IMMUNE-EPITHELIAL CROSSTALK IN INFLAMMATORY BOWEL DISEASES AND MUCOSAL WOUND HEALING

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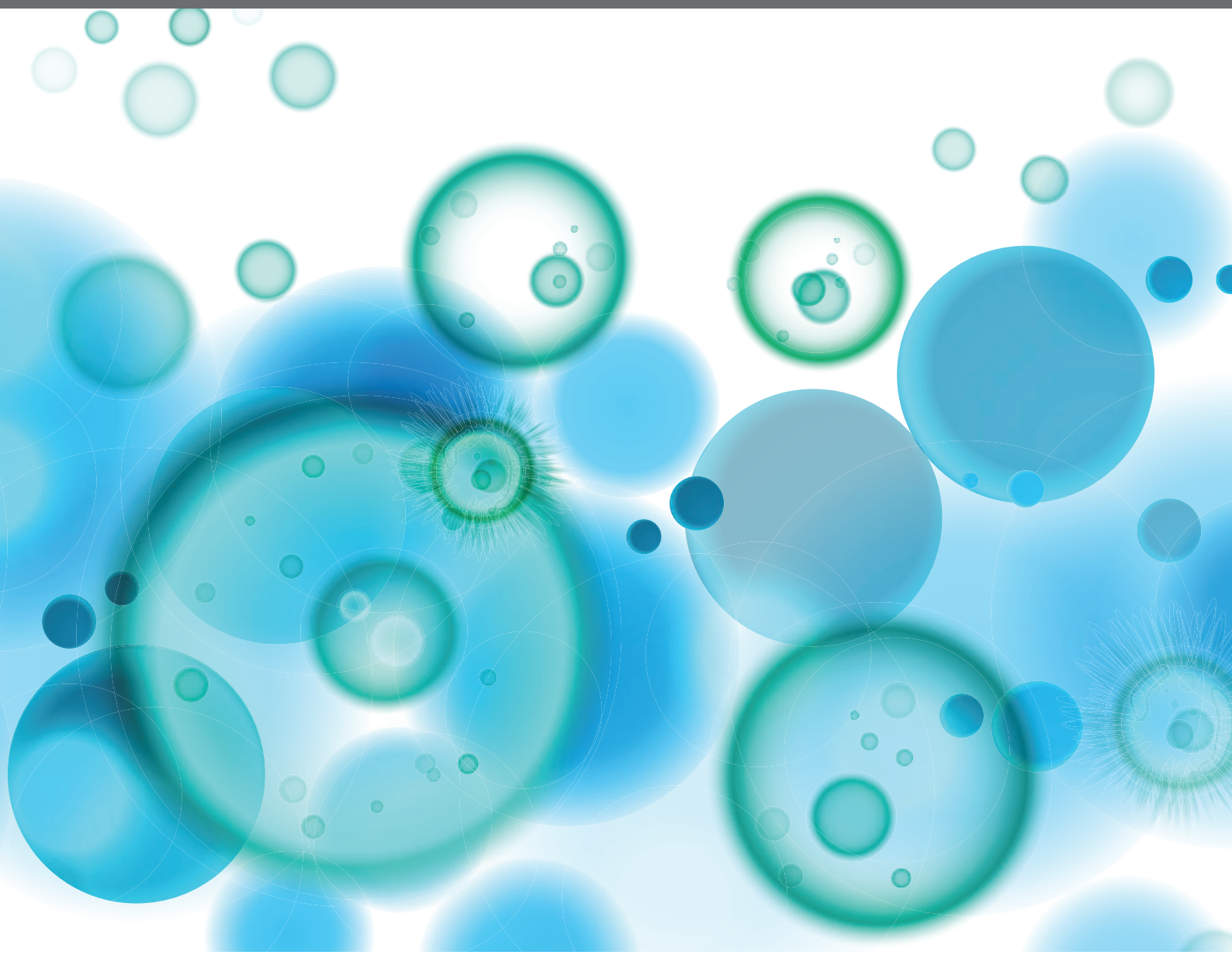

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IMMUNE-EPITHELIAL CROSSTALK IN INFLAMMATORY BOWEL DISEASES AND MUCOSAL WOUND HEALING

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EDITORIAL
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Editorial: Immune-Epithelial Crosstalk in Inflammatory Bowel Diseases and Mucosal Wound Healing

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Editorial on the Research Topic

Immune-Epithelial Crosstalk in Inflammatory Bowel Diseases and Mucosal Wound Healing

EPITHELIAL BORDER PATROL

The intestinal surface is covered by a single cell lining of columnar epithelial cells, which are perfectly equipped for tasks in nutrient absorption in the small intestine and water resorption in the colon. As these cells come into contact with a plethora of luminal constituents, the intestinal epithelium also needs to be considered as the gut's first line of defense under homeostatic conditions. The luminal microflora can be considered as a long neglected additional organ of the body, and alterations in the microbial composition have been implicated as driving elements of multiple intestinal and extraintestinal diseases (1–4). In the wake of this "microbiome era," it is of utmost importance to elucidate mechanisms, of how immune cells and epithelial cells, on the one hand, react to and, on the other hand, actively shape the intestinal microflora. In this research topic, we introduce the work of several research groups dealing with intestinal immune homeostasis. Epithelial cells are generated from intestinal stem cells at the bottom of the crypts and differentiate into distinct cell types specializing in tasks of either absorption or secretion, respectively: enterocytes are responsible for absorptive functions, whereas goblet cells and enteroendocrine cells fulfill secretory tasks (5). At the bottom of the small intestinal crypt, Paneth cells have been identified by their high granular content as distinct secretory cells, providers of antimicrobial effector molecules and crucial housekeepers of the intestinal stem cell niche (6). The group of Jan Wehkamp and Eduard Stange has substantially contributed to the concept that small intestinal Paneth cells may represent a critical cell type in the pathogenesis of ileal Crohn's disease. In this research topic, Armbruster et al. explore how monocytes direct the antimicrobial response of Paneth cells by Wnt ligands.

The highly dynamic cellular events of epithelial repopulation along the crypt–villus axis require adaptions of the epithelial cytoskeleton, cell migration, and polarity. GTPases of the Rho family direct actin network remodeling in the intestinal epithelium. Lopez-Posadas et al. have recently published a seminal study, which introduced a role of epithelial prenylation and Rho GTPases to epithelial homeostasis and implied a possible pathogenic role of these processes in inflammatory bowel diseases (IBD) (7). In this research topic, they discuss the regulation of the epithelial cytoskeleton and its adaptive response during inflammatory stress. Patterson and Watson have performed insightful studies on the regulation of intestinal epithelial shedding and its relation to cell death and shed light on this cellular process under homeostatic and inflammatory conditions (8).

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The intestinal epithelium represents the first responder to microbial assaults and is thus functionally equipped to detect microbial intruders. Coleman and (Haller) provide a concise overview on how epithelial cells sense microbial components on a molecular level and what we have learned from gnotobiotic mice. One possible consequence of pattern recognition is the assembly of multimeric protein complexes in epithelial cells, known as inflammasomes. Lei-Leston et al. focus on this specific host-protective mechanism of epithelial cells, which has raised a tremendous amount of interest in past years (9).

The epithelial response to inflammatory insults is not only governed by direct effects of pathogenic microorganisms. Khalil et al. discuss the role of transient receptor potential channels in guiding neuropeptide release and immune cell activation in experimental models of colitis (10). Furthermore, tissue-resident mesenchymal cells subjacent to the epithelial barrier fulfill multiple tasks in the cellular crosstalk at mucosal barriers. Here, Kurashima et al. shed light on various mechanisms of how tissue-resident mesenchymal cells instruct epithelia and educate the intestinal immune response.

INTESTINAL IMMUNE CELL POPULATIONS—VARIABLE REACTION FORCES

The human body is equipped with a plethora of humoral and cellular mechanisms on how to resist external hazards. The intestinal tract harbors an enormous quantity and various well-known and yet to be defined immune cell populations, which respond to microbial challenges (11). T cell populations have attracted abundant attention and represent the primary target of successful therapeutic strategies in the treatment of IBD (12). Various strategies have evolved and target activation (azathioprine, cyclosporine, and anti-TNF) and differentiation (anti-IL-12/IL-23) of effector T cells, as well as their homing to the intestinal mucosa (anti-integrins). In this part of the series, Konjar et al. discuss the contribution of intestinal CD8 T cells to intestinal immune homeostasis. Intestinal T cell responses are subject to tight checks and balances. Effector T cell responses are suppressed by regulatory T cell populations, which enforce intestinal immune homeostasis (13, 14). In this issue, Wiesinger et al. provide an update on efforts to restore the balance of effector and regulatory T cells in ulcerative colitis by adoptive transfer of *ex vivo* expanded patient-derived autologous regulatory T cells. Kempski et al. discuss how specific effector cells, $CD4^+$ Th17 cells, orchestrate epithelial adaptions to specific inflammatory and neoplastic cues (15). Before being able to give rise to tissue-destructive immune responses, T cells need to home to the mucosa by transendothelial migration (Zundler et al.). Zundler et al. focus on molecular and functional mechanisms of T cell homing to the intestinal mucosa and the effects of anti-integrin strategies (Fuchs et al.).

Apart from understanding disease-driving molecular mechanisms, it is instrumental to discover ways to resolve inflammation (16). Ungaro et al. emphasize the role of specific lipid mediators in this process that actively determine the resolution phase of inflammation.

A picture is worth a thousand words. Waldner et al. provide insights into state-of-the-art methods on how to visualize inflammation and immune–epithelial crosstalk both *ex vivo* and *in vivo* in clinical applications using advanced imaging techniques including multiphoton microscopy and endomicroscopy. They describe the current state of the art and novel translational efforts to make the most out of advanced optical tools and their use in predicting the response to therapy.

Taken together, in this research topic, we propose that IBD develop as the consequence of a dysregulated immune–epithelial communication. Insufficient handling of environmental stressors by the intestinal epithelium would thus induce a devastating T-cell-guided immunopathology. The integrated approach of this research topic, linking immunology to epithelial biology, highlights avenues on how to advance the field for the future benefit of affected patients.

AUTHOR CONTRIBUTIONS

ML drafted the manuscript. All the authors edited the manuscript.

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MINI REVIEW
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Functional Role of Transient Receptor Potential Channels in Immune Cells and Epithelia

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Transient receptor potential (TRP) ion channels are widely expressed in several tissues throughout the mammalian organism. Originally, TRP channel physiology was focusing on its fundamental meaning in sensory neuronal function. Today, it is known that activation of several TRP ion channels in peptidergic neurons does not only result in neuropeptide release and consecutive neurogenic inflammation. Growing evidence demonstrates functional extra-neuronal TRP channel expression in immune and epithelial cells with important implications for mucosal immunology. TRP channels maintain intracellular calcium homeostasis to regulate various functions in the respective cells such as nociception, production and release of inflammatory mediators, phagocytosis, and cell migration. In this review, we provide an overview about TRP-mediated effects in immune and epithelial cells with an emphasis on mucosal immunology of the gut. Crosstalk between neurons, epithelial cells, and immune cells induced by activation of TRP channels orchestrates the immunologic response. Understanding of its molecular mechanisms paves the way to novel clinical approaches for the treatment of various inflammatory disorders including IBD.

Keywords: colitis, macrophages, cytokines, neuropeptides, neurogenic inflammation, nociception

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INTRODUCTION

The transient receptor potential (TRP) ion channel family consists of 28 members, which are divided into six subsets: TRPC ("canonical"), TRPM ("melastatin"), TRPV ("vanilloid"), TRPA ("ankyrin"), TRPML ("mucolipin"), and TRPP (or PKD) ("polycystin") (1). TRP channels are membrane proteins with substantial cation permeability, preferentially high calcium ion permeability, and calcium signaling plays a central role in many physiological processes. TRP receptors are polymodal ion channels with an exceptional role in the integration of various environmental stimuli including mechanical, thermal, or chemical stimuli. Inhering this function they are likely to be sensors for monitoring specific responses to different exogenous and endogenous chemical noxious and physical stimuli. As such, various TRP channels play an essential role in somatic and visceral nociception (2, 3). Upon activation, TRP channels also control the release of immunomodulatory neuropeptides such as substance P (SP) and calcitonin gene-related peptide (CGRP), the so-called neurogenic inflammation. During recent years, increasing evidence has demonstrated an important role of many TRP channels outside the nervous system in the context of inflammation; findings that extend the role of TRP channels in the regulation of inflammation beyond neuropeptide release. To date, only little is known about the functional role of TRP channels in the immune system. Moreover, recent reports describe a fundamental role of TRP channels in epithelial cells in mediating cytokine/

chemokine release as well (4). In summary, TRP channel activation induces immunomodulatory effects on multiple levels. This review will focus on the role of TRP channels in immune cells (with a focus on macrophages and T cells) and epithelial cells in general, with an additional special focus on TRPs in intestinal inflammation. Recognizing the large family of TRP channels, this mini-review focuses on TRPA1, TRPM8, TRPV1, and TRPV4 (alphabetical order), which are the most relevant TRP channels in the present context based on published literature until today. Table S1 (available in supplementary material) gives an overview of receptor expression and resulting biological effects in the different cellular compartments.

TRP CHANNEL FUNCTION—GENERAL ROLE IN IMMUNE CELLS AND EPITHELIA

TRPA1

TRPA1 is an irritant receptor that belongs to the ankyrin subfamily and is highly co-expressed with TRPV1 in a subset of sensory neurons (5). Only very little is known to date about TRPA1 expression in immune cells. The role of TRPA1 in macrophages was recently investigated in the context of the pathogenesis of atherosclerosis. Oxidized low-density lipoprotein (oxLDL) and the prototypical TRPA1 agonist allyl isothiocyanate (pungent ingredient in garlic) induced calcium transients in bone marrow-derived macrophages *via* TRPA1. TRPA1 expression was found to be upregulated in macrophage foam cells in atherosclerotic aortas of apolipoprotein E-deficient ($apoE^{-/-}$) mice. Treatment with a selective TRPA1 antagonist HC030031 (HC) led to aggravation of oxLDL-induced lipid accumulation and subsequently exacerbated atherosclerotic lesions in $apoE^{-/-}$ mice. In addition, HC-treated $apoE^{-/-}$ mice showed increased levels of serum HDL, triglycerides, total cholesterol, and the pro-inflammatory cytokines IL-1β, TNF-α, MCP-1, IL-6, and macrophage inflammatory protein-2 (MIP-2), which suggested a crucial anti-inflammatory role of TRPA1 in the pathogenesis of atherosclerosis and cholesterol metabolism of macrophage foam cells (6). Previously, another group provided evidence about the functional expression of TRPA1 in peritoneal macrophages. LPS-stimulated cannabichromene-treated (CBC, cannabinoid TRPA1 agonist) peritoneal macrophages showed a significantly decreased level of nitrite (stable metabolite of nitric oxide) compared to LPS-stimulated peritoneal macrophages without CBC pretreatment. Nitric oxide acts as an abundant pro-inflammatory mediator, which indicates anti-inflammatory effects of TRPA1 activation by CBC in peritoneal macrophages. Interestingly, the TRPA1 antagonists AP-18 and HC had almost the same inhibitory effect on the nitrite production as TRPA1 activation, which indicated that the effect of TRPA1 agonists was due to receptor activation and subsequent desensitization (7). The TRPA1 agonists acrolein and crotonaldehyde were able to excite the release of TNF-α and IL-8 (CXCL8, a potent neutrophil chemoattractant) from the human macrophage cell line U937, whereas acrolein induced release of IL-8 from the THP-1 macrophage cell line and from human alveolar macrophages. In addition, the lipid peroxidation product 4-hydroxy-2-nonenal (4-HNE), a mediator of oxidative stress and a TRPA1 agonist was found to be upregulated in lungs of patients with chronic obstructive pulmonary disease (COPD), and induced release of IL-8 from U937 cells. Conversely, saturated aldehydes had no effect. This indicated that alpha,beta-unsaturated aldehydes such as 4-HNE (an ingredient of cigarette smoke) are likely to be pivotal in activating macrophages that may ultimately result in the destructive inflammatory reaction involved in the course of disease in COPD (8).

TRPA1 is also expressed in cultured human airway cells including epithelial cells, smooth muscle cells, and fibroblasts. *In vitro*, acrolein and cigarette smoke aqueous extract (CSE) (both TRPA1 agonists) induced the release of IL-8 TRPA1 dependently which was reduced by pharmacological TRPA1 blockade. TRPA1 expression was highly co-localized with TRPV1 expression in airway sensory nerves and the activation of both TRPA1 and TRPV1 channels induced the release of the pro-inflammatory neuropeptide SP. Interestingly however, the pro-inflammatory effects of acrolein and CSE were independent of sensory neuronal activation. After 24 h of intra-tracheal instillation with both compounds, neutrophil chemoattractant chemokine (KC) was increased in bronchoalveolar lavage (BAL) independent of pretreatment with a SP receptor/NK1 antagonist. In contrast, intra-tracheal instillation of capsaicin or SP had no effect on KC levels. Furthermore, pretreatment with a TRPA1 antagonist (HC) decreased KC release. Moreover, BAL from TRPA1-deficient mice did not show any release of acrolein- and CSE-induced KC. Thus, KC accumulation-derived inflammation was independent of neurogenic factors, and non-neuronal TRPA1 was shown to be essential in this model of inflammatory airway disorder (9).

In line with these observations of TRPA1 acting as an immune modulator, TRPA1 expression was detected by northern blot, western blot, and immunohistochemical methods in Jurkat T cells as well as in human splenocytes (10). Bertin et al. also confirmed the expression of mouse and human TRPA1 at mRNA and protein level in murine T cells (11).

TRPM8

TRPM8 is characterized in peripheral sensory neurons as a cold sensor (12, 13). Increasing evidence is accumulating that TRPM8 might also be implicated in inflammatory disorders. Previously, direct evidence for TRPM8 expression in macrophages was reported. TRPM8-like channels could be activated by the TRPM8 agonist icilin measured by the patch-clamp technique in RAW 264.7 macrophages (14). Recently, we observed evidence for TRPM8 expression in several populations of murine macrophages, which modulated inflammatory responses. *In vitro*, TRPM8 activation by menthol induced an anti-inflammatory cytokine profile in murine peritoneal macrophages (increased IL-10 and decreased TNF-α release) (15). Consistently, 1,8-cineol (eucalyptol) and L-menthol (both TRPM8 agonist) were able to inhibit the production of pro-inflammatory cytokines in human monocytes and lymphocytes *in vitro* (16, 17). In our own studies, activation of TRPM8 in wild-type (WT) but not in TRPM8-deficient peritoneal macrophages enhanced phagocytosis of zymosan beads. *In vivo*, phagocytic activity of peritoneal

macrophages was impaired in TRPM8-deficient mice compared to WT controls (15).

TRPM8 was also found in human lung epithelial cells. Activation of this channel increased the expression of several cytokines and chemokines, including TNF-α, IL-4, IL-13, IL-1α, and IL-1ß, that modulate the response of other resident cell types in the lung such as immune cells, smooth muscle cells, and sensory neurons (18).

TRPV1

TRPV1 is expressed in sensory neurons of dorsal root ganglia (DRG), trigeminal, and vagal ganglia (19). Due to its high abundance in nociceptive neurons, TRPV1 acts as a nociceptor marker (20). Most of the TRPV1 expressing DRG neurons co-express peptidergic markers such as SP and CGRP (20). TRPV1 is activated by noxious stimuli such as capsaicin, extracellular acidification, or heat and is sensitized or activated by inflammatory mediators *in vitro* (21–23). Zhao and colleagues found that oxLDL-stimulated bone marrow-derived macrophages showed an increased level of TRPV1 expression and oxLDL activated TRPV1 which led to intracellular Ca^{2+}-transients that were abolished by superfusion with the TRPV1 antagonist capsazepine. Capsazepine aggravated the oxLDL-induced lipid accumulation and induced the production of MCP-1 and IL-6 in macrophages. In contrast, pretreatment of bone marrow-derived macrophages with evodiamine or capsaicin (TRPV1 agonists) alleviated lipid accumulation and impaired the production of MCP-1, MIP-2, and IL-6 (24). Capsaicin application to LPS- and IFN-γ-stimulated RAW 264.7 macrophages exhibited inhibitory effects on iNOS and the production of NO, COX-2, and PGE_2 in a concentration-dependent manner. Capsazepine failed to abolish the effect of capsaicin but rather showed similar inhibitory effects even synergistic with capsaicin on PGE_2 released from LPS-stimulated peritoneal macrophages (25). In another report, capsaicin failed to modulate the protein/mRNA levels of COX-2, whereas capsazepine exhibited an inhibitory effect on the COX-2 levels produced from LPS-stimulated peritoneal macrophages (25). Both capsaicin and capsazepine decreased iNOS mRNA levels in LPS/IFN-γ-stimulated peritoneal macrophages in a concentration-dependent manner (25). Since iNOS and COX-2 are regulated by transcription factors like nuclear transcription factor kB (NF-kB) (26), it was examined, whether capsaicin or capsazepine regulated the activation of NF-kB. Both, capsaicin and capsazepine, blocked the degradation of IkB-a induced by LPS-stimulated peritoneal macrophages, reflecting that both capsaicin and capsazepine inhibit the activation of NF-kB. Due to the inability of capsazepine to block the effect of capsaicin, the authors suggested that peritoneal macrophages do not express TRPV1 but rather a TRPV1-like protein (25). Intriguingly, these results become more complicated in their interpretation since we were recently able to show that capsazepine is also a potent TRPA1 agonist (2).

In a sepsis model of cecal ligation and puncture, LPS-stimulated TRPV1-deficient peritoneal macrophages showed impaired phagocytosis compared to unstimulated controls (27). LPS/SP co-stimulated $TRPV1^{-/-}$ macrophages were shown to restore phagocytic activity, an effect that was abolished by pretreatment with a selective antagonist of the SP/NK1 receptor. In contrast, CGRP-stimulated TRPV1-deficient macrophages did not show a significant difference in response to LPS (27). Furthermore, a TRPV1 antagonist decreased phagocytosis of LPS-stimulated WT macrophages compared to control cells (27). Recently, a previously unknown role of the endocannabinoid system in regulating immune homeostasis TRPV1 dependently was reported. Activation of TRPV1 by capsaicin induced production of the endocannabinoid anandamide in myeloid cells and promoted the presence of immunosuppressive $CXCR1^{hi}$ macrophages *via* anandamide acting on CB2 receptors expressed in the enteric nervous system. Moreover, this mechanism also provided protection from experimental autoimmune diabetes (28).

Amantini et al. could demonstrate that distinct thymocyte subsets express TRPV1, which is required for capsaicin-induced apoptosis (29). Functional expression of TRPV1 was furthermore shown by Bertin et al. in primary murine $CD4^+$ T cells (30). The authors could show that the co-stimulatory molecules CD4 and TRPV1 were co-localized within the plasma cell membrane of $CD4^+$ T cells and stimulation with capsaicin triggered calcium ion influx.

In bronchial epithelial cells, TRPV1 was able to control the expression of pro-inflammatory cytokines such as IL-6 and IL-8 due to its modulation of calcium traffic between the intra- and extracellular compartment (31). IL-8 is an essential chemotactic protein produced by neutrophils in the lung (32), which provides the molecular basis for a vital epithelial–immune interaction.

TRPV4

TRPV4 was originally identified to be involved in the regulation of osmotic homeostasis (33). Furthermore, TRPV4 is activated by mechanical stress and non-noxious heat (34). The prominent role of TRPV4 in visceral nociception was highlighted by recent work (35, 36). Beyond TRPV4 function in neurons, several reports suggest an important physiological function of non-neuronal TRPV4. TRPV4 is expressed in lung and gut epithelial cells and immune cells including macrophages, monocytes, neutrophils, and T cells (4, 37). Functional TRPV4 expression was recently observed in bone marrow-derived macrophages. The selective TRPV4 agonist GSK1016790A increased the intracellular calcium ion concentration in a dose-dependent manner, an effect that was inhibited by TRPV4 siRNA or a pharmacological blocker and completely abolished in TRPV4-deficient bone marrow-derived macrophages. Using fluorometry *in vitro* and quantifying phagocyted particles *in vivo*, the authors also observed impaired phagocytosis after downregulation of TRPV4 with siRNA in LPS-treated bone marrow-derived macrophages (38).

TRPV4 expression was shown in human airway epithelial cell lines (A549, Beas 2B, and NCI-H292) and primary airway epithelial cells. Selective TRPV4 agonists were shown to trigger calcium influx in NCI-H292 and increased the release and secretion of IL-8 and PGE_2 in a time- and concentration-dependent manner (37). *In vivo,* intranasal administration of the TRPV4 agonist 4α-PDD enhanced the concentration of KC, which subsequently led to the recruitment of neutrophils in BAL fluids in WT but not TRPV4-deficient mice (37), indicating an *indirect*

epithelial–immunological interaction. However, a *direct* role for TRPV4 expression in the function of murine alveolar macrophages was also demonstrated. 4α-PDD triggered calcium influx and subsequently production of superoxide and nitric oxide in alveolar macrophages of WT but not TRPV4-deficient mice. Likewise, the adoptive transfer of TRPV4-expressing alveolar macrophages into lungs of TRPV4$^{-/-}$ mice restored hypersusceptibility in a model of ventilator-induced mechanical injury (39).

In line with these pro-inflammatory functions of TRPV4, Majhi and colleagues found endogenous expression of different TRPV isoforms in Jurkat, primary human T cells, and mouse T cells isolated from spleens. Moreover, they observed calcium influx in T cells upon treatment with different TRPV4 agonists. Furthermore, *in vitro* activation of TRPV4 in T cells resulted in upregulation of TRPV1 and TRPV4 channels, T cell proliferation, and production of the pro-inflammatory cytokines IFN-γ, TNF-α, and IL-2. This effect was blocked pharmacologically suggesting that TRPV4-mediated calcium influx might play a crucial role in T cell-mediated immune responses (40).

TRP CHANNELS IN IMMUNE CELLS AND EPITHELIA—ROLE IN MODELS OF INTESTINAL INFLAMMATION

The majority of published literature ascribes the role of TRP channels in modulation of experimental colitis to its capacity to attenuate neuropeptide release and subsequently neurogenic inflammation. In accordance with this, we have found that TRPA1 and the pro-inflammatory neuropeptide SP in extrinsic primary afferent neurons are fundamental for the development of TNBS colitis. Pharmacological blocking of TRPA1 attenuated chronic colitis through inhibition of neuropeptide release (41). However, the role of extra-neuronally expressed TRP channels in the pathogenesis of intestinal inflammation is emerging and results in a complex crosstalk of different cellular compartments (see **Figure 1**). A recent study stressed the important role of extra-neuronal TRPA1 and TRPV1 receptor expression in this context. TRPA1 and TRPV1 were expressed in macrophages and epithelial cells in both healthy and inflamed human and murine

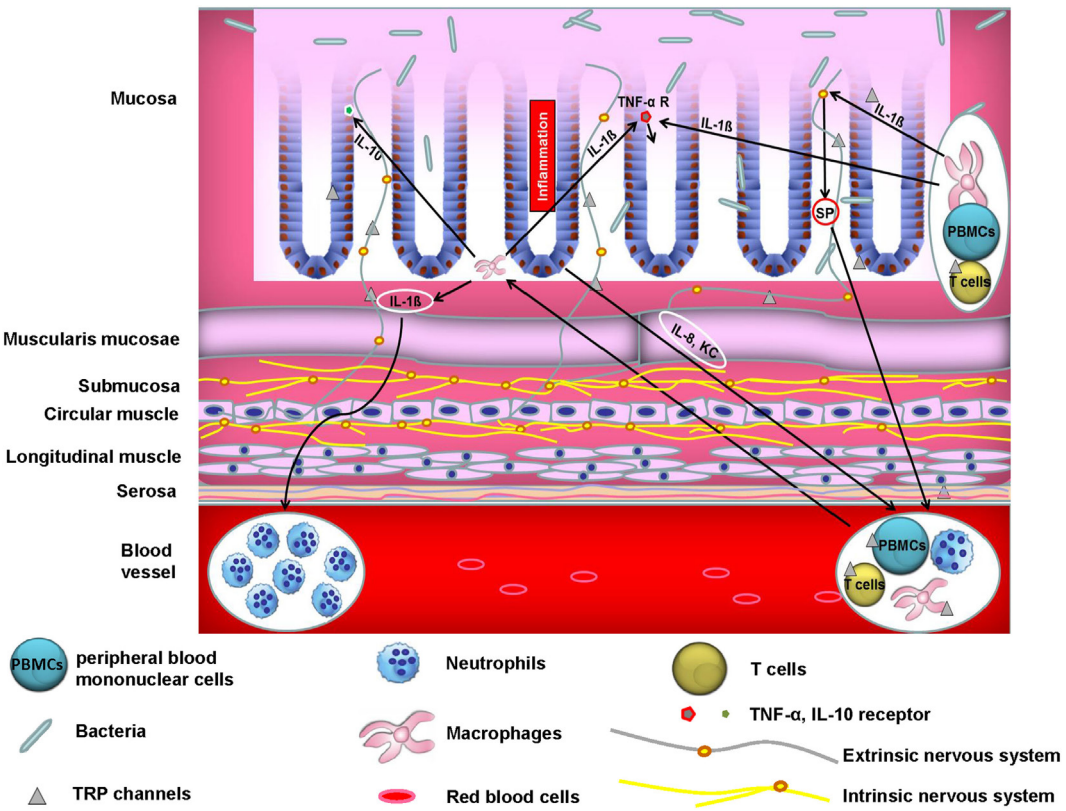

FIGURE 1 | Crosstalk between neurons, immune cells, and epithelial cells controls colonic homeostasis. A vital interplay between neurons, immune cells, and epithelial cells is crucial for colonic homeostasis. Aberrant function of one or more of these players may cause inflammation. For instance, activated macrophages release IL-1ß which among other cytokines acts as a chemoattractant to human peripheral blood mononuclear cells (PBMCs) (45). On the other hand, IL-1ß increases TNF-α receptor expression in epithelial cells which may promote TNF-α-mediated inflammatory responses and subsequently colitis. Moreover, IL-1ß acts on peptidergic sensory neurons through induction of pro-inflammatory substance P (SP) release. SP in turn induces migration of polymorphonuclear leukocytes *in vivo* (46). In addition, epithelial IL-8 and KC induce immune cell infiltration into the colonic wall. IL-10 is an essential immunoregulatory cytokine (47). Epithelial cells from murine small intestine and colon express the IL-10 receptor and its stimulation blocks IFN-γ-mediated pro-inflammatory effects (48). Moreover, not only epithelial cells but also enteric neurons express cytokines and chemokines such as IL-8, whose neuronal production is promoted by IL-1ß *via* MAPK signaling pathways (49).

colons (42). Since macrophages are major producers of TNF-α and activation of TRPA1 was able to impair the expression of TNF-α in distal colon homogenates during colitis, it is likely that TRPA1 in macrophages mediated the anti-colitogenic effect. In addition, employing different mouse models of colitis including IL-10 knockout mice as well as adoptive T cell transfer models of colitis, it was shown that the genetic deletion of TRPA1 in $CD4^+$ T cells caused intestinal inflammation *via* induction of the transcription factor Tbet which subsequently increased production of the pro-inflammatory cytokines IFN-γ and IL-2 (11) and resulted in a higher capacity to differentiate into TH1 effector cells. Additionally, $CD4^+$ T cells isolated from TRPV1 knockout mice showed impaired calcium influx upon T cell receptor stimulation, resulting in the inactivation of the transcription factors NFAT and NFkB. Likewise, TRPV1-deficient $CD4^+$ T cells failed to induce colitis in transfer models of colitis as TRPV1-deficient $CD4^+$ T cells showed decreased production of the pro-inflammatory cytokines IFN-γ, TNF-α, and IL-17 (30). An important extra-neuronal role of TRPV4 was recently demonstrated with regard to colonic inflammation. TRPV4 mRNA was found to be upregulated in colonic intestinal epithelial cells from mice with dextran sulfate sodium (DSS)-induced colitis. TRPV4 activation led to chemokine and cytokine release such as IL-8, IP-10, MIG, and MCP-1, which indicated a potential role of TRPV4 in activating pro-inflammatory signaling pathways that might induce the recruitment of macrophages and other immune cells. In addition, intrarectal enemas with the TRPV4 agonist 4α-PDD induced acute and chronic colonic inflammation in mice (4). Finally, we could recently show that mice that were reconstituted with TRPM8-deficient macrophages exhibited increased susceptibility to DSS, demonstrating a fundamental role of constitutive TRPM8 expression in macrophages in the context of colitis (15). Two recent reports, however, favor a dominant role for TRPM8 expression in controlling neuropeptide release and, thus, colitis development (43, 44). TRPM8 was shown to modulate the release of CGRP in the colonic microenvironment from peptidergic sensory neurons, which might have directed the protective effects of CGRP on $CD11^+$ dendritic cells (44).

CONCLUSION

The role of TRP channels reaches beyond the control of immunomodulatory neuropeptide release from sensory nerve endings. Many TRP channels are expressed in various immune cells, especially in macrophages and T cells. Here, they modulate many functions such as cytokine expression and release, migration, or phagocytic activity. Moreover, in a third compartment, the epithelial layer, TRP channel expression was also found to be relevant in the pathogenesis of many inflammatory disorders mainly through controlling chemokine/cytokine expression and release. Thus, a vital interplay between neurons, epithelia, and mucosal immune cells seems to maintain homeostasis in different organs, for example, the gut, the lung, and the vascular system and disruption of one or more of these players may induce disease (see **Figure 1**). Thus, targeting TRP channels and neuropeptide receptors might represent a promising new therapeutic approach in various inflammatory disorders such as inflammatory bowel disease, asthma, COPD, and atherosclerosis.

AUTHOR CONTRIBUTIONS

MK: conception and writing of the manuscript; KA: conception and writing of the manuscript; CW and CY: conception and writing of the manuscript; SW and CB: conception of the manuscript, revising it critically for important intellectual content, and final approval of the version to be published; ME: conception and writing of the manuscript, revising it critically for important intellectual content and final approval of the version to be published.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fimmu.2018.00174/full#supplementary-material.

TABLE S1 | Expression and biological effects of the different TRP channels in immune cells and epithelia.

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REVIEW
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Bacterial Signaling at the Intestinal Epithelial Interface in Inflammation and Cancer

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The gastrointestinal (GI) tract provides a compartmentalized interface with an enormous repertoire of immune and metabolic activities, where the multicellular structure of the mucosa has acquired mechanisms to sense luminal factors, such as nutrients, microbes, and a variety of host-derived and microbial metabolites. The GI tract is colonized by a complex ecosystem of microorganisms, which have developed a highly coevolved relationship with the host's cellular and immune system. Intestinal epithelial pattern recognition receptors (PRRs) substantially contribute to tissue homeostasis and immune surveillance. The role of bacteria-derived signals in intestinal epithelial homeostasis and repair has been addressed in mouse models deficient in PRRs and signaling adaptors. While critical for host physiology and the fortification of barrier function, the intestinal microbiota poses a considerable health challenge. Accumulating evidence indicates that dysbiosis is associated with the pathogenesis of numerous GI tract diseases, including inflammatory bowel diseases (IBD) and colorectal cancer (CRC). Aberrant signal integration at the epithelial cell level contributes to such diseases. An increased understanding of bacterial-specific structure recognition and signaling mechanisms at the intestinal epithelial interface is of great importance in the translation to future treatment strategies. In this review, we summarize the growing understanding of the regulation and function of the intestinal epithelial barrier, and discuss microbial signaling in the dynamic host–microbe mutualism in both health and disease.

Keywords: intestinal epithelium, intestinal microbiota, bacterial signaling, inflammation, colorectal cancer

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INTRODUCTION

The human gastrointestinal (GI) tract represents the most densely colonized organ of the body, with the highest microbial load of 10^{11} bacteria/mL content in the colon (1, 2). Bacteria dominate the microbial ecosystem in the GI tract, with more than 90% belonging to the phyla *Bacteroidetes* and the *Firmicutes* (3–5). Despite considerable progress the functional complexity of the microbiome is still unresolved, and to date, mechanisms of microbe–host interactions involve a pleiotropic network of immune, metabolic, and trophic functions (1, 6). Studies in germ-free animals recognized the essential role played by the intestinal microbiome in the development and regulation of the mucosal immune system during early life (7–12). While many organisms have been shown to fulfill protective functions in the GI tract and are critical for host physiology, complex shifts in the community structure and abundance of certain microbes have been associated with the onset of inflammatory

and tumorigenic diseases, such as inflammatory bowel diseases (IBD) and colorectal cancer (CRC) (6, 13–15).

Loss of epithelial barrier function and innate immunity are fundamental to the pathogenesis of inflammatory and infectious diseases. The intestinal immune system has the challenge of responding to pathogens, while remaining tolerant to food antigens and the commensal microbiota. The intestinal epithelium executes a compartmentalization between the lumen and the host, simultaneously acting as a selectively permeable first line of defense to fulfill its function of absorption, while maintaining an effective barrier against the intestinal microbiota, antigens and toxins. Intestinal epithelial cells (IECs) express pro-inflammatory cytokines in response to infectious invasive bacteria (16), but largely ignore non-pathogenic commensals (17). Certain intestinal pathogens (18, 19) and opportunistic commensals (20), however, can evade this first line of defense and enter IECs, suggesting that the existence of epithelial cell-intrinsic immune mechanisms for bacterial detection and limitation are essential. One key cell-autonomous mechanism of antibacterial defense is intestinal epithelial autophagy, shown to be activated following bacterial invasion through adaptor protein myeloid differentiation primary response gene-88 (MyD88) cell-intrinsic signaling, with autophagy-deficiency in mice causing increased dissemination of invasive bacteria (21), indicating that autophagy could have a broader role in inflammatory disease. IECs and innate immune cells of the lamina propria are able to differentiate self from non-self through a selective spatial and cellular expression of pattern-recognition receptors (PRRs) (22). Classically the detection of pathogen-associated molecular patterns (PAMPs) allows the intestinal epithelium to activate signaling pathways that induce the early host response to infection. The role of microbe-associated molecular patterns (MAMPs) in mediating innate recognition of the commensal "non-infectious" microbiota remains controversial. Paradoxically, recent progress in understanding IBD pathogenesis suggests that a defective innate immune system predisposes the host toward chronic inflammation (23, 24), supporting a protective role of PRR signaling in maintaining intestinal tissue homeostasis. Early work related to the activation of inflammation-related transcription factors, such as the nuclear factor kB (NF-kB), suggested a hormetic adaptation of the epithelium in response to commensal bacteria (25, 26), with elegant studies related to epithelial cell-specific inhibition of NF-kB activation validating the importance of this signaling pathway in maintaining tissue homeostasis (27). This paradigm shift was supported by Medzhitov and colleagues, demonstrating that microbiota-derived signals *via* the toll-like receptor (TLR)-related adaptor protein MyD88 protect mice from the development of colitis (28) and intestinal tumor formation (29). Thus, bacteria (dead or alive) and their metabolites form key mediators for the cross-talk between IECs and other mucosal cell types, through the interaction with host PRRs.

Although it is recognized that the intestinal microbiota has profound influences on health and disease, the understanding of the precise mechanism(s) by which this is exerted remains largely unknown (30). This review summarizes our knowledge of specific bacterial interactions and signaling mechanisms at the intestinal epithelial interface. We discuss bacterial signaling in inflammation and cancer, and reflect on how increasing knowledge of such mechanisms might be translated to the benefit of patient care.

THE INTESTINAL EPITHELIUM: OUR DYNAMIC PROTECTIVE BARRIER

In spite of the symbiotic nature of the microbe–host relationship, the close proximity of bacteria to intestinal tissue poses a considerable health challenge. An effective and dynamic intestinal epithelial barrier is therefore crucial to conserve a compartmentalized microbe–host interaction and tissue homeostasis (**Figure 1**). In the healthy organ, the epithelium maintains a distinct anatomical barrier relevant for a constant state of homeostasis, while being exposed to a myriad of environmental stimuli that include, but are not limited to, microbes, dietary products and inorganic materials (31). A single-cell layer of IECs forms a continuous physical barrier, with tight junctions connecting adjacent IECs and associating with cytoplasmic actin and myosin networks that regulate intestinal permeability (32). Long-lived pluripotent stem cells located at the base of intestinal crypts continuously produce tissue-specific precursor cells that transit through a differentiation pathway that gives rise to absorptive lineage cells (enterocyte/colonocyte) or secretory lineage cells (goblet, Paneth, enteroendocrine and tuft) (33). IECs represent not only a physical barrier but also contribute to intestinal health through the production of mucus (goblet cells) and the secretion of antimicrobial peptides (AMPs) (Paneth cells).

Goblet cells secrete mucin glycoproteins, of which Muc2 is the main constituent of the approximately 150-μm thick (in the mouse) colonic mucus layer (34). While the mucus layer in the small intestine consist of a single layer, in the colon, two structurally distinct mucus layers are formed; an inner mucus layer that is devoid of bacteria, and an outer mucus layer that forms a habitat for a large number of bacteria (35, 36). In addition to mucins, goblet cells secrete a range of bioactive molecules such as trefoil factor peptides (TFFs), resistin-like molecule β (RELMβ), and Fc-γ binding protein (37). Intestinal Paneth cells are the main source of AMPs that function in host defense and in establishing and maintaining the intestinal microbiota (38, 39). Secretory immunoglobulin A (sIgA) directed against intestinal bacteria and produced by Lipopeptide/lipoprotein (LP) plasma cells, binds the polymeric immunoglobulin receptor (pIgR), and transcytoses across the epithelium to prevent microbial translocation across the epithelial barrier (40, 41). This concerted interplay between plasma cells and IECs provides an adaptive immune element to the intestinal epithelial barrier. Also found scattered throughout the LP are T cells, stromal cells, and antigen presenting cells such as dendritic cells (DCs) and macrophages. Specialized IECs, called microfold (M) cells, and goblet cells facilitate the transport of luminal antigens and bacteria across the intestinal epithelial barrier to DCs, with macrophages sampling through trans-epithelial dendrites (42–44). Under steady-state conditions, the intestinal immune system detects commensal bacteria and provides basal signals without the full activation of adaptive immune responses (7).

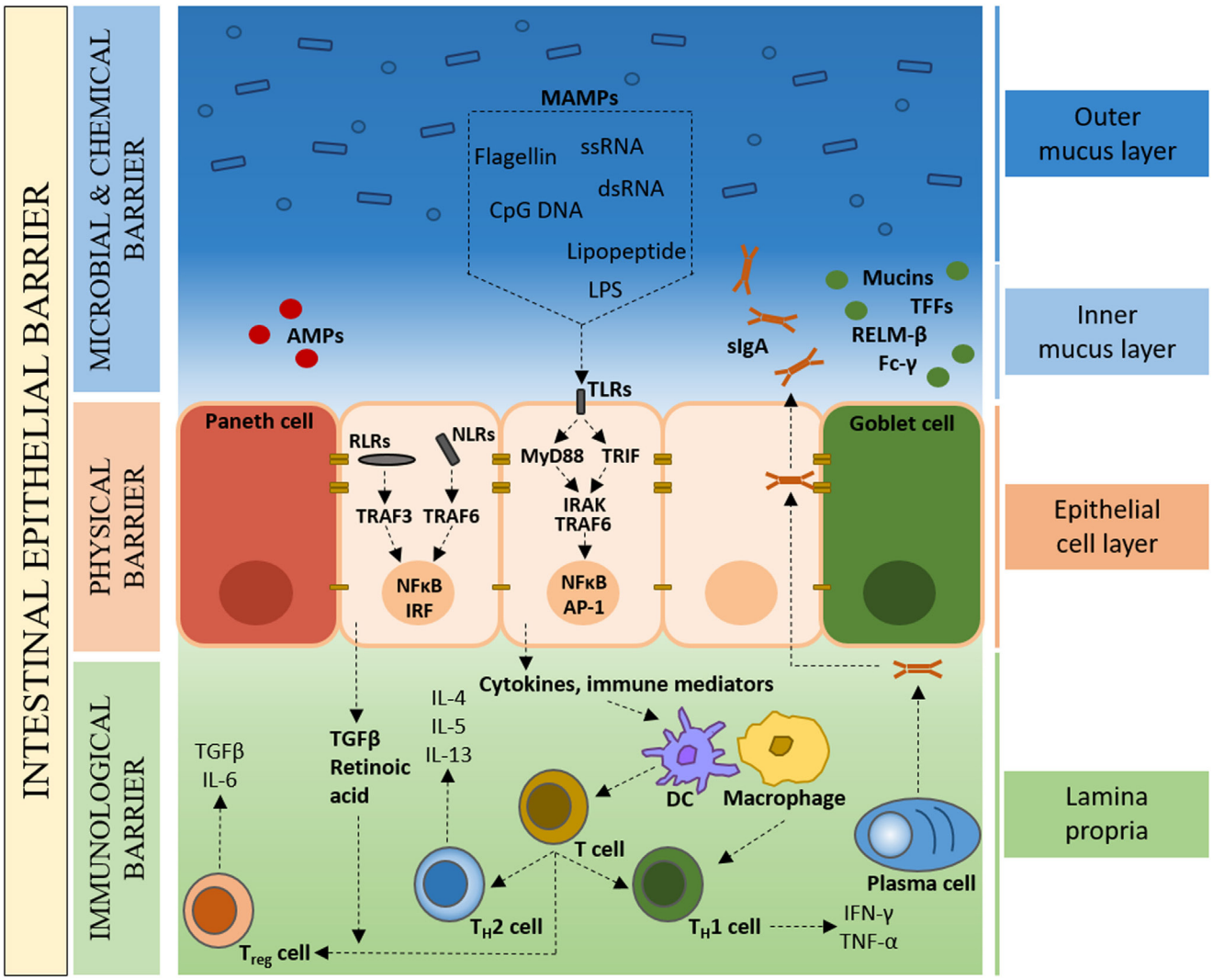

FIGURE 1 | The colonic intestinal epithelium as a dynamic protective barrier. The single-cell layer (10 μm) of intestinal epithelial cells (IECs), which is comprised of distinct subpopulations, separates the luminal intestinal microbiota from the underlying tissue, forming a physical barrier. Overlying the IECs is the microbial and chemical barrier, mainly composed of the mucus layer(s). Goblet cells secrete mucins, which form a proteoglycan gel to create an inner mucus layer that is devoid of bacteria, and an outer mucus layer that forms a habitat for the intestinal microbiota. The largely sterile inner mucus layer has a high concentration of secretory immunoglobulin A (sIgA), antimicrobial peptides (AMPs), microbe-associated molecular patterns (MAMPs), as well as other bioactive molecules such as trefoil factor peptides (TFFs), resistin-like molecule β (RELMβ), and Fc-γ binding protein. Underlying the IECs, the Lipopeptide/lipoprotein (LP) contains mainly plasma cells, macrophages, and dendritic cells that, in the healthy state, are of a naïve nature with limited expression of inflammatory cytokines.

The intestinal microbiota forms part of the intestinal barrier by limiting bacterial colonization and stimulating epithelial turnover (45). For example, *Bifidobacteria* species produce high concentrations of the short-chain fatty acid (SCFA) acetate, and can thereby prevent enteropathogenic *Escherichia coli* (EHEC) infection and its Shiga toxin release (46). Similarly, butyrate-producing *Fecalibacterium prausnitzii*, *Eubacterium rectale*, and *Roseburia* species directly target virulence gene expression to prevent bacterial infection (47). Studies have demonstrated that bacteria-dependent signals regulate the intestinal epithelial barrier and contribute to its effective functioning. Experiments in germ-free mice have shown that mucus layer thickness is reduced compared with conventionally housed mice, and that stimulation with lipopolysaccharide (LPS) and peptidoglycan (PGN) can reverse this to SPF-like levels of mucus thickness (48). Similarly, AMP and antimicrobial protein production, transcriptional- and post-translational regulation can be dependent on and enhanced by the presence of intestinal microbial signals (49–51). TLR, NOD-like receptor (NLR), RIG-like receptor (RLR), and C-type lectin receptor (CLR) family members provide distinct microbial signaling pathways in the intestinal epithelium (52–57). Despite evidence from mouse models deficient in PRRs and signaling adaptors (27, 52–56, 58), there is further need for epithelial-specific PRR knock-out mice to fully comprehend the role of bacteria-derived signals in intestinal epithelial homeostasis and repair.

BACTERIAL RECOGNITION AT THE INTESTINAL EPITHELIAL INTERFACE

Of the four signaling receptor families (TLR, NLR, RLR, and CLR), members of the TLR family of type I transmembrane proteins are the best-characterized receptors in the intestinal mucosa. NLRs are cytoplasmic receptors, of which nucleotide-binding oligomerization domain-containing protein 1 (NOD1) and NOD2 functions have been well characterized, that signal to elicit cytokine, chemokine, and defensing expression (59). RLRs recognize viral RNAs and induce innate antiviral responses (60). TLRs can be located at the cell surface or internal cell compartments, respond to specific ligands, and are associated with particular adaptors that activate downstream signaling cascades. Nearly all TLRs are expressed in the human colon, with the expression of TLR1, TLR2, TLR3, TLR4, TLR5, and TLR9 demonstrated in IECs of the human small intestine (61). Studies have identified four main adaptor molecules [MyD88, MyD88-adapter-like (Mal/TIRAP), TIR domain-containing adaptor-inducing interferon-β (TRIF), and TRIF-related adaptor molecule (TRAM)] that interact with TLRs in response to ligand stimulation (62, 63).

With the exception of TLR3, all TLRs signal *via* the adaptor protein MyD88, whose engagement triggers signaling cascades that ultimately lead to the activation of transcription factors such as NF-κB, interferon regulatory factor (IRF) and activator protein 1 (AP-1) (64). While the lack of MyD88 in certain mouse strains was shown to have a significant impact on the composition of the intestinal microbiota, linking TLR signaling to the structure of the microbial community (65), a study published the same year using MyD88- and TLR-deficient mice and wild-type littermates, demonstrates that colony and housing differences between laboratories make it difficult to clearly define the influence of innate immune signaling pathways on the microbiota (66). Here, Ubeda et al. found that MyD88 and TLR signaling does not detectably alter the composition of the intestinal microbiota, demonstrating the need for caution in the interpretation of microbiota analysis in mutant mice. It is important to bear in mind that observations in MyD88-deficiency do not imply a direct link to microbial signals, but may in fact be intrinsic. Besides TLR receptors, MyD88 associates with all receptors of the IL-1 cytokine family, and contributes to tissue homeostasis, including tissue repair and regeneration (28, 67, 68). Therefore, the inability of MyD88-deficient mice to respond to the IL-1 cytokine family is likely also involved. In the colon epithelium, for example, it was shown that the protective effect of MyD88 is, at least in part, mediated by the IL-1 cytokine family member IL-18 (69).

The monoassociation of germ-free mice with the prominent gut commensal *Bacteroides fragilis* revealed that this bacterium specifically signals through TLR2 on regulatory T cell *via* its polysaccharide A (PSA) symbiosis factor, to enable its niche-specific mucosal colonization (70). Similarly, the colonization of mice with *B. fragilis* protects against experimental colitis in a TLR2-dependent manner (70, 71). Monocolonization in germ-free rats with the commensal *Bifidobacterium lactis* was shown to cause TLR2-mediated MAPK and NF-κB pathway activation in IECs (72). Furthermore, The colonization of germ-free rodents with *Enterococcus faecalis* or *Bacteroides vulgatus* activate NF-κB signaling and induce chemokine expression in colonic IECs through TLR2 and TLR4 signaling, respectively (26, 73).

A study in TLR5-deficient mice showed that the cecal microbiota differed from wild-type littermates in >100 bacterial phylotypes (74), indicating that TLR signaling has implications in the regulation of the intestinal microbiota. This was also shown in MyD88-deficient mice that demonstrated higher levels cecal Rikenellaceae and Porphyromonadaceae families (75). In the healthy state, mice deficient in TLR signaling (MyD88-deficient, TLR4-deficient, MyD88/TRIF-knockouts) do not show any differences in proliferation and IEC barrier function compared with wild-type mice (76, 77). Under conditions of injury, however, MyD88-, TLR2-, and TLR4-deficient mice show increased susceptibility to dextran sodium sulfate (DSS)-induced colitis (28, 77, 78). Despite the importance of PRRs in the bidirectional crosstalk between the intestinal microbiota and the host, studies in PRR-deficient mice have shown that only those deficient in TLR5, NLRP6, or RIG-I develop spontaneous intestinal inflammation (79–81). This may suggest a major role of compensatory mechanisms, where PAMPs are recognized by multiple synergizing host PRRs that share key innate immune signaling pathways, resulting in a sufficient host response to commensal bacteria in PRR-deficient mice that do not show spontaneous phenotypes. It is important to consider that not all laboratories and animal colonies observe spontaneous basal inflammation in the above-mentioned PRR-deficient mice (82).

BACTERIAL SIGNALING MECHANISMS IN INTESTINAL INFLAMMATION

Despite difficulties in assigning the intestinal microbiota to the role of cause or consequence, chronic mucosal and, in particular, GI inflammation is linked to an imbalance of commensal bacteria and their gene products in patient groups with IBD (83–87). IBD is the collective name for multifactorial chronic relapsing inflammatory infections of the intestinal tract, which primarily includes Crohn's disease (CD) and ulcerative colitis (UC). IBD can be debilitating and may lead to life-threatening complications. The development of IBD is characterized by a change in the normal intestinal microbiota (dysbiosis), with a reduction in both bacterial quantity and bacterial diversity (83, 88–90). In the context of IBD, microbiota analyses have negatively associated *Faecalibacterium prausnitzii* and *Akkermansia muniphila* with the disease, whereas *Escherichia coli*, *Fusobacterium nucleatum*, *Haemophilus parainfluenzae*, *Veillonella parvula*, *Eikenella corrodens*, and *Gemella moribillum* were shown to be positively associated with the inflammatory disease (86, 91–94). Dysbiosis is associated with a breakdown of host–microbial mutualism, with accumulating evidence from numerous scientific disciplines firmly implicating such a breakdown in mutualism in the pathogenesis of IBD (95, 96).

Abnormal PRR signaling is implicated in the development of chronic intestinal inflammation. The cytosolic NLR NOD2 (also known as CARD15) recognizes bacterial PGN-derived muramyl peptide (MDP) to elicit NF-κB-mediated proinflammatory responses and AMP synthesis (97–99). *Nod2*-deficient mice harbor an elevated load of commensal resident bacteria,

display dysbiosis, and show a reduced ability to prevent intestinal pathogen colonization (100, 101). In turn, NOD2 expression is dependent on the intestinal microbiota, suggesting a feedback mechanism in the maintenance of intestinal homeostasis (101). In line with the above findings, Nod2 gene mutations were identified in patients with CD (102, 103), suggesting that Nod2 gene mutations may be associated with changes in the commensal microbiota that may facilitate disease progression.

The genetically engineered interleukin-10-deficient mouse (IL-10$^{-/-}$) provides a model of spontaneous intestinal inflammation (104) and has been extensively used as an experimental tool to mirror the multifactorial nature of IBD. Evidence for the requirement of resident enteric bacteria for the development of colitis in IL10$^{-/-}$ mice stemmed from studies in germ-free animals, where colitis development was not observed (105). It has been shown that the gram-positive intestinal bacterium *E. faecalis* drives distal colonic inflammation in IL-10$^{-/-}$ mice following monoassociation (106, 107). Furthermore, increased mucosal growth of, and specific antibody-titers against, *E. faecalis* have been shown in patients with UC, also correlating with disease severity (108, 109). Findings from our own group identified that the virulence factor gelatinase E (GelE) partially impairs intestinal epithelial barrier integrity in IL-10$^{-/-}$ mice (110), and that the colitogenic activity of *E. faecalis* was partially and almost completely abrogated when deficient for the enterococcal polysaccharide antigen (Δ*epaB*) and lipoproteins (Δ*lgt*) envelope structures, respectively (111). Monoassociation of IL-10$^{-/-}$ mice with the commensal bacteria *E. faecalis*, *E. coli*, or *Pseudomonas fluorescens* demonstrated that different commensal species selectively initiate distinct immune-mediated intestinal inflammation in the same host (107). Such results invite the hypothesis that particular microbial effectors, or a combination of effectors from different bacteria, are required to elicit pathogenesis or maintain the necessary barrier function for intestinal homeostasis. Additionally, not only the specific bacterium, but the susceptibility of the host plays a major role in disease progression, as shown by the induction of colitis by *Bacteroides vulgatus* in HLA/B27-β2m transgenic rats, but not in IL-2$^{-/-}$ mice (107, 112, 113).

Identifying bacterial gene products that drive protective rather than pathogenic inflammation in the intestine is crucial to rebalance homeostasis in inflammatory diseases and malignancies. *Lactobacillus* species, such as *Lactobacillus acidophilus*, are normal inhabitants of the intestinal microbiota and have received considerable attention as beneficial ecosystem members (114, 115). Several studies have shown that TLR2 regulates epithelial barrier function and enhances tight junction formation, as well as playing a crucial role in driving acute intestinal inflammation, but not chronic intestinal inflammation (116–118). *L. acidophilus* stimulates DCs through TLR2 *via* lipoteichoic acid (LTA) to trigger the production of inflammatory and regulatory cytokines (119–121). Deletion of the phosphoglycerol transferase gene (LBA0447) that synthesizes LTA generated an *L. acidophilus* derivative (NCK2025) that diminishes colitis when administered orally in a murine colitis model (122), confirming the role of LTA in inducing inflammation (123, 124). Of note here is that LTA, among others, may not present a true TLR2 ligand, as the large number of structurally diverse putative ligands may rather show their effects due to lipopeptide/lipoprotein (LP) contamination. In another example, *L. paracasei*, a single strain derived from the VSL#3 bacterial mixture clinically shown to reduce inflammation in IBD patients (125–127), was found to secrete the prtP-encoded protease lactocepin with anti-inflammatory effects *via* the degradation of proinflammatory chemokines (128, 129).

Collectively, the above findings support the notion that the colitogenic activity of opportunistic pathogens can be assigned to specific bacterial structures, and that such characterizations are indispensable in understanding host–microbe interactions relevant for the development of intestinal inflammation.

BACTERIAL SIGNALING MECHANISMS IN CRC

Colorectal cancer is one of the leading causes of death in the western society, being ranked third most lethal neoplasia in the United States in both men and women (130). Multiple lines of evidence show that the gut microbiota plays a major role in CRC development, both quantitatively and qualitatively. The significant role played by bacteria in inflammation-driven tumorigenesis is evident by the decreased tumor formation found in several CRC mouse models housed in germ-free conditions (131–133), or under antibiotic treatment (134). Accordingly, the inhibition of microbial recognition through the loss of PRR signaling or T-helper cell activation leads to a diminished neoplastic transformation (29, 131, 135, 136). Numerous bacterial species including, but not limited to, *Streptococcus bovis*, *Bacteroides fragilis*, and *E. coli* have been found in CRC samples. The best-known association is that of *S. bovis* bacteremia and CRC (137). It was demonstrated that *S. bovis* and its wall antigens induce IL-8 production, leading to the formation of nitric oxide (NO) and reactive oxygen species (ROS), which contribute to the neoplastic process (138). More recently, *Peptostreptococcus anaerobius* was identified as a candidate to be significantly enriched in the stool and mucosa of patients with CRC (139–141). A study assessed the association of *P. anaerobius* in stool and colonic tissue from patients with colorectal adenomas and adenocarcinomas, providing mechanistic insights that the actions of *P. anaerobius* are mediated *via* interaction with TLR2/4 on host cells to induce ROS production, increase cholesterol biosynthesis, and activate pro-oncogenic factors and pathways to promote CRC (142).

Approximately 80% of sporadic colorectal tumors are associated with mutations in the adenomatous polyposis coli (APC) gene (143); a central gatekeeper protein in CRC. Multiple intestinal neoplasia mice with a point mutation in *Apc* (*Apc*$^{Min/+}$) mimic sporadic cancer and familial adenomatous polyposis, and have been used to study the role of TLR signaling in intestinal tumorigenesis through the crossing with MyD88-deficient mice (MYD88-deficient × *Apc*$^{Min/+}$). While tumor incidence was similar in these mice compared with *Apc*$^{Min/+}$ mice, a reduction in tumor number and size was observed, which was linked to a reduced expression of the tumor growth-promotor COX2 (29, 144). These data suggest that TLR signaling is involved in tumor growth, but not tumor initiation. Further evidence for the contribution of TLR signaling to the development of sporadic

cancer, and colitis-associated cancer, stemmed from the use of TLR4-deficient mice that were protected against tumorigenesis following azoxymethane (AOM) and DSS treatment (145). Furthermore, TLR4 activation on tumor cells can prevent their lysis, thereby protecting cancer cells (146). This is of particular relevance with regard to cancer treatment strategies, as the immunosuppressant drug Rapamycin decreases TLR4 expression and its prostaglandin E2 production (147). Findings from animal models of CRC are corroborated by human studies; the TLR4/MyD88 co-receptor complex showed enhanced expression in approximately 20% of CRC patient samples, compared with normal mucosae and adenomas (148, 149).

Mechanistically, bacteria may promote tumorigenesis by numerous processes, including toxic metabolite production and genotoxic biosynthesis (150), thereby providing further CRC treatment tactics. One study aimed at inhibiting toxic effects of colibactin toxin-producing *E. coli*; frequent colonizers of CRC (151). Here, two identified boronic acid-based com pounds were shown to bind to the active site of the ClbP enzyme involved in the synthesis of colibactin, and shown to suppress DNA damage and tumorigenesis induced by *pks*-harboring [conserved genomic island coding for nonribosomal peptide synthetases (NRPS) and polyketide synthetases (PKS) bacteria (152)]. These findings not only confirm the role of colibactin toxin-producing *E. coli* in carcinogenesis but also provide a novel family of inhibitors to target pks-harboring bacteria in the treatment of CRC.

Injection of specific bacteria into tumor tissue may help eradicate tumors through the stimulation of inflammation and anti-tumor responses (153). In line with the above comment that a combination of multiple effectors may be necessary to maintain homeostasis or elicit pathogenesis: two bacteria can be better than one in cancer immunotherapy. A recent study applied an approach to cancer immunotherapy through the use of an attenuated *Salmonella typhimurium* strain engineered to secrete *Vibrio vulnificus* Flagellin B (FlaB) (154). Zheng et al. showed that FlaB-mediated tumor suppression is associated with TLR5-mediated host reactions and dependent on TLR4 and MyD88 signaling, as shown with TLR/MyD88 knockout mice and cell lines. Evidently it is feasible that non-virulent tumor-targeting bacteria can release multiple TLR ligands, and can be used as cancer immunotherapeutics.

In a latest study, Sahu et al. linked the dysbiotic behavior of a constitutively invasive variant of commensal non-pathogenic *E. coli* to CRC tumorigenesis (155). Aberrant host invasion leads to realignment of multiple host signal transduction cascades through reciprocal modulation of microbe sensing pathways Nod1/Rip2 and TLR/MyD88, leading to an expansion of the cancer stem cell population. This supports the notion that microbe-driven tumorigenesis may result from self-derived and contextual cues, which determine the role of such microbes in homeostasis and carcinogenesis, rather than strict correlations with commensal virulence.

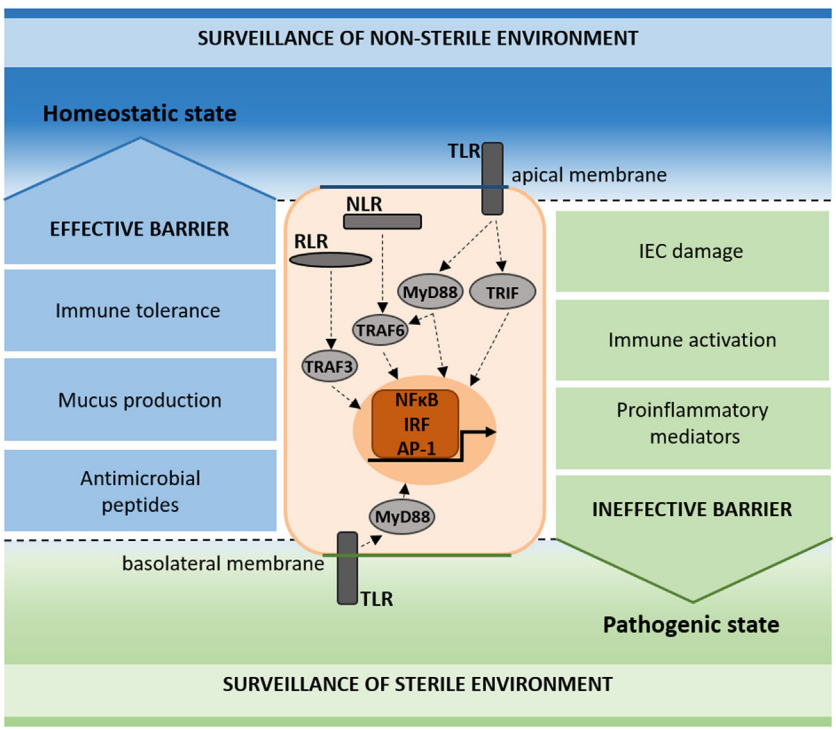

FIGURE 2 | Schematic representation of pattern recognition receptor (PRR) surveillance in the homeostatic and pathogenic state. PRRs (TLR, NLR, and RLR) signal on the apical and basolateral membrane of intestinal epithelial cells (IECs), contributing to the surveillance of the non-sterile (apical) and sterile (basal) environments. In the homeostatic state, immune tolerance, mucus production, and antimicrobial peptides add to the maintenance of an effective barrier (blue). In the pathogenic state, IEC damage, immune activation, and proinflammatory mediators result in an ineffective barrier (green).

Lactobacillus acidophilus NCK2025, discussed earlier with regards to the regulation of inflammation in a colitis model, was investigated in a mouse model of colonic polyposis (TS4Cre × APC^{lox468}) to assess its moderation of pathogenic inflammation within the tumor microenvironment (156). Khazaie et al. reported that oral treatment with the LTA-deficient *L. acidophilus* NCK2025 normalized innate and adaptive pathogenic immune responses, causing a regression of established precancerous colonic polyps. This work demonstrates the ability of the probiotic strain with anti-inflammatory properties to reverse preneoplasia, rendering this *L. acidophilus* strain as a potential candidate for regulating intestinal immunity in the protection against inflammation and CRC susceptibility. Additional investigations are key to further characterize bacterial gene products that can influence inflammation to restore intestinal homeostasis, to provide novel avenues for the treatment and prevention of inflammatory and cancer pathologies.

In light of high-sensitivity-detection of pre-cancerous lesions still posing a great challenge, the potential of fecal microbiota for the early-stage detection of CRC was recently investigated (157). In a metagenomic sequencing study to identify taxonomic markers in CRC patients, Zeller et al. found functional and taxonomic associations with CRC from noninvasive fecal sample readouts. Furthermore, general dysbiosis common to inflammation was addressed by including published metagenomes from IBD patients in the marker species classifier, showing that stronger associations were observed with CRC, with only modest influences by inflammation-related microbiota changes. This study demonstrates the possibility of CRC detection from fecal microbial markers, and the potential for further identification of cancer-associated differences in gene function, gene content and genomic variation through additional metagenomic data.

CONCLUDING REMARKS

Over the years, it has become evident that the intestinal microbiota is not merely a bystander in the complex events that regulate intestinal homeostasis, but that it plays a fundamental role in eliciting both beneficial and detrimental effects in the host. Collectively, the studies outlined in this review highlight the diverse and multifaceted roles of IECs and the intestinal microbiota in the maintenance of intestinal homeostasis, and the complexity of the relationship between the two. The diverse barrier functions of the intestinal epithelium play a crucial role in microbe–host mutualism. Cells of the intestinal epithelium express a range of PRRs that sense and respond to a variety of microbial signals to maintain an effective barrier and respond to pathogens (**Figure 2**). Evidence of the importance of PRR signaling stems from studies in mice with specific defects in such signaling pathways, which show increased susceptibility to developing disease (28). Regarding the host side of the mutualism, future studies to increase our understanding of how mucus, AMPs, and sIgA dynamics can be regulated to maintain barrier function will provide avenues to develop therapeutic interventions for preserving intestinal homeostasis. Probiotic and prebiotic treatment options available to consumers are currently drawn from a narrow range of organisms. Increasing knowledge of the intestinal microbiota with its constituents is changing this paradigm; however, due to the complex and dynamic nature of the intestinal ecosystem, the mechanistic understanding of the integration of bacterial signals remains a great challenge to this field. Antibiotics selectively targeting bacterial pathogens have been extensively used in the prevention and treatment of numerous diseases (158, 159). In light of antibiotics disrupting the composition of the enteric intestinal microbiota and promoting antibiotic resistance, future mechanistic experimental efforts to elucidate (yet) unidentified mechanisms of bacterial effector proteins to enable the development of novel drugs aimed at targeting rather than killing bacterial pathogens, seems like the logical step forward. To this end, animal models of inflammation and cancer provide useful approaches to demonstrate functionality, given the high interindividual variation and nature of studies using human cohorts.

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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Mucosal Mesenchymal Cells: Secondary Barrier and Peripheral Educator for the Gut Immune System

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Stromal connective tissue contains mesenchymal cells, including fibroblasts and myofibroblasts, which line the tissue structure. However, it has been identified that the function of mesenchymal cells is not just structural—they also play critical roles in the creation and regulation of intestinal homeostasis. Thus, mucosal mesenchymal cells instruct intestinal immune cell education (or peripheral immune education) and epithelial cell differentiation thereby shaping the local environment of the mucosal immune system. Malfunction of the mesenchymal cell-mediated instruction system (e.g., fibrosis) leads to pathological conditions such as intestinal stricture.

Keywords: intestinal stem cells, peripheral education, fibroblasts, mucosal healing, mesenchymal cells

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INTRODUCTION

Occurring below the mucosal mucus and membrane layer and at the forefront of host-environmental encounters, interactions between epithelial and immune cells are indispensable for the formation of the chemical, physical, and immunological barriers of the mucosal epithelium. Such interactions lead to immunophysiological functions—secretion of mucus containing anti-microbial peptides and secretory IgA antibodies, and enhancement of tight junctions—ultimately promoting intestinal homeostasis (1). These indispensable roles of the mucosal epithelial-immune cell barrier are well known due to functional studies demonstrating that disruption of barrier-associated genes (e.g., encoding MUC2 and E-cadherin) results in intestinal inflammation (2–4). Recently, however, focus has shifted toward the role of mesenchymal cell interactions with epithelial and immune cells and their effect on the formation and maintenance of intestinal homeostasis.

Mesenchymal cells are a large heterogenous population that includes fibroblasts, myofibroblasts, interstitial cells of Cajal, pericytes, many of which are within the mucosa (5). They are negative for common molecular markers for epithelial and hematopoietic cells (e.g., E-cadherin and CD45, respectively) but are positive for a combination of vimentin, CD90 (also known as THY1), S100A4, α-smooth muscle actin, desmin, smoothelin, platelet-derived growth factor (PDGF) receptor, and

c-kit (6, 7) (**Table 1**). Most notably, the expression of α-smooth muscle actin is used to distinguish between fibroblasts and myofibroblasts as the negative and positive cells, respectively [**Table 1**; (5)].

Although mesenchymal cells have various origins, they provide mechanical and structural support functions that are integral to intestinal morphogenesis, organogenesis, and homeostasis (8–10). In mice lacking PDGF, a necessary mesenchymal growth factor (8), intestinal myofibroblasts (pericryptal fibroblasts) are lost in the villous crypts during intestinal formation, leading to disorganization of the intestine (8). In organogenesis of lymph nodes [e.g., in Peyer's patches (PPs) and mesenteric lymph nodes], mesenchymal cells termed lymphoid tissue organizer aid in the accumulation of lymphocytes through stimulation by lymphoid tissue inducer cells (LTi or Group 3 innate lymphoid cells) (9, 10). Therefore, mesenchymal cells play multiple essential roles in developing and preserving gut anatomical homeostasis. In addition, interstitial cells of Cajal regulate gastrointestinal motility: loss of these through mutations of *KIT* cause abnormalities in intestinal peristalsis (5). Pericytes, or parietal cells, surround capillary vessels where they are responsible for regulating stretching and vascular permeability, and perform angiogenesis through interactions with endothelial cells, as reviewed elsewhere (5, 11). Fibroblasts and myofibroblasts, the main topic of this review, are essential for the formation of the higher-order structure of tissue (e.g., gastrointestinal tract) through production of extracellular matrix (ECM) (12), and therefore play an indispensable role in tissue regeneration and restoration (12).

In recent years, it has become apparent that mesenchymal cells act on various immunocompetent cells, such as dendritic cells and mast cells, to modulate differentiation, proliferation, and the function of these cells in peripheral tissues in a process we term "peripheral education" (13–15). Furthermore, mesenchymal cells regulate epithelial lineage development in intestinal infection (16). In colonic mucosa, the CD90-positive mesenchymal cell population expressing toll-like receptors and Nod-like receptors possesses phagocytic and antigen-presenting capabilities (17). Although their antigen-presenting capabilities are not as great as those of professional antigen-presenting cells, it is suggested that mesenchymal cells are involved in the direct induction or enhancement of mucosal acquired immune responses (17). Here, we provide an overview of recent advances concerning the role of mesenchymal cells in peripheral education and epithelial membrane repair for the creation of a healthy gut immune environment.

MESENCHYMAL REGULATORY SYSTEM FOR MUCOSAL FRONTLINE

Function of Mucosal Mesenchymal System in Epithelial Differentiation

Along the gut epithelial layer, which forms the first line of mucosal barrier by producing mucus containing antibacterial substances (1), microfold cells (M cells) are a gateway for the outside environment and are responsible for antigen uptake (or sampling) from the mucosal lumen (18). M cells are primarily located in the follicle-associated epithelium of PPs, a major organized lymphoid structure for the induction and regulation of the appropriate antigen-specific mucosal immune responses that confer protection and commensalism against pathogenic and beneficial antigens, respectively (9, 18). *In vivo* studies and *in vitro* organoid studies have shown that the cytokine RANKL (also known as TNFSF11) is essential for the induction of differentiation and maintenance of M cells located in the follicle-associated epithelium of PPs (19, 20). Mesenchymal cells located just below the follicle-associated epithelium are the main source of RANKL (19). A most recent study has shown that the unique type 6 collagen expressing mesenchymal cell populations producing RANKL are involved in the development of M cells (21). M cells are an entry site of antigens and luminal bacteria and antigen presentations were subsequently occurred for generating IgA in the PPs; therefore, RANKL induced M cell differentiation is imperative to the maintenance of host-microbe symbiosis (21). This type of mesenchymal instruction system for the development of mucosal immune system *via* the M cell induction is one of examples for the essential role of mesenchymal cell family for mucosal frontline upkeeping system (19, 20).

In the villi, mesenchymal cells guide epithelial cell (EC) lineage differentiation in both physiological and pathological conditions (6, 22). Under the homeostatic condition, epithelial stem cells primarily differentiate into absorptive ECs, which perform the primary physiological function of the gastrointestinal tract (1), however, upon infection, epithelial stem cells shift toward secretory EC differentiation (23). In the case of

TABLE 1 | Characteristics of surface molecules expressed by different mesenchymal cells.[a]

	Fibroblasts	Myofibroblasts	Pericytes	Smooth muscle	Interstitial cells of Cajal
Vimentin	+	+	+	−	+
CD90	+	+	±	−	−
S100A4	+	+	−	−	−
Alpha-smooth muscle actin	−	+	+	+	−
Desmin	−	−	+	+	−
Smoothelin	−	−	+	+	−
Platelet-derived growth factor receptor	+	+	+	+	?
c-kit	−	−	−	−	+

The expression molecules of mesenchymal cells (e.g., fibroblasts, myofibroblasts, pericytes, smooth muscle cells, and interstitial cells of Cajal) were defined.
[a]*The table was prepared by the data described in Ref. (6, 7).*

bacterial (e.g., *Salmonella*) infection, rapid differentiation and proliferation of secretory ECs such as Paneth cells (which secrete anti-microbial peptides, such as defensin and lysozyme) and goblet cells [which secrete mucin and anti-microbial proteins, such as TFF3 and resistin like β (RELMβ) (also known as FIZZ1)] is accelerated to clear the pathogens (23). This countermeasure shift in epithelial stem cell differentiation is mediated by pericryptal fibroblast-produced interleukin (IL)-33 (23) (**Figure 1**). Differentiation into secretory ECs is ordinarily repressed by Hes1 through the Notch signaling pathway (24, 25). But in the *in vitro* assessment with intestinal organoids IL-33 acts on epithelial stem cells *via* its receptor ST2, to suppress Notch signaling and thereby activate secretory EC differentiation (23) (**Figure 1**). IL-1β, IL-6, tumor necrosis factor (TNF)-α and bacterial cell components (e.g., lipopolysaccharide) are involved in the stimulation of IL-33 (23), but the extent of each of their roles is still unknown and needs further investigation.

Homeostatic maintenance of epithelial stem cells is generally understood to be maintained by neighboring Paneth cell production of Wnt3, Wnt5, and EGF (26). However, in the colon where Paneth cells are lacking, mesenchymal cell production of Wnt2b works to maintain epithelial stem cells (27). In addition, mesenchymal cells are responsible for secreting Wnt-activating growth factors such as R-spondin 3 during both homeostatic and non-homeostatic conditions (28, 29). A recent study indicates that, during inflammation, $CD34^+$ fibroblasts produce niche factors, including Wnt2b, Gremlin 1, and R-spondin 1, for maintenance of the intestinal stem cell niche (29) (**Figure 1**). The important role of mesenchymal cells in epithelial stem cell maintenance deepens their integral role in EC differentiation. These findings imply that the function of mesenchymal cells differs among location and reflects the surrounded tissues or microenvironments.

Mucosal Repair

The intestinal mucosa is frequently threatened by environmental substances (e.g., pathogenic microorganisms, and chemicals such as alcohol) or dysbiosis of commensal microorganisms. The gut is thus equipped with multiple innate and acquired defense mechanisms (e.g., mucus, anti-microbial peptides, IgA antibodies, and Th17 cells) (30). Although these systems are essential for host protection, they concurrently cause mucosal damage, and it is therefore crucial to simultaneously initiate the mucosal tissue repairing cascade (1), which involves various factors promoting epithelial restitution followed by epithelial regeneration and differentiation (31).

Epithelial restitution occurs early on in mucosal epithelial tissue that has suffered tissue damage due to inflammatory diseases (32, 33). ECs near the damaged region lose polarity and migrate rapidly to the epithelial-deficient region, restoring the epithelial layer (32). Epithelial restitution does not appear to involve proliferation of ECs from the crypt region (1, 32); rather the process occurs through covering or sealing of the denuded area by migrating ECs (33). IL-22 has been shown to promote myofibroblast mediated epithelial repair and defense as well as epithelial stem cell protection during inflammatory bowel diseases (34, 35). Upon inflammation, helper T cells and innate lymphoid cells near the site of inflammation secrete IL-22 (36). IL-22 then activates NF-κB and AP-1 transcription factors as well as MAP kinases of myofibroblasts (34, 35). IL-22 activated myofibroblasts

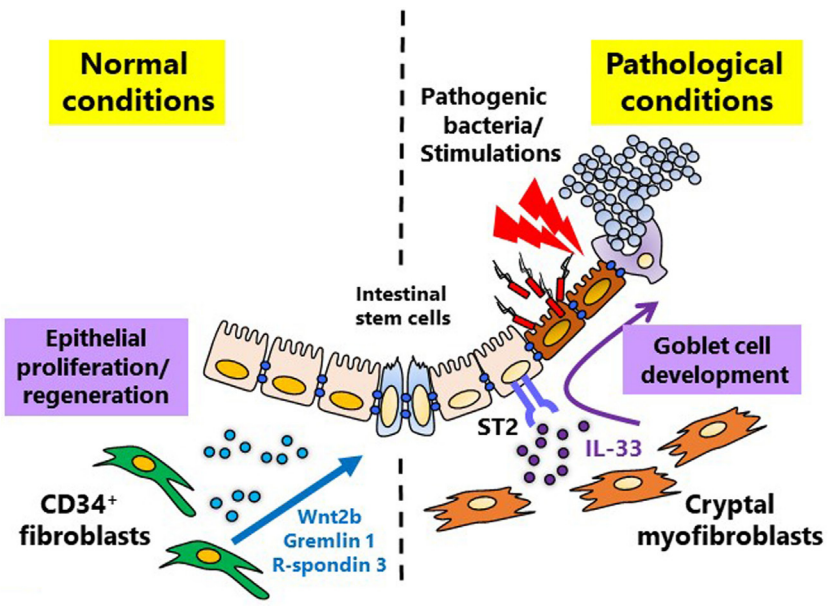

FIGURE 1 | Mesenchymal cell-instructed intestinal homeostatic and pathological conditions. Under normal conditions, mesenchymal cells promote mucosal homeostasis by maintaining physiological differentiation of absorptive epithelial cells from intestinal stem cells through the production of intestinal stem cell niche factors, including Wnt2b, Gremlin 1, and R-spondin 3. During pathological conditions, including inflammation and infection, mesenchymal cells can promote the essential switch from absorptive to secretory epithelial differentiation which is mediated by interleukin-33.

subsequently secreted proinflammatory cytokines (e.g., IL-6, IL-8, and IL-11) as well as MMP-1 and MMP-3 imperative to repair and remodeling (34). The IL-22 induced proinflammatory cytokines are necessary for the protection of epithelial stem cells and lack thereof has been linked to intestinal pathology and loss of epithelial barrier function (35). Additionally, chemokines (e.g., CXCL12) (37), and various other cytokines [e.g., IL-6 and transforming growth factor (TGF)- β1] (38, 39), and antimicrobial proteins (e.g., TFF3) (40) are suggested to play a role in epithelial restitution, the precise mechanism is still largely unknown. In addition, other studies have shown that during various other intestinal damages such as irradiation, Lgr5 positive cells are imperative to proper EC regeneration (41).

Alongside epithelial restitution, stimulation of fibroblasts near the inflammation site is an important process. Activation by immune cells (e.g., T cells and macrophages) and EC-produced TGF-β1 induces differentiation of fibroblasts into myofibroblasts expressing smooth muscle α-actin (αSMA) (6). Myofibroblasts specialize in the production of ECM molecules such as collagen and tenascin C, and together with fibroblasts, promote mucosal repair by appropriately adjusting the production and degradation of the ECM (42, 43). In addition, myofibroblasts produce growth factors (e.g., HGF), which induce EC proliferation, leading to migration of ECs to the repair site using ECM as a scaffold (44). Because efficient induction of myofibroblasts is essential for mucosal repair, several induction mechanisms exist other than development from activated conventional fibroblasts. For instance, differentiation from ECs (epithelial–mesenchyme transition) and endothelial cells (endothelial–mesenchyme transition) have been characterized in different tissues (e.g., kidney) (5, 45–47). Both epithelial– and endothelial–mesenchyme transitions induce migratory fibroblastic cells expressing vimentin and αSMA (5, 46). These processes are regulated by various cytokines, including TGF-β1, TNF-α, and IL-1β produced by immune cells and ECs (45).

In mucosal repair upon inflammatory bowel diseases (e.g., Crohn's disease), FGF2 and IL-17 produced from regulatory T cells and Th17 cells, respectively, as the result of stimulatory signals caused by dysbiosis of the intestinal flora have been shown to play a critical role (48). FGF2 and IL-17 synergistically promote expression of genes involved in intestinal mucosa healing (e.g., those encoding SPRR2, IL-6, and Arg2). IL-17 also strongly influences ECs and mesenchymal cells during the tissue disruption and healing process mentioned above (48).

Transforming growth factor-β1 is an essential cytokine for wound healing and enhancement of ECM production (49). It has been recently announced to be discontinued the phase III trial; however, patients with Crohn's disease have been treated with antisense oligonucleotides against SMAD7, which binds to the TGF-β receptor, blocking TGF-β1 signaling; inhibition of SMAD7 promotes TGF-β-induced activation of SMAD2 and SMAD3 signal transducers (50), thereby activating TGF-β1-mediated anti-inflammatory activities (50). However, chronic production of TGF-β1 continuously activates mesenchymal cells, especially fibroblasts and myofibroblasts, leading to organ fibrosis (51, 52). Fibrosis causes intestinal stricture and obstruction, and repeated intestinal resection results in short bowel syndrome (53). Although the mechanism of fibrosis induction is not fully understood and complex, excessive activation of the TGF-β1 pathway is generally considered to be a central causative element (54). Many patients with Crohn's disease undergo surgery to relieve fibrotic complications as their disease worsens (51, 52). Temporal and spatial activation of TGF-β1 is believed to lead to the wound healing; however, sudden wound healing may progress intestinal obstruction (53). Further analysis of mesenchymal cells provides promising strategies for the control of wound healing.

MUCOSAL PERIPHERAL EDUCATION

Mucosal Dendritic Cell Education

The intestinal tract is a special tissue that is constantly in contact with various stimuli such as microflora, foods, and metabolites. Since the intestinal tract acts as a gateway for environmental antigens and pathogenic microorganisms, the mucosal immune system must achieve the appropriate immunological balance between active and quiescent responses. The qualitative and quantitative adjustment of intestinal IgA antibody production is deeply involved in both the protection against pathogenic bacterial infection and the maintenance of the appropriate composition of commensal bacterial flora for a healthy gut environment (55). In steady state, secretary IgA antibodies are required to maintain healthy bacterial species, so called commensal mutualism (56) (**Figure 2**). Disruption of the mucosal immune system-mediated balancing act leads to the onset of various acute and chronic inflammatory diseases (57). In the induction of mucosal IgA antibody production, intestinal dendritic cells play a critical role by synthesizing retinoic acid (RA), which promotes antigen-specific mucosal T and B lymphocyte responses; this role is in addition to the classical role of dendritic cells in antigen presentation to T and B lymphocytes in organized inductive tissue (e.g., PPs) (58). RA-induced lymphocytes express gut-imprinting molecules such as the chemokine receptor CCR9 and the integrin α4β7, which are necessary for the preferential migration of antigen-specific lymphocytes from PPs to the lamina propria regions of intestinal tract where they produce IgA (59). RA production is peculiar to "mucosal-type" dendritic cells located in mucosa-associated lymphoid tissues (e.g., PPs), not splenic dendritic cells (58, 60). Furthermore, some mesenchymal cells can produce RA and GM-CSF (also known as CSF2), critical cytokine for generation of dendritic cells, in the vicinity of dendritic cells in the intestinal lamina propria (15) (**Figure 2**); from *in vitro* analysis, it has become obvious that the mesenchymal cells can convert spleen dendritic cells into "mucosal-type" dendritic cells (15). It is thus plausible to suggest the existence of a mucosal mesenchymal–dendritic cell cross-talk system that preferentially educates lymphocytes to produce IgA antibodies in the mucosa-associated tissues. Dendritic cells within the mucosal lamina propria can produce RA independently of intestinal bacteria, but RA produced from mesenchymal cells is dependent on stimulation from intestinal bacteria (15). These findings suggest that initial peripheral education machinery mediated by RA is orchestrated by the cross-communication between mesenchymal cells and commensal microbiota, which leads to the creation of a mucosal imprinting environment.

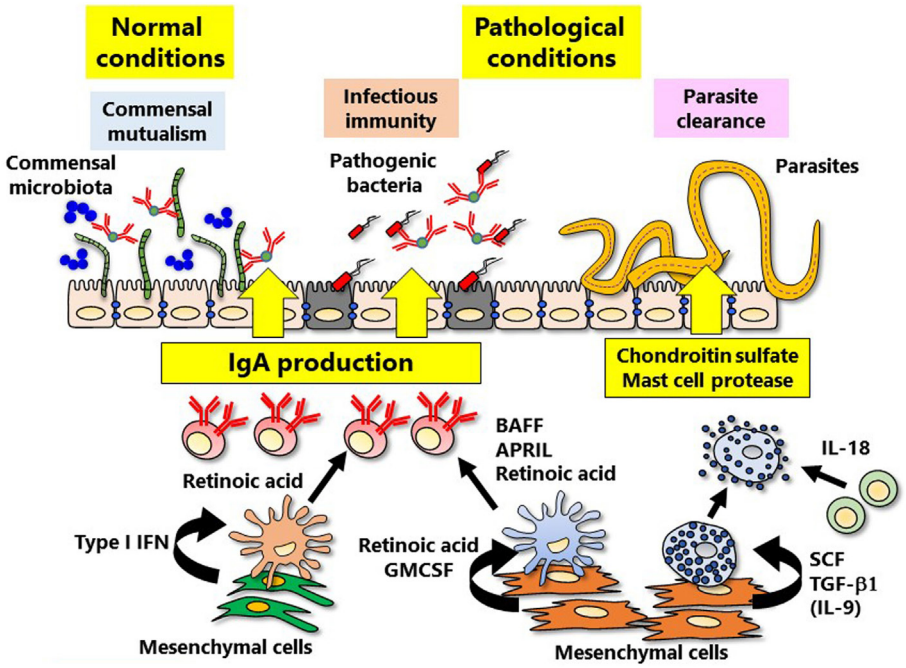

FIGURE 2 | Mesenchymal cell-instructed immune cell education. Mesenchymal cells induce peripheral immune education, thereby refining intestinal-specific immune responses. IgA is involved in the both normal (commensal mutualism) and pathological (the protection against bacterial infection) conditions. Induction of IgA is directly and indirectly regulated by mucosal mesenchymal cells *via* type I IFN and retinoic acid. In addition, the defense against parasite infection mediated by mast cells is also regulated by cytokines produced from mesenchymal cells.

In addition to RA, cytokines that promote IgA induction such as APRIL (also known as TNFSF13) and BAFF (also known as TNFSF13B) are produced by plasmacytoid dendritic cells, another subgroup of dendritic cells within the intestinal mucosa (61) (**Figure 2**). Type I IFN is deeply involved in the induction of mucosal plasmacytoid dendritic cells, and it has recently been reported that intestinal mesenchymal cells are the main source of type I IFN (61). Production of type I IFN from mesenchymal cells is stimulated by intestinal bacteria (61). It is thus necessary to further verify how and what kinds of gut bacteria and/or their derived factor(s) are involved in mucosal mesenchymal cell-instructed gut-imprinting and IgA production.

Mucosal Mast Cell Education

Mast cells undergo maturation after being distributed throughout the whole body, including gut mucosa, *via* blood from the bone marrow (13). The c-kit receptors on mast cells and the c-kit ligand (stem cell factor, SCF; also known as KITLG), are essential for maintaining mast cells; mice lacking either of these molecules have no mast cells (62). Mesenchymal cells, especially fibroblasts, are the main secretory source of SCF (13). The SCF–c-kit pathway works together with prostaglandin D2 and its receptor (DP1) pathway in the maturation of mast cells, including granule formation (63). Mast cell granules containing chondroitin sulfate and proteases (e.g., the chymase Mcpt1) are involved in the control of parasitic infections (64–66). In mice infected with an intestinal helminth, antigen–IgE complex and IL-18 activated mucosal mast cells to release chondroitin sulfate and Mcpt1 to achieve parasite expulsion. Chondroitin sulfate and Mcpt1 caused direct parasite damage and inhibited parasite invasion of ECs (67). However, inappropriate and unnecessary activation of mast cells within the mucosa, inflammation, and allergic reaction took place. For instance, proteases released from mast cells accelerate the influx of inflammatory cells (e.g., neutrophils) into the inflammatory site by weakening the tight junctions of endothelial cells (68).

Mast cells are classified into two subsets: "connective tissue type" and "mucosal type" (13). Mast cells that have heparin-containing granules are common in connective tissue, whereas those with chondroitin sulfate-containing granules are preferentially found in the intestine (13). In mast cells associated with the mucosal surface, expression of proteases Mcpt1 and -2 is particularly elevated (69). For the generation of "mucosal-type" mast cells, not only IL-9 producing T cells (so-called Th9 cells), but also gut mesenchymal cells have been shown to play a critical role (14). *In vitro* expression of heparin–Mcpt4 or chondroitin sulfate–Mcpt1 (representing "connective tissue type" or "mucosal type," respectively) is induced by co-culturing bone marrow-derived mast cell precursors with mesenchymal cells from skin dermis or intestinal mucosa, respectively (14). Because expression of Mcpt1 is induced by TGF-β1 and IL-9 (70), only intestinal, but not skin mesenchymal cells, were able to induce Mcpt1 expression (71) (**Figure 2**). Taken together, these results demonstrate the presence of an intestinal mesenchymal cell-instructed "mucosal-type" mast cell development system. Further, it is interesting to hypothesize that the mesenchymal

cells at different tissue locations (e.g., skin and gut) adopting the biological and anatomical characteristics of respective tissues are a major educator for the generation of "connective tissue type" and "mucosal-type" mast cells.

In summary, our new and advanced knowledge of the role of mesenchymal cell-instructed functional maturation of immunocompetent cells (e.g., dendritic cells and mast cells) will allow us to create novel strategies for the control of mucosal infection and inflammation in the near future.

FUTURE PERSPECTIVES

The functions of mucosal mesenchymal cells as the peripheral educator of immunological cells are critical in the development and maintenance of the intestinal homeostatic condition. Disruption of mucosal mesenchymal cell-instructed peripheral education system is likely a cause of gut pathological conditions. However, only a portion of the physiological, immunological, and pathological roles of these cells is clear, and detailed molecular and cellular mechanisms of the mucosal mesenchymal cell-instructed peripheral education system have yet to be elucidated.

Since mesenchymal cells are composed of a heterogeneous cell population, including fibroblasts, myofibroblasts, pericytes, interstitial cells of Cajal, adipocytes, and others, there remains a problem regarding the correct classification of subpopulations with specific molecular and morphological identification factors. Further investigations of the molecular role of mesenchymal cells in immune peripheral education, mucosal barrier formation, and fibrosis are required. It is thus important to elucidate the precise molecular interaction(s) between mesenchymal cells and immune cells to understand the bidirectional regulatory mechanisms. To this end, our current and future efforts aim to clarify the novel regulatory function of mesenchymal cells in the prevention of excess inflammatory reactions.

AUTHOR CONTRIBUTIONS

YK, DY, NS, SU, PE, TN, and HK conceived and wrote the manuscript.

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Good Manufacturing Practice-Compliant Production and Lot-Release of *Ex Vivo* Expanded Regulatory T Cells As Basis for Treatment of Patients with Autoimmune and Inflammatory Disorders

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In recent years, the exploration of regulatory T cell (Treg)-based cellular therapy has become an attractive strategy to ameliorate inflammation and autoimmunity in various clinical settings. The main obstacle to the clinical application of Treg in human is their low number circulating in peripheral blood. Therefore, *ex vivo* expansion is inevitable. Moreover, isolation of Treg bears the risk of concurrent isolation of unwanted effector cells, which may trigger or deteriorate inflammation upon adoptive Treg transfer. Here, we present a protocol for the GMP-compliant production, lot-release and validation of *ex vivo* expanded Tregs for treatment of patients with autoimmune and inflammatory disorders. In the presented production protocol, large numbers of Treg, previously enriched from a leukapheresis product by using the CliniMACS® system, are *ex vivo* expanded in the presence of anti-CD3/anti-CD28 expander beads, exogenous IL-2 and rapamycin during 21 days. The expanded Treg drug product passed predefined lot-release criteria. These criteria include (i) sterility testing, (ii) assessment of Treg phenotype, (iii) assessment of non-Treg cellular impurities, (iv) confirmation of successful anti-CD3/anti-CD28 expander bead removal after expansion, and (v) confirmation of the biological function of the Treg product. Furthermore, the Treg drug product was shown to retain its stability and suppressive function for at least 1 year after freezing and thawing. Also, dilution of the Treg drug product in 0.9% physiological saline did not affect Treg phenotype and Treg function for up to 90 min. These data indicate that these cells are ready to use in a clinical setting in which a cell infusion time of up to 90 min can be expected. The presented production process has recently undergone on site GMP-conform evaluation and received GMP certification from the Bavarian authorities in Germany. This protocol can now be used for Treg-based therapy of various inflammatory and autoimmune disorders.

Keywords: regulatory T cell, good manufacturing practice, autoimmunity, expansion, lot-release

INTRODUCTION

Regulatory T cells (Treg) play a critical role in maintaining immune homeostasis and limiting autoimmune responses by modulation of both innate and adaptive immunity (1). Classically defined Treg are characterized by their constitutive expression of CD4, CD25, and FoxP3 (2) and nearly absent expression of CD127 (3, 4). They can be divided in (i) natural Treg originating from the thymus and peripherally induced Treg, which differentiate from naïve T cells when self or non-self antigen is encountered under tolerogenic conditions (5, 6). Their existence in humans has first been described in 2001, when several groups were able to isolate (7–9) and expand (10) suppressive $CD4^+CD25^+$ T cells from human peripheral blood. Animal studies have shown that Treg successfully prevent type I diabetes, experimental autoimmune encephalitis, rheumatoid arthritis, inflammatory bowel disease, systemic lupus erythematosus, scurfy disease, graft-versus-host disease, and transplant rejection (11). As a result, the exploration of Treg-based cellular therapy has become an attractive strategy to induce tolerance in various clinical settings in patients (12). However, the main obstacle to clinical application of Treg in humans is their low number circulating in peripheral blood. Therefore, initial Treg enrichment and subsequent expansion protocols are necessary to generate clinical relevant Treg numbers. Treg enrichment from a peripheral blood product is challenging, since activated conventional human T cells may also express CD25 (13). As a result, isolation of Treg bears the risk of concurrent isolation of unwanted effector cells.

Currently, three main strategies to isolate and expand highly enriched Treg populations from a human blood product are exploited by several research groups. First, Treg can be isolated and expanded from a donor-derived umbilical cord blood product (14–17), yet this approach is not feasible in other settings than stem cell transplantation, since it cannot be excluded that allogeneic donor-derived Treg itself induce graft-versus-host like reactions in non-transplant patients. Alternatively, highly enriched Treg populations can be isolated using good manufacturing practice (GMP)-approved flow cytometry-based (FACS) cell sorters (18–20) or the mTOR inhibitor rapamycin can be added to the cell culture process to inhibit the proliferation of contaminating effector T cells (21–23) The latter is a calcineurin inhibitor that is widely used to prevent allograft rejection after transplantation (24). Previous animal studies showed that rapamycin decreases the number of $CD4^+$ cell subsets in mice, but increases the number of functional Treg (25). Based on these findings, Ogino et al. provided the proof-of-concept that mouse $CD4^+$ T cells can be expanded *ex vivo* in the presence of rapamycin (26). The addition of rapamycin to the cell cultures affected overall expansion efficiency but effectively inhibited the outgrowth of non-suppressive effector T cells. In addition, the rapamycin-expanded Treg ameliorated colitis in an SCID mouse model.

Safinia et al. (27) were the first to establish a GMP-compliant production protocol to expand $CD25^+$-enriched cells from peripheral blood in the presence of rapamycin with the intention to prevent rejection after liver transplantation. In their 36-day expansion protocol, multiple rounds of *in vitro* Treg stimulation are necessary to reach clinically relevant Treg numbers. This may result in loss of FoxP3 expression and epigenetic stability, thus increasing the risk of *in vivo* Treg conversion into unwanted inflammatory effector cells.

Here, we provide the $CD25^+$ enrichment protocol, *ex vivo* expansion protocol as well as the validated lot-release protocols that have been approved by the German regulatory authorities for a Treg drug product intended for clinical use in patients with autoimmune and inflammatory disorders. Treg produced by this 21-day protocol are epigenetically stable, suppressive and contain less than 0.1% of contaminating $CD8^+$ effector cells. Moreover, we demonstrate the stability of the Treg drug product both after storage for up to 12 months and after subsequent dilution in a 0.9% physiological saline infusion solution. Also, we show that the Treg drug product remains polyclonal after 21 days of expansion and expresses various receptors associated with lymphocyte trafficking to secondary lymphoid organs and sites of inflammation. The protocol is scheduled to produce Treg for a phase I dose-escalation in patients and serves as an add-on platform for the adoptive transfer of Treg in a broad range of autoimmune and inflammatory disorders.

MATERIAL AND METHODS

Ethical Considerations

This study was approved by the local Institutional Review Board (IRB) of the Friedrich-Alexander-Universität Erlangen-Nürnberg under IRB number 151_12 B. In agreement with IRB approval and in accordance with the Declaration of Helsinki, oral and written consent was obtained from all healthy donors who donated blood for this study.

Materials and Equipment

The following materials are used during the Treg production process:

Autologous leucapherisate	
Autologous plasma	
MACS® GMP ExpAct Treg Kit	Miltenyi Biotec (# 170-076-119)
Human serum albumin	Baxter (# PL 00116/0620)
MACS® GMP Rapamycin	Miltenyi Biotec (# 170-076-308)
CliniMACS® CD8 Reagent	Miltenyi Biotec (# 275-01)
CliniMACS® CD19 Reagent	Miltenyi Biotec (# 179-01)
CliniMACS® CD25 Reagent	Miltenyi Biotec (# 274-01)
L-Glutamine	Lonza (# BE 17-605 E)
CliniMACS® PBS/EDTA	Miltenyi Biotec (# 700-25)
IL-2 (Proleukin®)	Novartis Pharma (# PZN 02238131)
X-VIVO15	Lonza (# BE 04-744)
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich (# D2438)
Glucose solution 40% (Glucosteril 40%)	Frescenius Kabi Deutschland GmbH

Treg Manufacture

A detailed overview of the manufacturing process is provided in **Figure 1**. The complete manufacturing process is performed in the GMP facility of the department of dermatology at the Friedrich-Alexander Universität Erlangen-Nürnberg. The manufacturing process is approved by the Bavarian Authorities under number DE_BY_05_MIA_2017_0012/55.2-2678.3-41-4-16.

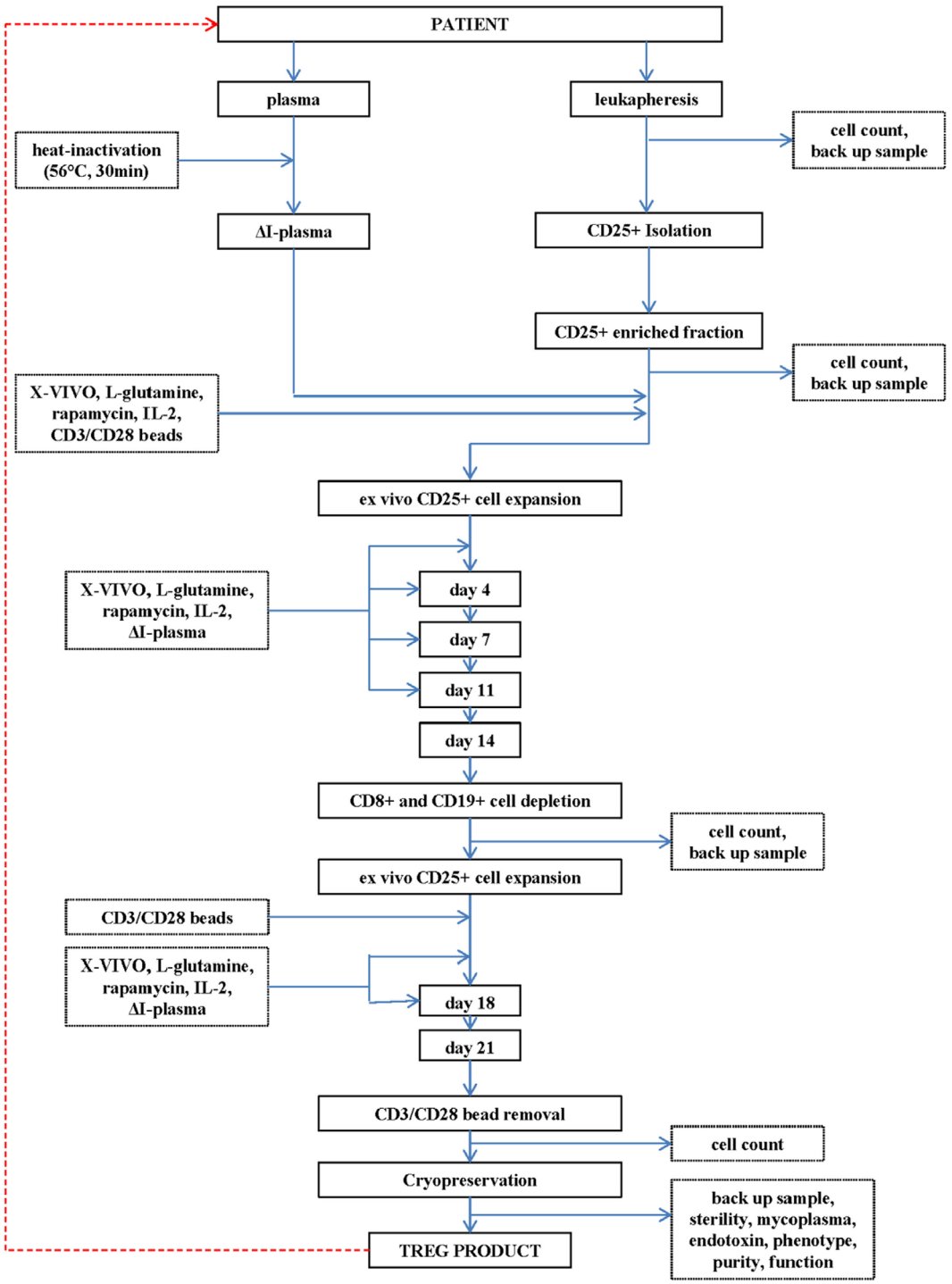

FIGURE 1 | Flowchart of the production of the regulatory T cell (Treg) drug substance and Treg drug product.

All cell purification steps are performed by using a CliniMACS® system (Miltenyi Biotec, Bergisch Gladbach, Germany) in conjunction with ISO certified CliniMACS® CD8 (Miltenyi Biotec, 275-01), CD19 (Miltenyi Biotec, 179-01), and CD25 (Miltenyi Biotec, 274-01) bead reagents. All purification steps are performed with GMP-grade CliniMACS® PBS/EDTA buffer (Miltenyi Biotec, 700-25) supplemented with clinical grade human serum albumin (Baxter, PL 00116/0620, PEI.H.03272.01-1). This buffer is hereafter called PBS–HSA–EDTA. All cell culture steps were performed in the presence of X-VIVO 15 medium without

gentamicin and phenol red (Lonza, BE 04-744) supplemented with heat inactivated autologous plasma, clinical grade IL-2 (1,000 IU/ml, Proleukin® S, Aldesleukin, Novartis Pharma, PZN 02238131), MACS® GMP rapamycin (100 ng/ml, Miltenyi Biotec, 170-076-308), and L-glutamine (200 mM, Lonza, BE 17-605 E). This medium is hereafter called complete autologous culture medium.

A leukapheresis product (department of Transfusion Medicine, Friedrich-Alexander Universität Erlangen-Nürnberg, Erlangen, Germany) is used as cell source for initial $CD25^+$ cell enrichment.

$CD25^+$ Cell Isolation

The operating procedures to enrich or deplete select cell subsets from a leukapheresis product are standardized and provided by the manufacturer of the CliniMACS® device (Miltenyi Biotec). Upon arrival in the GMP facility, the leucapherisate is diluted 1 + 3 with PBS–HSA–EDTA buffer and subsequently centrifuged at 200 *g* for 15 min at 22°C. After centrifugation, the supernatant is removed, the leucapherisate is resuspended in PBS–HSA–EDTA and centrifuged at 300 *g* for 15 min at 4°C. After this centrifugation step and subsequent removal of the supernatant, the leucapherisate is resuspended in 380 ml of cold PBS–HSA–EDTA and labeled with CliniMACS® CD25 reagent. The CliniMACS® CD25 bead reagent specifically labels up to 600×10^6 $CD25^+$ cells within a total population consisting of maximal 40×10^9 white blood cells. These CliniMACS® acceptance criteria are provided by Miltenyi Biotec. If one of these acceptance criteria is not met, a maximum of two portions, instead of one portion, of CliniMACS CD25 bead reagent are used to specifically label the leucapherisate. CD25-labeling is performed during 15 ± 2 min at 2–8°C at a cell shaker programmed at 25 rpm. After labeling is completed, the cell suspension is washed with PBS–HAS–EDTA, diluted in 100 ml PBS–HSA–EDTA and transferred into a cell collection bag. $CD25^+$ enrichment is performed by using the automatic CliniMACS® ENRICHMENT 3.2 program of the CliniMACS® device according to the manufacturer's instructions. The $CD25^+$ enriched cell fraction is used for further manufacturing.

Start of $CD25^+$ Cell Expansion on Day 0

Cell count and viability of the $CD25^+$ cells was determined on two samples of 40 µl by trypan blue staining according to European Pharmacopeia (Ph. Eur.) 2.7.29. Depending on cell number, $CD25^+$ cells were seeded at a density of 0.5×10^5 cells/ml in a 24-well or 6-well culture plate in complete autologous culture medium. To facilitate *in vitro* $CD25^+$ cell expansion, clinical grade IL-2 (1,000 IU/ml), rapamycin (100 ng/ml), and anti-CD3/anti-CD28 expander beads (MACS® GMP ExpAct Treg Kit, Miltenyi Biotec) were added at a bead-to-cell ratio of 4:1 to the cell cultures. Cell cultures were gently mixed and incubated at 37 ± 1°C, 5 ± 1% CO_2, >70% r.h. for 4 days.

Addition of Supplements at Day 4

At day 4, fresh IL-2 (1,000 IU/ml) and rapamycin (100 ng/ml) were added to the cell cultures to substitute for cellular consumption. Cell cultures were gently mixed and incubated at 37 ± 1°C, 5 ± 1% CO_2, >70% r.h. for 3 days.

Addition of Supplements and Medium on Day 7

At day 7, cell culture plates were collected from the incubator, and the total cell culture volume transferred into a T75 flask. Cell count and viability of the $CD25^+$ cells was determined on two samples of 40 µl by trypan blue staining according to Ph. Eur. 2.7.29. Depending on cell number, the cell density was adjusted to 0.5×10^6 cells/ml by adding fresh complete autologous culture medium, and the cell suspension was seeded in new culture plates. To substitute for cellular consumption, fresh IL-2 (1,000 IU/ml) and rapamycin (100 ng/ml) were added. Cell cultures were gently mixed and incubated at 37 ± 1°C, 5 ± 1% CO_2, >70% r.h. for 4 days.

Addition of Supplements and Medium on Day 11

Analogous to day 7, cell culture plates were collected from the incubator, and the cell suspension, depending on the volume, transferred into a T75 or 1 l cell culture flask. Cell count and viability of the $CD25^+$ cells was determined on two samples of 40 µl by trypan blue staining according to Ph. Eur. 2.7.29. Depending on cell number, the cell density was adjusted to 0.5×10^6 cells/ml by adding fresh complete autologous culture medium. Depending on total volume, the cell suspension was seeded in T75 or T175 cell culture flasks. To substitute for cellular consumption, fresh IL-2 (1,000 IU/ml) and rapamycin (100 ng/ml) were added. Cell cultures were gently mixed and incubated at 37 ± 1°C, 5 ± 1% CO_2, >70% r.h. for 3 days.

Depletion of $CD8^+$ and $CD19^+$ Cells on Day 14

At day 14, cell culture flasks were collected from the incubator, and the cell suspension, depending on the volume, transferred into a T75 or 1 l cell culture flasks. Cell count and viability of the $CD25^+$ cells was determined on two samples of 40 µl by trypan blue staining according to Ph. Eur. 2.7.29. To deplete potentially contaminating $CD8^+$ and $CD19^+$ cells, the $CD25^+$ cell product was magnetically labeled with CliniMACS® CD8 and CliniMACS® CD19 bead reagent according to the manufactures' instructions. Specifically, up to 4×10^9 $CD8^+$ cells and up to 5×10^9 $CD19^+$ cells within a total population consisting of maximal 40×10^9 cells may specifically be labeled by 7.5 ml of the CliniMACS® CD8 bead reagent and CliniMACS® CD19 bead reagent, respectively. These CliniMACS® acceptance criteria were provided by Miltenyi Biotec. In general, $CD25^+$ cell expansion on day 14 does not result in total cell numbers above 1×10^9 total cells, and the relative contamination with $CD8^+$ and/or $CD19^+$ cells is assumed to be below 25%. Therefore, a predefined aliquot of 1.875 ml of CliniMACS® CD8 bead reagent and CliniMACS® CD19 bead reagent is used to label up to 1.0×10^9 $CD8^+$ cells and up to 1.25×10^9 $CD19^+$ cells within a total population consisting of maximal 10×10^9 cells. For depletion of $CD8^+$ and $CD19^+$ cells, sterile CliniMACS® PBS/EDTA buffer supplemented with human serum albumin is used. Depletion of $CD8^+$ and $CD19^+$ cells is performed by using the automatic CliniMACS® DEPLETION 2.1 program of the CliniMACS® device. This program facilitates automated magnetic depletion of $CD8^+$ and $CD19^+$ cells in a closed, sterile system. Two cell fractions are collected into bags according to the instrumental settings of the CliniMACS® DEPLETION 2.1 program. The $CD8^+$- and $CD19^+$-depleted cell fraction 2 is used for further processing.

Cell number in fraction 2 is determined on two samples of 40 µl by trypan blue staining according to Ph. Eur. 2.7.29. $CD25^+/CD8^-/CD19^-$ cells of fraction 2 obtained from the CliniMACS® device are continued to be cultivated *in vitro*. Depending on cell number, the cell density was adjusted to 0.5×10^6 cells/ml by adding fresh complete autologous culture medium. Depending on total volume, the cell suspension was seeded in T75 or T175 cell culture flasks. To compensate for any anti-CD3/anti-CD28 bead removal during the $CD8^+$ and $CD19^+$ cell depletion process, anti-CD3/anti-CD28 beads (analogously to the amount of beads used at day 0) are added to the cell cultures. Finally, fresh IL-2 (1,000 IU/ml) and rapamycin (100 ng/ml) were added. Cell cultures were gently mixed and incubated at 37 ± 1°C, 5 ± 1% CO_2, >70% r.h. for 4 days.

Addition of Supplements and Medium on Day 18

Analog to days 7 and 11, cell culture plates or flasks were collected from the incubator, and the cell suspension, depending on the volume, transferred into a T75 or 1 l cell culture flasks. Cell count and viability of the $CD25^+$ cells was determined on two samples of 40 µl by trypan blue staining according to Ph. Eur. 2.7.29. Depending on cell number, the cell density was adjusted to 0.5×10^6 cells/ml by adding fresh complete autologous culture medium. Depending on total volume, the cells are subsequently seeded in T75 or T175 cell culture flasks. To substitute for cellular consumption, fresh IL-2 (1,000 IU/ml) and rapamycin (100 ng/ml) are added. Cell cultures are gently mixed and incubated (37 ± 1°C, 5 ± 1% CO_2, >70% r.h.) for an additional 3 days.

Harvesting of $CD25^+$ Cells at Day 21

At day 21, cell culture plates or flasks are collected from the incubator, and the total cell culture volume is transferred into a set of 50 ml centrifuge tubes. Culture flasks are washed once with approximately 10–20 ml of PBS/EDTA buffer supplemented with human serum albumin. Used washing buffer is also transferred into the 50 ml centrifuge tubes. Tubes are centrifuged, supernatants are discarded, and pellets are collected into a set of 50 ml centrifuge tubes by resuspending pellets with approximately 5 ml of PBS/EDTA buffer supplemented with human serum albumin. Cell count and cell viability of $CD25^+/CD8^-CD19^-$ cells are determined on two samples of 40 µl by trypan blue staining according to Ph. Eur. 2.7.29.

Anti-CD3/Anti-CD28 Expander Bead and CD25-, CD8-, and CD19-Labeling Bead Removal on Day 21

A maximum of 2.04×10^{10} beads can be depleted from a maximum of 4×10^{10} $CD25^+$ cells in a final concentration of 20×10^6–400×10^6/ml during the bead removal process. These CliniMACS® acceptance criteria are provided by Miltenyi Biotec. In general, $CD25^+$ cell expansion on day 21 does not result in total cell numbers above 4×10^{10} total cells. Therefore, the assumed maximum amount of added beads is 0.48×10^{10} [four times the maximum amount of isolated $CD25^+$ cells at day 0 ($1{,}200 \times 10^6$)]. For bead removal, sterile CliniMACS PBS/EDTA buffer supplemented with human serum albumin is used. Bead removal is performed by using the automatic CliniMACS® DEPLETION 2.1 program of the CliniMACS® device. The CliniMACS® device facilitates automated magnetic depletion of anti-CD3/anti-CD28 expander beads and CD25-, CD8-, and CD19-labeling beads in a closed, sterile system. Two cell fractions are collected into bags according to the instrumental settings of the CliniMACS® DEPLETION 2.1 program. The bead-depleted cell fraction 2 is used for filling and storage.

Filling and Storage of the $CD25^+$ Cells at Day 21

The bead-depleted cells are transferred into a set of 50 ml centrifuge tubes. Tubes are centrifuged, supernatants are discarded, and pellets are collected into 100 ml of PBS/EDTA buffer supplemented with human serum albumin. The final cell number in cell fraction 2 after bead removal is determined on two samples of 40 µl by trypan blue staining according to Ph. Eur. 2.7.29. Before filling and storage, a freezing medium is freshly prepared in a sterile bottle. The total volume is calculated based on the cell counting results obtained after the first centrifugation step after the anti-CD3/anti-CD28 bead removal step. The freezing medium consists of human serum albumin, DMSO, and 40% glucose solution (hereafter called freezing medium). After definition of the final $CD25^+$ cell number, cells are centrifuged and depending on cell number resuspended in an appropriate volume of human serum albumin at a final concentration of 20×10^6 viable cells/ml. Five hundred microliters of cell suspension are filled into each cryovial (1.0 ml total volume). After addition of 500 µl freezing medium (55.5 Vol.-% human serum albumin, 25.0 Vol.-% DMSO, and 20.0 Vol.-% glucose) to every vial, the closed vials are mixed gently and transferred immediately into a freezing container. The container is than stored immediately at −75 ± 10°C for 4–18 h. The vials are transferred to the gas phase of liquid nitrogen (≤−150°C) for up to 2 years.

Treg Lot-Release

Assessment of Treg Drug Product Identity and Cellular Composition

Throughout the manuscript, Treg are defined as $CD4^+CD25^+CD127^-$ cells (=Treg drug product identity) based on previously published data (3, 4). Treg drug product identity was determined before and after 21 days of expansion by staining with directly conjugated mouse antihuman antibodies (mAbs) against CD4 (FITC, clone RPA-T4), CD25 (FITC, clone M-A251), and CD127 (PE, clone RDR5). Treg drug product cellular composition was determined before and after 21 days of expansion by staining with directly conjugated mouse antihuman mAbs against CD8 (FITC, clone SK1), CD19 (FITC, clone SJ25C1), CD16 (PE, clone 3G8), and CD56 (PE, clone B159). Corresponding $IgG_{1,\kappa}$ mouse isotype controls (FITC, clone MOPC-21 and PE, clone MOPC-21) were included to assess unspecific binding. Potential dead cells were excluded by labeling with propidium iodide (PI) (BD Biosciences) according to the manufacturer's instructions. Cells were acquired using a FACS Calibur (BD Biosciences), and data were analyzed using CellQuest™ Pro (BD Biosciences) software. For information-only purposes, cells were intracellularly stained with FoxP3 (clone PCH101) using a FoxP3/Transcription Staining Buffer Set (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions.

In general, flow cytometry is performed as described in Ph. Eur. 2.7.24. The specific method for flow cytometry was validated, since it is not in detail described in the Ph. Eur. In brief, thawed dendritic cells were used to validate the method for flow cytometry. Thawed dendritic cells, thawed lymphocytes, thawed Treg, and thawed $CD25^-$ cells were used to determine specificity. Lymphocytes were single stained with CD4, CD127, or CD8 antibody, respectively, fixed with 1% formaldehyde, washed twice and subsequently mixed with unstained lymphocytes of the same preparation. To determine specificity of the CD25 antibody, Treg were single stained with the CD25 antibody, fixed with 1% formaldehyde, washed twice and subsequently mixed with unstained Treg of the same preparation The FACS result for stained lymphocytes or Treg was set to 1 (=100% positive). For specificity purposes, optimal positive and negative cells for each antibody characterizing the Treg drug product were measured. Nominal negative cells shall show less than 0.04 (=4%) positive cells, and nominal positive cells shall show more than 0.04 (=4%) positive ones. Summarized results are shown in **Table 1**. In addition, the stringent cutoff of less than 0.1% contaminating $CD8^+$ cells in the final Treg product was validated separately. To avoid false negative $CD8^+$ values in the final Treg product, the number of to acquire cells was increased to 300,000. A total of six acquisitions were performed with mature monocyte-derived dendritic cells, which represented the $CD8^-$ cell subset. The SD (*s*) was calculated from all acquired "$CD8^+$" cells within the $CD8^-$ mature dendritic cell subset, and the quantification limit (QL) was calculated based on the following formula: $QL = 10s/a$ with *a* set to 0.973 (=linear regression value derived from the flow cytometry validation report) (data not shown).

Assessment of Treg Drug Product Purity

Regulatory T cell drug product purity was determined after anti-CD3/anti-CD28 expander bead removal by staining with directly conjugated mouse antihuman $IgG_{3,\kappa}$ Labeling Check PE (clone AC146, Miltenyi Biotec) and APC (clone AC146, Miltenyi Biotec). Samples were acquired using a FACS Calibur (BD Biosciences), and data were analyzed using CellQuest™ Pro (BD Biosciences) software.

The method to measure anti-CD3/anti-CD28 expander bead and CD25-, CD8- and CD19-labeling bead contamination was validated since it is not described in a Ph. Eur. Specifically, thawed lymphocytes were mixed with predefined amounts of anti-CD3/anti-CD28 expander beads and incubated for 1 h at 37°C at 5% CO_2. After 1 h of incubation, cells were lysed using saponin 0.2% (Sigma-Aldrich, St. Louis, MI, USA) to also capture potential intracellular beads. Subsequently, beads were stained by flow cytometry with Labeling Check Reagent PE and Labeling Check Reagent APC as described above. To determine bead contamination, all samples were acquired with the use of Trucount® tubes

TABLE 1 | Validation results of analysis by flow cytometry.

Validation part	Parameter	Acceptance	Result		Passed
Reproducibility	Coefficient of variation	≤5%	0.3 0.5 0.7	2.0% 2.5% 0.8%	X yes ☐ no
Intermediate precision	Deviation of mean	≤10%	0.3 0.5 0.7	8.7% 4.9% 7.1%	X yes ☐ no
	Coefficient of variation	≤8%	0.3 0.5 0.7	5.6% 3.8% 4.0%	X yes ☐ no
Linearity	Correlation	≥0.9	1,000		X yes ☐ no
	Linear regression	n.a.	$y = 0.973x + 0.002$		X yes ☐ no
Range	Cell fraction	0.04–1	0.04–1		X yes ☐ no
Accuracy	Deviation from actual value	0.3 ± 0.03 0.5 ± 0.05 0.7 ± 0.07	0.3 0.5 0.7	0.022 0.019 0.014	X yes ☐ no
	Recovery	100 ± 10%	0.3 0.5 0.7	92.8% 96.1% 98.1%	X yes ☐ no
Limit of detection	Quantification limit (QL) (10,000 cells)	≤0.04	0.016		X yes ☐ no
Limit of detection CD8	QL (300,000 cells)	≤0.001	0.001		X yes ☐ no
Specificity	Fluorescence in channel 1 or 2	Meets specificity	Meets specificity for all antibodies tested		X yes ☐ no
Efficiency of antibodies	Fraction of positive cells	Within range of development	All antibodies within range		X yes ☐ no

Positive cells and negative cells were mixed at the indicated fractions 0.3, 0.5, and 0.7 to represent 30, 50, and 70% of the final cell mixture. For reproducibility, triplicate analysis of three different fractions of positive cells was statistically analyzed by coefficient of variation. The analysis of a different person on a different day was used to determine the intermediate precision. To determine linearity, 10 different fractions of positive cells were analyzed by correlation factor and linear regression. These results were compared with the theoretical real fraction contents to determine accuracy. The limits of detection were determined by analyzing six negative cell fractions and calculated with the formula: 10s/a (s indicating SD and a indicating slope of regression). Antibody specificity and efficiency were determined by using positive and negative cell fractions, respectively.

(BD Biosciences) to assure standardized acquisition. Nominal samples negative for beads should show less than 400 beads, nominal samples positive for beads should show more than 400 beads. At least 100×10^6 Treg drug product cells should be lysed, labeled with Labeling Check Reagent PE and Labeling Check Reagent APC and subsequently measured to define bead contamination in the final Treg drug product. The results of the validation are summarized in **Table 2**.

Assessment of Treg Drug Product Function

The method to assess Treg drug product function was validated, since it is not described in the Ph. Eur. Specifically, cryopreserved autologous $CD25^-$ cells were thawed, washed, and labeled with 5 μM carboxyfluorescein succinimidyl ester (CFSE) (ThermoFisher Scientific, Carlsbad, CA, USA). Next, CFSE-labeled $CD25^-$ cells (containing $CD4^+$ and $CD8^+$ cells; hereafter called responder cells) were cocultured with thawed day 21 Treg drug product cells (hereafter called Treg) at a Treg to responder cell ratio of 1 + 1, 1 + 5, and 1 + 10. Cocultures were stimulated with anti-CD3 and anti-CD28 coated beads (MACS GMP ExpAct Treg Kit, Miltenyi Biotec) at a bead-to-cell ratio of 1 + 1. Negative controls included responder cells alone and Treg + responders cells at a ratio of 1 + 1 without the addition of anti-CD3 and anti-CD28 coated beads. The positive control included responder cells alone with the addition of anti-CD3 and anti-CD28 coated beads at a bead + responder cell ratio of 1 + 4. The absolute cell concentration and cell density at the beginning of the coculture was 1×10^6/ml and $1 \times 10^6/cm^2$ per well, respectively.

Cocultures were routinely performed in triplicates in 48-well plates and harvested after 60–72 h of incubation at 37 ± 1°C, 5 ± 1% CO_2, >70% r.h. After 60–72 h of incubation, conditions were harvested, stained with PI and CD8 as described under flow cytometry and acquired using a FACS Calibur (BD Biosciences). Data were analyzed using CellQuest™ Pro (BD Biosciences) software. Treg-mediated suppression was calculated based on the percentage of divided cells in the first cell generation with the positive control set to 100%. One cell generation was defined to contain at least ≥10% divided cells. Based on all cell generations, negative control cells should show less than 5% proliferated cells, and positive control cells should show more than 30% proliferated cells. Lot-release is based on the amount of Treg-mediated suppression in cocultures with a Treg to responder cell ratio of 1 + 1, 1 + 5, and 1 + 10, respectively. For validation, more Treg to responder cell ratios were included. The results of the validation are summarized in **Table 3**.

TABLE 2 | Validation results of analysis of anti-CD3/anti-CD28 bead contamination.

Validation part	Parameter	Acceptance	Result		Passed
Reproducibility	Coefficient of variation	≤25	750 1,000 3,000	7.1 8.7 22.7	X yes ☐ no
Intermediate precision	*F*-value	<9.28	750 1,000 3,000	1.54 5.50 2.49	X yes ☐ no
	Deviation from mean	≤15%	750 1,000 3,000	6.5 14.1 7.3	X yes ☐ no
	Coefficient of variation	≤25	750 1,000 3,000	14.0 13.3 17.6	X yes ☐ no
Linearity	Coefficient of correlation	≤0.98	0.996		X yes ☐ no
Range	Detection range	300–6,000	300–6,000		X yes ☐ no
Accuracy	Mean	637–863 850–1,150 2,400–3,600	750 1,000 3,000	689 1,105 3,420	X yes ☐ no
	Retrieval rate	±15% ±15% ±20%	750 1,000 3,000	8.1% 10.5% 14.0%	X yes ☐ no
Limit of detection	QL	<400	390		X yes ☐ no
Specificity	Positive particle	>400	1,105		X yes ☐ no
	Negative particle	<400	38		X yes ☐ no

A total of 750, 1,000, and 3,000 anti-CD3/anti-CD28 expander beads were mixed with 100 × 10^6 peripheral blood mononuclear cells. For reproducibility, triplicate analysis of indicated cell-bead mixtures was statistically analyzed by coefficient of variation. The analysis of a different person on a different day was used to determine the intermediate precision. To determine linearity, 10 different cell-bead mixtures were analyzed by correlation factor and linear regression. These results were compared with the theoretical real fraction contents to determine accuracy. The limits of detection were determined by analyzing six bead-negative cell mixtures and calculated with the formula: 10s/a (s indicating SD and a indicating slope of regression). Specificity was determined by using bead-positive and bead-negative cell mixtures, respectively. QL, quantification limit.

Assessment of the Treg Drug Product Concerning Viability, Cell Number, Cell Concentration, Sterility, Bacterial Endotoxins, and Mycoplasma DNA

Cell number, cell concentration, and cell viability are determined by trypan blue staining and microscopic examination using a hemocytometer according to the method described in Ph. Eur. 2.7.29. Sterility testing is routinely performed according to Ph. Eur. 2.6.1 by Bioservice Scientific Laboratories (BSL) GmbH, Planegg, Germany. Testing on bacterial endotoxins is routinely performed according to Ph. Eur. 2.6.14 by BSL. Testing on mycoplasma DNA is achieved by PCR by an in-house validated method by BSL.

T Cell Receptor (TCR) Vβ Repertoire Analysis

The TCR Vβ repertoire of *ex vivo* generated $CD25^+$ cells was determined by using the IO Test Beta Mark TCR Vβ Repertoire kit (Beckman Coulter, France) as previously published (28). Day 0 derived $CD25^+$ cells and day 21 $CD25^+$ cells were stained and analyzed for TCR Vβ specificity according to the manufacturer's instructions.

Epigenetic Analysis

Genomic cellular DNA was isolated using a high pure PCR template preparation kit (Roche). Next, sodium bisulfite conversion of the purified DNA was performed by using the EpiTect® Fast DNA Bisulfite Kit (Qiagen) according to the manufacturer's

TABLE 3 | Validation results of analysis of regulatory T cell (Treg)-mediated suppression.

Validation part	Sample	Specificity	Result	Passed
Reproducibility				
Coefficient of variation	2 + 1	≤5%	0.3	X yes ☐ no
	5 + 1	≤5%	1.0	X yes ☐ no
	10 + 1	≤5%	2.2	X yes ☐ no
	30 + 1	≤5%	4.5	X yes ☐ no
	50 + 1	≤10%	4.9	X yes ☐ no
Intermediate precision				
Deviation from mean	2 + 1	≤±8%	5.0	X yes ☐ no
	5 + 1	≤±12%	7.2	X yes ☐ no
	10 + 1	≤±20%	12.5	X yes ☐ no
	30 + 1	≤±30%	−5.6	X yes ☐ no
	50 + 1	≤±40%	−39.8	X yes ☐ no
Coefficient of variation	2 + 1	≤10%	2.8	X yes ☐ no
	5 + 1	≤10%	3.8	X yes ☐ no
	10 + 1	≤10%	5.4	X yes ☐ no
	30 + 1	≤15%	4.8	X yes ☐ no
	50 + 1	≤15%	8.0	X yes ☐ no
Linearity				
Coefficient of correlation in ascending range	100 + 1 to 5 + 1	≥0.8	0.908	X yes ☐ no
Range	All samples	≤10–≥90%	2.4–97.7%	X yes ☐ no
Accuracy				
Retrieval rate	1 + 1	100 ± 5%	99.7%	X yes ☐ no
	5 + 1	100 ± 5%	98.4%	X yes ☐ no
	10 + 1	100 ± 10%	108.8%	X yes ☐ no
Limit of detection				
QL proliferation		≤1%	0.4%	X yes ☐ no
QL suppression		≥95%	99.99%	X yes ☐ no

Responder cells and Treg were mixed at the indicated ratios of 2 + 1, 5 + 1, 10 + 1, 30 + 1, and 50 + 1. For reproducibility, triplicate analysis of responder cell to Treg ratios was statistically analyzed by coefficient of variation. The analysis of a different person on a different day was used to determine the intermediate precision. To determine linearity, 10 different responder cell to Treg ratios (1,000 + 1, 200 + 1, 100 + 1, 50 + 1, 30 + 1, 10 + 1, 5 + 1, 2 + 1, 1 + 1, and 1 + 2) were analyzed by correlation factor and linear regression. These results were compared with the theoretical real fraction contents to determine accuracy. The limits of detection were determined by analyzing six non-stimulated responder cell to Treg mixtures (1 + 1) and calculated with the formula: 10s/a (s indicating SD and a indicating slope of regression).
QL, quantification limit.

instructions. The following primers and probe enabled us to specifically detect methylated FoxP3 (29):

5′-TGTCGATGAAGTTCGGCGTAT-3′ (forward)
5′-CCCCCGACTTACCCAAATTT-3′ (reverse)
6FAM-5′-CGGTCGTTATGACGTTAATGGCGGA-3′-TAMRA (probe)

Primers and probes for the detection of unmethylated FoxP3 were designed accordingly:

5′-TGTTGATGAAGTTTGGTGTAT-3′ (forward)
5′-CCCCCAACTTACCCAAATTT-3′ (reverse)
6FAM-5′-TGGTTGTTATGATGTTAATGGTGGA-3′-TAMRA (probe).

Quantitative PCR (qPCR) was performed by using the maxima probe qPCR master mix (ThermoFisher Scientific) and a C1000TM Thermal Cycler (Bio-Rad). Percentage of methylation in cells was calculated as Meth. [%] = $100/[1 + 2^{\Delta Ct(meth-unmeth)}]$% as recently described (30). ΔCt(meth − unmeth) represents the difference between the Ct value of methylated FoxP3 signal and Ct value of unmethylated FoxP3 value. Hypomethylation was calculated as hypometh. [%] = 100% − meth. [%]. Since the male FoxP3 Treg-specific demethylated region (TSDR) is described to be fully demethylated, and the female TSDR shows hemimethylation (31), the above described calculation would underestimate the relative number of hypomethylated Treg in cells derived from human donors. To circumvent an underestimation, the methylation index for female-derived probes was corrected using the following formula: meth. $[\%]_{female}$ = meth. [%] − (100 − meth. [%]). Similarly, hypomethylation in female derived probes was calculated as hypometh. [%] = 100% − meth. $[\%]_{female}$.

Assessment of Homing Markers

The homing potential of day 0 $CD25^+$ cells and day 21 Treg drug product cells was assessed by staining with directly conjugated mAbs against CCR4 (clone L21H4), CCR8 (clone 191704), CD62L (clone DREG-56), CD103 (clone Ber-ATC8), CXCR3 (clone G025H7), PSGL-1 (clone 688101), CCR9 (clone L053E8), CCR5 (clone HEK/1/85a), alpha4 Integrin (clone MZ18-24A9), beta7 Integrin (clone FIB27), and the purified mAb GPR15 (clone 367902) with subsequent staining with specific mouse secondary IgG2b APC labeled antibody. Corresponding mouse isotype controls were included to assess unspecific binding. Cells were acquired using an LSR Fortessa (BD Biosciences), and data were analyzed using FlowJo software.

Statistical Analysis

Statistical differences as measured by a two-sided paired Student *t* test were calculated using Excel v2010, based on the number of experiments as indicated in the figure legends. Differences were considered to be significant at a *P* value less than 0.05.

RESULTS

Treg Drug Product Expansion, Viability, and Cell Number

To validate the established GMP-complaint production protocol and the predefined lot-release criteria, we performed four subsequent Treg productions with a healthy donor-derived leukapheresis product. Such a production is also called "consistency run" (hereafter called Con) and is executed by exactly following the predefined protocol and predefined lot-release criteria. A minimum of three consecutive Cons who pass all predefined lot-release criteria is required to obtain official GMP production approval from the German authorities. With use of the protocol, leukapheresis-derived $CD25^+$ cells expanded greater than 2 orders of magnitude with an average cell number of 113×10^6 at day 7 (range 34×10^6–199×10^6), 501×10^6 at day 11 (range 158×10^6–849×10^6), 635×10^6 at day 14 (range 234×10^6–$1{,}020 \times 10^6$), 986×10^6 at day 18 (range 159×10^6–$2{,}074 \times 10^6$), and $1{,}076 \times 10^6$ (range 528×10^6–$1{,}440 \times 10^6$) at day 21 after

anti-CD3/anti-CD28 expander bead removal (**Figures 2A,B**). The exact starting Treg number and exact expansion rate per consistency run are shown in File S1 in Supplementary Material. In addition, cell viability met the predefined limit of ≥75% viable cells at day 7 (range 94.7–98.3%), day 11 (range 93.5–98.7%), day 14 (range 95.8–97.6%), day 18 (range 94.3–98.8%), and day 21 (range 92.9–98.3%) after anti-CD3/anti-CD28 expander bead removal in every consistency run (**Table 4**).

Treg Drug Product Sterility, Bacterial Endotoxins, and Mycoplasma DNA

One thawed vial of the day 21 Treg drug product from each consistency run was tested for its sterility and the presence of bacterial toxins and mycoplasma DNA. As confirmed by BSL, no bacterial growth was contaminating the Treg drug product after 21 days of expansion. Also, no bacterial endotoxins and no mycoplasma DNA could be detected (**Table 4**).

Treg Drug Product Identity and Cellular Composition

The presence of CD4 (≥90.0%) and CD25 (≥80.0%) in combination with low to absent surface expression of CD127 (≤10.0%) is used to phenotypically discriminate Treg from effector T cells. This approach has been elected based on studies by Liu et al. who reported that CD127 surface expression inversely correlates with FoxP3 and suppressive function of human $CD4^+$ Treg[3]. A cutoff of more than 80.0 and 90.0% was chosen for CD25 and CD4, respectively. A cutoff of less than 10.0% was chosen for CD127. Moreover, the Treg drug product cellular composition is guaranteed by the stringent cutoff criteria for potential CD8 (≤0.1%), CD19 (≤1.0%), and CD56/CD16 (≤1.0%) cells contaminating the Treg drug product. These cutoff criteria were based on the results of several clinical studies in our department with dendritic cells (32, 33). In these studies, a cellular contamination, based on the sum of $CD3^+$ T cells, $CD19^+$ B cells,

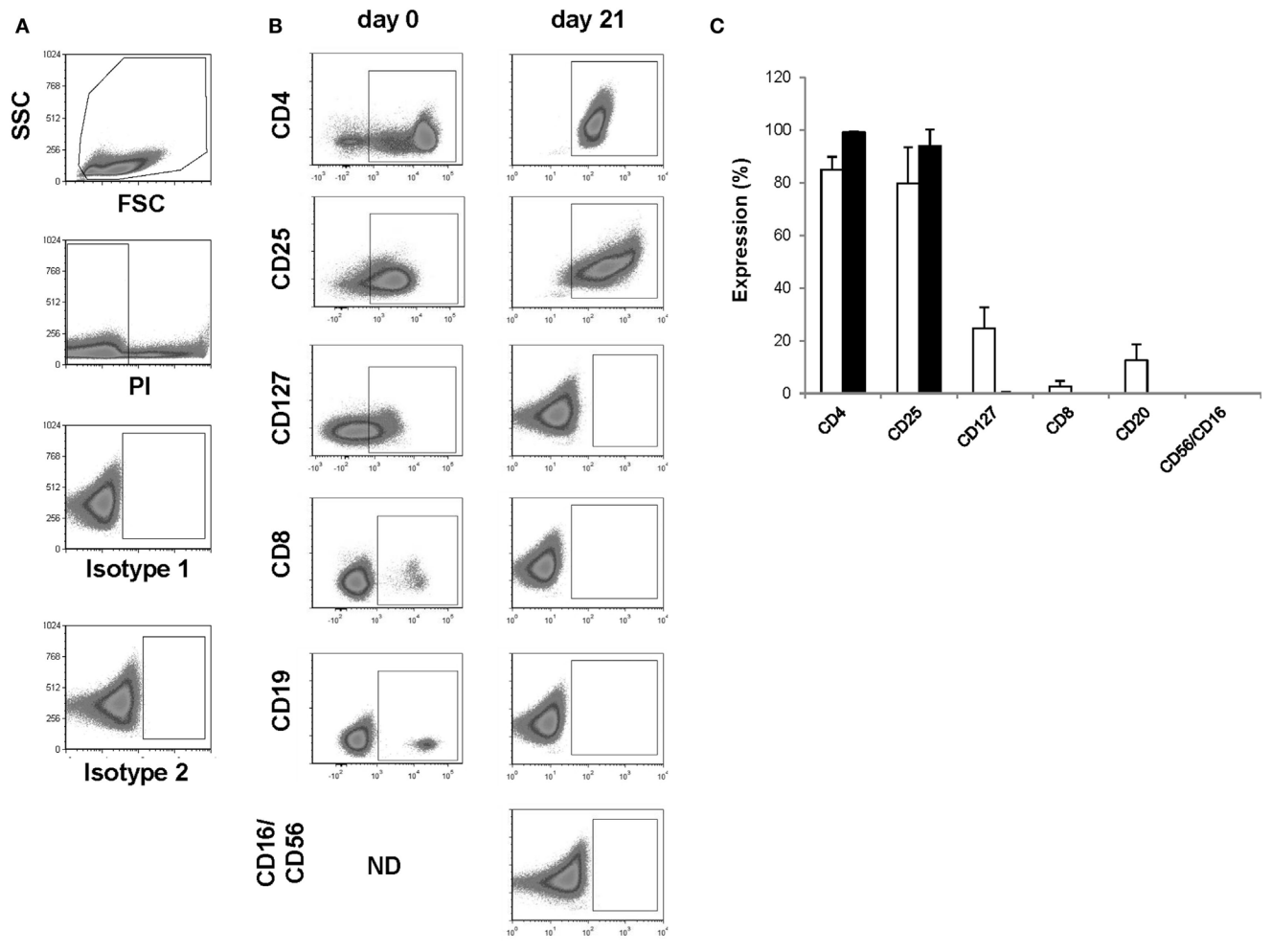

FIGURE 2 | Assessment of identity and cellular composition. **(A)** Representative FACS plots showing the gating strategy. **(B)** Representative FACS plots gated on PI^- cells showing CD4, CD25, CD127, CD8, CD19, and CD56/CD16 expression after $CD25^+$ cell enrichment at day 0 and after thawing the day 21 regulatory T cell (Treg) drug product. **(C)** Proportion of PI^- cells expressing CD4, CD25, CD127, CD8, CD19, and CD56/CD16 after $CD25^+$ cell enrichment at day 0 ($n = 4$, white bars) and after thawing the day 21 Treg drug product ($n = 4$, filled bars). ND, not determined.

TABLE 4 | Good manufacturing practice-compliant production process evaluation.

Parameter	Limit	Con1	Con2	Con3	Con4
Viable cells/ml	$\geq 5.0 \times 10^6$	10.5×10^6	6.3×10^6	10.8×10^6	8.2×10^6
Cell viability (%)	≥75	96.7	92.8	96.8	96.9
Sterility	No growth	No growth	No growth	No growth	No growth
Endotoxin (IU/ml)	≤30	≤30	≤30	≤30	≤30
Mycoplasma	Negative	Negative	Negative	Negative	Negative
CD4 (%)	≥90.0	99.6	99.2	98.6	99.4
CD25 (%)	≥80.0	98.9	98.6	85.5	93.3
CD127 (%)	≥10.0	0.00	0.05	0.68	0.00
CD8 (%)	≤0.10	0.00	0.00	0.06	0.00
CD19 (%)	≤1.0	0.00	0.00	0.00	0.00
CD56/16 (%)	≤1.0	0.00	0.00	0.06	0.00
Labeling check reagent PE (%)	≤400	≤400	≤400	≤400	≤400
Labeling check reagent APC (%)	≤400	≤400	≤400	≤400	≤400
Suppression at ratio 1 + 1 (%)	≥80.0	99.5	81.4	98.2	99.9
Suppression at ratio 1 + 5 (%)	≥60.0	96.1	60.4	97.2	99.4
Suppression at ratio 1 + 10 (%)	≥50.0	85.4	52.5	61.6	83.2

Four successive regulatory T cell (Treg) productions were performed with healthy donor-derived leukapheresis products. Productions passed the indicated limits for release. Viable cells per milliliter indicate the total number of life retrieved Treg in the final Treg product. Viability indicates the percentage of life cells in the final Treg product. Sterility indicates bacterial growth contaminating the final Treg product. CD4, CD25, CD127, CD8, CD19, and CD56/CD16 indicate surface expression on the final Treg product. Labeling check reagent PE and APC indicate the remaining number of anti-CD3/anti-CD28 beads in the final Treg product. Suppression at ratio 1 + 1, 1 + 5, and 1 + 10 indicate the amount of suppression within the first generation of proliferating $CD8^+$ responder cells by the final Treg product.

and $CD56^+$ NK-cells, of up to 10% of the total infused cells was tolerated well by patients with cutaneous or ocular melanoma. In the case of *ex vivo* expanded autologous Treg, contamination with $CD8^+$ T cells potentially induces inflammation in patients with autoimmune disorders. Therefore, a cutoff of less than 0.1% contaminating $CD8^+$ cells was chosen for the release of the Treg drug product and a cutoff of less than 1.0% was chosen for contaminating CD19 and CD56/CD16 cells. Importantly, to reliably show less than 0.1% $CD8^+$ cell contamination in the final Treg product, a total of 300,000 cells must be acquired by flow cytometry. As shown in **Figure 2** and **Table 4**, the performed consistency runs passed the predefined lot-release criteria for product identity and cellular composition by the validated flow cytometry method. Specifically, an average of 99.2% CD4 (range 98.6–99.6%), 94.1% CD25 (range 85.5–98.9%), 0.18% CD127 (range 0.0–0.68%), 0.02% CD8 (range 0.0–0.06%), 0.0% CD19 (range 0.0–0.0%), and 0.2% CD56/16 (range 0.0–0.06%) was determined in the thawed Treg drug products. In addition, as an internal scientific in-process control, intracellular FoxP3 expression was determined on enriched day 0 $CD25^+$ cells and day 21 expanded $CD25^+$ cells (File S2 in Supplementary Material).

Treg Drug Product Purity

The anti-CD3/anti-CD28 expander beads are removed from the cell product after 21 days of expansion using the CliniMACS® system. Although the CD25-, CD8-, and CD19-labeling beads generally are metabolized during the expansion process, potential remaining labeling beads will simultaneously be removed from the Treg drug product. As defined by our in-house validated bead removal method and shown in **Figure 3** and **Table 4**, bead removal was efficient, since in a total of 100×10^6 lysed Treg product cells less than 400 beads could be retrieved by flow cytometry.

Treg Drug Product Function

Currently, the intracellular expression of FoxP3 is the most recognized marker to define Treg in human, yet intracellular FoxP3 staining mostly shows high intra-sample variation. In addition, consensus on which antibody clone to include in staining protocols is inconclusive (32–34). We, therefore, elected to omit FoxP3 expression as a separate lot-release criterion. To compensate for the lack of a highly specific Treg marker as part of our lot-release criteria, we established a biologic assay confirming the suppressive nature of the thawed Treg drug product. The objective was to reach ≥80.0% suppression within the first generation of divided cells at a responder cell to Treg ratio of 1 + 1, ≥60.0% suppression within the first generation of divided cells at a responder cell to Treg ratio of 1 + 5, and ≥50.0% suppression within the first generation of divided cells at a responder cell to Treg ratio of 1 + 10. In addition, suppression within the first generation of divided cells at a responder cell to Treg ratio of 1 + 1 had to exceed suppression within the first generation of divided cells at a responder cell to Treg ratio of 1 + 5. Likewise, suppression within the first generation of divided cells at a responder cell to Treg ratio of 1 + 5 had to exceed suppression within the first generation of divided cells at a responder cell to Treg ratio of 1 + 10. As shown in **Figure 4** and **Table 4**, an average responder cell proliferation of 21.3% was observed within the first generation of divided cells when no Treg were added to the coculture. In the presence of Treg at a ratio of 1 + 1, responder cells showed an average of 1.09% of proliferation within the first generation of divided cells, whereas 2.37 and 6.62% of proliferation was observed at a ratio of 1 + 5 and 1 + 10, respectively (**Figure 4C**). The Treg-mediated suppression within the first generation of divided cells was calculated with the positive control set to 100%, resulting in an average suppression of 94.7% at a responder cell to Treg ratio of 1 + 1 (range 81.4–99.9%), 88.3% suppression at a responder cell to Treg ratio of 1 + 5 (range 60.4–99.3%) and 70.7% suppression

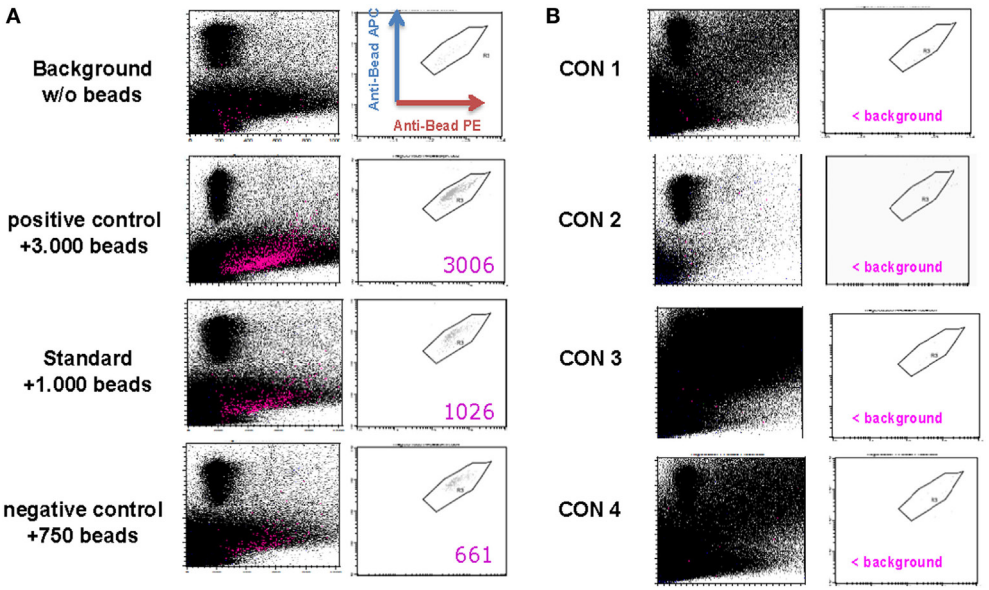

FIGURE 3 | Assessment of regulatory T cell (Treg) drug product purity. **(A)** Representative FACS plots gated on PE$^+$/APC$^+$ anti-CD3/anti-CD28 expander beads in a control sample containing no anti-CD3/anti-CD28 expander beads (=background sample), a control sample containing 750 anti-CD3/anti-CD28 expander beads (=negative control sample), a control sample containing 1,000 anti-CD3/anti-CD28 expander beads (=standard sample), and a control sampler containing 3,000 anti-CD3/anti-CD28 expander beads. **(B)** FACS plots gated on PE$^+$/APC$^+$ anti-CD3/anti-CD28 expander beads in a thawed Treg drug product from consistency run 1 (=Con1), consistency run 2 (=Con2), consistency run 3 (=Con3), and consistency run 4 (=Con4).

at a responder cell to Treg ratio of 1 + 10 (range 52.5–85.5%) (**Figure 4D**). In addition, average suppression within the first generation of divided cells at a responder cell to Treg ratio of 1 + 1 exceeded suppression within the first generation of divided cells at a responder to Treg ratio of 1 + 5. Likewise, average suppression within the first generation of divided cells at a responder cell to Treg ratio of 1 + 5 exceeded suppression within the first generation of divided cells at a responder to Treg ratio of 1 + 10. Moreover, based on all proliferated CD8$^+$ cells, including all cell generations, a gradual increase in mean fluorescence intensity (MFI) was observed with increasing Treg to responder cell ratios with a mean MFI of 522.8 (range 440–705) at a Treg to responder cell ratio of 1 + 10 and a mean MFI of 667.5 (range 539–893) at a Treg to responder cell ratio of 1 + 1 (File S3A–C in Supplementary Material). Likewise, a reduction in the total number of cell generations was observed with increasing Treg to responder cell ratios (File S3D in Supplementary Material).

Treg Drug Product Stability

Stability data were acquired with Treg drug products that were continuously stored in the gas phase of liquid nitrogen ≤−150°C for at least 12 months. Stored Treg drug products were thawed and analyzed according to the criteria defined for lot-release. Microbial testing, phenotyping, cellular composition, viability, recovery, and function met the limits of the drug product in every case for up to 12 months (**Table 5**). Therefore, guaranteed stability for the Treg drug product was set at 12 months.

Moreover, Treg drug products stored for at least 12 months from each consistency run were thawed, diluted with 0.9% sodium chloride solution and incubated for 90 min at 30 ± 1°C to mimic the preparation of the Treg drug product for adoptive transfer in clinical settings. After 90 min of incubation at 30 ± 1°C in a 0.9% sodium chloride solution, phenotype, cellular composition, viability, recovery, and function met the preset limits of the drug product in all tested products (**Table 6**).

Treg Drug Products Are Polyclonal, Hypomethylated, and Express Various Markers Associated with Tissue Homing

To rule out the outgrowth of monoclonal Treg clones during the GMP-compliant production process, Treg drug products were thawed and stained with a panel of 24 distinct TCR Vβ monoclonal antibodies, which cover approximately 70% of the human TCR Vβ repertoire. As shown in **Figure 5A**, both enriched day 0 CD25$^+$ cells and day 21 Treg expressed all 24 TCRs, indicating that the produced Treg remain polyclonal. In addition, GMP-compliant Treg production does not affect hypomethylation of the Treg at intron 1 of the FoxP3 locus, since no significant difference in hypomethylation was found between enriched day 0 CD25$^+$ cells and day 21 Treg drug products ($P = 0.5111$, **Figure 5B**).

Moreover, as shown in **Figure 6**, the Treg drug product expressed various receptors associated with lymphocyte trafficking into tissues and to sites of inflammation. Specifically, expanded Treg expressed moderate to high levels of PSGL-1, α4β7 integrin, CD103, CCR4, and CD62L and expression levels significantly increased during the expansion process with $P = 0.0022$ for PSGL-1, $P = 0.0073$ for α4β7 integrin, $P = 0.0138$ for CD103, $P = 0.0248$ for CCR4, and $P = 0.0036$ for CD62L.

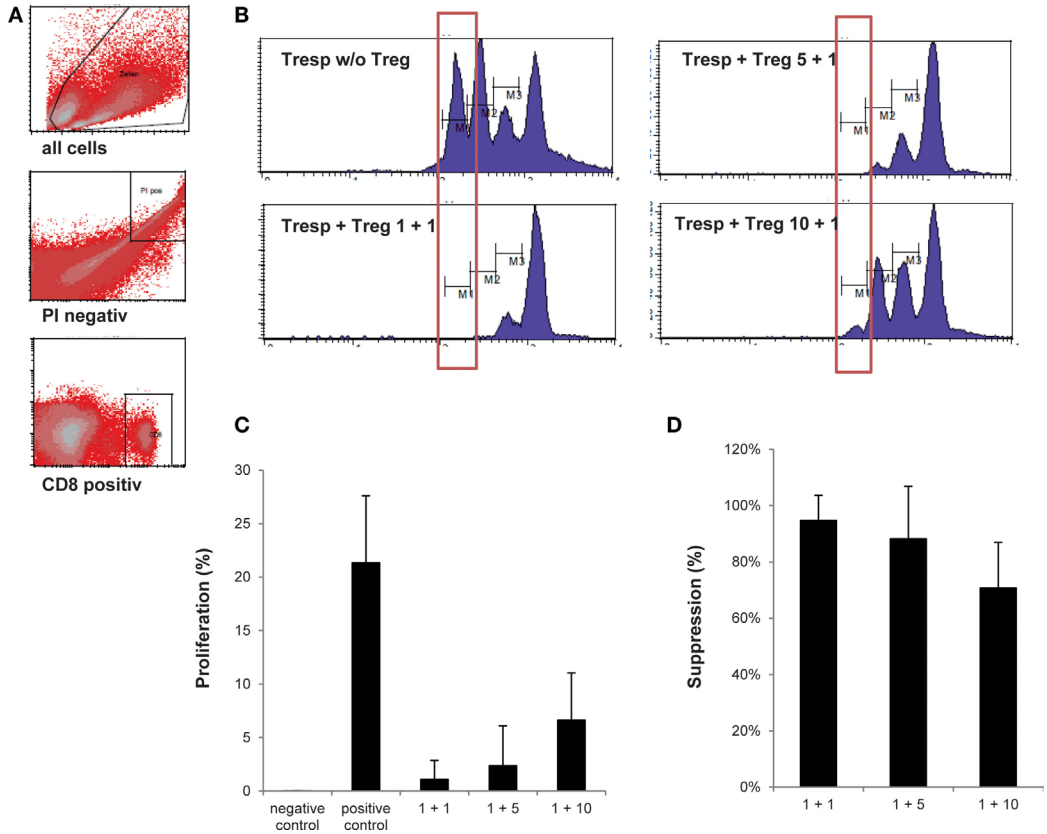

FIGURE 4 | Assessment of regulatory T cell (Treg) drug product function. **(A)** Representative FACS plots showing the gating strategy defining $CD25^-$ responder cell proliferation. **(B)** Representative histograms gated on $CD8^+/CFSE^+$ responder cells showing percentage of responder cell proliferation within the most divided cell generation in the presence of the thawed day 21 Treg drug product cells at a Treg to responder cell ratio of 1 + 1, 1 + 5, and 1 + 10, respectively. **(C)** Proportion of the first generation of responder cells ($n = 4$) showing proliferation in the presence of no anti-CD3/anti-CD28 beads (=negative control), in the presence of anti-CD3/anti-CD28 beads (=positive control) and at a Treg to responder cell ratio of 1 + 1 (mean 1.09%), 1 + 5 (mean 2.37%), and 1 + 10 (mean 6.62%), respectively. **(D)** Amount of suppression in the first generation of divided cells at a Treg to responder cell ratio of 1 + 1 (mean 94.7%), 1 + 5 (mean 88.3%), and 1 + 10 (mean 70.7%).

TABLE 5 | Results of regulatory T cell (Treg) drug product stability testing after at least 12 months of storage.

Parameter	Con1		Con2		Con3		Con4		Acceptance criteria	Criteria passed
	Release	>12 months	Release	>12 months	Release	>12 months	Release	>12 months		
CD4 (%)	99.6	99.7	99.2	99.2	98.6	98.7	99.4	99.3	≥90.0	Yes
CD25 (%)	98.9	95.7	98.6	98.3	85.5	81.9	93.3	93.8	≥80.0	Yes
CD127 (%)	0.0	1.4	0.1	2.5	0.7	1.6	0.0	1.2	≤10.0	Yes
CD8 (%)	0.0	0.1	0.0	0.0	0.1	0.1	0.0	0.1	≤0.5	Yes
CD19 (%)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	≤1.0	Yes
CD56/16 (%)	0.0	0.4	0.0	0.0	0.0	0.0	0.0	0.0	≤1.0	Yes
Suppression at ratio 1 + 1 (%)	99.5	95.2	81.4	99.4	98.2	96.1	99.9	98.4	≥80.0	Yes
Suppression at ratio 5 + 1 (%)	96.1	80.8	60.4	85.9	97.2	66.9	99.4	79.2	≥60.0	Yes
Suppression at ratio 10 + 1 (%)	85.4	66.5	52.5	65.9	61.6	61.8	83.2	70.8	≥50.0	Yes
viable cells/ml ($\times 10^6$)	10.5	9.3	6.3	9.1	10.8	9.8	8.2	7.9	≥5.0	Yes
Cell viability (%)	96.7	78.7	92.8	79.7	96.8	84.1	96.9	74.1	≥50.0	Yes
Sterility	ng	ng	ng	ng	ng	ng	ng	ng	ng	Yes
Endotoxin (IE/ml)	<30	<30	<30	<30	<30	<30	<30	<30	<30	Yes
Mycoplasma	neg	neg	neg	neg	neg	neg	neg	neg	neg	Yes

Treg were thawed after a minimum of 24 h after production or after at least 12 months of storage at ≤−150°C and analyzed for lot-release. CD4, CD25, CD127, CD8, CD19, and CD56/CD16 indicate surface expression on the final Treg product. Suppression at ratio 1 + 1, 1 + 5, and 1 + 10 indicate the amount of suppression within the first generation of proliferating $CD8^+$ responder cells by the final Treg product. Viable cells per milliliter indicate the total number of life retrieved Treg in the final Treg product. Viability indicates the percentage of life cells in the final Treg product. Sterility indicates bacterial growth contaminating the final Treg product.
ng, no growth; neg, negative.

TABLE 6 | Results of regulatory T cell (Treg) drug product stability testing in the clinical application solution.

Parameter	Con1		Con2		Con3		Con4		Acceptance criteria	Criteria passed
	0.5 × 10⁶/ml	20 × 10⁶/ml	0.5 × 10⁶/ml	20 × 10⁶/ml	0.5 × 10⁶/ml	20 × 10⁶/ml	0.5 × 10⁶/ml	20 × 10⁶/ml		
CD4 (%)	93.2	98.9	97.3	98.2	98.7	98.4	99.5	99.4	≥90.0	Yes
CD25 (%)	89.1	87.5	86.1	96.6	81.1	86.0	95.9	94.6	≥80.0	Yes
CD127 (%)	0.2	0.1	0.9	0.0	6.8	4.8	3.3	1.9	≤10.0	Yes
CD8 (%)	0.0	0.1	0.0	0.0	0.1	0.1	0.0	0.1	≤0.5	Yes
CD19 (%)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	≤1.0	Yes
CD56/16 (%)	0.0	0.4	0.0	0.0	0.0	0.0	0.0	0.0	≤1.0	Yes
Suppression at ratio 1 + 1 (%)	97.7	96.5	98.8	97.6	97.0	96.5	93.5	96.6	≥80.0	Yes
Suppression at ratio 5 + 1 (%)	90.3	91.7	94.7	96.2	91.7	76.1	82.5	92.6	≥60.0	Yes
Suppression at ratio 10 + 1 (%)	81.6	87.1	70.3	70.3	78.5	63.6	56.9	55.2	≥50.0	Yes
Viable cells/ml (×10⁶)	7.3	6.7	6.1	5.7	6.0	5.5	7.1	5.7	≥5.0	Yes
Viability (%)	76.1	70.5	78.0	70.6	77.2	79.1	74.9	76.3	≥50.0	Yes

Treg were thawed at least 12 months after storage at ≤−150°C and analyzed for stability in a 0.9% sodium chloride solution at indicated Treg concentrations. CD4, CD25, CD127, CD8, CD19, and CD56/CD16 indicate surface expression on the final Treg product. Suppression at ratio 1 + 1, 1 + 5, and 1 + 10 indicate the amount of suppression within the first generation of proliferating CD8⁺ responder cells by the final Treg product. Viable cells per milliliter indicate the total number of life retrieved Treg in the final Treg product. Viability indicates the percentage of life cells in the final Treg product.

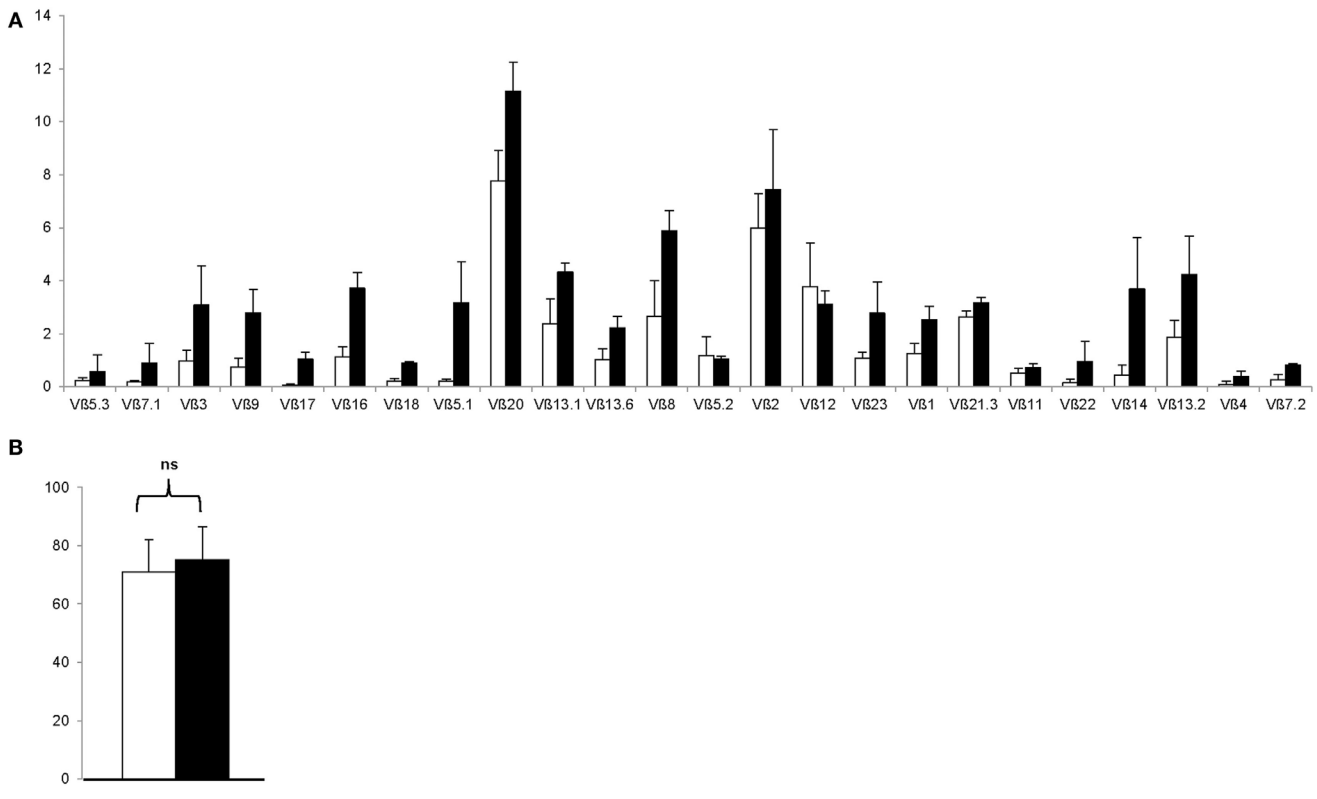

FIGURE 5 | The regulatory T cell (Treg) drug product is polycloncal and hypomethylated at intron 1 of the FoxP3 locus. **(A)** Proportion of Treg expressing indicated T cell receptor Vβ subtype after CD25⁺ cell enrichment at day 0 ($n = 4$, white bars) and after 21 days of *ex vivo* expansion ($n = 4$, filled bars). **(B)** Percentage hypomethylation at intron 1 of the FoxP3 locus after CD25⁺ cell enrichment at day 0 ($n = 4$, white bar) and after 21 days of *ex vivo* expansion ($n = 4$, filled bar). NS, not significant.

Also, high levels of CCR5 and CXCR3 were expressed on both day 0 CD25⁺ enriched cells and day 21 Treg, whereas only little to moderate expression levels of CCR8, CCR9, and GPR15 were detected.

Risk Management and Limits of Detection

A major risk in the presented protocol is the detection of human immunodeficiency virus (HIV), hepatitis B virus (HBV), or hepatitis C virus (HCV) antigens or microbiological impurities

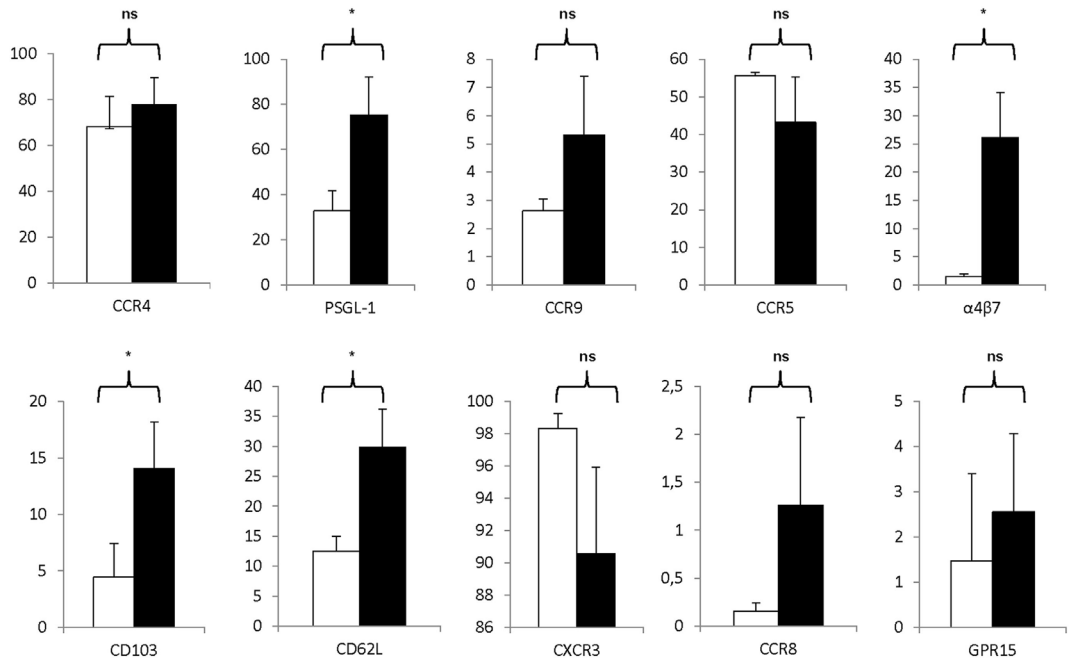

FIGURE 6 | Markers associated with homing are significantly expressed on the regulatory T cell (Treg) drug product. Proportion of $CD25^+$ cells at day 0 (white bars) and day 21 Treg drug product cells (filled bars) expressing CCR4 ($n = 4$), PSG-1 ($n = 4$), CCR9 ($n = 4$), CCR5 ($n = 4$), α4β7 ($n = 4$), CD103 ($n = 4$), CD62L ($n = 4$), CXCR3 ($n = 4$), CCR8 ($n = 4$), and GPR15 ($n = 4$). NS, not significant, $^*P < 0.05$.

in the patient material or final Treg product. When the leucapherisate is tested positive for HIV, HBV, or HCV, the production process is stopped immediately, and cell cultures will be destroyed accordingly. When the final drug product is tested positive for microbiological impurities, the product will be placed into quarantine, and is not released for clinical treatment. Confirmation tests will be performed to define if the product remains positive, and will be destroyed or was tested false positive.

Other Treg production risks include a low number of Treg after 21 days of expansion. In this case, the amount of cells regularly used for lot-release (120×10^6) could be reduced accordingly. Any out of specification result for Treg phenotype or Treg function results in the non-release of the Treg product. However, if the number of $CD8^+$ cells, $CD19^+$ cells, and/or anti-CD3/anti-CD28 expander beads exceeds the specification, the $CD8^+/CD19^+$ cell depletion and/or anti-CD3/anti-CD28 expander bead removal step could be repeated. Applicable limits of detection and quantification are provided in **Tables 1–3**, respectively.

DISCUSSION

Here, we show, for the first time, an official authority GMP-approved protocol to produce large numbers of *ex vivo* rapamycin-expanded $CD25^+$ cells intended to treat inflammatory and autoimmune disorders. In addition, we provide the complete testing and validation of the lot-release of the final Treg drug product after freezing and thawing. Moreover, we extended traditional lot-release criteria and added a functional biological assay assuring the suppressive nature of the produced Treg cells at different Treg-to-effector T cell ratios. Thus far, published clinical studies testing adoptively transferred Treg included classical suppression assays as part of the immune-monitoring assays (18) after treatment of the patients to correlate clinical outcome with *in vitro* Treg function, but the delivered Treg product did not undergo potency testing before administration to the patient. By contrast, this Treg product is not released for clinical treatment unless *in vitro* suppression is proven at various cell ratios for each batch of Treg.

All the consistency runs met the specifications of the process and the product, including sterility, Treg phenotype, non-Treg cellular contamination, anti-CD3/anti-CD28 expander bead purity, and Treg function after freezing and thawing. This contrasts the general consideration that cryopreservation of Treg products is challenging (35) and that stimulation and expansion steps are necessary to restore Treg function after thawing (36).

Stability data were acquired with Treg, which were continuously stored in the gas phase of liquid nitrogen at ≤150°C for at least 12 months. Stored Treg were shown to retain Treg phenotype and function. Therefore, as advocated by Singer et al. (12), our Treg drug product could facilitate an "on demand" treatment for an acute inflammatory disease or acute allograft rejection without the time delay required for Treg enrichment and expansion. Moreover, since stability is warranted for at least 12 months, multiple Treg doses could be administered to the patient at different time points.

Besides Treg drug product stability after freezing and thawing, the Treg also remained stable after dilution in a 0.9% physiological saline infusion solution for up to 90 min. This is an important

assurance, since the Treg drug product will be transferred intravenously through continuous infusion using 50 ml syringes in a perfusion pump.

Importantly, this Treg expansion protocol has several differences compared with the Treg expansion protocol intended to treat patients after liver transplantation (27). First, the presented production protocol reaches clinically relevant Treg numbers after 21 days of expansion without the need to re-stimulate the expanding Treg. Second, less than 0.1% of contaminating $CD8^+$ effector cells were present in the released Treg product. This is a fraction of the allowed 10% $CD8^+$ effector cells in the Treg product intended to prevent liver rejection. Third, expanded Treg remain hypomethylated at intron 1 of the FoxP3 locus, confirming their epigenetic stability. Finally, the produced Treg show suppressive function against autologous $CD8^+$ effector cells at various Treg-to-effector cell ratios; whereas the Treg produced to prevent liver rejection showed suppression of allogeneic effector cells at one Treg-to-effector cell ratio.

In the past years, several clinical studies employing expanded Treg have been conducted. The majority of studies included patients at risk for GvHD (14–16) or organ rejection (37, 38) after transplantation. These studies either infused *ex vivo* expanded Treg (14–16) or freshly isolated non-expanded Treg cells (39, 40). Treg infusions were well tolerated and no dose-limiting toxicities were reported (14, 15). In addition, the onset of both acute and chronic GvHD was favorably affected compared with historical controls and no adverse effects on non-relapse mortality or relapse were detected within a minimum follow-up of 2 years (14, 15). In addition, the possibility of adoptive Treg to ameliorate insulin dependency in both children and adults has been reported (17, 18). In these studies, Treg transfer was safe and not associated with serious adverse events in the treated children (17). By contrast, four serious adverse events were reported in the treated adults (18). Specifically, one patient experienced three episodes of serious hypoglycemia 14, 248, and 463 days after Treg treatment, and one patient experienced an episode of diabetic ketoacidosis 67 days after Treg treatment. Interestingly, by labeling with [6,6-2H2] glucose, Treg were demonstrated to persist in the peripheral circulation for up to one year after transfer (18). Finally, the safety and efficacy of *ex vivo* expanded ovalbumin-specific IL-10-producing Treg has been assessed in patients with Crohn's disease (CD). The safety profile in this pilot study showed good tolerability and adverse events reflected the underlying CD. Moreover, a clinical significant improvement of disease symptoms was noted 5 weeks after Treg infusion in 40% of patients (41).

In conclusion, Treg produced by the presented method have broad clinical potential. Based on the fact that the presented Treg drug product is polyclonal and expressing various receptors associated with (i) lymphocyte trafficking into the skin (42, 43) (e.g., CCR4 and CD103), (ii) homing into lymphoid organs (44, 45) (e.g., PSGL-1 and CD62L), (iii) homing to the intestinal mucosa (46, 47) (e.g., CCR9 and α4β7 integrin), and (iv) sites of inflammation (45, 48, 49) (e.g., PSGL-1, CD103, and CXCR3), the Treg could be effective in clinical studies aiming to treat various autoimmune-based and inflammatory disorders such as skin diseases, rheumatic diseases, intestinal inflammation, and graft-versus-host disease.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the local Review Board (IRB) of the Friedrich-Alexander Universität Erlangen-Nürnberg under IRB number 151_12B with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the local IRB of the Friedrich-Alexander Universität Erlangen-Nürnberg.

AUTHOR CONTRIBUTIONS

MW, DS, SR, CL, AF, IA, and CV performed experiments and analyzed data. GS and MN initiated the project. GS and BS-T acted as advisors during the GMP implementation. CV drafted the manuscript. MW, DS, SR, CL, AF, RA, CN, IA, AS, BS-T, MN, and GS critically revised the manuscript for intellectual content.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/article/10.3389/fimmu.2017.01371/full#supplementary-material.

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T_H17 Cell and Epithelial Cell Crosstalk during Inflammatory Bowel Disease and Carcinogenesis

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The intestine is colonized by hundreds of different species of commensal bacteria, viruses, and fungi. Therefore, the intestinal immune system is constantly being challenged by foreign antigens. The immune system, the commensal microbiota, and the intestinal epithelial surface have to maintain a tight balance to guarantee defense against potential pathogens and to prevent chronic inflammatory conditions at the same time. Failure of these mechanisms can lead to a vicious cycle in which a perpetual tissue damage/repair process results in a pathological reorganization of the normal mucosal surface. This dysregulation of the intestine is considered to be one of the underlying causes for both inflammatory bowel disease (IBD) and colorectal cancer. T_H17 cells have been associated with immune-mediated diseases, such as IBD, since their discovery in 2005. Upon mucosal damage, these cells are induced by a combination of different cytokines, such as IL-6, TGF-β, and IL-1β. T_H17 cells are crucial players in the defense against extracellular pathogens and have various mechanisms to fulfill their function. They can activate and attract phagocytic cells. Additionally, T_H17 cells can induce the release of anti-microbial peptides from non-immune cells, such as epithelial cells. The flip side of the coin is the strong potential of T_H17 cells to be pro-inflammatory and promote pathogenicity. T_H17 cells have been linked to both mucosal regeneration and inflammation. In turn, these cells and their cytokines emerged as potential therapeutic targets both for inflammatory diseases and cancer. This review will summarize the current knowledge regarding the T_H17 cell-enterocyte crosstalk and give an overview of its clinical implications.

Keywords: Th17 cells, inflammatory bowel diseases, colorectal cancer, enterocytes, cytokines

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INFLAMMATORY BOWEL DISEASE (IBD) AND CARCINOGENESIS

The gastrointestinal tract is essential for the absorption of nutrition and serves as a crucial barrier to protect the host against pathogens. It is colonized by up to 3.8×10^{13} microorganisms such as bacteria and fungi (1). The immune system of the intestine and commensal bacteria maintain a delicate and well-regulated homeostasis. However, when this balancing act is disrupted, chronic inflammatory conditions, such as IBD, can occur. The most common manifestations of IBD are Crohn's disease (CD) and ulcerative colitis (UC). Their symptoms share common hallmarks such as diarrhea, abdominal pain, and relapsing inflammation in the intestine (2). The inflammation

in UC patients is limited mostly to the colon with continuous inflammation of the mucosa and submucosa. CD patients suffer from patchy inflammation that causes deep ulcerations and can affect the whole gastrointestinal tract (3).

Even though the prevalence of IBD especially in western countries such as the USA is high (1.3%) and the resulting costs to the health systems are increasing (4), the underlying cause of IBD is still unknown. Twin studies revealed that genetic predispositions to develop IBD exist; however, the concordance rate for IBD in monozygotic twins does not exceed 50%, highlighting the importance of environmental factors, referred to as Exposome, for disease development (5–7). Furthermore, the intestinal microbiome in combination with the immune system seems to play a crucial role in IBD. Several studies describe a reduced diversity of commensal bacteria species in patients suffering from IBD (8, 9). Additionally, several bacterial pathogens have been implicated in the onset or progression of the disease (10). Furthermore, mouse studies indicate that changes in the microbiota composition can occur prior to colitis development (11, 12), suggesting that microbial changes might be involved in the development of IBD. However, whether dysbiosis is a cause or a consequence of IBD in humans remains to be solved. Nevertheless, barrier defects which are typically present in IBD seem to cause a dysregulated immune response against so far unknown components of the commensals. Especially the adaptive immunity, more specifically $CD4^+$ T cells, seems to be inappropriately activated in response to commensal microorganisms in IBD. Classically, CD used to be associated with a chronic T_H1 immunity, whereas during UC T_H2 immunity has been thought to be implicated (13). However, after the discovery of the involvement of IL-23 and T_H17 cells in autoimmune inflammation of the nervous system, further mouse studies revealed a prominent involvement of these cells during intestinal inflammation in CD and UC (14–16). Furthermore, already in 2003, T_H17 cell-associated cytokines were reported to be upregulated in tissue biopsies and serum of patients with IBD (17, 18). Moreover, chronic inflammation predisposes IBD patients to the development of colorectal cancer (CRC) (19). The chronic inflammation and mucosal injury can trigger long-lasting healing responses that are not terminated, leading to tissue dysfunction and finally to carcinogenesis (20). T_H17 cells and T_H17 cell-associated cytokines are also involved during CRC in humans. T_H17 cells were found elevated in tumors of CRC patients and an increased T_H17 cell immune response correlates with advanced stages of CRC (21–23).

In summary, four main components lead to the pathology of IBD: genetic predisposition, environmental factors, intestinal microbiome, and a dysregulated immune system. So far, the main therapeutics broadly available to treat IBD are based on suppressing the immune response. But these therapies are unable to reset the intestinal homeostasis and do not directly treat the underlying cause of the chronic inflammation. Therefore, patients suffering from IBD mostly require lifelong treatment. T_H17 cells could be the link between these four components. Thus, a better understanding of the interactions of these four components and T_H17 cells is a main focus of current research, with the aim of developing more specific and efficient therapies. This review aims to summarize the current knowledge about the interactions of T_H17 cells and T_H17 cell-associated cytokines with the mucosal surface in the intestine and in the microbiota.

T_H17 CELLS AND THEIR ASSOCIATED CYTOKINES

T_H17 cell-mediated immunity is essential for the clearance of extracellular bacteria and fungi by attracting neutrophils and inducing the release of anti-microbial peptides from epithelial cells (24–28). T_H17 cell cytokines include IL-17A, IL-17F, TNF-α, and IL-22 (15, 26). It has been demonstrated in mice that intestinal T_H17 cells are induced by segmented filamentous bacteria (SFB), which are gram-positive, spore-forming bacteria located in the terminal ileum of the small intestine. Accordingly, T_H17 cells are mainly located in this part of the intestine under physiological conditions (29). A critical feature of SFB is its ability to adhere to the intestinal epithelial cells (IEC). SFB and other bacteria with the same ability such as *Citrobacter rodentium* and *Escherichia coli* induce the production of serum amyloid A (SAA) from epithelial cells (30). Subsequently, SAA induces the release of IL-6, TGF-β, and IL-1β from intestinal cells, especially dendritic cells, which leads to the differentiation of T_H17 cells (29–31). IL-6 signaling leads to the activation of STAT3 and subsequent induction of RORγt, one of the key transcription factors of T_H17 cells, and of other T_H17 cell-related factors such as IL-17A/F and IL-23R (25, 32–34). IL-1β is crucial for the differentiation of T_H17 cells (35). Besides other effects, IL-1β induces the expression of the transcription factor IRF4, which is needed for the expression of RORγt (36). The role of TGF-β for T_H17 cell differentiation is still controversial. T_H17 cells can occur in the absence of TGF-β in the gut mucosa (37). However, TGF-β can negatively regulate T_H1 and T_H2 while promoting T_H17 cell differentiation and therefore favors the contribution of T_H17 cells (38). Due to the presence of microbiota in the intestine, the T_H17 cell differentiation differs in comparison to sterile organs. One essential alteration is the activation of the transcription factor aryl hydrocarbon receptor (AHR) by ligands derived from food or intestinal microbiota (39, 40). AHR is highly expressed already in early stages of T_H17 cell differentiation (41, 42). AHR expression is not essential for T_H17 cell differentiation. However, it is nonetheless non-redundant for the secretion of IL-22 by T_H17 cells, a cytokine vital for the anti-microbial properties of T_H17 cells (42–44).

IL-23 is an important cytokine for T_H17 cell biology. However, the IL-23 receptor is absent on naïve T cells. Accordingly, research led to the discovery that IL-23 is essential for the effector properties of T_H17 cells rather than their induction (45, 46).

In the following sections, we want to outline the effects of T_H17 cell-associated cytokines such as IL-17A, IL-22, and TNF-α on epithelial cells during IBD and carcinogenesis.

IL-17A DURING INFLAMMATION AND CARCINOGENESIS

IL-17A is a member of the IL-17 family consisting of IL-17A, IL-17-B, IL-17-C, IL-17-D, IL-17E, and IL-17F (47). Both IL-17A and IL-17F signal through the IL-17RA–IL-17RC complex and

activate the NF-κB and MAPK pathways (48). IL-17A is produced mainly by T_H17 cells although production by many other cell types including $CD8^+$ T cells, γδ T cells, NK cells, NKT cells, and innate lymphoid cells (ILCs) has been described. Initial studies have shown increased *IL17A* mRNA expression and increased numbers of T_H17 cells in the inflamed tissue of IBD patients compared to healthy mucosa (18, 49, 50). Furthermore, the amount of IL-17A producing PBMCs correlates with disease severity in patients with UC (51). These results imply a pathogenic role of those cells in the intestine in IBD. IL-17A induces the recruitment and activation of granulocytes and locally promotes the production of other pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β (52, 53). In line with these findings, a blockade of IL-23 and IL-21 in murine models of colitis results in decreased numbers of T_H17 cells and in a favorable disease outcome (54, 55). Surprisingly however, blockade or genetic deletion of IL-17A resulted in aggravated disease severity in the DSS-induced colitis, a mouse model of IBD (55, 56). Interestingly and in contrast with the data obtained using IL-17A knock-out mice, IL-17F knock-out mice show a less severe DSS-induced colitis (57). As IL-17F binds to the IL-17RA-IL-17RC complex with lower affinity than IL-17A, the different activation strength of the receptor complex might explain those opposing results. These findings do emphasize the need for strict distinction between the functions of T_H17 cells and one of their signature cytokines, IL-17A. The protective function of IL-17A in mouse IBD models can be explained by the effect of this cytokine on enterocytes. Similar to IL-22, IL-17A induces their proliferation and tight-barrier formation and therefore promotes the integrity of the epithelial barrier (55, 58). The other side of the coin of those regenerative and physiological effects is the potential of IL-17A to promote carcinogenesis in the colon. Following a barrier defect, bacterial translocation and IL-23 production by innate immune cells, such as DCs and macrophages (Mφ), induce high levels of IL-17A which in turn can favor intestinal tumorigenesis (59). Importantly, an enterocyte-specific knock-out of the IL-17RA decreased tumor formation in mice, suggesting a direct tumorigenic function of IL-17A (60). Moreover, increased frequencies of IL-17A producing cells were found in human CRC and high *IL17A* mRNA expression correlates with a poor prognosis in these patients (60–62). Although these results do indicate a potential therapeutic benefit of targeting IL-17A, the heterogeneity of T_H17 cells makes it difficult to conclude their exact function in human CRC and further research is needed before initiating clinical trials.

IL-22 DURING INFLAMMATION AND CARCINOGENESIS

IL-22 is a member of the IL-10 family that has gained considerable attention in the last years due to its role in linking inflammation and regenerative processes. Although originally described as a T_H1 cytokine (63), it can be produced by a variety of immune cells including $CD4^+$ (T_H1, T_H17, T_H22), $CD8^+$ T cells, γδ T cells, NK cells, NKT cells, and group 3 ILCs. Innate lymphoid cells represent a newly described cell type which belongs to the lymphoid lineage but is characterized by the absence of antigen-specific T- or B-cell receptors. Group 3 ILCs represent a subgroup of innate lymphoid cells that are defined by the expression of the master transcriptional factor RORγt and the capacity to produce IL-17A and IL-22. They are therefore widely regarded as the innate counterpart of Th17 cells. Based on the expression of NKp46 (NCR1), group 3 ILCs can be further subdivided into ILC3s and lymphoid-tissue inducer cells. IL-22 signals through a heterodimeric receptor consisting of the ubiquitously expressed IL10R2 and the more specifically expressed IL22R1 (64, 65). Upon binding of IL22, the IL-22–IL-22R1–IL-10R2 complex signals mainly through STAT3 and the AKT-MAPK pathway, although activation of STAT1 and STAT5 has also been shown in certain cells (66, 67). The need for tight control of IL-22 activity is emphasized by the existence of an endogenous antagonist, called IL-22 binding protein (IL-22BP), which binds IL-22 and prevents binding to the membrane bound IL-22 receptor. In the intestine, IL-22 signaling elicits multiple responses that aim to maintain the integrity of the mucosal barrier, trigger antimicrobial responses, and promote wound healing (68, 69). In mice, levels of IL-22 increase upon chemically induced tissue damage and are crucial for tissue regeneration. Interestingly, levels of IL-22BP decrease in parallel, further increasing the activity of IL-22 (70). Similar results have been described in patients with acute diverticulitis (71). Surprisingly however, IL-22BP levels were recently shown to be elevated in patients with IBD and thus limit the tissue-healing effects of IL-22, suggesting that a disturbed IL-22–IL-22BP axis might be one of the mechanisms contributing to the chronification of inflammation (**Figure 1**) (71, 72). Although usually regarded as a tissue-protective cytokine, pro-inflammatory effects of IL-22 in the intestine have also been described. For instance, NCR-ILC-3, which coproduce IL-17A and IL-22, have been shown to be pathogenic in mouse models of IBD in a microbiota-dependent fashion and are increased in patients with IBD (73, 74). On the other hand, NCR-ILC-3 decreased in these patients and depletion of IL-22 producing ILC-3 makes mice more susceptible to *C. rodentium* infection (75–77). Similarly, T cell-derived IL-22 was shown to be both pathogenic and protective in murine models of IBD (78, 79). The distinct functions of ILC and T cell-derived IL-22 in IBD are still not completely understood. It can be hypothesized that ILC-derived IL-22 is crucial in the initial phases of inflammatory responses, whereas in chronically inflamed tissue, T cell become the major producers as suggested by Basu et al. (80).

As mucosal healing is one of the major aims in the therapy of IBD, IL-22 is widely regarded as a potentially beneficial cytokine in those diseases. On the other hand, a relationship between regenerative agents and tumorigenesis has been known for a long time and patients with IBD are known to have an increased risk for the development of CRC. Indeed, prolonged or excessive IL-22 signaling promotes tumorigenesis in the intestine of both mice and humans. In murine models of colon cancer, knock-out of the IL-22BP resulted in accelerated tumor growth, which was IL-22 dependent (70). In line with these results, levels of IL-22 are increased in patients with CRC and IL-22 can directly promote growth of cancer cell lines *in vitro* (81). Although ILCs have been shown to promote tumor growth in mice through IL-22, data in humans indicate $CCR6^+$ T_H17 cells to be the major source (81, 82). Further research is required to

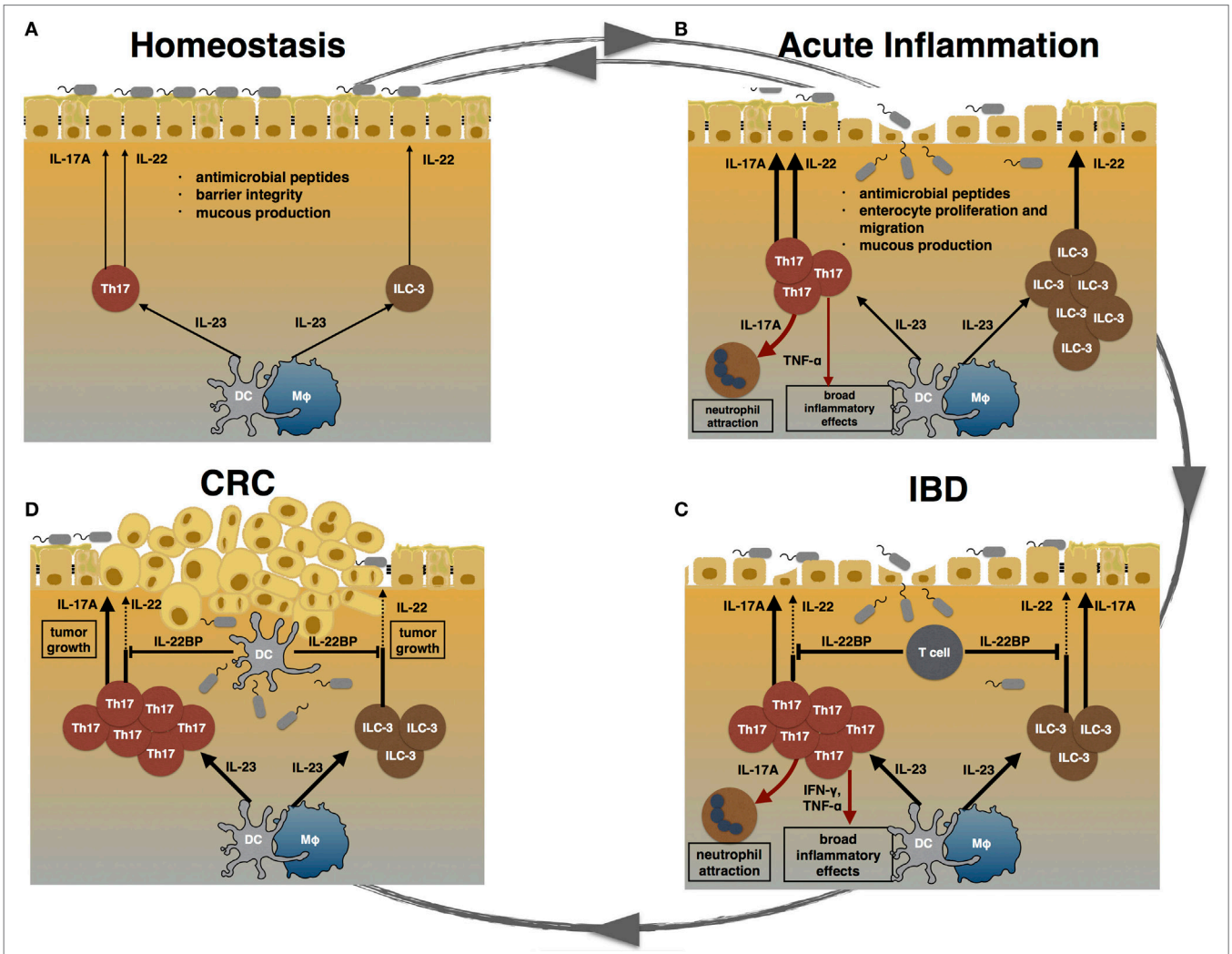

FIGURE 1 | TH17 cells during homeostasis, inflammation, and carcinogenesis. **(A)** During homeostasis, TH17 cells and ILC3 are induced by certain species of the commensal microbiota. IL-17A and IL-22 promote epithelial barrier integrity, mucus production, and the release of anti-microbial peptides. **(B)** Acute inflammation can be caused by pathogenic bacteria. Invading pathogens induce the expansion of TH17 cells and ILC3. TH17 cell-associated cytokines attract neutrophils and trigger a pro-inflammatory response in order to clear the invading agent. Furthermore, IL-17A and IL-22 promote enterocyte proliferation and migration, thereby promoting mucosal healing. After clearance of the pathogen, the intestinal immune system returns to homeostasis. **(C)** Failure to terminate an intestinal immune response can lead to chronic inflammation. In inflammatory bowel disease (IBD), highly pathogenic TH17 cells expand and secret pro-inflammatory cytokines such as IL-17A, TNF-α, and IFN-γ. Especially, TNF-α and IFN-γ cause a broad inflammatory response. The regenerative effects of IL-22 are counter regulated by high levels of T cell-derived IL-22 binding protein (IL-22BP) in IBD patients. **(D)** Chronically elevated levels of IL-17A and IL-22 can promote carcinogenesis. Hereby, IL-22 can be controlled by DC-derived IL-22BP. However, whether this mechanism fails in human colorectal cancer (CRC) is currently unknown.

understand the distinct effects of ILC vs. T cell derived IL-22 in human carcinogenesis.

TNF-α DURING INFLAMMATION AND CARCINOGENESIS

The cytokine TNF-α has been linked to inflammatory responses for a long time. TNF-α can be produced by a multitude of immune cells such as macrophages, $CD4^+$ lymphocytes, NK cells, neutrophils, mast cells, and eosinophils. A soluble form and transmembrane bound form of TNF-α exist (83). Both the soluble and the membrane-bound TNF-α can interact with two TNF receptors, TNF-R1 (TNFRSF1A) and TNF-R2 (TNFRSF1B), expressed on IEC (84). TNF-α signaling leads to the release of other pro-inflammatory molecules and can provoke both pro- and anti-apoptotic signals in the IEC (85). An involvement of TNF-α in the pathology of IBD was first assumed after assessing TNF-α levels in serum of children suffering from IBD (86). Further studies revealed elevated levels of TNF-α in stool and mucosal tissue also (87, 88). Based on the knowledge of the pro-inflammatory properties and these observations, TNF-α became an interesting target for new therapeutic approaches. The development of anti-TNF-α antibodies and their application in humans is one of the best examples of how the concept, "from

bench to bedside" can be successfully employed. Already in 1995 Dullemen et al. reported the successful use of a monoclonal antibody cA2 (infliximab) in CD patients (89). Since then, anti-TNF-α therapy has greatly improved the management of IBD. Furthermore, it has been demonstrated that TNF-α can promote colitis-associated CRC, a long-term consequence of IBD. In a mouse model of colitis-induced carcinogenesis, the blockade of TNF-α led to reduced mucosal injury and in turn to decreased tumor formation (90). Accordingly, elevated expression of TNF-α in tumors of CRC patients is associated with advanced cancer stages in humans (91). However, the treatment with anti-TNF antibodies causes high costs to health care systems and can cause some severe side effects such as opportunistic infections (92). Finally, around 10–30% of patients with IBD do not respond to anti-TNF-α treatment and 20–40% of patients lose response over time (93). Moreover, a prognostic factor that can predict the response to this therapy is missing.

NEW THERAPEUTIC STRATEGIES AND PATIENT MANAGEMENT

T_H17 cells are highly enriched in IBD, and several genetic risk loci being associated with IBD are linked to T_H17 cells. Thus, several drugs were developed with the intention to manipulate T_H17 cell development or function in patients with IBD. First clinical trials using monoclonal antibodies targeting cytokines related to T_H17 cells, such as IL-17A, show similar results to those obtained in murine IBD models and highlight the need to make a clear distinction between the biological functions of IL-17A and T_H17 cells in general. As described above, despite the potential pro-inflammatory properties of T_H17 cells, their signature cytokine IL-17A was shown to also have beneficial effects. IL-17A can promote enterocyte proliferation, tight-barrier formation and epithelial barrier integrity in the intestine (54, 55). A clinical trial with secukinumab, an anti-IL-17A antibody, further highlighted the importance of IL-17A for mucosal homeostasis. Although being highly effective in psoriasis, blockade of IL-17A resulted in aggravated disease course in IBD patients (94–96). In contrast to blockade of IL-17A, antibodies against IL-23 were effective in preventing colitis in mouse models (55). Similarly, ustekinumab proved to be effective in patients with CD (97). Ustekinumab targets the p40 subunit of IL-12, which is part of IL-12 and IL-23. Therefore, it affects both T_H1 (together with ILC-1) and T_H17 (together with ILC-3) lineages. Interestingly, a first trial involving the blockade of the p19 subunit of IL-23 (and therefore not affecting IL-12 signaling) using risankizumab also delivered promising results in patients with moderate-to-severe CD and might represent a future therapy in IBD (98). In contrast to the above-mentioned approaches, anti-TNF therapy is already well established in the therapy of both CD and UC. Surprisingly, we have recently shown that the effectiveness of this therapy is to some extent dependent on the IL-22–IL-22BP axis in both mice and humans (71). Direct therapeutic intervention into the IL-22–IL-22R1–IL-22BP system might therefore represent a novel strategy in patients with IBD. Importantly, as IL22R1 is expressed exclusively on non-hematopoietic cells, such a therapy would not lead to systemic immunosuppression, a common side-effect of most currently used medications. Nevertheless, the oncogenic properties of IL-22 could represent a major obstacle in the development of safe and effective drugs targeting the IL-22–IL-22R1–IL-22BP axis.

AUTHOR CONTRIBUTIONS

LB and JK wrote the manuscript and equally contributed to the manuscript; NG and SH supervised and revised the manuscript.

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REVIEW
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Actors and Factors in the Resolution of Intestinal Inflammation: Lipid Mediators As a New Approach to Therapy in Inflammatory Bowel Diseases

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In the last few decades, the pathogenesis of inflammatory bowel disease (IBD) in genetically predisposed subjects susceptible to specific environmental factors has been attributed to disturbance of both the immune and non-immune system and/or to the imbalanced interactions with microbes. However, increasing evidences support the idea that defects in pro-resolving pathways might strongly contribute to IBD onset. The resolution of inflammation is now recognized as a dynamic event coordinated by specialized pro-resolving lipid mediators (LMs), which dampen inflammation-sustaining events, such as angiogenesis, release of pro-inflammatory cytokines, clearance of apoptotic cells, and microorganisms. Among these pro-resolving molecules, those derived from essential polyunsaturated fatty acids (PUFAs) have been shown to induce favorable effects on a plethora of human inflammatory disorders, including IBD. Here, we offer a summary of mechanisms involving both cellular and molecular components of the immune response and underlying the anti-inflammatory and pro-resolving properties of PUFAs and their derivatives in the gut, focusing on both ω-3 and ω-6 LMs. These fatty acids may influence IBD progression by: reducing neutrophil transmigration across the intestinal vasculature and the epithelium, preventing the release of pro-inflammatory cytokines and the up-regulation of adhesion molecules, and finally by promoting the production of other pro-resolving molecules. We also discuss the numerous attempts in using pro-resolving PUFAs to ameliorate intestinal inflammation, both in patients with IBD and mouse models. Although their effects in reducing inflammation is incontestable, results from previous works describing the effects of PUFA administration to prevent or treat IBD are controversial. Therefore, more efforts are needed not only to identify and explain the physiological functions of PUFAs in the gut, but also to unveil novel biosynthetic pathways of these pro-resolving LMs that may be dysregulated in these gut-related disorders. We suppose that either PUFAs or new medications specifically promoting resolution-regulating mediators and pathways will be much better tolerated by patients with IBD, with the advantage of avoiding immune suppression.

Keywords: resolution of inflammation, pro-resolving lipid mediators, inflammatory bowel disease, polyunsatured fatty acids, pathogenesis, mucosal inflammation, tissue homeostasis

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INTRODUCTION

Inflammatory bowel diseases (IBDs), encompassing ulcerative colitis (UC), and Crohn's disease (CD) are immunologically mediated inflammatory disorders of the gut, whose prevalence and incidence are dramatically increasing worldwide. Although clinical manifestations of these diseases are different, they share common features. In fact, both UC and CD are characterized by epithelial barrier damage that allows commensal bacteria and microbial products to translocate into and colonize the intestinal wall. This event triggers the release of cytokines, chemokines, and eicosanoids which thanks to regulatory mechanisms, mediate the physiological self-limiting immune-response (1, 2). Furthermore, both immune and non-immune components of the intestinal mucosa have been shown to exert a key role in IBD pathogenesis (3). In terms of immune components, the innate and the adaptive immune system are essential to chronic intestinal inflammation. In fact, innate immune cells (e.g., neutrophils, monocytes, and macrophages) hold the capability to remodel the response of adaptive T cells during the inflammatory process (4). Concomitantly, studies of the intestinal microbiota, environmental factors, and genetics have identified a significant contribution of non-immune components to the pathogenesis of IBD, which include: breach in the epithelial wall, that is, the first line of gut defense against bacteria and other microorganisms (5–7); defects in the biological activities of stromal cells, which hold immune-modulatory actions and the capability to clear chemokines and cytokines from the inflammatory milieu to re-establish mucosal homeostasis (8, 9); defective endothelial cell functions, crucial for the angiogenic process but also for the regulation of leukocyte adhesion, and trafficking across the hematic and lymphatic barriers (10–14). Activities of both immune and non-immune cells need to be finely modulated and constantly balanced, in order to avoid chronicity of inflammation and tissue damage.

Another key component of IBD pathogenesis is represented by the gut microbiota (15). In fact, the gastrointestinal tract hosts the largest microbial community of the organism that can be shaped by environmental factors, diet, and hygiene during childhood (16), whereas in adulthood this is more stable with a defined composition of bacteria (17, 18). In healthy subjects, homeostasis exists between the intestinal microbiome, mucosal barrier, and immune system. In IBD, this homeostasis is altered causing a "dysbiosis," disrupted barrier function as well as immune system activation (15).

Although many efforts have been made to delineate the causes underlying the exact etiopathogenesis of IBD, so far our knowledge does not fully clarify what causes its onset. It is currently well accepted that at the basis of IBD pathogenesis (19, 20) there is an imbalance between pro- and anti-inflammatory signals (1). This suggests that defects in the proper release of pro-resolving molecules during the acute phase of inflammation may characterize IBD onset. For decades, the resolution of inflammation has been considered a passive event, in which pro-inflammatory signals simply dilute over time. This concept was overturned when Serhan and colleagues discovered for the first time that a specific class of lipids, known as eicosanoids and docosanoids, promotes, and orchestrates the resolution process (21, 22).

This discovery gave rise to a new field of research studying the mechanisms and the factors involved in the resolution phase of the inflammatory response, which is finely and temporally regulated by specialized pro-resolving mediators, named lipoxins (LXs), resolvins (Rv), protectins, and maresins. These resolving bioactive lipids are synthesized from ω-6 and ω-3 polyunsaturated fatty acids (PUFAs) and have been demonstrated to exert potent immune-resolving effects (2). However, this line of research is still at its infancy in the IBD field.

In fact, the vast majority of therapies currently in use for IBD aims at blocking key inflammatory mediators that are triggered during the early stages of acute inflammation. However, targeting infiltrating immune cells does not always lead to remission or stable resolution. Indeed, conventional anti-inflammatory agents do not alter the course of IBD because the naturally occurring resolution programs are likely to be subverted. For this reason, promotion and maintenance of the resolving milieu may represent a good alternative therapeutic approach to dampen chronic inflammation in IBD. In addition, defects in the production of resolving molecules may strongly contribute to IBD onset, thus expanding our understanding of what triggers these gut-related diseases.

This review aims to describe how the resolution process plays a fundamental role in the gut both at the physiological and pathological level. After a brief overview on IBD pathogenesis, we will emphasize which cellular and molecular components govern the resolving phase of intestinal inflammation and we will discuss the state of the art of preclinical and clinical studies employing PUFA-derived pro-resolving lipid mediators (LMs) in IBD.

RESOLUTION OF INFLAMMATION AND PRO-RESOLVING LMs: A BRIEF OVERVIEW

For decades, anti-inflammatory treatments have been used to treat chronic inflammatory conditions because of the concept that the chronically established inflammation was caused by an exaggerated immune response rather than a failure in the resolution of inflammation (23). Indeed, for years the resolution process has been considered a passive event where inflammatory signals progressively dissipate (2, 24). In contrast to this assumption, during the last decade resolution of inflammation has been conclusively recognized as an active and tightly regulated process orchestrated by pro-resolving LMs, which have been found to dampen inflammation-sustaining events such as cell proliferation, migration, and clearance of apoptotic cells and microorganisms (2, 25).

At tissue and cellular level, the key steps that characterize the resolution process are (i) clearance of the inciting stimuli, (ii) silencing of pro-inflammatory and local survival signals, including chemokine gradients, (iii) polymorphonuclear (PMN) efferocytosis and clearance by tissue and monocyte-derived macrophages, and (v) recirculation of macrophages *via* lymph flow. LMs represent the key signaling molecules in this process, which regulate the inflammatory profile and promote the return of affected tissues to homeostasis (26).

In this context, ω-3 and ω-6 PUFAs are specialized LMs that have the capability of influencing the inflammatory processes, such as those governing IBD. They are essential fatty acids that need to be obtained from the diet; in fact, since mammals lack of endogenous enzymes necessary for ω-3 PUFA desaturation, they cannot be synthesized by humans (27).

Polyunsaturated fatty acid metabolism is recognized as an important factor in immune regulation and disease control. In particular, the metabolic balance between ω-6 and ω-3 PUFAs is widely held to be important in human health and diseases (27–30). PUFA-derived bioactive metabolites are formed *in vivo* by enzymatic oxidation through the action of cyclooxygenases (COXs), lipoxygenases (LOXs), and cytochrome P450 (CYP450) monooxygenases. From ω-6 PUFAs, e.g., arachidonic acid (AA), the COX pathway leads to the formation of prostanoids, such as prostaglandins (PGs) and thromboxanes (TXs), the LOX pathway leads to leukotrienes (LTs) and LXs, and the CYP450 pathway gives rise to hydroxy-eicosatetraenoic acids (HETEs) and epoxy-eicosatrienoic acids (**Figure 1A**) (2, 24, 31, 32). Except for LXs (33), ω-6 PUFAs are conventionally involved in the initiation of inflammatory responses. On the contrary, ω-3 PUFAs seem to promote resolution of inflammation (34). α-linolenic acid (ALA) is an ω-3 PUFA and is categorized with the ω-6 linoleic acid (LA) as an essential fatty acid. As ω-6 LA can be metabolized into AA, ALA can be converted into precursors for long chain ω-3 PUFAs such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Both EPA and DHA, which can be found in some fish oils, are good substrates for LOX and CYP, thus being efficiently converted into bioactive metabolites such as E-series resolvins (RvEs), D-series resolvins (RvDs), protectins, and maresins that act as potent pro-resolving endogenous mediators in a wide range of human inflammatory disorders, including IBD (**Figure 1B**) (35–44). A large number of studies sustain the anti-inflammatory potential of EPA and DHA and their derivatives [for a recent review, see Ref. (35, 39)]. Nevertheless, the molecular mechanisms by which these essential fatty acids exert their anti-inflammatory effects remain controversial, particularly in the gut.

Inflammatory bowel disease patients may display a deficiency in essential fatty acids and/or a defect in PUFA biosynthesis and metabolism. This is why the intake of ω-3 PUFAs may benefit patients with both UC and CD by a series of beneficial events such as inhibition of natural cytotoxicity, and improvement of oxidative stress (35, 45–47). This concept is strengthened by the fact that the intestinal mucosa seems to be highly responsive to ω-3 long-chain PUFAs (47–49).

ACTIONS OF PRO-RESOLVING PUFAs AND TARGET CELL TYPES IN THE GUT

Active resolution of inflammation is characterized by a sequential series of events that starts from building an adequate inflammatory response against inciting agents, to minimizing local tissue damage. In this context, pro-resolving PUFAs act with various signals and mechanisms to different cell compartments, with the final purpose to remodel and clear healed tissues of unnecessary immune cells, thus bringing the inflamed organ to the original homeostasis.

The intestinal epithelium is a key coordinator of both inflammation and resolution. Thanks to tight junctions (TJs), intestinal

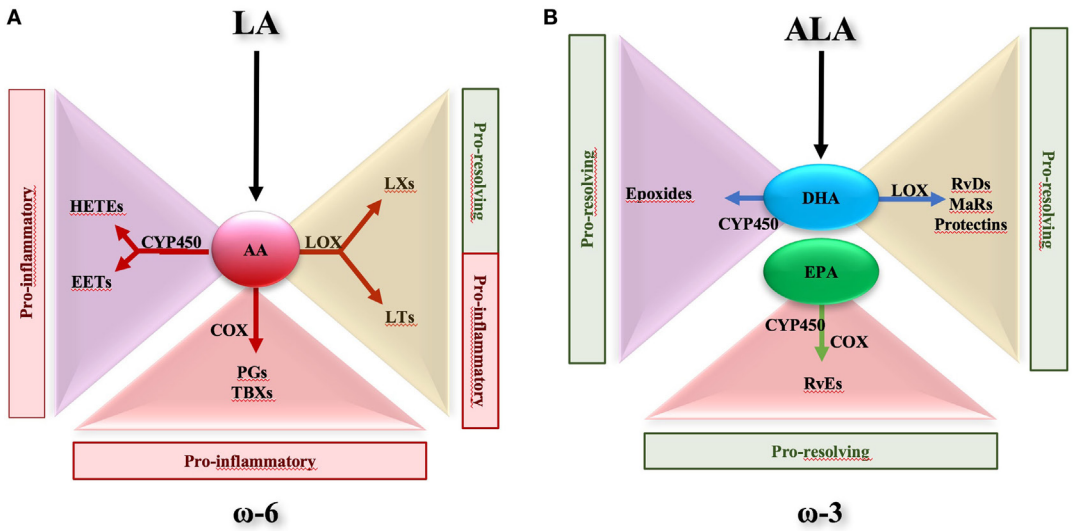

FIGURE 1 | Metabolic route of ω-6- and ω-3-derived lipid mediators. **(A)** Essential fatty acid linoleic acid, classified as ω-6 polyunsaturated fatty acid, can be converted into arachidonic acid (AA). In turn, AA is metabolized in hydroxy-eicosatetraenoic acids (HETEs) and epoxy-eicosatrienoic acids (EETs) *via* cytochrome P450 (CYP450). *Via* lipoxygenase (LOX) pathway, AA is converted to lipoxins (LXs) and leukotriens (LTs), whereas *via* cyclooxygenase it is metabolized in prostaglandins (PGs) and tromboxanes (TBXs). HETEs, EETs, PGs, TBXs, and LTs are all pro-inflammatory, while LXs are considered pro-resolving mediators. **(B)** Essential fatty acid α-linolenic acid is converted to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). EPA and DHA may be substrate of CYP450, resulting into production of E-series resolvins (Rv) and epoxides, respectively. In addition, DHA is metabolized *via* LOX to D-series Rv, maresins, and protectins. All these EPA- and DHA-derived mediators are recognized to harbor pro-resolving properties.

epithelial cells form a dynamic barrier protected by a thick mucus layer which controls what can reach the lamina propria from the lumen (50, 51). In IBD pathogenesis, altered intestinal barrier functions, in terms of decreased mucous production (52) and reduced expression of TJs (53), have been associated with increased gut permeability, which facilitates the absorption of microbial products and triggers an excessive response, eventually leading to mucosa injury in both CD and UC (54, 55). In order to counteract pathogen infections, epithelial cells are able to produce and release in the luminal mucus antibacterial and endotoxin-neutralizing molecules called bactericidal permeability-increasing protein (BPI). BPI damages the membranes of Gram-negative bacteria, neutralizes endotoxin, and opsonizes bacteria for neutrophil phagocytosis (56). BPI is transcriptionally up-regulated by LXs and resolvin E1 (RvE1) (57). In addition, it was observed that RvE1 significantly upregulates the expression of intestinal alkaline phosphatase (ALPI), an enzyme whose activity is critical for the maintenance of bacterial homeostasis (57): for its luminal location, ALPI has been demonstrated to block Gram-negative growth such as *Escherichia coli* and strongly neutralizes LPS through dephosphorylation of moiety in lipid A (58). This was confirmed in the mouse model of dextran sodium sulfate (DSS)-induced colitis, during which the *in vivo* induction of ALPI by RvE1 positively correlated with the resolution process (57).

Lipoxins have also demonstrated to exert an *ex vivo* cytoprotective role on intestinal epithelial cells (59). Goh and colleagues showed that administration of LXs significantly ameliorates TNF-α-induced mucosal inflammation and reduces epithelial cell apoptosis. However, the mechanisms through which LXs exert these cytoprotective effects remain yet to be defined (33).

Polyunsaturated fatty acids have been shown to modulate other biological activities of intestinal epithelial cells. It is known that pro-resolving LMs exert their functions by binding with cell surface receptors, the majority of which belongs to G protein-coupled receptors (GPRs) (60). Among these receptors, GPR120 has been found to be the most abundantly expressed in the gut, particularly on epithelial cells and macrophages (59, 60). A study from Mobraten and colleagues shows that DHA, EPA, or AA are able to trigger GPR120 in Caco-2 cells, initiating multiple and independent signaling processes with different kinetics and intensity; these are (i) the activation of MAP kinases, (ii) the inhibition of IL-1β induced NF-κB activation, and (iii) the cytosolic accumulation of Ca^{2+} (61). Another group shows differential effects of activation of GPR120 by DHA in human intestinal Caco-2 and murine STC-1 cells, two different cell lines representing the mammalian intestinal epithelial layer. In this study, GPR120 stimulation by ω-3 PUFAs increased β-arrestin2 interaction with TAB 1 and attenuated TNFα-induced inflammatory effects by association of TAB 1 with TAK1, which resulted in reduced activation of NF-kB (59). Anti-inflammatory effects exerted by PUFAs through GPR120 were confirmed *in vivo* by Zhao et al., who demonstrated that triggering of GPR120 by DHA treatment ameliorate the experimental colitis in IL-10 deficient mice (62). Interestingly, transcription of *GPR120* in intestinal epithelial cells was found tremendously increased by bacteria belonging to the *Firmicutes, Bacteroides and Proteobacteria phyla* (63), all classified as microorganisms harboring anti-inflammatory properties. This is intriguing, because the dysbiosis observed in patients with IBD is basically caused by a diminished diversity of *Firmicutes* (64). This suggests that reduced expression levels of GPR120 may be one of the causes underlining IBD pathogenesis, and that targeting this receptor may represent a new therapeutic strategy in IBD; however, to date there are no studies that deeply characterize and quantify GPR120 in the inflamed mucosa of IBD patients and further studies to elucidate this aspect are needed. The effects of PUFAs on intestinal epithelial cells are schematically summarized in **Figure 2A**.

Neutrophils (PMN leukocyte) are the first cell type of the innate immune system to reach inflamed areas and hold the essential role of limiting the invasion of microorganisms (65). In fact, upon transmigration through activated endothelial cells, PMNs infiltrate the intestinal epithelium, and once reached the apical portion of epithelial cells, they come into contact with tons of bacterial stimuli, which further sustain PMN activation. PMN accumulation within the intestinal crypts has been associated with transepithelial resistance (66, 67) and epithelial barrier integrity (68), and in IBD the persistent and prolonged PMN flux across the epithelium has been shown to cause mucosal ulceration and barrier disruption, ultimately facilitating microorganism entry into the submucosa (69), and contributing to the clinical syndrome of malabsorption and diarrhea in these patients (31, 68). However, PMNs are also recognized as important players in the first stages of the resolution program. For example, they release pro-inflammatory LMs (e.g., PGI2 and LTB4) during early inflammation, before producing pro-resolving molecules, such as LXs, Rvs, and protectins at the onset of resolution (21). Due to this dual role, PMN activity needs to be finely regulated in order to reduce tissue damage and avoid chronicity of diseases (70, 71). LXs deriving from the metabolic route of AA, have been shown to inhibit PMN flux across the epithelial barrier (21, 72). In line with this, patients with severe UC display colonic deficiency in LX biosynthesis, which causes low to absent production of lipoxin A4 (LXA4) (73). Accordingly, LXA4 analogs dampen colitis induced by 2,4,6-trinitrobenzene sulfonic acid (TNBS) or DSS (74, 75). RvE1 has been also shown to inhibit PMN transepithelial migration, and TNBS-induced colon damage (**Figure 2B**) (36, 57). These LMs, that include protectin D1, not only also support phagocytosis of apoptotic PMNs (76), but also mediate the overexpression of C–C chemokine receptor type 5 receptors on apoptotic neutrophils, thus sequestering inflammatory chemokines such as chemokine (C–C motif) ligand 3 and CCL5, and promoting PMN clearance at sites of inflammation (77).

During intestinal inflammation, PMNs not only represent the target cell type of many pro-resolving PUFAs, but they are also the main producers of many molecules. In fact, a number of recent studies (78–81) clearly indicate that activated PMNs generate crucial anti-inflammatory and pro-resolving mediators that characterize the onset of resolution (82, 83). This aspect has been convincingly demonstrated *in vivo*, by depletion of circulating PMNs with anti-Gr1 antibodies, which resulted in the exacerbation of colitis in various mouse models of IBD, implicating PMNs as a key protective factor in ongoing intestinal inflammation (84). This may justify the controversial role exerted by neutrophils to

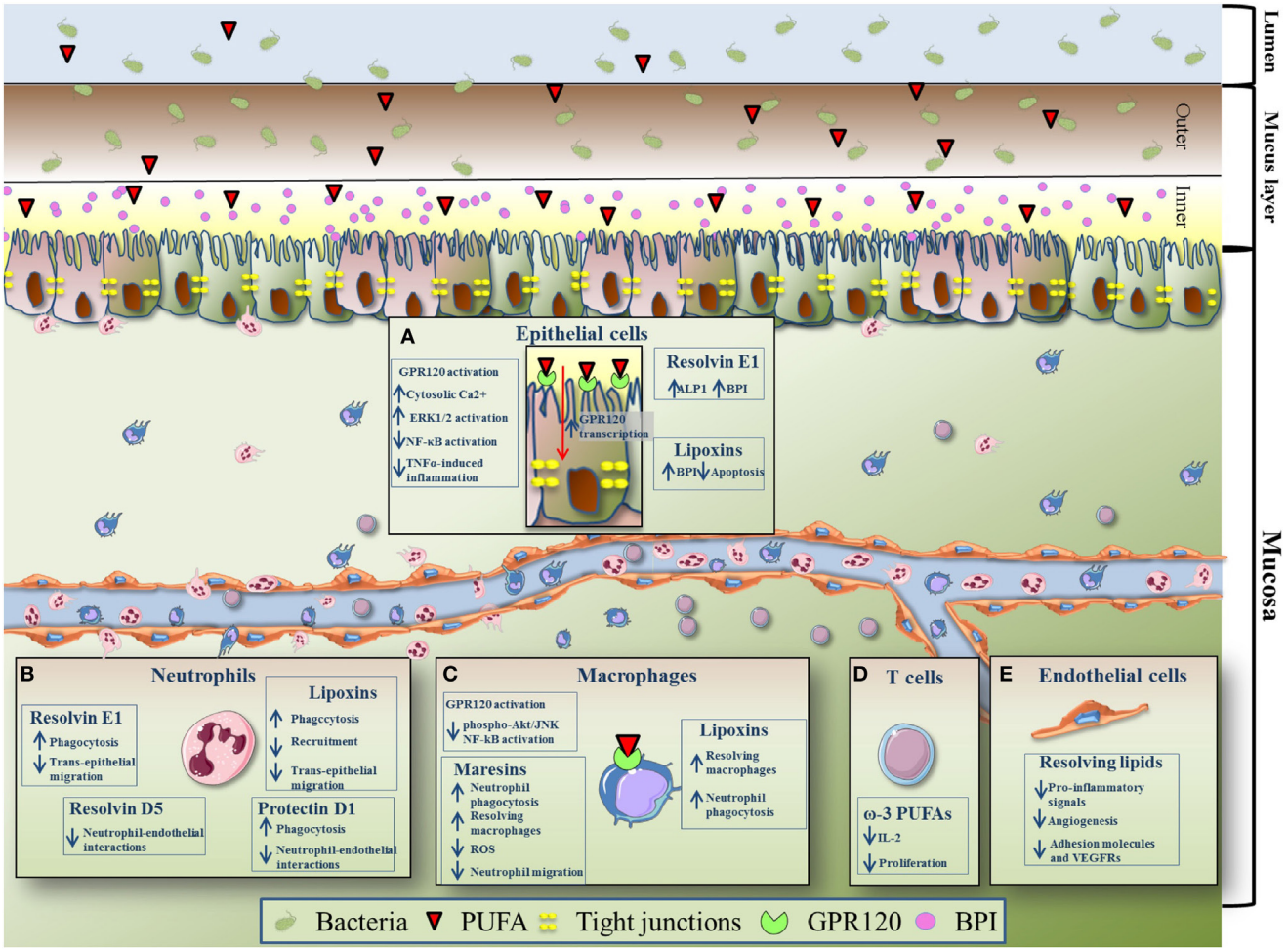

FIGURE 2 | Effects of pro-resolving polyunsaturated fatty acids (PUFAs) on immune and non-immune intestinal components. **(A)** Thanks to tight junctions, intestinal epithelial cells form a dynamic barrier protected by a thick mucus layer (inner and outer) which controls what can reach the lamina propria from the lumen. In order to counteract pathogen infections, epithelial cells are able to produce and release in the luminal mucus antibacterial and endotoxin-neutralizing molecules called bactericidal permeability-increasing protein (BPI). BPI is transcriptionally up-regulated by lipoxins (LXs) and resolvin (Rv) E1. In addition, it was observed that resolvin E1 (RvE1) significantly upregulates the expression of intestinal alkaline phosphatase. Moreover, LXs inhibit epithelial cells apoptosis. G protein-coupled receptor (GPR)120 activation by PUFAs [eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and arachidonic acid] leads to accumulation of cytosolic Ca^{2+}, activation of MAP kinase ERK1/2, inhibition of IL-1β-induced NF-κB activation, and TNFα-induced inflammation. Transcription of *GPR120* is increased by bacteria belonging to the bacteroides, proteobacteria, and firmicutes phyla. **(B)** Neutrophils (polymorphonuclear) are the first immune cells recruited to the site of inflammation, but are also important players in the first stages of the resolution program. LXs reduce neutrophil recruitment to the inflamed tissue, transepithelial migration, and phagocytosis. Protectin D1 promotes neutrophil phagocytosis. Similar to LXs, RvE1 reduces neutrophil transepithelial migration and induces neutrophil phagocytosis. Moreover, both protectin D1 and RvD5 have been shown to reduce neutrophil–endothelial interaction. **(C)** Macrophages, important for the resolution of intestinal inflammation, express high level of GPR120. EPA- and DHA-dependent activation of GPR120 has been shown to repress Akt/JNK phosphorylation and NF-kB induction. LXs enhance non-phlogistic phagocytosis of apoptotic neutrophils by macrophages. Treatment with LXs may also polarize intestinal macrophages into a resolving phenotype, thus promoting resolution of inflammation. Maresins exert potent pro-resolution and anti-inflammatory activities, ultimately leading to reduced neutrophil migration and increase macrophage phagocytic activities. Maresins induces also the resolving phenotype of macrophages and inhibit reactive oxygen species production. **(D)** EPA and DHA (ω-3 PUFAs) inhibit T cell proliferation and reduce IL-2 production. **(E)** Pro-resolving lipid mediators (DHA, α-linolenic acid-derived) exert anti-inflammatory and anti-angiogenic effects on the gut endothelium. They reduce the production of IL-6, IL-8, GM-CSF PGE-2, and LTB-4 (pro-inflammatory signals), decrease the levels of adhesion molecules (intercellular adhesion molecule 1 and vascular cell adhesion protein 1), and vascular endothelial growth factor receptor 2, thus suppressing the angiogenic component of inflammation.

the pathogenesis of IBD, and why their contribution may differ between CD and UC (85). In fact, while in patients with active UC it has been observed a correlation between the extent of PMN infiltration and the severity of the disease (86), several other studies have reported PMN dysfunction in patients with CD (87–89).

Resident macrophages, located in the sub-epithelial layers of the gut, are designated for protecting the host against pathogens and for regulating mucosal responses to commensal bacteria. For this reason, they are considered important players in the resolution of inflammation (90). These cells of the innate immune

system have the characteristic to express various GPRs, including GPR120 (19, 91). EPA- and DHA-dependent activation of GPR120 has been shown to have anti-inflammatory activities in both RAW 264.7 monocytes and primary intraperitoneal macrophages; these effects were abolished by GPR120 silencing (92). In another study, PUFA-dependent signaling cascade that follows GPR120 activation in the gut was observed also in macrophages, where the stimulation of this receptor led to the repression of Akt/JNK phosphorylation and NF-kB-mediated cyclooxygenase-2 (COX-2) induction (92–95). Blood-derived macrophages, that in chronic IBD are known to secrete inflammatory cytokines and tissue-degrading proteases (96, 97), and that well differentiate from resident macrophages, are recognized as either perpetuator of inflammation or effectors of the resolution process (33). Treatment of monocyte-derived macrophages with LXA4 and its analogs induced a strong enhancement in phagocytosis of apoptotic neutrophils (98), thus attributing to these PUFA derivatives an additional role in the resolution of intestinal inflammation.

Following studies on macrophages during the resolution process, a new pathway capable of producing potent mediators from DHA has been uncovered and the resulting metabolites have been coined maresins (MaR1 and MaR2), which exert potent pro-resolution and anti-inflammatory activities, ultimately leading to reduced neutrophil migration and increased macrophage phagocytic activities (99–102). Marcon and colleagues recently showed that MaR1 may cause a switch in the macrophage phenotype from the pro-inflammatory "classically activated M1" to the pro-resolving "alternatively activated M2," as well as direct blockade of PMN transmigration and reactive oxygen species production, which could explain, at least in part, the beneficial actions of this LM in experimental colitis (103). The effects of PUFAs on macrophages are schematically summarized in **Figure 2C**.

Studies on the effects of PUFA derivatives on the adaptive immune system in the gut are still in their infancy. In general, both DHA and EPA were observed to reduce *in vitro* T cell proliferation and to decrease the expression of both Th1 and Th2 cytokine IL-2 (**Figure 2D**). Recent works have also unveiled the effects of ω-3 PUFAs on Th17 cells (104–106). However, only few *in vivo* studies have shown a real effect of pro-resolving LMs in T cell reactivity in the gut; these will be described in the paragraph on animal studies.

The excessive transfer of various immune cell types from the peripheral blood to the affected gastrointestinal tracts of IBD patients, depends not only on surface molecules expressed by activated leukocytes, but also on high levels of adhesion molecules expressed by endothelial cells (14). Endothelial cells are key regulators of the inflammatory response, not only providing in the steady state an anti-inflammatory and anti-coagulatory surface, but also controlling which cell type enters the site of inflammation (107). Thus, alterations of endothelial cells may cause an imbalance between initiation of pro-inflammatory mechanisms and those that promote resolution and restitution of tissue homeostasis, ultimately leading to chronic inflammatory disorders, such as IBD. Patients with IBD are indeed characterized by increased vascularization, and excessive release of angiogenic factors (108, 109). Resolving LMs were observed to exert anti-inflammatory and anti-angiogenic effects on the gut endothelium. For example, Ibrahim and colleagues demonstrated that DHA is able to decrease vascular cell adhesion protein 1 (VCAM-1), TLR4, COX-2, and vascular endothelial growth factor receptor 2 (VEGFR-2) expression and reduce the production of IL-6, IL-8, GM-CSF, and PGE-2 in intestinal microvascular endothelial cells (HIMEC) stimulated with IL-1β. Moreover, administration of ALA during the TNBS model resulted in the decrease of intercellular adhesion molecule 1 (ICAM-1), VCAM-1, and VEGFR-2 expression levels, thus leading to suppression of angiogenesis in the inflamed colon (**Figure 2E**) (110). Interestingly, Ungaro et al. demonstrated that the Major Facilitator Superfamily Domain containing 2A (MFSD2A) may act as a new player in the resolution of intestinal inflammation, likely promoting endothelial production of DHA-derived pro-resolving mediators (20). In this study, lentiviral induction of MFSD2A conferred anti-angiogenic properties to HIMEC, reducing *in vitro* capillary formation and proliferation, and significantly inhibited TNFα-triggered inflammatory machinery of NF-kB signaling, *via* production of pro-resolving DHA-derived metabolites. These findings suggest that stimulating MFSD2A activity in intestinal endothelial cells could be a novel and powerful therapeutic approach to treat IBD.

Overall we have reported that the main modes of action of PUFAs in the inflamed gut are: (i) inhibition of leukocyte chemotaxis, reduced expression of adhesion molecules, and diminished leukocyte-endothelial adhesive interactions, (ii) modulation of epithelial biological functions and interactions with PMN, (iii) suppression of macrophage phagocytic activities, (iv) production of inflammatory cytokines, and (v) modulation of endothelial functions and T-lymphocyte reactivity. However, there are other mechanisms of action that have not been described in the intestine, but that may be crucial for further studies in IBD. For example, it has been demonstrated that resolving ω-3 PUFAs, such as EPA and DHA, can compete with the enzymes that convert AA into pro-inflammatory eicosanoids, thus inhibiting the release of inflammatory cytokines (e.g., TNF-α and IL1-β) (111). Furthermore, administration of ω-3 PUFA derivatives may benefit IBD patients by change in the lipid composition of intestinal cell membranes, activation of anti-inflammatory proteins such as the transcription peroxisome proliferator activated receptor γ (PPAR-γ), and reduction in the gut production of pro-inflammatory molecules, like NF-κB, LTs, and PGs (35, 45–47, 112, 113).

ROLE OF PUFAs IN ANIMAL MODELS OF IBD

One of the first studies unveiling the contribution of PUFAs in IBD progression was done by Hudert et al., who exploited a transgenic mouse carrying *Caenorhabditis elegans fat-1* gene, encoding for a specific desaturase capable of producing ω-3 PUFAs from ω-6 PUFAs (114). As a consequence, this transgenic mouse is characterized by a low ratio of ω-6/ω-3 fatty acids in its tissues and organs (115). They showed that *fat-1* transgenic mice subjected to the DSS protocol of chemically induced experimental colitis, had significantly reduced signs of colon inflammation, in terms of both clinical manifestation and pathology, than

wild-type littermates. Such amelioration was positively correlated with the production of anti-inflammatory ω-3 PUFA derivatives, reduced levels of pro-inflammatory cytokines and a concomitant increase of mucus-specific factors in their colons. Moreover, beside a reduced number of Th17 cells in lymphoid tissues, they also observed a reduced expression of Th17 cell type-specific cytokines and chemokine receptors specifically in the colonic mucosa, indicating a role for ω-3 PUFAs on T cell reactivity. The reduced susceptibility to chemically induced colitis in *fat-1* mice is likely to result from reduced activation of the NF-κB pathway and decreased expression of TNFα, IL-1β, and inducible NO synthase. Conversely, the enhanced protection conferred by a thicker mucus layer in these mice was probably due to the concomitant up-regulation of trefoil factor 3, toll-interacting protein, and zonula occludens-1.

Initial studies on the efficacy of PUFAs in animal models of IBD considered the use of PUFA precursors instead of single metabolites. One of the first fatty acid used in experimental colitis was conjugated LA (CLA), a mixture of 28 isomers of LA (116); this has been tested in pig models of colitis. Animal treated with CLA showed reduced signs of intestinal inflammation, accompanied by decreased serum levels of TNF-α and NF-κB, and increased amount of transforming growth factor β and PPAR-γ (117). These findings were confirmed in two different experimental mouse models of colitis, either chemically (DSS)- or CD4-induced (118).

Other studies have focused their attention on the ω-6/ω-3 PUFA ratio. During the DSS-induced colitis model, mice administered with ALA-enriched diet, consequently resulting in a decreased uptake of LA, showed less severe colitis, with a markedly alleviated intestinal inflammation (119). The beneficial effects exerted by the ALA-enriched diet was probably due to the reduced PMN influx into the colonic mucosa, because of the decreased activity of both myeloperoxidase (MPO) and alkaline phosphatase. In addition, ALA supplementation blocked TNF-α and IL-1β up-regulation, by comparison with the control group.

Following studies were designed to use specific PUFA metabolites rather than precursors, with ω-3 EPA- and DHA-derived LMs as main candidates for both animal and clinical trials.

The first work involving ω-3 PUFA derivatives and IBD were conducted by using both TNBS- and DSS-induced colitis. Arita and colleagues demonstrated that RvE1 exerts protective effects in TNBS-induced intestinal inflammation, in terms of reduced body weight loss, colon shortening, and tissue damage, by reducing PMN flux into the colonic mucosa, and, at the same time, by limiting either the production of TNFα and IL-12, or the expression of pro-inflammatory enzymes, like COX-2. The authors also showed that the expression of the RvE1 receptor *ChemR23* was up-regulated in colonic mucosa of TNBS-treated animals (36).

Similar effects were observed in the DSS-induced model of colitis by Ishida et al., who demonstrated that repeated administrations of RvE1 were able to dampen colitis severity in terms of body weight loss, colon shortening, and histological score (41). Concomitantly, they observed a reduction in NF-kB phosphorylation, TNFα, IL-1β, and IL-6 levels in colonic tissues, along with higher levels of *ChemR23* mRNA, supporting a possible role for this receptor in the pathogenesis of intestinal inflammation (41). Other groups confirmed these findings additionally indicating that an interplay might exist between ALPI and RvE1 that ultimately leads to resolution of intestinal inflammation.

In 2011, Bento et al. showed that aspirin-triggered (AT)-RvD1 and RvD2 protect mice against both TNBS- and DSS-induced colitis (47). In this study, the preventive administration of these resolvins significantly ameliorated clinical manifestations, such as body weight loss, disease activity index, colonic damage, and colon shortening. Beside these clinical findings, they showed these mice to produce reduced levels of pro-inflammatory cytokines, and diminished activation of NF-kB pathway and expression of VCAM-1, ICAM-1, and leukocyte function-associated antigen-1. Finally, the authors demonstrated that blockage of LXA4 receptor (ALX), reversed the (AT)-RvD1protective effects in DSS-induced colitis, concluding that (AT)-RvD1 action may depend on ALX activation.

Other DHA-derived pro-resolving mediators, such as maresins, have also shown fundamental properties in experimental IBD. In fact, preventive or therapeutic administration of MaR1 (103) demonstrated for the first time that this DHA metabolite protects mice against both acute and chronic DSS-induced colitis, reducing disease activity index, colon shortening, body weight loss, and MPO activity. In addition, the authors demonstrated that MaR1 inhibited the production of pro-inflammatory cytokines like IL1-β, IL-6, TNF-α, and IFN-γ in colon tissue, together with down-regulation of NFk-B activation and diminished neutrophil transmigration in the inflamed mucosa (103). Similar results were obtained with the TNBS-induced model of colitis.

A very recent work from Gobbetti et al. shows that exogenous administration of LMs derived from ω-3 docosapentaenoic acid (ω-3 DPA), an intermediary product between EPA and DHA, named protectin $D1_{n-3\ DPA}$ ($PD1_{n-3\ DPA}$) and resolvin $D5_{n-3\ DPA}$ ($RvD5_{n-3\ DPA}$), was effective in preventing the acute model of DSS-induced colitis, in terms of reduced colon length, and microscopic damage score (120). These protective effects were partially linked to reduced granulocyte trafficking and PMN–endothelial interactions, which may occur downstream adhesion molecule activation. The translational impact of these data was determined not only by the ability of $PD1_{n-3\ DPA}$ and $RvD5_{n-3\ DPA}$ to reduce human neutrophil adhesion onto TNF-α-activated human endothelial monolayers, but also to the identification of ω-3 DPA metabolites in human colon biopsies. Using targeted LC-MS/MS-based LM metabololipidomics on colonic biopsies from controls and IBD patients they observed that LTB_4, PGE_2, and TX B_2 were significantly increased in inflamed tissues in comparison with controls. Notably, they showed that $RvD5_{n-3\ DPA}$ and $PD1_{n-3\ DPA}$ were augmented in tissue biopsies from IBD patients compared with those from control. This finding on human IBD samples is in contrast with the fact that $RvD5_{n-3\ DPA}$ and $PD1_{n-3\ DPA}$ exert protective effects against chemically induced acute colitis, and warrants further investigation. There may be a dysfunctional susceptibility of cells targeted by these mediators in IBD. Moreover, it would be interesting to distinguish the effects of $RvD5_{n-3\ DPA}$ and $PD1_{n-3\ DPA}$ in patients with UC versus CD.

The last study that needs to be mentioned has been done by Meister and Ghosh, who treated IBD patient-derived biopsies with fish oil. They found reduced inflammation in terms of high IL-1a/IL-1b ratio in tissues derived from patients with UC, but

not in tissues from patients with CD. These contrasting outcomes indicate that variations in diet composition may influence the success of a nutritional therapy for UC or CD patients (121). All mentioned animal studies are summarized in **Table 1**.

It is worth of note that although the majority of pre-clinical studies on animal model of IBD are promising and provide strong or mild anti-inflammatory properties of ω-3 PUFAs (122–131), other works revealed that an abundant intake of dietary ω-3 PUFAs could even worsen the clinical signs of colitis (132–135). This discrepancy may be explained by different treatment and dose regimen, by different animal facility conditions, and different racemic mixture that could have been used to treat mice. In any case, this must be taken into consideration when animal studies need to be translated into clinical management of IBD.

CLINICAL APPLICATION

As for animal models, many attempts have been done to prove ω-3 PUFA efficacy in human studies. The great therapeutic potential of ω-3 PUFAs has been also encouraged by some works reporting alterations in the production of pro-resolving LM. For example, Pearl and colleagues revealed the ω-6/ω-3 PUFA composition were altered in the inflamed gut mucosa of patients with active UC, in comparison with healthy samples (136). Additionally, Masoodi et al. reported that pro-inflammatory PUFA metabolites (PGD2, PGE2, TXB2, 5-HETE, 11-HETE, 12-HETE, and 15-HETE) not only were increased in the inflamed mucosa of patients with active UC, but their levels also correlated with the disease activity (137). Interestingly, our group recently characterized colonic biopsies isolated from patients with active UC showing that the production of pro-resolving DHA-derived metabolites was defective in inflamed mucosa in comparison with colon tissues from patients with UC in remission and healthy controls. This indicates that pro-resolving mechanisms are deficient in patients with active UC (20), suggesting that ω-3 PUFA administration can be exploited as a novel therapeutic approach to treat IBD.

The majority of studies that have been performed so far uses diet as way of delivery of ω-3 PUFAs, in combination or not with the conventional IBD therapies (138). John et al. found that the intake of dietary EPA and DHA was conversely correlated with the risk of developing incident UC (139). Similarly, in a cohort of patients with CD, the dietary DHA intake was conversely correlated with the development of incident CD, with statistical significance (140). Moreover, clinical trial for CD and UC revealed the beneficial effects of ω-3 enriched diet (141–151) in terms of clinical and histological parameters. Among these, Belluzzi et al. showed that in patients with CD in remission, fish-oil enriched diet is effective in decreasing relapse frequency (146). In another multicenter, randomized, double-blind, clinical trial the beneficial role of fish-oil administration in patients with UC was demonstrated. The positive clinical outcome was expressed in terms of reduced rectal leukotriene B4 (LTB4) levels, improvements in histological scores, and gain of weight.

Omega-3 PUFA administration may also be effective in pediatric patients. In children with CD treated with mesalazine, diet supplementation with ω-3 PUFAs significantly reduced the frequency of relapse within 1-year observation in comparison with patients receiving placebo, consisting in olive oil (145).

However, in a clinical trial (EPIC-1 and -2) conducted by Feagan et al. the efficacy of a mixture of ω-3 PUFA was revised; in fact, the treatment was not effective in preventing relapse and maintaining remission in CD patients (152). All clinical studies are summarized in **Table 2**.

The opportunity of clinical application for PUFAs has been evaluated by few systematic reviews and meta-analyses. For example, the study by Turner and colleagues found significant positive effects of ω-3 PUFA supplementation in CD patients. However, these conclusions derived from only six trials that are highly heterogeneous. Analysis of three clinical trials on ω-3 PUFA administration in patients with UC described no significant outcome. Thus, the authors concluded that data available were insufficient to prescribe the use of ω-3 PUFAs for the maintenance of remission in CD and UC (153, 154).

Overall, the studies conducted so far are elusive and displayed no real evidence of efficacy (138, 155–159). This might be due to different reasons: (a) the ω-3-based diet needs to be tightly controlled

TABLE 1 | Polyunsaturated fatty acid (PUFA) administration in animal models of IBD.

Study reference	Administered PUFA	Model of colitis	Outcomes
Viladomiu et al. (116)	CLA	DSS (pig)	Reduction of: DAI, TNFα, increase of: TGFβ and PPARγ
Bassaganya-Riera et al. (118)	CLA	DSS, $CD4^+$ transfer (mouse)	Reduction of: inflammation, TNFα, increase of: TGFβ and PPARγ
Tyagi et al.(119)	Decreased LA/ALA ratio	DSS (rat)	Reduction of: DAI, intestinal inflammation, TNFα, and IL1β levels
Arita et al. (36)	RvE1	TNBS and DSS (mouse)	Reduction of: weight loss, colon shortening and tissue damaging, PMN infiltration, IL-12, TNFα, and COX-2
Ishida et al. (41)	RvE1	DSS (mouse)	Reduction of: weight loss, colon shortening and tissue damaging, NFk-B activation, TNFα, IL-1β, and IL-6
Bento et al. (47)	AT-RvD1, RvD2	TNBS and DSS (mouse)	Reduction of: weight loss, DAI, colon damage and shortening, pro-inflammatory cytokines, NFk-B activation, and adhesion molecules
Marcon et al. (103)	MaR1	DSS (mouse)	Reduction of: DAI, colon shortening, weight loss, myeloperoxidase activity, pro-inflammatory cytokines, NFk-B activation, and neutrophil transmigration
Gobbetti et al. (120)	PD1 and RvD5	DSS (mouse)	Reduction of: colon length and pro-inflammatory cytokines, leukocyte–endothelial interaction

TABLE 2 | Clinical studies with the use of polyunsaturated fatty acids (PUFAs) in inflammatory bowel disease.

Study reference	Treatment	Disease	Outcome
Romano et al. (145)	ω-3	CD	Lower relapse than placebo
Belluzzi et al. (146)	ω-3	CD	Maintenance of remission compared with placebo
Feagan et al. (152)	ω-3	CD	No effects
Stenson et al. (142)	ω-3	UC	No changes compared with placebo
Barbosa et al. (141)	ω-3	UC	Decreased oxidative stress compared with placebo
Lorenz-Meyer et al. (157)	ω-3 and low carbohydrate diet	CD	No amelioration compared with placebo
Nielsen et al. (158)	ω-3 and ω-6, arginine and ribonucleic acids, and prednisolone	CD	No significative reduction of Crohn's disease activity index (CDAI) compared with placebo
Geerling et al. (147)	ω-3 and antioxidant	CD	Increase of antioxidants; better resolving PUFA profiles in treated compared with placebo
Nielsen et al. (148)	ω-3	CD	Reduced pro-inflammatory cytokines and CDAI
Eivindson et al. (159)	ω-3 and corticosteroids	CD	No difference between groups
Brunborg et al. (149)	ω-3	UC/CD	Reduced joint pain
Bjørkkjaer et al. (150)	ω-3	UC/CD	Reduced disease activity compared with placebo
Seidner et al. (151)	ω-3, fiber, and antioxidant	UC	Reduced use of prednisone compared with placebo
Salomon et al. (143)	ω-3	UC	Improvement in seven patients

in IBD patients; (b) the administration of ω-3 PUFA (DHA or EPA) through diet is not effective because of insufficient intestinal absorption due to ulcers or because of biochemical modification of PUFAs when they are in the systemic circulation; (c) EPA and DHA are general precursors of a plethora of specific pro-resolving lipids, that, by definition, are locally and timely regulated. Therefore, the administration through the diet does not help to finely control such metabolism; (d) patients may harbor genetic predisposition impeding the correct DHA or EPA metabolism, thus leading to insufficient production of bioactive pro-resolving LMs.

CONCLUDING REMARKS

In IBD patients, diet and lifestyle changes, conventional or newly identified drugs, do not always resolve inflammation and relieve symptoms of the disease. One of theories formulated in the last few years is that anti-inflammatory agents do not alter the course of the disease, because naturally occurring resolution programs may have been subverted. Few studies, including findings from our group, showed that eventual dysfunctions in resolution pathways and/or deficits in precursors of pro-resolving mediators, such as ω-3 PUFAs, may lead to persistent inflammation and provoke alteration in gut mucosa homeostasis, thus being part of IBD pathogenesis. For this reason, the use of pro-resolving PUFAs, particularly the ω-3 ones, brings new possibilities to the treatment of IBD, and could be of great interest to pharmacological industry.

Although numerous pre-clinical and clinical studies employing the use of PUFAs, either as fatty acid precursors or single metabolites, showed controversial results, there is still much more to discover about the beneficial effects of these molecules, particularly in the IBD field. It would be important not only to uncover new cellular and molecular processes modulated by PUFAs under gut inflammatory conditions, but also to unveil novel biosynthetic pathways of these pro-resolving LMs that may likely be dysregulated in IBD. Ways of delivery, safety, dosage, and regimen treatment, and interaction with other drugs should also be further addressed in order to establish the most efficient replacement therapy. We suppose that either PUFAs or new medications specifically promoting resolution pathways will be much better tolerated by patients with IBD, mimicking the physiological processes through which inflammation naturally occurs in the organism, with the advantage of avoiding immune suppression.

AUTHOR CONTRIBUTIONS

FU, FR, SD, and SDA conceived and wrote the manuscript, and realized the figure.

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REVIEW
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Imaging of Mucosal Inflammation: Current Technological Developments, Clinical Implications, and Future Perspectives

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In recent years, various technological developments markedly improved imaging of mucosal inflammation in patients with inflammatory bowel diseases. Although technological developments such as high-definition-, chromo-, and autofluorescence-endoscopy led to a more precise and detailed assessment of mucosal inflammation during wide-field endoscopy, probe-based and stationary confocal laser microscopy enabled *in vivo* real-time microscopic imaging of mucosal surfaces within the gastrointestinal tract. Through the use of fluorochromes with specificity against a defined molecular target combined with endoscopic techniques that allow ultrastructural resolution, molecular imaging enables *in vivo* visualization of single molecules or receptors during endoscopy. Molecular imaging has therefore greatly expanded the clinical utility and applications of modern innovative endoscopy, which include the diagnosis, surveillance, and treatment of disease as well as the prediction of the therapeutic response of individual patients. Furthermore, non-invasive imaging techniques such as computed tomography, magnetic resonance imaging, scintigraphy, and ultrasound provide helpful information as supplement to invasive endoscopic procedures. In this review, we provide an overview on the current status of advanced imaging technologies for the clinical non-invasive and endoscopic evaluation of mucosal inflammation. Furthermore, the value of novel methods such as multiphoton microscopy, optoacoustics, and optical coherence tomography and their possible future implementation into clinical diagnosis and evaluation of mucosal inflammation will be discussed.

Keywords: endoscopy, mucosal inflammation, inflammatory bowel disease, Crohn's disease, ulcerative colitis, narrow-band imaging, confocal endomicroscopy, multiphoton microscopy

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INTRODUCTION

Inflammatory bowel diseases (IBDs), which include Crohn's disease (CD) and ulcerative colitis (UC), affect an estimated 3.1 million people in the United States and about 2.5 million people in Europe. They result in a chronic disabling mucosal inflammation of the gastrointestinal tract (1–3). Affected patients suffer from abdominal pain, diarrhea, hematochezia, weight loss, nausea, etc. and are exposed

to an increased risk for complications such as abscess formation, perforation, or the development of colorectal cancer (CRC).

For the clinical diagnosis and management of IBD patients, endoscopic and non-invasive imaging techniques have gained increasing importance for the evaluation of mucosal inflammation during recent years. Although the initial diagnosis of IBD is based on several parameters including clinical, laboratory, endoscopic, radiologic, and histologic features, especially endoscopic results frequently provide essential information for the definitive diagnosis of IBD and the differentiation between CD and UC. Furthermore, endoscopic evaluation of mucosal inflammation vs. mucosal healing is regarded as gold standard for the evaluation of disease activity and therefore the therapeutic management of IBD (3).

Besides inflammation-associated complaints, the increased risk for the development of CRC poses a severe treat for IBD patients. The risk for CRC has been associated with the duration, severity, and extend of colonic inflammation. Independent risk factors include the presence of primary sclerosing cholangitis (PSC) or a family history of CRC. For UC, a cumulative risk of 1.6% after 10 years, 8.3% after 20 years, and up to 18.4% after 30 years has been reported (4). Although recent studies report lower risk rates, for instance Jess et al. described a 2.4-fold increase of CRC risk after 14 years in UC patients (5), it is still widely accepted that long-standing colitis poses a risk factor for CRC development. As a matter of fact, most national and international guidelines on the management of UC recommend repeated endoscopy for CRC surveillance. In patients with CD, an increased risk has been reported in patients with Crohn's colitis (6). Although data are more limited in comparison to UC, surveillance endoscopy is also recommended for CD patients with long-standing colonic inflammation.

Recent technological developments critically improved the diagnostic accuracy and enabled new applications for endoscopy in various types of diseases and organs. These technologies include wide-field endoscopes with high-definition optical resolution, dye-based or virtual chromoendoscopy, or autofluorescence-endoscopy and also endomicroscopic techniques such as endocytoscopy or confocal laser endomicroscopy (CLE), which provide *in vivo* microscopic information of the mucosal surface during endoscopy. In addition to endoscopic imaging techniques, also non-invasive imaging such as computed tomography (CT), magnetic resonance imaging (MRI), scintigraphy, and ultrasound (US) provide valuable information about disease activity that supplements endoscopic imaging techniques. In this article, we will discuss current data supporting the use of these technologies for the evaluation of mucosal inflammation and provide an outlook on future developments that might further improve the diagnosis and management of IBD.

CURRENT ENDOSCOPIC TECHNIQUES FOR THE DIAGNOSIS AND FOLLOW-UP OF MUCOSAL INFLAMMATION

High-definition video endoscopy is technically mature, widely accepted, and available and therefore considered to be the gold standard for the detection and characterization of mucosal inflammation during initial diagnosis and for evaluating the disease activity in patients with established CD or UC. Whereas conventional white light endoscopy seemed to be sufficient enough for initial and short-term follow-up procedures, more advanced techniques like dye-based and virtual chromoendoscopy or magnification endoscopy are helpful for the evaluation of mucosal healing and in the long-term follow-up during surveillance of IBD.

Diagnosis and Assessment of Disease Activity

As a first diagnostic step in patients suspicious of IBD, ileocolonoscopy plays a crucial role for the differentiation of UC and CD. As complementary examinations, upper GI endoscopy, magnetic resonance tomography, small bowel capsule endoscopy (7), or enteroscopy (8) may give additional information on the extent of the disease. In patients with suspected, known or relapsed CD, capsule endoscopy is recommended in those with negative findings in ileo-colonoscopy or gastroscopy (9). The role of colon capsule endoscopy as a surveillance technique, however, is far away from clinical routine and will therefore not replace regular colonoscopies in patients with long-standing IBD in the near future. Except for perianal CD, endosonography of the upper or lower GI tract cannot contribute to the extent of the disease neither for initial diagnosis nor for further evaluation in patients with established diagnosis for IBD. Index-colonoscopy should include a segmental inspection and biopsy of any visible lesion or inflammation in combination with the acquisition of biopsies of non-inflamed mucosal areas (10, 11). The morphological aspect and extent of the inflamed mucosa is of central importance for determining the underlying disease and for distinguishing other inflammatory causes.

For an optimal therapeutic management of IBD, regular evaluation of disease activity is mandatory. Endoscopic evaluation of mucosal healing has been shown to provide good correlation with the clinical course of disease and therefore is currently considered as gold standard for evaluating disease activity (**Figure 1**). In this regard, endoscopic disease activity scores are helpful for the prediction of the disease progression or for evaluation of the treatment success by follow-up procedures after initiation of immunosuppressive therapy. The UC endoscopic index of severity (12), the simplified endoscopy score for CD (13) and the Rutgeerts-Score (14) for the postoperative situation are the most common used scores for documentation of the disease activity in IBD patients. A >50% decrease in Simple Endoscopic Score in Crohn's Disease (SES-CD) or a Rutgeerts Score i0-i1 is the definition for endoscopic response (15). However, none of these scores has so far been uniformly accepted as standard for endoscopic evaluation of disease activity.

Besides white-light endoscopy, the determination of the disease activity was evaluated in a prospective study with the use of virtual chromoendoscopy with narrow band imaging (NBI) versus white light endoscopy and a special mucosal vascular pattern was noticed with NBI. The vascular pattern showed a good correlation to histology indicating a more precise grading during ongoing endoscopy with NBI (16). Another study found similar results when comparing high-definition white light

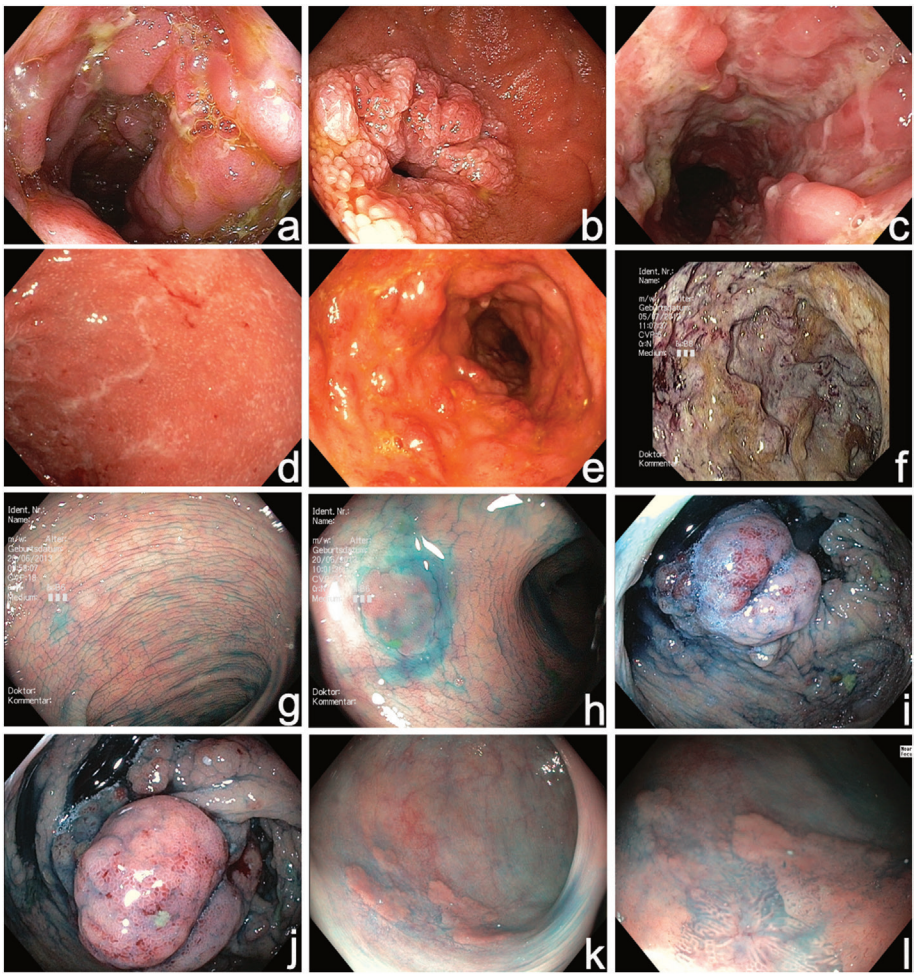

FIGURE 1 | High-resolution video endoscopy used for initial diagnosis of Inflammatory bowel disease (IBD) **(a–f)** and in combination with chromoendoscopy (diluted solution of indigocarmine 0.1%) during surveillance colonoscopy **(g–l)**. **(a)** Acute Crohn's disease (CD) in the terminal ileum, **(b)** Crohn's stenosis in the duodenum, **(c)** segmental fissural ulcerations in the left colon SES-CD 32, **(d)** mild active UC UCEIS 3, **(e)** moderate active UC UCEIS 5, **(f)** severe UC UCEIS 8, **(g)** normal chromoendoscopy with uniformly distributed contrast dye, **(h)** identification of a small flat lesion (hyperplastic polyp) with chromoendoscopy, **(i,j)** chromoendoscopy-guided evaluation of pseudopolyps during surveillance colonoscopy, **(k,l)** identification of an inhomogeneous flat polypoid area, and **(l)** with near focus mucosal irregularities are visible indicating high grade intraepithelial neoplasia. SES-CD, simplified endoscopy score for Crohn's disease; UCEIS, ulcerative colitis endoscopic index of severity.

endoscopy with i-scan virtual chomoendoscopy in patients with IBD (17). In a recent study, optical enhancement with i-scan was combined with magnification endoscopy and a good correlation with histological scores of acute and chronic inflammation was found indicating that this technique might be able to adequately evaluate mucosal healing (18).

However, further data are required to clearly evaluate the usefulness of these techniques for clinical routine endoscopy of IBD diagnosis and monitoring disease activity.

CRC Surveillance

Although the risk of CRC in IBD nowadays is considered to be lower than previously assumed (19), the overall risk of CRC in IBD patients remains higher in comparison to the general population (20). Therefore, much strength has been made to detect early changes of mucosal alterations in between the active disease periods. Advanced endoscopy techniques, especially dye-based endoscopy, are recommended for the detection of intraepithelial neoplasia (IEN), which has a high risk of progression to IBD-associated CRC. This is not only relevant for high-grade IEN, but also low-grade IEN, which was found to develop infrequently into more advanced neoplasia (21), but has a substantial risk of progression into advanced cancer (22).

For colon cancer screening in the general population, virtual chromoendoscopy (NBI, i-scan, FICE) is not recommended as a standard technique, because comparative studies with high-definition video endoscopy showed controversial results (23–25). No difference was seen when conventional and high-definition white light endoscopy was compared for polyp detection in the general population (26). Classical dye-based pan-chromoendoscopy, mostly used with diluted indigocarmine solution (0.1–0.5%), however, is superior to white light endoscopy and markedly

enhanced the detection rate of adenomas in the average risk population (27). Unfortunately, dye-based chromoendoscopy is not used for screening colonoscopy in most Western countries, because this technique is time-consuming, not reimbursed and therefore not well accepted in general practice. Previous studies have also shown a learning curve by using virtual chromoendoscopy techniques for the detection of colorectal neoplasia (23) and further technical improvements like blue laser imaging (28) or linked-color imaging (29) will bring new data regarding CRC screening with virtual chomoendoscopy in the general population.

Although a substantial number (17–28%) of patients with IBD develop CRC before the initiation of a structured surveillance program (30), follow-up colonoscopies are recommended after 8–10 years of extensive colitis or 15–20 years of left-sided colitis (10). A risk stratification should be made for those patients with severe inflammation, colitis-associated PSC, or familiar history of CRC (10). In the surveillance of IBD, dye-based chromoendoscopy with acquisition of targeted biopsies is recommended according to several guidelines (10, 11, 31) and has largely replaced classical random biopsy protocols in most countries. The cost-effectiveness and the efficiency of this surveillance strategy was shown in several studies (32–34). A combination of chromoendoscopy with magnification endoscopy was investigated in very few studies and found a better prediction of disease extent in UC (35). In most Western countries, magnification endoscopy is not used for routine surveillance endoscopy with a more widespread use in the Eastern part of the world. Overall, there is a recognizable tendency toward more sophisticated improvements with the potential to contribute to a better identification and differentiation of colorectal lesions. For instance, first data on full-spectrum endoscopy, a technology providing a field of view of 330°, and the impact on surveillance colonoscopy in IBD patients was published. The authors reported a superior detection rate of cancer precursors and found a large miss rate in forward-viewing endoscopes (36). However, additional studies are still required to fully evaluate the potential of these technologies for IBD surveillance.

Although 10–20 years ago the detection of IEN associated with IBD in many cases directly led to proctocolectomy, published data over the last decade have induced a paradigm-shift toward endoscopic resection techniques, if technically feasible (37). After detection of dysplasia, lesions should be fully resected by endoscopy following the guidelines of the SCENIC consensus conference (38, 39). The development of endoscopic resection techniques and newer data on their safety and efficiency has justified this strategy.

ENDOMICROSCOPY OF THE GASTROINTESTINAL TRACT: *IN VIVO* HISTOLOGY OF INFLAMMATORY DISEASES

Principles and Technical Background of CLE

Confocal laser endomicroscopy has been introduced in 2003 and since then, has emerged as a cross-sectional high resolution technique that allows to precisely visualize and characterize gastrointestinal pathology *in vivo* (40–44) at (sub)cellular level (45). Technically, CLE utilizes low-powered blue laser with a wavelength of 488 nm that is directed through a pinhole onto a defined point of the intestinal mucosa. Upon reaching the tissue, an autofluorescence signal is produced which is reflected and refocused on the detection system. Importantly, this reflected light again passes through a pinhole while scattered light from outside the plane of interest is not detected. This results in increased spatial resolution of the images obtained. The region of interest is scanned in both, the horizontal and vertical planes and thereby provides data on signal intensity for each individual point of interest inside the tissue. The fluorescence signal of each point is then converted into a 2D or 3D image using a computer algorithm enabling histologic imaging with 1,000-fold magnification *in vivo* in real time.

Since CLE depends of the fluorescence signal from the tissue, the application of contrast agents either intravenously or topically is required. Among the intravenous contrast agents, fluorescein is most commonly utilized and usually administered immediately before imaging. Optimal image contrast is achieved with 2.5 to 5 mL of fluorescein and images can be obtained within 30 s up to 60 min after injection (46). Administration of fluorescein results in microscopic visualization of the vasculature, the lamina propria, and the intracellular spaces of the tissue while cell nuclei are not stained with fluorescein. Nuclear staining usually requires topical contrast agents such as acriflavine and cresyl violet which can be applied through a spraying catheter (47, 48). However, there is increasing concern over mutagenic potential conferred by topical contrast agents due to their DNA intercaling properties.

To date, two different CLE systems are available and used in clinical routine, both of which are FDA-approved and CE-certified (49) (**Table 1**): (i) a probe based CLE system which can be used with virtually any existing endoscope with a working channel ≥2.8 mm diameter (pCLE, Cellvizio, Mauna Kea Technologies, Paris, France) and (ii) an endoscope-based CLE which integrated into a high-resolution endoscope (eCLE; Pentax, Tokyo, Japan) (50–52). However, the eCLE system is no longer commercially available. As a common feature, both eCLE and pCLE emit blue laser light with an excitation wavelength of 488 nm and detect the reflected light between 205 and 585 nm. With eCLE images are acquired with a scan rate of 1.6 frames/s and a resolution of 1,024 × 512 pixels, or at 0.8 frames/s with a resolution of 1,024 × 1,024 pixels. With eCLE, laser power and depth of scanning is manually adjustable (depth: 0–250 μm, power: 0–1,000 μW). The acquired images have a confocal image field of view of 475 μm × 475 μm with lateral and axial resolution of 0.7 and 7 μm, respectively.

The pCLE system uses stand-alone confocal miniprobes that are compatible with any endoscope with a working channel ≥2.8 mm diameter. Typically, a single probe can be used for 20 different applications and specific probes are available for various organs within the gastrointestinal tract. With pCLE laser power and imaging plane depth are fixed. Depending on the miniprobe utilized, lateral resolution can range from 1 to 3.5 μm and with field of view of 240 to 600 μm. All probes have image scan rates of 12 frames/s with a 30.000 pixels scanning field, thereby enabling real-time videos of the intestinal mucosa.

TABLE 1 | Technical characteristics of probe based and endoscope-based CLE devices.

	Endoscope-based CLE	Probe-based CLE			
	eCLE	GastroFlex	GastroFlexUHD	ColoFlex	ColoFlexUHD
Image-plane depth (µm)	0–250	70–130	55–65	70–130	55–65
Lateral resolution (µm)	0.7	3.5	1	3.5	1
Field-of-view (µm)	475 × 475	600 × 600	240 × 240	600 × 600	240 × 240
Frames per second	0.8 –1.6	12	12	12	12
Magnification	1,000-fold	1,000-fold	1,000-fold	1,000-fold	1,000-fold
Required operating channel (mm)		≥2.8	≥2.8	≥2.8	≥2.8
Length (cm)	120 and 180	300	300	400	400

eCLE, endoscope-based confocal laser endomicroscopy; pCLE, probe-based confocal endomicroscopy; UHD, ultrahigh definition.

Based on these technical characteristics, both CLE systems have specific advantages which are for eCLE its higher resolution, the adjustability of the imaging plane depth, and the possibility to simultaneously obtain biopsies for standard histopathology, whereas the pCLE system can be readily used with virtually any endoscope throughout the entire gastrointestinal tract and also allows to obtain videos of the intestinal mucosa in real time.

CLE for Assessment of Intestinal Inflammation

The technical application CLE as well as the assessment and interpretation of CLE mages for the evaluation of mucosal inflammation can be rapidly learned. Studies have shown that the performance of individual investigators constantly increases over time and leads to a decreased acquisition time and improved diagnostic accuracy after the first three examinations of pCLE (53). In a recent study by Chang et al., the diagnostic accuracy and learning curve for the identification of mucosal barrier function and mucosal integrity was assessed (54). For this purpose, a total of 180 endoscopic CLE images of the terminal ileum were evaluated for increased intestinal permeability (IP) as assessed by cell-junction enhancement, fluorescein leak (FL), and cell dropout (CDO) by experienced and inexperienced analysts as well as pathologists after a 30-min teaching session (54). As shown in this report, the identification of IP requires only a short learning curve after which a high diagnostic accuracy is achieved.

Various studies have demonstrated that increased IP and barrier dysfunction can well be visualized with CLE. As originally described by Kiesslich et al. (55), epithelial gaps are the morphologic equivalent of shedded epithelial cells and these epithelial gaps have been shown to be of utmost importance for the assessment of inflammatory activity with CLE in IBD patients.

Endomicroscopic characteristics of impaired intestinal mucosal barrier function have been described as the following: (i) FL, in which fluorescein spills into the lumens between two or more shedded or eroded enterocytes, (ii) cell junction enhancement, which, as an apical accumulation of fluorescein between two epithelial cells, morphologically represents an impairment of tight-junction proteins and can therefore be regarded as a precursor of final breakage of the basal tight junction (leading to FL), and (iii) CDO as defined as shedding of apoptotic cells into the luminal space, where they often can be found as cell detritus (**Figure 2**). Of note, all of the features are functional features and can only be observed with dynamic imaging with CLE. Hence, they do not have a histopathologic equivalent.

In a prospective pilot study in 58 IBD patients in clinical remission, Kiesslich et al. were able to show that increased cell shedding with FL can predict subsequent disease relapse within 12 months after the endomicroscopy (56). Specifically, the sensitivity, specificity and accuracy of an endomicroscopic grading system evaluating cell shedding and local barrier dysfunction (the so-called Watson score) to predict a flare were 62.5, 91.2, and 79%, respectively (56).

Similarly, as shown by Liu et al., the epithelial gap density is significantly higher in patients with CD compared to controls (57) and both UC and CD patients with elevated gap density have been shown to be at significantly higher risk for hospitalization or surgery (58). In a recent study, Lim et al. evaluated CLE images of the duodenum of 35 patients (15 CD, 10 UC, and 10 controls) for the number of epithelial gaps, cell shedding and the degree of FL into the lumen (59). In all patients, the duodenum was macroscopically normal and histopathology showed mild and unspecific duodenitis in 7 out of 15 CD patients while all UC patients had histologically normal duodenal mucosa. Importantly, both UC and CD patients exhibited an increased number of epithelial gaps, epithelial cell shedding, and luminal FL compared to controls, thereby suggesting disease activity otherwise not apparent on conventional endoscopy or histopathology (59). In their totality, these data convincingly illustrate that increased IP and local barrier dysfunction can be visualized by CLE and that the appearance of the later is directly associated with disease outcome.

Confocal laser endomicroscopy also has been proven to be able to precisely assess the degree of mucosal inflammation *in vivo* in real-time in IBD and to discriminate between active disease. As shown for UC, colonic crypts appear small and round with an irregular arrangement in remission upon CLE. In contrast, active disease leads to large, irregularly shaped crypts with a chaotic arrangement and an increased numbers of lamina propria capillaries (60).

When grading inflammatory activity as observed during CLE with a four-grade classification system that combines changes of the crypt and microvascular architecture with FL in patients with UC, Li et al. were able to show that these parameters correlated with histology (61). Interestingly, over 50 percent of patients with endoscopic remission had active disease upon histology. In contrast, remission based on CLE was not associated with active disease on histology. Thus, CLE seems to provide more

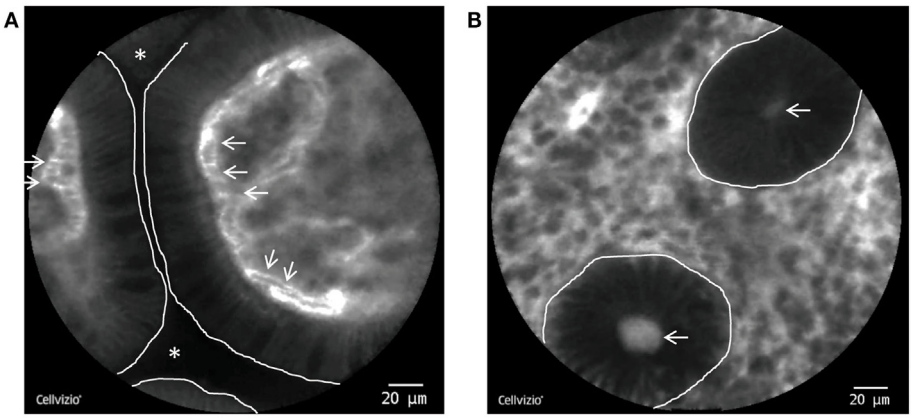

FIGURE 2 | pCLE of the terminal Ileum and the colon. **(A)** Single villi in the terminal ileum as visualized by pCLE. The enterocytes do not exhibit gaps or leakage and the intestinal lumen is not contrasted, consistent with an intact epithelial barrier. White line: border of the enterocytes to the intestinal lumen. White stars: intestinal lumen. White arrows: erythrocytes inside fluorescein containing capillaries. **(B)** Inflamed colonic mucosa from a patient with Crohn's disease (CD). The dark round structures represent single crypts (white line) with a fluorescein leakage into the lumen (white arrows).

reliable information on UC activity than white light endoscopy in UC (61). Similar observations can be made in CD: Results from our Erlangen group indicate that active CD is characterized by an increased tortuosity of colonic crypts, enlargment of crypt lumens, increased vascularity, microerosions, and higher lamina propria cell infiltrates on CLE, whereas CD in remission is associated with a higher number of crypts and goblet cells in comparison to controls (62). When these criteria were systematically evaluated using a scoring system [Crohn's Disease Endomicroscopic Activity Score (CDEAS)], endomicroscopic distinction of patients with quiescent and active disease was possible with a median CDEAS score of 2 in quiescent CD and 5 in active CD (62). In their totality, these data demonstrate that CLE allows to precisely assess the degree of mucosal inflammation in IBD patients.

Apart from that and very consistent with the known histomorphological differences between UC and CE, CLE can also be utilized for the *in vivo* differentiation between these two diseases. Specifically, CD is characterized by significant discontinuation of inflammatory signs such as cryptitis and crypt tortuosity on CLE to UC. UC, in contrast, has been shown to appear with a serious and prevalent crypt distortion, reduced number or density of crypts, and an irregular surface during CLE (44).

Another central field of interest is the detection of dysplasia in IBD and particularly in UC, several studies have investigated the value of CLE during surveillance colonoscopy. In a landmark trial published in 2007 by Kiesslich et al., 161 patients with longstanding UC in clinical remission were randomized to get either conventional white light colonoscopy or chromoendoscopy with endomicroscopy (63). For the detection of dysplasia as the primary outcome, random as well as targeted biopsies were obtained in the WLE group whereas in the endomicroscopy group, circumscribed mucosal lesions were first identified by chromoendoscopy and biopsy specimens were taken only in the presence of *in vivo* mucosal irregularities on CLE (63). Strikingly, by using chromoendoscopy with endomicroscopy, 4.75-fold more neoplasias could be detected than with conventional colonoscopy while at the same time 50% fewer biopsies were required (63).

Soon thereafter, a study on 36 patients with a recent diagnosis of polypoid or non-polypoid lesions showed an overall accuracy (97%) and excellent agreement with histology (kappa value = 0.91) when using CLE to distinguish colitis-associated polypoid lesions from sporadic adenoma. These data suggest that CLE might well be utilized for patient stratification into those suitable for endoluminal resection versus those that would require immediate referral for proctocolectomy (64).

Importantly, the aforementioned studies were performed with eCLE. In a pilot study on 22 UC patients, 48 lesions were compared to 87 random locations with by high-definition WLE, NBI, and pCLE. As demonstrated in a report on 22 UC patient with 48 visible lesions, pCLE is feasable with reasonable diagnostic accuracy for dysplasia surveillance in UC (65).

Although not analyzed systematically, the typical appearance of colitis-associated polypoid lesions has been described as dark cells with mucin depletion, goblet cell and a reduced crypt density, a denticulated irregular epithelial layer, distortion and dilatation of the microvasculature, and increased vascular permeability (45, 66).

At the same time, a recent prospective, cohort study including 61 patients with CD from five centers showed only an incremental increase in the diagnostic accuracy when performing eCLE after chromoendoscopy compared to chromoendoscopy alone while the dysplasia rate was generally low in this study (67).

Overall, these aforementioned studies reliably indicate that CLE allows to assess the microscopic degree of inflammation in patients with IBD and thereby enables real-time *in vivo* histology. Importantly, the microscopic evaluation of mucosal inflammation is a central aspect for the assessment of mucosal healing, which serves as an important prognostic and therapeutic parameter in IBD patients (3). Hence, in order to facilitate and optimize both the medical therapy as well as the dysplasia and cancer surveillance of IBD patients, develop into a widely used diagnostic modality in the near future. Further, cumulating

evidence suggesting that the evaluation of the intestinal barrier by CLE can to be used prospectively identify patients that are under risk of experiencing a disease flare and therefore enables a risk-tailored patient care.

MOLECULAR IMAGING OF GUT INFLAMMATION AND PREDICTION OF THERAPEUTIC RESPONSE

As discussed above, the field of gastrointestinal endoscopy has experienced a rapid technological development in recent years, leading to advanced imaging methods that enhance the visibility of mucosal structures and mucosal inflammation. Nevertheless, there is still the unmet clinical need for better visualization of specific mucosal lesions. This necessity is especially evident in the detection of precancerous lesions in cancer surveillance. The sensitivity of the aforementioned endoscopic methods is limited by their reliance to solely detect structural alterations, which can often be minuscule, making them impossible for the detection on the anatomical level. The identification of mucosal lesions could be markedly improved by the visualization and characterization of biological processes that occur at the cellular level, which would add a major new dimension to our current diagnostic possibilities. Imaging of certain biological properties could enable the detection of otherwise not identifiable lesions (68–72).

Endoscopic molecular imaging is based on *in vivo* visualization of disease-specific perturbations at the molecular level. This approach aims to not only broaden our diagnostic capabilities but also provides novel insights into the pathogenesis of various diseases of the digestive tract.

Requirements for Endoscopic Molecular Imaging

The prerequisite for the successful application of molecular imaging procedures is the identification of molecular targets that represent the answer to the posed clinical question. These targets are often the result of basic science research activities that lead to the successful identification of specific cellular proteins critically involved in the immunopathogenesis of diseases. The epitopes that have so far been targeted in molecular imaging studies include Cathepsin B, epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2 (HER2), Claudin-1, and tyrosine-protein kinase Met (c-Met) for the enhanced detection of colonic adenoma, and EGFR and vascular endothelial growth factor (VEGF) for CRC. In the stomach, MG7 was identified as a marker for gastric cancer and Periostin for esophageal squamous cell cancer. Furthermore, HER2, certain glycans and cyclophilin A (CypA) were used for better detection of Barrett's neoplasia in the esophagus (70). These research findings of molecular targets that are specific for diseases build the basis for the translational transfer into preclinical and clinical implementation. Another important requirement are molecular probes that elicit specific interactions with the chosen target structure. The ideal molecular probe would possess high target affinity, rapid binding kinetics, deep tissue penetration, low immunogenicity, safe toxicity profile, *in vivo* stability, low cost, and rapid clearance form non-targeted tissue, which would guarantee maximal specificity for the signal (73). Different probes have so far been used in preclinical or even clinical applications. The most common ones are lectins, peptides, antibodies or affibodies. These dyes are then often labeled by bright fluorescent dyes as optical reporters (68, 74). The most common dyes used in the field of molecular imaging are high-affinity fluorophores like Cyanine 5.5, fluorescein isothiocyanate (FITC), or Alexa Fluor 488 that provide a distinct fluorescence emission spectrum from 422 to 900 nm, which can be detected by dedicated fluorescence endoscopes in real-time. Activatable enzymes represent another highly attractive probe class that has so far only been used in preclinical mouse models. They are optically dormant in the absence of disease and generate a bright fluorescence signal in the presence of proteolytic enzymes that are only overexpressed in neoplastic lesions (75, 76). The probes can be applied systemically, which allows distribution throughout the entire body and deep tissue penetration at the cost of a heightened probability of toxic reactions and the requirement of a lead-time prior to examination. Another alternative is topical administration of the probe *via* a spray catheter into the digestive tissue, which allows application in higher doses to achieve an improved image contrast, while markedly reducing the risk of systemic toxicity. The limitation of this administration route is its restriction to the detection of focal disease only (77).

The most suitable endoscopic system for molecular imaging incorporates a wide-field endoscope that allows to precisely distinguish changes of mucosal architecture and to detect fluorescent molecular probes for further on-site characterization. Several devices have been developed and used in various molecular imaging studies. These include custom fiber optical endoscopes with narrowband filters or blue light sources for excitation (78, 79). Also, CLE has recently become one of the most widely used endoscopic devices for microscopic molecular imaging studies (45). It is currently available as a flexible fiber-optic bundle device that can pass through the instrument channel of the endoscope. It provides real-time images with cellular and even sub cellular resolution *in vivo*. The technique has been described in detail in this manuscript before and uses laser light with a wavelength of 488 nm, which matches the peak absorption of FITC, or 660 nm for excitation. The focus of the laser light is directed to a thin imaging plane inside the tissue. The intensity of the light reflected off a given point, which would be the fluorescent probe in the setting of molecular imaging, is then measured in order to compute a virtual image from these data.

Preclinical Intestinal Endoscopic Molecular Imaging Studies

The visualization of molecular targets in the colon has been the subject of numerous preclinical studies addressing a variety of clinically relevant problems.

In vivo preclinical studies regarding the colon have primarily focused on the detection of neoplastic lesions. Impressive results could be provided by Mitsunaga et al., who topically administered an enzymatically activatable fluorescent probe to detect gamma-glutamyl transpeptidase, which is selectively expressed in colonic neoplasia. Using a modified wide-field fluorescence

endoscope, it could be shown that the probe detected most high-grade dysplasias and cancer in mice treated with axoxymethane and dextran (80). This approach of visualizing tumor-specific enzyme activity was also applied in xenograft-bearing mice, in which lysine–lysine cleaving proteases were detected in neoplastic tissue with a high tumor-to-background ratio (81). Another study topically applied a near infrared octapeptide specific for colonic dysplasia in an adenoma mouse model and here again successful identification of the neoplastic lesions was achieved (82).

Other studies used fluorescent labeled antibodies for preclinical detection of malignant tumors. First, a fluorescent antibody targeting EGFR antibody was tested against human xenograft tumors in mice. CLE was able to accurately identify EGFR expression in this experimental setting. Possible application in patients was suggested by topical application of this probe to human colonic specimen *ex vivo*, where differentiation between malignant and non-neoplastic tissue was also proven (83). The same group also applied an Alexa Fluor 488-labeled anti-VEGF antibody in murine tumor xenograft models and surgical human CRC specimen. A handheld confocal instrument allowed successful identification of neoplastic tissue (84). In a subsequent approach, the fluorescent-labeled therapeutic anti-EGFR antibody cetuximab was tested. Prediction of response to monoclonal anti-EGFR antibody treatment with cetuximab was shown in a xenograft model of human CRC cells with high or low expression of EGFR injected into nude mice. The CLE-assessed fluorescence intensity after injection of a labeled cetuximab test dose was able to predict response to subsequent targeted therapy with this monoclonal anti-EGFR antibody (85).

Clinical Intestinal Endoscopic Molecular Imaging Studies

Endoscopic molecular imaging has already made the transfer into clinical studies and evidence for the feasibility of this approach is continuously growing. A first proof-of-principle study for the detection of intestinal tumors was done with an optical probe built from a monoclonal antibody against carcinoembyonic antigen conjugated with fluorescein. The fluorescent antibody was applied topically during colonoscopy in patients with colorectal polyps or tumors. Patients were examined with a wide-field endoscope with an increased optical range through the use of narrow-band filters. The group was able to identify 19 of 25 tumors and importantly no adverse events or immunological side effects were observed (78). Another pivotal landmark trial using CLE to visualize neoplastic cells was done by topical application of a short peptide sequence isolated from a phage peptide library generated from human adenomas, which was conjugated with fluorescein. The topically applied fluorescein-conjugated peptide showed increased binding to neoplastic cells with a sensitivity and specificity over 80% (86).

A recently published study by Burggraaf et al. elegantly examined the ability of an intravenously injected Cy5-labeled GE-37 peptide to detect dysplastic lesions in 15 patients with high-risk for CRC. The peptide was able to specifically bind to c-Met, which is overexpressed in dysplastic crypts. The examination was done with a modified fiber-optic colonoscope that provided fluorescence images 3 h after injection of the peptide. There was an increased uptake of the probe by colonic polyps. Final analysis demonstrated that all 47 tubular adenomas found, showed increased fluorescence intensity, as did 33/42 hyperplastic lesions and 8/41 of the normal mucosa taken in the study. A total of nine additional adenomas were found by this diagnostic method, which were not found by fiber-optic white light examination. Importantly, there was no systemic side effect visible (87).

Another recently published study was able to impressively detect sessile serrated adenomas (SSAs). SSAs have flat, subtle features and are therefore difficult to detect with conventional colonoscopy. Using phage display, the group of Joshi et al. identified a peptide that preferentially binds to SSAs. Performing *in vivo* fluorescence endoscopy in patients, the authors reported that SSAs had a 2.43-fold increased mean fluorescence intensity compared to healthy colonic mucosa. Fluorescence labeling distinguished SSAs from normal colonic mucosa with 89% sensitivity and 92% specificity. The peptide had no observed toxic effects in the study (88). The same group also demonstrated the ability of a multimodal video colonoscope to collect *in vivo* real-time wide-field images of nonpolypoid colonic adenomas using fluorescently labeled peptides (89).

Apart from the early detection of CRC, molecular imaging procedures were recently used for the prediction of therapeutic efficacy of biological therapies in IBD patients. Reliable prediction of therapeutic response is essential in clinical practice in order to avoid exposure of non-responders to an inefficient biological therapy and the associated potential side effects of this treatment. This would moreover enable the treating physician to directly introduce the patient to the best suited biological therapeutic option, which would enable an improved and time-efficient control of disease for the patient. Recently, a Good Manufacturing Practice (GMP)-conform version of an anti-TNF antibody was topically applied in an investigator initiated trial with 25 CD patients to predict response to subsequent anti-TNF therapy (90). As anti-TNF antibodies appear to induce their anti-inflammatory effect primarily by binding to membrane bound TNF (mTNF) on mucosal target cells (91), the identification of such mTNF-expressing cells in the mucosa was used to identify patients responding to subsequent anti-TNF therapy. The number of mTNF positive cells in the inflamed mucosa was quantified *in vivo* using CLE. In this study, patients with increased numbers of mTNF positive mucosal cells had a superior clinical response at 12 weeks (11/12 patients) compared to patients with lower numbers of mTNF mucosal cells (2/13 patients). Clinical efficacy was sustained in the observed follow-up period of 12 months and was associated with the induction of mucosal healing (90). In the field of antiadhesion molecule therapies, a similar approach was tested *ex vivo* in CD patients. Here, CLE was used in conjunction with a topically applied fluorescein-labeled antiadhesion molecule antibody to visualize mucosal integrin expression *ex vivo* in the mucosal tissue of CD patients to predict response to subsequent anti-adhesion molecule therapy. This approach was again based on the assumption of an association between the expression

levels and the response to biological therapy directed against a target molecule. In the study, mucosal biopsies of five CD patients with anti-TNF refractory disease were taken for *ex vivo* molecular imaging with the fluorescent anti-adhesion molecule antibody vedolizumab, to visualize the mucosal expression of its target molecule, the α4β7 integrin. CD patients who responded with sustained clinical and endoscopic remission to vedolizumab therapy showed markedly higher expression of the α4β7 integrin than non-responders (92). These results might open new avenues for personalized medicine in the treatment of CD patients and might serve as a possible model approach for other inflammatory disorders that are treated with biologics.

Although endoscopic molecular imaging procedures are currently at an early stage of development in clinical procedures, the feasibility of this method has been impressively proven by various clinical studies. Possible applications include enhanced detection of neoplastic mucosal lesions, identification of dysplasia in inflamed mucosa, and prediction of therapeutic responses to molecular targeted treatment. The major challenge for further application of these studies is regulatory approval, as fluorescent probes are regarded as new investigational drugs by the authorities and therefore require extensive preclinical efficacy and safety data. Furthermore, facilities that provide GMP-compliant environments are need for the synthesis of the fluorescent probes. Nevertheless, the available exciting data of the first molecular imaging studies clearly emphasize the potential of this method that might have an impressive impact on improved future diagnostic and therapeutic algorithms.

NON-INVASIVE IMAGING OF INTESTINAL INFLAMMATION

Due to the invasiveness of endoscopy and the associated risk for complications, there is an enduring demand for non-invasive modalities to assess mucosal inflammation. Accordingly, standard imaging techniques including CT, MRI, scintigraphy, and US have not only been used for the detection of stenosis and penetrating lesions such as fistula and abscesses but also for the evaluation of disease activity in IBD patients (93). CT is usually performed as CT enterography with oral and i.v. contrast for the detection of bowel wall pathology and abnormal contrast enhancement (94, 95). Parameters used to assess disease activity include wall thickening, enhancement of the mucosa or intestinal wall, mural stratification, comb sign, and enlargement of regional lymph nodes (95). Similarly, MRI is performed following administration of oral contrast as MR enterography (MRE) and wall thickness, increased contrast uptake, edema, and ulcerations are assessed (96). A quantitative index, the Magnetic Resonance Index of Activity, has been developed that incorporates MRI-based features of disease activity based on logistic regression and shows good correlation with endoscopic disease activity. Scintigraphy is mostly performed with 99mTc-HMPAO or 111In-oxine-labeled white blood cells, which accumulate at sites of active disease (97). Regarding transabdominal US, thickening of the intestinal wall, color doppler-based assessment of vascularization, reduced bowel stratification and peristalsis, or compressibility are used as parameters for the evaluation of disease activity (95). As all of these imaging techniques offer a limited spatial resolution, evaluation of inflammation limited to the mucosa is barely feasible.

In fact, most data from clinical trials are available for the evaluation of CD activity, which can be detected more easily due to transmural inflammation. Regarding the evaluation of upper gastrointestinal tract and small bowel disease activity in CD patients, all techniques have been shown to provide comparable results for the evaluation of terminal ileitis. In comparison to CT, MRI, and scintigraphy, coverage of the entire length of the small bowel is limited with US (97). For the evaluation of Crohn's colitis, MRI and CT provide high accuracy, although data for CT are limited. The diagnostic accuracy of US in Crohn's colitis depends on the experience of the investigator and the affected location. It has been reported to be comparable to MRI and CT for the evaluation of the sigmoid/descending colon, whereas accuracy is lowest in the rectum (98).

Overall, data on the evaluation of disease activity in UC with non-invasive imaging techniques are limited. Despite the low spatial resolution, current data are promising for MRE. For instance, Oussalah et al. evaluated disease activity in 96 patients with IBD (UC = 35, CD = 61) using MRI with diffusion-weighted imaging (DWI-MRI) (99). In this study, diagnostic accuracy of DWI-MRI to detect endoscopic inflammation was even superior in patients with UC (sensitivity = 89.47%; specificity = 86.67%; AUROC = 0.920) in comparison to CD (sensitivity = 58.33%; specificity: 84.48%; AUROC = 0.779). Further studies also show valuable results for US, whereas data on CT only show moderate correlation with disease severity (97). Therefore, further studies are urgently required to fully estimate the value of non-invasive imaging in UC.

In addition to traditional tomographic imaging techniques, also new technological developments have recently entered clinical research. A promising technique for the evaluation of disease activity in IBD patients is multispectral optoacoustic tomography (MSOT) (100). MSOT allows a precise localization of specific molecules in tissues up to several centimeters of penetration depth through the photoacoustic effect. The photoacoustic effect describes the observation that light absorbed by molecules is inducing thermoplastic expansion, which can be detected as US waves with very high spatial resolution. By subsequently exciting a tissue with several wavelengths, spectral unmixing techniques can be used to calculate the relative contribution of specific molecules to the overall signal with MSOT. In this way and based on their characteristic absorption, oxygenated, and deoxygenated hemoglobin have been shown to be easily detectable by MSOT. In a recent study, Knieling et al. evaluated the use of non-invasive transabdominal MSOT for the evaluation of CD activity (101). Performing MSOT in 108 patients with active CD and remission, the authors could show that MSOT-based measurements of total hemoglobin in the intestinal wall show excellent correlation with the endoscopic degree of inflammation assessed with the SES-CD. In comparison to US, MSOT was superior regarding differentiation of remission and low-grade disease activity. Although these data are encouraging, further studies are needed to evaluate the full potential of this new technique in IBD patients.

FUTURE DIRECTIONS FOR THE ENDOSCOPIC EVALUATION OF MUCOSAL INFLAMMATION

In recent years, a number of new optical technologies have been evaluated with regard to their diagnostic value in endoscopic assessments of organs and tissues. First, there are various imaging techniques to resolve the morphological structure of tissues and also to gain functional information about cellular processes in some cases. Second, there are a number of spectroscopy techniques that resolve the spectral composition of detected light signals with sensitivity to the molecular composition of the sample. Each of the technologies is based on specific interactions of light with matter: elastic and inelastic scattering, absorption, and fluorescence.

Imaging techniques include optical coherence tomography (OCT), multiphoton microscopy (MPM), coherent anti-stokes Raman scattering (CARS) microscopy, and fluorescence lifetime imaging; the main spectroscopy techniques are Raman spectroscopy and Fourier transform infrared spectroscopy.

Optical coherence tomography can be used to image structural features of tissue. Contrast is based on light absorption and reflection in the tissue at interfaces with refractive index changes. The detection principle is interference of light from a reference path with light reflected from the tissue. Changing the length of the reference path by a moveable mirror tunes the focal position in the sample and encodes the axial position of the signal in the so-called time domain OCT technique. Additional scanning of the light beam in the lateral plane allows for three-dimensional tissue imaging. Alternatively, interference patterns can be detected on a spectrometer and be converted to spatial positions by Fourier transform in spectral domain OCT, which has become the current standard for most commercial systems. Typically, resolutions in the range of 10 µm and imaging depths in the range of 2 mm can be achieved.

First results on the evaluation of gastrointestinal diseases with OCT were provided already 20 years ago (102). In 2004, Shen et al. exposed tissue samples from 48 patients with IBD to OCT *ex vivo* (103). According to this study, OCT enabled the identification of transmural inflammation and thereby allowed the differentiation between CD and UC with excellent correlation to histopathology. This study was subsequently supported by *in vivo* endoscopic OCT data in 40 patients with CD and 30 patients with UC, again proposing that OCT can aid the discrimination of CD and UC based on the detection of transmural inflammation (104). Although these data are encouraging, subsequent confirmation in follow-up studies is missing so far. As there is a continuous research in further improving endoscopic OCT and more advanced devices are under development (105), more data on the use of OCT for the evaluation of mucosal inflammation can be expected.

In MPM, a fluorescent molecule is excited by interacting with two or more photons at the same time. Excitation wavelengths are typically in the near infrared range for fluorescence emission in the visible spectrum. Infrared light is less scattered in the tissue than light of shorter wavelengths, thus larger penetration depth can be achieved for imaging. Multiphoton effects are only observed in the small region of the objective focus, where the energy density is highest. Fluorescence signals can therefore be collected close to the back aperture of the objective without the need for a confocal pinhole that blocks light from out-of-focus planes. This way of detection usually achieves a better signal-to-noise ratio and improved image contrast in tissue imaging, especially in deeper layers of the tissue. Penetration depths are typically limited to 150 or 200 µm in dense tissues like colon mucosa, but may also reach to more than 500 µm in brain imaging. MPM is well suited for label-free imaging of tissue based on autofluorescence [two-photon excited autofluorescence (TPEF)] of endogenous molecules like NADH or FAD, which are present in all cells in different quantities. In addition, the extracellular matrix can be visualized through second harmonic generation (SHG) from collagen-I, which is another specific two-photon effect that can be observed only in a few very regular and non-centrosymmetric filament structures. By combining the detection of TPEF and SHG signals, MPM provides a detailed information of biological tissues without the requirement of labeling.

Regarding intestinal tissue, label-free multiphoton imaging can provide a subcellular resolution of the mucosal surface enabling the identification of epithelial cell nuclei, goblet cells, interstitial collagen, etc. (106). Safdarian et al. could show that label-free MPM can be used to detect and quantify eosinophil infiltration in eosinophilic esophagitis *ex vivo* (107). In addition, we have previously shown that label-free MPM can also be used to display mucosal inflammation in tissue samples from IBD patients *ex vivo* (**Figure 3**) (108). Recent technological progress enabled continuous miniaturization of MPM devices leading to

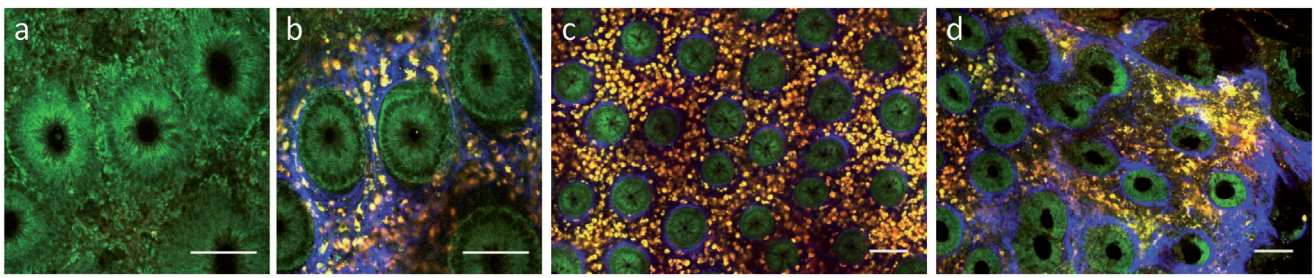

FIGURE 3 | Label-free multiphoton microscopy of Crohn's disease in human colon biopsies. **(a)** Epithelial layer at 10 µm depth. **(b)** Upper lamina propria at 40 µm depth. **(c)** Weak inflammation. **(d)** Strong active inflammation. Scale bars: 100 µm.

the development of first MPM endoscopy systems, which have been demonstrated in preclinical studies (109, 110). Once clinical MPM devices are available, it will be interesting to see, if this technology can provide further benefit for clinical diagnostics of mucosal inflammation.

Raman spectroscopy is a technique that allows point measurements in biological tissues providing a detailed information about the molecular composition through the detection of inelastic scattering. Inelastic scattering is based on the Raman effect, which can occur due to changes of vibrational, rotational, or electronic energy of a molecule following excitation (111). As individual molecules have characteristic Raman signals, spectroscopic evaluation of these signals provides information about individual molecular components in a tissue sample similar to a molecular "fingerprint." This information has already been used to the diagnosis of IBD *ex vivo* (112, 113). In a recent study, Addis et al. used Raman spectroscopy to assess disease activity vs. mucosal healing in tissue biopsies of patients with UC *ex vivo* (114). As Raman spectroscopy can be performed with a fiber optic probe, it can easily be integrated into an endoscopy setup. As a proof of principle, Pence et al. used colonoscopy-coupled Raman system in a pilot study in IBD patients *in vivo* (115). The data from this study are encouraging for a future use of Raman spectroscopy in the diagnosis and monitoring of IBD.

In addition to single point measurements, the Raman effect can also be used for tissue imaging through CARS or Stimulated Raman Scattering (SRS) microscopy (116). These technologies probe vibrational molecular transitions, for example C-H bonds. The coherent excitation of these vibrational transitions is achieved by combined excitation with two lasers (called pump and Stokes laser) at two different wavelengths. The energy difference of these two wavelengths is chosen to exactly match the energy of the vibrational bond to be probed in the tissue. The nonlinear interaction of photons from the laser source with molecular oscillators in the sample leads to generation of shorter wavelength photons detected in CARS microscopy and a stimulated Raman loss or gain in scattered light intensities at the original laser wavelengths, which can be probed by lock-in amplifiers in SRS microscopy. In comparison to standard Raman spectroscopy, CARS uses only one Raman frequency for excitation at a time, but with a much higher yield in detected photons that allows for tissue imaging with a laser-scanning microscope. In a recent study, Chernavskaia et al. used label-free non-linear multimodal combining TPEF, SHG, and CARS imaging to evaluate disease activity in tissue samples from IBD patients *ex vivo* (117). Comparing results from these non-linear imaging approaches with histopathological results, the authors could identify a feature set for automatic prediction of disease activity with high diagnostic accuracy. Although these data are preliminary, they propose that non-linear label-free multimodal imaging approaches might be valuable tools for the assessment of mucosal inflammation.

CONCLUSION

Endoscopic evaluation of mucosal inflammation has made a significant progress during recent years. New wide-field approaches such as high-definition endoscopy, dye-based chromoendoscopy, or magnifying endoscopy have not only improved the diagnosis and monitoring of disease activity but also cancer surveillance in IBD patients. For the first time, CLE enabled *in vivo* histology of mucosal surfaces providing real-time information about the microscopic state of disease. Despite these improvements, there are still unmet needs for clinical management of IBD patients. First data for molecular imaging approaches or new optical technologies are promising to cover these needs. However, as these techniques are only on the way for clinical translation, future studies will need to show their benefit for the evaluation of mucosal inflammation.

AUTHOR CONTRIBUTIONS

MW, TR, SS, CB, and RA together prepared the concept, wrote, and reviewed the manuscript.

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REVIEW
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Intestinal Barrier Interactions with Specialized CD8 T Cells

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The trillions of microorganisms that reside in the gastrointestinal tract, essential for nutrient absorption, are kept under control by a single cell barrier and large amounts of immune cells. Intestinal epithelial cells (IECs) are critical in establishing an environment supporting microbial colonization and immunological tolerance. A large population of $CD8^+$ T cells is in direct and constant contact with the IECs and the intraepithelial lymphocytes (IELs). Due to their location, at the interphase of the intestinal lumen and external environment and the host tissues, they seem ideally positioned to balance immune tolerance and protection to preserve the fragile intestinal barrier from invasion as well as immunopathology. IELs are a heterogeneous population, with a large innate-like contribution of unknown specificity, intercalated with antigen-specific tissue-resident memory T cells. In this review, we provide a comprehensive overview of IEL physiology and how they interact with the IECs and contribute to immune surveillance to preserve intestinal homeostasis and host-microbial relationships.

Keywords: mucosal immunology, intraepithelial lymphocytes, inflammatory bowel disease, $CD8^+$ T-lymphocytes, epithelial cells

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INTRODUCTION

The intestinal epithelia are a single cell layer of large surface. Together with a mucus layer, the epithelia form a dynamic physical barrier between the host and its environment. Estimates are up to 100 trillion microorganisms, including pathogens, have made the gastrointestinal tract their home (1), which makes the intestine the largest potential port for microbial invasion. However, a proportion of the microorganisms in the intestine can contribute to the hosts' health and immunity. These commensal bacteria compete for resources with pathogenic microorganisms and provide metabolic capacity to digest food products by generating important compounds (e.g., vitamin K) or by assisting other microorganisms with supportive roles. The delicate nature of the single cell epithelial barrier, the essential function of the gastrointestinal tract to absorb nutrients and liquids, and the balance to maintain beneficial microbes, while offering protection against invasion and avoiding tissue damage, requires an effective and robust, yet tolerant, immune system.

The intestinal immune surveillance network is an integrated part of the organ, which enables it to swiftly pick up cues regarding its health status and contributes to tissue homeostasis as well as repair. Immune surveillance links rapid activation of innate immune cells to the more delayed recruitment of adaptive immune cells (2), ultimately resulting in immunological memory. Part of the innate system is the intestinal epithelial cells (IECs) themselves as well as classical innate immune cells. Mostly, macrophages, monocytes, and dendritic cells (DCs) migrate to the intestine from the bone marrow *via* blood (3). Following infection, interactions between antigen presenting cells and lymphocytes

can take place in specialized structures, unique to the intestine, such as isolated lymphoid follicles and Peyer's patches (4).

T-lymphocytes recognize foreign particles (antigens) by their surface expressed T cell receptor (TCR). With each T cell expressing a nearly unique TCR, collectively T cells can recognize nearly all foreign antigens. From the two major types of T cells found in blood and secondary lymphoid organs (SLO), CD4 expressing helper T (T_H) cells are generated in the thymus as precursors without a defined function. They recognize antigens presented in major histocompatibility complexes class II (MHCII) after processing by antigen presenting cells. T_H cells have an important orchestrating role, differentiating into effector cells with distinct supportive functions in type 1 (T_H1), type 2 (T_H2), and type 3 (T_H17) immunity and high levels of flexibility (5, 6). Specialized regulatory T cells can curtail responses and form part of a carefully balanced immune system (7). CD8 expressing cytotoxic T cells similarly derive from the thymus as naive cells. They mainly recognize antigens resulting from the target cells' transcriptional machinery and degradation of cytosolic proteins by the proteasome presented in MHCI, such as those resulting from viral infections as well as intracellular bacterial infections. Upon encountering their cognate antigen, $CD8^+$ T cells differentiate into effector cells, classically thought to be part of type 1 immunity due to their high potential for interferon (IFN)γ production.

The maintenance of effector T cells is metabolically costly. Rapidly dividing cells require large amounts of energy for the production of cellular building blocks and secretion of effector molecules. These cells can potentially contribute to chronic inflammation and immunopathology. To avoid such possible danger and energy expense, the majority of effector cells undergo apoptosis after pathogen clearance, re-establishing homeostasis. Yet, some persist as memory cells, providing protection against re-infection. Memory CD8 T cells are a heterogeneous population, varying in phenotype, function, and localization (8) (**Figure 1**). This facilitates a swift and tailored response to a broad array of potential insults. In addition, the intestinal immune system has another important population of specialized $CD8^+$ T-lymphocytes known as intraepithelial lymphocytes (IELs) (9). Intriguingly, IELs have characteristics of naive, effector, and memory cells require bidirectional cross-talk with IECs (10) (**Figure 1**), with one murine IEL estimated to be present for every 4–10 IECs (11, 12).

Aberrant immunity has severe consequences, especially in the intestine where a single epithelial cell layer forms the barrier between the host and a very high amount of microorganisms. Immunity against commensal bacteria can result in chronic inflammation, such as observed in inflammatory bowel diseases (IBDs). In this review, we focus on CD8 expressing T cells, particularly IELs, which, located in the very top layer of the intestinal barrier, are ideally positioned to monitor the intestinal microbiota. They may contribute to modulating immunity toward microbes as well as immunopathology, and are involved in tissue homeostasis and epithelial repair. We will discuss some of the properties of IELs and speculate on their role in the intestinal immune surveillance network.

Conventional CD8 T Cells

The initiation of an adaptive immune response requires several myeloid and lymphoid cell types. These cells need to be brought together and act in a strictly orchestrated manner in time and space to license immune cell activation (13). Critical interactions are those between antigen presenting cells, especially DCs and T cells (14). In order to become fully activated, naive T cells require signaling through TCR (signal 1) as well as costimulatory receptors (signal 2), such as CD28 and CD40. Additional cues (signal 3) provide inflammatory context and involve cytokines and chemokines (15, 16).

During the initiation phase, naive $CD8^+$ T cells rapidly proliferate and differentiate into cytotoxic T-lymphocyte effector cells thereby gaining the ability to kill target cells by releasing perforin and granzymes, and secrete large amounts of cytokines, such as tumor necrosis factor (TNF) and IFNs (17) (**Figure 1**). The rapid proliferation during the expansion phase ensures that a limited number of precursor cells can counter infectious agents. Effector cells migrate to most tissues in the body to ensure the removal of all infected cells and pathogens (18). However, such a response cannot be sustained and proximally 95% of effector cells die in a contraction phase upon pathogen clearance (19).

A limited number of cells develop into memory cells, returning to a state of quiescence with slow cell turn over and effector molecule transcription. Despite this they are able to rapidly reactivate, proliferate, and express effector molecules upon reencounter with a similar pathogen (18, 20–23). How memory T cells develop remains incompletely understood. There are different signals influencing T cells upon and after encountering their cognate antigen that influence the size and quality of the T cell memory pool (8).

Three subtypes of memory T cells are recognized, they are; effector memory (T_{EM}) T cells, central memory (T_{CM}) T cells (18, 24, 25), and tissue-resident memory (T_{RM}) cells expressing CD69 and CD103 (26–29) (**Figure 1**). Differences in cell localization, recall ability, and effector functions provide intersecting levels of protection against re-infection (30). Memory cells found circulating through blood, lymph, and SLO are referred to as T_{CM} cells and express CD62L and CCR7, which enable entry in lymphoid organs and circulation (31–34). Those cells primarily found in non-lymphoid tissues are T_{EM} cells (18, 22).

Although migration of T cells is a pillar of successful immune defense, experiments using defined tissue grafts from ganglia, skin, and intestine as well as the use of parabiosis have defined a residential population of memory T cells (27, 35–37). At epithelial barrier sites such as the skin, lungs, reproductive organs, and gastrointestinal tract, a unique memory population is found; T_{RM} cells. These cells share characteristics with T_{EM} cells, expressing CD44 and low levels of CD62L (**Figure 1**). They are found at the initial site of infection, providing very regional immune surveillance and protection against re-infection (35, 38, 39), and do not recirculate (40). The discovery of T_{RM} cells and subsequent detailed analysis have resulted in a paradigm shift that most memory T cells are an integral part of non-lymphoid tissues (41). But, these cells did not settle on empty ground to fill a previously non-existent niche. T_{RM} cells compete, successfully, with innate or

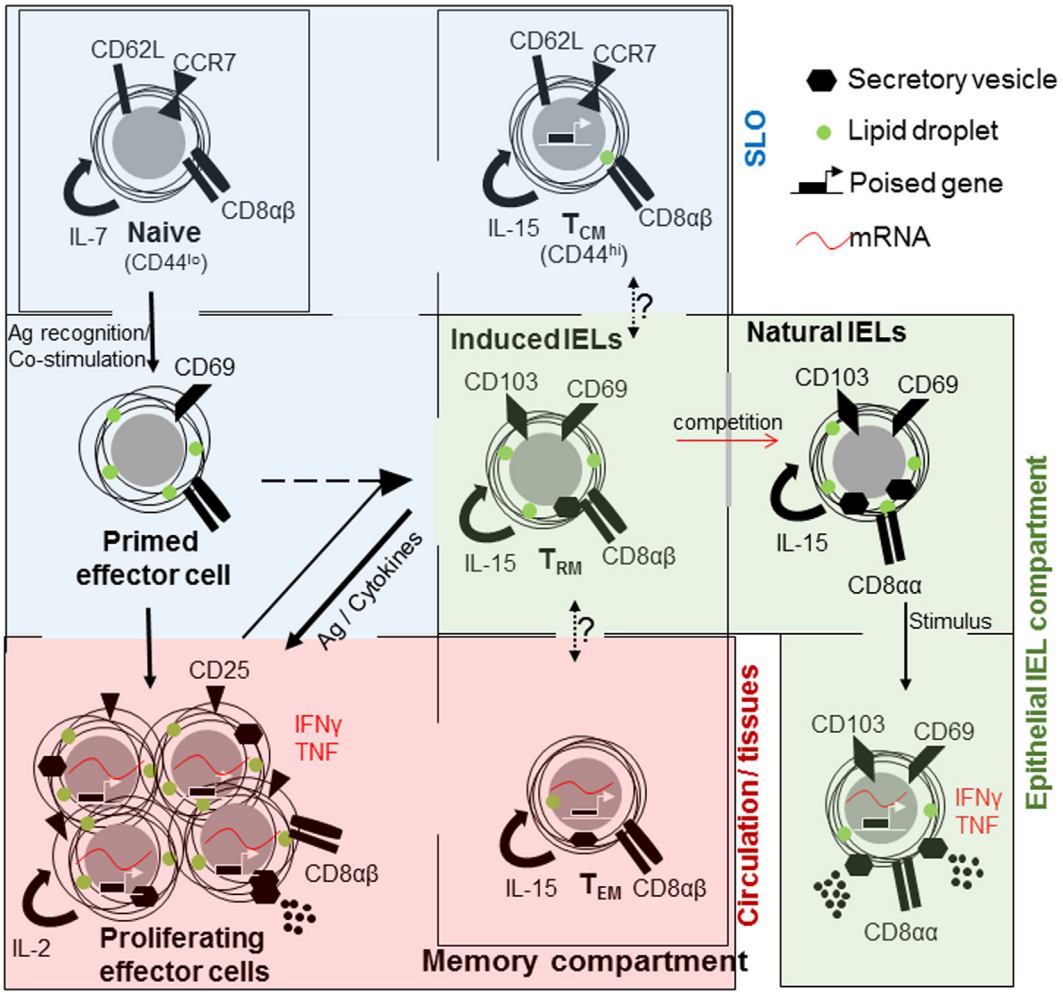

FIGURE 1 | The relationships between CD8+ T cell populations in the small intestine. Naive CD8+ T cells (top left) are maintained in a quiescent state within their own compartment under homeostatic control. They mainly circulate through the secondary lymphoid organs (SLO). Upon encountering antigen, T cells are primed, acquire cellular building blocks such as lipids, and express CD69. Thereafter, they undergo rapid proliferation and express CD25 [high affinity interleukin (IL)-2 receptor], cytokines such as tumor necrosis factor (TNF) and interferon (IFN)γ and can release cytolytic factors, as effector T cells. Large proportions or effector T cells will die by apoptosis. Memory cells are derived from primed or effector T cells of which three subsets are distinguished; central memory T cell (T_{CM}) that is present in the SLO, effector memory T cells (T_{EM}) that are circulating and rapidly acquire effector functions and tissue-resident cells (T_{RM}) in tissues, especially barrier sites, such as the skin and intestine. All memory cells rely on IL-15 for their maintenance. At barrier sites T_{RM} cells compete with natural intraepithelial lymphocytes (IELs), both maintained in a semi-activated state expressing CD69 and CD103 and metabolically charged.

innate-like lymphocytes, which are already present at the original site of infection (39).

Intraepithelial Lymphocytes

A variety of innate or innate-like lymphoid cell types reside in tissues, including natural killer (NK) cells, innate lymphoid cells, and T cells expressing the γδ TCR chains (γδ T cells), homodimers of CD8α or a semi-invariant TCRαβ such as NKT cells, and mucosal associated invariant T cells. The top layer of the epithelia, in murine and human intestine as well as murine skin, contains large populations of such innate-like T cells within the IEL population.

Intestinal IELs express the prototypical tissue-resident integrin CD103 (integrin αE), with which they interact with IECs (10), as well as C-Type lectin and early activation marker CD69, and the NK cell inhibitory receptor 2B4 (CD244) (42). Antibody staining for CD8α, CD69, and CD103 in lymphocytes sourced from the intestinal intraepithelial fraction provides a homogenous cell population (42). However, IELs can be divided into subsets based on their activation mechanism and on the antigens, which they may recognize. Induced or adaptive IELs are derived from conventional CD8αβ T cells, which recognize non-self antigens in the context of MHCI. They home to the intestinal barrier upon encountering their cognate antigen in the intestine as T_{RM} cells (9, 43). Induced IELs accumulate with age (44), replacing natural IELs (39).

Natural or innate-like IELs also originate in the thymus where they acquire homing factors and identity upon selection on self-antigens and seed the intestine as a precursor population

(45–51). They express CD8αα homodimers, in contrast to conventional CD8αβ T cells (52) (**Figure 1**). They express either the conventional αβ TCR or the non-conventional γδ TCR. In the small intestine around 60% of all IELs express TCRγδ, in marked contrast to SLO in which γδ T cell represent less than 1% (53). In humans, natural IELs predominantly express TCRVδ1 with a contribution from TCRVδ3, the majority of which express CD8 (54, 55). In contrast to murine cells, human γδ T cells may process and present antigen (56).

Contrary to T cells found in SLO and in line with T_{RM} cells, IELs do not circulate through blood and lymph and are tissue-resident (57). IELs seem to respond to a broad range of inflammatory cues, but the precise identity of these signals remains unknown. They modulate epithelial cell homeostasis and local immune responses by targeting other immune cells, viruses, and bacteria (9, 58, 59). The majority of IELs hold cytoplasmic granules containing large amounts of granzymes, cytokines, and chemotactic factors (42, 43, 60–62). At the first sight, the cytotoxic properties of IELs suggest, they can cause damage to the epithelial barrier by powerfully attacking infected cells, particularly IECs (63). However, IELs are well adapted to the intestinal environment in order to survive and perform their functions in protecting the delicate epithelial layer. In recent years, several studies of IELs have revealed distinct characteristics regarding their maintenance, activation, and contribution to the host immune response to preserve a healthy epithelial barrier.

Maintaining IELs

Intraepithelial lymphocytes develop pre-birth, occupy the epithelia before microbial colonization, and play an important role in immune protection during early life (64). It remained debatable for a considerable time if natural IELs take up residence at the intestinal epithelia as precursor naive-like cells or as antigen-experienced memory-like CD8+ T cells poised for activation or reactivation. The later would suggest that a priming step may be required, post-thymic development in the SLO, before seeding in IEL compartment. Transcriptional analysis, comparing IELs harvested under non-inflammatory conditions with memory CD8+ T cells, revealed paradoxical findings of their activation status (42, 43). IELs constitutively express transcripts of genes associated with activated cytotoxic T cells [granzyme A, granzyme B, serglycin, Fas ligand (FasL), and CCL5]. Yet, at the same time, IELs highly express transcripts of genes involved in immune regulation. These include cytotoxic T-lymphocyte associated protein 4, Ly49E-G, the NK cell inhibitory receptor Ig superfamily-related gp49B, and programmed cell death 1 (42). Factors involved in microbe-toxicity, such as regenerating islet-derived protein 3 gamma (Reg3γ), are readily detected in IELs under steady state conditions (65). In addition, several transcripts have been translated, and proteins are present and stored in secretory vesicles, e.g., granzymes (66). IELs home are retained in a poised activation state in mice lacking most secondary organs (67, 68), suggesting priming in secondary organs for natural IELs is not essential and the IEL activation status may be maintained by factors in the local epithelial environment.

Natural IELs are present in axenic mice. However, reduced numbers (of induced IELs) and decreased cytotoxicity of IELs from germ-free mice indicate that signals from the microbiota or other environmental stimuli are required to maintain intestinal CD8+ T cells and their function (51, 69, 70). The ligand activated transcription factor, arylhydrocarbon receptor is critical for IEL maintenance (59, 71). Of interest, the provision of ligands can be achieved *via* food intake, especially green vegetables, and may also be obtained from the microbiota (59, 72). Curiously, 30–50% of IELs from conventional standard pathogen-free mice express the marker Thy1 (CD90), but those found in axenic mice do not. Colonization of germ-free mice results in the generation of Thy1-expressing IELs (69), but as yet no functional differences have been attributed to the expression of Thy1.

Intraepithelial lymphocyte maintenance and activation also critically relies on interactions between IECs and microorganisms. Myeloid differentiation primary response gene 88 (MyD88), the adapter protein used by many toll-like receptors (TLRs), interleukin (IL)-1R, and IL-18R activate the transcription factor nuclear factor-κB, is required for IEL maintenance *via* the production of IL-15 (65, 73) (**Figure 2**). IL-15 production signals *via* the type 1 transcription factor, Tbox expressed in T cells (Tbet) to maintain IEL precursors (51). TLR2 may be at least one of the pattern recognition receptors involved in IEL maintenance, *via* IL-15 induction, its absence resulting in marked reduction of intestinal IELs (74, 75). Although IL-15 may be induced by microorganisms, they may not be essential for its production as axenic mice have reportedly higher levels of *Il15* transcripts, and no differences in numbers of natural IELs were observed (51). Nucleotide-binding oligomerization domain-containing protein (NOD)2, an intracellular sensor for microbial products has also been shown to be important for IEL maintenance (76). IELs in NOD2-deficient mice show reduced proliferation and increased levels of apoptosis. Once again, NOD2 signaling, *via* recognition of gut microbiota, results in IL-15 production (**Figure 2**). Of interest, NOD2 is able to tune the signaling of TLR2 dose dependently (77). Although these results have been achieved using whole body knock outs for MyD88, TLR2, or NOD2, and therefore, the exact role of IECs remains to be determined, they indicate that microorganisms may play an important role in IEL maintenance.

IEL Activation Status

Due to the positioning of IELs just underneath the single epithelial layer and their potential involvement in modulating intestinal pathology, the activation status of IELs is intensively studied. Transcriptional data of IELs foretells puzzling semi-activation of IELs that could enable them to deal with a broad range of pathologies quickly, with reduced requirement for immediate energy absorption and new gene expression (78). Unlike conventional CD8+ T cells, IELs express high levels of Tnfsf6 transcripts during steady state (42, 43), but do not express the encoding FasL protein on their surface until additional activation takes place (61). Despite their poised state and effector-like or T_{EM} cell characteristics, IELs do not contain transcripts for cytokines, which they secrete during conditions of inflammation (43). This suggests IELs require additional cues to initiate part of their effector function capacity.

Understanding the activation properties of IELs is essential to gain insight into mechanisms of local immunity and events

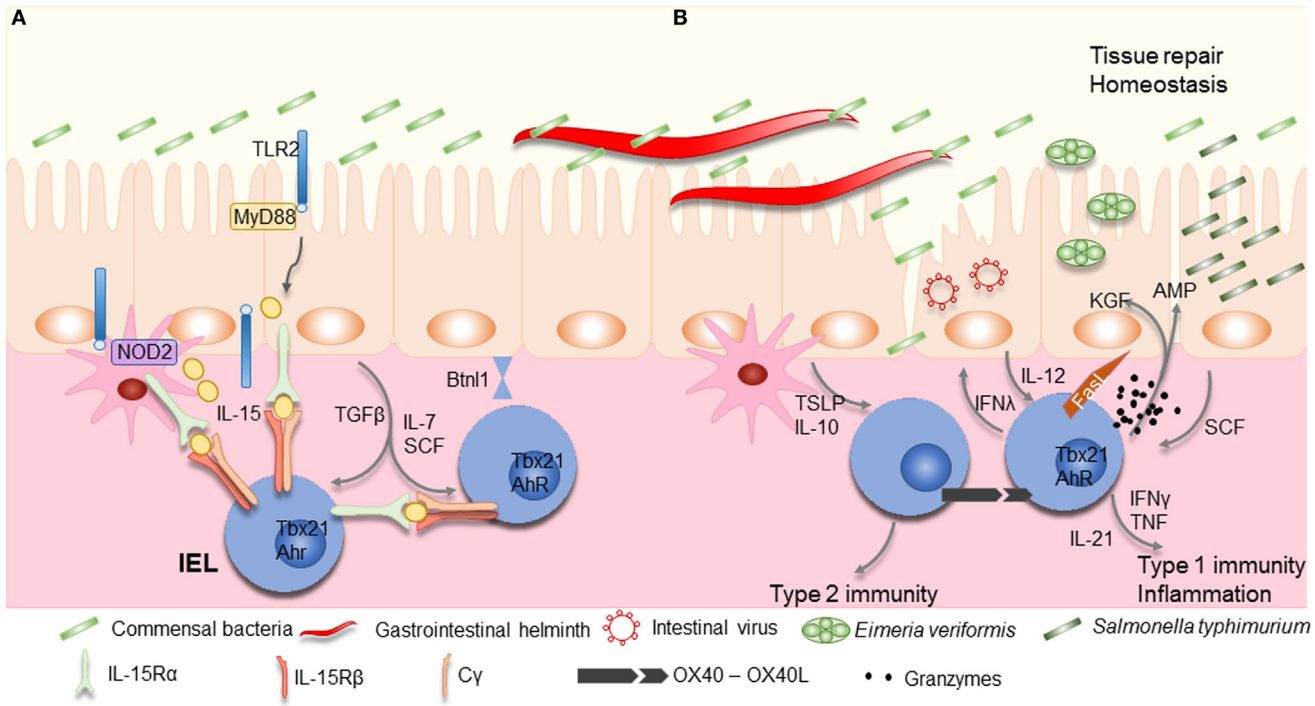

FIGURE 2 | Maintenance and activation of intraepithelial lymphocytes (IELs). **(A)** Commensal bacteria can contribute to IEL maintenance. Signaling *via* TLR2 and myeloid differentiation primary response gene 88 (MyD88) increases interleukin (IL)-15 production, an important survival factor for IELs. Antigen presenting cells, such as dendritic cells (DCs) or macrophages, also produce IL-15 in a NOD2 dependent manner. IL-15 is bound to the IL-15Rα on the producing cells, and is presented *in trans* to the IEL, which carry the IL-15Rβ/Cγ chain receptor complex, and signals *via* the transcription factor Tbx21. IL-7 and stem cell factor (SCF) are additional examples for IEC derived cytokines important for IEL survival, while arylhydrocarbon receptor expression (AhR) and tissue-specific factors, such as butyrophilin-like 1 (Btnl1), play an additional role in maintaining IELs. **(B)** Infections cause disruption or damage to the epithelial barrier. Dependent on the type of insult, IEC and DCs produce cytokines like thymic stromal lymphopoietin (TSLP), IL-10, IL-12, or SCF, thereby directing the type of immune response. Additional stimulation may be derived from IEL–IEL cross-talk, such as *via* OX40–XO40L interactions. IELs produce pro-inflammatory cytokines such as interferons (IFNs) and tumor necrosis factor (TNF), and cytotoxic factors such as Fas ligand (FasL) and granzymes, as well as antimicrobial peptides (AMPs) to contain the infection and contribute to wound healing and restoration of homeostasis by secreting growth factors such as KGF. Aberrant IEL activation and potentiation by cytokines might be involved in the development of chronic inflammation and IBD.

associated with tolerance, chronic inflammation, and immunopathology. Intestinal mucosa resected from patients with IBD (79) or celiac disease (80) contains increased numbers of activated T cells, a hallmark of intestinal inflammatory disorders (81). Yet, in a chemically induced colitis model, dextran sulfate sodium, 2,4,6-trinitrobenzene sulfonic acid (TNBS) or T cell transfer colitis, and IELs were found to be protective (60, 76, 82–84). This raises questions regarding the role of IELs in the intestinal immune network, whether they can, at least in part, contribute to chronic inflammation and pathology, or if they have a more tolerogenic or regulatory role. Furthermore, although cytotoxicity and microbicidal activity are an important part of IEL activity, it is not clear if their potential to produce cytokines and chemokines can be tailored to the level or identity the microbial threat.

Transcriptional data also suggested that IELs are metabolically prepared for swift action. IELs, compared with memory CD8$^+$ T cells, contain increased levels of mRNA for metabolic enzymes, especially those involved in the generation of fatty acids and cholesterol esters (42, 43). In line with the expression of CD69, IELs seem arrested in a semi-activated state. Yet, in stark contrast to effector cells, IELs survive for a considerable period of time. For example, murine skin IELs are generated only during embryogenesis, but can be found throughout adult life and into old age. The skin and intestinal epithelia are lipid-rich, but availability of other nutrients may be limited (85). This may explain why skin T_{RM} cells appear to use mitochondrial β-oxidation of exogenous lipids, mediated by intracellular transport proteins, including fatty-acid-binding protein-4 and -5, supporting their longevity and protective function (78). Similarly, natural IELs highly express surface molecules involved in lipid uptake, such as apolipoprotein E and low-density lipoprotein receptor (42). The increased presence of receptors and enzymes involved in lipid metabolism in IELs compared with conventional T cells suggests that altered metabolic processes may be involved in maintaining their poised activation status. However, it remains to be determined if the increase in lipid metabolism sets IELs apart or if it reflects their semi-activated status, since recently activated conventional T cells utilize the same pathways (86). Lipids are also required for the differentiation of CD8$^+$ memory T cells, the formation of which requires metabolic reprogramming

characterized by enhanced mitochondrial fatty-acid oxidation (87). Although, cell-intrinsic lipolysis is implicated in memory cell formation, suggesting the acquisition of fatty acids from the external environment is not critical, these lipids may have been obtained during the initial priming stage. These data imply that the metabolism of IELs reflects, in part, that of recently activated T cells and that lipolysis may be inhibited in IELs to arrest them in a poised activation status, thereby preventing progression to a quiescent memory status.

Role for the TCR in IEL Activation

The understanding of the role of the TCR on IEL development, differentiation, homing, and activation has long been hampered by the absence of a known selective or activating ligand. Recent work has identified tissue-specific ligands expressed in the thymus, driving the development and homing of either murine skin IELs or intestinal TCRγδ IELs, not peptide-MHC or lipid-CD1 complexes, but the butyrophilin-like molecules skint-1 and butyrophilin-like 1 (Btnl1), respectively (88–90). The role of the TCRαβ expressed on natural CD8αα IELs remains unknown (91). The collective data from these studies strongly suggest that the TCRγδ is required for both thymic selection and imprinting of IEL identity as well as their maintenance in specific tissue niches. However, butyrophilins, part of the immunoglobulin superfamily [for detailed review see Rhodes et al. (92)], are, unlike MHC or CD1 molecules, not known to present ligands. Thus, it remains unclear if IELs are stimulated *via* their TCR or if they sense other cues, such as inflammatory, tissue damage or cell stress factors provided by IECs or accessory cells (93), or are under the influence of metabolic alterations as a result of cell damage or bacterial growth in the microenvironment which can enhance T_H cell subset differentiation (94).

Agonist-driven positive selection of IELs in the thymus suggests that mature IELs at the epithelial barriers could subsequently be activated by specific TCR ligands. IEL TCR activation may be achieved by cell surface receptors, such as non-classical MHC molecules (95, 96). The parameters required for an agonist to activate IELs upon conditions of inflammation or tissue damage exclude constitutively expressed surface molecules, such as skint-1 and Btnl1, unless a gradient reaching a critical activation threshold can be achieved. If an agonist able to activate IELs exists, it does not preclude direct IEL activation by microbial products such as observed for γδ T cells in both man and mouse (97–99). Such direct activation is commonly observed in conventional $CD8^+$ T cells that have been pre-selected in the thymus and successfully primed and expanded in the periphery. Reactivation of memory $CD8^+$ T cells is readily achieved by cytokines and TLR ligands, resulting in secretion of IFNγ from polyclonal T cells that bridge innate and adaptive immunity (100). Similarly, peripheral γδ T cells can be directly activated by TLR ligands and combinations of cytokines (99, 101).

Administration of anti-CD3ε antibodies, which directly stimulate the TCR signaling complex thereby bypassing TCR-specific ligation, has often been used as a proxy to stimulate IELs in mice. However, its systemic activity, due to indiscriminate total T cell activation in all tissues, results in "cytokine release syndrome," increasing serum levels of IL-2, TNF, and IFNγ, and leading to intestinal phenotypes, such as diarrhea (102, 103). The small intestines from mice treated with anti-CD3 show increased epithelial ion transport, altered spontaneous muscle activity, and reduced IEC viability (104). The effect of anti-CD3 is rapid, with DNA fragmentation observed after 30 min in the areas most enriched with IELs, followed within hours by IEC shedding into the lumen (105). Similar effects on IEC viability were observed upon administration of anti-TCRγδ antibodies, but not with those stimulating TCRαβ (106). The effect on IEC shedding, however, was fully dependent on TNF receptor signaling and may not necessarily depend on IEL activation since conventional T cells can also secrete large amounts of TNF.

Following anti-CD3 stimulation, poised IELs acquire aspects of fully activated effector T cells with higher expression of CD44, Ly-6C, OX40, FasL, and CD25 and reduced expression of CD45RB protein, accompanied by expression of cytotoxic mediators as well as cytokine transcripts (61, 107). Effects of anti-CD3 on IEC viability appear to correlate well with the cytotoxic capacity of IELs, especially since release of granzyme B is observed upon anti-CD3 stimulation (62). However, DNA fragmentation is independent of the pore forming protein perforin (62). This suggests that IECs are non-specifically targeted by their proximity to activated T cells or by their susceptibility to soluble mediators. The accumulative data postulate that *in vivo* activation of IELs can at least in part be achieved *via* TCR ligation. IEL activity can have a major impact on intestinal physiology, altering electrolyte balance and IEC viability. However, their potential to damage IECs markedly contrast with the requirement to maintain an intact single cell intestinal barrier to efficiently protect the host and questions if TCR stimulation accurately recapitulates the physiological role of IELs. IEC–IEL bidirectional interactions are instrumental to maintain IELs, but it remains unknown if IECs directly contribute to IEL activation and, if they do, what the identities of the activating cues are?

IEL Activation by Microbes

Commensal bacteria can invade tissues when opportunity arises. Such opportunities occur upon initial microbial invasion of new-borns before species-specific adaptive immunity has fully developed or when the host is immune compromised (108). Since, activating IELs may not require antigen processing or rely on presentation by MHC-like molecules, it remains possible that IELs recognize molecular patterns generated by bacterial non-peptide antigens or conserved unprocessed protein antigens produced by bacteria or released by epithelial cells upon damage or cell stress (109).

Invasion of pathogens or tissue damage could create the conditions for commensal microorganisms to invade the intestinal tissues. Innate immunity relies on the detection of highly conserved pathogen-associated molecular patterns (110). Receptors involved in the detection of invasion will respond to the microbial components present in both pathogen and commensal microorganisms. But it has become clear that not all microorganisms evoke a similar response. Indicators of viability, such as the presence of prokaryotic mRNA invoke a much stronger immune response (111). The balance of immunity and tolerance at the epithelial interphase is also illustrated by the production of IgA,

the predominant antibody isotype critical at mucosal sites (112). IgA mainly coats pathogenic bacteria, which can confer colitis in axenic mice (113). IgA is a poor activator of the immune system; in line with the idea that strong immunity at mucosal sites is best avoided. IELs have been implicated in coordinating IgA response. TCRδ-deficient mice, harboring reduced IEL numbers, show reduced IgA levels in serum, saliva, and fecal samples. TCRδ-deficient mice also produce much lower levels of IgA antibodies upon oral immunization (114).

Role for IECs in IEL Activation; Co-stimulation

A requirement for co-stimuli or T_H cell help is linked with the need for clonal expansion and differentiation, creating a significant delay in adaptive immunity. Immune surveillance by tissue-resident lymphocytes requires a swift response without prior cell expansion or differentiation, observed for γδ T cells and memory T cells, such as T_{RM} cells (27, 115). This is in line with the hypothesis that the IEL response primarily serves to contain a potential threat, not necessarily resulting in microbe eradication or the establishment of immunological memory, thereby limiting microbial or toxin dissemination and keeping the single cell barrier intact by avoiding intestinal pathology.

Homing of IELs to the small intestine seems consistent with oligoclonal activation by commonly encountered antigens. The poised activation status of IELs could ensure their rapid activation without the need for an array of instructive signals. IELs seem not to depend on signal 2, required for conventional T cell activation and protective immunity (116). CD28 as well as additional co-receptors, such as CD2 and CD5, appear reduced or absent from IELs (43, 117–119). Furthermore, expression of MHC molecules or the costimulatory B7 proteins on at least keratinocytes is not required for activation of skin IELs (97). However, since the triggering of IELs could contribute to immunopathology, their activation is likely controlled on several levels, such as by signals derived from inflammation or tissue damage. The absence of a requirement for classic costimulatory signals for IEL activation suggests that close interactions with IECs play a prominent role.

OX40 (CD134, TNFRSF4) is expressed by activated T cells controlling cell expansion (120), including IELs (121). Its expression correlates well with T cell activity observed in patients with IBD, active celiac disease, Crohn's disease (CD), and ulcerative colitis (UC) (122, 123). *In vitro* activation of IELs with anti-CD3ε antibodies results in the expression of both OX40 and its ligand (OX40L) (121). Of note, OX40L is not expressed upon activation of conventional T cells. Additional ligation of OX40 seems to boost IEL activity and reduce the secretion of IL-10. This suggests that accumulation of IELs at sites of inflammation may alter their potential and that such co-stimulation may not necessarily depend on OX40L expression by IECs or myeloid cells (**Figure 2**).

Skin and intestinal IELs express the junctional adhesion-like molecule-1 (JAML-1), which provides co-stimulation upon ligation with the coxsackie-adenovirus receptor (CAR) (124, 125). JAML signaling results in cytokine production from skin IELs and may provide additional context for full IEL activation, presumably as response to infection or tissue damage. However, the latter requires its expression to be regulated upon insult or microbial invasion, which remains to be determined. Furthermore, its ligation by neutrophil-derived soluble JAML compromises intestinal barrier integrity and reduces wound repair through decreased IEC proliferation (126). Thus, the role of JAML—CAR in barrier defense remains to be clarified.

Role for IECs in IEL Activation; Cytokines

Intestinal IELs can express receptors for TNF, leukemia inhibitory factor, thymic stromal lymphopoietin (TSLP), stem cell factor (SCF; c-Kit ligand), transforming growth factor (TGF)β, IL-12, IL-15, and IL-21 (43, 127). TGFβ, most likely derived from IECs upon microbial stimulation, is required to maintain natural CD8αα IELs and to induce CD103 expression. The absence of TGFβ or its receptor results in markedly reduced numbers of IELs, while over expression increased the IEL population (50). How TGFβ influences IEL activity remains unknown.

Interleukin-15 plays a central role in maintenance of natural IELs and emphasizes the close interactions between IECs and IELs (128–131). IL-15 is presented in *trans* to IELs by epithelial cells, in the thymus, skin, and the intestine, which express both the IL-15Rα and IL-15 (132). IL-15R signaling induces the expression of anti-apoptotic molecules, Bcl-2 and Bcl-xL by IELs (133). The production of IL-15 is regulated, at least in part, by contact with microbial components. MyD88- and TLR2-derived signals are required for IEL maintenance *via* the induction of IL-15 production (65, 73, 75). T_{RM} cells are similarly dependent on IL-15-mediated signals (134), whereby high levels of IL-15 can TCR-independently trigger $CD8^+$ T cells to become cytotoxic (135, 136). Upon IEC damage, IL-15 production increases (65), as observed during celiac disease, and correlates strongly with IEL activity (133, 137). IL-15 stimulation of IELs results in increased IFNγ and TNF production, granzyme-dependent cytotoxicity, NK receptor expression, and increased survival (138). Of note, the increase in IL-15 production in conjunction with additional cues, such as retinoic acid can stimulate DCs, thereby inducing the secretion of pro-inflammatory factors and indirectly activate IELs. IL-15 induces the secretion of IL-21 by IELs, observed in celiac disease, which may be part of a self-sustaining feed-forward loop as observed in Th17 cells, enhancing IEL activation and cytotoxicity (139, 140).

Another important cytokine involved in T cell homeostasis is IL-7 (141). It is secreted by non-hematopoietic cells, especially thymic and IECs, with enhanced expression observed upon tissue damage (142–144). IEL development requires IL-7R signaling in the thymus, but local IL-7 expression by IECs can restore the γδIEL subset, not other γδ T cell subsets, suggesting extrathymic development or maturation of γδIELs may take place in the intestinal compartment (51, 142, 145). The use of acute IL-7 reporter mice indicates that production of IFNγ by T cells, such as IELs, can modulate the level of IL-7 and IL-15 produced by IECs, thereby regulating IEC homeostasis, absorptive function as well as the composition of the microbiota (144, 146). Vice versa, IEC derived IL-7 can regulate IEL survival and proliferation, particularly induced CD8αβ IELs (147). Overexpression of IL-7 results in lymphoid expansion and colitis (148).

Intestinal epithelial cells show a basal production of TSLP that is important for host protection during helminth infections (149). Similar to IL-15, TSLP receptor stimulation of $CD8^+$ T cells enhances expression of Bcl-2 (150), and may play a role in IEL survival. TSLP seems to enhance type 2-mediated immunity (**Figure 2**). Upon TSLP encounter, IECs and DCs produce IL-10 and reduce IL-12 production, thereby reducing type 1 immunity (151). IELs are known to be able to secrete IFNγ and loss of intestinal integrity results in IEC-produced IL-12 (152), the prototypical driver of type 1 immunity. In the absence of TSLP, mice are more susceptible to colitis and have increased levels of IFNγ producing cells. *Salmonella typhimurium* infection increases the expression of SCF produced by IECs (153). Its receptor, c-Kit, is expressed by IELs (127). The absence of SCF results in marked reduction of IEL numbers in mice, while its presence seems to play a role in IEL activation (154). Whether SCF and TLSP act as instructive cues initiating divergent IEL activation profiles remains to be investigated.

Containing Invasive Microbes

An ascending bacterial load exists from duodenum to jejunum and ileum, accumulating in very high numbers in the cecum and colon. Of note, IEL numbers are descending from duodenum to ileum, with few found in the colon (4). The causality of this striking inverted relationship remains unknown. IELs can produce antimicrobial factors and tissue repair factors in response to bacteria that penetrate the intestinal epithelium (60). IELs play an important role in the regulation and differentiation of epithelial cells at the base of the crypts (58, 155). IELs thereby help to preserve the integrity of damaged epithelial surface by providing the localized delivery of an epithelial cell growth factor (60). The mucosal protection afforded by IELs is of critical importance particularly during the first hours after bacterial exposure (156), in line with the hypothesis that IELs function to contain microbes upon invasion and initiate barrier repair thereby reducing immunopathology. With respect to pathogenic infections, in the majority studied, IELs offer protection against a wide variety of intestinal species, including *Eimeria vermiformis* (64, 157, 158), *Toxoplasma* (159, 160), *Encephalitozoon cuniculi* (161), Norovirus (107), and *Salmonella* (159, 160). Interestingly, infections with at least the pathogens *Salmonella* and *Toxoplasma* have indicated that IELs migrate to the site of infection and into the lateral intra-intestinal space (160), possibly initiating IEL–IEL co-stimulation (121). Collective release of preformed antimicrobial peptides (65, 156, 162) could directly contribute to microorganism containment and clearance (163).

Upon intestinal infectious challenges, protection and pathology are a result of the interplay between the microbes, the IELs, conventional T cells, and other immune cells. During *Eimeria* infection, IELs' production of IFNγ and TNF is instrumental in protective immunity, and expression of junctional molecules to preserve epithelial barrier integrity (158). However, elevated IFNγ and TNF levels in the intestinal mucosa also contribute to the pro-inflammatory cascade involved in barrier disruption and pathology (164). γδIELs are able to reduce pathology and their absence exaggerates mucosal injury upon *Eimeria vermiformis* infection (157). The absence of αβ T cells results in reduced capacity to clear the parasite, in part compensated by the adoptive transfer of $CD4^+$ T cells. In the absence of γδIELs, *Salmonella* or *Toxoplasma* infection result in increased microbe transmigration due to reduced epithelial barrier integrity. This increased transmigration leads to increased immunity mediated by conventional T cells (159). This indicates that IELs are not ultimately responsible for microbial clearance, but can modulate the initial response and recruitment of immune cells in order to moderate the risk of immunopathology.

Intraepithelial lymphocytes can contribute to viral immunity. Viral control depends largely on conventional T cells but, at least, αβIELs take part in viral clearance in the mucosa (165–167). Intestinal viral challenge, such as the non-enveloped RNA virus norovirus (MNV) present in many laboratory animal facilities, results in infection of IEC and myeloid cells (168). The infection can be controlled by IFNs, particularly IFNλ (169). Of note, the IFNλ (IFN type III) response seems to be operating particularly at epithelia barriers. This indicates that barrier immunity is kept local, to avoid systemic responses in which type I IFNs play a dominant role (170, 171). IELs, upon stimulation with plate-bound anti-CD3, can transcribe IFN genes, type I, II, and III, and the supernatant of *in vitro* activated IELs reduces viral infection (107, 172). *In vivo* anti-CD3 administration as well as culture supernatant from activated IELs results in IFN type I/III receptor-dependent expression of IFN responsive genes in intestinal IECs. Administration of anti-CD3 antibodies before intestinal viral challenge with murine norovirus can reduce viral load (107). However, due to the polyclonal stimulation of all T-lymphocytes, it remains unclear what contribution IELs provide and which properties may be uniquely attributed to them. It remains unknown if IELs are stimulated upon intestinal viral invasion and if so, how such invasion would enable the activation of IELs.

Besides microbicidal and cytotoxic activity, IELs produce cytokines and chemokines. Some chemokine transcripts are already present in IELs under steady state conditions, such as CCL5 and XCL1, but not those encoding for cytokines, such as IFNγ and TNF (43). This suggests that the recruitment of additional immune cells and release of these powerful cytokines and other chemoattractants such as CXCL1, CXCL2, and CXCL9 (65), might be delayed compared with cytotoxicity. The clearance of pathogens and the instigation of immunological memory involve careful orchestration of various cellular components. At epithelial sites, pathogen containment by innate-like T-lymphocytes may precede recruitment of myeloid cells and subsequent involvement of the adoptive arm of the immune response.

IELs in IBDs

The initiation of an immune response is not taken lightly, especially at the intestinal barrier. When immune activation does take place, pathogen clearance, pathology, and the need to maintain tissue integrity and its repair are offset. The consequences of microbial invasion or aberrant immunity can be severe (81). IBD can affect any part of the intestine and present with extra-intestinal manifestations. Despite advances in IBD understanding, the cause(s) and mechanism(s) remain unknown and disease incidence is increasing with changes in the environment and life style likely

making a contribution (173). Disease occurrence and severity are further compounded by diet, dehydration, and antibiotic use (174) as well as age (175).

There are indications that IBD results from alterations in innate immunity resulting in excessive adaptive immune activation (176, 177). IBD seems to result from immune-genetic predispositions and environmental factors, especially a dysregulated response to microorganisms (178). The presence of micro-infections or patches of affected intestinal tissue, associated with bacterial presence, next to seemingly unaffected tissue suggest localized immune activation or inefficient immune resolution (179). Several immunologic and histopathologic features of IBD, such as the presence of activated T cells secreting IFNγ and IL-17 and immunopathology (180), can be explained as a defect in mucosal immune regulation and as a consequence of persistent mucosal T cell activation (181). Several studies have shown that IL-12 production is increased in inflammatory lesions of patients with CD (182). In line with activated T cells, UC patients have an increased level of IL-7 and IL- 15, which may perpetrate additional T cell activation (183, 184).

There is limited data for a role of IELs in IBD, either disease enhancing or reducing. Accumulation of γδIELs in inflamed areas of IBD patients have been reported, and associated with increased levels of IL-15 and TSLP (133, 151). Although identifying lamina propria migrated γδ T cells and γδIELs is difficult, these cells constitutively produce IFNγ in patients suffering from either CD or UC (185). It remains to be determined if reduced numbers of IELs contribute to increased susceptibility to inflammation or are a consequence of ongoing inflammation, such as due to an increase in αβ effector T cells or activation-related cell death. Murine studies have demonstrated potential protective roles for γδIELs in intestinal inflammation as well as IBD models (59, 82, 186), but others have suggested that IEL expansion and activation can exacerbate the progression of colitis (187, 188). Lesions in IBD are mainly found in areas of reduced IEL density and highest load of bacteria, the colon, and ileum (178). The numbers of IELs correlate inversely with disease severity, and IEL numbers are restored to levels observed in healthy controls upon treatment with anti-TNF (189). This indicates vulnerability at those sites with altered immune surveillance and intense bacteria–IEC interactions, in line with the presence of adherent-invasive *Escherichia coli* at IBD lesions (179).

Loss-of-function mutations of NOD2 are strongly associated with CD (190–193). This correlates with the loss of IELs seen in mice deficient for NOD2 (76), but detailed insights are lacking and NOD2 plays an important role in other cell populations such as DCs, able to influence type 1 and type 3 immunity (194). Reduced proportions of γδIELs at the intestinal mucosa of CD patients suggested a protective role for these cells (189, 195, 196). IELs show increased activity at inflammatory sites in IBD patients, secreting IFNγ and TNF (189). Furthermore, IELs may enhance the production of IFNγ in the human colon (197). Nevertheless, murine IELs have been shown to be able to reduce the production of IFNγ production by conventional CD4 T cells, indicating their capacity to reduce type 1 immunity (198). In line with IELs forming an important part of the first line of defense is the increased susceptibility to infection of CD patients, which have reduced IEL numbers, with the intracellular parasite microsporidia (199). Unfortunately, the data on a role for IELs in preventing or reducing susceptibility to IBD remain inconclusive. The majority of IELs are found in the small intestine, a site not easily accessible, and IELs as a population of human T-lymphocytes are not well defined with respect to their identity and location. Often γδ T cells, such as those found in the circulation, are used as a proxy for IELs. However, murine studies and data on human TCRδ-chain usage have shown IELs to be very different from γδ T cells found in other tissues [(55, 59) #1587].

CONCLUSION

Intraepithelial lymphocytes are an integral part of the epithelial barrier. They do not exist as isolated cells monitoring the front line, but have close bidirectional interactions with IECs and possibly other immune cells. This close interaction enables the reception and provision of signals at very close range to maintain epithelial integrity. This interaction may be crucial in initiating local repair and containing low level of microbial invasion. Localized cues of potentially low dose would enable the activation of poised IELs without necessarily alerting the adaptive immune system. The single cell epithelial barrier is under constant threat of assault. Containing such threats locally with minimal immune activation is of great benefit to limit immunopathology and maintain optimal nutrient uptake.

How IELs, displaying many characteristics of effector T cells, are maintained in a poised state remains poorly understood. Differences identified in metabolic pathways may reflect their partial activation status or indicate differential metabolic wiring of IELs. The cues that enable full IEL activation remain ill-defined and the activity of IELs, the existence of different modes of action, are unknown. With technological advantages, such as multicolor flow cytometry and microscopy new players at the mucosal sites have been identified. This may now help us to unravel the complex multiplayer immune surveillance network of the mucosal immune response with real potential to discover novel targets for therapies to alleviate or even cure the different forms of IBD and celiac disease.

AUTHOR CONTRIBUTIONS

All authors wrote the review and contributed to the figures.

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Interplay of GTPases and Cytoskeleton in Cellular Barrier Defects during Gut Inflammation

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An essential role of the intestine is to build and maintain a barrier preventing the luminal gut microbiota from invading the host. This involves two coordinated physical and immunological barriers formed by single layers of intestinal epithelial and endothelial cells, which avoid the activation of local immune responses or the systemic dissemination of microbial agents, and preserve tissue homeostasis. Accordingly, alterations of epithelial and endothelial barrier functions have been associated with gut inflammation, for example during inflammatory bowel disease (IBD). The discriminative control of nutriment uptake and sealing toward potentially pathological microorganisms requires a profound regulation of para- and transcellular permeability. On the subcellular level, the cytoskeleton exerts key regulatory functions in the maintenance of cellular barriers. Increased epithelial/endothelial permeability occurs primarily as a result of a reorganization of cytoskeletal–junctional complexes. Pro-inflammatory mediators such as cytokines can induce cytoskeletal rearrangements, causing inflammation-dependent defects in gut barrier function. In this context, small GTPases of the Rho family and large GTPases from the Dynamin superfamily appear as major cellular switches regulating the interaction between intercellular junctions and actomyosin complexes, and in turn cytoskeleton plasticity. Strikingly, some of these proteins, such as RhoA or guanylate-binding protein-1 (GBP-1) have been associated with gut inflammation and IBD. In this review, we will summarize the role of small and large GTPases for cytoskeleton plasticity and epithelial/endothelial barrier in the context of gut inflammation.

Keywords: epithelium, endothelium, vascular, barriers, gut, junction proteins, inflammation, inflammatory bowel disease

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INTRODUCTION

Epithelia at mucosal surfaces represent the first barrier preventing potentially harmful environmental factors to invade the host. In the intestine, the epithelium does not only represent a simple physical obstacle against pathogen invasion but it also regulates nutrient uptake and innate immune function by avoiding the activation of mucosal immune responses (1). Thereby, maintenance of epithelial integrity is a key aspect in order to preserve homeostasis and to impair the development of inflammation in mucosal tissues (2). In addition to the epithelium, the gut–vascular barrier (GVB) has been recently described as a new anatomical structure which builds a second protective barrier preventing the microbiota to enter the bloodstream while allowing the translocation of immune cells and antigens (3). Barrier function of the epithelium as well as of the endothelium is

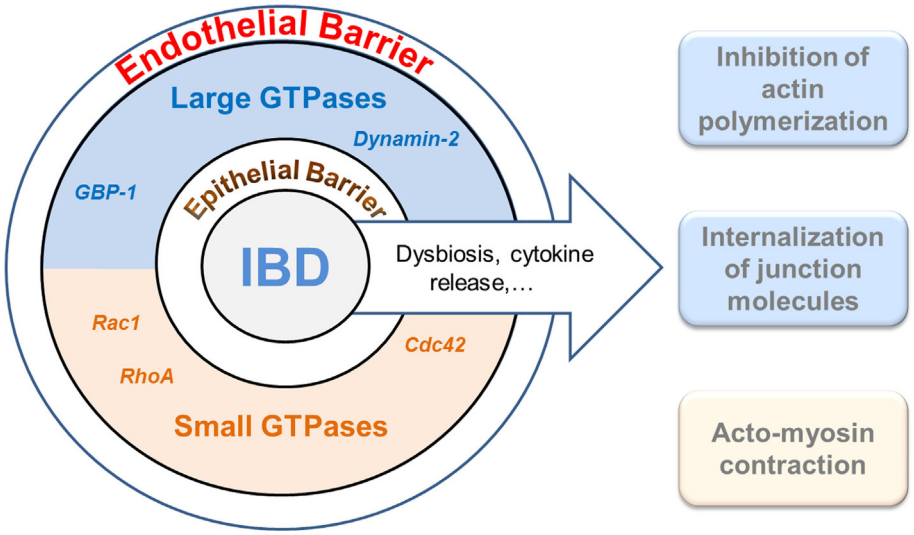

FIGURE 1 | Interplay of GTPases and the cytoskeleton in cellular barrier defects during gut inflammation. The intestinal epithelium and the endothelium establish two coordinated physical and immunological barriers. Increased barrier permeability is pathogenetically associated with inflammatory bowel diseases (IBDs). Different members of the families of small (lower brown) and large (upper blue) GTPases have recently been shown to regulate junctional and cytoskeletal dysfunctions both in epithelial and endothelial cells and, accordingly, may play an important role in IBD. It warrants further studies to determine whether cooperative, antagonistic, or redundant functions are exerted by the different GTPases.

dependent on a complex cytoskeletal organization and, in particular, on the formation of stable cell–cell junctions (4–6). These structures undergo profound changes during inflammation (7). Accordingly, increased paracellular permeability and epithelial/endothelial barrier dysfunction have been linked to the pathogenesis of chronic inflammatory disorders, such as inflammatory bowel diseases (IBDs) (2, 8, 9). IBD is defined as an idiopathic, chronic, and relapsing inflammation of the gastrointestinal tract. Two main clinical manifestations, Crohn's disease (CD) and ulcerative colitis (UC), affect a rather young population whose quality of life is significantly reduced. Despite intensive research, the pathogenesis of IBD is not completely understood. Here, we discuss the role of small and large GTPases in the cytoskeletal rearrangements induced in intestinal epithelial and endothelial barriers during inflammation **(Figure 1)**.

INTERCELLULAR JUNCTIONS IN EPITHELIUM AND ENDOTHELIUM

Apical junction complexes (AJC) built by tight junctions (TJs) and adherens junctions (AJs) enable the connection between adjacent cells, both in intestinal epithelium and endothelium. The AJC contribute to barrier function by controlling selective diffusion of molecules or cells, maintaining cell polarity and allowing intercellular communication (10). TJs consist of occludins, claudins, and junctional adhesion molecules (JAMs) (6, 11). AJs are composed of cadherins and nectins (12, 13). Both represent specialized zipper-like structures which enable the sealing of the paracellular space within the epithelial or endothelial layer (14). These intercellular junctions are connected to the actomyosin cytoskeleton *via* cytoplasmatic adaptors, such as zonula occludens (ZO) proteins, and catenins (6, 15, 16), which supports the mechanical strength of the junctions. For instance, in the resting endothelium, the cortical actin network ensures the necessary tension for the formation of stable interactions at AJs (17). AJs and TJs have been shown to influence each other's assembly and maintenance in a reciprocal manner (18, 19). In the presence of permeability-inducing molecules, actin reorganizes into stress fibers, which increases traction forces and leads to the uncoupling of AJC from the actin cytoskeleton resulting in the formation of gaps between adjacent cells (20, 21). Contraction of a perijunctional actomyosin ring further regulates permeability in a myosin light-chain kinase- dependent manner (22). In addition, TJ and AJ molecules can be removed from the cell surface by internalization and/or by proteolytic cleavage resulting in extracellular domain shedding (18). Thus, the interaction between cytoskeleton and intercellular junctions is crucial for maintenance of epithelial/endothelial barrier function (23).

Intercellular junction composition and abundancy are tissue-dependent. Within the intestinal epithelium, TJs proteins can be categorized in three families: claudins (claudin-1, 2, 3, 4, 5, 7, and 15) (24), tight junction-associated Marvel proteins (Occludin, Marvel D3, and tricellulin) (25), and cortical thymocyte marker of the *Xenopus* (CTX) (JAM-A, CAR, and CLMP) (26). The composition and structure of endothelial TJs can vary according to the type of vessel or organ (27). In intestinal endothelial cells (EndoCs), TJs are composed of occludin, JAM-A, ZO-1, and cingulin, while claudin-5 was mostly associated with gut lymphatic EndoCs (3). Epithelial AJs are composed of α- and β-catenin and E-cadherin, while AJs within EndoCs are formed by VE-cadherin and β-catenin (3). The formation of VE-cadherin adhesions at AJs is the primary event regulating EndoC-cell interactions during vasculogenesis, and this depends on intracellular tension generated by the actin cytoskeleton (18).

EPITHELIAL BARRIER REGULATION DURING INTESTINAL INFLAMMATION

Epithelial integrity in the gut has to be tightly regulated. In order to build up a protective barrier against luminal content, a precise and complex cell turnover warranties the renewal of the epithelium without compromising its tightness. Stem cells at the crypt bottom proliferate and differentiate into several IECs subtypes with specialized biological functions (28). Then, most of the differentiated IECs migrate upwards to the villus tip, where aged cells die and are shed into the lumen (29, 30). During this sophisticated process, the tightness of the epithelial layer is achieved by the intimate connection between epithelial cells, which is primarily mediated by intercellular junctions connected to the actin cytoskeleton (6). Focusing on cell shedding, the maintenance of epithelial integrity is warranted by the redistribution of junctional proteins along lateral membranes in a cytoskeleton and membrane trafficking-dependent molecular mechanism (31, 32).

The complex cytoskeleton network in IECs (4, 23, 31) orchestrates key cellular and molecular events during epithelial morphogenesis and renewal (12, 33). On a cellular level, the cytoskeleton defines cell shape and polarity which are important for nutrient uptake, anchoring of IECs to the basal membrane and communication with the sub-epithelial compartment (34, 35). Cytoskeletal plasticity within IECs is relevant to maintain barrier integrity and tissue homeostasis. Accordingly, breakdown of epithelial integrity has been observed after disruption of intercellular junctions and cytoskeleton rearrangement, e.g., in the context of infection or inflammation (36–38).

Increased epithelial TJ permeability is a hallmark of tissue alterations observed in the gut of IBD patients (39–43). Although a correlation between permeability and disease activity could be shown in CD patients, for instance (44, 45), the triggering event involved in the breakdown of gut homeostasis is still a matter of controversy. Mouse studies demonstrated that deficiency of single TJ proteins is not associated with pathology due to compensatory mechanisms (46, 47), except for claudin-15 (48). By contrast, it is well accepted that inflammation-derived mediators mediate TJ dysfunction and thereby contribute to the breakdown of epithelial integrity in experimental colitis and IBD. These mediators include cytokines, such as IL-6 (49), IL-13 (50, 51), TNF (52), and type II Interferon (IFN-γ) (53–55). Then, increased intestinal permeability in IBD patients might be secondary to the release of cytokines within the gut mucosa (56, 57). These cytokines then affect paracellular permeability *via* myosin light-chain II-mediated contraction of the prejunctional actin ring, as shown for TNF in IBD patients (23). These observations support the assumption that epithelial integrity breakdown is indeed a consequence of inflammation.

However, recent studies in IBD patients demonstrated that flares of the disease are preceded by increased permeability, which argues for a causative role of the epithelium in the development of intestinal inflammation (41, 58–60). Interestingly, even healthy relatives (61–63) and non-inflamed gut areas in CD patients (64) showed an elevated intestinal permeability. Accordingly, new therapy strategies based on epithelial restoration led to promising results in IBD patients. For instance, therapeutically induced decrease of epithelial permeability by vitamin D (65, 66) or probiotics (67–69), IL-22-triggered mucus production (70) or maintenance of epithelial cell integrity by butyrate (71, 72), or anti-TNF antibody treatment resulted in a clinical amelioration of chronic colitis (73, 74). The remaining open question is which mechanism might regulate cytoskeleton remodeling and epithelial permeability.

VASCULAR BARRIER REGULATION DURING INTESTINAL INFLAMMATION

The endothelium consists of a continuous monolayer of EndoCs lining the wall of blood and lymphatic vessels (75). It represents a semipermeable barrier between the bloodstream and the interstitium which regulates nutrient transport, tissue fluid homeostasis, immune cell transmigration (75), and restricts the transport of proteins in an organ-dependent manner (18). Similar to the epithelium, cell–cell junctions are crucial for the barrier role of the endothelium. The loss of EndoC-cell junctions causes a flux of proteinaceous fluid from the bloodstream into tissues, resulting in the development of edema. In addition to cell–cell junctions, coverage of the EndoC layer by pericytes is involved in the endothelial barrier function and was found to regulate permeability of the blood–brain barrier (76, 77).

The intestinal vascular endothelium represents a specialized vascular bed (3, 78). In the intestine, the capillaries are located directly underneath the epithelial layer and organized in gut–vascular units composed of EndoCs, pericytes, and enteric glial cells (3). Interestingly, the resting gut blood endothelium displays different levels of permeability depending on its localization. In the lamina propria, the endothelial permeability is increased compared to the submucosa, allowing the translocation of nutrients and antigens into the bloodstream while limiting enteric bacteria penetration (3).

During IBD, the intestine undergoes profound histological changes, including massive leukocyte infiltration, increased blood vessel density, and edema, which are all linked to vascular function (79–81). During inflammation, the vasculature is activated by inflammatory cytokines (ICs), such as TNF, interleukin-1 β (IL-1β), or IFN-γ, which leads to the expression of leukocytes adhesion molecules and fosters immune cell transmigration. In addition, neo-angiogenesis is induced and correlates with disease severity. More precisely, elevated levels of vascular endothelial growth factor (VEGF) can be found in the inflamed mucosa and in the blood during active IBD (80, 82–84) and vessel density is increased in the intestinal mucosa during IBD and in mouse model of colitis (9). However, inflammatory mediators such as ICs exhibit antiangiogenic activity and the concomitant presence of angiogenic and angiostatic molecules may disturb the physiologic regulation of angiogenesis (85–87). This might explain the disorganized intestinal vasculature observed in IBD, which is characterized by reduced vessel coverage, increased vessel leakiness, edema, and stenosis (81). Furthermore, vessel permeability strongly increases in both acute and chronic DSS-colitis mouse models compared to healthy animals (9). Interestingly, both ICs and VEGF have been shown to increase paracellular permeability of EndoC monolayers in culture (53, 88–90). In particular, high

levels of IFN-γ and markers of IFN-γ-activated endothelium, such as ICAM1, VCAM1, MAdCAM, CXCL10, or guanylate-binding protein-1 (GBP-1), can be detected in the gut mucosa of mice during DSS-induced intestinal inflammation (9). In this model, neutralization of IFN-γ resulted in an increased vessel density while vessel permeability decreased (9). Hence, the vascular effects of IFN-γ during IBD might contribute to disease severity by limiting angiogenesis and increasing vessel permeability, ultimately leading to the loss of GVB function. At the molecular level, endothelial (and epithelial) cells treated with IFN-γ undergo remodeling of the actin cytoskeleton and cell–cell junctions, the latter associated with a decrease of ZO-1 expression and internalization of TJ and AJ proteins (55). Further studies are necessary to understand the exact mechanisms of barrier function regulation by IFN-γ.

ROLE OF LARGE AND SMALL GTPases IN THE REGULATION OF CYTOSKELETON REMODELING DURING INTESTINAL INFLAMMATION

Large and small GTPases are molecular switches transducing signals from the extracellular compartment to the intracellular machinery. By means of a GTP–GDP-mediated activation cycle (91), these proteins are involved in numerous biological processes, with dramatic impact on cell biology. Most functions of GTPases depend on their association with cellular membranes. The localization of the protein in close proximity to cellular membranes requires a specific posttranslational modification named prenylation. Prenylation consists of the binding of an isoprenoid at the C-terminal end of the target protein and impacts on protein physicochemical properties, subcellular localization, and function (92, 93). New findings demonstrated the important role of large and small GTPases as major cytoskeleton interacting partners and in the regulation of actomyosin dynamics and intercellular junctions (94). Changes in the GTPase activity promote actomyosin dysregulation associated with pathological conditions in several organs (95–97).

Proteins belonging to the Ras superfamily are defined as small GTPases because of their low molecular weight. The Ras superfamily of proteins consists of five families (Ras, Rho, Ran, Rab, and Arf) and more than 160 different members (98). They participate in the regulation of cell proliferation, cytoskeletal dynamics/morphology, membrane trafficking, cellular adhesion, vesicular, and nuclear transport (99–101). Besides the well-described superfamily of small GTPases, the dynamin superfamily of large GTPases represents a group of enzymes involved in pathogen resistance, budding of transport vesicles, division of organelles, cytokinesis, and cytoskeletal rearrangements (102). It comprises dynamins, Mx proteins, OPA, mitofusins, atlastins, and guanylate-binding proteins (GBPs). Large GTPases are characterized by the ability to oligomerize and harbor an oligomerization-dependent GTPase activity (102).

In the following, we will summarize the role of small and large GTPases in cytoskeleton remodeling, epithelial and endothelial integrity, and their relevance in maintenance of barrier functions in the gut.

Small GTPases

Impaired small GTPase function in the intestinal epithelium is associated with junctional and cytoskeletal dysfunctions (103–105). Numerous *in vitro* studies demonstrated Rho-mediated regulation of the cytoskeleton within epithelial cells (106–111); both up- and downregulation of Rho protein function can alter actomyosin contractility and in turn impair barrier function (112, 113). Actomyosin contraction due to phosphorylation of MLC2 by ROCK is involved in epithelial RhoA signaling, which is required for pathological as well as physiological epithelial cell extrusion (32, 114). The link between RhoA and intestinal inflammation was first shown in 2003, when increased RhoA activation in experimental colitis and patients suffering from IBD was identified (115). In a subsequent study, it was found that Rho-GDP dissociation inhibitor alpha expression was upregulated in CD and UC patients (116). We recently showed that IBD seems to be associated with impaired RhoA function (117). Inflamed areas in the gut of IBD patients depicted an accumulation of RhoA in the cytosol of IECs. This altered subcellular localization could presumably be a sign of RhoA dysfunction, since association to the plasma membrane is required for GTPase activation (118, 119). Furthermore, IEC-restricted lack of RhoA in mice resulted in the development of spontaneous inflammation (117). Interestingly, another recent study demonstrates that lack of Arhgap17, a RhoGTPase activating protein, causes increased epithelial permeability, not leading to spontaneous colitis but increasing the severity of DSS-induced colitis in mice (120). Taking together, RhoA can be considered as an important regulator of epithelial cytoskeleton and homeostasis in the gut. However, the mechanism and regulation of this process is still controversial. Actomyosin contraction due to phosphorylation of MLC2 by ROCK is involved in epithelial RhoA function, but whether RhoA inhibition, activation or both would modify epithelial integrity and permeability is still unclear.

Rac1 and Cdc42 also appear as attractive targets for the regulation of epithelial barrier function. *In vivo* genetic deletions of Cdc42 or Rac1 within IECs are associated with defects on epithelial cell proliferation and/or differentiation (121–124). Interestingly, genetic deletion of Cdc42 in mice resulted in an intestinal phenotype which resembled human microvillus inclusion disease. In the latter, cytoskeleton remodeling appears as a complementary mechanism to Paneth cell differentiation defects, leading to apical junction disorientation and increased intestinal paracellular permeability (123, 124).

Considering the relevance of regulated small GTPase function for cytoskeleton remodeling within IECs, prenylation has emerged as an attractive candidate target in epithelial restoration. Interestingly, IECs from IBD patients show decreased expression of the prenylation-catalyzing enzyme GGTase-Iβ (117). The link between GGTase-I-mediated prenylation and inflammation was confirmed by the dramatic intestinal distortion observed in mice with GGTase-Iβ-deficient IECs, which was ameliorated upon local induction of Rho activation (117). The destruction of intestinal architecture upon epithelial *Pggt1b*, the gene encoding for GGTase-Iβ (geranylgeranyltransferase1 beta subunit) deletion goes along with cytoskeleton remodeling, cell shedding alterations, and increased intestinal permeability. In conclusion, prenylation

may represent a novel relevant pathway for maintenance of gut homeostasis and epithelial integrity. Future studies are needed in order to further elucidate the molecular mechanisms related to Rho GTPases and other targets of prenylation within the intestinal epithelium. In this context, a recent study showed that the commensal microbiota can increase intestinal epithelial permeability through the small GTPase ARF4 (125). The expression of ARF4 led to a decrease in the expression of TJ proteins by a mechanism which still has to be determined (125). These results open new perspectives for the understanding of the role of the microbiome in the regulation of intestinal barrier function and in the onset of colitis.

Similar to their function in the epithelium, small GTPases play an essential role in the regulation of the endothelial barrier function through their impact on actin dynamics (126). RhoA activation and subsequent Rock-mediated actomyosin contractility decreases endothelial barrier function upon permeability-inducing compounds, such as thrombin (127). On the other hand, Rac1 and Cdc42 signals are able to counterbalance an increase of endothelial permeability by stabilizing intercellular junctions, decreasing actin contractility, and in turn facilitating the contact between adjacent EndoCs (128, 129). A complex interplay between opposite effects from RhoA and Cdc42/Rac1 and their functional cooperation defines Rho-mediated regulation of endothelial integrity. This crosstalk between RhoA and Rac1 is of particular importance in the context of chronic inflammation. TNF is well known to induce endothelial actin cytoskeleton reorganization and intercellular gaps through a sequential activation of Cdc42, Rac and RhoA (130). In addition, novel findings demonstrated that endosomoal RhoB also controls Rac1-mediated stabilization of the endothelial barrier (131). Despite these observations, so far, little is known about the role of Rho GTPases and prenylation in EndoCs during intestinal inflammation.

Large GTPases

Among large GTPases, two molecules (dynamin-2 and GBP-1) are of particular importance in the regulation of barrier function. Dynamins are involved in transcellular and paracellular permeability (132). Both, paracellular and transcellular permeabilities are increased in the intestinal epithelium during IBD (133) and are co-regulated in the microvascular endothelium through a compensatory mechanism, involving Rac, Dynamin-2 and actin (132). In general, transcellular permeability is regulated by vesicular transcytosis, which allows the transfer through a cell of macromolecules, such as albumin, by vesicle-mediated endocytosis and exocytosis (134). During transcytosis, invaginations of the plasma membrane (caveolae) are formed and coated by clathrin and actin. Dynamin finally achieves the scission of the nascent vesicle under GTP hydrolysis (134). In addition, Dynamin-2 regulates paracellular permeability through modulation of TJs and AJs. Dynamin-2 is able to bind several AJ and TJ proteins, to link them with the actin cytoskeleton and to ensure the stability of TJs and AJs in the epithelium and the endothelium (135). Furthermore, Dynamins directly interact with actin, foster actin polymerization, and induce actin bundles formation (136). Dynamin-2 is also involved in the maintenance of the apical constriction and the recycling of E-cadherin (137, 138). Dynamin-2 plays a role in barrier maintenance during TNF-induced epithelial shedding (32) and is also involved in the maintenance of the vascular barrier function under hypoxia, by inducing the activity of eNOS (139). Hence Dynamin-2 represents an important regulator of epithelial and endothelial permeability as well as vascular homeostasis.

Members of the human GBP family are involved in immune response against intracellular pathogens and inflammation (140). GBP-1 is the best characterized protein of the seven-member family (140–143). GBP-1 expression is strongly induced by ICs, notably by IFN-γ and has been detected in the inflamed mucosa during IBD (9, 143, 144). GBP-1 has been found to mediate the inhibitory effects of IFN-γ on cell proliferation, migration, and invasion and to inhibit tumor growth and angiogenesis *in vivo* (85,86,145–148). More precisely, GBP-1 can reorganize intracellular actin cytoskeleton in epithelial, endothelial, and T-cells (149, 150). GBP-1 directly interacts with β-actin and inhibits actin stress fiber formation, while co-localizing with cortical actin (149, 151). Actin depolymerization, for instance by latrunculin, has been shown to induce Occludin internalization (152). In addition, GBP-1 was found to localize at TJs both in intestinal crypts of patients with CD and UC and in human IEC lines treated with IFN-γ (144). In this model, the silencing of GBP-1 expression led to increased apoptosis, indicating that it exerts a protective role in epithelium homeostasis (144). However, the role of GBP-1 on cell–cell permeability and junction regulation is still not well understood.

Taken together, large and small GTPases, as well as prenylation, represent novel key players for maintenance of gut homeostasis, regulating epithelial and endothelial integrity under physiological and inflammatory conditions **(Figure 1)**. Despite the here described current knowledge in the field, some still open questions encourage the scientific community in this field to fulfill the description of the molecular mechanism behind these observations. It still remains to be determined to which extend the endothelial barrier participate to IBD pathogenesis and whether angiogenesis or endothelial activation contributes the most to the disease. On the other hand, the description of the role of other Rho GTPases, such as Rac1 or Cdcd42, for epithelial integrity; as well as molecular mechanisms regulating prenylation within IECs, should be further investigated. More detailed studies on inflammation-associated cytoskeleton remodeling within IECs and EndoCs might help in the identification of new target structures for an optimized treatment or early diagnosis of IBD.

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RL-P, MS, and NB-L wrote the manuscript. IA and MN were critically involved in the design of the work and the discussion of the content. All the authors approved the final manuscript.

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In the Wnt of Paneth Cells: Immune-Epithelial Crosstalk in Small Intestinal Crohn's Disease

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Paneth cells, specialized secretory epithelial cells of the small intestine, play a pivotal role in host defense and regulation of microbiota by producing antimicrobial peptides especially—but not only—the human α-defensin 5 (HD5) and HD6. In small intestinal Crohn's disease (CD) which is an entity of inflammatory bowel diseases, the expression of HD5 and HD6 is specifically compromised leading to a disturbed barrier and change in the microbial community. Different genetically driven but also non-genetic defects associated with small intestinal CD affect different lines of antimicrobial Paneth cell functions. In this review, we focus on the mechanisms and the crosstalk of Paneth cells and bone marrow-derived cells and highlight recent studies about the role of the Wnt signaling pathway in this connection of ileal CD. In summary, different lines of investigations led by us but also now numerous other groups support and reconfirm the proposed classification of this disease entity as Paneth's disease.

Keywords: Paneth cell, Crohn's disease, defensins, Wnt signaling, monocytes

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INTRODUCTION

In approximately 70% of Crohn's disease (CD) patients, the small intestine is affected, the remainder have colonic disease only (1). Mostly due to refluxing colonic contents, the small intestine also harbors large populations of microbes with a complex repertoire of various bacterial species, although their density is much lower than in the colon by about 3 orders of magnitude (2). Specializing in the production of different antimicrobial peptides (AMPs), Paneth cells are responsible for the host defense in this part of the intestinal tract. Generally, AMPs are the first line of defense of the human body. These innate immune effector molecules serve as endogenous antibiotics protecting the host against the multitude of commensals and pathogens with their antimicrobial activity. In humans and mammals, AMPs consist of various protein families consisting of two major families: the defensins and cathelicidins. They are small cationic peptides with amphipathic characteristics (3). Human defensins are characterized by their beta-sheet structure and are subdivided in the two main groups of α- and β-defensins by the structure of the six disulfide-connected cysteines (4). Different defensins and AMPs are expressed by all barrier-epithelial cell tissues throughout the entire body but they are also found in circulating immune cells playing an important role in host defense (e.g., most abundant content of neutrophils). While human β-defensins are produced by all epithelial surfaces (including the skin, gastrointestinal, respiratory, and urogenital tract), human α-defensin 5 (HD5) and HD6 are expressed dominantly by small intestinal-epithelial-secretory Paneth cells (5, 6).

In this review, we focus in particular on the pivotal role of the Wnt signaling pathway in the immune-epithelial crosstalk in small intestinal CD. Reflecting the complexity of gut homeostasis,

epithelial Paneth cells are closely linked to bone marrow-derived cells and monocytes are directly controlling Paneth cells *via* Wnt signaling. Besides ileal CD, this is likely also relevant for graft-versus-host diseases.

PANETH CELLS

Paneth cells are specialized secretory epithelial cells located in the small intestine on the bottom of the crypts of Lieberkühn. They originate from crypt stem cells and are filled with secretory granules containing large quantities of antimicrobial proteins and peptides. Besides the human α-defensins HD5 and HD6, these include lysozyme, regenerating islet-derived 3 gamma (Reg3γ), and secretory phospholipase A_2. Taken together, Paneth cells play an important role in the maintenance of the intestinal barrier function (6, 7). The expression levels of the α-defensins HD5 and HD6 are about three to one and exceeds those of other peptides including lysozyme or phospholipase A_2 up to 100-fold (8, 9). The α-defensins HD5 and HD6 are constitutively expressed with varying levels in different diseases, whereas the Reg3γ production by Paneth cells is induced in the presence of microbes in the intestinal lumen (10, 11). Furthermore, Paneth cells have an influence on the microbial composition of the small intestine. With their expression of various antimicrobials, they protect the intestine from pathogens and limit the number of commensals in the crypts (6). In addition, Paneth cells are implicated in stem cell regulation (1). The expression of the pattern-recognition receptor nucleotide-binding oligomerization domain containing 2 (NOD2) and its activation secures stem cell survival essential for tissue regeneration and healing processes (12).

CROHN'S DISEASE

Inflammatory bowel disease (IBD) is a chronic inflammation of the gastrointestinal tract characterized by an infiltration of various immune cells as a result of a pathological interaction of the commensal microbiota within the mucosa. IBD is classified in ulcerative colitis (UC) and CD. UC is restricted to the colon and typically shows continuous mucosal inflammation, whereas CD potentially arises all along the gastrointestinal tract characterized by a patchy discontinuous inflammation (13). Depending on the localization of the lesions, CD is subdivided into ileum only (L1), colon only (L2), or both ileum and colon (L3) (14), and this phenotype of CD location is remarkably stable over time (15).

CD—Imbalance of Microbiota and Innate and Adaptive Immune Response

During the relapsing course of their disease, CD patients predominantly suffer from abdominal pain and diarrhea (14). In the healthy gut, immune homeostasis prevails with a gut microbiota that is in balance with intestinal epithelial cells producing AMPs and releasing immune modulatory cytokines that drive naïve dendritic cells (DCs) to differentiate into tolerogenic DCs that trigger the priming of regulatory T cells. In contrast when an imbalance exists between an under-protected mucosa and an altered, usually less diversified microbiota naïve DCs may differentiate into immunogenic DCs initiating the priming of effector T cells leading to inflammation (16).

Link of Ileal CD and Paneth Cells: "Paneth's Disease"

It has been shown that in ileal CD a reduced expression of mucosal AMPs leads to inflammation and an attenuated antimicrobial defense by the mucosa (17). In a mouse model of CD-like ileitis, Schaubeck et al. showed a loss of the Paneth cell product lysozyme (18). Patients with ileal CD showed a decreased constitutive expression of the α-defensins HD5 and HD6 produced by Paneth cells. The reduction of HD5 and HD6 was further associated with mutations in the NOD2 receptor (10). It was the first evidence pointing toward the Paneth cell when Lala et al.

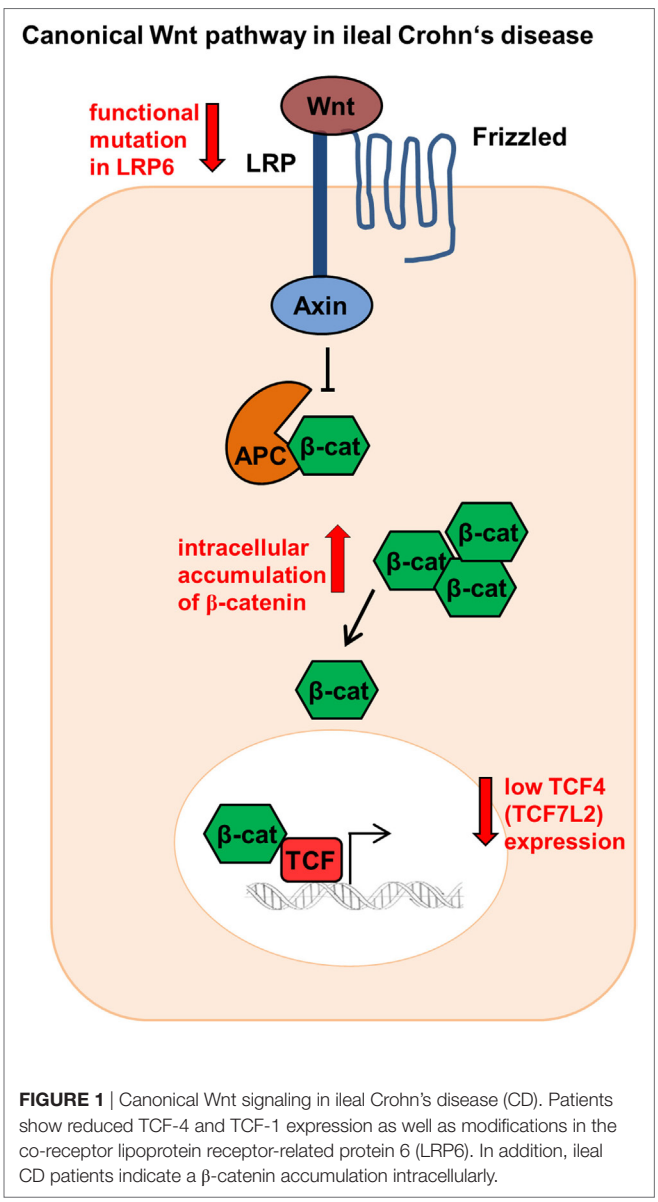

FIGURE 1 | Canonical Wnt signaling in ileal Crohn's disease (CD). Patients show reduced TCF-4 and TCF-1 expression as well as modifications in the co-receptor lipoprotein receptor-related protein 6 (LRP6). In addition, ileal CD patients indicate a β-catenin accumulation intracellularly.

discovered that in the intestinal mucosa not only macrophages but also Paneth cells express high levels of NOD2 (19). Several mutations in the NOD2 gene were detected, so that this intracellular receptor for bacterial muramyl-dipeptide was the first susceptibility gene in ileal CD and about 30% of patients who suffer from ileal CD carry this mutation (20).

However, CD is related to many other genetic defects that also lead to impaired Paneth cell function (1) and to Paneth cell necroptosis (21). Further examples are the autophagy gene autophagy-related 16-like 1 (Atg16L1) that plays an important role in CD pathogenesis by affecting Paneth cell granule exocytosis in patients with an ileal phenotype (22) or the transcription factor X-box binding protein 1 (XBP1) of the endoplasmic reticulum stress response activated during an inflammation. XBP1 defect leads to Paneth cell disturbance and increased susceptibility to IBD (23). In the IBD linkage region on chromosome 19q13, the calcium-mediated potassium channel subfamily N member 4 (KCNN4) is situated. The KCNN4 encoded protein plays a pivotal role in Paneth cell secretion and showed reduced expression levels in NOD2-mutated ileal CD patients (24). Furthermore, in the mucosa of ileal CD patients, adherent bacteria are present (25), probably caused by compromised antimicrobials Paneth cell function. Consequently, a compromised antibacterial defense due to reduced α-defensin expression or secretion explains many features of ileal CD, even though the mechanisms are complex and vary in term of pathways and origin.

Wnt SIGNALING IN ILEAL CD

The Wnt signaling pathway plays a pivotal role in the gut mucosal homeostasis and therefore in the intestinal epithelium. Wnt is an important element keeping intestinal epithelial stem cells in a proliferating status, enabling stem cell maintenance (26), and provoking the differentiation and maturation process of Paneth cells, thereby regulating the expression of the alpha-defensins HD5 and HD6 (27). The canonical Wnt pathway is activated when Wnt ligands released from epithelial cells, Paneth cells, or mesenchymal cells bind to the cell surface receptor "Frizzled" (28). They ultimately mediate the stabilization of β-catenin that can then transfer into the nucleus. Nuclear β-catenin binds to the transcription factors, T-cell factor 4 (TCF-4 or TCF7L2) and lymphoid enhancer factor, and enables target gene transcription such as the antibacterial defensin genes alpha 5 and 6 (*DEFA5* and *DEFA6*), the genes for HD5 and HD6 (29) (**Figure 1**).

Previously our group could show that in ileal CD there is a link between the reduced expression of Paneth cell α-defensins

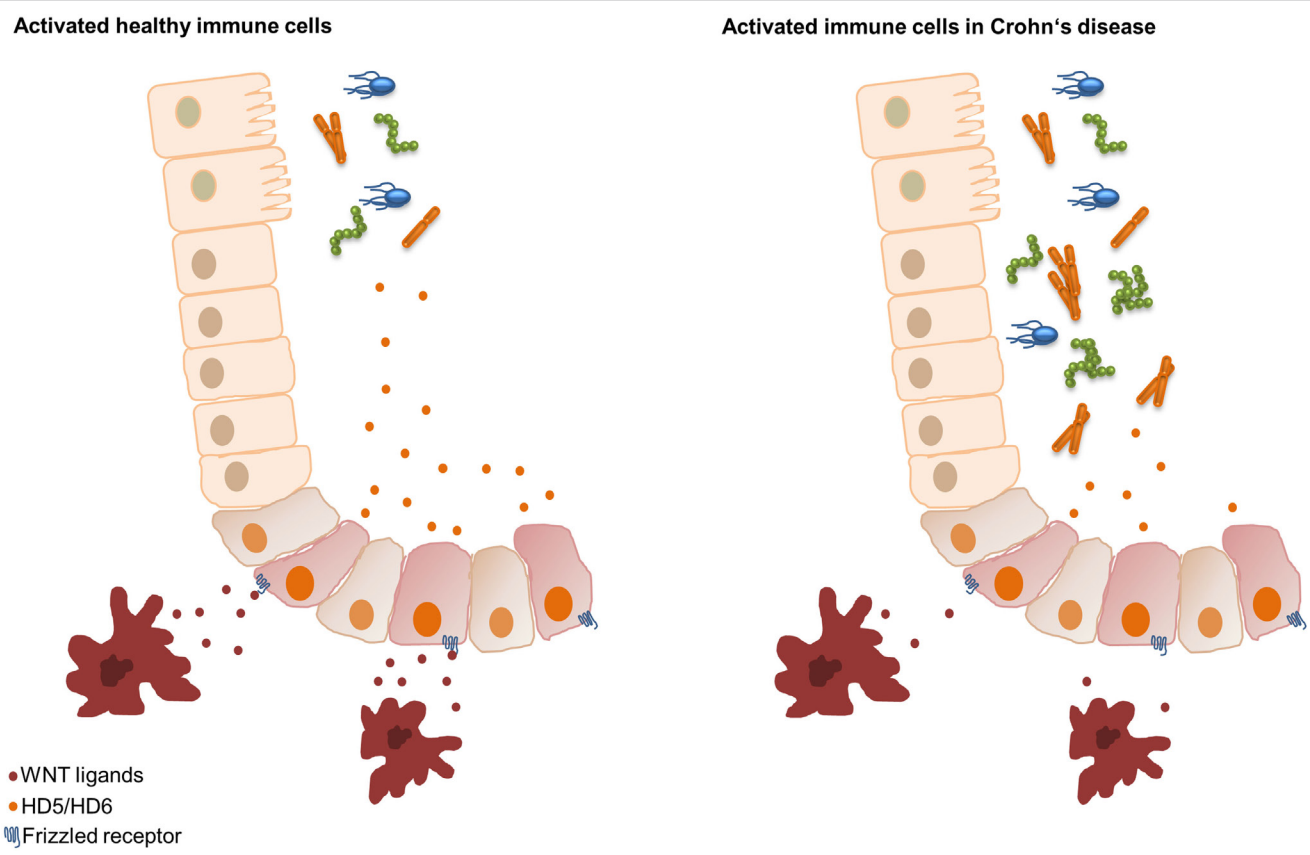

FIGURE 2 | Hypothesis of the Wnt signaling Paneth cell connection in ileal Crohn's disease (CD). In healthy controls, classical monocytes produce Wnt ligands leading to an inducible upregulation of human α-defensin 5 (HD5) and HD6. Classical monocytes of patients with ileal CD show reduced Wnt ligand expression, resulting in an impaired inducibility of HD5 and HD6, leading to bacterial invasion.

HD5 and HD6 and the Wnt transcription factor TCF-4 (TCF7L2). Ileal CD patients showed diminished TCF-4 expression irrespective of the inflammation status (25). However, not only TCF-4 is involved, the expression levels of the Wnt signaling effector TCF-1 were also reduced in ileal CD patients (30). Furthermore, the co-receptor low-density lipoprotein receptor-related protein 6 (LRP6), a further Wnt factor playing a key role in the cytoplasmic stabilization of β-catenin, was also modified in CD leading to diminished HD5 expression (31). Ileal CD patients diagnosed under the age of 18 showed a 10.63% higher mutation rate for the single-nucleotide polymorphism LRP6 rs2302685 (31). This turns LRP6 into an appealing therapeutic target toward early onset ileal CD. Not only variations of co-receptors or transcription factors are impaired in CD. A further study demonstrated that both β-catenin and E-cadherin accumulate intracellularly in an unusual fashion and showed an altered localization in the plasma membrane in CD patients (32) (**Figure 1**).

In addition, we recently found that peripheral blood mononuclear cells (PBMCs) of healthy controls reconstituted the decreased HD5 and HD6 levels of ileal CD patients. We demonstrated that the driving force of the PBMC effect was the Wnt ligand expression and not the cytokine release. The monocytes of CD patients expressed significantly lower values of the canonical Wnt ligands Wnt3, Wnt3a, Wnt1, and the wntless Wnt ligand secretion mediator (33). At the same time, measured cytokines did not show significant differences. So, a further very essential mechanism in CD is the connection between Paneth cells and bone marrow-derived monocytes characterized by an attenuated intestinal barrier function through a reduced Wnt ligand expression in PBMCs. Therefore, we hypothesize that in ileal inflammation circulating immune cells, probably classical monocytes as the main subset (3), supply the necessary Wnt ligands that lead to the production of HD5 and HD6 in Paneth cells resulting in an enhanced intestinal barrier function. However, in ileal CD, the monocytes show reduced Wnt ligand expression, thereby negatively affecting the secretion of the AMPs HD5 and HD6 and leading to bacterial infiltration and chronic inflammation (33) (**Figure 2**). But, the potential mechanisms of reduced Wnt delivery from monocytes still remain unknown.

CONCLUSION

Here, in this review, we emphasized the important role of ileal defensins of the Paneth cell in host defense mechanisms of the small intestine. Our group could show that in ileal CD patients, the defective barrier leads to an invasion of different bacteria around the mucosa. This is a direct consequence of the various Paneth cell defects leading to a reduced α-defensin expression in ileal CD. However, we recently found out that the infiltrating monocytes in ileal CD patients showed a compromised Wnt ligand production leading to an impaired defensin-inducing capacity. In conclusion, there is a defect interaction between Paneth cells and monocytes in ileal CD but the exact mechanisms of the regulation of Wnt ligand expression in monocytes have to be investigated to further develop new therapeutic strategies in intestinal disorders.

AUTHOR CONTRIBUTIONS

NA, ES, and JW wrote and discussed the manuscript.

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Epithelial Cell Inflammasomes in Intestinal Immunity and Inflammation

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Pattern recognition receptors (PRR), such as NOD-like receptors (NLRs), sense conserved microbial signatures, and host danger signals leading to the coordination of appropriate immune responses. Upon activation, a subset of NLR initiate the assembly of a multimeric protein complex known as the inflammasome, which processes pro-inflammatory cytokines and mediates a specialized form of cell death known as pyroptosis. The identification of inflammasome-associated genes as inflammatory bowel disease susceptibility genes implicates a role for the inflammasome in intestinal inflammation. Despite the fact that the functional importance of inflammasomes within immune cells has been well established, the contribution of inflammasome expression in non-hematopoietic cells remains comparatively understudied. Given that intestinal epithelial cells (IEC) act as a barrier between the host and the intestinal microbiota, inflammasome expression by these cells is likely important for intestinal immune homeostasis. Accumulating evidence suggests that the inflammasome plays a key role in shaping epithelial responses at the host–lumen interface with many inflammasome components highly expressed by IEC. Recent studies have exposed functional roles of IEC inflammasomes in mucosal immune defense, inflammation, and tumorigenesis. In this review, we present the main features of the predominant inflammasomes and their effector mechanisms contributing to intestinal homeostasis and inflammation. We also discuss existing controversies in the field and open questions related to their implications in disease. A comprehensive understanding of the molecular basis of intestinal inflammasome signaling could hold therapeutic potential for clinical translation.

Keywords: inflammasome, IL-18, IL-1β, intestinal epithelial cells, NOD-like receptor, pyroptosis, inflammatory bowel disease

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INTRODUCTION

Intestinal homeostasis is governed by complex interactions between the host immune system, the vast constitutive antigenic load in the lumen, and the epithelial barrier. Breakdown in this molecular dialog can lead to the development of chronic pathologies, such as inflammatory bowel diseases (IBD). The precise etiology of IBD remains unclear, although it is likely multifactorial involving a number of elements, such as host genetic susceptibility, environmental factors (e.g., smoking), and the composition of the microbiome (1). These factors contribute to the disturbance of homeostasis leading to the generation of chronic inflammation and development of IBD, including Crohn's disease (CD) and ulcerative colitis (UC). IBD are debilitating, relapsing diseases affecting approximately 1:400 people. With no cure available, IBD patients are consigned to long-term anti-inflammatory and immune suppressive therapies, and surgery is often required. Thus, there

is an urgent, unmet need to further understand the molecular mechanisms underlying IBD, to inform the development of new potential therapies. Genome-wide association studies (GWAS) revealed that inflammasome-associated genes were linked to IBD susceptibility (2), suggesting that this family of proteins is important for maintenance of intestinal homeostasis.

The inflammasome is a multimeric protein complex involved in inflammation. It comprised of an intracellular Pattern Recognition Receptors (PRR), usually a NOD-like receptor (NLR), and is activated in response to exogenous pattern-associated molecular patterns (PAMP) or endogenous danger-associated molecular patterns (DAMP) (3). NLR are highly conserved throughout evolution attesting to their important role in host defense (4). NLR possess three domains: the N-terminal effector domain that may be a caspase recruitment domain (CARD), a pyrin (PYD) domain, or a baculovirus inhibitor of apoptosis repeat (BIR) domain; the central nucleotide-binding oligomerization domain (NOD); and the C-terminal domain comprised of leucine rich repeat sequences (LRR) (5). Based on their N-terminal domains, NLR can be divided into four main families (**Table 1**). Different NLR have been linked to the detection of different signals, for example, NLRC4 recognizes bacterial flagellin (6, 7) whereas NLRP1 has been implicated in the sensing of anthrax lethal toxin (8), but the specific molecular ligands for a majority of NLRs remain uncharacterized. In some cases, the LRR of the C-terminal bind directly to the PAMP (5); however, the precise mechanism of agonist activation of NLR remains to be determined, as other reports have postulated an auto-inhibitory role for the LRR (9).

Upon sensing of endogenous or exogenous danger signals, some NLR oligomerize *via* their NOD domains. If the NLR contains a CARD domain this can facilitate the recruitment of the inactive enzyme pro-caspase-1, through direct CARD–CARD interactions. However, inflammasome-forming NLR lacking a CARD domain use their PYD domain to recruit the adaptor protein Apoptosis-associated speck-like protein containing CARD (Asc)—comprising a PYD and a CARD domain, and this serves as a scaffold, bridging the interactions between the NLR and pro-caspase-1. This "canonical" inflammasome formation results in the autocatalytic activation of caspase-1. Caspase-1 has two main functions, cleavage of pro-IL-1β and pro-IL-18 into their active forms for secretion (53, 54), and the induction of a specialized form of inflammatory cell death known as pyroptosis (55–57). Another form of inflammasome has been described which does not require a member of the NLR family, but instead contains members of the PYHIN family (PYD and HIN domain containing). For example, the PYHIN family member absent in melanoma 2 (AIM2) can directly bind to its stimulus, double-stranded DNA (dsDNA), which may be present in the cytosol during infection, to form a caspase-1 containing inflammasome (41).

Of emerging interest in the field is the formation of "non-canonical" inflammasomes by caspase-11 and caspase-8. Caspase-11 was originally discovered to be important in caspase-1 and -3 activation (58) and has been found to indirectly increase processing of pro-IL-1β and pro-IL-18 by promoting NLRP3 inflammasome activation (59). Indeed, it was shown that caspase-11 can detect intracellular LPS, and some intracellular bacteria, leading to cell death (60, 61). The human orthologs of murine caspase-11, namely, caspase-4 and -5, appear to serve similar functions (46, 62). Recently, an inflammasome formed by NLRC4, Asc, and potentially caspase-8 was described in a model of enteric *Salmonella enterica* serovar Typhimurium (*S.* Tm) infection, and this inflammasome was required for expulsion of infected intestinal epithelial cells (IEC) (**Table 2**) (24). There has also been a report of caspase-8 driving caspase-1 cleavage and downstream pro-IL-1β cleavage during *Yersinia pestis* infection of macrophages (63). Although immune cells and IEC express both "canonical" and "non-canonical" inflammasome components, how these complexes interact with one another upon stimulation

TABLE 1 | NLR family members and other inflammasome components.

NLR/inflammasome component		Ligand/agonist	Expression in IEC
NLR family			
NLRA (acidic activation domain)	CIITA	Unknown	Yes (10, 11)
NLRB1 (BIR domain)	NAIP1, NAIP2	T3SS (12, 13)	Yes (14–16)
	NAIP5, NAIP6	Flagellin (12, 13)	Yes (14–16)
NLRC (CARD domain)	NLRC1 (NOD1)	iE-DAP (17)	Yes (18)
	NLCR2 (NOD2)	MDP (19, 20)	Yes (18, 21, 22)
	NLRC4	Flagellin, T3SS rod proteins (*via* NAIP) (6, 7, 23)	Yes (24–26)
	NLRC3 + 5	Unknown	ND
NLRP (PYRIN domain)	NLRP1	Anthrax lethal toxin, ATP, and MDP (8, 27)	Yes (28)
	NLRP3	ATP, MSU, toxins, oxidized mitochondrial DNA, alum, silica, UV radiation, amyloid β (5, 29), and SCFA (acetate) (30)	Yes (26, 31)
	NLRP6	Metabolites (e.g., taurine, spermine, and histamine) (32)	Yes (33–35)
	NLRP7	Microbial lipopeptides (36)	ND
	NLRP9b	dsRNA (37)	Yes (37)
	NLRP12	*Yersinia pestis* (38)	ND
	NLRP 2, 4, 5, 8, 10, 11, 13 + 14	Unknown	ND
Unclassified	NLRX1	ssRNA, dsRNA, and poly (I:C) (39)	Yes (40)
Inflammasome components			
AIM2		dsDNA (41)	Yes (42)
Asc		NA	Yes (16, 43)
Caspase-1		NA	Yes (26, 44, 45)
Human caspase-4/ murine caspase-11		LPS (46)	Yes (44, 46–48)
Caspase-8		ND	Yes (24)
IL-1β		NA	Yes (44)
IL-18		NA	Yes (44, 49–52)

SCFA, small chain fatty acids; ND, not determined; NA, not applicable; T3SS, type 3 secretion system; IEC, intestinal epithelial cells; CARD, caspase recruitment domain; AIM2, absent in melanoma 2; dsDNA, double-stranded DNA; NLR, NOD-like receptor.

TABLE 2 | Inflammasome components and intestinal inflammation.

Mutant strain	Trigger	Effect	Reference
Inflammasome components			
Asc$^{-/-}$	DSS	Increased pathology	(33, 42, 52, 131, 164)
		Decreased IL-18 levels	(33, 52)
		Decreased AMP levels	(32)
		Treatment with taurine rIL-18 ameliorated disease	
	C. rod	Increased bacterial colonization	(34, 43, 103)
		Increased pathology	(43, 103)
		Decreased IL-18 levels	(43)
		Decreased mucus secretion by goblet cells	(34)
	Rotavirus	Increased viral load	(37)
Casp1$^{-/-}$ Casp11$^{-/-}$	DSS	Increased pathology	(33, 51, 52, 164)
		Decreased IL-18 levels	(51, 52)
		Phenotype rescued by rIL-18	
	C. rod	Increased bacterial colonization	(34)
	FlaTox	Decreased IEC pyroptosis	(24)
	NSAID-induced SI damage	Decreased pathology	(165)
		Decreased IL-1β levels	
Caspase1$^{-/-}$	DSS	Decreased pathology	(142)
		Decreased IL-18 levels	
	Rotavirus	Increased viral load	(37)
Casp1$^{\Delta IEC}$	DSS	Decreased pathology	(142)
		Decreased IL-18 levels	
Casp1$^{\Delta IEC}$	Rotavirus	Increased viral load	(37)
Caspase11$^{-/-}$	DSS	Increased pathology	(47, 48)
		Increased IL-18	(48)
		Decreased IL-18 and IL-22	(47)
		Phenotype rescued by rIL-18	
	S. Tm	Decreased IL-18 levels	(44)
		Decreased pathology	
		Increased intraepithelial bacterial burden	
		Decreased IEC extrusion	
gasdermin D$^{-/-}$	FlaTox	Decreased IEC pyroptosis	(24)
gasdermin D$^{-/-}$	Rotavirus	Increased viral load	(37)
		Decreased IEC death	
Casp1$^{-/-}$Casp8$^{-/-}$ Ripk3$^{-/-}$	*S.* Tm FlaTox	Decreased IEC extrusion	(24)
NLR proteins			
NAIP1–6$^{\Delta/\Delta}$	*S.* Tm	Increased intraepithelial bacterial loads	(14)
		Decreased IEC expulsion	
NAIP1–6$^{\Delta/\Delta IEC}$	*S.* Tm	Increased intraepithelial bacterial loads	(14)
NLRC4$^{-/-}$	DSS	Increased pathology	(30)
	C. rod	Increased bacterial colonization	(25)
		Increased pathology	
		Decreased IL-18 at steady state	
	S. Tm	Increased intraepithelial bacterial loads	(14)
iNLRC4^{+}Vil-Cre^{+}	*S.* Tm FlaTox	Comparable bacterial burden	(24)
		Comparable IL-18 and PGE_2 levels	
		Comparable caspase-1 and caspase-8 activation	
NLRP1$^{-/-}$	DSS	Increased pathology	(131)
		Rescued by treatment with rIL-1β or rIL-18 or antibiotics	
NLRP3$^{-/-}$	DSS	Increased pathology	(30, 42, 52, 164)
		Decreased pathology	(166)
		Decreased IL-1β	
	C. rod	Increased pathology	(43, 103)
		Increased bacterial colonization	(43, 103)
	T cell transfer colitis	Increased pathology upon transfer of NLRP3$^{-/-}$ T cells into lymphopenic hosts	(167)
		Increased Th17 cells and decreased Th1 cells	
	NSAID-induced SI damage	Decreased pathology	(165)
		Decreased IL-1β levels	
NLRP6$^{-/-}$	DSS	Increased pathology	(33)
		Decreased IL-18 levels	(32, 33)
		Decreased AMP levels	(32)
	C. rod	Increased bacterial colonization	(34)
		Decreased mucus secretion by goblet cells	
		Decreased autophagosome formation	
NLRP9b$^{-/-}$	Rotavirus	Increased viral load	(37)
		Decreased IEC death	
NLRP9b$^{\Delta IEC}$	Rotavirus	Increased viral load	(37)
NLRP12$^{-/-}$	DSS	Increased pathology	(168–170)
NLRX1$^{\Delta IEC}$	DSS	No change in pathology	(40)
		Increased IEC proliferation	
PYHIN sensors			
AIM2$^{-/-}$	DSS	Increased pathology	(42, 129)
		Decreased IL-1β levels	(129)
		Decreased IL-18 levels	(42, 129)
		Decreased IL-22BP levels	(42)
		Dysregulated AMP levels	(42, 129)

AMP, antimicrobial peptides; C. rod, Citrobacter rodentium; FlaTox, Legionella pneumophila flagellin fused to the N-terminal domain of Bacillus anthracis lethal factor; NAIP5, ligand delivered to cytosol; IEC, intestinal epithelial cells; NSAID, non-steroidal anti-inflammatory drugs; SI, small intestine; S. Tm, Salmonella Typhimurium; DSS, dextran sodium sulfate; rIL-18, recombinant IL-18; NLR, NOD-like receptor. Mutant strain: Casp1$^{\Delta IEC}$, caspase-1-deficient IEC; NAIP1–6$^{\Delta/\Delta IEC}$, NAIP1–6-deficient IEC; iNLRC4^{+}Vil-Cre^{+}, NLRC4 only expressed in IEC; NLRP9$^{\Delta IEC}$, NLRP9b-deficient IEC; NLRX1$^{\Delta IEC}$, NLRX1-deficient IEC.

and tailor their responses (e.g. pro-inflammatory cytokine secretion versus pyroptosis) remains to be elucidated (**Figure 1**).

Innate immune recognition at mucosal surfaces, in particular the intestine, is a critical mediator of homeostasis (64). Indeed, in the gut, PRR sensing has been implicated in several key processes, such as maintenance and repair of the epithelial barrier and

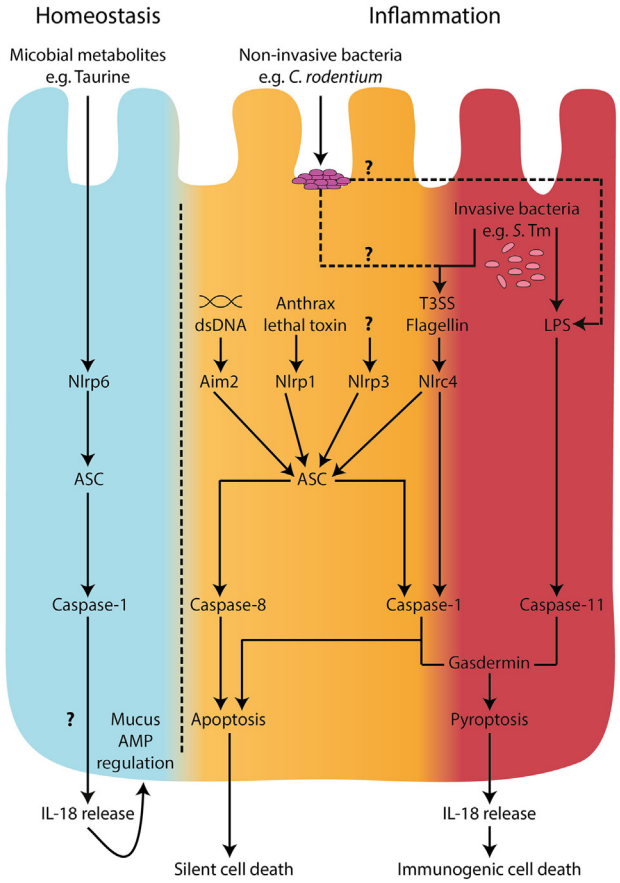

FIGURE 1 | Inflammasomes in intestinal epithelial cells. During homeostatic conditions, in the absence of inflammation, IL-18 is released from epithelial cells and is involved in epithelial repair, proliferation, and maturation (33, 34). A metabolomics screen identified microbiome-derived metabolites, including taurine, that are capable of modulating NLRP6 inflammasome activation and subsequent IL-18 secretion (32). However, the mechanisms of release of IL-18 during homeostatic conditions are undefined. In the context of microbial invasion and pathogen-associated molecular pattern stimulation, inflammasome activation in intestinal epithelial cells has been described to engage both "canonical," caspase-1-mediated and "non-canonical," caspase-11 pathways (14, 24, 44). Recently, caspase-8 was also shown to be involved in inflammasome responses downstream of NLRC4 engagement with intracellular flagellin (24). Both caspase-1 and caspase-11 can lead to cell death by pyroptosis accompanied by IL-18 secretion; however, caspase-1 and caspase-8 were shown to lead to a non-lytic form of cell death upon NLRC4 sensing of intracellular flagellin (24). These observations raise the possibility of a distinction between a pro-immunogenic cell death signal driven by caspase-11 and GsdmD, a pro-silent cell death driven by caspase-8, and perhaps a threshold-dependent cellular decision between non-lytic and lytic forms of cell death involving caspase-1. Under low stress levels, it would be desirable to deal with the invading threat in an immunologically silent way. However, when the threat is high, an immunogenic cell death could recruit inflammatory cells to help clear the microbial insult.

production of antimicrobial peptides (AMP) (65–67). Aside from basal roles at steady state, effective PRR signaling also protects against enteric pathogens by initiating immune responses **Tables 2** and **3** (68–70). To date, the majority of work has focused on the role of the hematopoietic compartment in microbial detection and inflammation, but non-hematopoietic cells, particularly IEC, are now appreciated to be important contributors to PRR sensing circuits in the gut (71).

Intestinal epithelial cells face a unique challenge as they constitute the first cellular border between the complex contents of the gut lumen and the largely sterile subepithelial compartment. This intestinal epithelial surface area is greatly increased by gland like invaginations called crypts, as well as projections of small finger like protrusions in the small intestine, known as villi. IEC are composed of various specialized cell types; enteroabsorptive cells, goblet cells, Paneth cells, neuroendrocrine cells, tuft cells, and stem cells. Due to the constant epithelial turnover, stem cells are responsible for replenishing any lost cells *via* Notch-mediated epithelial cell differentiation (72). Goblet cells secrete heavily glycosylated mucins which form a mucus matrix (73) into which Paneth cells secrete antimicrobial peptides (AMP) (74–76), together providing a physical and chemical barrier between the epithelial cell layer and the luminal contents. This barrier is further fortified by the secretion of IgA dimers into the mucus layer which act to sterically hinder any potential threats (77). In addition, goblet cells have been reported to deliver luminal antigens to subepithelial antigen-presenting cells enabling screening of the luminal contents (78). Thus, there are numerous antimicrobial mechanisms employed by the epithelium to limit access of potentially inflammatory stimuli.

During homeostasis, interactions with the microbial and dietary antigens induce a non-inflammatory IEC state that promotes immune tolerance. However, luminal content occasionally carries pathogenic microorganisms or toxic particles capable of causing mucosal damage and, in severe cases, systemic disease. Accumulating evidence suggests that the inflammasome plays a key role in modulating epithelial responses at the host–lumen interface. Data generated on purified IEC, *in situ* detection, or cell-specific ablation have revealed an expression of an array of inflammasome components within IEC including; NAIP, NLRC4, NLRP1, NLRP6, AIM2, caspase-1, caspase-4/5 (human), caspase-11 (mouse), Asc, and IL-18 (**Tables 2** and **3**) (79, 80). This review will discuss the functional importance of the inflammasome and its components within the context of epithelial cells and intestinal inflammation.

INFLAMMASOMES AND THEIR SOLUBLE MEDIATORS IN INTESTINAL HOMEOSTASIS

Inflammasome formation and caspase-1 activation lead to cleavage and secretion of the active forms of IL-1 family member cytokines, such as IL-1β and IL-18. These cytokines play a central role in immunity due to their diverse array of biological functions and broad range of target cells. IL-1β is a potent pro-inflammatory cytokine exerting a plethora of systemic and local effects. IL-1β promotes the recruitment of immune cells to the site of inflammation *via* induction of adhesion molecules and chemoattractants (81, 82). Stimulation with IL-1β promotes the activation and effector functions of dendritic cells, macrophages, and neutrophils (83). In addition, IL-1β plays a role in adaptive

TABLE 3 | Soluble mediators of inflammasome activation and intestinal inflammation.

Mutant strain	Trigger	Effect	Reference
IL-1R1 signaling pathway			
IL-1αβ$^{-/-}$	*S*. Tm	No effect on intraepithelial bacterial load	(14)
IL-1β$^{-/-}$	DSS	Decreased pathology Hematopoietic expression (monocytes)	(98)
	C. rod	Increased bacterial colonization Increased pathology	(103)
IL-1R1$^{-/-}$	DSS	Increased pathology	(104)
	C. rod	Increased pathology	(104)
	T cell transfer colitis	Decreased pathology upon transfer of IL-1RI$^{-/-}$ T cells into lymphopenic hosts Decreased Th17 cell survival	(101)
IL-18R signaling pathway			
IL-18$^{-/-}$	–	Increased intestinal Th1 and Th17 effector cells Non-hematopoietic expression [intestinal epithelial cells (IEC)]	(49)
	DSS	Increased pathology	(33)
	C. rod	No effect on bacterial colonization Increased bacterial colonization Increased pathology	(104) (103)
	S. Tm	No effect on intraepithelial load	(14)
	Rotavirus	Comparable viral load	(37)
IL-18Tg	DSS	Increased pathology	(123)
IL-18$^{\Delta IEC}$	DSS	Decreased pathology	(110)
IL-18$^{\Delta/HE}$	DSS	Decreased pathology	(110)
IL-18R$^{-/-}$	–	Increased intestinal Th1 and Th17 effector cells Decreased intestinal Treg function	(49)
	C. rod	Increased bacterial colonization Increased pathology	(43)
IL-18r$^{\Delta IEC}$	DSS	Decreased pathology	(110)
IL-18r $^{\Delta/HE}$	DSS	No difference in pathology	(110)
IL-18bp$^{-/-}$	DSS	Increased pathology Increased goblet cell loss	(110)
IL-18bp$^{-/-}$ IL-18r$^{\Delta/HE}$	DSS	No difference in pathology	(110)

C. rod, Citrobacter rodentisum; S. Tm, Salmonella Typhimurium; Treg, T regulatory cells.

Mutant strain: IL-18Tg, IL-18 transgenic: overexpression of IL-18; IL-18$^{\Delta IEC}$, IL-18-deficient IEC; IL-18$^{\Delta/HE}$, IL-18-deficient hematopoietic cells; IL-18r$^{\Delta IEC}$, IL-18R-deficient IEC; IL-18r $^{\Delta/HE}$, IL-18R-deficient hematopoietic cells; IL-18bp$^{-/-}$, IL-18 binding protein-deficient; IL-18bp$^{-/-}$IL-18r$^{\Delta/HE}$, IL-18bp$^{-/-}$ with IL-18R-deficient hematopoietic cells.

immunity driving T cell activation and survival (84), and acting in concert with other cytokines to promote Th17 cell differentiation (85). Due to these highly pro-inflammatory properties, IL-1β release is tightly regulated *via* a two-step process, namely, TLR-induced production of an inactive ~31–34 kDa precursor pro-IL-1β, followed by caspase-1 dependent cleavage and secretion of the active form (86).

Several clinical studies reported high levels of IL-1β production by the lamina propria mononuclear cells from active colonic lesions of IBD patients (87–89). IL-1β levels in the colon correlated with disease activity suggesting an important role for this cytokine in driving local inflammation (90, 91). Elevated colonic IL-1β levels are also characteristic of many animal IBD models (92–94), and strategies blocking IL-1β signaling were beneficial in ameliorating acute models of intestinal inflammation (95–98). Moreover, genetic alterations of key innate immune molecules, such as NOD2 and Atg16l1, resulted in over production of IL-1β by macrophages and enhanced susceptibility to dextran sodium sulfate (DSS)-mediated intestinal injury (99, 100). In addition, IL-1β augmented the recruitment of granulocytes and the activation of innate lymphoid cells during *Helicobacter hepaticus*-triggered intestinal inflammation, and IL-1R signaling in T cells controlled the early accumulation and survival of pathogenic Th17 cells in the colon during T cell transfer colitis (101). The role of IL-1β in promoting intestinal inflammation has also been confirmed in infection studies, as blocking IL-1β ameliorated pathology in both *Clostridium difficile*-associated colitis and *Salmonella* Typhimurium-induced enteritis (68, 102). However, alternative studies suggest a protective role for IL-1β during *Citrobacter rodentium* induced intestinal inflammation, as IL-1R1$^{-/-}$ and IL-1β$^{-/-}$ animals suffered from increased bacterial loads and pathology (**Table 3**) (103, 104).

Although IL-1β signaling appears to play a predominant role in mediating intestinal inflammation, IEC do not produce significant levels of IL-1β themselves (44, 105). Interestingly, stratified epithelia at other sites produce considerable amounts of IL-1β upon activation of their NLRP3 inflammasome (106, 107). The significance of differential IL-1β expression between epithelial cell types in distinct tissues remains incompletely explored. In the gut, it appears that lamina propria phagocytes constitute the main source of IL-1β during intestinal inflammation (101, 108).

In contrast, there is substantial evidence for the expression and secretion of IL-18 by the intestinal epithelium. Notably, at steady state in the intestine IEC appear to be the primary source of IL-18 (44, 49, 50, 109). The inactive 24 kDa precursor pro-IL-18 is constitutively expressed by IEC, primed for release upon inflammasome activation (44, 49, 50, 109, 110). Akin to IL-1β, IL-18 has been shown to induce a diverse array of immune responses. Originally termed IFNγ-inducing factor, IL-18 is typically considered a Th1 promoting cytokine due to its ability to elicit IFNγ production by T cells (111). However, in the presence of the correct co-stimuli, IL-18 can also drive Th2 cytokine production (112), or IL-17 production by γδ T cells (113). In addition, IEC derived IL-18 can drive perforin production by NK cells during enteric infection with *S.* Typhimurium, revealing an important role for IEC in coordinating acute mucosal responses (114).

Genome-wide association studies have linked mutations within the IL-18R1-IL-18RAP locus with susceptibility to IBD (115–117). Furthermore, increased IL-18 levels were detected in the biopsies of CD patients (50, 118). Using immunohistochemical analysis, IL-18 localized to the epithelium of non-inflamed regions, whereas in involved regions IL-18 was detected in cells morphologically described as macrophages (50). However, this altered IL-18 distribution was specific to CD, as UC patients displayed an epithelial distribution of IL-18 regardless of severity

(50). Moreover, the bioactivity of mature IL-18 is regulated by the production of IL-18 binding protein, levels of which are also elevated in CD patients (119, 120). Thus, although the contributions of IL-18 to clinical intestinal inflammation remain unclear, evidence suggests that dysregulated IL-18 signaling could influence intestinal inflammation.

In murine models, different studies have drawn conflicting conclusions on whether IL-18 plays a predominantly pathogenic or protective role in intestinal inflammation. Early studies using biochemical inhibition of IL-18 signaling revealed a detrimental role for the cytokine in intestinal inflammation mediated by DSS (121, 122). Furthermore, overexpression of *IL-18* in IL-18 transgenic mice resulted in increased severity of DSS-mediated intestinal injury (**Table 3**) (123). Hyperproduction of IL-18 in mice deficient in Atg16l1, a key autophagy adaptor molecule, was also associated with increased susceptibility to DSS, a phenotype which was rescued by antibody-mediated blockade of IL-18 (100). This exacerbated inflammation associated with IL-18 may be due to its ability to induce pro-inflammatory effector T cell activation, even in the absence of T cell receptor engagement (111, 113, 124, 125). In fact, intestinal T cells express significantly greater amounts of IL-18R than those found in systemic lymphoid tissues, suggesting that they may be particularly sensitive to IL-18 signaling (49). Indeed, blocking IL-18 signaling protected mice against colitis mediated by transfer of naive T cells into lymphopenic hosts (126).

Conversely, independent studies using IL-18- and IL-18R-deficient mice revealed a beneficial role for IL-18 signaling during DSS colitis (**Table 2**) (127, 128). In addition, caspase-1$^{-/-}$ animals were more susceptible to DSS-mediated colitis, which was associated with decreased epithelial cell proliferation and IL-18 secretion (51). This was corroborated by Zaki et al., who also observed increased susceptibility to DSS colitis in the absence of caspase-1 (**Table 2**) (52). Interestingly, this exacerbated phenotype could be rescued through administration of recombinant IL-18 (rIL-18), but not by adoptive transfer of myeloid cells, suggesting that IL-18 expression in the non-hematopoietic compartment was essential for protection (51, 52). Similarly, non-hematopoietic NLRP6 expression was found to be necessary to protect against DSS colitis, an effect that was again associated with impaired IL-18 production (33). In addition, deficiencies in NLRP6 were associated with a dominant dysbiosis (33) and decreased microbiota diversity (32), with rIL-18 treatment ameliorating this effect by increasing AMP production by IEC (32). Furthermore, a metabolomics screen identified potential microbiome-derived metabolites capable of modulating NLRP6 inflammasome activation and subsequent IL-18 secretion (32). Thus, deficiencies in NLRP6 expression are associated with reduced IL-18 production and the emergence of a dysbiotic microbiome that sensitizes mice to exacerbated DSS-mediated intestinal inflammation. In addition, deficiency in the cytosolic dsDNA sensor AIM2 also led to increased pathology upon DSS administration, which was again associated with decreased IL-18 signaling (**Table 2**) (42, 129).

In fact, DSS colitis is ameliorated in antibiotic treated genetically susceptible mice (33, 42, 98, 129–131), or exacerbated in mice receiving transfers of pathobionts (98), signifying the importance of the microbiota composition in this model. Microbiota sensing may also mediate protective effects against DSS colitis as evidenced by reports of exacerbated disease in germ-free mice (132) and Myd88$^{-/-}$ mice (133). A key caveat of many studies using DSS colitis models in mice with genetic deficiencies is that they did not employ appropriate co-housing strategies to minimize any potential effects of the microbiota. As such, variations or "dysbiosis" in the microbiota may have occurred as a result of long-term microbial divergence due to extended isolation of breeding cohorts, as was reported for TLR-deficient mice (134). Therefore, studies in which inflammasome-deficient strains were compared to independent breeding cohorts of wild type mice must be interpreted with caution. In addition, these conflicting results emphasize the importance of using littermate controls to evaluate potential differences in susceptibility to experimental colitis in genetically modified mice.

Epithelium-derived IL-18 has also been implicated in protecting against infection-associated intestinal inflammation. For example, IL-18-deficient or IL-18R-deficient mice were more susceptible to colonization and inflammation upon infection with *C. rodentium* (**Table 3**) (43, 103, 109). Similarly, caspase1$^{-/-}$ animals suffered from increased susceptibility to *C. rodentium* infection which was associated with increased inflammatory responses and decreased IL-18 secretion, suggesting a protective role for IL-18 in this model (103). Consistent with these findings, mice deficient in NLRP3 or Asc also suffered from exacerbated *C. rodentium* infection and pathology (43). Furthermore, non-hematopoietic cells were the source of this protective NLRP3 and Asc circuit, with strong Asc expression evident in the IEC (43). However, although *C. rodentium*-infected Asc$^{-/-}$ animals almost completely lacked IL-18 in the intestine, the absence of NLRP3 did not affect IL-18 secretion (43). Thus, NLRP3 signaling may be mediating alternative protective pathways aside from IL-18 production (43). NLRC4 expression in IEC is also important for protection against *C. rodentium* induced intestinal inflammation, and NLRC4 deficiency was associated with decreased basal IL-18 levels and increased early pathogen colonization of the epithelium (25). Thus, the discrepancies in intestinal IL-18 production between the NLRP3- and Asc-deficient mice may be explained in part by compensation of the NLRC4 inflammasome in the absence of NLRP3 expression. Finally, NLRP6 inflammasome expression was also reported to protect against *C. rodentium* induced inflammation, and this was linked to effective mucin granule exocytosis by goblet cells (**Table 2**) (34). In addition, NLRP6 inflammasome formation and subsequent IL-18 secretion also enhanced AMP production by IEC (32). The non-redundant requirement for several NLR in protection from attaching and effacing pathogens like *C. rodentium* suggests that distinct NLR may mediate slightly different protective responses in IEC and/or that activation of NLR in additional cell types may contribute to epithelial protection. In addition, whether and how different inflammasomes interact during *C. rodentium* infection remains to be fully elucidated, although there is some evidence for the interaction of NLRP3 and NLRC4 inflammasomes during *S.* Typhimurium infection (135).

The epithelial protective effects of IL-18 may be explained by its roles in wound healing (127, 136) and in driving IL-22 (109), a cytokine important for AMP production and mucosal barrier

integrity (137, 138). Of note, IL-22 expression has been shown to protect mice against several models of IBD (139, 140). In fact, administration of rIL-18 to IEC decreased their production of IL-22 binding protein allowing for greater amounts of IL-22 signaling (42). Interestingly, co-administration of IL-22 and IL-18 induced reprogramming of IEC gene expression, not observed with either cytokine alone, which correlated with protection against rotavirus infection, suggesting that these cytokines may act in concert in the intestine to promote antimicrobial responses (141). In addition, IL-18 has also been demonstrated to promote optimal T regulatory cells responses in the gut, with the lack of IL-18 associated with increased proinflammatory T effector cells (49).

Such studies have led to the conclusion that epithelial-derived IL-18 promotes barrier integrity and maintains a healthy microbiota, which contributes to protection against intestinal injury and inflammation. However, this function of IL-18 has been inferred from complete deletion of inflammasome components, as well as the cytokine itself, alongside bone marrow chimera studies. Recently, studies have been conducted using IEC-specific IL-18 knockouts (IL-18$^{\Delta IEC}$) (110) and IEC-specific caspase-1 knockouts (Casp1$^{\Delta IEC}$) (**Tables 2** and **3**) (142). These studies reported that caspase-1 activation and consequent IL-18 secretion by IEC during DSS colitis was associated with exacerbated inflammation and decreased goblet cell maturation (110, 142). These findings are somewhat surprising, as NLRP6 deficiencies were previously associated with both decreased IL-18 levels (33) and goblet cell mucus secretion (34), which led to increased susceptibility to DSS-mediated intestinal injury. In addition, several studies demonstrated that rIL-18 administration rescued inflammasome-deficient phenotypes from hypersusceptibility to DSS colitis (32, 47, 51, 52, 131). Considering these publications, the authors argue that extrapolation of IL-18 functions from mice fully deficient in inflammasome components should be interpreted with caution, as such deletions may affect the myeloid compartment beyond the scope of IL-18 production (i.e., there could be confounding effects on IL-1β production and pyroptosis). However, numerous bone marrow chimera experiments pointed to the importance of non-hematopoietic inflammasome expression in mediating protection against intestinal inflammation (25, 43, 51, 52, 129). As noted above, it is very likely that the microbiota is a key confounding factor, therefore repeating DSS colitis in IL-18$^{\Delta IEC}$ mice housed in alternative vivariums could help clarify the contribution of genotype versus microbiota. Clearly, further studies using mice with cell-type specific ablation of inflammasome components (or effector molecules) need to be carried out to better understand their diverse functions in IEC.

In addition to IL-1 family cytokines, inflammasome activation affects the release of alternative bioactive factors by immune cells. The alarmin high-mobility group box 1 (HMGB1) was originally identified as a nuclear DNA-binding protein. Upon infection or injury, inflammasomes were shown to mediate extracellular release of HMGB1 from stimulated immune cells triggering inflammation (143, 144). In the context of epithelial cells, LPS transfection of IEC led to HMGB1 release (46) and infection of gingival epithelial cells with *Fusobacterium nucleatum* drove release of HMGB1 alongside Asc and IL-1β secretion (107), suggesting that inflammasomes may be involved in the active secretion of HMGB1 from IEC. Caspase-1 activation has also been hypothesized to play a role in unconventional protein secretion of leaderless peptides such as IL-1α and FGF_2 from macrophages (145). Others have postulated that AMP may be regulated *via* post translation modification by an effector downstream of inflammasome activation (146). The lipid inflammatory mediators, eicosanoids, have also been linked to inflammasome-dependent unconventional secretion (147, 148). In fact, the eicosanoid prostaglandin PGE_2 was secreted by murine IEC upon NLRC4 inflammasome activation (24). Examination of the downstream soluble mediators of inflammasome activation, aside from IL-1β and IL-18, remains comparatively understudied in IEC compared to classical immune cells. Future work will need to address this by systematically examining the inflammasome-dependent secretome of activated IEC, and its downstream activities.

INFLAMMASOMES AND CELL DEATH: PYROPTOSIS AND APOPTOSIS

Inflammasome functional studies to date have largely focused on the secretion of downstream soluble mediators. However, there is much emerging interest in the role of inflammasome-dependent cell death, termed pyroptosis, an inflammatory form of cell death (149, 150). Pyroptosis takes place following engagement of "canonical" (caspase-1) or "non-canonical" (caspase-11) inflammasomes. "Non-canonical" triggering of pyroptosis occurs by intracellular LPS engagement with caspase-11 and has mainly been described in macrophages (151, 152). Identification of the "non-canonical" pathway followed from the finding that 129SvEv mice carried a passenger mutation that truncated the *caspase-11* gene (59), meaning that the original *caspase-1* knockout mice, which were generated on a 129SvEv background, were deficient in both *caspase-1* and *caspase-11*. Using caspase-11 complementation, Kayagaki et al. showed that macrophages underwent caspase-1-independent "non-canonical" cell death in response to several inflammasome activating stimuli, including Gram-negative bacteria such as *Escherichia coli*, *Vibrio cholerae*, and *C. rodentium,* as well as LPS co-treatment with cholera toxin subunit B (59). Subsequently, it was found that macrophages that were loaded with intracellular LPS activated caspase-11 and died by pyroptosis, and that mice lacking caspase-11 were protected from LPS-induced endotoxemia and pyroptosis (59–61). Finally, two independent studies identified caspase-11 as the key intracellular receptor for LPS (46, 153).

Caspase-11-driven pyroptosis has been shown to be key for protection against intracellular pathogens, particularly those that can escape from phagocytic vacuoles, such as *S.* Tm (151, 154, 155). However, studies with phagocytes and embryonic fibroblasts reported that caspase-1 "canonical" inflammasomes were required for efficient processing of IL-1β and IL-18, even in the context of direct caspase-11 activation, which was only able to lead to cytokine cleavage *via* indirect activation of caspase-1

(156–159). Nevertheless, caspase-11-dependent activation of IL-18 has also been reported, for instance, cecal tissue explants from *S.* Tm-infected *caspase-11*-deficient mice were also defective in IL-18 but not IL-1β secretion (44). Furthermore, colonic tissue explants from *C. rodentium*-infected *caspase-11*-deficient mice also had decreased IL-18 secretion (160). This caspase-11-dependent IL-18 processing was proposed to occur in IEC, contrary to the caspase-1-dependent cleavage of IL-18 and IL-1β observed in myeloid cells (161).

The importance of "canonical" and "non-canonical" inflammasomes may vary depending on the nature and characteristics of the pathogenic threat and the cell types involved. For example, upon challenge with flagellin-deficient *Salmonella,* caspase-1-deficient macrophages died in a similar manner to WT macrophages, whereas caspase-11-deficient macrophages were resistant to cell death (158, 161). In contrast, both caspase-1 and caspase-11 were required for cell death in macrophages infected with WT *Salmonella* (158). This highlights the fact that *Salmonella* can activate both the "canonical" inflammasome, through flagellin–NAIP–NLRC4 interactions, and the "non-canonical" inflammasome, through direct LPS–caspase-11 interactions (**Figure 1**). The complementary roles of "canonical" and "non-canonical" inflammasomes are especially important in the context of bacterial infections. Bacterial evasion strategies can counteract inflammasome responses, such as inhibition of epithelial caspase-11 *via* NleF, a type 3 secretion system effector protein produced by *E. coli* and *C. rodentium* (160). In a caspase-11-deficient scenario, however, pyroptosis may still proceed due to intact caspase-1 activation, highlighting potential redundancy of these two caspases (162). It seems logical that the intestinal epithelium, as a first line of defense, would have intrinsic mechanisms to warn the immune system of an invading threat. Indeed, as noted above, caspase-11 in mice (an ortholog of human caspases-4/5) is important for the recognition and clearance of *S.* Tm, and mice lacking caspase-11 harbor increased loads of *S.* Tm in the intestinal epithelium (14, 44). Furthermore, siRNA knockdown of caspase-4 in human colonic IEC led to reduced cell death upon *E. coli*, *S.* Tm, and *Shigella flexneri* infection (44, 163), and this was accompanied by increased *S.* Tm intracellular load, and reduced IEC shedding (44, 161).

Recent studies, in addition to highlighting the importance of "non-canonical" inflammasomes in innate immune defense in IEC, have also shed some light on the mechanisms involved in IEC-intrinsic restriction of *S.* Tm invasion. The innate immune sensor NLRC4 and its NAIP adaptors were shown to be essential for the extrusion of infected IEC into the intestinal lumen following *S.* Tm challenge of streptomycin-treated mice (14). IEC extrusion may represent a cell-intrinsic defense mechanism that serves to limit the rate of pathogen colonization of the intestinal epithelium. In this study, it was unclear whether IEC extrusion was linked to pyroptosis, as plasma membrane integrity of extruded enterocytes seemed to be maintained (14). However, by using an inducible construct to drive the expression of NLRC4 specifically in the intestinal epithelium, Rauch et al. showed that IEC-specific NLRC4 activation by FlaTox (*Legionella pneumophila* flagellin fused to the N-terminal domain of *Bacillus anthracis* lethal factor to drive cytosolic delivery) was sufficient to drive pathology, IEC death and IL-18 release (24).

Moreover, in agreement with the findings of Sellin et al., FlaTox activation of NLRC4 in IEC also limited *S.* Tm colonization of intestinal tissues and drove IEC death and extrusion (14). However, FlaTox-induced expulsion of IEC was accompanied by lytic cell death with plasma membrane permeabilization, resembling pyroptosis (24) (see **Table 4** for morphological features of pyroptosis). From these studies, it becomes clear that, upon NLRC4 activation, IEC can undergo cell death and expulsion from the intestinal epithelium. In parallel, experiments in which caspase-1 expression was selectively induced in IEC, it was found that caspase-1 could drive pyroptosis in response to NLRC4 activation by FlaTox. On the contrary, caspase-1-deficient IEC did not undergo lytic cell death but were expelled from the epithelial layer with intact plasma membranes, indicating that caspase-1 was required for pyroptosis but not for IEC extrusion (24). Furthermore, they also observed that caspase-1-independent IEC extrusion following NLRC4 activation was dependent on both Asc and caspase-8 (24). Taken together, these studies show that various inflammasome-dependent responses are triggered in IEC during *S.* Tm infection, and these encompass activation of NLRC4, caspase-1, caspase-11 and possibly caspase-8 (14, 24, 44) (see **Figure 1**). However, it is unclear how the different inflammasome responses are regulated in IEC and if they

TABLE 4 | Characteristic features of different cell death pathways.

Characteristic of the dying cell	Apoptosis	Necrosis	Pyroptosis	Necroptosis
DNA fragmentation	+ (171–173)	+/– (171, 172)	+ (174–177)	? (See necrosis)
Nuclear condensation	+ (171–173)	(172, 178)	+ (179)	– (172, 180)
Nuclear integrity maintained	– (171–173)	+ (171, 172)	+ (181)	+ (172, 180)
Cell swelling	– (171–173)	+ (171, 172)	+ (175)	+ (172, 180)
Lysis/membrane permeability	– (171–173)	+ (171, 172)	+ (175)	+ (178)
Membrane blebbing and shedding	+ (171–173)	– (171, 172)	– (182)	? (See necrosis)
Membrane pore formation	–	–	+ (183–185)	+ (186, 187)
DAMP release	–	+ (188)	+ (179)	+ (178)
IL-1β and IL-18 release	–	–	+ (179)	–
Main caspases	casp-3 and casp-7	Non-caspase mediated	casp-1 and casp-11 (mouse) casp4 and casp-5 (humans)	Non-caspase mediated (189)

+, present; –, absent; +/–, present to a limited degree; ?, not yet assessed.
GsdmD, gasdermin D; DAMP, danger-associated molecular pattern.

are redundant, complementary, or interdependent. In addition, further studies are required to better define the precise kinetics and interconnections between downstream responses, such as IEC expulsion and cell death.

The detailed role of pyroptosis *in vivo* remains largely unexplored due to limited knowledge of the downstream targets of caspase-1 and caspase-11 culminating in cell death. Recently, however, gasdermin D (GsdmD) was identified as a direct downstream target of caspase-1 and caspase-11 and was shown to be required for pyroptosis upon "canonical" and "non-canonical" inflammasome engagement (150, 183, 184, 190, 191). Indeed, upon GsdmD cleavage by caspase-1 or caspase-11, its ~30 kDa N-terminus embeds itself in the plasma membrane, forming 10–14 nm pores and ultimately leading to lytic cell death (184, 185, 192). Interestingly, GsdmD is highly expressed in the intestinal epithelium, suggesting that GsdmD may also be involved in pyroptosis in IEC (192, 193). Consistent with this hypothesis, IEC pyroptosis in response to *in vivo* administration FlaTox did not proceed in gasdermin D-deficient mice (24). A very recent study using a mouse model of rotavirus infection reported that activation of a novel NLR inflammasome that recognizes viral dsRNA, NLRP9b, contributed to the restriction of rotavirus replication in IEC organoids, at least partly through gasdermin D-induced pyroptosis (37). Furthermore, mice deficient in either GsdmD or NLRP9b displayed increased susceptibility to rotavirus infection *in vivo* (37). Collectively, these reports suggest that different IEC inflammasomes converge on GsdmD-induced pyroptosis to restrict pathogen load in infected IEC.

Pyroptosis shares a number of morphological features with both apoptotic and necrotic forms of cell death (**Table 4**). Akin to necrosis, in pyroptosis, nuclear integrity is maintained, and the cell undergoes cytoplasmic swelling due to membrane permeabilization that ultimately terminates in cell lysis (174, 179). Akin to apoptosis, pyroptotic cells exhibit DNA fragmentation and are TUNEL positive, as well as presenting nuclear condensation (174–176, 179). Before the acknowledgment of pyroptosis as a new form of cell death (57), its similarities to necrosis and apoptosis led researchers to attribute inflammasome-driven cell death to only apoptosis and/or necrosis (174, 176, 179). It is partly for this reason that the interconnections between the different types of cell death upon inflammasome activating stimuli remain poorly understood. The discovery of GsdmD as a key player in pyroptosis should help elucidate the molecular pathways involved (150, 191, 192).

In addition to pyroptosis, inflammasome responses in various cell types have also been linked to apoptotic cell death. For instance, ectopic expression of NLRC4 and Asc in HEK293T cells (which lack caspase-1) showed that these molecules can engage with caspase-8 to drive apoptosis (194). Furthermore, both apoptosis and pyroptosis have been observed in macrophages following NLRP3 or AIM2 activation (194, 195). Interestingly, macrophages lacking GsdmD were reported to undergo cell death upon LPS plus *S.* Tm or nigericin treatment, through a poorly defined mechanism that was independent of caspase-1, were delayed compared to pyroptosis, and had some features of apoptosis (192).

The literature also suggests some cross-regulation between pyroptosis and apoptosis as THP-1 cells treated with etoposide, an apoptosis inducing drug, resulted in the cleavage of GsdmD into a ~43 kDa fragment, different from the 30 kDa fragment observed in pyroptosis, that occurred independently of caspase-1 (196). The generation of the 43 kDa fragment was observed upon caspase-3 and -7 activation during apoptosis. This suggests that the apoptosis and pyroptosis pathways may compete for the same substrate and that cells may not be able to simultaneously undergo both forms of cell death. The authors speculated that the alternative cleavage of GsdmD by apoptotic caspases-3 and -7 may prevent apoptotic cells from becoming pyroptotic, thus maintaining and immunologically silent cell death (196).

Other studies suggest that differing thresholds may operate between the two cell death pathways following inflammasome activation. For example, in macrophages, for caspase-8 dependent apoptosis to occur upon AIM2 activation, the concentrations of DNA required were much lower than for pyroptosis (195). Under low stress levels, it would be desirable to deal with the invading threat in an immunologically silent way to avoid hyper inflammation, thus apoptosis would be favored. However, when the threat is high, an inflammatory response could help deal with the microbial insult, therefore pyroptosis may be beneficial. However, it is important to stress that it remains to be demonstrated if this threshold-dependent decision controls differential cell death pathways following inflammasome activation *in vivo* and in cells other than macrophages.

LINKING INFLAMMASOME EFFECTOR MECHANISMS

Both IL-1β and IL-18 lack signal peptides and therefore are not secreted through the conventional ER–Golgi pathway (197–199). For IL-1β, the better described cytokine of the two, several routes of release have been proposed, including secretory lysosomes, exosomes, and microvesicles (200–204). The secretory exosome pathway was proposed through the observation that IL-1β in monocytes was localized in endosomal-like vesicles that are normally targeted for degradation, but can be redirected to the extracellular space (202, 205). In addition, microvesicle-mediated rapid secretion was proposed after observing vesicles associated with bioactive IL-1β as early as 2 min post-ATP stimulation in activated monocytes (203). However, studies on these secretory routes were often contradictory and employed different cell systems, thus these models of secretion remain controversial (206). The mechanisms of secretion of IL-18 are generally assumed to follow the mechanisms of IL-1β secretion but are much less investigated.

However, pyroptosis has now been proposed to be responsible for the release of IL-1β and IL-18 to alert the immune cells of the imminent danger, leading to the onset of inflammatory responses (207). This was first suggested by the observation of caspase-1-dependent pores in the plasma membrane of *Salmonella*-infected macrophages, ultimately leading to cell swelling and osmotic lysis (175). This was supported by more recent studies of ATP-stimulated BMDM, in which pharmacological inhibition

of membrane permeabilization—a hallmark of pyroptosis—abolished IL-1β secretion, but not processing (200). The recent discovery of GsdmD and its requirement for pyroptosis offers a potential mechanistic explanation linking pyroptosis and cytokine secretion. Both caspase-1 and -11 are able to cleave GsdmD, releasing the active N-terminus that mediates pore formation and lytic cell death (184, 185, 191, 192) (**Table 3**). Consistent with the concept that pyroptosis facilitates cytokine secretion, macrophages lacking GsdmD exhibit defective IL-1β secretion in response to various "canonical" and "non-canonical" inflammasome activators, including intracellular LPS, Gram-negative bacteria and nigericin (150, 185, 191, 192). However, there is also evidence in the literature of IL-1β release in the absence of cell death in peritoneal macrophages, human monocytes, and neutrophils (208, 209). In particular, neutrophils were able to secrete IL-1β in response to *Salmonella* infection through a mechanism that was dependent on NLRC4 and caspase-1 but was independent of cell lysis (210). The mechanisms of secretion of inflammasome-processed cytokines may therefore be dependent on the cell type and the nature of the activatory signals.

It is again important to emphasize that inflammasome effector responses have largely been studied in leukocytes, particularly phagocytic cells. Whether the discoveries made in these cell types are applicable to tissue cells, including IEC, remains to be determined. For instance, classical activation of the inflammasome has long been viewed as a two-step process, starting with the transcriptional regulation of the inflammasome components. Thus, caspase-11 induced cell death in macrophages was dependent on priming by TLR4 ligands through TRIF, but not on Myd88 signals (157, 158, 211). Indeed, LPS administration in mice, rapidly induced *caspase-11* expression in various tissues including thymus, spleen, and lung (161, 212). Conversely, IL-1β release in phagocytic cells depended on Myd88-mediated transcriptional priming (3, 213). These requirements appear to be somewhat different in the intestinal epithelium, for example, although TLR4 signaling is downregulated in IEC (214), caspase-11-dependent responses still occur. This suggests that *caspase-11* is constitutively expressed in the intestinal epithelium and can be rapidly activated upon pathogen invasion (48). This "ready-to-go" phenotype of IEC inflammasome components is further supported by the observations that *NLRC4* and *pro-IL-18* are constitutively expressed by IEC and may not require priming (25, 49). Furthermore, the constitutive colonization of commensal Gram-negative bacteria in the intestine could explain the constitutive elevated expression of *caspase-11* and *IL-18* in the gut compared to other tissues (www.proteinatlas.org) (161).

It is also worth noting that during homeostatic conditions, and thus in the absence of inflammation, the inflammasome-dependent cytokine IL-18 is released from IEC and is believed to have functions in epithelial repair, proliferation and maturation (33, 34). The mechanisms of secretion of IL-18 by IEC during homeostatic conditions are not well understood and whether pyroptosis occurs in IEC under physiological conditions *in vivo* remains to be determined (215). Although there is increasing evidence that IEC-intrinsic inflammasome activation plays a key role in early innate defense against pathogens that target the intestinal epithelium (14, 24, 25, 43, 44), much remains to be learned on how inflammasomes and their downstream effector responses are regulated in IEC. Due to their constitutive exposure to microbial PAMP, inflammasome circuits and thresholds in IEC may be quite different to those primarily identified in macrophages and dendritic cells. Nevertheless, the constitutive secretion of IL-18 by IEC indicates that inflammasomes are active under homeostatic conditions in the intestinal epithelium. However, the precise signals or thresholds that determine when this may be superseded by the induction of pyroptosis or alternative cell death pathways remain to be determined. For example, it will be important to assess the role of GsdmD in IL-18 secretion and IEC turnover during steady-state conditions. Furthermore, it will also be vital to understand how inflammasome responses in IEC are modulated during pathogenic attack or during inflammatory conditions, where an optimal balance between apoptosis, pyroptosis, and cytokine release may be required to control potential pathogens and restore homeostasis.

CONCLUSION AND PERSPECTIVES

High expression levels of many inflammasome proteins are enriched in the steady-state intestinal mucosa implicating their importance in barrier maintenance and immune monitoring. The spatial location of the IEC, directly facing the lumen, in combination with their primed phenotype, implies that inflammasomes are key sensors of intestinal insults. Indeed, as discussed throughout this review, deletion of these components is primarily associated with increased susceptibility to injury and infection. Thus, we can conclude that epithelial inflammasomes are critical for a healthy gut, both at steady state and during acute infection or injury. However, the molecular mechanisms orchestrating epithelial inflammasome activation remain incompletely understood, representing a key area for further research.

Frustratingly, the literature contains numerous examples of conflicting data pertaining to the functional impact and cellular sources of inflammasome components in various models of intestinal infection and inflammation. To better define these, the field needs to implement stringent lines of investigation that properly control for key environmental factors. Variation of the intestinal microbiome is likely responsible for most of the inconsistent findings reported the literature. For example, recent studies have identified protozoa (216) and microbial metabolites (32) as novel environmental factors capable of influencing inflammasome activation in the intestinal epithelium and in modulating susceptibility to intestinal inflammation. Therefore, standardized use of littermate controls for *in vivo* experiments should be implemented to circumvent misinterpretations resulting from differences in microbiota composition and baseline mucosal immune activation across distinct breeding cohorts. Furthermore, as different animal facilities will harbor their own distinct microbiotas, it would be advantageous if key experiments were reproduced in different vivariums.

To further assess the specific locations important for inflammasome function, tissue- and cell-specific deletion approaches represent an important approach, for example, the IL-18$^{\Delta IEC}$ line specifically lacking IL-18 production in IEC (110). In addition, complementary studies using inducible knockouts will be

useful for understanding acute responses while ruling out any developmental disadvantages. The increasing application of primary intestinal epithelial "organoid" cultures will complement the *in vivo* genetic approaches, enabling analysis of acute responses, as well as offering a tool for molecular manipulation of IEC (217). Moreover, transitioning from murine studies into humans will be bolstered by these new *ex vivo* techniques (218).

Murine bone marrow-derived macrophages have served as the gold standard for a majority of inflammasome research, contributing significantly to our understanding of inflammasome signaling and effector responses. However, it is likely that IEC inflammasomes are regulated differently to classic hematopoietic cells, due to the unique intestinal environment. Thus, we need to address how inflammasome activation and regulation in IEC differs from that described in myeloid cells and the resulting implications. For example, we can already surmise from the literature that IEC produce comparatively little IL-1β (44, 105) and constitutively express IL-18 (49). It is likely that within IEC there is a different composition of inflammasome machinery to tailor their immune responses. In addition, IEC could be capable of producing other potential secretory factors besides IL-18 upon inflammasome activation, for example, prostaglandin production by IEC was recently associated with NLRC4 activation (24). The signaling circuitry and relationship between different effector responses also needs to be elucidated. For example, are there distinct activation thresholds or can different inflammasome components work in concert, as has been described for NLRC4 and NLRP3 during *S.* Typhimurium infection of macrophages (219).

Our understanding of what specific agonists activate IEC inflammasomes is limited and warrants further investigation. Aside from microbial signals, how do dietary antigens interact with the intestinal epithelium? Evidence already exists for the capacity of dietary ligands to induce inflammasome activation [e.g., high fat and high cholesterol diets (79)] or dampen inflammasome activation [e.g., ketones (220)]. However, further investigation is required to delineate whether these dietary factors act directly and/or indirectly (e.g., through modulation of the microbiota) (30). Indeed, a recent study reported that a high fiber diet conferred protective effects in the DSS colitis model both by reshaping the gut microbiota and by increasing release of SCFAs that activated NLRP3 inflammasomes in a non-hematopoietic cell population.

Finally, inflammasome activation in IEC has been described to result in IEC extrusion and cell death (14, 24). Further investigation needs to be carried out into the role of pyroptotic cell death in mucosal immune responses. The regulation of different forms of cell death in IEC and the consequences for infection or inflammatory diseases also requires further characterization. For example, does too little IEC death result increased potential for invasive infection due to lack of cell extrusion and does too much IEC death perpetuate unnecessary inflammation? Finally, what function does dysregulated inflammasome activation and pyroptosis play in IBD? IBD patients are known to have necrotic lesions and increased levels of IL-18 and IL-1β in the inflamed intestine, but their relative contributions to chronic intestinal pathology remain incompletely understood.

Despite these challenges and limitations understanding gut-associated inflammasome signaling, its role in regulating dietary–microbiome–host immune interactions constitutes a critical component in maintaining homeostasis and mediating various immune-mediated disorders. Encouragingly, the identification of small molecules capable of targeting specific inflammasome components (44) could represent an opportunity for novel clinical interventions to tackle these currently incurable disorders.

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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Anti-Adhesion Therapies in Inflammatory Bowel Disease—Molecular and Clinical Aspects

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The number of biologicals for the therapy of immunologically mediated diseases is constantly growing. In contrast to other agents that were previously introduced in rheumatologic or dermatologic diseases and only later adopted for the treatment of inflammatory bowel diseases (IBDs), the field of IBD was ground breaking for the concept of anti-adhesion blockade. Anti-adhesion antibodies selectively target integrins controlling cell homing to the intestine, which leads to reduction of inflammatory infiltration to the gut in chronic intestinal inflammation. Currently, the anti-α4β7-antibody vedolizumab is successfully used for both Crohn's disease and ulcerative colitis worldwide. In this mini-review, we will summarize the fundamental basis of intestinal T cell homing and explain the molecular groundwork underlying current and potential future anti-adhesion therapies. Finally, we will comment on noteworthy clinical aspects of anti-adhesion therapy and give an outlook to the future of anti-integrin antibodies and inhibitors.

Keywords: inflammatory bowel diseases, ulcerative colitis, Crohn's disease, vedolizumab, natalizumab, etrolizumab, gut homing, integrins

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INTRODUCTION

Inflammatory bowel diseases (IBDs), such as Crohn's disease (CD) and ulcerative colitis (UC), are characterized by chronically relapsing inflammation of the gut and are associated with considerable morbidity and reduced quality of life (1). The pathogenesis of IBD is still incompletely understood. However, environmental factors, genetic susceptibility, changes in the intestinal microbiome, and altered immune signaling in the gut have been identified to play an essential role during IBD development (2, 3). In particular, infiltration of various immune cells in the inflamed gut in IBD is a prominent feature of both CD and UC. These cells are targeted by most "traditional" IBD therapies including immunosuppressive agents and anti-tumor necrosis factor (TNF) antibodies. Yet, a significant portion of patients does not respond to such therapies, loses response or experiences side effects, underscoring the need for additional treatment concepts.

One such concept is anti-adhesion therapy. T lymphocytes are a crucial part of the intestinal immune (4, 5) system, and their numbers are mainly controlled by the balance of proliferation and apoptosis (6, 7) as well as by cell recruitment of circulating T cells from the bloodstream. The clinical use of antibodies like natalizumab or vedolizumab, which block surface molecules on T cells called integrins regulating their capacity to home to the gut, has conferred considerable attraction to intestinal T cell trafficking and the concept of anti-adhesion therapies. Meanwhile, several additional antibodies and compounds targeting distinct T cell trafficking steps are under

development, and one or the other might soon contribute to a growing family of anti-trafficking drugs for the treatment of IBD.

In this mini-review, we will give an overview of the basic principles underlying intestinal T cell trafficking and summarize the translational relevance of these principles by highlighting the most important molecular and clinical aspects of current and future anti-adhesion therapies.

MECHANISMS OF T CELL TRAFFICKING

A central event in the pathogenesis of T cell-dependent chronic intestinal inflammation is the homing of T lymphocytes to the gut (**Figure 1**). Homing describes a multistep process consisting of cell tethering to and rolling along activated endothelial cells, subsequent activation and firm adhesion of T cells, finally leading to their para- or transcellular transmigration from high endothelial venules (HEVs) into the tissue (8). To ensure that antigen-experienced T cells can reach their designated destination, a "zip code" like system of specific molecules controls homing to the intestinal lamina propria (LP). The expression of these molecules is primed during activation and expansion of naïve T cells after contact with their cognate antigen in the gut-associated lymphoid tissues. There, dendritic cells (DCs) (9), characterized by expression of CD103, not only present intestinal antigens to T cells and co-stimulate them, if applicable, but also produce retinoic acid (RA) through retinal aldehyde dehydrogenase. RA leads to upregulation of unique gut-homing markers including the integrin α4β7 and CC-chemokine receptor (CCR) 9 and, in turn, to loss of naïve T cell homing markers such as CCR7 (10–12).

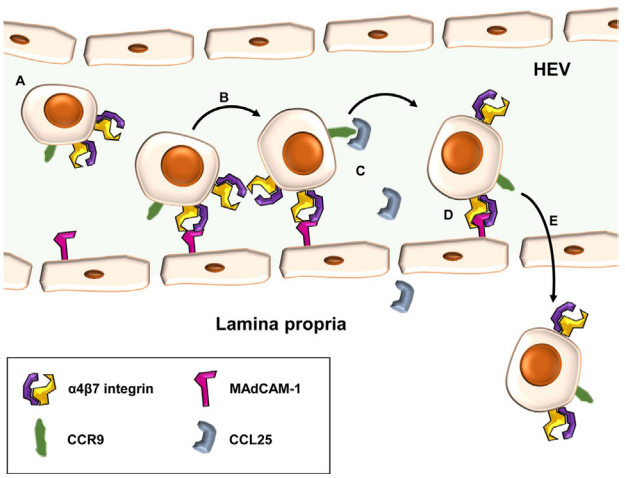

FIGURE 1 | Principle of α4β7-mediated cell adhesion in the intestine. Gut-homing T cells carrying the α4β7 integrin and CC-chemokine receptor (CCR) 9 **(A)** may role along high endothelial venules (HEVs) of the gut by low-affinity interactions of α4β7 with mucosal vascular addressin cell adhesion molecule (MAdCAM)-1 **(B)**. Upon CCR signaling, e.g., *via* CC-chemokine ligand (CCL)-25 and CCR9 **(C)**, integrin-affinity modulation of α4β7 allows tight interaction with MAdCAM-1 and leads to firm adhesion of cells at the endothelial wall **(D)**. Subsequently, T cells may home para- or transcellularly to the lamina propria **(E)**.

After this switch in integrin expression, primed T cells can leave lymphoid organs to reenter the systemic circulation and adhere to intestinal HEVs expressing the addressin mucosal vascular addressin cell adhesion molecule (MAdCAM)-1 (13). MAdCAM-1 is the natural interaction partner of α4β7 integrin and, thus recognizing the "zip code" of gut-homing T cells (14). Unlike constitutively expressed selectins, integrins on T cells have to be activated in a process known as integrin-affinity modulation, before they can establish firm binding (15). This results in a conformation highly affinitive for the respective addressin. In contrast to other organs, where rolling is mainly mediated by selectins, weak and dynamic interactions of the low-affinity conformation of α4β7 with MAdCAM-1 are sufficient to induce tethering and rolling of T cells in the gut.

Rolling reduces the velocity of circulating T cells in the blood stream creating the basis for further homing and transmigration steps. Affinity modulation required for conformational change of α4β7 to its high-affinity state and subsequent firm adhesion is initiated by cell activation through chemokine receptor signaling. For instance, the CCL-25 secreted by LP cells in the small intestine, binds to CCR9, which is specifically expressed on gut-homing T cells. Subsequently, integrin heterodimers change from a folded position, in which the headpiece of the molecules is bent toward the plasma membrane and the addressin binding pocket is hidden, to an open conformation increasing not only the accessibility of the binding domain but also fully opening the pocket and enhancing its affinity (15, 16).

In addition to α4β7, other integrins like α4β1 may also contribute to adhesion of T cells to intestinal HEVs (17). Upon firm arrest of T cells, interactions of integrins with junctional adhesion molecules expressed on HEVs like JAM-1 contribute to para- or transcellular extravasation into the inflamed tissue (18).

Once homed to the gut, T cells contribute to immunological events depending on their designated role, such as T helper (Th) 1, Th2, Th9, Th17, cytotoxic T cells, or regulatory T cells (Tregs). However, trafficking of these cells is not necessarily finished, e.g., CCR7-dependent recirculation *via* lymphatic vessels (19) or sphingosine-1 phosphate-dependent exit to the blood stream has been described (20) and is reviewed elsewhere (21). Moreover, transforming growth factor β may trigger the upregulation of αEβ7 integrin, which cooperates with E-cadherin in the gut epithelial layer retaining T cells in or near the epithelium (22, 23).

It has been recognized more than two decades ago that all these mechanisms are not only academically interesting but also translationally relevant and allow targeted interference with the gut-homing process. Accordingly, targeted treatments for IBD interfering with the gut homing process have been developed and molecular and clinical aspects of these therapies will be discussed in the following paragraphs.

MOLECULAR ASPECTS OF ANTI-ADHESION THERAPIES

T cell trafficking includes a multitude of events such as priming, homing, recirculation, or retention, and all these steps are potential targets of therapy. So far, strategies impeding

integrin-dependent cell adhesion to addressins have been most successful (24), and we will thus focus on these anti-adhesion therapies. Most importantly, the anti-α4-antibody natalizumab and the anti-α4β7-antibody vedolizumab reached clinical approval after large phase III studies (25–27).

Yet, the divergent fate of these antibodies illustratively underscores redundancies and specificities (**Figure 2**) in integrin-dependent homing to the gut and other organs, based on the heterodimeric composition of integrins. An α and a β chain pair form an αβ heterodimer with most single chains combining with different other chains to form several distinct heterodimers. Thus, targeting integrins by antibodies on the monomer and heterodimer level results in coverage of a set of integrins or only one specific representative, respectively.

Both anti-α4 and anti-α4β7 strategies were initially evaluated in the cotton-top tamarine model of colitis, where they protected these animals from UC-like disease (28, 29), prior to testing of humanized antibodies in clinical trials. Soon after approval of natalizumab for CD, a report of progressive multifocal leukoencephalopathy (PML) was published (30), a severe infectious side effect deemed to arise from concurrent inhibition of α4β1-dependent homing *via* vascular cell adhesion molecule (VCAM)-1 to the central nervous system (31). Consistently, this has led to strong limitation or complete abandoning of natalizumab use in CD. On the other hand and matching with the current knowledge about T cell homing, vedolizumab, which is specific for the α4β7 heterodimer, has been successfully used for the treatment of both CD and UC for several years (32, 33) and has not been associated with infectious side effects in the central nervous system. The higher specificity of vedolizumab, however, also results in missing out alternative homing pathways as demonstrated by a study suggesting that homing *via* α4β1 might at least partially compensate for α4β7 blockade in CD patients treated with vedolizumab (17).

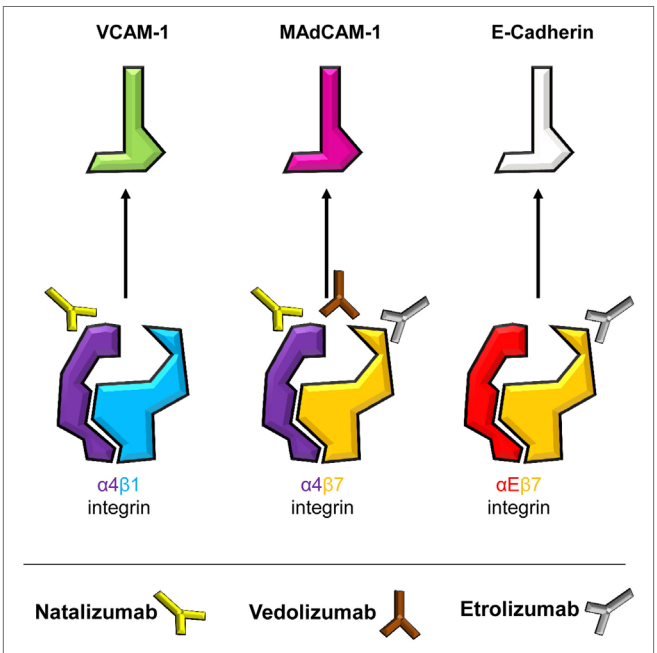

FIGURE 2 | Specificities of current and potential future anti-adhesion antibodies. Integrins are heterodimers with an α- and a β-chain. Dimers containing α4 and β7 chains, i.e., α4β1, α4β7, and αEβ7 integrins, mediate intestinal T cell trafficking. By targeting monomers or heterodimers, different specificities of anti-integrin antibodies are achieved. Integrins and their respective ligands are indicated, and the antibodies natalizumab (anti-α4), vedolizumab (anti-α4β7), and etrolizumab (anti-β7) are depicted next to their respective target(s).

With the ongoing clinical studies of the anti-β7-antibody etrolizumab we are currently facing a new attempt to block α4β7 together with another integrin (34). Pan-β7 inhibition provides hopes that dual targeting of αEβ7 and α4β7 might increase therapeutic effects by additionally blocking intestinal retention of pathogenic T cells through E-cadherin (35). However, less gut specificity might be observed with β7 blockade since αEβ7 is also expressed by T cells in other tissues and might be important for the control of local infections there (36, 37). It will thus be an important task to determine potential infectious side effects of etrolizumab in the ongoing phase III trials. Moreover, it is not clear, whether anti-β7 antibodies impact $CD103^+$ intestinal DCs (38). Since such DCs were proposed to be responsible for the induction of Tregs with anti-inflammatory properties under homeostatic conditions (39), it cannot be excluded that pan β7 inhibition reduces intestinal Treg cell numbers. However, it has also been shown that intestinal inflammation alters the role of these DCs switching their function to inducers of effector-like T cells (40), thus rather suggesting that anti-β7 treatment could help to reduce inflammation beyond the T cell level.

Taken together, the molecular mechanisms of targeting α4, α4β7, or β7 integrins in IBD show that it is not easy to find the optimum between the poles of maximally efficient gut homing blockade (i.e., inhibition of a plurality of responsible molecules) and selectivity (i.e., maximum safety). Therefore, further translational and empirical research is needed for elucidation of these challenging questions.

Such considerations get even more complicated when also taking the addressin side into account. Regarding the success of α4β7 inhibition, it seems logical that antibodies to MAdCAM-1 should result in similar clinical benefit. Yet, this black-and-white thinking does not match the myriads of grayscales in human biology since α4β7 is not only cooperating with MAdCAM-1 but also contains epitopes to bind to VCAM-1 and to fibronectin (41). This might be one explanation for the impression provided by early clinical studies that anti-MAdCAM-1 might not be as effective as vedolizumab (42, 43), although it has been claimed that vedolizumab does not interfere with α4β7 binding to VCAM-1 (41).

Another interesting molecular aspect of anti-adhesion therapies that is only beginning to be understood is the marked difference in the expression of integrins like α4β7 and αEβ7 on specific Th cell populations (35, 44). While Th2 and Th17 cells seem to express high levels of α4β7, Th1 and Th9 cells have low expression of α4β7. In contrast, αEβ7 is high in Th9 and Th17 but low in Th2 and Th1 cells (35). Since it is considered that CD is marked by Th1 and UC by Th2-like signaling (4, 45), differential expression of α4β7 might be one piece in the puzzle to

explain, why the proportion of UC patients responding to treatment with vedolizumab seems to be higher compared with CD (25, 26). Moreover, it seems possible that assessment of individual or disease-specific Th cell profiles might help to optimize treatment by choosing antibodies most closely covering the respective subsets.

In conclusion, our understanding of the molecular mechanisms of gut homing has facilitated the development of novel therapies for IBD, but we are far away from a profound conceptual comprehension that includes an exact perception of the role of integrins and addressins in different tissues, with regard to different cellular subpopulations and concerning less prominent or rather overlooked "cross-interactions" between different homing pathways.

CLINICAL ASPECTS OF ANTI-ADHESION THERAPIES

Blocking the migration of inflammatory cells into the target tissue is, as outlined earlier, an intriguing concept. The field was clinically implemented with the α4-antibody natalizumab. Clinical efficacy was proven first in a pilot study in CD (46), and subsequently in a phase III trial (47, 48). Here, patients with moderate to severe CD and an increase in C-reactive protein were randomized to receive 300 mg natalizumab or placebo at weeks 0, 4, and 8. Response by week 8, as indicated by a ≥70-point decrease from baseline in the CD activity index, sustained through week 12 in 48% of natalizumab-treated patients and in 32% of placebo-treated patients. This was statistically highly significant, and hence the primary endpoint of the study was met (48). These observations led to the approval of natalizumab for CD in North America. The enthusiasm for α4 blockade came to a sudden halt, when a fatal JC virus-related PML was reported upon natalizumab treatment (30), preventing the drug from approval in the European Union. The explanation for this side effect is rather obvious since anti-α4 equally hinders $\alpha4\beta1^+$ immune cells from not only infiltrating the gut but also the brain, hence impeding appropriate cerebral antiviral immunity.

Subsequently, the field moved on by developing more specific anti-adhesion strategies. The first and at this point only one with EMA approval for IBDs is the α4β7-antibody vedolizumab. Two large phase III trials led to approval (**Table 1**) (25, 26). Briefly summarized for UC, the primary endpoint at week 6, clinical response, showed significant differences (47.1% vedolizumab group vs. 25.5% placebo group) (25). Of the patients who responded to induction therapy at week 6, 88% were in remission after 104 weeks and 96% after 152 weeks of treatment (49). In CD, clinical remission showed a significant difference at week 6 (14.5% vedolizumab group vs. 6.8% placebo group) (26). Of all patients responding in week 6 who received vedolizumab continuously, 83 and 89% of patients were in remission after 104 and 152 weeks, respectively (50).

Besides these, initial phase III trials several real life registries from various countries have reported comparable efficacy (34, 49, 50, 52, 53).

In a German cohort with 212 consecutive patients with either CD or UC, clinical remission at week 14 was assessed (33). 23.7% of patients with CD and 23.5% with UC achieved clinical remission. One has to recognize that during the initial time period after approval mostly more refractory patients were exposed to vedolizumab. The cohort was then followed for 30 and 54 weeks, respectively, and included 67 CD and 60 UC patients. Primary endpoint was clinical remission at week 54, which was achieved in 21% of CD and 25% of UC patients, respectively (54).

It should also be mentioned that in comparison with anti-TNF antibodies, it seems that vedolizumab needs longer to manifest full effects (25, 26, 55). Regarding the abovementioned mechanistic aspects, it is tempting to speculate that this might be due to preserved function of T cells already present in the LP during the initial phase of vedolizumab treatment, while homing inhibition might only then lead to marked effects on T cell function, when a significant portion of these LP T cells undergoes apoptosis and replenishment is impeded.

Several other strategies are currently under clinical investigation including the anti-β7-antibody etrolizumab where a recent phase II trial for UC showed promising results and initiated a broad phase III study program (34). In a double-blind, placebo-controlled, randomized, phase II study including patients with moderately to severely active UC that did not respond to conventional treatment were randomized (1:1:1) to receive either etrolizumab 100 mg at week 0, 4, and 8 with placebo at week 2, 420 mg etrolizumab loading dose at week 0 followed by 300 mg at weeks 2, 4, and 8 or placebo. 124 patients were included and none of the placebo group patients reached the primary endpoint of clinical remission at week 10, whereas 21% of the etrolizumab 100 mg group and 12% in the 300 mg group met the endpoint. The authors conclude that etrolizumab showed clinical efficacy and hence α4β7 as well as αEβ7 might provide future therapeutic targets. Beside efficacy, the remarkable part of the study was that it provided for the first time a predictive biomarker for the responsiveness to an anti-inflammatory biological since αE expression in the intestinal mucosa correlated with a better response to

TABLE 1 | Overview of clinical data from randomized-controlled studies on natalizumab, vedolizumab and etrolizumab in CD and UC.

	Efficacy		Important safety aspects
	CD	UC	
Natalizumab	Phase III: + 16% clinical response after 8 weeks vs. placebo (in patients with elevated CRP) (48)		Risk of PML (30)
Vedolizumab	Phase III: + 7.7% clinical remission after 6 weeks vs. placebo (26)	Phase III: + 21.6% clinical response after 6 weeks vs. placebo (25)	Nasopharyngitis, surgical site infection? (25, 26, 51)
Etrolizumab		Phase II: + 21% clinical remission after 10 weeks vs. placebo (34)	Influenza-like illness, arthralgia, and rash (34)

Differences in the primary endpoint vs. placebo group are indicated, and most important side effects are noted. See text for details.
CD, Crohn's disease; UC, ulcerative colitis; CRP, C-reactive protein; PML, progressive multifocal leukoencephalopathy.

etrolizumab treatment (34). In a follow-up study, these findings were specified and showed that high granzyme A and αE mRNA expression levels in colon biopsies revealed patients with UC more likely to respond to etrolizumab treatment (56).

Several other strategies target migration; one is approaching MAdCAM-1 on the endothelial site. A first dose-finding study indicated safety and efficacy in patients with UC (57). Very recently, the results of a phase II follow-up study were published. In this trial, patients were treated with subcutaneous injections of one of four doses (7.5, 22.5, 75, or 225 mg) of the anti-MAdCAM-1 antibody PF-00547659 or placebo. The primary endpoint was remission at week 12. This was met in three of the four verum groups (7.5, 22.5, or 75 mg), the highest difference in efficacy compared to placebo was observed in the 22.5 mg group (58).

SAFETY

After the fatal complications observed under natalizumab treatment, none of the other strategies currently approved or studied revealed a new case of PML. A recent publication summarizes the collected safety data (May 2009–June 2013) from six studies of vedolizumab. Any patient that received ≥1 infusion of vedolizumab or placebo was included, and results were expressed as exposure-adjusted incidence rates with the number of patients experiencing the event per 100 person-years of exposure. The analysis included 2,830 patients with 4,811 person-years of exposure. Remarkably, no increased risk for any infection was associated with vedolizumab. Most important, up-to-date, no case of PML has been reported within this review or outside (59). The limitation of the study is the number of patients, while 2,789 had been exposed to ≥1 dose of vedolizumab, only 906 were exposed for ≥24 months and only 40 were exposed for ≥48 months (59).

Somewhat surprisingly, extra-intestinal symptoms in patients receiving vedolizumab are observed and are more common in those patients who respond to therapy (60). Recent data indicate that a shift in integrin expression under α4β7 neutralization toward a β1 upregulation results in an altered migrational behavior of immune cells in non-intestinal tissue including skin, joints, and lung (61, 62).

CONCLUSION AND OUTLOOK

The discussed data indicate that anti-migrational strategies have found their way into clinical practice and the development of further anti-adhesion compounds together with other concepts like Janus kinase inhibitors, anti-IL-23p19 antibodies, or Smad7 blockade might provide optimized IBD treatment in the future. However, as outlined in the first paragraphs of this mini-review, a more detailed understanding of localized integrin expression is required to perform a more personalized treatment and identify the responding patients early on. However, first data indicate that this might become feasible.

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MINI REVIEW
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Deciphering the Complex Signaling Systems That Regulate Intestinal Epithelial Cell Death Processes and Shedding

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Intestinal epithelial cells play a fundamental role in maintaining homeostasis. Shedding of intestinal cells in a controlled manner is critical to maintenance of barrier function. Barrier function is maintained during this shedding process by a redistribution of tight junctional proteins to facilitate closure of the gap left by the shedding cell. However, despite the obvious importance of epithelial cell shedding to gut health, a central question is how the extrusion of epithelial cells is achieved, enabling barrier integrity to be maintained in the healthy gut and restored during inflammation remains largely unanswered. Recent studies have provided evidence that excessive epithelial cell shedding and loss of epithelial barrier integrity is triggered by exposure to lipopolysaccharide or tumor necrosis factor alpha. Subsequent studies have provided evidence of the involvement of specific cellular components and signaling mechanisms as well as the functionality of microbiota that can be either detrimental or beneficial for intestinal barrier integrity. This review will focus on the evidence and decipher how the signaling systems through which the mucosal immune system and microbiota can regulate epithelial cell shedding and how these mechanisms interact to preserve the viability of the epithelium.

Keywords: intestinal epithelial cells, shedding, apoptosis, necroptosis, pyroptosis

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INTRODUCTION

The intestinal barrier separates the body from the contents of the intestine. It comprises several elements: a mucus layer containing antibacterial peptides covering the luminal surface of the epithelium; the epithelial cell monolayer, junctional proteins, and intraepithelial lymphocytes (IELs); and a subepithelial layer of extracellular matrix and mesenchymal cells including myofibroblasts and fibroblasts. A central element of this intestinal barrier is the epithelial cell (1). In health, there is a continuous shedding of epithelial cells from villus tip or colonic surface as a result of migration of the epithelial cell up the crypt–villus axis from stem cells at the base of the crypt (**Figure 1A**). The shedding of epithelial cells is counter-balanced by cell division in the crypt region of the villi to maintain homeostasis and a strict single layer epithelium and integrity of the crypt–villus axis (2–4). In physiological conditions, epithelial cells undergo apoptosis during the shedding process though it remains unclear whether apoptosis initiates the shedding process or is secondary to detachment from the basement membrane (3) (**Figure 1B**). In contrast to physiological cell shedding, tumor necrosis factor alpha (TNFα)-induced apoptotic cell shedding often results in the shedding of multiple adjacent

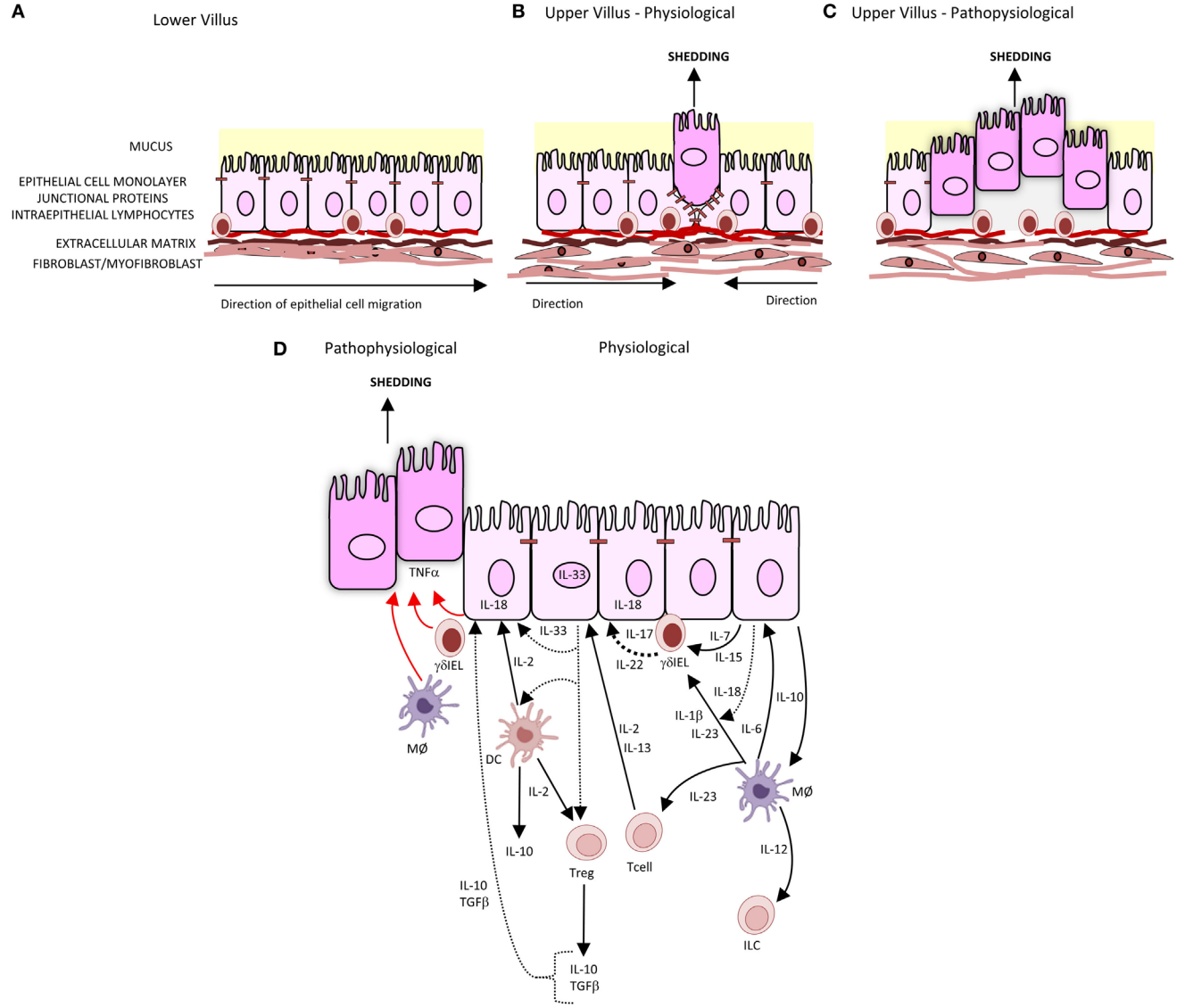

FIGURE 1 | Intestinal epithelial cell shedding. **(A)** In health, epithelial cells migrate up the villus from the base of crypt to the tip. This is achieved through the crawling movement of the epithelial cells through epithelial–substratum interactions between integrins, heparin sulfate proteoglycans, and extracellular matrix. **(B)** At the villus tip, physiological cell shedding with redistribution of tight junctional proteins. **(C)** Pathophysiological cell shedding with multiple cells shed from a single site leading to barrier loss. **(D)** Immunological regulation of cell shedding. Under pathophysiological conditions, TNFα is released by γδIELs, macrophages (MØ), and intestinal epithelial cells (represented by red arrow) resulting in cell shedding. Pathways of cytokines secreted by intestinal epithelial cells, T cells, T regulatory cells (Tregs), and dendritic cells (DC) involved in intestinal epithelial barrier integrity are represented by black arrows, with dashes representing cytokines which have been identified but specific role in barrier integrity and subsequent regulation of cell shedding not yet defined.

cells causing a breach in the epithelial monolayer too large to be sealed with subsequent loss of barrier function (5) (**Figure 1C**).

BALANCE OF CELL DIVISION, MIGRATION, AND SHEDDING MAINTAINS BARRIER INTEGRITY

Epithelial migration is intimately coupled to cell shedding as the two processes must be coordinated to maintain a steady number of epithelial cells on the crypt/villus axis. Until recently, little has been known about the cellular and molecular mechanisms of intestinal epithelial cell migration. This migration is a complex of mechanisms through which each component is intricately balanced. The crawling movement of the epithelial cell along the crypt–villus, axis relies on epithelial cell–substratum interactions regulated by the expression of integrins (6, 7), heparan sulfate proteoglycans (8), growth factor (9), cytokine (10), and chemokine receptors (11) as well as extracellular matrix such as laminins and collagen IV (12).

When the epithelial cell is shed, a discontinuity or gap in the villus epithelial monolayer is created, which could potentially

compromise the epithelial barrier. However, in health, normal cell shedding never causes a breach in the epithelial barrier because this gap is plugged by redistribution of tight junction proteins, which include occludin, ZO-1, and the adherens junction protein E-cadherin (13). This redistribution mechanism of tight junction proteins has also been reported in TNFα-induced cells shedding at sites where the gap created by cell shedding has been successfully sealed (14). A further refinement to the extrusion mechanism has been added by the observation that the extrusion of the dying cell is initiated by tension of the dying cell on its neighbors transmitted through cortical contractile actin and a myosin ring at the apex of the dying epithelial cell (15). The redistribution of tight junction proteins results in the modulation of actin filaments, either through actin polymerization with the formation of lamellipodial or actin–myosin interactions forming a ring or a combination of both (16, 17). The mechanics of actin polymerization and lamellipodial formation and actin–myosin interactions are not only dependent on GTPases, Rac1 and Rho (18), respectively, and Cdc42 (19), and trefoil factors (9, 20), but also on many factors including regulation of actin-binding proteins such as villin (21–26), the locality and density of the cell shedding (17, 27), substratum extracellular matrix (28), gap formation (29), and cytokine signaling pathways. Cytokines such as IFNγ and TNFα are involved not only in regulating the remodeling of the junctional proteins (30) but can also be regulated by junctional proteins (31). These cytokines can also act synergistically through the convergence of the β-catenin signaling pathways. IFNγ regulates intestinal epithelial cell proliferation and apoptosis through AKT–β-catenin pathways and Wnt–β-catenin signaling pathways, with TNFα activation of the β-catenin signaling through P13K-AKT and NF-κB signaling (32).

To untangle these complexities, computational modeling of cell division and migration as well as the use of *in vivo* and *in vitro* models using epithelial cell lines and keratinocytes have been used (4, 15, 33–38). The morphological properties of the cells selected for the cellular models are monolayer formation and contractility including the ability to undergo cell division, morphogenesis, and migration to close gap formation caused by injury (39, 40). This has provided an insight into how epithelial cells that line many organs surface operate but how that information can be applied to understand the mechanisms of cell homeostasis and repair within the intestine. Wong and colleagues (33) focused on the migratory positioning and velocity of cells within the crypt and developed a model demonstrating this through the expression and interactions of Eph receptors and ephrins and their regulation cell adhesion. The study highlighted the importance of the cell–cell, cell–substratum, and cytoskeletal organization for maintaining cell migration along the crypt. Parker and colleagues (4) demonstrated how the proliferation of cells within the crypt is the primary force for driving cell migration up the villus and by implication cell shedding. Maintenance of epithelial homeostasis and response to injury is regulated through the expression of signal transduction pathways such as WNT (41, 42) and NOTCH (43, 44) and JAK/STAT pathways and interaction with cytokines. The pathways are highly complex with multiple interactions. For example, JAK3/IL-2/IL2R can result in regulation of villin (45), the STAT5 pathway regulates cellular proliferation of intestinal stem cells (46), and STAT3/IL-22/IL-22R pathways regulate cellular regeneration (47).

The factors determining whether an individual intestinal epithelial cell is shed is not understood. In epithelial cells of the Zebrafish fin, it has been found that the overcrowding and physical stretching of the epithelial cell as it reaches the tip of the fin is sensed by the stretch activated cation selected ion channel Piezo-1. This stimulates extrusion of the epithelial cells through sphingosine 1-phosphate signaling and Rho kinase (37). Furthermore, it has recently been demonstrated that cellular crowding sensed through Piezo1 increases epithelial proliferation in the Zebrafish larvae to preserve overall epithelial homeostasis (38). It is not known whether similar mechanisms occur in the mammalian intestine.

A recent study has suggested that the actin regulatory protein villin might direct the site of intestinal epithelial apoptotic cell shedding on the villus. It regulates cell turnover through the regulation of caspase-3 and caspase-9 apoptotic pathways and regulating actin polymerization and depolymerization (21). Recent data have demonstrated that villin is not only anti-apoptotic but also has pro-apoptotic functions. This function is dependent on the cleavage of villin by proteolytic enzymes. These enzymes, such as meprin, a matrix metalloproteinase, cleaves the villin into fragments, of which the N-terminal villin fragment is pro-apoptotic at the villus tip and can reorganize the actin filaments resulting in cell shedding (48).

TYPES OF CELL DEATH INDUCING CELL SHEDDING

A number of types of cell death have been reported intestinal epithelial cells. TNFα-induced apoptotic cell shedding has been studied in some detail. However, it is becoming appreciated that pyroptosis and necroptosis also play a role in intestinal epithelial cell injury (**Table 1**).

Apoptosis is mediated through either intrinsic or extrinsic pathways (49, 50). In the intrinsic pathway, cellular injury triggers the release of cytochrome *c* from mitochondria to form an apoptosome in cytosol, comprising cytochrome *c*, apopotic protease factor 1 (APAF-1), and procaspase-9, which triggers activation of a cascade of proteases called caspases which kill the cell. In the extrinsic pathway, apoptosis is triggered by the binding of external proteins such as TNFα or FasL to their cognate receptors expressed on the surface of the target cell. The binding

TABLE 1 | Intestinal epithelial cell death processes involved in cell shedding (49–56).

Apoptosis	Necroptosis	Pyroptosis	Necrosis
Caspase-3 +ve	Caspase-3 –ve	Caspase-3 –ve	Caspase-3 –ve
Caspase-1 –ve	Caspase-1 +ve	Caspase-1 +ve	Caspase-1 –ve
Tunnel +ve	Tunnel +ve	Caspase-11 (mouse) +ve	Annexin V +ve
Annexin V +ve	RIP3 +ve	Caspase-4 (human) +ve	Propidium iodide +ve
Propidium iodide –ve	RIPK-3 +ve	Caspase-5 (human) +ve	*Tunnel +ve/–ve*
Caspase-8 +ve/–ve	Caspase-8 +ve	Gasdermin D +ve	

of the ligand to the receptor stimulates the activation of caspase-8 through a series of intermediate proteins to cause apoptosis (51). In a mouse model of rapid small intestinal epithelial cell shedding and apoptosis developed by Watson and colleagues (5, 14, 16, 52), it has been demonstrated that TNFα release in the lamina propria caused cell shedding *via* the TNF receptor 1. The TNFα then activates NF-κB pathway. A differential sensitivity of cell shedding to NF-κB pathways was observed with NF-κB1 decreasing sensitivity, while NF-κB2 increases the sensitivity of epithelial cell shedding to lipopolysaccharide (LPS). Studies of the mechanism of cell shedding have shown that activation of caspase-3 by TNFα cleaves and activates Rho-associated protein kinase (ROCK1) and the phosphorylation of myosin light chains resulting in the membrane blebbing formation in apoptotic cells. Inhibition of either of these enzyme activities arrests cell shedding after its initiation such that the shedding process is incomplete (14, 15). In addition, it has been reported that synthesis of sphingosine-1-phosphate by dying cell binds to the S1P(2) receptor in neighboring cells to activate myosin contraction to extrude the dying cell out of the epithelial monolayer (53).

Ubiquitin-dependent signaling activated by pattern recognition receptors (PRRs) mediates activation of NF-κB transcription factors as well as the MAP kinases p38 and JNK. NF-κB1 and MAPK expression reduces cell shedding, while NF-κB2 increases shedding. NF-κB is required for expression of downstream cytokines and chemokines such as TNFα, IL-6, IL-1β, and CCL20. Data to date demonstrate an action of PRRs in intestinal inflammation and epithelial apoptosis; therefore, it is plausible that aspects of the innate immune system may regulate cell shedding.

The mode of cell death is dependent on the activation of various cellular signaling pathways after initial cytokine stimulation. The differences have been highlighted recently by the groups of Günther et al. and Rauch et al. (52, 54). In the absence or inactivation of caspase-8, TNFα induces necroptosis at the base of the crypt with loss of Paneth cells *via* RIP-3 kinase. This is relevant to Crohn's disease as necroptosis occurs in the intestinal crypt (55). Caspase-8 acts as a type of switch. When functional, it initiates apoptosis which is a benign form of cell death from the point of view of the whole animal. However, when caspase-8 is not functional, cell death still occurs but *via* RIP3-kinase-dependent necroptosis which affects multiple cell types in a number of organs with increased mortality (52). Rauch and colleagues demonstrated the induction of apoptosis through caspase-8 activation and interaction with inflammasomes. Inflammasomes in inflammation regulate cell death through the activation of caspase-1 resulting in the expulsion of cells or pyroptosis. This mode of action can be induced through microbial ligands binding to NAIP family members of the inflammasome complex (56).

BACTERIAL ENTRY AND EPITHELIAL CELL SHEDDING

When shedding of multiple adjacent apoptotic cells creates gaps that are too large to be plugged by the redistribution of apical junctional proteins, as frequently occurs when TNF concentrations are high, the epithelial barrier is breached at the shedding site (14). In clinical studies using confocal endomicroscopy, this has been shown to trigger relapse of inflammatory bowel disease (14). This allows the entry of bacteria such as *Listeria* (57), antigens, and toxins from the lumen, which act to amplify inflammatory reactions within the lamina propria. However, apoptotic cell shedding can be an important mechanism to expel epithelial cells invaded by pathogenic bacteria and thereby reducing the chance of bacterial colonization as well as localizing inflammatory reactions. To this end, pathogenic bacteria, such as *Shigella*, *Citrobacter*, and *Salmonella*, have evolved to prevent cell shedding through the production of bacterial effector proteins. One effector protein secreted by these bacteria is the protein OspE that enhances epithelial cell–matrix interactions through binding of the integrin-linked kinase of the epithelial cell to the cells actin cytoskeleton resulting in increased integrin expression and thereby increased focal adhesions to the extracellular matrix (58, 59). This evasive mechanism results in bacterial colonization and inflammatory reactions within the intestine. However, this bacterial evasive mechanism relies on an interaction between the epithelial cell and underlying matrix *via* the integrin-linked kinase, which can only take place in the crypt and lower villus (58, 59). Although *Salmonella* can inhibit cell shedding, and thereby interfere with the epithelial cell response to bacterial infection, it is not the only mechanism of defense by the epithelial cells. This mechanism is through the formation of inflammasomes complexes, caspase-1 activation, and the production of cytokines and ultimately pyroptotic cell death (60), although recent work has demonstrated that this mechanism can result in apoptotic and pyroptotic cell death *via* caspase-8 activation (52, 61).

REGULATION OF CELL SHEDDING BY THE MUCOSAL IMMUNE SYSTEM

Intraepithelial lymphocytes within the epithelial monolayer have normally been associated with celiac disease; however, recent date indicate that they may have a central role in epithelial barrier function. Interestingly, recent data from Edelblum and colleagues (62) have demonstrated that γδ-IELs can migrate along the epithelium by an occludin-dependent mechanism. Given that occludin is redistributed to surround the shedding cell during expulsion, it is an attractive hypothesis that the IELs might participate in the regulation of cell shedding through occludin-dependent mechanisms. IELs could initiate epithelial cell restitution by stimulating epithelial cell migration into the gap created by cell shedding. They might also signal to the epithelial cells adjacent to the shedding cells to stimulate cytoskeletal reorganization. Migration of IELs within the epithelium can also be regulated by the chemokine–chemokine receptor interaction such as CCL25–CCR9 (63) as well as through the expression of chemokine receptors CCR5, CX3CR1, and CCR3 (64). Chemokine regulated migration of IELs could potentially direct IELs to sites of cell shedding. IELs could potentially also regulate the responses of other cell populations, such as subepithelial myofibroblasts and macrophages. Such subepithelial

responses may be important in the prevention of paracellular migration of opportunistic pathogenic (65, 66) and commensal bacteria (67).

Both innate and adaptive immunity are hypothesized to regulate or respond to cell shedding. Within the innate immune system that comprises monocytes/macrophages, dendritic cells, innate lymphoid cells, and epithelial cells, microbes are recognized by PRRs such as toll-like receptors and nucleotide oligomerization domains (NODs) expressed on these cells. We have found that *Bifidobacterium breve* significantly reduce LPS and TNFα-induced epithelial cell shedding through a NOD2-dependent mechanism that requires the exopolysaccharide of the Bifidobacteria (68).

Although there that been innumerable studies of components of the adaptive and innate immune systems regulating mucosal damage, only few studies that specifically investigated the regulation of epithelial cell shedding (**Figure 1D**). Mechanistic studies have demonstrated a role for T regulatory cells in both adaptive and innate immunity. Production of cytokines IL-10, IL-4, and IL-13 is critical for suppression of pro-inflammatory cytokine responses from other immune cells such as monocytes/macrophage and thus could reduce TNFα-induced cell shedding. IL-13 also downregulates the effects of LPS-induced endotoxin. Its effects of LPS-induced cell shedding have not been reported. IL-13 has been shown to modulate intestinal epithelial tight junctions, claudin-2, and apoptosis and therefore potentially cell shedding (69). The cytokines, such as IL-10, IL-21, IL-22, IL-23, and IL-6, activate STAT3 and, in addition to IL-13, are also regulated through STAT3. Inhibition of STAT3 blocks the anti-apoptotic activity of IL-6 (70); therefore, it is possible that inhibition of STAT3 may also disrupt the immunosuppressive action of IL-13 and IL-10, which in turn modulates TNFα production and thereby epithelial shedding and apoptosis.

CONCLUSION

Important advances have been made in our understanding of the maintenance of epithelial integrity in health and disease. The mechanisms of extrusion of epithelial cells are now being unraveled though it remains unclear what the determinants are of an individual epithelial cell being shed. A number of studies of cytokines and chemokines have demonstrated their importance in epithelial integrity they have not specifically addressed their role in the regulation of cell shedding itself. It is now appreciated that a number of types of cell death can trigger epithelial extrusion with increasing examples of necroptosis and pyroptosis being reported in addition to apoptosis. There is now also an increasing understanding that epithelial cell shedding can be a protective mechanism against infection through expulsion of invading pathogens. Further studies are likely to reveal therapeutic targets for inflammatory and infective bowel disease.

AUTHOR CONTRIBUTIONS

AP collected and analyzed data, drafted the manuscript, and contributed toward and approved the final manuscript. AW drafted the manuscript and contributed toward and approved the final manuscript.

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Clinical Response to Vedolizumab in Ulcerative Colitis Patients Is Associated with Changes in Integrin Expression Profiles

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Background: Despite large clinical success, deeper insights into the immunological effects of vedolizumab therapy for inflammatory bowel diseases are scarce. In particular, the reasons for differential clinical response in individual patients, the precise impact on the equilibrium of integrin-expressing T cell subsets, and possible associations between these issues are not clear.

Methods: Blood samples from patients receiving clinical vedolizumab therapy were sequentially collected and analyzed for expression of integrins and chemokine receptors on T cells. Moreover, clinical and laboratory data from the patients were collected, and changes between homing marker expression and clinical parameters were analyzed for possible correlations.

Results: While no significant correlation of changes in integrin expression and changes in outcome parameters were identified in Crohn's disease (CD), increasing α4β7 levels in ulcerative colitis (UC) seemed to be associated with favorable clinical development, whereas increasing α4β1 and αEβ7 correlated with negative changes in outcome parameters. Changes in α4β1 integrin expression after 6 weeks were significantly different in responders and non-responders to vedolizumab therapy as assessed after 16 weeks with a cutoff of +4.2% yielding 100% sensitivity and 100% specificity in receiver-operator-characteristic analysis.

Discussion: Our data show that clinical response to vedolizumab therapy in UC but not in CD is associated with specific changes in integrin expression profiles opening novel avenues for mechanistic research and possibly prediction of response to therapy.

Keywords: inflammatory bowel diseases, ulcerative colitis, T cells, vedolizumab, integrins

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INTRODUCTION

Inflammatory bowel diseases (IBD) with the main entities of Crohn's disease (CD) and ulcerative colitis (UC) arise from a complex pathogenesis that crucially involves pro-inflammatory T cells (1–3). Most available therapies including the monoclonal anti-α4β7 integrin antibody vedolizumab

prominently target these T cells and mediate their beneficial effect on chronic intestinal inflammation by controlling numbers and function of intestinal T cells (4).

While this in some cases includes the promotion of T cell apoptosis (5) or inhibition of pro-inflammatory differentiation (6), vedolizumab is thought to reduce replenishment of intestinal T cells by impeding α4β7 integrin-dependent gut homing (7, 8). Gut homing is a multistep-process facilitating the access of effector and effector memory T cells that have been primed in the gut-associated lymphoid tissue in the presence of retinoid acid to the intestinal lamina propria (9, 10). This process crucially depends on tight adhesion of T cell-expressed α4β7 integrin to endothelial mucosal vascular addressin cell adhesion molecule (MAdCAM)-1 and, consistently, recent *in vitro* and *in vivo* data have shown that vedolizumab mechanistically blocks adhesion of α4β7-expressing T lymphocytes to endothelial MAdCAM-1 (11–13). This is thought to lead to reduced infiltration of pro-inflammatory T cells to the gut with subsequent decrease in inflammation (14).

While vedolizumab has developed to a new mainstay in the therapy of IBD and is successfully used throughout the world (15–17), deeper insights into the immunological effects of α4β7 blockade are still scarce. In particular, the reasons why some patients show no clinical response are still unclear and the factors influencing mucosal healing in vedolizumab-treated patients are largely unknown. Moreover, several pieces of evidence suggest that the efficacy in CD and UC might be different (7, 8, 18), and only partial explanations for these observations are available.

In the present study, we reasoned that different degrees of clinical response to vedolizumab therapy might reflect in different changes in the expression of α4β7 integrin and related T cell surface markers. Accordingly, we sequentially analyzed integrin expression profiles in CD and UC patients receiving clinical vedolizumab therapy and show that several clinical features of disease activity are correlated with specific changes in integrin expression in UC but not CD, which might even serve for prediction of therapeutic response.

MATERIALS AND METHODS

IBD Patients

Patients with established diagnosis of UC ($n = 17$) and CD ($n = 19$) were treated with vedolizumab according to established clinical protocols (7, 8) at the Department of Medicine 1 of the University Hospital Erlangen. Peripheral blood samples were sequentially collected before each treatment from treatment one (T1) up to treatment six to eight (T2–T6/8) with T1–T3 administered at weeks 0, 2, and 6 and T4–T8 administered in intervals of between 4 and 8 weeks depending on clinical response (Figure S1A in Supplementary Material). **Table 1** summarizes the patients' clinical data. Gut samples from control and IBD patients came from surgical specimens or biopsies obtained during routine colonoscopy.

All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Ethics Committee of the University Hospital Erlangen.

TABLE 1 | Patient characteristics.

		Crohn's disease	Ulcerative colitis
Number		19	17
Age (Ø)		41.7 (20–64)	44.7 (24–68)
Female (%)		68.4	47.1
Harvey–Bradshaw index (Ø)		8.5 (2–21)	
Mayo c.s. (Ø)			3.8 (1–6)
Adjunctive therapy (%)	Immunosuppressants	15.8	17.6
	Steroids	26.3	76.5
	Mesalazin	21	70.5
Previously received anti-TNF therapy (%)		100	88.2
Localization (%)		L1: 10.5 L2: 5.3 L3: 42.1 L4+: 36.8 n.d.: 5.3	Proctitis: 5.9 Proctosigmoiditis: 17.6 Left-sided colitis: 5.9 Extended colitis: 5.9 Pancolitis: 64.7

TNF, tumor necrosis factor; n.d., not determined.

Flow Cytometry

Using density gradient centrifugation with Pancoll (Pan Biotech), peripheral blood mononuclear cells were isolated and stained with antibodies against CD4 (VioBlue, VIT4; Miltenyi Biotec), CD8 (AF647, SK1; Biolegend), α4 integrin (FITC, MZ18-24A9; Miltenyi Biotec), αE integrin (PE/Cy7, Ber-ACT8; Biolegend), β1 integrin (AF647, TS2/16; Biolegend), β7 integrin (PerCP/Cy5.5, FIB27; Biolegend), CCR2 (BV605, K036C2; Biolegend), or CCR6 (PE/Cy7, Ber-ACT8; Biolegend) and fixed with the FoxP3/Transcription Factor Staining Buffer Set (eBioscience). Flow cytometric analyses (Figures S1B,C in Supplementary Material) were performed on an LSR Fortessa instrument (BD).

Immunohistochemistry

For fixation, cryosections of gut samples were incubated with 4% paraformaldehyde. Subsequently, avidin/biotin blocking reagent (Vector Laboratories), protein-blocking reagent (Roth), and goat serum were used for blockade of unspecific binding sites. Slides were incubated with primary antibodies specific for E-cadherin (36/E; BD) and αE integrin [EPR4166(2); Abcam] with subsequent treatment with biotin-conjugated goat anti-mouse antibody (Vectorlabs) and a streptavidin-Dylight 488 conjugate (Biolegend) or a Cy3-labeled goat anti-rabbit antibody (Merck), respectively. After counterstaining of cell nuclei with Hoechst dye (molecular probes), confocal microscopy (LSM SP8) was used for analysis.

Clinical Parameters

Clinical data documented by the attending physician before treatment initiation or on the occasion of vedolizumab treatments of the analyzed patient cohort were retrospectively collected from the electronic patient files. Particularly, these data included weight (in kilograms), abdominal pain (patient-reported numeric rating scale intensity ranging from 0 to 10), stool frequency (stools per day) and consistency (1—solid, 2—soft, 3—pasty, 4—liquid), presence of blood in the stool, laboratory parameters [C-reactive

protein (CRP), hemoglobin], and well-established disease activity indices [Harvey–Bradshaw index (HBI) for CD (19) and Mayo clinical subscore (MCS) for UC (20)].

Statistics

To correlate changes in integrin expression with clinical parameters, flow cytometric and clinical data from T2 to T8 were analyzed in comparison to the baseline value obtained before T1. Absolute differences compared with T1 (e.g., Δ HBI vs. T1), or relative differences compared with T1 expressed as % of the baseline value (e.g., % α4β1 expression compared with T1) were calculated. Accordingly computed values for integrin and chemokine receptor expression were correlated with the listed clinical parameters in GraphPad Prism, and Pearson's *r* was calculated. Where reasonable, changes in categorial variables were grouped to "decrease," "no change," and "increase," and corresponding integrin expression changes were compared with one-way ANOVA and Newman–Keuls *post hoc* or Student's *t*-test.

For the analysis of relation between α4β1 expression changes at T3 and clinical response at T5, UC patients were classified as "responders," when the MCS had dropped by two or more points from T1 to T5 and as "non-responders," when the MCS had increased, remained the same, or dropped by not more than one point. Integrin expression changes in these groups were compared by Student's *t*-test, and a receiver-operator characteristic (ROC) was compiled.

Levels of significance are indicated by asterisks ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

RESULTS

Significant Correlation of Changes in Integrin and Chemokine Receptor Expression under Vedolizumab Therapy

We analyzed changes in the expression of integrins and chemokine receptors in a cohort of 19 patients with CD and 17 patients with UC (**Table 1**).

Since the factors regulating integrin and chemokine receptor expression in different T cell subsets substantially intersect (21, 22), we reasoned that tracking expression of such markers in patients over time should reveal concordant changes between different subsets or different markers. Thus, we started our analyses with according explorations. We found a significant correlation of changes in the α4β7 integrin expression on $CD4^+$ with that on $CD8^+$ T cells both in CD (**Figure 1A**) and in UC (Figure S2A in Supplementary Material). A similar finding was made for the correlation of changes in the expression of CCR2 with CCR6 on $CD4^+$ T cells (**Figure 1B**; Figure S2B in Supplementary Material). Moreover, changes in αEβ7 integrin expression on $CD4^+$ and $CD8^+$ T cells were correlated with each other and an association of changes in α4β1 expression with both CCR2 and CCR6 was found in CD (Figures S2C–E in Supplementary Material and data not shown), confirming that cues regulating integrin expression in T cells have similar impact on the $CD4^+$ and the $CD8^+$ subset and suggesting that there is considerable overlap in the signals regulating expression of homing markers.

Changes of α4β7 Integrin Expression Are Related to Clinical Presentation of Vedolizumab-Treated Patients in UC but Not in CD

In addition, we correlated the changes in the expression of integrins and chemokine receptors over the course of vedolizumab therapy to changes in clinical parameters.

For α4β7 integrin, we found that increasing expression on $CD4^+$ T cells from patients with UC during vedolizumab therapy was associated with decreasing abdominal pain reported by the patients as numeric rating scale intensity (**Figure 2A**). This might reflect successful blockade of α4β7-dependent gut homing leading to an increasing percentage of α4β7-expressing T cells in the peripheral blood and, consistently, to reduced intestinal symptoms.

Unexpectedly, however, no such association could be identified for patients with CD (**Figure 2B**). This is consistent with the notion that response or non-response to vedolizumab therapy in CD does not go along with specific alterations of α4β7 integrin expression and suggests that differences between the mechanistic impact of vedolizumab therapy in CD and UC exist.

In addition, we wondered whether response or non-response to vedolizumab might be associated with different pretreatment levels of α4β7-expressing $CD4^+$ T cells. Surprisingly, it appeared that IBD patients with a clinical response after 16 weeks (defined as a decrease of at least two points in the HBI or MCS) had lower initial frequencies of α4β7-expressing T cells than patients without clinical response (**Figure 2C**). While this finding requires prospective validation in larger cohorts, it might indicate that low α4β7 expression increases the likelihood that α4β7-dependent homing of disease-relevant T lymphocytes to the gut is completely blocked.

Dynamic Expression of αEβ7 Integrin on T Cells Is Associated with Clinical Outcome Parameters in UC

Moreover, an association of rising αEβ7 expression with worse development of clinical parameters was noted in UC: there was a coherence of increases in αEβ7 expression on $CD4^+$ T cells with increasing levels of the inflammation marker CRP (**Figure 3A**) and a trend toward looser stools when αEβ7 expression increased (Figure S3A in Supplementary Material). Such association of rising αEβ7 with poorer clinical presentation was even clearer when analyzing αEβ7 on $CD8^+$ T cells. Here, relative αEβ7 expression compared with T1 was significantly increased in patients with mounting scores in the MCS "rectal bleeding score" component compared with patients with declining scores (**Figure 3B**). This was backed up by a highly significant correlation of increasing αEβ7 on $CD8^+$ T cells with looser stool consistency and increasing CRP. Moreover, a strong trend for a positive coherence with increasing abdominal pain was noted (Figure S3B in Supplementary

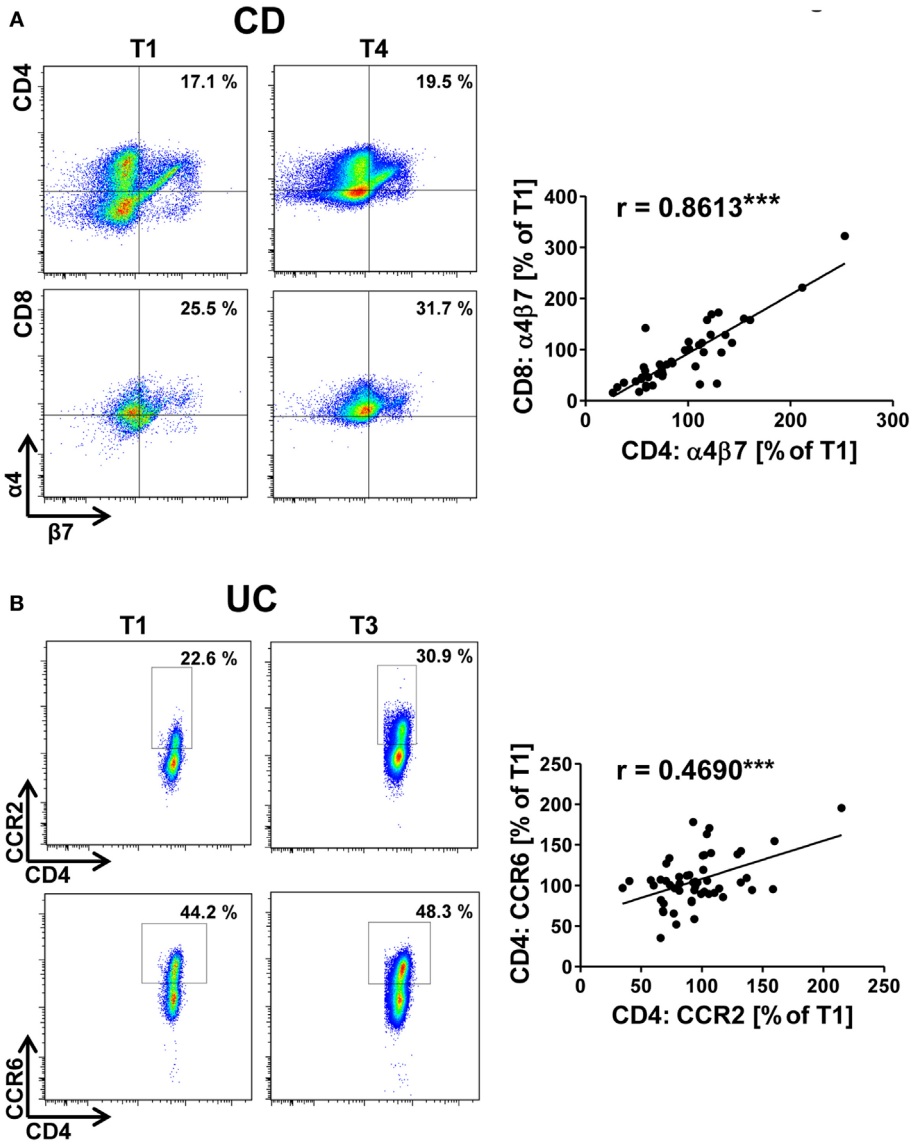

FIGURE 1 | Correlation of dynamic changes in integrin and chemokine receptor expression in patients under vedolizumab treatment. Correlation of changes vs. baseline (T1) observed before treatment 2–8 (T2–8) in flow cytometric α4β7 expression on peripheral CD4+ and CD8+ T cells from Crohn's disease (CD) patients **(A)** and of changes in flow cytometric CCR2 and CCR6 expression on peripheral CD4+ T cells from ulcerative colitis (UC) patients **(B)** treated with vedolizumab. Left panels: representative plots from one patient showing the percentage of $\alpha4^+\beta7^+$ among CD4+ and CD8+ T cells **(A)** and the percentage of CCR2+ and CCR6+ among CD4+ T cells **(B)** at baseline (T1) and before treatment 3 or 4 (T3/T4) as indicated. Right panel: pooled data from 12 **(A)** and 15 patients **(B)** depicting the changes vs. T1 observed before T2 to T8. Pearson's *r* and significances are indicated.

Material). These observations proposed that increasing αEβ7 might have a negative impact on the outcome of vedolizumab therapy in UC. Once again, no similar correlations could be identified in CD (data not shown).

Some of these observations for the correlation of αEβ7 with clinical data suggested a link of αEβ7 with intestinal epithelial barrier integrity, since normal consistency and frequency of bowel movements as well as the absence of blood in the stool require an intact epithelium to allow resorption of nutrients and foods as well as to preserve the integrity of deeper layers of the gut wall.

Accordingly, we performed immunohistochemical stainings for αE integrin and its ligand, the epithelial cell marker E-cadherin. As expected, we could demonstrate αE^+ cells occurring in close proximity to epithelial cells both in the healthy and inflamed gut (**Figure 3C**) and, furthermore, also in patients receiving vedolizumab (**Figure 3D**). Although our sequential measurements confined to the peripheral blood, this indicated that the reason for specific association of dynamic αEβ7 expression changes with clinical development under vedolizumab therapy might be due to an impact of αEβ7-expressing T cells on the intestinal epithelium.

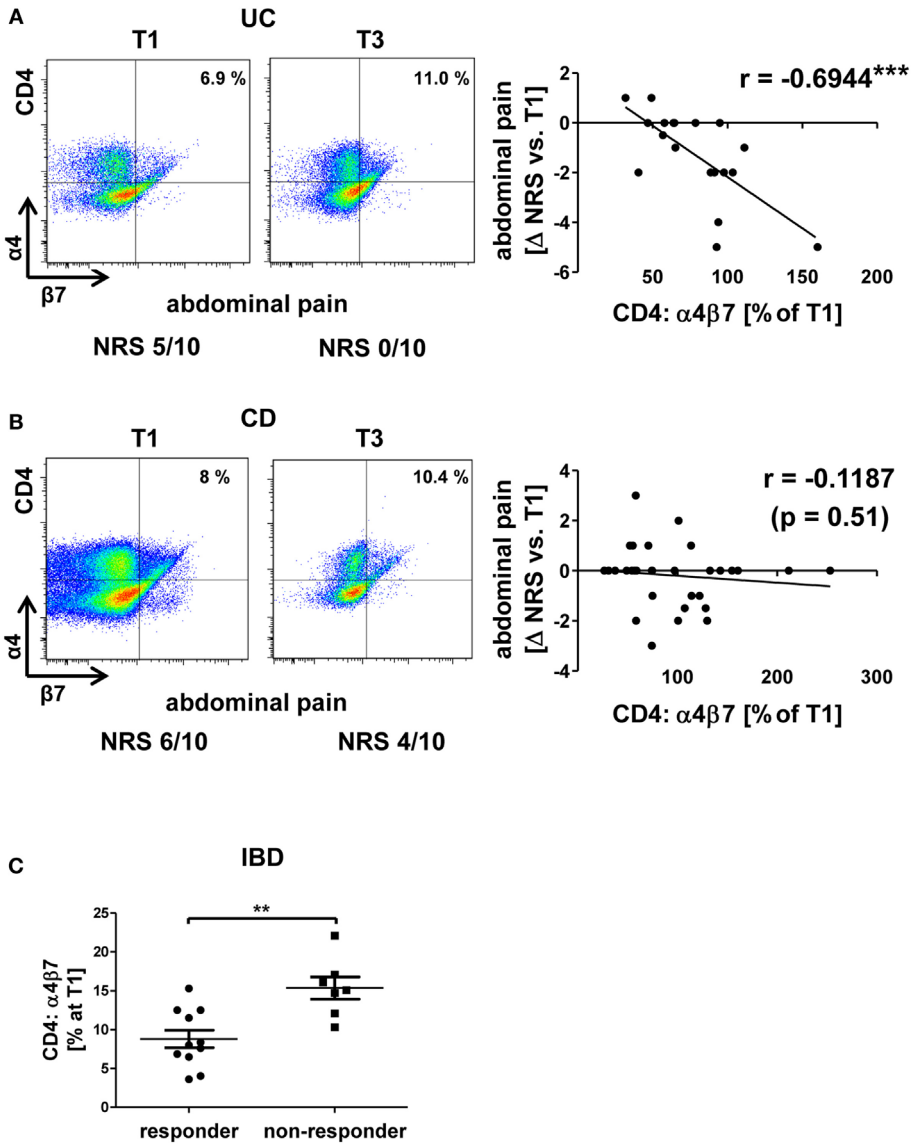

FIGURE 2 | Correlation of dynamic changes in α4β7 integrin expression with clinical parameters in patients under vedolizumab therapy. **(A,B)** Correlation of changes vs. baseline (T1) observed before treatment 2–8 (T2–8) in flow cytometric α4β7 expression on peripheral CD4+ T cells with changes in patient-reported abdominal pain in patients with UC **(A)** and CD **(B)**. Left panels: representative plots from one patient showing the expression of α4β7 on CD4+ T cells before the mentioned treatments and indicating the corresponding abdominal pain rating below. Right panels: pooled data from 7 **(A)** and 11 patients **(B)** depicting the changes vs. T1 observed at T2–T8. Pearson's *r* and significances are indicated. IBD, inflammatory bowel diseases; NRS, numerical rating scale; CD, Crohn's disease; UC, ulcerative colitis. **(C)** Flow cytometric expression of α4β7 integrin at baseline in IBD patients with a clinical response (defined as decrease of at least two points in Mayo clinical subscore or Harvey–Bradshaw index) after 16 weeks. Significance is indicated.

Changes in α4β1 Expression after 6 Weeks Vedolizumab in UC Are Correlated with Clinical Response after 16 Weeks

A similar pattern of association of dynamic integrin expression with clinical outcome parameters as for αEβ7 integrin was identified for α4β1 integrin in UC since increases in α4β1 expression were correlated with worse development of clinical parameters in vedolizumab-treated patients. Particularly, when α4β1 rose, patients experienced a higher frequency of bowel movements (**Figure 4A**). Moreover, when patients reported of looser stools compared with T1, they were more likely to have increased levels of α4β1 expression compared to T1, resulting in a significant correlation of these parameters (**Figure 4B**). This is also consistent with the finding that in patients, in which the partial "physician global assessment score" of the MCS dropped, relative α4β1 expression compared to T1 was lower than in those with increasing physician global assessment scores (**Figure 4C**).

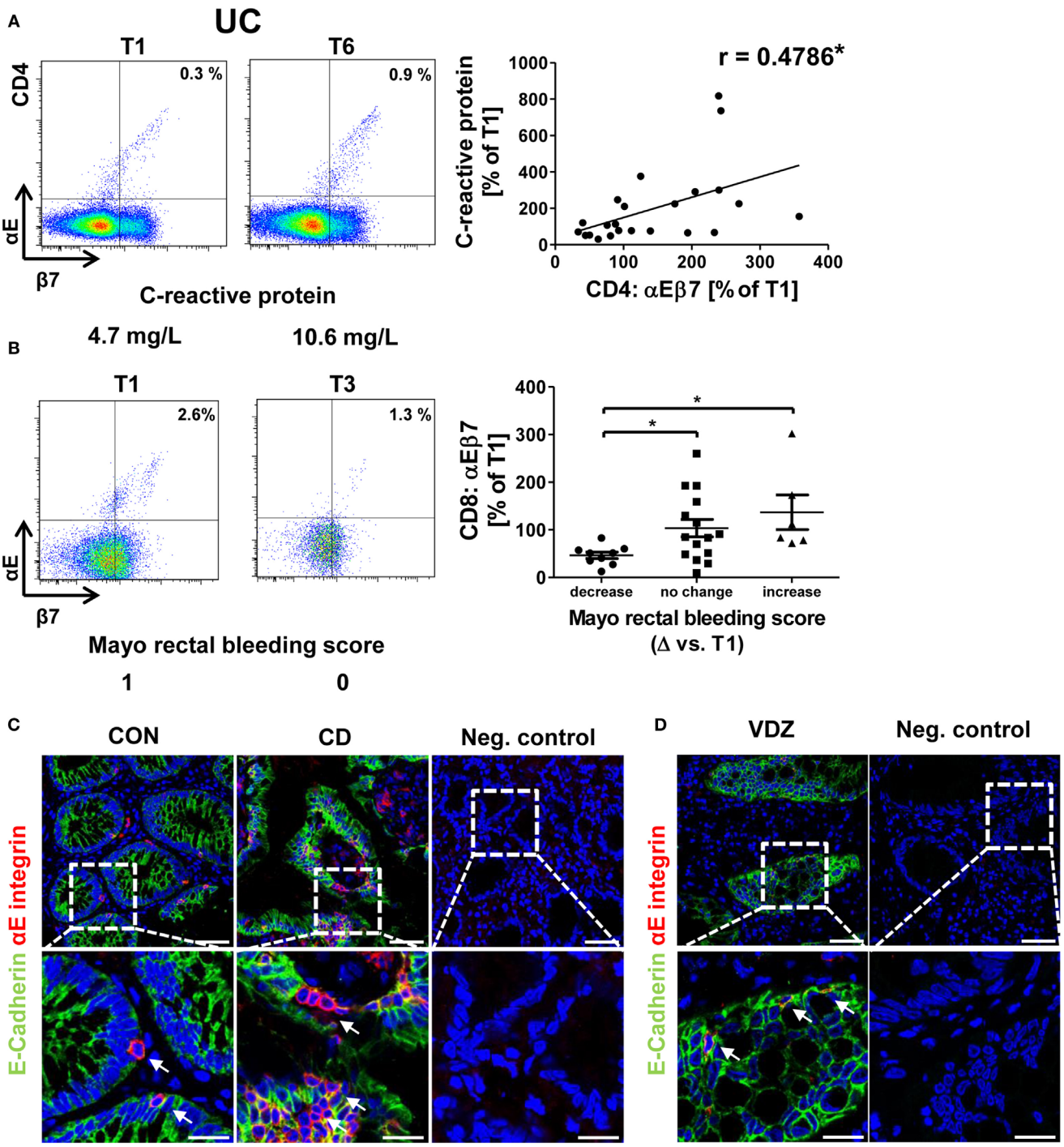

FIGURE 3 | Correlation of dynamic changes in αEβ7 integrin expression with clinical parameters in patients under vedolizumab therapy. **(A)** Correlation of changes vs. baseline (T1) observed before treatment 2–8 (T2–8) in αEβ7 expression on peripheral CD4+ UC T cells with changes in C-reactive protein levels. **(B)** Changes in αEβ7 expression on peripheral CD8+ UC T cells in patients with decreasing, unchanged, or increasing Mayo rectal bleeding score. Left panels: representative plots from one patient showing the expression of αEβ7 on CD4+ or CD8+ T cells before the mentioned treatments and indicating the corresponding clinical parameters below. Right panels: pooled data from 9 patients depicting the changes vs. T1 observed at T2–T8. Pearson's *r* and significances are indicated. **(C,D)** Representative images showing immunohistochemistry of gut cryosections for αEβ7 (red) and epithelial E-cadherin (green) in a non-IBD patient (CON) and a CD patient **(C)** as well as in a patient treated with vedolizumab **(D)**. White arrows indicate αEβ7+ cells in contact with E-cadherin+ epithelial cells. VDZ, vedolizumab; IBD, inflammatory bowel diseases; CD, Crohn's disease; UC, ulcerative colitis.

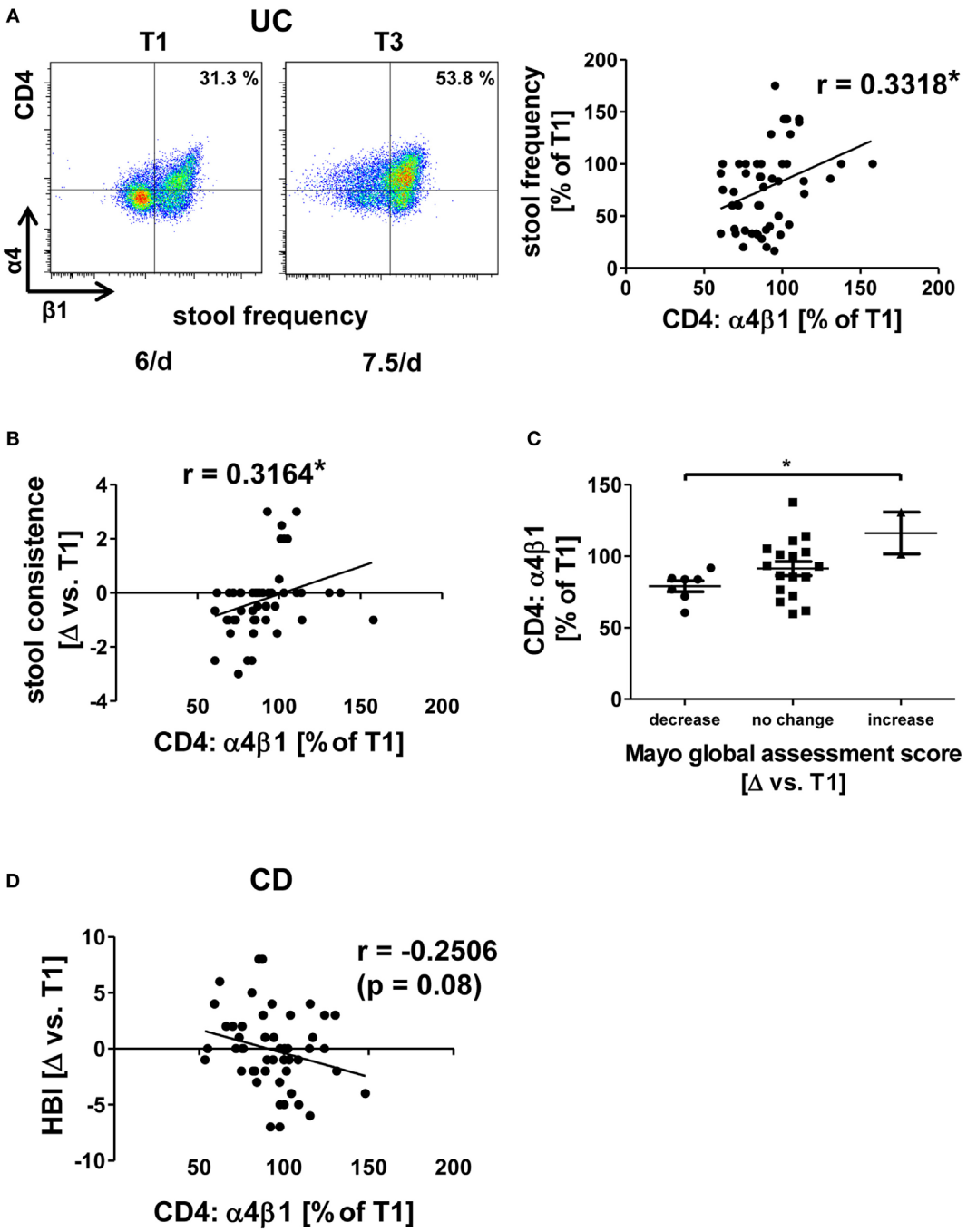

FIGURE 4 | Correlation of dynamic changes in α4β1 integrin expression with clinical parameters in patients under vedolizumab therapy. **(A,B,D)** Correlation of changes vs. baseline (T1) observed before treatment 2–8 (T2–8) in flow cytometric α4β1 expression on peripheral CD4+ T cells with changes in stool frequency **(A)** and stool consistence **(B)** in ulcerative colitis (UC) patients and Harvey–Bradshaw index (HBI) in Crohn's disease (CD) patients **(D)**. Representative plots from one patient **(A)** show the expression of α4β1 on CD4+ T cells before the mentioned treatments, and the corresponding stool frequency is indicated below. Graphs **(A,B,D)** show pooled data from 13 to 14 patients depicting the changes vs. T1 observed at T2–T8. Pearson's *r* and significances are indicated. **(C)** Changes vs. T1 in flow cytometric α4β1 expression on peripheral CD4+ T cells observed before T2–T8 in UC patients with decrease, no change, or increase of the Mayo global assessment subscore. Significance is indicated.

However, no significant correlation between clinical changes and α4β1 could be identified in CD. Yet, there was a trend ($p = 0.08$) suggesting that decreasing α4β1 expression might be associated with increasing HBI scores in CD patients (**Figure 4D**). Taken together, dynamic changes in all α4β7-related integrins were significantly related to dynamic changes of clinical outcome

parameters in UC, which is compatible with the perception that individual (counter-)regulatory pathways might affect outcome of vedolizumab therapy in UC by mediating the expression of integrins. On the other hand, while the findings for α4β1 integrin rather suggested a differential regulation compared with UC, no such significant correlations were identified in CD supporting the idea that molecular differences in the homing pathways implicated in CD and UC exist.

Like for other drugs, the response to vedolizumab treatment cannot be predicted in single patients so far leading to a significant portion of patients, which are treated without success and have to be assigned to another therapy. In this light, we explored whether any of the above depicted findings might be used to identify an early marker of successful vedolizumab treatment in UC. To this end, we compared integrin expression changes after 6 weeks of vedolizumab treatment (i.e., before T3) with clinical outcome before T5 (i.e., 16.1 ± 0.2 weeks), and patients were classified as "responders" and "non-responders" based on the MCS as described in the Section "Materials and Methods."

Indeed, we found that patients with a clinical response had decreasing α4β1 levels after 6 weeks compared with baseline, while patients without clinical response had increasing levels compared with baseline (**Figure 5A**).

This was the case in all 11 patients that could be included into this analysis, and the distribution was statistically significant. An ROC analysis showed that a cutoff of +4.2% change in α4β1 integrin from T1 to T3 had 100% sensitivity and 100% specificity for the allocation of patients from our cohort to the responder or non-responder group at T5 (**Figure 5B**). Of note, no association of initial levels of α4β1-expressing $CD4^+$ T cells with response could be observed, and only one of seven responders already fulfilled the respective criterium of at least two points drop in MCS at T3, indicating that changes in α4β1 expression are indeed preceding clinical outcome manifestation.

DISCUSSION

The approval of vedolizumab for clinical therapy of both UC and CD has substantially increased the therapeutic armamentarium in IBD (7, 8). Meanwhile, efficacy and safety have not only been documented in randomized clinical trials but also in real-world settings (15–17). This has been accompanied by mechanistic investigations elucidating *in vivo* effects of vedolizumab on T cell homing (13, 23). However, a number of questions regarding the immunological effects of vedolizumab remain. For instance, it remains elusive why vedolizumab lacks effect in a portion of patients and why this portion seems to be larger in CD compared with UC (7, 8, 18). Moreover, this also includes questions addressing the immunological sequelae of α4β7-dependent homing disruption, e.g., regarding the expression and functionality of other homing molecules in view of their effects on the equilibrium of peripheral blood and intestinal T cell populations (14).

Our present study was conducted with the aim to bring some light into these uncertainties and, therefore, we systematically analyzed associations of changes in α4β7 and related integrins (24) as well as in chemokine receptors with changes in clinical parameters over the course of vedolizumab therapy. For the first time, our data show that several parameters of patient-reported and physician-documented response to vedolizumab treatment are associated with specific changes in the expression of integrins but not chemokine receptors in UC providing new insights into the mechanisms of vedolizumab therapy and fueling hopes for their use in prediction of response to therapy.

While several significant correlations between integrin expression changes and clinical parameter changes were identified in UC, none could be identified in CD. On a molecular level, this further substantiates the empirical clinical observation that differences in the efficacy of vedolizumab treatment seem to exist between UC and CD (18). The only correlation that was approaching significance was that of changes in α4β1 expression with changes in HBI score, suggesting that decrease of the former might go along with increase of the latter parameter. This is in line with earlier observations in an *in vivo* mouse model showing that compensatory homing *via* the α4β1/vascular cell adhesion molecule (VCAM)-1 pathway might bypass α4β7 blockade in CD (23) and matching to rodent data that propose considerable redundancy in different homing pathways (25, 26) and VCAM-1-dependent homing as an important pathway in CD-like experimental colitis (27). Accordingly, decreasing $\alpha4\beta1^+$ CD4 T cells in the peripheral blood might reflect increased gut homing of such cells triggering increased intestinal inflammation.

In UC, increase in α4β7 seemed to be associated with favorable clinical development, while increase of α4β1 or αEβ7 expression were correlated with worsening of several clinical parameters. The specificity of these findings for integrins was supported by the fact that changes in CCR2 and CCR6 expression, which are primarily unrelated to α4β7, did not correlate with any of the parameters analyzed.

While these observations are undoubtedly interesting, they are raising new questions regarding the underlying mechanisms. A possible explanation of our findings could be that patients in which α4β7 blockade by vedolizumab sufficiently works have both more peripheral blood T cells expressing α4β7 and amelioration of clinical symptoms due to preclusion of $\alpha4\beta7^+$ T cells from the gut tissue. On the other hand, upregulation of α4β1 and αEβ7 might be a sign of upregulation of rescue pathways, which has also been proposed to be responsible for extraintestinal side effects observed under vedolizumab therapy (28). In particular, cells might upregulate the expression of alternative integrins in an attempt to ensure access to or positioning in the lamina propria *via* alternative pathways beyond the blocked α4β7–MAdCAM-1 axis, which could subsequently lead to severer or maintained inflammation. However, especially since the coherence of α4β1 expression changes with changes in clinical parameters seems to be different in CD und UC, this remains speculative and underscores that additional translational research is necessary to better understand the alterations in integrin-expressing cell subsets at the interface of the peripheral blood and the intestine. Yet, in light of the above remarks, such differences between CD and UC must not be surprising but should be interpreted as another cue illustrating differences in therapeutic interference with homing in CD and UC. It has also to be taken into account that our cohort mainly consisted of patients previously exposed to anti-tumor necrosis factor (TNF)-α antibodies, and results

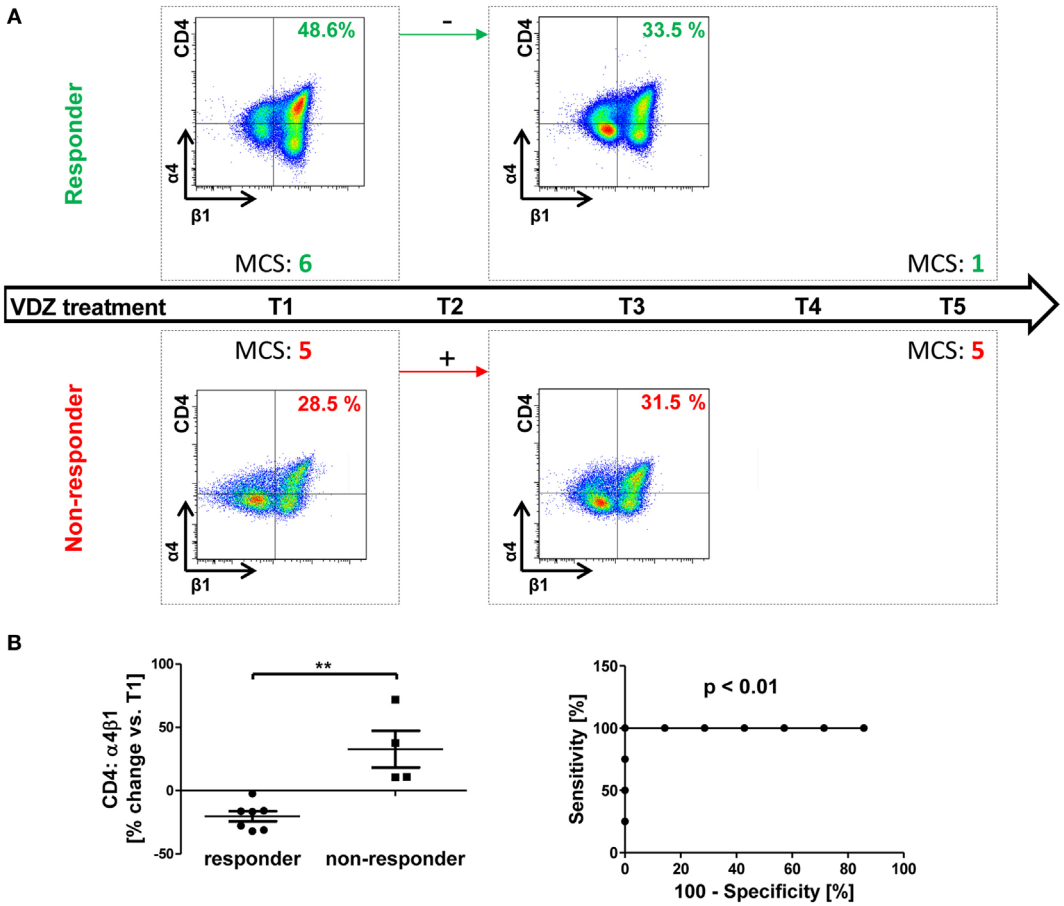

FIGURE 5 | Possible prediction of response to vedolizumab therapy by changes in α4β1 integrin expression in ulcerative colitis (UC). **(A)** Schematic drawing representatively depicting the association of changes in α4β1 expression from baseline (T1) to treatment three (T3) with the response to vedolizumab therapy from T1 to T5 in UC patients. Upper panels: in patients with Mayo clinical subscores (MCS) decreasing by at least two points (responders, green) α4β1 expression on CD4+ T cells decreased. Lower panels: in patients without clear decrease in the MCS, α4β1 expression increased. **(B)** Left panel: pooled statistic of the evaluated cohort (n = 11). Right panel: receiver-operator-characteristic analysis for prediction of clinical response before T5 by changes in α4β1 observed before T3. Significances are indicated.

might not show such a difference in anti-TNF naïve CD and UC collectives.

No comparable data for the use of anti-adhesion antibodies have been reported so far. However, one study sequentially assessed the peripheral blood of IBD patients under therapy with the anti-TNF-α antibody infliximab for expression of regulatory T cell (Treg) markers. The authors showed that infliximab responders and non-responders had differential development in peripheral Treg profiles (29). Thus, although infliximab is believed to mediate its effect predominantly by inhibition of increased TNF-α signaling in the lamina propria, associated changes could be noted in the blood. Since vedolizumab blocks α4β7 integrin on T cells in the peripheral blood, such analyses even assess the changes of immunological markers at the point of action vedolizumab.

The findings reported for αEβ7 match with a recent report from our group suggesting that a subset of αEβ7+ T cells does not express α4β7 (22), and αEβ7+ cells might accumulate in the gut *via* additional or alternative pathways. Of note, αEβ7 itself has been proposed to mediate gut homing independently of α4β7 *via* a so far unknown ligand (30). Moreover, it has to be mentioned that additional αEβ7+ T cells have been shown to be induced in the gut in response to epithelium-released transforming growth factor-β (31), and the only known ligand for αEβ7 is E-cadherin expressed on the intestinal epithelium (32–34). Thus, it seems possible that cells deprived of α4β7 compensatorily upregulate αEβ7 on their surface in search of another homing pathway to reach the gut or—more general—in search of possibilities to ensure positioning in the lamina propria (whether by homing or by epithelial retention). As we show, many αEβ7-bearing cells can be found in close contact with E-cadherin-expressing epithelium. Thus, it is very likely that αEβ7+ T cells communicate with the epithelium. Independent reports have recently shown that αEβ7 is enriched in pro-inflammatory T cell subsets (22, 35) and that αEβ7+ T cells express higher levels of granzyme A than αEβ7− T cells (36). *In vivo*, this might result in deleterious effects of such αEβ7-expressing on epithelial cells, which is supported by some of our data showing correlations of changes in αEβ7

integrin with changes in clinical parameters that are indicative of intestinal epithelial barrier function.

Taken together, these correlation analyses indicate so far unknown associations between clinical and immunological parameters, while the exact mutual dependencies need to be clarified in further research. Though, from a clinical perspective the questions whether such associations or initial expression levels might be exploited for monitoring or even prediction of therapeutic response to vedolizumab obtrudes and, thus, we performed respective analyses as detailed above. Unexpectedly, we observed that IBD patients with a clinical response after 16 weeks had lower initial levels of α4β7-expressing $CD4^+$ T cells than non-responders. This is intriguing since one could have assumed that higher α4β7 expression is a sign of higher importance of α4β7-dependent homing, and it might thus be more promising to block α4β7 in patients with higher expression. Yet, the explanation for our finding could be that even low numbers of α4β7-expressing T cells are crucial for disease pathogenesis and low initial expression might raise the odds of completely preventing these T lymphocytes from homing to the gut. It will be an important task of future studies to prospectively validate this preliminary observation. Moreover and most interestingly, we also found a surprisingly clear association of changes in α4β1 expression on $CD4^+$ T cells after 6 weeks with clinical response after 16 weeks. While these pilot data—like the whole study—are limited by the rather small patient number and retrospective collection of clinical data, thus requiring confirmation in larger multicenter studies, it is nevertheless an observation that disserves further investigation and raises hopes that 2 months of ineffective treatment could be saved in some patients by measurement of the α4β1 expression profile at baseline and after 6 weeks of treatment. Although this would not be a prediction marker that can be assessed before beginning therapy like it was conceptually shown for membrane-bound TNF-α receptor in therapy with anti-TNF-α antibodies (37) or intestinal αE expression in therapy with the experimental anti-β7 integrin antibody etrolizumab (38), it could yet accelerate the assessment of individual response to vedolizumab.

In conclusion, our results suggest that individual response to vedolizumab treatment in UC might be reflected by specific changes in integrin profiles in the peripheral blood. Further studies are required to confirm the translational potential of these observations for the prediction of response to therapy.

ETHICS STATEMENT

All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Ethics Committee of the University Hospital Erlangen.

AUTHOR CONTRIBUTIONS

FF, DS, and SZ performed the experiments; RA, SH, SF, CN, IA, MN, and SZ provided clinical samples, protocols, reagents, or designed experiments; FF, DS, CN, IA, MN, and SZ analyzed and interpreted the data; SZ drafted the manuscript; all authors critically revised the manuscript for important intellectual content.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.00764/full#supplementary-material.

FIGURE S1 | Study outline. **(A)** Schematic sketch of the study design. Patients treated with vedolizumab were followed up from treatment one to six to eight, and blood samples were sequentially collected before each treatment for subsequent flow cytometric analysis of integrin and chemokine receptor expression on $CD4^+$ and $CD8^+$ T cells. Clinical and laboratory data from the respective patients were retrospectively collected. Changes of clinical and flow cytometric parameters were correlated. **(B,C)** Gating strategy for the measurement of integrin and chemokine receptor expression on T cells. After exclusion of doublets and gating on lymphocytes in the forward/sideward-scatter, $CD4^+$ **(B)** or $CD4^+$ and $CD8^+$ T cells were selected, and the expression of $\alpha4^+\beta1^{high}$, $CCR2^+$, and $CCR6^+$ **(A)** or $\alpha4^+\beta7^+$ and $\alpha E^+\beta7^+$ cells **(B)** were quantified, respectively.

FIGURE S2 | Correlation of dynamic changes in integrin and chemokine receptor expression in patients under vedolizumab therapy. Correlation of changes in flow cytometric α4β7 expression on peripheral $CD4^+$ and $CD8^+$ T cells from ulcerative colitis (UC) patients **(A)**, of changes in flow cytometric CCR2 and CCR6 expression on peripheral $CD4^+$ T cells **(B)**, of changes in flow cytometric αEβ7 expression on peripheral $CD4^+$ and $CD8^+$ T cells **(C)**, and of changes in flow cytometric α4β1 expression with changes in CCR2 **(D)** and CCR6 **(E)** expression on peripheral $CD4^+$ T cells in Crohn's disease (CD) patients treated with vedolizumab. **(C)** Contains representative plots showing the percentage of $\alpha E^+\beta7^+$ among $CD4^+$ and $CD8^+$ T cells before the indicated treatments. Panels include pooled data from 12 to 18 patients. Pearson's *r* and significances are indicated.

FIGURE S3 | Correlation of dynamic changes in integrin expression with clinical parameters in ulcerative colitis (UC) patients under vedolizumab treatment.

Correlation of changes in flow cytometric expression of αEβ7 on CD4+ T cells **(A)** and CD8+ T cells **(B)** with changes in the indicated clinical parameters. Pearson's *r* and significances are indicated. Panels include data from 9 to 15 patients.

TABLE S1 | Correlation of changes in integrin expression with changes in clinical parameters in ulcerative colitis patients. Pearson's *r* values for the correlation of changes in expression of the different integrins (in lines) with the changes of a representative panel of clinical parameters (in columns) are noted. Dark green color indicates significant correlations matching with the overall picture mentioned in the text. Light green indicates correlations not reaching significance matching with the overall picture mentioned in the text. Pale green indicates correlations not further supporting the overall picture mentioned in the text. Here, *p* values are additionally indicated to show that these correlations were not essential for overall interpretation.

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