epithelial cell barrier activity in the gut through these activities. In this framework, it is interesting that MMP-14 was also identified by our meta-analyses as angiocrine mediator. MMP-14 was reported as an angiocrine factor in lung regeneration and as a member of the membrane-type matrix metalloproteinases that are not inhibited by TIMP-1 (114, 135). In addition, CXCL10, regarded as a major driver in IBD pathogenesis (6), was also identified as angiocrine mediator in our meta analyses. In the DSS-model blockade of CXCL10 enhanced crypt cell survival (136) and mice with a knock out of the CXCL10 receptor CXCR3 showed considerably lower crypt damage (137). Based on these findings it was suggested that CXCL10 may exert direct effects on epithelial cells in the gut (138).

The bioinformatical analysis showed that the overlap of genes retrieved from the different studies was low. This is well in agreement with the high variation of activation and organ-dependent plasticity of endothelial cells. In this framework, the six genes identified in endothelial cells from colorectal carcinoma may exhibit the highest relevance for IBD

TABLE 1 | Angiocrine barrier-modulating candidate genes in inflammatory bowel disease.

Gene	Full name (according to GeneBank)	Alias (GeneBank)	GeneID (GeneBank)	Burghoff et al. (126)	Tunica et al. (129)	Kwon et al. (127)	Jambusaria et al. (130)	Baggetta et al. (128)	Naschberger et al. (119)
CLU	Clusterin	AAG4, APO-J, APOJ, CLI, CLU1, CLU2, KUB1, NA1/NA2, SGP-2, SGP2, SP-40, TRPM-2, TRPM2	1191	x					
CST3	Cystatin C	ARMD11, HEL-S-2	1471	x					
FBN2	Fibrillin 2	CCA, DA9, EOMD	2201	x					
GDF15	Growth differentiation factor 15	GDF-15, MIC-1, MIC1, NAG-1, PDF, PLAB, PTGFB	9518	x					
MGP	Matrix Gla protein	GIG36, MGLAP, NTI	4256	x					x
EDN1	Endothelin 1	ARCND3, ET1, HDLCQ7, PPET1, QME	1906	x					
IGF2	Insulin like growth factor 2	C11orf43, GRDF, IGF-II, PP9974, SRS3	3481	x			x		
TIMP1	TIMP metallopeptidase inhibitor 1	CLGI, EPA, EPO, HCI, TIMP, TIMP-1	7076	x	x		x		
LOXL2	Lysyl oxidase like 2	LOR, LOR2, WS9-14	4017	x	x				
CST1	Cystatin SN	-	1469			x			
A2M	Alpha-2-macroglobulin	A2MD, CPAMD5, FWP007, S863-7	2			x			
MMP14	Matrix metallopeptidase 14	MMP-14, MMP-X1, MT-MMP, MT-MMP 1, MT1-MMP, MT1MMP, MTMMP1, WNCHRS	4323		x				
FBLN1	Fibulin 1	FBLN, FIBL1	2192		x				
VWF	Von Willebrand factor	F8VWF, VWD	7450		x		x		
PDIA3	Protein disulfide isomerase family A member 3	ER60, ERp57, ERp60, ERp61, GRP57, GRP58, HEL-S-269, HEL-S-93n, HsT17083, P58, PI-PLC	2923		x				
WFDC2	WAP four-disulfide core domain 2	EDDM4, HE4, WAP5, dJ461P17.6	10406				x		
BSG	Basigin (Ok blood group)	5F7, CD147, EMMPRIN, EMPRIN, HAb18G, OK, SLC7A11, TCSF	682				x		
CXCL10	C-X-C motif chemokine ligand 10	C7, IFI10, INP10, IP-10, SCYB10, crg-2, gIP-10, mob-1	3627				x		
PTGDS	Prostaglandin D2 synthase	L-PGDS, LPGDS, PDS, PGD2, PGDS, PGDS2	5730				x		
SAA2	Serum amyloid A2	SAA, SAA1	6289				x		
SAA1	Serum amyloid A1	PIG4, SAA, SAA2, TP53I4	6288				x		
ICAM1	Intercellular adhesion molecule 1	BB2, CD54, P3.58	3383				x	x	
SPARCL1	SPARC like 1	MAST 9, MAST9, PIG33, SC1, hevin	8404				x		x
VWA1	Von Willebrand factor A domain containing 1	WARP	64856				x		x
FGFR1	Fibroblast growth factor receptor 1	BFGFR, CD331, CEK, ECCL, FGFBR, FGFR-1, FLG, FLT-2, FLT2, HBGFR, HH2, HRTFDS, KAL2, N-SAM, OGD, bFGF-R-1	2260						x
PTGS1	Prostaglandin-endoperoxide synthase 1	COX1, COX3, PCOX1, PES-1, PGG/HS, PGHS-1, PGHS1, PHS1, PTGHS	5742						x
CTSH	Cathepsin H	ACC-4, ACC-5, ACC4, ACC5, CPSB	1512						x
TNFRSF1B	TNF receptor superfamily member 1B	CD120b, TBPII, TNF-R-II, TNF-R75, TNFBR, TNFR1B, TNFR2, TNFR80, p75, p75TNFR	7133						x

(see **Table 1**). Interestingly, SPARCL1, which has been shown to affect susceptibility to experimental colitis in mice was part of this group (122). In summary, this analysis identified several interesting candidates, which may participate in the angiocrine inter-barrier communication in IBD. These factors may provide putative new targets for treatment of the disease. The specific impact of most of these factors on the epithelial barrier functions has to be determined in future studies.

CONCLUSION

First evidence exists that the gut-vascular barrier (GVB) communicates via angiocrine signals with the epithelial barrier during IBD. The molecules involved in this communication may provide new targets for clinical monitoring and treatment of the disease. In-depth elucidation of the underlying effects and the specific mechanisms warrants further studies.

AUTHOR CONTRIBUTIONS

MS, SK, and EN: analyzed the literature and wrote the manuscript. MK performed the bioinformatical analysis. All authors approved the final version of the manuscript.

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Effects of Vitamin D-Deficient Diet on Intestinal Epithelial Integrity and Zonulin Expression in a C57BL/6 Mouse Model

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Background and Aims: Vitamin D (VD) plays an important role not only in mineral balance and skeletal maintenance but also in immune modulation. VD status was found correlated with the pathophysiology and severity of inflammatory bowel diseases and other autoimmune disorders. Epithelial barrier function is primarily regulated by the tight-junction (TJ) proteins. In this study, we try to establish an animal model by raising mice fed VD-deficient diet and to investigate the effects of VD-deficient diet on gut integrity and zonulin expression.

Methods: Male C57BL/6 mice were administered either VD-deficient [VDD group, $25(OH)_2D_3$ 0 IU/per mouse] or VD-sufficient [VDS group, $25(OH)_2D_3$ 37.8 IU/per mouse] special diets for 7 weeks. Body weight and diet intake were recorded weekly. Serum VD levels were detected. After sacrifice, jejunum and colon specimens were collected. The villus length and crypt depth of the jejunum as well as mucosa thickness of the colon were measured. Various serum pro-inflammatory cytokines and intestinal TJ proteins were assessed. The serum level of zonulin and the mRNA expression of jejunum zonulin were also investigated.

Results: We found that mice fed a VDD diet had a lower serum level of VD after 7 weeks ($p < 0.001$). VDD mice gained significant less weight ($p = 0.022$) and took a similar amount of diet ($p = 0.398$) when compared to mice raised on a VDS diet. Significantly decreased colon mucosa thickness was found in VDD mice compared with the VDS group ($p = 0.022$). A marked increase in serum pro-inflammatory cytokine levels was demonstrated in VDD mice. All relative levels of claudin (CLD)-1 ($p = 0.007$), CLD-3 ($p < 0.001$), CLD-7 ($p < 0.001$), and zonulin-1 (ZO-1, $p = 0.038$) protein expressions were significantly decreased in the VDD group when compared to the VDS group. A significant upregulation of mRNA expression of jejunum zonulin ($p = 0.043$) and elevated serum zonulin ($p = 0.001$) were found in the VDD group.

Conclusions: We successfully demonstrated that VDD could lead to impaired barrier properties. We assume that sufficient VD could maintain intestinal epithelial integrity and prevent mucosal barrier dysfunction. VD supplementation may serve as part of a therapeutic strategy for human autoimmune and infectious diseases with intestinal barrier dysfunction (leaky gut) in the future. To our knowledge, this is the first study to demonstrate that VDD could lead to a significant upregulation in mRNA expression of the jejunum zonulin level and also a marked elevation of serum zonulin in a mouse model.

Keywords: vitamin D, gut integrity, tight junction, zonulin, leaky gut

INTRODUCTION

In addition to its principal function as a calcium regulator in facilitating the absorption and metabolism of calcium and bone health, vitamin D (VD) can affect cell and tissue morphology. VD also has immune regulatory functions and contributes to the homeostasis in the body (1). VD protects the gut barrier by regulating tight-junction (TJ) proteins and inhibiting intestinal apoptosis. VD deficiency (VDD) is associated with a number of diseases, such as allergic diseases, inflammatory bowel disease (IBD), and autoimmune disorders (2–4). In addition, serum VD levels are inversely correlated with the degree of non-alcoholic steatohepatitis and fibrosis in children with non-alcoholic fatty liver disease (5, 6). We have also demonstrated that VD levels are inversely associated with the severity of fibrosis of the native liver in patients after Kasai's portoenterostomy for biliary atresia (7).

The gastrointestinal tract is the largest immunological organ in the body and has a central role in immune homeostasis (8, 9). Epithelial barrier function is primarily regulated by the TJ proteins. VD is involved in the regulation of the epithelial barrier functions (10, 11). VD is an important mediator of intestinal epithelial defenses against infectious agents, and VDD predisposes to severe intestinal injury (12, 13). Mice with simple VDD are susceptible to colitis because of impaired colonic antimicrobial activity and homeostasis of enteric bacteria (14). VDD is related to the high incidence of colorectal cancer, and VD supplementation may inhibit the development of colorectal cancer (15). VD may reverse colorectal cancer through regulating intestinal flora, especially *Akkermansia muciniphila*, and maintaining colon barrier integrity (15).

Mice fed a high-fat and VDD diet have an increased amount of pathogens (*Helicobacter hepaticus*) in the gnawer ileum, but the amount of symbiotic bacteria (*Akkermansia muciniphila*) markedly decreased (16). Epithelial barrier function is primarily regulated by the TJ proteins. We can foresee the translocation of pathogens to intestine once dysfunction of TJ occurs. These functions require a highly organized TJ morphology which may be modified by VD supplementation. Besides, zonulin is the eukaryotic counterpart of the *Vibrio cholerae* zonula occludens toxin (17). Human zonulin is identical to prehaptoglobin-2 and binds to the epidermal growth factor receptor and protease-activated receptor 2 in the intestinal epithelium. This complex initiates the phosphorylation of zonula occludens proteins and leads to the small intestine's TJ disassembling (18). According to previous research studies, zonulin is the only measurable blood protein that reflects the intestinal permeability, and increased zonulin level is considered to be a marker of impaired intestinal barrier (19, 20).

Investigators in most studies look at the effect of VD on the recovery of intestinal injury in infected mouse models (21–23). They seldom investigate the roles of VD on the integrity of gut morphology and function of TJ proteins. We hypothesized that VD was involved in the regulation of the epithelial barrier functions and VDD might predispose mice to intestinal injury, since zonulin is the only measurable blood protein that reflects the intestinal permeability and increased zonulin level should be observed in mice fed with a VDD diet. In this study, we try to establish an animal model by raising mice fed a VDD diet in order to elucidate the roles of VD in gut morphology and barrier functions. We also investigate the enterocyte microstructures, inflammatory cytokines, and TJ protein expressions in this mouse model. The serum level of zonulin and the mRNA expression of jejunum zonulin were also investigated.

MATERIALS AND METHODS

Animals' Experiment and Ethics Statement

Male C57BL/6 mice (3–4 weeks of age) were used in all experiments and were approved by the Institutional Animal Care and Use Committee (IACUC) of MacKay Memorial Hospital (IACUC number: MMH-A-S-107-026). IACUC has been accredited, approved, and authorized by the government office, Agriculture and Food Agency Council of Agriculture, Executive Yuan, Taiwan. All methods were performed in accordance with the relevant guidelines and regulations in this animal study.

To produce standard VD-3 [$25(OH)_2D_3$] concentrations (1,500 IU/kg diet) in the circulation, mice were fed a ssniff R/M-H diet (E15312-24; VD deficient, normal vitamin D&P, ssniff Spezialdiäten GmbH, Soest, Germany) containing either 37.8 IU supplement/per week based on the consumption of 25.2 g diet/per mouse weekly at the age of 6 weeks (adult mouse) or saline solution by oral gavages once a week (24).

We used cholecalciferol VD-3 [$25(OH)_2D_3$] as the VD diet source in this mouse model study. Body weight and diet intake were recorded weekly. After 7 weeks, the serum 25-hydroxyvitamin D3 [$25(OH)_2D_3$] concentrations were measured

in the mice using the ELISA kit. Blood samples were obtained *via* cardiac puncture and were centrifuged to yield serum.

Cytokine Analysis

The serum (50 μl) was analyzed by the Bio-Plex Pro™ Mouse Cytokine Multiplex Panel kit (Bio-Rad Laboratories Inc., Hercules, CA, USA). Targets of cytokines included IL-1β, IL-6, IL-10, IL-12, IFN-γ, MCP-1, and TNF-α. Extracted serum utilized the Bio-Plex 200 system (Luminex Co., Austin, TX, USA). The tissues lysates were extracted from the jejunum.

Immunofluorescent Localization of TJ Proteins

The location of TJ proteins was studied using immunohistochemistry. Samples of jejunum were embedded in paraffin, cut into 3-μm sections, mounted on slides glasses, and deparaffinized by standard protocols. For antigen retrieval, the tissues were treated with Tris/EDTA solution buffer (10 mM Tris, pH 9; 1 mM EDTA). Incubation with primary rabbit anti-ZO-1 antibody, anti-claudin (CLD)-1, anti-CLD-3 (Sigma, Merck, Germany), anti-CLD-7 (Abcam, Cambridge, UK), and mouse anti-occludin (OCDN) (Life Technologies, Carlsbad, CA, USA) was conducted at 4°C overnight followed by PBS washing. DyLight 488-conjugated anti-rabbit antibody and DyLight 549-conjugated anti-mouse antibody (Jackson, Bar Harbor, ME, USA) were used as secondary antibodies. Then, three more washes were performed. The fluorescence of the TJ proteins (ZO-1 and CLD-1) was examined using a confocal microscope (MRC 600; Olympus, Tokyo, Japan) with a krypton argon laser. The fluorescence of the TJ proteins (CLD-3, CLD-7, and OCDN) was examined using a fluorescence microscope (AX10, Zeiss, Jena, Germany). The images collected had an optical thickness of 3 microns for the jejunum. The images shown represented a projection of the sections made for each villus.

Histological Analysis

Histological analysis was performed according to the standard protocol that was published in the literatures (25, 26). Briefly, the tissues of jejunum and colon were processed and fixed in 10% buffered neutral formalin. The tissues of jejunum and colon were processed and fixed in 10% buffered neutral formalin. Then, the tissues were further processed and embedded into paraffin. The samples were cut into 3-mm-thick sections. All sections were deparaffinized and stained with hematoxylin and eosin (H&E) according to standard procedures. The sections were photographed by using a TissueFAXS automatic scanning system, captured by a digital camera, and analyzed by HistoQuest software (TissueGnostics, Vienna, Austria). Four mice in each group were sacrificed for parameter determination. Measurements of villus height and crypt depth of the small intestine were determined for whole well-orientated villi and crypts per small intestinal tissue section per mouse, and the values were averaged. We also assessed the colonic specimens for histological changes and mucosa thickness measurements. The villus height, crypt depth, villus height/crypt depth ratio of each jejunal tissue section and the muscular layer and mucosa thickness of each colon tissue were measured to determine if gut integrity was whole and well-oriented.

Western Blot of TJ Proteins

Jejunum tissues were lysed in an ice-cold lysis buffer including Tris–HCl, NaCl, $MgCl_2$, glycerol, NP-40, SDS, aprotinin, leupeptin, PMSF, and pepstatin A and placed on ice for 10 min as described in our previous study (27). The supernatant of lysed samples was collected after centrifugation. The total protein concentration was quantified by BCA protein assay kit (Thermo, USA). The protein was added to an equal volume of 2× Laemmli sample buffer and boiled for 10 min and then run at 8% polyacrylamide gel at 100 V for 1.5 h. The treated protein was transferred to Immunoblot PVDF membranes (Bio-Rad). After overnight blocking (PBS/Tween supplemented with 0.05% non-fat dry milk), blots were incubated with rabbit polyclonal antibodies to CLD-1, CLD-3, CLD-7, OCDN, ZO-1, and GAPDH in 0.05% Tween 20/TBS for 4 h. GAPDH was used as a loading control. The secondary antibodies were horseradish peroxidase conjugated anti-mouse IgG. Immunoreactive bands were visualized by chemiluminescence reagents and exposed to an X-OMAT film. Band densities were determined using XnView software. A ratio of variety TJ proteins to GAPDH in the control band was calculated for each sample.

Measurement of Serum Zonulin Level and Intestinal Zonulin mRNA Expression

Serum zonulin as a marker of leaky gut was measured by the zonulin competitive ELISA kit (MyBioSource, San Diego, CA, USA). The assay sensitivity was 0.1 μg/ml according to the manufacturer's instructions. Quantitative real time PCR (qRT-PCR) was used to determine the expression of zonulin at the level of mRNA. Pairs of oligonucleotide primers specific to zonulin used in our study included the following (28):

forward primer, 5′-TCATCACGGCGCGCCAGG-3′
reverse primer, 5′-GGAGGTCTAGAATCTGCCCGAT-3′.

As mentioned in our previous study, total RNA was isolated from jejunum specimens using the TRIzol reagent (Invitrogen) and was then used for cDNA synthesis with random hexamers. DNA detection and amplification were also detected by qPCR using an ABI 7500 Fast System v1.4.0 (Applied Biosystems). Gene expression was normalized to the GAPDH expression levels (29).

Statistical Analysis

The quantitative data were expressed as mean ± standard error (SE) for triplicate measurements. Statistical analyses were performed with an independent *t*-test using SPSS 12.0. Statistical significance was defined as a $p < 0.05$.

RESULTS

Effects of VDD on Weight Gain and Diet Intake

After completion of the experiment, all mice tolerated well and no animal exhibited signs of marked adverse effects such as bloody stool passage or cachexia. No mortality was noted. The mice were weighed daily, and the results of the two groups were compared.

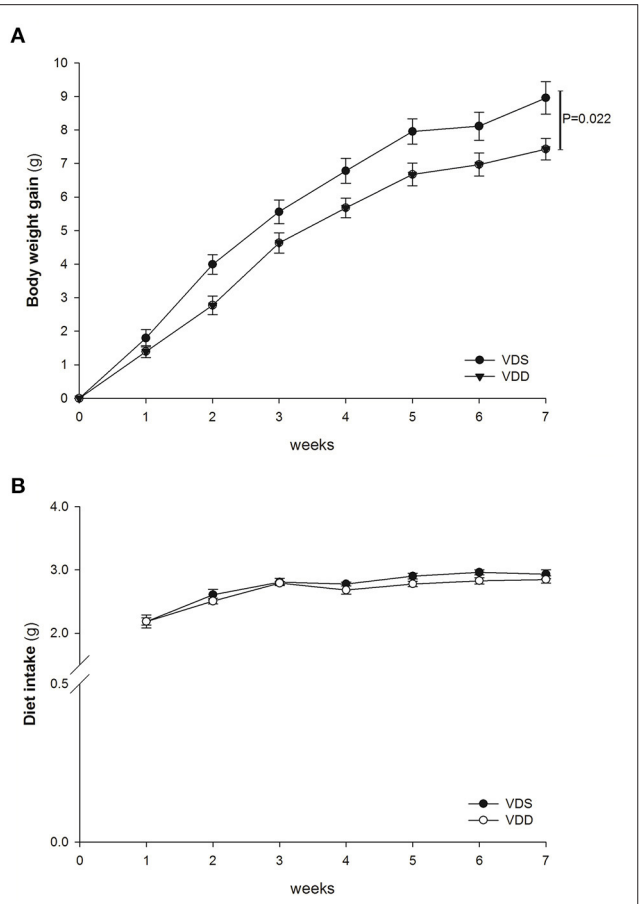

FIGURE 1 | Weight gain and diet intake. The average weight gain and diet intake in both VDD and VDS groups were shown. **(A)** Average body weight gain. VDD mice gained less weight, and a significant difference was found between the two groups ($p = 0.022$ at the 7th week). **(B)** Average amount of diet intake. VDD mice had a similar amount of diet intake compared to the VDS mice during the experiment ($p = 0.398$). The statistical analysis was performed by the independent *t*-test. Five mice in each group for parameter determination.

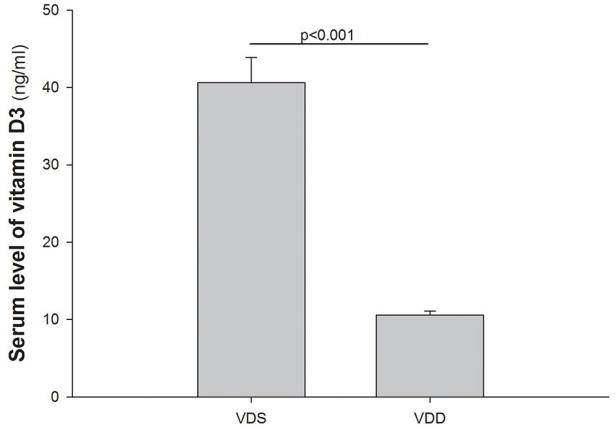

FIGURE 2 | Serum levels of $1,25(OH)_2D_3$. Serum levels of $25(OH)_2D_3$ in the VDD and VDS groups are shown. The serum level of $25(OH)_2D_3$ was 10.45 ± 0.61 ng/ml after 7 weeks in the VDD group while the serum level of $25(OH)_2D_3$ was 40.62 ± 3.24 ng/ml during the study period in the VDS group ($p < 0.001$). The statistical analysis was performed by the independent *t*-test. Five mice in each group for parameter determination.

In the beginning of the study, we used male C57BL/6 mice (3–4 weeks of age) in all experiments, so the initial average diet intake amount (2.19 g) was lower than the standard adult level; however, it increased gradually to 2.9 g at the end of the experiment. VDD mice gained significantly less weight (VDD 147.14 ± 2.96% vs. VDS 161.24 ± 4.91%, $p = 0.022$) (**Figure 1A**) when compared to mice raised on a VDS diet after 7 weeks. The average amounts of diet intake of both groups are shown in **Figure 1B**. The difference on feed intake did not affect the study result since VDD mice took a similar amount of diet during the experiments when compared to mice raised on VDS diet after 7 weeks.

VDD Affects the Serum Vitamin D_3 Level

The serum levels of VD [$25(OH)_2D_3$] in the VDD and VDS groups are shown in **Figure 2**. In the VDD group, the serum level of $25(OH)_2D_3$ was 10.45 ± 0.61 ng/ml after 7 weeks. On the contrary, the serum level of $25(OH)_2D_3$ in the VDS group was significantly higher (40.62 ± 3.24 ng/ml) than the VDD group during the studied period ($p < 0.001$).

VDD Causes Intestine Histology and Damage

The effect of the VDD diet on the intestine histology is shown in **Figure 3**. The HE stain shows a typical irregular edge and lining of the jejunal mucosa, distorted enterocytes, and significant inflammatory cell infiltration in VDD mouse. However, the intestinal villi of the mouse in the VDS group were more uniform and inflammatory cell infiltration was absent (**Figure 3A**). We found no significant differences in the villus height level, crypt depth level, and villus height/crypt depth ratio of the jejunum between the two groups (**Figure 3B**). Besides, we found that the muscular layer thickness of both groups was similar. VDD mice had slightly higher jejunal mucosa thickness compared with VDS mice but did not reach significant difference (data not shown). Similar histological damages were found in the colonic histology in the VDD group, including decreased mucosa thickness, crypt hyperplasia, loss of epithelial integrity, and inflammatory cell infiltration (**Figure 4A**). Both groups had similar thickness of the muscular layer (**Figure 4B**). However, VDD mice had significantly decreased colonic mucosa thickness compared with mice raised on VD-sufficient diet (VDD 165.09 ± 3.69 μm vs. VDS 187.03 ± 5.80 μm, $p = 0.022$) (**Figure 4C**).

VDD Affects Intestine TJ Protein Structure

The effects of VDD on the jejunal TJ proteins' (ZO-1, CLD-1, CLD-3, CLD-7, and OCDN) structure and distribution are shown in **Figures 5**, **6**. Immunofluorescence studies can provide general information on specific proteins (in this case ZO-1, CLD-1, CLD-3, CLD-7, and OCDN) concerning their overall pool (thickness of the signal) and distribution. A sharp signal means accumulation in specific cellular sites like the cell boundaries,

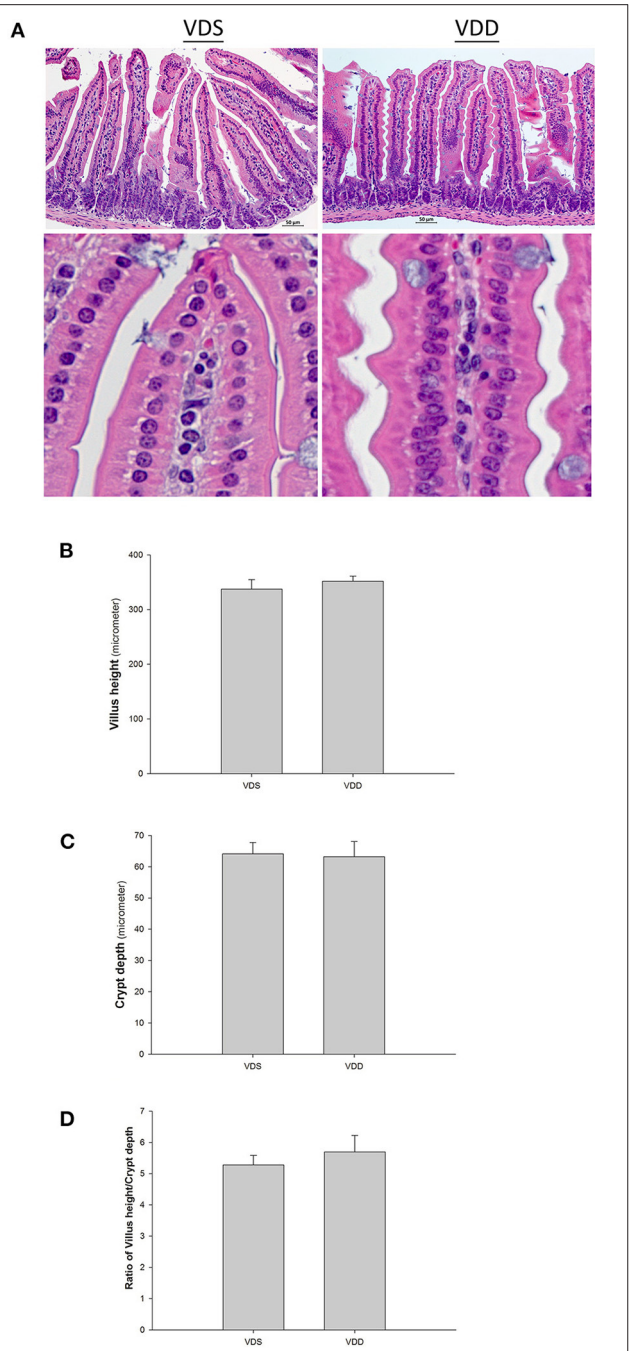

FIGURE 3 | Effect of vitamin D deficiency on the intestinal histology. **(A)** Representative histological images (HE stains) of the jejunum in both groups. VDD mice showed worsened histologic damage with typical irregular edge lining of the jejunal mucosa, distorted enterocytes, and significant inflammatory cell infiltration. Intestinal villi of the mice in the VDS group were more uniform, and inflammatory cell infiltration was absent. Lower photo: magnification 750%. Segments of the jejunum were taken for measurement of the villus height **(B)**, crypt depth **(C)**, and villus height/crypt depth ratio **(D)** per mouse. The levels in villus height, crypt depth, and villus height/crypt depth ratio of the jejunum were compared, and no significant changes were demonstrated between the two groups. Values were represented as mean ± SEM and were analyzed using the independent *t*-test. Four mice in each group sacrificed for parameter determination. More than 15 villi and crypts in each staining of the slice in both groups.

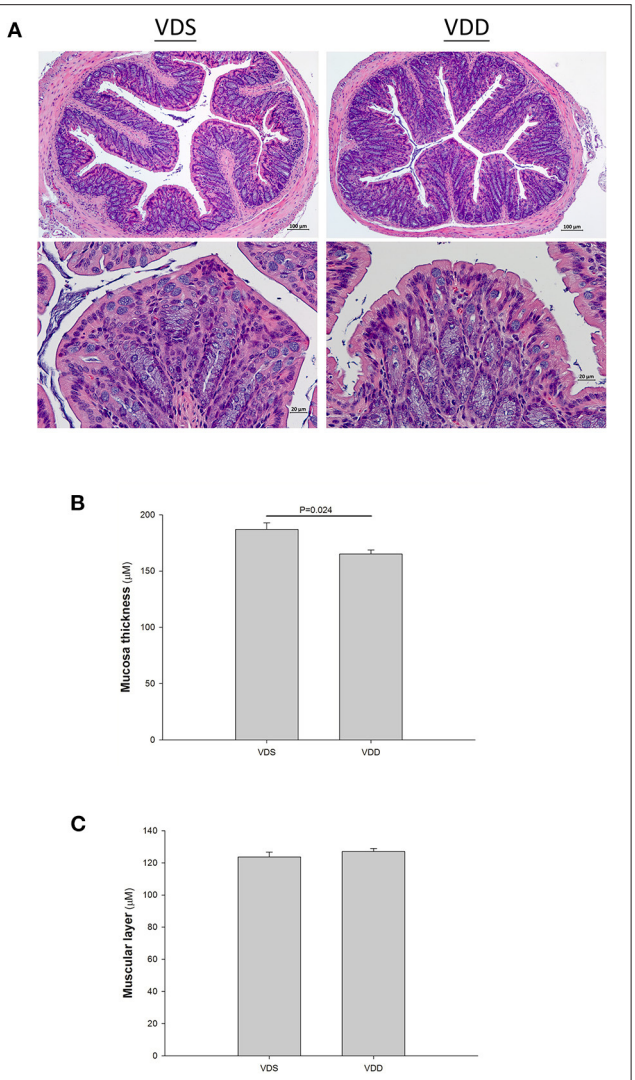

FIGURE 4 | Effect of vitamin D deficiency on the colonic histology. **(A)** Representative histology of colonic specimens with H&E stains in mice. Marked histological damages were found in the colonic specimens in the VDD group. Lower photo: magnification 750%. Segments of colon were taken for measurement of the muscular layer **(B)** and mucosa thickness **(C)**. Both groups had similar thickness of muscular layer ($p = 0.395$). However, VDD mice had significantly decreased colonic mucosa thickness compared to mice raised on VDS diet ($p = 0.024$). Values were represented as mean ± SEM and were analyzed using the independent *t*-test. Four mice in each group sacrificed for parameter determination. More than 15 fields for each staining of the slice in both groups.

suggesting that the structure of TJ is maintained, while a blurry signal suggests a more diffuse distribution within the cell cytoplasm and implies a dysfunction of TJ. In our study, the fluorescence lines for ZO-1, CLD-3, and OCDN proteins staining were clear and sharp in the VDS group but blurry, cloudy, and irregular in the VDD group (**Figures 5A**, **6A**). Cell nuclei were found located at the baseline of enterocytes in the VDS group, but irregular arrangements of cell nuclei were observed in the

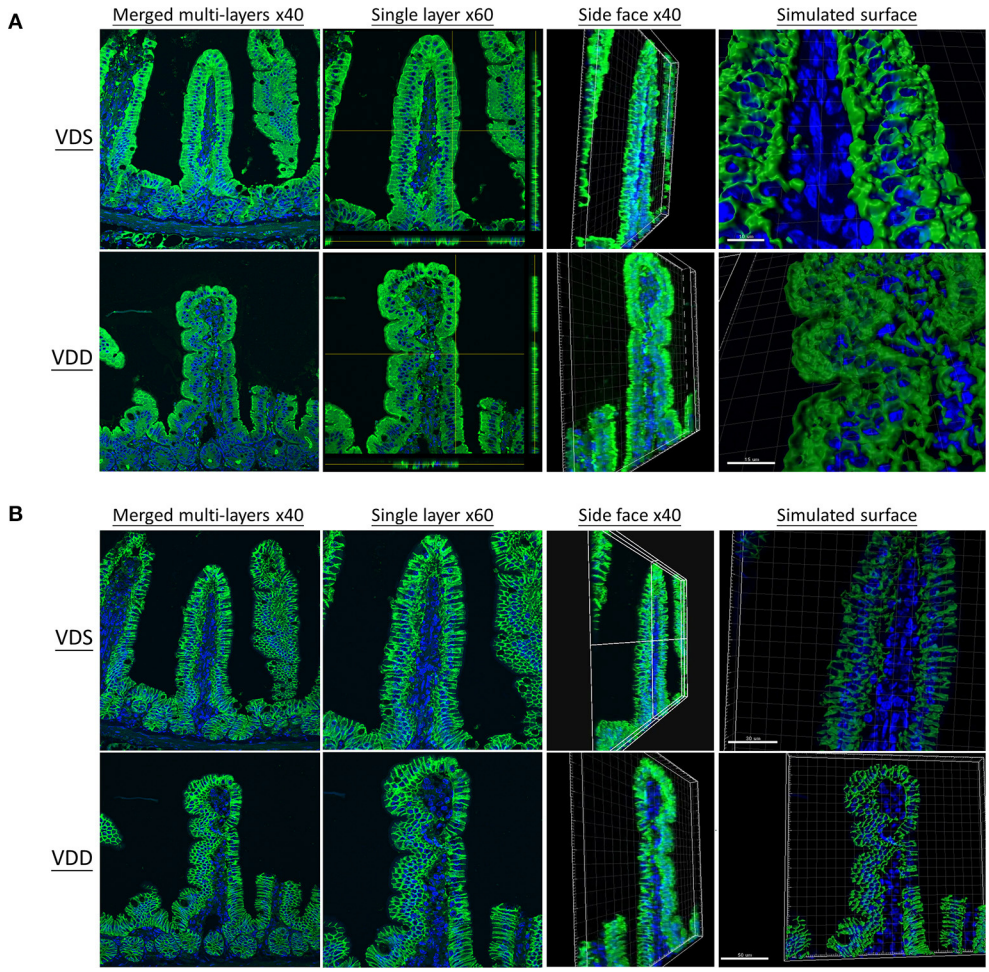

FIGURE 5 | Vitamin D maintains intestinal tight junction expressions in VDD mice by both en face view MPM and reflectance confocal microscopy (RCM) of the jejunum. DAPI was used for nucleus labeling (blue). Front, side face, and simulated surface were displayed. The distribution of tight junctions was visualized by the zonula occludens-1 (ZO-1) fusion protein (DyLight 488, green) **(A)** and claudin-1 (CLD-1) (DyLight 488, green) **(B)**. The fluorescence line was clear and sharp in the VDS group but blurry, cloudy, and irregular in the VDD group at ZO-1 and CLD-1 distributions. Cell nuclei (blue dot) were found located at the baseline of enterocytes in the VDS group but irregular arrangement in the VDD group.

VDD group. Similar findings were observed in CLD-1 and CLD-7 staining (**Figures 5B**, **6B**). The surface of villi was straight and smooth in the VDS group but winding and irregular in the VDD group. OCDN was demonstrated rich in the apical villi in the VDS group as shown in the merged figure (**Figure 6**). However, we noticed that the colonic specimen texture in the VDD group was less elastic when compared with the VDS group while mice were sacrificed in this study. We compared the morphology of the colon to the jejunum and noticed that there were no obvious typical irregular edge and lining of the colon mucosa as we demonstrated in jejunal tissue.

VDD Affects Intestine TJ Protein Expression

The effects of VDD on the jejunal TJ protein expression are shown in **Figure 6**. All relative levels of CLD-1 (VDD 0.10 ± 0.01 vs. VDS 1.00 ± 0.23, $p = 0.007$), CLD-3 (VDD 0.18 ± 0.05 vs. VDS 1.00 ± 0.05, $p < 0.001$), CLD-7 (VDD 0.04 ± 0.02 vs. VDS 1.00 ± 0.02, $p < 0.001$), and ZO-1 (VDD 0.55 ± 0.07 vs. VDS 1.00 ± 0.17, $p = 0.038$) protein expressions were significantly decreased in the VDD group when compared to the VDS group. However, we found no significant difference in the relative level of OCDN between the two groups (**Figures 7A,B**).

VDD Upregulates Serum Inflammatory Cytokine Expressions

The effects of VDD on the serum inflammatory cytokine expressions are shown in **Figure 8**. Upregulations of IL-1β (VDD 20.55 ± 1.96 pg/ml vs. VDS 6.31 ± 2.17 pg/ml, $p < 0.001$), IL-6 (VDD 5.93 ± 1.00 pg/ml vs. VDS 1.75 ± 0.36 pg/ml, $p = 0.005$), IL-10 (VDD 26.84 ± 4.42 pg/ml vs. VDS 25.36 ± 4.10 pg/ml, $p = 0.037$), IL-12 (VDD 281.43 ± 40.94 pg/ml vs. VDS 143.23 ± 44.98 pg/ml, $p = 0.038$), and TNF-α (VDD 82.53 ± 8.05 pg/ml vs. VDS 64.91 ± 12.58 pg/ml, $p = 0.007$) were found in VDD mice. All these serum inflammatory cytokines were significantly higher in the VDD group when compared to the VDS group. However, we

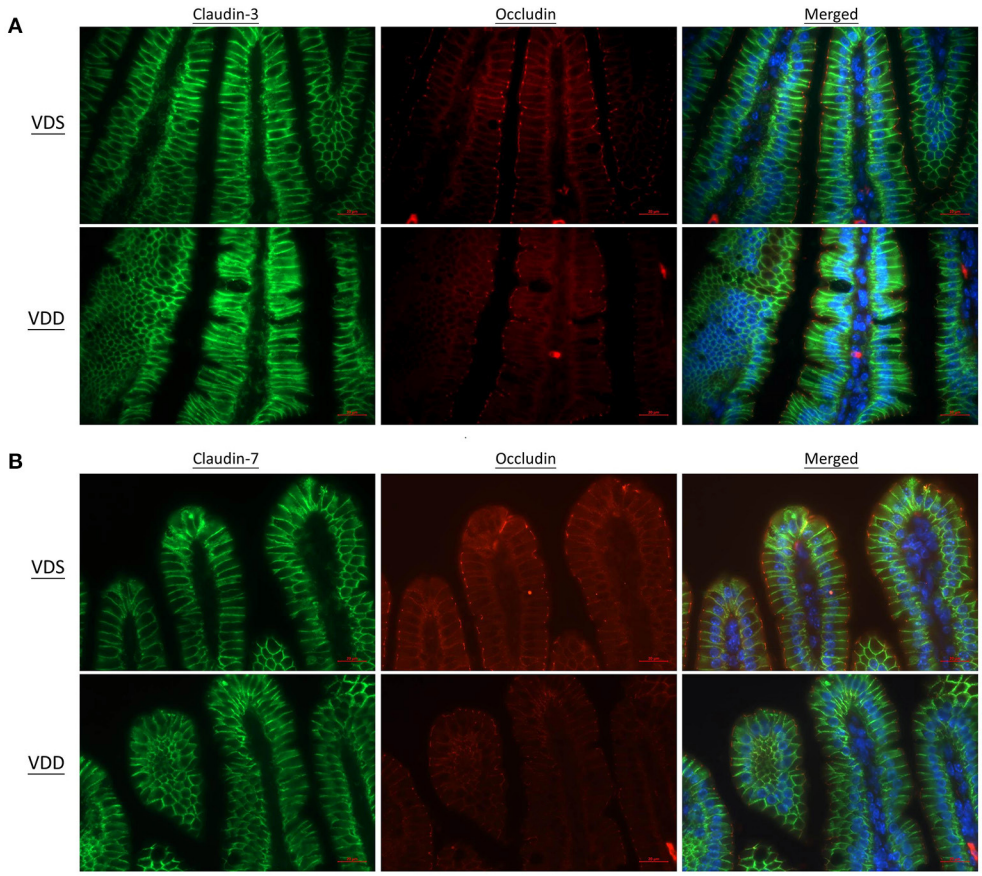

FIGURE 6 | Vitamin D maintains intestinal tight junction expressions in VDD mice by fluorescence microscopy of the jejunum. DAPI was used for nucleus labeling (blue). Distribution of tight junctions was visualized by the claudin (CLD)-3 (DyLight 488, green) and occludin (OCDN) (DyLight 549, red) fusion proteins. CLD-3, OCDN, and DAPI were merged. **(A)** CLD-7 (DyLight 488, green), OCDN, and DAPI were merged. **(B)** The fluorescence line was clear and sharp in the VDS group but blurry, cloudy, and irregular in the VDD group at CLD-3, CLD-7, and OCDN distributions.

found no significant differences in IFN-γ and MCP-1 between the two groups.

VDD Affects the Serum and Intestinal Zonulin Levels

The effects of VDD on the serum zonulin level and intestinal zonulin expression are shown in **Figure 9**. A significant difference in serum zonulin levels was found between the two groups (VDD 2.20 ± 0.09 μg/ml vs. VDS 1.53 ± 0.12 μg/ml, $p = 0.001$) (**Figure 9A**). Similarly, a significantly higher relative level of mRNA expression of jejunum zonulin was observed in the VDD group (VDD 1.44 ± 0.11 vs. VDS 1.03 ± 0.13, $p = 0.043$) (**Figure 9B**).

DISCUSSION

VD has demonstrated multifaceted effects on gut health and has been shown to target three major components of the gastrointestinal tract: intestinal epithelial barrier, gut immunity, and gut microbiota (30). Previous studies had shown that VDD was strongly correlated with gut integrity and immune response (12, 31). However, the mechanisms underlying the protective effects of VD on intestinal barrier function remain essentially unclear. In this study, we successfully established an animal model by raising C57BL/6 wild-type mice fed the VDD diet and elucidated the roles of VD on gut morphology and barrier functions. C57BL/6 wild-type mice were useful and popular mouse strains in gastrointestinal tract studies (32, 33). In our study, the serum level of VD was significantly reduced in the VDD group when compared with the VDS group and showed a deficient level according to human criteria. We also investigated the enterocyte microstructures, inflammatory cytokines, and TJ protein expressions with promising results. For the first time, we successfully demonstrated that VDD diet could lead to a significant upregulation in the mRNA expression of the jejunum zonulin level in a mouse model.

Effects of VDD on Jejunum and Colon Histology

In this mouse model, we demonstrated that VD did have a protective effect in the development of intestinal epithelial and colonic cells. We found that a typical irregular edge and lining

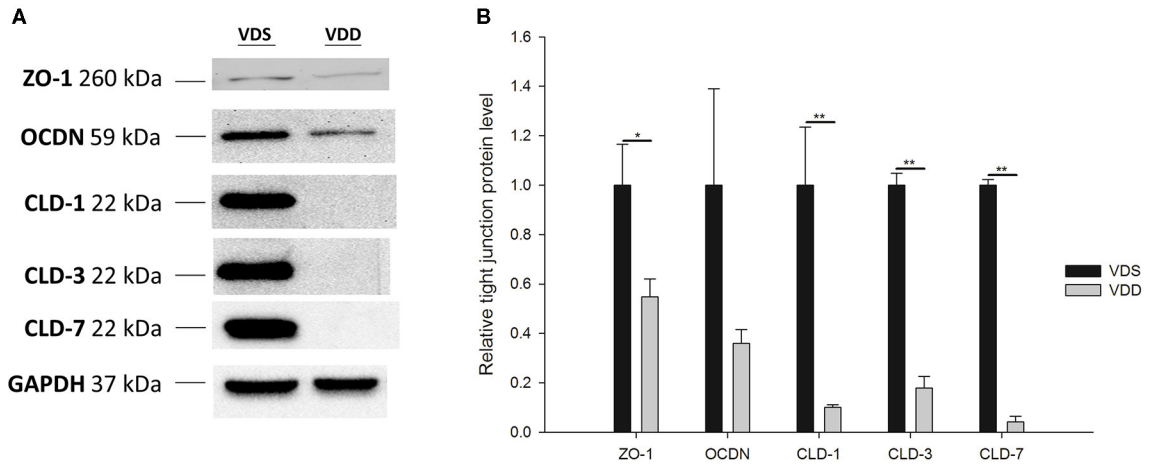

FIGURE 7 | Effects of vitamin D deficiency on the jejunum tight-junction protein (CLD1, CLD3, CLD7, OCDN, and ZO-1) expressions. **(A)** Representative tight-junction protein levels by Western blot analysis. **(A)** The lines indicated the positions of CLD1, CLD3, CLD7, OCDN, and ZO-1, respectively. GAPDH served as a control of protein lysate loading. **(B)** The band densities of various tight-junction proteins were quantified by Image Lab™ software. All relative levels of CLD1, CLD3, CLD7, and ZO-1 protein expressions were significantly decreased in the VDD group when compared to the VDS group. No significant difference in relative level of OCDN was found between the two groups ($p = 0.200$). Values were analyzed using the independent *t*-test. (*$p < 0.05$, **$p < 0.001$). Four mice in each group for parameter determination.

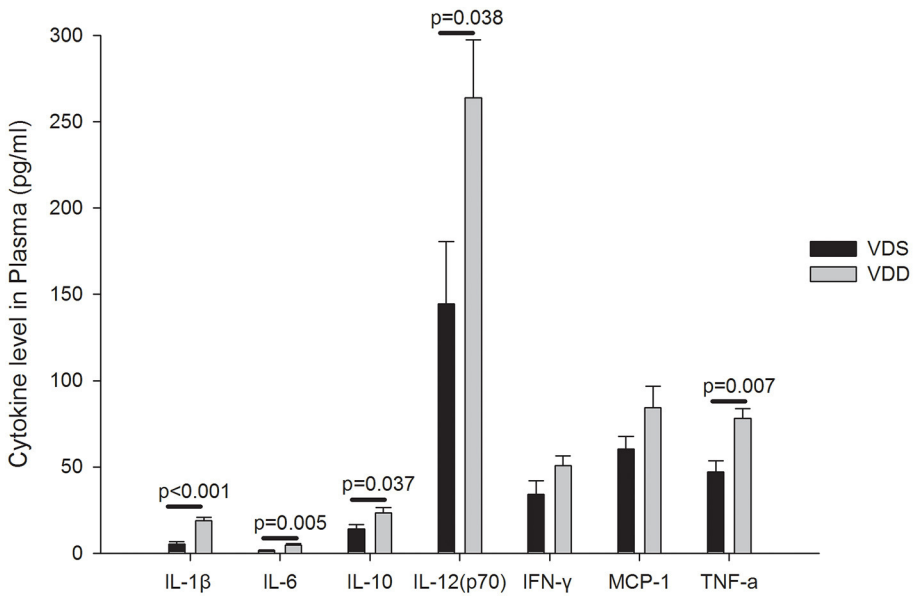

FIGURE 8 | Effects of vitamin D deficiency on the plasma inflammatory cytokine expression. Upregulations of IL-1β, IL-6, IL-10, IL-12, and TNF-α were found in VDD mice. All these plasma inflammatory cytokines were significantly higher in the VDD group when compared to the VDS group. *p*-values were shown inside the figure. No significant differences in IFN-γ ($p = 0.116$) and MCP-1 ($p = 0.119$) were found between the two groups. Statistical analysis was performed by the independent *t*-test. Four mice in each group for parameter determination.

of the jejunal mucosa and distorted enterocytes with significant inflammatory cell infiltration causing mucositis were revealed in VDD mice. However, the intestinal villi of the mouse in the VDS group were much uniform and no inflammatory cell infiltrations were found. We noticed that villus height, crypt depth, and villus height/crypt depth ratio of the jejunum were similar in both groups and no significant differences were found. Similarly, histological damages were found in the colonic histology in the VDD group, including crypt hyperplasia, loss of epithelial integrity, and inflammatory cell infiltration. VDD mice had significantly decreased colonic mucosa thickness compared to mice raised on VDS diet after 7 weeks.

Besides distorted morphology, VDD caused significant intestinal inflammation compared to the control group in

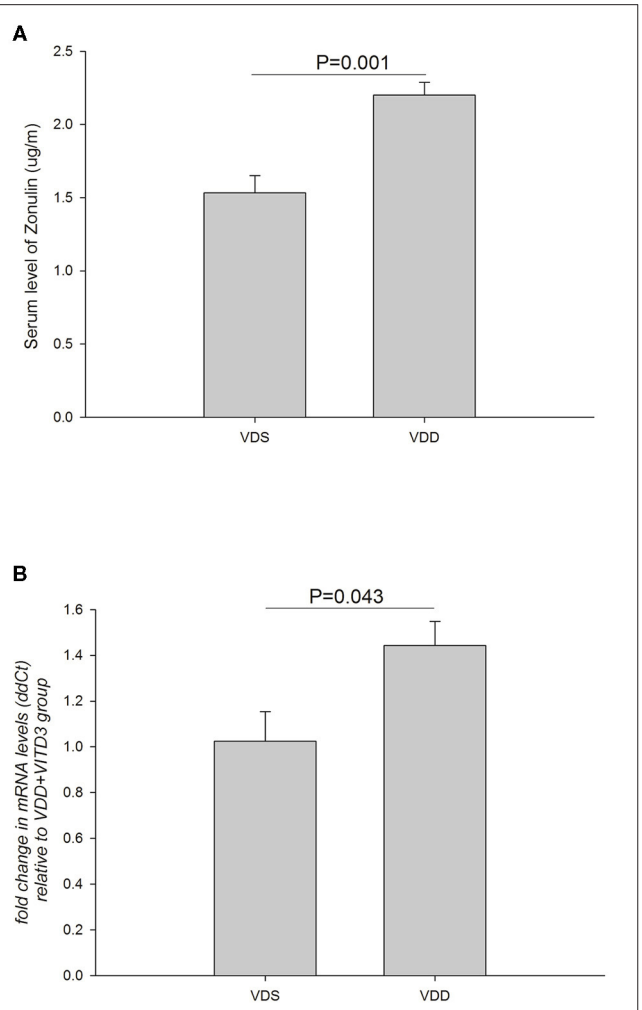

FIGURE 9 | Effects of VDD on the serum and intestinal zonulin levels. **(A)** Serum zonulin levels between the VDD and VDS groups. A significant difference in the serum zonulin level was found ($p = 0.001$). **(B)** A significantly higher level of mRNA expression of jejunum zonulin was observed in the VDD group ($p = 0.043$). Statistical analysis by independent *t*-test. Four mice in each group for parameter determination.

our study. Ryz et al. observed that VDD mice showed more infiltrating macrophages and neutrophils in the cecal tissues, particularly in the submucosal regions when compared with VDS mice (21). In a previous mouse model study, Assa et al. demonstrated a hyperplastic response in sham-infected VDD mice when compared with sham-infected VDS mice (12). Wang et al. also found that proliferation and apoptosis of intestinal epithelial cells played critical roles in cirrhosis-associated intestinal mucosal barrier dysfunction (34). Their results showed that VD restored the proliferative ability of crypt cells in the intestines, inhibited enterocyte apoptosis, maintained the normal intestinal epithelial turnover, and improved the integrity and function of the intestinal epithelial barrier in CCl_4-induced liver cirrhotic rats.

Effects of VDD on Intestine TJ Proteins Structure and Expression

We showed that the fluorescence lines for ZO-1, CLD-1, CLD-3, CLD-7, and OCDN protein staining were clear and sharp in the VDS group but blurry, cloudy, and irregular in the VDD group. Cell nuclei were found located at the baseline of enterocytes in VDS mice, but irregular arrangements of cell nuclei were observed in the VDD group. Besides, levels of CLD-1, CLD-3, CLD-7, and ZO-1 proteins were significantly decreased in the VDD group when compared to the VDS group. Zhao et al. demonstrated that when compared to TJ expressions in the control group, there were significantly reduced expressions in ZO-1, OCDN, and CLD-1 in the VDD group (13). They also found that TJ marker expressions in the VD-treated group were also significantly higher than those in the VDD group, which suggested the importance of VD to maintain the integrity of the TJ complex. Consistent with previous studies, our findings demonstrated that significant intestinal morphological alterations and TJ protein loss occurred in VDD mice. We also showed that ZO-1, CLD-1, CLD-3, CLD-7, and OCDN were highly expressed in jejunum tissues in the VDS group.

However, not all the research studies demonstrated beneficial effects of VD on intestinal barrier function, Mandle et al. in their randomized controlled trial concluded that no evidence was found for incremental effects of supplemental VD on CLD-1, OCDN, and MUC12 levels in the normal colorectal mucosa of patients at increased risk for colorectal cancer (35). Their findings do not support that VD alone substantially affects the expression of the three biomarkers.

Effects of VDD on the Serum Inflammatory Cytokine Expressions

One of the most significant impacts of VD in our study was its effect on the host inflammatory response within the intestine. Even under uninfected conditions, VDD mice showed a higher intestinal inflammatory condition with elevated intestinal expressions of various pro-inflammatory cytokines. Soares et al. suggested two principles of mucositis development including firstly the generation of reactive oxygen species which directly damaged cells, tissue, and blood vessels and secondly the upregulation of pro-inflammatory cytokines including TNF-α, IL-1β, and IL-6 which caused further mucosal injuries (36). In our mouse model, all measured serum cytokines except IFN-γ and MCP-1 were significantly higher in the VDD group when compared to the VDS group. We showed that VD played important roles in the immune modulation and processed anti-inflammatory effects. VD has been shown to modulate a wide variety of immune responses. Assa et al. found that VD exerted its effect on the host inflammatory response within the intestine. VDD mice had elevated expressions of IL-17A and IL-17F in the distal colon compared with VDS mice (12). Uninfected VDD mice expressed higher mRNA transcripts for both pro- and anti- inflammatory cytokines in colonic homogenates. Blaschitz et al. reviewed that local immune responses serve to contain infections by pathogens to the gut while preventing pathogen dissemination to systemic sites. Several subsets of T cells in

the gut contribute to the mucosal response to pathogens by secreting a subset of cytokines including IL-17A, IL-17F, IL-22, and IL-26. These cytokines induce the secretion of chemokines and antimicrobial proteins, thereby orchestrating the mucosal barrier against gastrointestinal pathogens (37). Additionally, IL-1β also plays a crucial role in the activation of the NF-kB pathway, even working with TNF for a synergistic effect in kickstarting the inflammatory response of endothelial adhesion molecules (38). Proinflammatory cytokines including TNF-α, IL-1β, and IL-6 were shown to play important roles in amplifying the severity of chemotherapy-induced intestinal mucositis (39). In our previous study, we demonstrated that those mice in the 5-FU-induced intestinal mucositis group had significantly higher levels of circulating pro-inflammatory cytokines which decreased significantly after probiotic administration (26). We found similar results in using SCID/NOD mice as animal model, suggesting that innate immunity plays a role in the pathogenesis of intestinal mucositis (40). It seems that VD also exerts similar anti-inflammatory effects through the inhibition of pro-inflammatory cytokine expressions according to the results of this mouse model study.

Effects of VDD on the Serum and Intestinal Zonulin Levels

In the recent decade, Fasano et al.'s serial studies led to the discovery and characterization of zonulin as the only human protein discovered to date that is known to reversibly regulate intestinal permeability by modulating intercellular tight junctions (41–43). They have generated evidence that the small intestine exposed to enteric bacteria secreted zonulin (44). Following the release of zonulin, the intestine showed increased permeability (leaky gut) and disassembly of ZO-1 from the TJ complex (45). A systematic review of the literature revealed that zonulin has been reported as a biomarker of several pathological conditions, including autoimmune diseases, diseases of the nervous system, and neoplastic conditions (44, 45).

Emerging data have led to the hypothesis that VD plays a role in promoting epithelial barrier function. However, the relationship of VD and serum and intestine zonulin levels was seldom discussed. In a prospective study to document the relationship between the admission vitamin D deficiency and markers of intestinal permeability in hospitalized patients who were critically ill, Eslamian et al. showed that median plasma endotoxin and zonulin decreased with increasing serum levels of VD categories in the overall study population (46). Their finding suggested a relationship between VDD and early alterations in intestinal permeability. Increased intestinal permeability causing a leaky gut phenomenon has been shown to play a crucial role in the pathogenesis of IBDs. In humans, serum and fecal zonulin were found to be elevated in patients with active Crohn's disease but not with ulcerative colitis (47). In a recent study, serum zonulin concentration was found to be higher in both diseases, and an inverse correlation was observed between serum zonulin concentration and disease duration (48).

In this study, we looked at the effects of VDD on the serum and intestine zonulin levels. We observed that there was a significantly higher level of mRNA expression of jejunum zonulin in the VDD group. Similarly, a marked increase in serum zonulin level was found in the VDD group. Our findings suggested that VDD diet did induce mucosal barrier dysfunction and initiate the release of zonulin in the jejunum. Mucosal injury thus caused a significant rise in serum zonulin level in this mouse model. Asmar et al. stated that zonulin is usually triggered to release when the small intestine is exposed to enteric pathogens and gluten (42). Our findings suggested that zonulin could be released whenever there was mucosa barrier injury or leaky gut conditions even in a non-infected VDD mouse model. We also demonstrated that serum zonulin could reflect the level of zonulin in the intestinal tract and assessment of the serum zonulin level is desirable and clinically more feasible. Whether the fecal zonulin level correlates with the severity of intestinal mucosa injury was not studied in our study but warrants further investigation.

However, we recognize that zonulin is secreted not only from enterocytes; it has been found in several extra-intestinal tissues, e.g., adipose tissue, brain, heart, immune cells, liver, lungs, kidney, and skin (49, 50). Thus, the levels of zonulin in serum reflect not only intestinal secretion but also secretion from other organs. Nevertheless, to date it is impossible to elucidate the exact origin of the serum zonulin level and the studies on the roles of zonulin in extra-intestinal tissues are limited. Serum zonulin levels are supposed to reflect mainly the intestinal permeability and act as a marker of gut integrity.

Studies on the association of intestinal zonulin expression and TJ composition are few in the literature. Feng et al. demonstrated that dietary bisphenol A (BPA) uptake destroys the morphology of the colonic epithelium and increases the pathology score (51). The levels of endotoxin, diamine peroxidase, D-lactate, and zonulin are significantly elevated in both plasma and colonic mucosa. The expression of TJ proteins (ZO-1, occludin, and claudin-1) in the colonic epithelium of BPA mice decreased significantly, and their gene abundance was also inhibited.

Roles and Mechanisms of VD on TJ Proteins and Gut Integrity

TJ are the most apical junctional complex connecting both neighboring epithelial and endothelial cells. They comprised various transmembrane proteins. VD plays a crucial role in protecting the integrity of the intestinal epithelial barrier against infectious and inflammatory insults. Gubatan et al. have suggested that VD can enhance innate immunity by inducing antimicrobial peptides and regulate adaptive immunity by promoting anti-inflammatory T cells and cytokines (30). Besides, Kong et al. have proved that VDD may compromise the mucosal barrier, leading to increased susceptibility to mucosal damage and increased risk of IBD (11). An increased permeability in the TJ may provide a major site for both infection and establishment of inflammation in the gut (52–54). Bacterial translocation is believed to occur *via* a paracellular pathway through the epithelial cells causing a leaky gut phenomenon.

VD was found to be able to protect the intestinal barrier from injuries induced by multiple reagents (13, 39, 55).

Several mechanisms by which VD exerts anti-inflammatory effects have been suggested. Using Caco-2 monolayers as *in vitro* models and a gluten-sensitized mouse model as an *in vivo* model, Dong et al. recently investigated the protective effect of 1,25-dihydroxyvitamin D3 on pepsin–trypsin-resistant gliadin-induced tight-junction injuries (56). They successfully demonstrated that, both *in vitro* and *in vivo*, VD3 significantly attenuated the TJ injury-related increase in intestinal mucosa barrier permeability. VD3 treatment upregulated the TJ protein expression levels and significantly decreased the MyD88 expression and zonulin release signaling pathway.

Zhao et al. showed that VD might have a protective effect on barrier integrity by maintaining the expression of TJ proteins, thereby reducing the severity of gut inflammation (13). Using the VDD mouse model, Zhang et al. successfully demonstrated that both the differentiation of Th1 cells and the production of relative cell cytokines (IL-2, IFN-γ, and TNF-β) were inhibited by $1,25(OH)_2D_3$. The addition of $1,25(OH)_2D_3$ has direct effects on CD4+ T cells and supports its potent immunosuppressive benefits in the treatment of a number of other autoimmune diseases (57). Other studies have shown that VD protects the gut epithelial barrier by suppressing gut epithelial cell apoptosis (58, 59). In this mouse model, our data showed that VD might have a protective effect on barrier integrity by maintaining the expression of TJ proteins, thereby reducing the severity of gut inflammation.

This study adds to previous reports that the bioavailability of VD is an important contributing factor for determining the epithelial integrity. Once the mucosal barrier is breached, the submucosa is exposed to a vast pool of luminal antigens, including food and bacteria, thereby engaging the innate immune responses including increased production in proinflammatory cytokines TNF-α and IFN-γ. We demonstrated that VDD caused a significant destruction of the intestinal morphology and an increase in the circulating pro-inflammatory cytokines such as IL-1, IL-6, TNF-α, and IFN-γ. The reduction in these cytokines by VD may be either due to its direct suppressive effect on the expression of these pro-inflammatory cytokines or due to the effect on maintenance of epithelial barrier function, leading to a reduction in foreign luminal antigenic load, and a full activation of the innate immune system.

In summary, our data suggest that VD may be effective in the maintenance of gut integrity, decreasing histological inflammation, enhancing epithelial cell resistance to injury, and suppressing pro-inflammation responses to luminal antigens. Previous studies seldom emphasized and investigated the role of VD on gut morphology and TJ proteins. In this study, we successfully established an animal model by raising mice fed a VDD diet to elucidate the roles of VD on gut morphology and barrier function. To our knowledge, this is the first study to demonstrate that VDD could lead to a significant upregulation in mRNA expression of jejunum zonulin in a mouse model.

There are several limitations in our study. One limitation is that the study period was indeed short and the observation of possible pathological changes might be inadequate. Various doses of VD supplementation were not assessed to determine their possible contributions to the observed effects. Another limitation is that we did not address the possible mechanisms by which VD exert their beneficial outcomes such as the effects on intestinal permeability and transepithelial electrical resistance and the influence on the composition and diversity of the intestinal microbiota. These areas should be investigated in future experiments. Besides, we only assess the TJ proteins ZO-1, CLD-1, CLD-3, CLD-7, and OCDN in this study. The expressions and roles of other TJ proteins such as pore-forming CLD-2 and−15 warrant further investigation. In this study, we do not investigate the roles of microbiota and the consequences of dysbiosis that may probably occur in VDD mice. It would be also helpful to complement our study with the gene expression of the same TJ proteins to establish whether VDD causes a decreased gene expression of these proteins or an increased degradation of their protein pool. Nevertheless, the greatest challenge for an animal model is the difficulty in translating results obtained from the current model to the wide range of human patient groups with varying ages and diagnoses. More clinical works are needed to demonstrate the beneficial effects of VD and to elucidate the correct dosing regimens for the management of various human disorders with intestinal barrier dysfunction.

CONCLUSIONS

We successfully demonstrated that VDD could lead to impaired barrier properties. We assume that sufficient VD could maintain intestinal epithelial integrity and prevent mucosal barrier dysfunction. Thus, VD supplementation may serve as part of therapeutic strategy for human autoimmune or infectious diseases with intestinal barrier dysfunction (leaky gut phenomenon) in the future. To our knowledge, this is the first study to demonstrate that VDD could lead to a significant upregulation in mRNA expression of jejunum zonulin level and also a marked elevation of serum zonulin in a mouse model.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of MacKay Memorial Hospital (IACUC number: MMH-A-S-107-026).

AUTHOR CONTRIBUTIONS

C-YY, W-TC, J-SC, C-YL, C-WC, and H-CL conceived and designed the experiments. C-YY, M-LC, and J-SC performed the experiments. C-YY, W-TC, C-BJ, S-WC, M-LC, and C-YL analyzed the data. C-YY, W-TC, C-BJ, S-WC, M-LC, C-YL, C-WC, and J-SC contributed the reagents/materials/analysis tools. C-YY, W-TC, C-BJ, S-WC, M-LC, C-YL, J-SC, C-WC, and H-CL wrote the paper. All authors contributed to the article and approved the submitted version.

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REVIEW
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Rules of Engagement: Epithelial-Microbe Interactions and Inflammatory Bowel Disease

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Inflammatory bowel diseases (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), are complex, multifactorial disorders that lead to chronic and relapsing intestinal inflammation. The exact etiology remains unknown, however multiple factors including the environment, genetic, dietary, mucosal immunity, and altered microbiome structure and function play important roles in disease onset and progression. Supporting this notion that the gut microbiota plays a pivotal role in IBD pathogenesis, studies in gnotobiotic mice have shown that mouse models of intestinal inflammation require a microbial community to develop colitis. Additionally, antimicrobial therapy in some IBD patients will temporarily induce remission further demonstrating an association between gut microbes and intestinal inflammation. Finally, a dysfunctional intestinal epithelial barrier is also recognized as a key pathogenic factor in IBD. The intestinal epithelium serves as a barrier between the luminal environment and the mucosal immune system and guards against harmful molecules and microorganisms while being permeable to essential nutrients and solutes. Beneficial (i.e., mutualists) bacteria promote mucosal health by strengthening barrier integrity, increasing local defenses (mucin and IgA production) and inhibiting pro-inflammatory immune responses and apoptosis to promote mucosal homeostasis. In contrast, pathogenic bacteria and pathobionts suppress expression and localization of tight junction proteins, cause dysregulation of apoptosis/proliferation and increase pro-inflammatory signaling that directly damages the intestinal mucosa. This review article will focus on the role of intestinal epithelial cells (IECs) and the luminal environment acting as mediators of barrier function in IBD. We will also share some of our translational observations of interactions between IECs, immune cells, and environmental factors contributing to maintenance of mucosal homeostasis, as it relates to GI inflammation and IBD in different animal models.

Keywords: IBD, microbiota, dog, mouse, intestinal permeability, epithelial barrier

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INTRODUCTION

The inflammatory bowel diseases (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), are complex, multifactorial inflammatory diseases affecting the gastrointestinal (GI) tract (1, 2). IBD is an immune-mediated disorder comprising two distinct phenotypes having varying clinical, endoscopic, immunologic and histopathologic features (3, 4). Crohn's disease is characterized by

patchy, transmural inflammation that primarily affects the terminal ileum but can also involve the small intestine. Ulcerative colitis causes diffuse superficial mucosal ulcerative inflammation restricted to the rectum and colon. The cause for IBD remains unknown but it is likely that genetically susceptible individuals develop an aberrant immune response to their microbiota, leading to chronic inflammation and repetitive injury to the intestines (2). The onset of IBD typically occurs in the second or third decade of life but rising incidence worldwide suggest a prominent role for environmental factors (5).

The intestinal epithelium is composed of a monolayer of columnar epithelial cells that communicate continually with the luminal microbiota and an underlying network of innate and adaptive immune cells. This mucosal barrier normally prevents the entry of pathogenic microbes and toxins while regulating the absorption of nutrients, electrolytes, and water from the lumen into the systemic circulation (6). There is a growing body of data indicating that dysfunction of the intestinal barrier is a causative factor in the pathogenesis of IBD. For example, numerous IBD genetic risk loci affect pathways active in epithelial cells involved in essential functions such as innate immunity, autophagy and endoplasmic stress (7). Moreover, epithelial barrier dysfunction secondary to chronic inflammation and recurring "flares" is characteristic of IBD (8). During active disease, inflammatory mediators (cytokines/bacterial products) released in the intestinal mucosa progressively damage the epithelium and expose mucosal immune cells to luminal antigens that amplify the inflammatory response (3, 9). Finally, the intestinal epithelium is actively involved in repair mechanisms that promote mucosal healing through re-epithelialization to patch defects and maintain mucosal homeostasis (10, 11). Also contributing to maintenance of the mucosal barrier is the controlled replenishment of intestinal epithelial cell (IEC) subtypes (e.g., columnar cells, goblet cells, enteroendocrine cells and Paneth cells) from LGR5 intestinal stem cells (12). In this review, we will focus on the role of IECs and the luminal environment (including the microbiota) to act as mediators of barrier function in IBD. We will also share some of our translational observations of interactions between IECs, immune cells, and environmental factors (including the gut microbiota) contributing to loss of mucosal homeostasis as it relates to GI inflammation and IBD in different animal models.

Intestinal Barrier and Mucosal Homeostasis

Structural Components of the Epithelial Barrier

The term *mucosal barrier* was first proposed by Cummings in 2004 and describes the complex structure that separates the luminal environment from the internal milieu (13). The intestinal mucosal barrier is a functional entity consisting of separate but interlinked components, including physical elements (e.g., the underlying vascular endothelium, epithelial cells, and the mucus layer), along with a chemical layer composed of digestive secretions, immune molecules, and cellular products (cytokines, inflammatory mediators, and antimicrobial peptides). Apart from these layers, the microbiota also contributes to barrier integrity along with immune functions and GI motility. The intestinal epithelium is composed of a single layer of columnar cells and different specialized cell subtypes: enterocytes, goblet cells, Paneth cells, enteroendocrine cells and immune cells, including intraepithelial lymphocytes (IELs) and dendritic cells (**Table 1**; **Figure 1**) (15). Three types of junctional complexes [tight junctions (TJ), adherens junctions (AJ) and desmosomes] provide mechanical cohesion to these columnar cells and seal the paracellular space to regulate the movement of water ions and small molecules across the intestinal mucosa (16–18).

Tight junctions form the most apical adhesive (JP) and are continuous around the IEC at the border between apical and lateral membrane regions (16–18). They function as a semi-permeable paracellular barrier that move ions and solutes through the intercellular space while excluding luminal antigens, bacteria and their toxins. Within TJ complexes are integral transmembrane proteins, occludin and members of the claudin family, that link adjacent cells to the actin cytoskeleton to regulate paracellular permeability (16). Claudins represent a family of TJ proteins that regulate the movement of water and electrolytes through sealing molecules and pores. Experimental

TABLE 1 | Components of the intestinal epithelial barrier and their perturbation in IBD.

Components	Function	Known defects
Physical Barrier		
Mucus layer	Adherent and loose layers, contain AMPs and microbiota (loose layer)	Reduced thickness to mucus layer, bacterial biofilm with CD, altered composition to mucus layer
Enterocytes	Digestion, macromolecule transport, secrete β-defensins	Defective defensin production, mucosal ulceration/erosions
Goblet cells	Secrete mucin and trefoil factors	Decreased number of goblet cells
Paneth cells	Secrete α-defensins, Reg3 proteins, lysozyme, BMPs for ISC niche	Reduced antimicrobial activity
Enteroendocrine cells	Produce serotonin and 5-HT; sense microbial metabolites	Altered enteroendocrine secretion
Intercellular junctions	Intercellular transport, regulate barrier function	Altered expression and localization
Intra-epithelial lymphocytes (IELs)	Immune surveillance, cytotoxic activity	Imbalance in IEL cytolytic and regulatory functions
Dendritic cells	Antigen sampling	Increased activation promoting inflammation
Plasma cells	Produce secretory IgA (sIgA), help maintain ISC niche	Increased in number, increased granzyme B and cytotoxic activities
Chemical Barrier		
Digestive secretions	Degrade nutrients and bacteria	Altered secretions
Anti-microbial peptides (AMPs)	Bacterial degradation and exclusion	Reduced antimicrobial activity
Cytokines, inflammatory mediators	Promote inflammation	Increased production contributing to repetitive mucosal injury

BMPs, bone morphogenic proteins; 5-HT, 5 hydroxytryptamin; ISC, intestinal stem cell; CD, Crohn's disease.

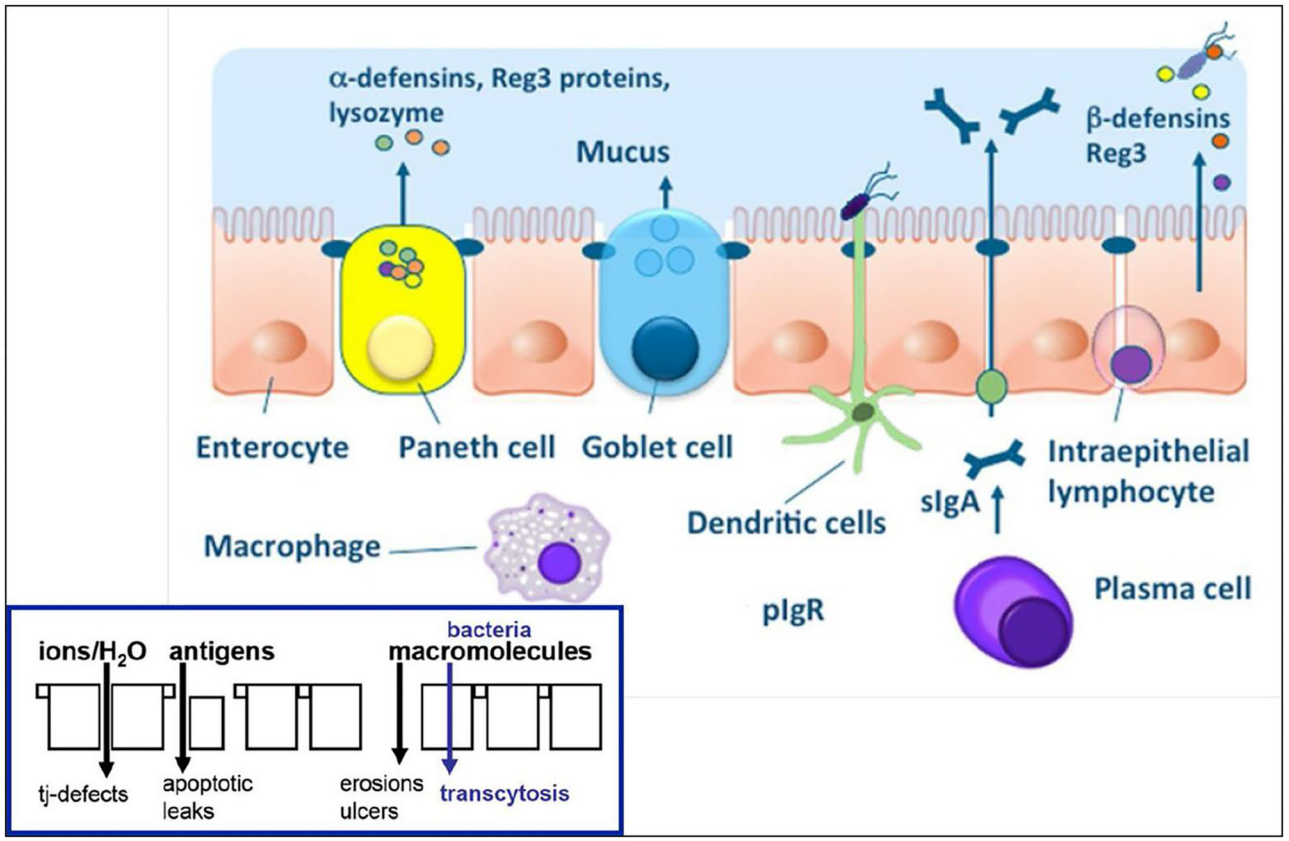

FIGURE 1 | Physical and chemical components of the intestinal epithelial barrier. See text for component specifics. With epithelial barrier dysfunction (insert), intestinal permeability increases which allows for antigens and macromolecules (bacteria) to pass into the lamina propria where innate and acquired immune cells reside. From: (14) with permission.

studies indicate that differential claudin expression (either up- or down-regulation) is associated with impaired barrier function (19, 20). The important TJ adapter proteins, zonulin occludens (ZO) -1, -2 and -3, connect the cytoskeleton to the transmembrane TJ proteins. Underneath the TJs are the AJs that are important for cell-to-cell signaling and epithelial restitution, while desmosomes provide structural stability between the IECs (16, 21). Summarizing, the intestinal epithelium maintains its selective barrier function through the formation of complex protein-protein networks that mechanically link adjacent IECs to selectively seal the intercellular space.

The expression pattern of JPs is tightly regulated and varies by intestinal compartment (small vs. large intestines), villus/crypt location, and cell membrane location (apical, lateral or basolateral). The expression of AJ and TJ proteins is a dynamic process that is steadfastly regulated by phosphorylation causing both beneficial and harmful consequences (22–24). For example, phosphorylation can either promote TJ protein formation to enhance barrier function or alternatively it can disrupt and redistribute TJ and AJ proteins to increase intestinal permeability (25, 26).

The human intestinal epithelium constantly renews itself every 4–5 days under normal homeostasis, with the pace of renewal increasing following damage. Regulating this process are pluri-potential stem cells that give rise to all GI epithelial cell lineages and can generate whole intestinal crypts (12). At the tips of villi and along the epithelia of the colon, mature cells undergo apoptosis and are normally shed into the GI lumen. Intestinal stem cells (ISCs that express LGR5) can differentiate into four specialized cell types, including columnar cells (enterocytes and colonocytes), goblet cells, enteroendocrine cells and Paneth cells (the latter cell type found only in the small intestine) (15). Columnar cells are the most abundant epithelial cell found in the small and large intestines and are involved in absorption. Goblet cells produce and secrete mucin (e.g., mucin-2) which covers the surface of the intestinal epithelium. Antimicrobial peptides and lysozyme further fortify the antimicrobial properties of the mucus compartment to promote antigen elimination. Paneth cells produce lysozyme and several antimicrobial peptides to protect against microbial infection including α-defensins and Reg3 proteins (27, 28). They also reside adjacent to ISCs and provide the necessary growth factor (e.g., Wnt, EGF or Notch) signals to the ISCs and constitutes the stem cell's niche (12). Epithelial cells secrete β-defensins in response to sensing of microbes by their pattern recognition as either commensal bacteria or pathogens. Secretory immunoglobulin

A (sIgA) is produced by plasma cells to mediate protection at mucosal surfaces by binding bacteria and viruses to prevent their attachment to or invasion of IECs (i.e., immune exclusion) (29). Finally, the resident bacteria provide a deterrent to microbial invasion and maintenance of mucosal homeostasis through competitive exclusion, nutrient utilization, niche localization and their production of bacteriocins (30).

Intestinal Barrier Permeability Pathways

The intestinal epithelium serves as the primary compartment of the mucosal barrier and uses both transcellular and paracellular mechanisms to transport substances from the lumen into the lamina propria. The transcellular pathway primarily transports nutrients and compounds having high molecular weight (>600 Da) by means of endocytosis or carrier-dependent transport systems. The protein complexes interconnecting enterocytes (i.e., TJ, AJ, and desmosomes) are dynamic key modulators that allow for the paracellular transport of water, small solutes and electrolytes between enterocytes while restricting the passage of microbes and large molecules (31, 32). Since paracellular transportation occurs through the space between cells, it is less selective as compared to the transcellular pathway which is regulated by membrane channels. Taken together, these two pathways selectively regulate the degree of permeability for substances having different physiochemical properties, such as variable size and ionic charge, into the lamina propria. Any impairment in the integrity or function of these transporting routes increases intestinal permeability which is implicated in the pathogenesis of several GI and extra-GI diseases (i.e., having local or systemic manifestations) such as IBD, celiac disease, type I diabetes, and emotional stress (33, 34).

The gut microbiome, which contains 10^{14} bacteria and 100-fold more genes than the entire human genome, has a pivotal role in development of the host immune system and metabolism (35). A well-balanced symbiotic relationship between the gut microbiota and the host is required for maintenance of mucosal homeostasis. There are approximately 1,000 different bacterial species within five dominant phyla (i.e., *Bacteroidetes, Firmicutes, Actinobacteria, Proteobacteria, and Verrucomicrobia)* that comprise the healthy human fecal microbiota (36). In contrast, the core gut bacteria in the feces of specific pathogen free (SPF) mice contains 37 genera (37). In this group, *Anaerostipes* spp were present in all mice and are an important butyrate producing bacterial species contributing to mucosal barrier integrity. Another murine microbe with high prevalence is *Parabacteroides* spp which are important in stimulating host immunity. The other dominant murine bacteria include carbohydrate-utilizing and lactate and/or acetate-producing microbes such as *Bifidobacterium* spp and *Lactobacillus* spp. These observations suggest that the composition of a core microbiome within a species is essential for maintaining gut homeostasis and are reflective of overall host health to a variable extent.

Methods to Investigate the Intestinal Epithelial Barrier

The intestinal epithelial barrier remains selectively permeable if its integrity is not compromised. Following mucosal barrier disruption, intestinal permeability increases and delivers phlogistic dietary and/or microbial products to the mucosal immune system which provoke host responses. Therefore, the normally tolerogenic crosstalk between the host and the microbiota becomes perturbed resulting in the generation of an overactive immune response. Overtime, this continuous immune stimulation gives rise to intestinal inflammation which triggers the onset of chronic GI disease, such as IBD. Longitudinal studies in patients with IBD indicate that altered intestinal permeability precedes relapse of CD, suggesting a pathogenic role for barrier dysfunction in IBD as well as an indicator of impending symptoms (38). There are several methods for assessment of intestinal permeability via administration of oral probes, *in vitro* or tissue measures, and endoscopic evaluation of the intestinal epithelial barrier (mucosa) in humans (**Table 2**) (14, 21).

Our own work using a defined microbiota [colonized with the altered Schaedler flora (ASF)] mouse model shows that healthy ASF mice have increased intestinal permeability as compared to conventionally reared (CONV) mice. Using RNA *in situ* hybridization, we provide evidence that greater concentrations of bacteria (EUB probe) and/or their products translocate into the cecal lamina propria vs. bacterial products that translocate in CONV mice (**Figure 2**). Furthermore, ASF mice demonstrated greater IgG antibody response against members of their resident microbiota when compared to the antibody response directed against these same bacteria in CONV mice (unpublished observations). Our findings are in accordance with previously published data confirming that mice harboring a less diverse gut microbiota have an altered mucosal barrier and increased intestinal permeability (40).

Microbiota Alterations in IBD

Observations in Human IBD

Abundant clinical studies indicate a dysfunctional interaction between the gut microbiota and the host response in the onset and pathogenesis of IBD. Increased risk of IBD is associated with changes in composition/structure of the intestinal microbiota or genetic predisposition that impairs normal microbial sensing, both of which can cause altered host-microbe interactions (2, 3, 41–48). CD and UC are not considered single gene disorders, as over 240 susceptibility and IBD risk loci have now been identified (49, 50). Twin studies showed that while there is a genetic basis for IBD, it is not inherited in a simple Mendelian fashion (51). Genetic linkage analysis studies have identified nine disease loci, five of which meet the most stringent linkage analysis criteria, the remaining of which were at least suggestive (49). Mutations in several genes responsible for innate immune sensing of the intestinal microbiota, including NOD2/CARD15, IL-23R and ATG16L1, can also lead to increased risk for IBD (52–54). CARD15/NOD2 was the first IBD susceptibility gene that was identified emphasizing the importance of mucosal-microbial disturbances in the pathogenesis of IBD (53). CARD15 encodes an intracellular protein expressed in multiple immune system components including Paneth cells, monocytes, tissue macrophages and intestinal epithelial cells (52, 55–57). In Paneth cells, NOD2 mediates activation of NF-κB that leads to the

induction of defensins. With NOD2 mutations in CD, selective α-defensin production is attenuated which predisposes intestinal epithelial cells to microbial infection (58). Additionally, two autophagy genes, ATG16L1 and IRGM—both of which have roles in the processing of microbial antigens as part of the innate immune system—were identified as susceptibility genes (54, 59). Polymorphisms in these genes promote deranged innate immune responses leading to persistent intracellular bacterial infection that promote the development of IBD. IBD has also been linked to IL-10 deficiencies in humans. In the study by Glocker et al., investigators found that mutations in either IL10RA or IL10RB are associated with severe early onset enterocolitis in children (60). In a separate study, investigators reported NOD2 mutations in patients with IBD that were linked to inhibition of IL-10 in human monocytes (61).

The host microbiota plays an important role in the pathogenesis of IBD as evidenced by numerous clinical studies. Antibiotic use, both in early childhood and in adults, has been associated with increased risk for development of IBD (62). Moreover, the risk for IBD increases following an episode of infectious gastroenteritis (63). There are other observations implicating the microbiota including reports that mucosal inflammation is localized to gut segments with the greatest bacterial loads (2, 42). Furthermore, antibiotic treatment may be effective in a subset of IBD patients (post-surgical, CD and in pouchitis patients). Antibiotics have been used with varying degrees of success and longevity of response in patients with CD having luminal disease, fistulizing disease, and secondary septic complications such as post-operative infections (64). Results from large scale clinical trials and meta-analyses have been mixed with some analyses finding mild to moderate benefits in disease activity scores (65, 66) and others finding no benefit (67). Furthermore, probiotics and fecal microbiota transplant (in UC patients) may induce or maintain remission in some IBD patients (68–70).

Importantly, many studies have shown consistent alterations in microbial communities characterized by reduced microbial diversity in patients with IBD compared to controls (41, 71). The fecal microbiota of both CD and UC patients contains a depletion of Bacteroidetes and Firmicutes phyla (in particular *Clostridium* spp), which are the dominant normal fecal microbiota, and an increased abundance in Proteobacteria (42, 45, 72). Moreover, a metagenomic analysis of microbiomes demonstrated 25% fewer mucosal microbial genes from IBD patients compared with the microbiomes of healthy controls, suggesting that lower microbial diversity is present and contributing to disease (73). Several studies have found decreased abundance of *Faecalibacterium prausnitzii* (74), a major butyrate producing bacteria in the gut, and an increase in sulfate-reducing bacteria (SRB) which cause decreased expression of epithelial TJPs to increase intestinal permeability in IBD (75).

Still other studies have focused on the role of the mucosal microbiota that is different than the fecal microbiota between controls and patients with IBD. Using fluorescence *in situ* hybridization, high concentrations of bacteria were shown adherent to the epithelium of IBD patients as a thick biofilm, mainly composed of *Bacteroides fragilis* (43). In one seminal study, a depletion of Lactospiraceae and Bacteroidetes but increased abundance of Proteobacteria and Actinobacter were present in colonic biopsy specimens from both CD and UC patients, relative to control tissue samples (45). The distribution of operational taxonomic units (OTUs) was associated with disease state but not anatomy (small vs. large intestine) or gross pathology. Furthermore, the microbiome collected in multiple GI locations from a large cohort of treatment naïve patients with new-onset CD found

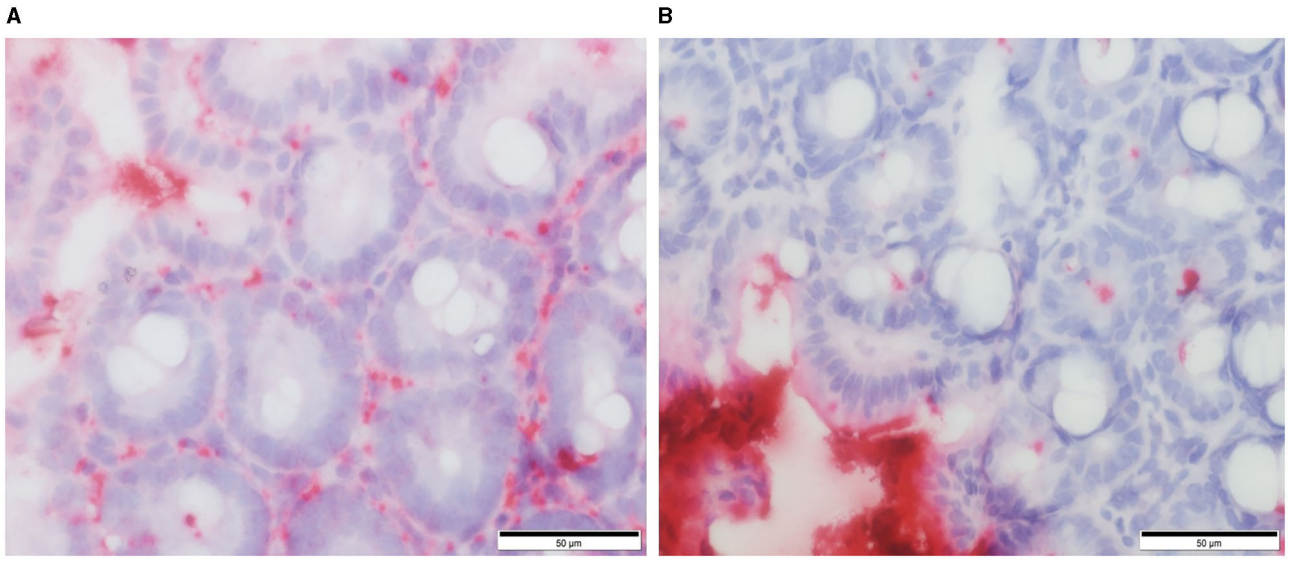

FIGURE 2 | RNA *in situ* hybridization for total bacteria (EUB probe) in murine cecal tissue specimens. Red staining indicates the presence of bacteria and/or their products within the cecal lamina propria of ASF colonized mice **(A)** and conventional mice **(B)**. From: Parvinroo et al. (39) with permission.

an increased abundance of Enterobacteriaceae, Pasteurellacaea, Veillonellaceae and Fusobacteriaceae and a reciprocal decrease of Erysipelotrichales, Bacteroidales and Clostridiales in pediatric IBD samples as compared to controls (45). These changes also correlated with disease status, that is, inflammation had a significant impact on microbial composition. Since several of the underrepresented bacterial phyla in IBD patients are butyrate-producing microbes, depletion of these organisms might reduce butyrate production, which is an important energy source for colonic epithelial cells and may enhance epithelial barrier integrity and mediate GI immune responses (42). Loss of significant quantities of these bacteria that provide key metabolic products (i.e., short chain fatty acids) to the host could exacerbate some forms of IBD (76–78).

TABLE 2 | General means for assessment of intestinal permeability in humans and animals.

Method	Human	Animal	Material needed	Comments
Orally administered probes				
Lactulose/mannitol	X	X	Urine	Time consuming
FITC-dextran		X	Serum	Time consuming
51Cr-EDTA	X	X	Urine	Time consuming; radiation hazard
***In vitro*/tissue measures**				
Ussing chamber	X	X	Biopsies	Invasive; requires specialized equipment
TEER	X	X	Biopsies	Invasive; requires specialized equipment
Histology	X	X	Biopsies	Invasive; permits specialized testing (IHC, confocal microscopy) for TJP expression
Scanning electron microscopy	X	X	Biopsies	Invasive; specialized fixative; expensive
DNA/RNA extraction	X	X	Biopsies	Invasive; permits qPCR for TJP expression
Biomarkers				
LAL assay (LPS)	X	X	Plasma	May have technical limitations
Citrulline	X	X	Plasma	Reliability in the dog is questioned
FABP	X	X	Plasma	ELISA performed on plasma or urine
Endoscopic measures				
Confocal endomicroscopy	X	X*		*As performed in dogs; specialized equipment; expensive
Endoscopic mucosal impedance	X			Directly measures duodenal impedance; specialized equipment; expensive

Modified from references 20 and 38; TEER, trans-epithelial electrical resistance; LPS, lipopolysaccharide; FABP, fatty acid binding protein; IHC, immunohistochemistry; TJP, tight junction protein.

**denote that this intervention is only used in dogs.*

Pathobionts, such as adherent-invasive *Escherichia coli* (AIEC), are present within the mucosa in 21–62% of patients with ileal CD and 0–19% of healthy individuals (79, 80). Dysbiosis is associated with increased levels of oxygen in the intestinal lumen (81), possibly due to increased intestinal permeability and/or mucosal inflammation (82). In the inflamed gut, increased colonic oxygen levels restrict obligate anaerobic populations (e.g., Firmicutes) and increase the abundance of facultative anaerobes, including members of the family Enterobacteriaceae (83). Patients with CD have specific NOD2 variants that lead to defective innate sensing, autophagy, and immune responsiveness to CD-associated AIEC (7, 84). The adhesion molecule CEACAM6 is over expressed in ileal CD patients which also makes individuals more susceptible to mucosal colonization by AIEC (85). AIEC pathobionts strongly adhere to and invade IECs inducing robust pro-inflammatory cytokine secretion (e.g., IFN-γ, TNF-α) which causes direct damage to the intestinal barrier and promotes inflammation. Once within the ileal mucosa, AIECs can reside and replicate within macrophages, leading to an increased pro-inflammatory response (86). In contrast, AIEC colonization does not occur in colonic CD and the lack of AIEC mucosal translocation in UC patients would suggest that *E. coli* does not play a primary role in UC pathogenesis (87).

Observations in Murine Models of Intestinal Inflammation

Most different mouse models support a role for the microbiota in experimental intestinal inflammation. Early studies in mice treated with dextran sodium sulfate (DSS), a chemical irritant that disrupts the colonic intestinal epithelial barrier to contribute to the development of colitis, reported significant increases in intestinal *Bacteroidaceae* and *Clostridium* spp, in particular *Bacteroides distasonis* and *Clostridium ramosum*, in both acute and chronic colitis DSS models (88). In another study, increased numbers of colonic mucin-degrading *Akkermansia muciniphila* and Enterobacteriaceae were correlated to disease activity in DSS-treated mice resembling UC (89). Interleukin-10 knockout (IL-$10^{-/-}$) mice develop spontaneous colitis that is entirely dependent on gut bacteria (90), and where colonic inflammation is attenuated when treated with antibiotics before disease onset (91) or is eliminated altogether in mice housed in a germ free environment (92). Animal models have also shown that intestinal inflammation is transferable through the intestinal microbiota. Germ-free IL-$10^{-/-}$ mice colonized by the intestinal microbiota of IBD patients exhibit increased colitis as compared to mice colonized with the intestinal microbiota derived from healthy human controls (93). In IL-$10^{-/-}$ mice, loss of regulatory IL-10 secretion results in intolerance to their intestinal microbiota, unbalanced pro-inflammatory responses contributing to mucosal barrier disruption, and the development of spontaneous colitis.

The administration of broad or narrow spectrum antibiotics shows different therapeutic activities in various regions of the colon in SPF colonized IL-$10^{-/-}$ mice. Narrow spectrum antibiotics, such as ciprofloxacin or metronidazole, prevented cecal and colonic inflammation in IL-$10^{-/-}$ mice following SPF colonization. Ciprofloxacin was most effective in treating cecal inflammation by reducing aerobic bacteria, including, *E. coli*

and *E. faecalis*; whereasmetronidazole was superior in reducing colitis and eliminated anaerobic bacteria (e.g., *Bacteroides* spp) in both the cecum and colon (94). Importantly, while ciprofloxacin and metronidazole prevented the induction of typhlocolitis in IL-$10^{-/-}$ SPF-colonized mice, these antibiotics had little effect after the onset of intestinal inflammation. In contrast, the broad-spectrum combination antibiotic vancomycin-imipenem decreased total luminal bacteria and prevented and treated both cecal and colonic inflammation. Taken together, these studies demonstrate that gut bacteria have differing inflammatory roles with some species initiating onset of intestinal inflammation while other microbe subsets drive chronic colitis (95).

Additional evidence supporting the role of the microbiota in colitis development is provided by studies using transfer animal models of colitis induced by deficiency of T-bet in innate immune cells. T-bet is a transcription factor that plays a crucial role in development of Th1 cells and in the regulation of innate and adaptive immunity (96). In certain murine models, loss of T-bet in mice lacking B and T cells (*T-bet*$^{-/-}$/*RAG-1*$^{-/-}$) results in transmissible colitis in conventionally raised wild-type mice by co-housing, presumably caused by microbiota transmission (97). In similar fashion, *Casp3/11*-deficient mice, which are normally protected against DSS-induced colitis, lose this protection and become more sensitive to DSS on co-housing with WT mice (98).

Specific pathogenic bacteria have been associated with the development of intestinal inflammation in murine models. *Proteus mirabilis* and *Klebsiella pneumoniae* correlate with colitis in *T-bet*$^{-/-}$/*Rag2*$^{-/-}$ mice, a mouse model resembling UC (97). Different *Helicobacter* spp, including infection with *H. hepaticus* and *H. bilis* or exposure to their antigens, trigger IBD-like disease in susceptible mice. For example, *H. hepaticus* induces chronic colitis in SPF-housed IL-$10^{-/-}$ mice accompanied by increased expression of pro-inflammatory biomarkers IFN-γ, TNF-α and nitric oxide (99). In a separate study, the combination of *H. hepaticus* infection and CD45RB high CD41 T-cell reconstitution resulted in marked disease expression in severe combined immunodeficiency (*SCID*) mice similar to that observed in human IBD (100). Still other experiments employing targeted infection with *H. hepaticus* were able to produce colitis and sometimes colonic tumors in different mouse strains having defects in immune function and/or regulation (101). Our group has previously shown that defined microbiota [i.e., altered Schaedler flora (ASF)] mice are a useful tool to investigate the impact of specific members of the Proteobacteria (e.g., *E. coli*, *Helicobacter* spp) on the development of colitis. The induction of typhlocolitis in ASF mice colonized with either *H. bilis* or *Brachyspira hyodysenteriae* was accompanied by induction of ASF-specific antibody (102). Using a "multiple-hit" mouse model of colitis, we have shown that colonization of ASF mice with *H. bilis* increased host susceptibility to onset of severe colitis following low dose (1.5%) DSS administration (i.e., inflammatory trigger) (103). An analysis of the molecular/cellular mechanisms revealed increases in mucosal gene expression involving lymphocyte activation and inflammatory cell chemotaxis, with infiltration of more mucosal immune cells in *H. bilis*-colonized mice prior to DSS treatment vs. DSS treatment alone. A subsequent study with a similar experimental design used microarray analysis to demonstrate differential mucosal gene expression associated with alterations in fatty acid metabolism and detoxification in a time course following *H. bilis* colonization (104). This latter study provided preliminary evidence as to the types of factors or changes in the intestinal mucosa (i.e., alterations in housekeeper genes) that potentially predispose the host to the development of typhlocolitis.

Citrobacter rodentium is an attaching and effacing (non-invasive) bacterial pathogen that primarily causes acute typhlocolitis in mice, except when barrier function is impaired or in animals that are genetically susceptible to inflammation where infection can trigger chronic disease (105). The *C. rodentium* infection model was one of the first mouse models to show that composition of the intestinal microbiota influences susceptibility to infection (106), and that infection can alter the composition and spatial distribution of the resident microbiota post-infection (107). Finally, *Fusobacterium varium* isolated from the colonic mucosa of patients with UC was shown to induce experimental ulcerative colitis in mice (108). Collectively, these experimental studies provide compelling evidence that individual resident species are capable of inducing colitis in susceptible mouse models.

Novel Animal Model Observations Implicating Epithelial Barrier Dysfunction in IBD

Here we relate some of our own work utilizing different animal models to investigate host-microbe interactions mediating chronic intestinal inflammation and the role of the mucosal barrier in these different model systems.

The Dog as a Naturally Occurring Model of Chronic Inflammatory Enteropathy

Dogs represent a well-recognized large animal model that naturally develops CIE (also referred to as idiopathic IBD in the veterinary literature), sharing remarkable similarities in etiology, clinical course, histologic lesions and interventional strategies to human IBD (**Table 3**) (109–116). The obvious advantages of the dog in relation to other common animal models (e.g., rodents, zebra fish) include their large body size, longer life span, and they possess a GI tract of similar size, structure and function to that of humans. Of key importance for translational studies, pet dogs are exposed to the same environmental conditions and even share similar microbiota composition with their owners (117, 118). *Clostridialis*, *Fusobacteria*, *Bacteroides* and *Proteobacteria* are the dominant bacteria comprising the healthy canine fecal microbiota (119, 120). Metagenomic analyses in a small cohort of healthy dogs indicate that diet induced changes in microbial composition are not associated with changes in function, and that the fecal microbiota of dogs, mice and humans exhibit a high degree of metabolic and phylogenetic similarity (121). Considering the common microbiota and environmental exposures with humans, there is growing interest in whether similar mechanisms of CIE pathogenesis are shared between species (122).

Certain dog breeds show a predisposition to the development of CIE suggesting a role for host genetics in this disorder.

TABLE 3 | Comparative features of IBD in different animal models.

Feature	Human	Dog	Rodent
Genetic basis	Yes	Yes	Engineered
Etiology	Multifactorial and complex	Multifactorial and complex	+/- Multifactorial
Intact immune system	Yes	Yes	+/-
Resident microbiota role	Yes	Yes	Yes
Blood in stool	Yes	Yes	Yes
Diarrhea	Yes	Yes	Yes
Disease activity measures	Clinical indices, biomarkers	Clinical indices, biomarkers	Laboratory markers
Definitive diagnosis	GI mucosal biopsy	GI mucosal biopsy	GI mucosal biopsy
Longitudinal studies	Yes: endoscopy + histology	Yes: endoscopy + histology	Difficult to perform
Primary therapy	Anti-inflammatory drugs	Diet + anti-inflammatory drugs	Anti-inflammatory drugs
Disease heterogeneity	Yes	Yes	Variable
Spontaneous GI flares	Yes	Yes	+/-

GI, gastrointestinal.

The German shepherd dog, Soft-coated wheaten terrier and Boxer dog/French bulldog have an increased incidence of CIE clinically that has been linked to mutations in innate immune genes, including TLR5, NOD2, and autophagy gene NCF2 (123, 124). Importantly, several of the same breeds (i.e., German shepherds, Boxer/French bulldog) show positive clinical response to administration of antimicrobials, indicating a potential interaction of host susceptibility with the intestinal microbiota in affected dogs. Intestinal biopsies are required to confirm histopathologic inflammation of CIE, with GI endoscopy being the preferred modality to visually inspect the GI mucosa and to acquire targeted biopsy samples. Mucosal lesions of erosions, friability and increased granularity are observed most frequently during endoscopy and correlate best to histopathologic inflammation (**Figure 3**) (113, 126). Lympho-plasmacytic enteritis of varying severity is the most common type of inflammation often accompanied by changes in mucosal architecture, including villous atrophy/fusion, erosions, ulceration, cryptal changes and/or depletion of colonic goblet cells (**Figure 4**) (127). Mixed cellular infiltrates are also observed in dogs with epithelial disruption (neutrophils) or in response to invasive mucosal bacteria (macrophages) as occurs with granulomatous colitis.

Like experimental models and human IBD, the intestinal microenvironment is implicated in the development of CIE in dogs. Numerous studies have shown that intestinal inflammation in dogs is accompanied by dysbiosis, where the proportions of *Clostridiales*, *Fusobacteria*, *Bacteroidetes* and *Prevotellaceae* are decreased, but the proportion of *Proteobacteria*, including

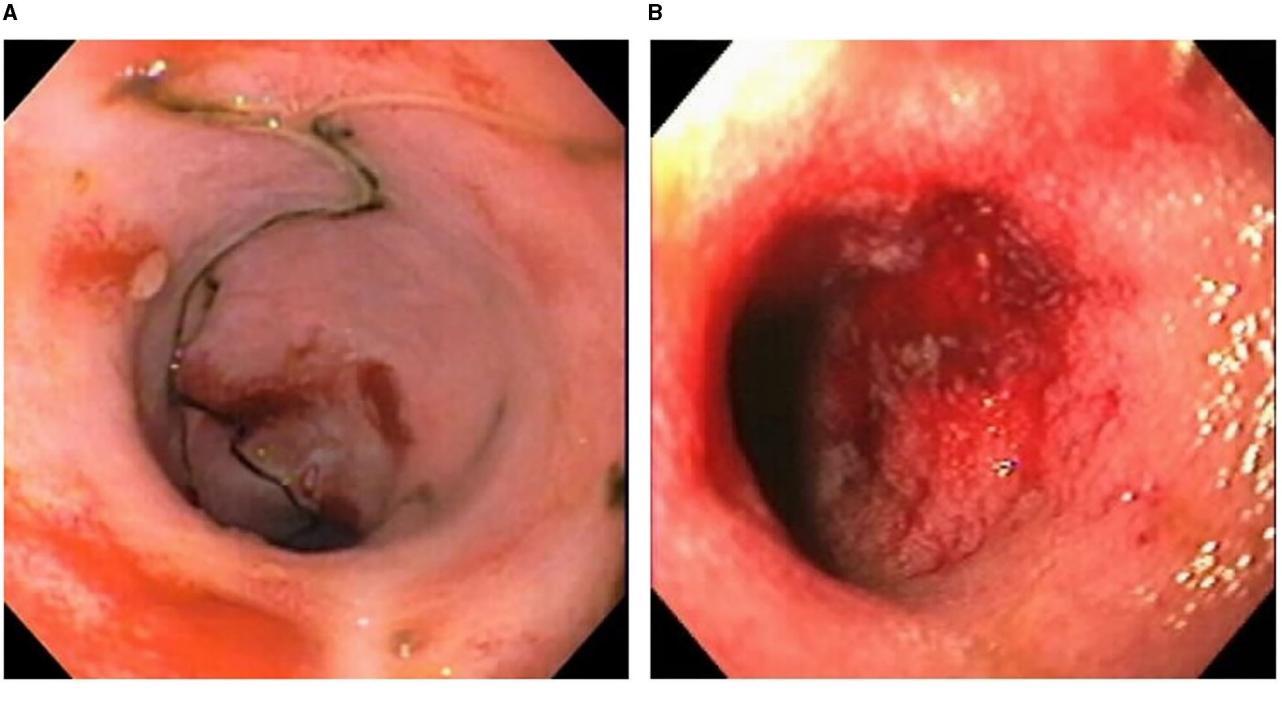

FIGURE 3 | Endoscopic evidence of intestinal barrier disruption in dogs with CIE. Multifocal erosions are evident within the ileal **(A)** and colonic **(B)** mucosae of different dogs with moderate-to-severe CIE. From: Jergens et al. (125), with permission.

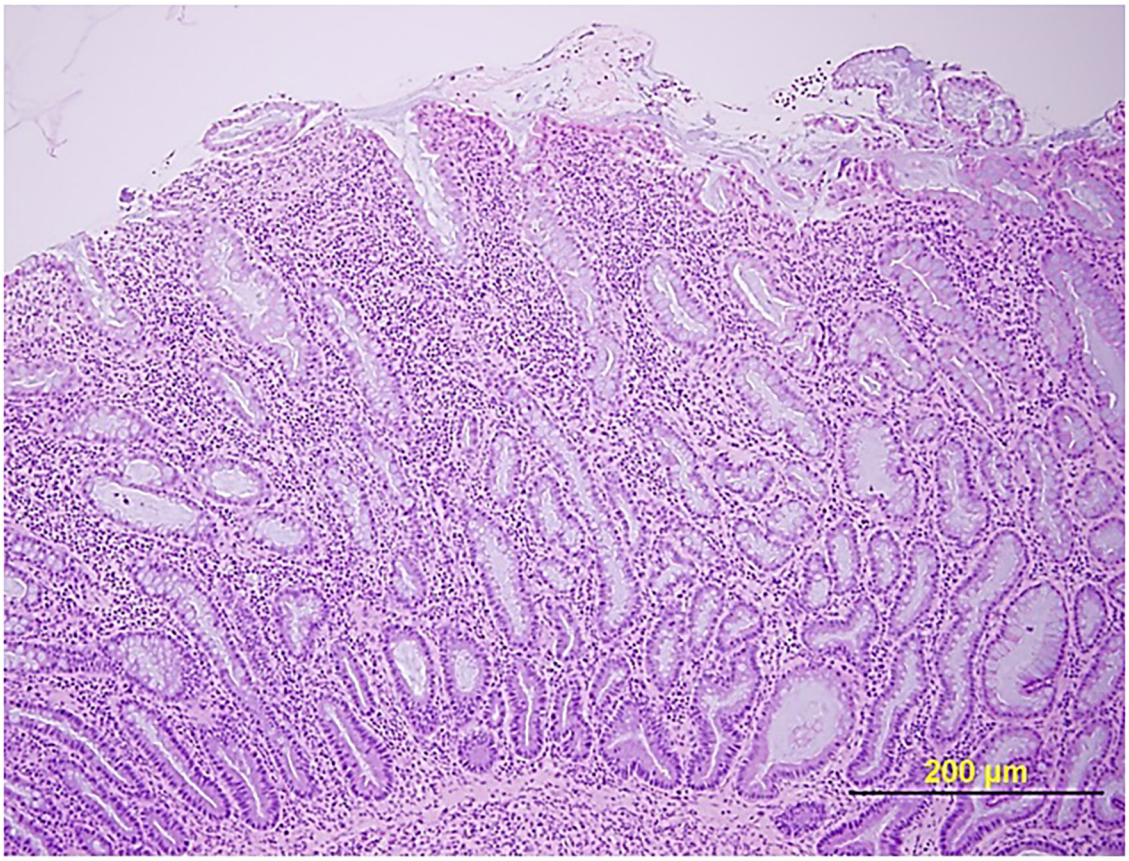

FIGURE 4 | Histopathologic evidence of intestinal barrier disruption. Duodenal biopsy showing focally extensive villus erosions covered by neutrophils and cell debris in a dog with CIE. Hematoxylin and eosin (HE) stain. From: (110), with permission.

Enterobacteriaceae, is significantly increased compared to healthy dogs (128–130). Mucosal associated *E. coli* are significantly increased with intestinal inflammation of CIE, granulomatous colitis and colorectal cancer (adenocarcinoma) in dogs (114, 131). Granulomatous colitis (GC) is a unique variant of CIE, causing chronic colitis with small volume diarrhea, straining, hematochezia and mucoid feces in predominantly young Boxer dogs. Here, a possible genetic defect in innate immune sensing confers increased susceptibility to *E. coli* invasion of colonic tissues (124). With this immune defect, ineffective respiratory burst impairs the host's ability to eliminate intracellular pathogens, including catalase-positive bacteria. A diagnosis of canine GC is confirmed by mucosal culture and/or fluorescence *in situ* hybridization that identify invasive *E. coli* within the colonic mucosa of affected dogs (**Figure 5**). In Boxers with GC, long-term remission is observed with antimicrobial eradication of mucosally invasive *E. coli*, suggesting a causal relationship between this bacterial strain and clinical disease (131). Of interest, the observed phylotype of *E. coli* isolated from Boxer dogs with GC bears strong phylogenetic resemblance to the pathobiont *E. coli* strain isolated from CD patients (132).

The intestinal barrier of dogs with CIE has been investigated to a limited extent. Using duodenal biopsy samples obtained endoscopically from healthy dogs and dogs with CIE, the mucosal expression of claudin-1, -2, -3, -4, -5, -7, and -8; E-cadherin; and β-catenin was determined by immunoblotting and compared between dog groups (133). Results showed no difference in expression of each claudin and β-catenin between healthy dogs and dogs with CIE; while the expression of E-cadherin was reduced in dogs with CIE. Immunofluorescence microscopy (in a subset of CIE dogs) showed decreased intensity of E-cadherin labeling in the apical villi of dogs with CIE. In humans with IBD, a significant correlation between low E-cadherin expression and disease activity has been previously demonstrated (134). In another study, the ratio of IL-1β to IL-1 receptor antagonist (Ra), and the effect of IL-1β on occludin mRNA expression in the duodenal and colonic mucosa were investigated in healthy dogs and dogs with CIE (135). The ratio of IL-1β to IL-1Ra in the colonic mucosa was higher in dogs with CIE vs. healthy dogs. *Ex vivo* cultures of duodenal and colonic biopsies incubated with IL-1β showed reduced expression of occludin mRNA in colonic, but not duodenal, cultures of dogs with CIE. These findings are similar to observations in humans where both occludin mRNA and protein concentrations are reduced in the intestines of CD and UC patients (136). Finally, another study investigated intestinal pro- and active metalloproteinase (MMP) -2 and -9 activities in healthy dogs and dogs with chronic enteropathy (CE) using gelatin zymography.

In dogs with CE, there was a greater number of samples positive for pro- and active MMP2 and -9 in the duodenal, ileal and colonic mucosa as compared to healthy dogs (137). Similar findings of elevated matrix metalloproteinases have been reported in dogs with CIE and in humans with IBD (138, 139).

Clinical trials evaluating drug or probiotic therapy have provided indirect evidence on the role of the intestinal barrier in canine CIE. In one trial, the effects of a hydrolyzed diet and oral prednisone on the spatial distribution of mucosal bacteria in dogs with CIE was investigated using FISH (140). Medical therapy was associated with beneficial changes in microbial community structure and enhanced mucosal junctional protein expression in dogs with CIE. The spatial distribution of mucosal bacteria differed with increased numbers of *Bifidobacteria*, *Faecalibacteria* and *Streptococci* found within adherent mucus of dogs with CIE post-treatment compared to healthy dogs. Using immunohistochemistry (IHC), the expressions of occludin and E-cadherin were increased but zonulin decreased in dogs with CIE following prednisone therapy. Still other studies using multi-strain probiotics for the treatment of canine CIE have shown potential beneficial alterations in junctional proteins that are associated with remission. In one trial, probiotic therapy with VSL#3 was investigated in comparison to combination treatment with prednisone and metronidazole administered continuously to dogs with CIE for 90 days (115). Dogs treated with probiotic showed remission accompanied by changes in beneficial mucosal responses (i.e., increased numbers of FoxP3+ and TGF-β+ cells) and increased mucosal expression of occludin. Another probiotic trial using FISH to investigate the mucosal microbiota showed that remission of dogs with CIE was associated with changes in beneficial bacterial species and up-regulated expression of junctional proteins following 6 weeks of probiotic therapy (141). Both probiotic and standard therapy for CIE (e.g., hydrolyzed diet + oral prednisone) were associated with rapid remission without improvement in histopathologic inflammation. Probiotic therapy was associated with increased expression (IHC) of junction proteins E-cadherin, occludin and zonulin vs. dogs with CIE that received standard therapy (**Table 4**; **Figure 6**). Collectively, these observations of increased barrier integrity in dogs receiving glucocorticoid or probiotic therapy for CIE are in broad agreement with studies in UC patients and experimental models of intestinal inflammation (142–145).

TABLE 4 | Probiotic therapy modulates TJP expression in dogs with IBD.

Colon	Claudin-2	E-cadherin	Occludin	Zonulin
Healthy dogs	91*	1,031*	1,119*	371*
Pre-VSL #3 IBD	1,212^	575	131	61
Post-VSL #3 IBD	82	902^	859^	326^

**P < 0.05 for healthy dogs vs. Pre-VSL #3 IBD dogs; ^P < 0.05 for Pre-VSL #3 IBD dogs vs. Post-VSL #3 IBD dogs; TJP, tight junction protein. From reference 129.*

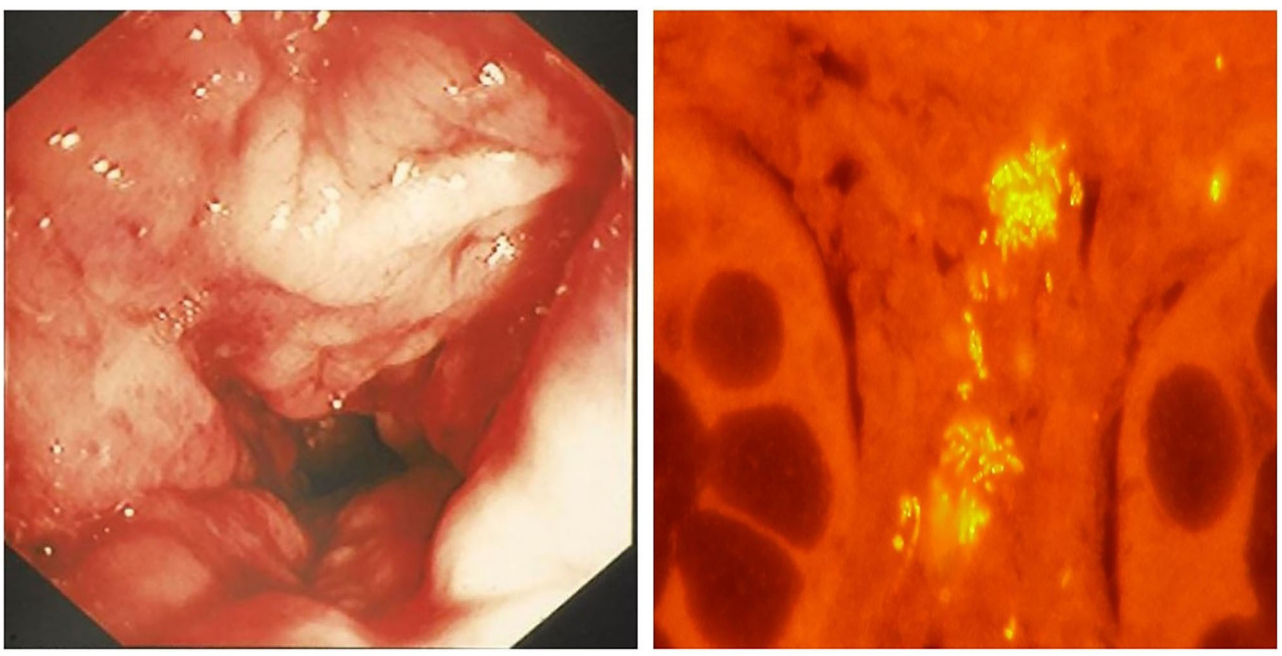

FIGURE 5 | Granulomatous colitis in a 2-year-old English bulldog. **(A)** Endoscopic image of severe colonic granularity (increased texture) involving the descending colon. **(B)** Colonic biopsy from this dog shows clusters (yellow fluorescence) of mucosal associated *E. coli* following fluorescence *in situ* hybridization. From: Jergens et al. (125), with permission.

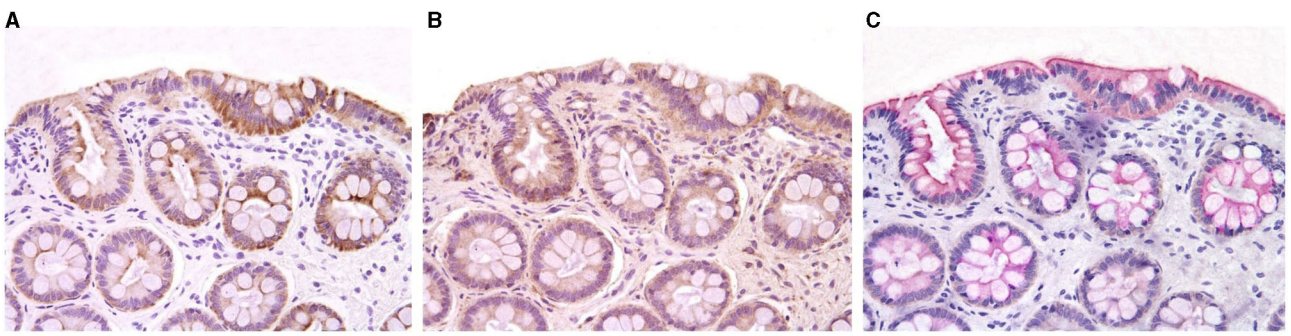

FIGURE 6 | Immunohistochemistry for expression of tight junction proteins in colonic biopsies of healthy dogs and dogs with CIE before and after probiotic VSL #3 therapy. Healthy dogs generally express increased TJPs as compared to dogs with CIE at diagnosis (pre-VSL #3). A reciprocal increase in TJP expression is observed in dogs with CIE following probiotic treatment (post-VSL #3). **(A)** Claudin expression; **(B)** E-cadherin expression; **(C)** Occludin expression. All images at 20X magnification. From: White et al. (141), with permission.

Other Murine Model Observations

Brachyspira hyodysenteriae is a Gram-negative anaerobic spirochete and is the causative agent of swine dysentery. The pathogenesis of disease has been studied in mice and pigs and has been shown to rely on the presence of a resident microbiota (146, 147), production of a ß-hemolysin (148), local inflammatory response of the host (149, 150), and recruitment of host inflammatory cells (151). With respect to the need for other resident bacteria, our own research has shown that the colonization of GF mice with *B. hyodysenteriae* failed to induce typhlocolitis in mice, even when mice were observed for 110 days post-colonization. The need for at least one member of the resident microbiota was demonstrated by administering *Bacteroides vulgatus* to GF mice previously colonized with B. hyodysenteriae (i.e., no disease) and typhlocolitis developed within 5 days. This result suggested that the presence of *B. vulgatus* either enhanced the virulence of *B. hyodysenteriae* or induced host innate immune responses that contributed to the resultant inflammatory response. Furthermore, treatment of mice with an antibiotic cocktail to which the spirochete was resistant in their drinking water for 7 days, prior to colonization with *B. hyodysenteriae*, prevented the onset of disease even though the numbers of spirochetes colonizing the cecum and colon were like that of untreated mice with typhlocolitis. In these conventionally reared mice, the role of the resident microbiota was further shown by replacing the antibiotic-containing drinking with normal drinking water and the severe typhlocolitis developed within 15 days. It was shown that the antibiotics significantly reduced the numbers of bacteria in the feces and cecal contents by six to seven $\log_{10}$ with the dominant bacterial types remaining being Gram-negative facultative anaerobes and strict anaerobes. One conclusion to be drawn from these results would suggest that the crosstalk between the host and the resident microbiota contributes to disease susceptibility and the severity of the inflammatory response (152, 153).

It has also been shown that disease caused by *B. hyodysenteriae* can be inhibited by treating mice orally with an extract (i.e., hypoxoside) from *Hypoxis hemerocallidea* corm (also known as *Hypoxis rooperi,* African Potato). Beginning seven days prior to challenge, the oral administration of hypoxoside did not prevent the colonization of *B. hyodysenteriae,* but prevented the onset of typhlocolitis as evidenced by the lack of inflammatory cell infiltration, absence of crypt hyperplasia, and reduction in the expression of cytokine-specific genes regulated by NF-kB activation (149). As with the administration of antibiotics mentioned above, the administration of hypoxoside prevented disease and expression of TNF-α-specific mRNA when treatment began at least 7 days prior to colonization with *B. byodysenteriae.* The need to initiate treatment 7 days prior to colonization with *B. hyodysenteriae* coincides with the turnover of colonic epithelial cells and suggests that the host inflammatory set-point can be altered in the new epithelial cells by affecting which bacteria are present (i.e., antibiotic use) or by changing the responsiveness of the epithelial cells to phlogistic stimuli (i.e., hypoxoside). In this regard, administration of hypoxoside also inhibited crypt epithelial cell hyperplasia following colonization with *B. hyodysenteriae* (**Figure 7**). The ability to affect epithelial cell responsiveness was further demonstrated by adding conjugated linoleic acid (CLA) to the diet of pigs prior to colonization with *B. hyodysenteriae.* It has been shown that CLA is a ligand for peroxisome proliferator-activated receptor gamma (PPAR-g) and that the activation of PPAR-g promotes mucosal epithelial health by suppression of inflammation and facilitating metabolic reprogramming (i.e., oxidative phosphorylation) of colonic epithelial cells associated with the use of SCFAs derived from microbial metabolism (150, 154). To further demonstrate that the interaction of *B. hyodysenteriae* with the colonic epithelium-induced inflammatory cell recruitment, mice that were treated with anti-CD18 or anti-CD29 to prevent extravasation of neutrophils from blood failed to develop typhocolitis (151). Using *B. hyodysenteriae* as a model of bacterial induced colitis, these studies have demonstrated that the colonic epithelium in association with the resident microbiota is a key contributor of mucosal health or disease.

In the context of IBD, epithelial barrier function is a critical component of maintaining mucosal homeostasis and tissue health. It has been shown that mice (i.e., mdr1a$^{-/-}$) lacking the multiple drug resistance gene P-glycoprotein 170 (Pg-170)

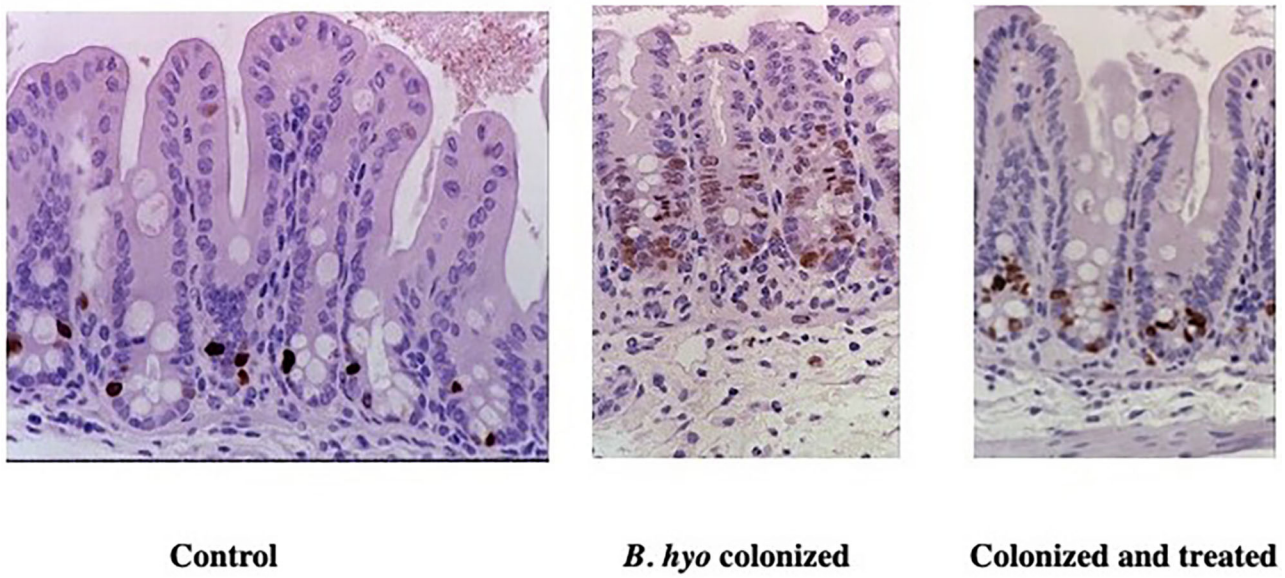

FIGURE 7 | Immunohistochemical detection of proliferating epithelial cells in mice treated with hypoxoside. Mice were either sham treated orally with sterile drinking water (Control) or hypoxoside (Colonized and treated). The mice treated daily with either saline or hypoxoside (15 mg) beginning 8 days prior to colonization with *Brachyspira hyodysenteriae (B. hyo)*. Mice were necropsied 3 days after colonization. One h prior to necropsy, mice received an IP injection of BrDU. Proliferating epithelial cells were identified by labeling their DNA with anti-BrDU using immunohistochemistry. From: (149), with permission.

develop spontaneous colitis between 8 and 30 weeks of age associated with epithelial barrier dysfunction. As an efflux pump, Pg-170 is highly expressed in colonic epithelial cells and contributes to the removal of xenobiotics and phlogistic compounds from the cytosol (155). As with many murine models of colitis, GF mdr1a$^{-/-}$ mice do not develop colitis and administration of metronidazole in the drinking water ameliorates the colitis, indicating a role for the resident microbiota in the disease process (156, 157). Like the studies performed using hypoxoside, we have shown that treating mdr1a$^{-/-}$ mice with botanical extracts from either *Prunella vulgaris* or *Hypericum gentianoides* prevented or significantly attenuated colitis in mdr1a$^{-/-}$ mice (158, 159). The reduction in colonic inflammation was consistent with the reduction of NF-kB regulated cytokines and chemokines (e.g., CXCL1, CXCL9, CCL2, CCL20, and TNF-α). In companion studies, we demonstrated that administration of caffeic acid to mice increased the expression to *Cyp4b1* (i.e., cytochrome P450) in the colonic mucosal and ameliorated DSS-induce colitis (160). Analogous to Pg-170, CYP4B1 controls the metabolism of proinflammatory compounds in the GI epithelium and contributes to maintenance of the mucosal barrier. Again, this demonstrates the central role colonic epithelial cells have in the attenuation of mucosal inflammation induced by microbial compounds and in the maintenance of mucosal homeostasis and GI health.

As IECs are also able to take up antigen and PRR ligands, they contribute to the maintenance of mucosal immunity and intestinal health. The importance of the epithelial response to luminal antigens was elegantly demonstrated by examining the inflammatory response in MyD88$^{-/-}$ mice (161). Initially, the authors had reasoned that since much of the mucosal inflammation associated with IBD was associated with production of pro-inflammatory cytokines; the absence of MyD88 should reduce the severity of disease due to impaired recognition of MAMPs derived from the microbiota. However, these authors demonstrated that the MyD88$^{-/-}$ mice developed more severe colitis than the wild-type counterparts. These observations indicated that there is a cytoprotective aspect to the local inflammatory response that is key to mucosal homeostasis. As mentioned above, we had reported that the administration of anti-CD18 or anti-CD29 attenuated lesion severity in mice colonized with *B. hyodysenteriae*. However, if mice were administered a cocktail containing both anti-CD18 and anti-CD29 or neutrophils were depleted, lesions were more severe than in sham treated mice colonized with *B. hyodysenteriae* (151). Like the MyD88$^{-/-}$ mice, the inability to recruit inflammatory cells resulted in a more severe lesion supporting the importance of epithelial cell responses to inflammatory stimuli, at least in moderation. Similarly, the administration of hypoxoside likely had a beneficial effect in inhibiting the typhlocolitis associated with *B. hyodysenteriae* colonization because it attenuated the local inflammatory responses as opposed to inhibiting that response, thus, retaining the cytoprotective benefit of the residual inflammatory response.

The role of the epithelial cells to support antigen uptake and maintenance of mucosal tolerance is partially mediated by the induction of regulatory T cells (Tregs) and the secretion of IgA (sIgA) in the the GI lumen. Functionally, one of the features of the sIgA is to provide for immune exclusion which would reduce, but not eliminate, microbial antigen interactions with epithelial cells and underlying immune cells (162). To this end,

we evaluated the ability of orally administered serum-derived bovine immunoglobulin (SBI) to inhibit DSS-induced murine colitis (163). The SBI would function to bind to bacterial antigens and reduce the innate and/or adaptive immune activation contributing to colitis. Results demonstrated that mucosal inflammation was significantly reduced, there was a decrease in secretion of pro-inflammatory cytokines and a reduction in intestinal fatty acid binding protein and serum amyloid A. As with the use of botanical extracts, dietary CLA and attenuation of neutrophil recruitment, the use of SBI to reduce mucosal inflammation by lessening the phlogistic potential of luminal content on the mucosa while allowing for the beneficial (i.e., cytoprotective) expression of host inflammatory responsiveness.

CONCLUSIONS

Host-microbe interactions play important roles in maintaining homeostasis of the mucosal epithelial barrier as well as contributing to the development of IBD. The concept that the intestinal epithelium serves as a "translator" between the intestinal microbiota and the immune system seems both logical and plausible (164). Here, the epithelium is responsive to signals from the microbiota by means of pathogen recognition receptors and translates these messages into signals that direct mucosal immune cells. Conversely, IECs receive signals from the underlying immune system and translate them into signals that shape intestinal barrier function and the structure and function of the gut microbiota. Dysregulation of the intestinal barrier is a salient feature of IBD in humans and animal models of inflammation, regardless of species. As such, treatment approaches that aim to support gut barrier function have been identified and are currently under review, including nutritional approaches (avoidance of Western-style diet, precision (FODMAP) diet, prebiotics/fibers]; probiotic approaches (select probiotics, multi-strain probiotics, symbiotic preparations); and drug/other approaches (short chain fatty acids, metformin, fecal microbiota transplantation) (14, 21, 165, 166).

AUTHOR CONTRIBUTIONS

AJ, SP, JK, and MW: review design and input, data/narrative analysis, preparation, and review/editing the manuscript. AJ, SP, and MW: performance of experiments. All authors contributed to the article and approved the submitted version.

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