CYTOKINES AND INTESTINAL MUCOSAL IMMUNITY

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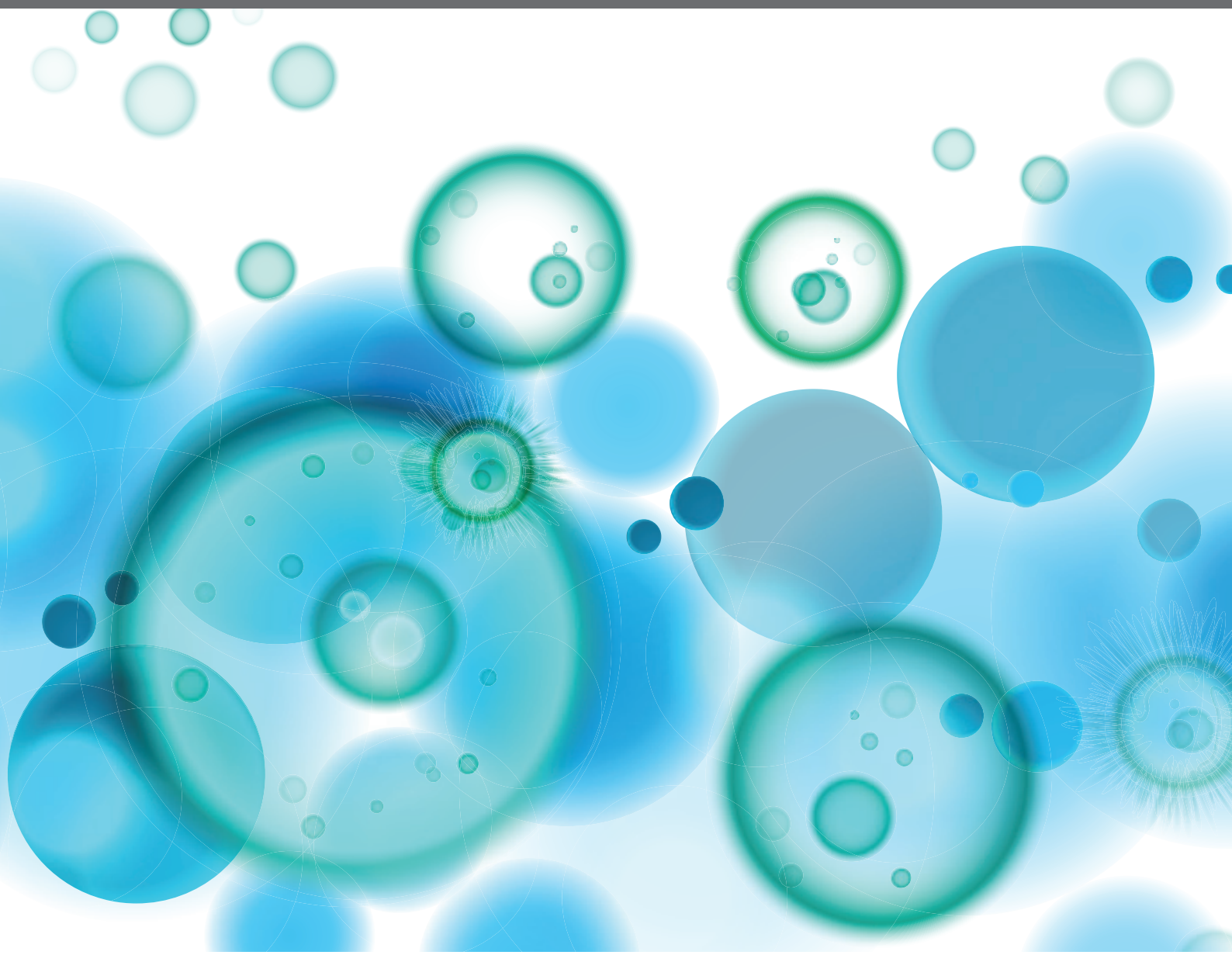

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CYTOKINES AND INTESTINAL MUCOSAL IMMUNITY

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EDITORIAL
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Editorial: Cytokines and Intestinal Mucosal Immunity

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Editorial on the Research Topic

Cytokines and Intestinal Mucosal Immunity

Since discovery of the prototypic cytokines, interleukin-1 (IL-1) and tumor necrosis factor-α (TNF), almost 50 years ago (1, 2), an explosion of information has followed regarding the biology of cytokines and their critical role(s) during health and disease. To date, 41 interleukins and more than 18 TNF superfamily (TNFSF) members have been described. Notably, in 1990, our group was one of the first to show that blockade of a single cytokine, *i.e.*, IL-1, was effective in markedly reducing the severity of experimental colitis (3), laying the foundation to conceptualize that targeting of an individual cytokine could successfully impact the development and progression of a specific disease. The role of cytokines, in fact, has been particularly important in the gastrointestinal tract, both in maintaining homeostasis and during chronic inflammatory disorders, such as inflammatory bowel disease (IBD), wherein many cell types have the ability to both react to, and produce, cytokines in response to a variety of antigenic stimuli, dietary products, microbial components, and toxic agents. This wealth of new information has led to the approval of different anti-cytokine therapies, such as anti-TNF and anti-IL-12/23 monoclonal antibodies, for the treatment of both Crohn's disease (CD) and ulcerative colitis (UC), the two main forms of IBD. In addition, novel small molecule inhibitors, such as those targeting the JAK/STAT pathway, and which possess broad anti-cytokine activity, are now available in the armamentarium of gastroenterologists to treat IBD.

In this Research Topic, the role of canonical, and more novel, cytokines are discussed in the context of intestinal immunity and chronic gut inflammation. Three articles focus on the role(s) of TNFSF members (Li et al.; Valatas et al.; Giles et al.). Specifically, Li et al. and Valatas et al. report the importance of the TL1A (TNF-like ligand 1A, TNFSF15)/DR3 (death receptor 3,TNFRSF25) ligand-pair, for which increasing evidence suggests a critical role not only in the pathogenesis of IBD, but also in the development of gut fibrosis/fibrostenotic disease. These papers highlight TL1A/DR3's pleiotropic functions in regulating the balance between T effector and T regulatory cells (Tregs), as well as innate lymphoid cells (ILCs), thereby serving as a vital rheostat during IBD. Notably, monoclonal antibodies against TL1A are currently in clinical trials for the treatment of CD and UC, and will shortly reveal the efficacy of anti-TL1A/DR3 strategies in IBD.

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Abbreviations: CD, Crohn's disease; DR3, death receptor 3; IL-, interleukin-; ILC, innate lymphoid cell; TGFβ1, transforming growth factor-beta1; Th, T-helper; TL1A, TNF-like ligand 1A; TNF, tumor necrosis factor-α; TNFSF, TNF super family; Treg, T-regulatory cell; UC, ulcerative colitis; VDZ, vedolizumab.

Additionally, Giles et al. show that LIGHT (TNFSF14) can have differential effects on the outcome of experimental colitis, depending on engagement of, and signaling through, its various cognate receptors, further emphasizing the complexity of the TNFSF and the function of its members in IBD.

Also included in the Research Topic are comprehensive reviews highlighting the contribution of key populations of cytokine-producing, and -responding, gut mucosal cells(Andrews et al.; De Salvo et al.; Weidinger et al.). Intestinal epithelial cells play a central role in maintaining homeostasis throughout the gastrointestinal tract as collectively, they represents the host's first line of defense against the external environmental, while balancing their response to the underlying gut mucosal immune system. Dysregulation of these finely-tuned interactions can compromise barrier function and result in uncontrolled, chronic inflammation. Andrews et al. provide important information regarding how epithelial-derived cytokines, as well as cytokines affecting the gut epithelium, orchestrate crucial functions during health and disease states. Furthermore, ILCs are a relatively new family of heterogenous immune cells driven by specific transcription factors that exhibit distinct cytokine profiles and are particularly enriched at mucosal barriers. As their name implies, ILCs provide necessary innate immunity to the host; however, based on their strategic location, they must also be able to temper and quickly alter response(s) to their dynamic and everchanging surroundings. In this context, De Salvo et al. summarize what is currently known regarding the cytokines that enable ILC plasticity, and their ability to readily transdifferentiate, leading to either protective or pathogenic functions within the gut. Interestingly, the original article from Christodoulou-Vafeiadou et al. underscores the important contributions of epithelial versus innate immune cells, reporting that the RNA binding protein, HuR, that post-transcriptionally regulates mRNAs encoding, among others, cytokines, confers both convergent as well as divergent functions in the gut depending on its cellular source and what type of challenge is presented to the host (*e.g.*, colitis-associated cancer vs. infectious colitis). Finally, a newly emerging area of interest in cytokine biology is the role adipokines, which are soluble factors released by fat tissue. Their role in IBD is especially relevant since CD is often characterized by hyperplasia of the mesenteric fat situated around affected, inflamed segments of the small intestine, and notably, this so-called 'creeping fat' has also been implicated in fibrostenotic CD, with recent work demonstrating its regulation by live bacteria derived from the gut microbiome (4). Weidinger et al. provide important information regarding adipokines, its regulation of creeping fat, and put forth a working model as to how these factors participate in gut health and disease.

The remaining contributions focus on various aspects of targeting cytokines to treat IBD. Anti-$\alpha4\beta7$ integrin therapy using vedolizumab (VDZ) was introduced a decade ago as an effective, alternative option for IBD patients that were refractory to either conventional treatments or TNF inhibitors. Ironically, Rath et al. show that increased activation of TNF is observed in IBD patients unresponsive to VDZ, and that anti-TNF therapy may actually be more effective in VDZ refractory patients. In regards to newer anti-cytokine strategies, blocking the IL-12/IL-23 axis has proven to be effective for both CD and UC patients. Interestingly, although Th17 cells are known to expand in response to IL-23, anti-IL-17 treatment is reported to be ineffective in treating CD, and in fact, worsens disease (5). In support of disease specificity for anti-cytokine therapy, Buchele et al. show that blockade of the IL-23/Th17 pathway is ineffective in preventing colitis in a mouse model of graft-vs.-host-disease, suggesting that targeted anti-cytokine therapy is disease specific, rather than organ-specific, and that the underlying etiology is critical to consider. Regulation of another cytokine recently tested in CD is TGFβ1, whose anti-inflammatory activity is restored by administering Mongersen, an oral SMAD7 antisense oligonucleotide, but with controversial outcomes. Troncone et al. review this important cytokine pathway in intestinal immunity that, despite negative results of Phase III clinical trials (6), remains an interesting therapeutic target for IBD, perhaps using a different drug formulation. In closing, Abo et al. contribute an original article demonstrating the efficacy of combination IL-2 immunocomplex and anti-IL-5 in experimental colitis through a mechanism that involves expansion of Foxp3$^+$ Tregs. Although premature to consider for IBD treatment, the concept of combination therapy is certainly an attractive strategy, particularly for complex, multi-pathway disease processes that are prevalent in disorders like IBD. We anticipate that the field of cytokines in intestinal immunity will continue to grow and evolve, which will lead to a better understanding of pathogenic mechanism(s) and discovery of novel anti-cytokine treatments for chronic gut inflammation.

AUTHOR CONTRIBUTIONS

TTP, FC and CAD contributed equally to conceptualizing, writing, and editing the final submitted manuscript. CAD contributed to editing the final submitted manuscript. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Death Receptor 3 Signaling Controls the Balance between Regulatory and Effector Lymphocytes in SAMP1/YitFc Mice with Crohn's Disease-Like Ileitis

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Death receptor 3 (DR3), a member of the tumor necrosis factor receptor (TNFR) superfamily, has been implicated in regulating T-helper type-1 (T_H1), type-2 (T_H2), and type-17 (T_H17) responses as well as regulatory T cell (T_{reg}) and innate lymphoid cell (ILC) functions during immune-mediated diseases. However, the role of DR3 in controlling lymphocyte functions in inflammatory bowel disease (IBD) is not fully understood. Recent studies have shown that activation of DR3 signaling modulates T_{reg} expansion suggesting that stimulation of DR3 represents a potential therapeutic target in human inflammatory diseases, including Crohn's disease (CD). In this study, we tested a specific DR3 agonistic antibody (4C12) in SAMP1/YitFc (SAMP) mice with CD-like ileitis. Interestingly, treatment with 4C12 prior to disease manifestation markedly worsened the severity of ileitis in SAMP mice despite an increase in $FoxP3^+$ lymphocytes in mesenteric lymph node (MLN) and small-intestinal lamina propria (LP) cells. Disease exacerbation was dominated by overproduction of both T_H1 and T_H2 cytokines and associated with expansion of dysfunctional $CD25^-FoxP3^+$ and ILC group 1 (ILC1) cells. These effects were accompanied by a reduction in $CD25^+FoxP3^+$ and ILC group 3 (ILC3) cells. By comparison, genetic deletion of DR3 effectively reversed the inflammatory phenotype in SAMP mice by promoting the expansion of $CD25^+FoxP3^+$ over $CD25^-FoxP3^+$ cells and the production of IL-10 protein. Collectively, our data demonstrate that DR3 signaling modulates a multicellular network, encompassing T_{regs}, T effectors, and ILCs, governing disease development and progression in SAMP mice with CD-like ileitis. Manipulating DR3 signaling toward the restoration of the balance between protective and inflammatory lymphocytes may represent a novel and targeted therapeutic modality for patients with CD.

Keywords: Crohn's disease, inflammatory bowel disease, death receptor 3, SAMP1/YitFc, ileitis, regulatory T cells, innate lymphoid cell, TL1A, $CD25^{+/-}$ T cells

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INTRODUCTION

Crohn's disease (CD) is an inflammatory bowel disease (IBD) characterized by chronic and relapsing inflammation of gut intestinal segments. Although the cause of the disease is still unknown, an exaggerated immune response against commensal bacteria in individuals with a genetic predisposition has been postulated as a key mechanism (1). Pharmacological treatment of the disease is

generally based upon suppression of the immune system using non-specific drugs and blockade of inflammatory processes by biological therapy, such as antibodies targeting the cytokine TNF-α (2). However, a significant percentage of patients fail to improve or maintain remission for prolonged periods. For these individuals very limited options currently exist. As a result, more than 70% of patients require surgical removal of the affected intestinal segments. Furthermore, surgery does not necessarily provide long-lasting resolution of the inflammatory process, and recurrence after surgery occurs in the majority of patients with CD (3). Thus, to date there remains no cure for this devastating disease.

Death receptor 3 (DR3) (TNFRSF25), a member of TNFR superfamily expressed primarily on lymphocytes and innate lymphoid cells (ILCs), is a receptor for the cytokine TL1A (TNFSF15) secreted by dendritic cells, monocytes, macrophages, plasma cells, synovial fibroblasts, and endothelial cells (4–12). Preclinical and clinical studies have clearly shown a fundamental role for the TL1A/DR3 cytokine/receptor pair in the pathogenesis of inflammatory diseases, including rheumatoid arthritis (13–15), diabetic retinopathy (16), pulmonary sarcoidosis (17), asthma (10, 18), and, especially, IBD (19). Precisely, TL1A and DR3 expression is significantly increased, in an inflammation-specific manner, in both serum and inflamed tissues in IBD patients and in murine experimental ileitis (19). Genome-wide association studies have identified polymorphisms associated with IBD risk in the gene that encodes for TL1A protein (20–24). Finally, studies in animal models of intestinal inflammation have demonstrated that sustained expression of TL1A leads to chronic small-intestinal inflammation, whereas blockade of the TL1A/DR3 axis suppresses murine colitis (7, 12). Our laboratory has previously identified a novel role of TL1A/DR3 system in modulating lymphocyte functions and in preserving gut homeostasis in dextran sodium sulfate (DSS)-induced acute colitis (25). Specifically, following DSS treatment, TL1A- and DR3-deficient mice displayed an increase in disease severity mediated by defective suppressive function of regulatory T cells (T_{regs}), and a concomitant expansion of pro-inflammatory T-helper type-1 (T_H1), type-2 (T_H2), and type-17 (T_H17) (25). These results provided compelling evidence that TL1A/DR3 signaling exerts pleiotropic effects on lymphocyte homeostasis, cell proliferation, activation, function, and differentiation, mediating the balance between inflammatory and T_{reg} responses. Additional data supporting the role of DR3 in T_{reg} functionality consist in the observation that treatment with 4C12, an agonistic antibody to DR3, induces selective expansion of T_{regs} and reduces activation of conventional T cells in an allergic lung mouse model (26), in cardiac allografts (27) and in graft vs. host disease mouse model (28). This demonstrates that modulation of DR3 signaling may be a potential therapeutic target in immune-mediated disease, hence leading to appealing applications in CD therapy.

Recent findings have demonstrated that DR3-expressing ILCs could be an integral part of the DR3 signaling network (8, 10, 11). As effectors of innate immunity and regulators of tissue modeling, ILCs have been shown to play an important role in inflammatory diseases in the skin, lung, and gut (29). It is thought that the identified ILC populations, including group 1 (ILC1), group 2 (ILC2), and group 3 (ILC3), have a cytokine expression pattern that resembles that of T_H1, T_H2, and T_H17/T_H22 cells, respectively (30). ILC1 subset, found enriched in inflamed intestine of CD patients, expresses the transcription factor T-bet and responds to interleukin 12 (IL-12) by producing interferon-γ (IFN-γ) (31–33). The development and function of ILC2 cells depend on the transcription factor Gata-3 and produce the type-2 cytokines IL-5 and IL-13. The important role of ILC2s in virus-induced experimental models of airway hyperactivity and in allergic lung responses has been recognized (34–36). Regulated by the transcription of retinoic acid receptor-related orphan receptor-γt (RORγt), ILC3s produce IL-17 and IL-22 in response to IL-23 and IL-1β (37–39). ILC3s play a protective role in the healthy gut, by modulating epithelial cell regeneration through IL-22 secretion (40, 41). Nevertheless, innate sources of IL-17 were found significantly elevated in the intestinal mucosa of CD and Ulcerative colitis (UC) patients, suggesting a contribution of ILC3s to intestinal inflammation in IBD (38).

Recent data from our group supports a pro-inflammatory role of DR3/TL1A signaling mediated through activation of effector T cells during chronic inflammation, underscoring the importance of this cytokine–receptor pair in promoting gut immunopathology (Cominelli *et al.,* unpublished data). However, the discovery that DR3 promotes T_{reg} expansion (26–28) has led to the hypothesis of whether T_{reg} proliferation prior to disease initiation can revert CD-like ileitis. Therefore, in the current work, we investigated whether treatment with 4C12 prior to disease onset could delay or even ablate ileitis in SAMP mice, and whether DR3 is a master regulator of lymphocyte functions. We evaluated the distribution of total T_{regs} ($CD4^+FoxP3^+$), of $CD25^+$ and $CD25^-$ T_{reg} subsets, and of ILCs in mesenteric lymph node (MLN) and lamina propria (LP) cells. Interestingly, our results indicate that DR3 stimulation accelerates and exacerbates ileitis onset by triggering T_H1 and T_H2 responses, and by mitigating anti-inflammatory processes. In addition, our data suggest that DR3 signaling pathway promotes the expansion of non-regulatory $CD25^-$ T cells and ILC1s concomitant to the reduction of $CD25^+$ T_{regs} and ILC3s.

MATERIALS AND METHODS

Antibodies and Reagents

Agonistic anti-DR3 (4C12) monoclonal Ab and control Armenian Hamster IgG isotype (IgG) were purchased from BioLegend (San Diego, CA, USA). Anti-CD3e (2C11), anti-CD28 (37.51), anti-IL-17A (TC11-18H10), and CD16/CD32 (2.4G2) Abs were purchased from BD Biosciences (San Diego, CA, USA). T_{regs} were stained by using the $FoxP3^+T_{reg}$ staining kit following the manufacturer's instructions (eBioscience, San Diego, CA, USA). Collagenase and DNase were obtained from Sigma-Aldrich (St. Louis, MO, USA), and dispase from Roche (Mannheim, Germany). RPMI-1640 cell culture medium (RPMI), fetal bovine serum (FBS), penicillin, and streptomycin (P/S) were all purchased from Invitrogen (Grand Island, NY, USA). Cytokines and other reagents were purchased from the following vendors: TGF-β1 and IL-6 (R&D Systems, Minneapolis, MN, USA), IL-2 (eBioscience), and PMA,

ionomycin and GolgiStop (BD Biosciences). All ELISA kits were purchased from eBioscience.

Experimental Animals

An equal number of male and female 5-week-old SAMP and age/gender-matched AKR/J (AKR) mice, and 10-week-old SAMP × DR3$^{-/-}$ (DR3$_{KO}$) and age/gender-matched SAMP × DR3$^{+/+}$ (DR3$_{WT}$) mice were used in each experiment, with a mean body weight of 26.3 g on the day of sacrifice. Mice were housed and maintained in ventilated micro-isolator cages (Allentown Inc.) with 1/8-inch corn bedding and cotton nestlets for environmental enrichment (Envigo), kept on 12-h light/dark cycles, and maintained under specific-pathogen-free conditions in the Animal Resource Center of Case Western Reserve University (CWRU). All mice had *ad libitum* access to water and were fed with standard laboratory rodent diet P3000 (Harlan Teklad) throughout the experiments. Mice were genotyped by PCR-based assays of genomic tail DNA. All experimental procedures were approved by the Institutional Animal Care and Use Committee of CWRU and were in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care guidelines. All experiments were conducted in a blinded manner, without prior knowledge of treatments and mouse groups by the experimenter. Mice were randomized to different interventions using a progressive numerical number. The code for each mouse was known only to the animal caretaker and was revealed at the end of the study.

Treatment

Five-week-old SAMP and AKR mice were given intraperitoneal injections of 10 μg of 4C12 (or IgG) in 100-μL PBS, weekly, for 4 weeks, as previously described elsewhere (26). Mice were sacrificed at the beginning of the fifth week.

Histology

Mouse ilea were collected, rinsed with phosphate-buffered saline (PBS), fixed in Bouin's fixative solution (Fisher Scientific, Pittsburgh, PA, USA), embedded in paraffin, and sectioned. Histological evaluation of inflammation severity was determined in hematoxylin and eosin-stained 5-μm-thick sections, by using a semi-quantitative scoring system as previously described (42). Briefly, scores ranging from 0 (normal histology) to 3 (maximum severity of histologic changes) were used to evaluate histologic indices for (1) active inflammation (infiltration with neutrophils), (2) chronic inflammation (lymphocytes and plasma cells in the mucosa and submucosa), (3) monocyte inflammation (macrophages in the mucosa and submucosa), (4) villous distortion (flattening and/or widening of normal villus architecture), and (5) transmural inflammation. The total inflammatory index represents the sum of all five individual components. Histological scoring was performed by a single trained pathologist in a blinded fashion.

Stereomicroscopy

Ileal tissue abnormalities (i.e., cobblestone lesions) and normal mucosa were investigated by examining the cellular structural pattern of ileal tissue *via* stereomicroscopy, cm by cm, using a reference catalogue of lesions, as previously described (43). Starting from the distal end, 10 cm of ileum were collected, fixed in Bouin's solution overnight, and then transferred to 70% ethanol for stereomicroscopic analysis. Both healthy and cobblestone-like areas were calculated per cm using ImageJ software (NIH, Bethesda, MD, USA).

Isolation and Culture of Mesenteric Lymph Node Cells

Mesenteric lymph node cells were removed aseptically at the time of sacrifice, and cells were gently dispersed through a 70-μm cell strainer to obtain single-cell suspensions. Note that 1×10^6 resulting cells were cultured in RPMI-1640 with 10% FBS and 1% P/S for 72 h in the presence of 1-μg/mL anti-CD3/CD28 monoclonal Ab, as previously described (7). For measurement of *de novo* IL-17 protein in cell supernatants, MLN cells were placed in a culture medium supplemented with 1-ng/mL TGF-β1, 20-ng/mL IL-6, and 20 U/mL IL-2 for 72 h, and then stimulated with 50-ng/mL PMA, 1-μg/mL ionomycin, and 1 × GolgiStop for 4 h at 37°C (25). After the incubation period, the cells were collected for flow-cytometry assay, as described below, and supernatants were collected for IL-10, IL-13, IL-17, TNF-α, and IFN-γ analysis by ELISA, according to the manufacturer's instructions.

Isolation of Lamina Propria Mononuclear Cells

Ilea were collected from experimental mice, rinsed in ice-cold PBS, and cut into pieces of approximately 0.5 cm. To remove epithelial cells and intraepithelial lymphocytes, tissues were placed in 25-mL Ca^{2+}- and Mg^{2+}-free HBSS supplemented with 5-mM EDTA and 1-mM DTT, and shaken for 30 min at 250 rpm at 37°C. The remaining tissues were finely fragmented, placed in 30-mL RPMI medium supplemented with 10% FBS, 0.8-μg/mL dispase and 0.1-μg/mL collagenase D, and digested for 1 h at 37°C. Cells were collected by centrifugation at 1,300 rpm for 5 min at room temperature (RT). Cell pellets were then analyzed by flow cytometry.

Quantitative Real-time RT-PCR

Total RNA was isolated from homogenized ileal tissues using the RNeasy Mini kit (Qiagen, Valencia, CA, USA). cDNA was generated from 1 μg of RNA with maize mosaic virus random hexamers (Invitrogen). Semi-quantification of the target genes was carried out by real-time RT-PCR using SYBR Green methodology. Relative expression of each target gene was calculated by the ΔΔCt method (44). The expression of FoxP3, IL-17A, and β-actin mRNA was evaluated by using the following primer sequences: FoxP3, (5′-CCCAGGAAAGACAGCAACCTT-3′ and 5′-TTC TCACAACCAGGCCACTTG-3′); IL-17A (5′-TTTAACTCCCT TGGCGCAAAA-3′ and 5′-CTTTCCCTCCGCATTGACAC-3′); β-actin (5′-CAGGGTGTGATGGTGGGAATG-3′ and 5′-GTAG AAGGTGTGGTGCCAGATC-3′).

Flow Cytometry

To identify T_{regs}, freshly isolated LP or cultured MLN cells were stained with a mouse T_{reg} staining kit, according to the

manufacturer's instructions. Briefly, lymphocytes were blocked for 10 min on ice with anti-mouse CD16/CD32 Abs, and then stained with anti-mouse CD4 and CD25 Abs, for 30 min at 4°C in the dark. After washing, cells were stained with live/dead Fixable Violet Dead Cell Stain Kit (Thermo Scientific, Waltham, WA, USA) to determine cell viability, followed by incubation with a fixation/permeabilization buffer (eBioscience) for 30 min at 4°C in the dark. Cells were then washed with permeabilization buffer and stained with anti-mouse FoxP3 and IL-17A Abs for 30 min at 4°C in the dark. To collect live ILCs from MLNs, viability stain was used as indicated above. Cells were then stained with a combination of fluorescently conjugated monoclonal Abs optimized in a previous work (45) for 30 min at 4°C or at RT, to detect cell surface and intracellular proteins, respectively. Flow-cytometric acquisition was performed on a BD FACS LSR II instrument for T_{regs}, and on a FACSAria sorter for ILCs. Data were subsequently analyzed using FlowJo_V10 software (Tree Star) by gating on live cells based on forward vs. side scatter profiles, then gating on singlets using forward scatter area vs. height, followed by dead-cell exclusion and then cell subset-specific gating (Figures S2 and S3 in Supplementary Material). CountBright™ absolute counting beads (Thermo Scientific) were used to determine absolute cell number of ILCs by flow cytometry, according to the manufacturer's instructions.

Statistical Analysis

Data reported in the current work are representative of three independent experiments. For comparisons made between any given two groups with normal or not normal distribution, Student's *t*-test (two-tailed) or Mann–Whitney test was used, respectively. Provided the data fulfilled the assumptions for parametric statistics, comparison between more than two groups was carried out by two-way ANOVA with Bonferroni's *post hoc* test. All data were expressed as median ± interquartile range with ≥95% confidence intervals. An alpha level of 0.05 was considered significant. All statistical analyses were performed using GraphPad Prism (version 7.03; GraphPad Software, San Diego, CA, USA).

RESULTS

Death Receptor 3 (DR3) Stimulation Exacerbates Ileitis in SAMP Mice

Converging animal studies have shown that treatment with 4C12 prevented the development of allergic lung inflammation (26), promoted the survival of cardiac allografts (27), and reduced graft vs. host disease (28), through the expansion of T_{regs}. Hence, we sought to test whether 4C12 treatment could delay or even ablate disease onset in a well-characterized mouse model of CD-like ileitis, i.e., SAMP1/YitFc (SAMP). SAMP mice spontaneously develop chronic ileitis that resembles human CD, characterized by severe inflammation in the terminal ileum, skip lesions, transmural inflammation, granulomas, crypt hyperplasia, infiltration of both acute and chronic inflammatory cells, spontaneous skin lesions, and in some instances perianal fistulas (46–48).

In this study, we administered 4C12 or control Ab weekly to SAMP mice prior to disease manifestation (5-week-old), along with AKR littermates. After 4 weeks, 4C12-treated SAMP mice exhibited significantly increased ileitis severity (3.7-fold) compared with IgG-treated mice (17.7 ± 1.6 vs. 4.8 ± 1.6, $P = 0.0022$, **Figures 1A,B**). SAMP mice that received 4C12 were characterized by higher inflammation scores compared with controls, including 3.4-fold increase in active inflammation index (4.0 ± 0.0 vs. 1.2 ± 0.4, $P = 0.0022$, **Figure 1C**), fourfold increase in chronic inflammation index (4.0 ± 0.0 vs. 1.0 ± 0.0, $P = 0.0022$, **Figure 1D**), and fivefold increase in transmural inflammation index (1.7 ± 0.5 vs. 0.3 ± 0.5, $P = 0.0065$, **Figure 1E**). Additionally, distorted villous architecture, such as broadening and blunting (3.5-fold increase, 3.5 ± 0.8 vs. 1.0 ± 0.0, $P = 0.0022$, **Figure 1F**), and inflammatory infiltrates in the LP (3.4-fold increase, 4.5 ± 0.8 vs. 1.3 ± 0.5, $P = 0.0022$, **Figure 1G**) were significantly elevated in 4C12-treated mice compared with controls. Using an established protocol (43), we performed stereomicroscopic 3D-pattern profiling analysis of ileal tissue, revealing that 4C12-treated SAMP mice harbored a higher number and wider area of cobblestone lesions per cm, compared with controls (1.7-fold increase, 11.2 ± 2.4 vs. 6.6 ± 2.6, $P = 0.0229$, **Figures 1H,I**). Stimulation of DR3 in AKR mice did not have any effects on the health of the rodents (Figure S1 in Supplementary Material). These findings suggest that activation of DR3 signaling prior to disease manifestation accelerates inflammation occurrence, worsening ileitis development in a susceptible host.

DR3 Stimulation Increases FoxP3$^+$ Regulatory T-Cell Population but Has No Effects on T-Helper Type-17 (T_H17) Subset

Stimulation of DR3 with 4C12 leads to the expansion of *bona fide* $CD4^+FoxP3^+T_{regs}$ ($FoxP3^+$ T_{regs}), which are able to dampen host autoaggression in health (26–28). In addition to modulating this T-cell subpopulation, activation of DR3 pathway may trigger a signaling cascade that plays a role in T_H17 cell network (49, 50). Considering that T_{reg} and T_H17 cells share key mediators essential for cell differentiation, such as TGF-β1 (51), we can infer that DR3 signaling may modulate the homeostasis of both subsets. Hence, first, we asked whether treatment with 4C12 could enrich $FoxP3^+$ T_{regs} in SAMP mice. Second, we investigated whether the effect of DR3 activation on $FoxP3^+$ T_{regs} was coupled with a modulation of the expression of IL-17 in our system. We observed a significant increase in the proportion of $FoxP3^+$ T_{regs} in the MLN of 4C12-treated SAMP mice compared with those from the IgG-treated group (1.6-fold increase, 8.0 ± 1.8 vs. 5.0 ± 0.9, $P = 0.0134$, **Figure 2A**). Also, these data positively correlate with mRNA relative abundance of FoxP3 gene in ileal specimens (3.5-fold increase, 6.5 ± 1.0 vs. 1.9 ± 0.2, $P < 0.0001$, **Figure 2B**). On the other hand, administration of 4C12 did not significantly alter the frequency of $CD4^+IL\text{-}17^+$ cells (T_H17s), although a contraction of T_H17 frequency was observed in 4C12-treated SAMP mice compared with controls (0.7-fold decrease, 6.5 ± 1.1 vs. 9.3 ± 1.4, $P = 0.1748$, **Figure 2C**). IL-17 mRNA expression was significantly upregulated in the ileum of SAMP mice in comparison to that of AKR mice; however, we did not detect any differences in each mouse strain with regard to 4C12 administration (**Figure 2D**). Our data indicate that DR3 signaling expands

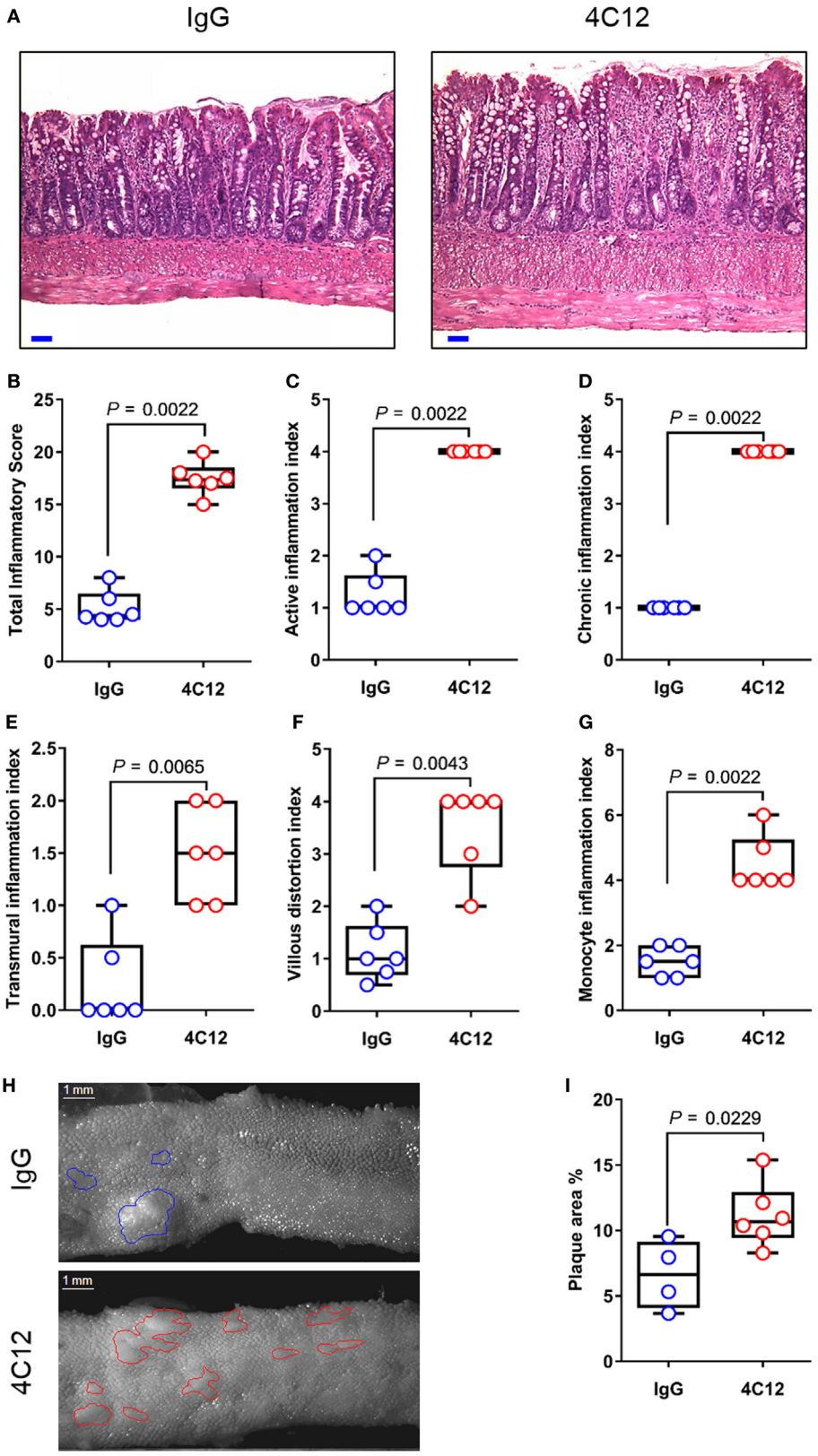

FIGURE 1 | Continued

FIGURE 1 | DR3 stimulation accelerates ileitis development in SAMP mice. **(A)** Representative photomicrographs of ileal sections of SAMP mice treated with control IgG isotype (left panel) or with 4C12 (right panel). Scale bar is 50 μm. **(B)** Total histologic score represents the sum of five indices including **(C)** active inflammation, **(D)** chronic inflammation, **(E)** transmural inflammation, **(F)** villous distortion, and **(G)** monocyte inflammation. Data presented as median ± interquartile range and analyzed by Mann–Whitney test, $n = 6$. **(H)** Mucosal architecture of fixed postmortem ileal specimens collected from 4C12-treated and IgG-treated SAMP mice was examined by stereomicroscopy. **(I)** Cobblestone area expressed as percentage of total specimen calculated in the ileum of 4C12-treated and IgG-treated SAMP mice. Data presented as median ± interquartile range and analyzed by two-tailed unpaired *t*-test, $n = 4$–6. Data are representative of three independent experiments.

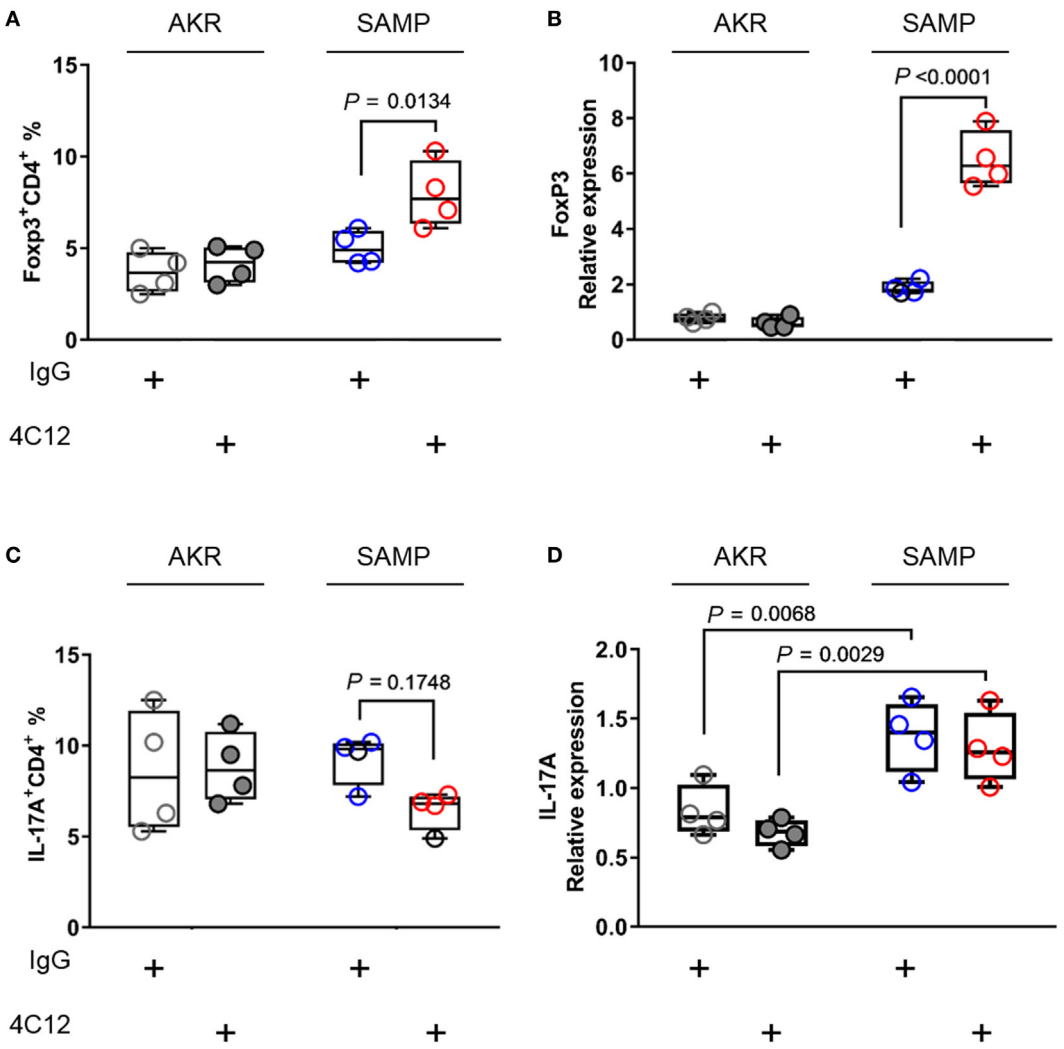

FIGURE 2 | DR3 stimulation increases FoxP3$^+$ T_{regs} without altering T_H17 cell population during chronic ileitis. **(A)** Flow-cytometric analysis of mesenteric lymph node cells from IgG- and 4C12-treated SAMP or AKR mice after staining with specific anti-CD4 and anti-FoxP3 Abs or **(C)** anti-CD4 and anti-IL17 Abs. **(B)** Relative expression of FoxP3 mRNA and **(D)** of IL-17A mRNA was measured in total tissue RNA extracted from the terminal ilea of SAMP or AKR mice treated with IgG or 4C12 (10-week-old, $n = 4$). The relative expression of each target gene was normalized to the relative expression of β-actin in the sample. Data presented as median ± interquartile range and analyzed by two-way ANOVA, with Bonferroni's *post hoc* test. Data are representative of three independent experiments.

FoxP3$^+$ T_{regs}, but not T_H17s, in MLN and LP cells of a susceptible host, but not of a healthy individual.

DR3 Stimulation Enriches CD25$^-$FoxP3$^+$ at the Expense of CD25$^+$FoxP3$^+$ Cells

Despite having FoxP3$^+$ T_{regs} enriched in MLN (**Figure 2A**) and FoxP3 mRNA upregulated in the ileum, 4C12-treated SAMP mice exhibited a more severe ileitis compared with IgG-treated controls (**Figure 1**). CD4$^+$CD25$^+$FoxP3$^+$ T_{regs}, which play a central role for the maintenance of immune homeostasis, are known to be generated in the thymus (thymus-derived cells or natural T_{reg}) and in the periphery (peripheral-derived cells or inducible T_{reg},) (52). Of note, peripheral-derived T_{regs} include a major subset of CD4$^+$CD25$^-$FoxP3$^+$ and a relatively small subset of

$CD4^+CD25^+FoxP3^+$ cells. CD25 is the α-chain of the IL-2 receptor (IL-2R) and it is a T-cell activation marker as well as a T_{reg} marker. It interacts with the β- and γ-chains of IL-2R, to form a high-affinity receptor, which promotes cell proliferation and functions (53). It has been shown that, when TL1A recruits DR3 on T cells, they become highly responsive to endogenous IL-2 *via* IL-2R, resulting in cell proliferation. With these premises and with the intent to identify T_{reg} subtypes, we further investigated the contribution of CD25 to the regulatory pool in our model. Our data indicated that, in SAMP mice upon 4C12 treatment, MLN and LP lymphocytes are partially depleted (twofold) of $CD25^+FoxP3^+$ cells compared with controls (1.6 ± 0.4 vs. 3.3 ± 1.1, $P = 0.0128$, **Figure 3A**; 1.3 ± 0.6 vs. 2.9 ± 0.9, $P = 0.0070$, **Figure 3C**). This phenomenon was accompanied by a concurrent enrichment (2.5-fold) of $CD25^-FoxP3^+$ cells (8.3 ± 0.6 vs. 3.4 ± 1.3, $P < 0.0001$, **Figure 3B**; 5.5 ± 1.0 vs. 2.2 ± 0.4, $P = 0.0022$, **Figure 3D**). Our results demonstrate that, in a susceptible host, DR3 activation correlates to the reduction of $CD25^+FoxP3^+$ in favor of $CD25^-FoxP3^+$ cells. To get further insights into this observation, we carried out extensive immunophenotyping of $CD25^+FoxP3^+$ and $CD25^-FoxP3^+$ cells in unmanipulated mice. Our analysis revealed that, by expressing specific markers for T_{reg} activation and suppressive functions (i.e., Nrp-1, Helios, CTLA-4, GITR, Icos, CD103, and CD73) and by producing IL-10, $CD25^+FoxP3^+$ cells may exert regulatory functions, which are instead impaired in $CD25^-FoxP3^+$ cells (Tables S1 and S2 in Supplementary Material). Interestingly, the frequency of DR3-expressing cells was significantly elevated in the $CD25^+FoxP3^+$ subset compared with $CD25^-FoxP3^+$ subpopulation, in MLNs of both AKR and SAMP mice ($p \leq 0.0052$, Tables S1 and S2 in Supplementary Material), suggesting a higher dependency of $CD25^+FoxP3^+$ cells on DR3 signals. Taken together, these results suggest that DR3 activation in SAMP mice promotes the switch of regulatory $CD25^+FoxP3^+$ cells to a non-regulatory $CD25^-FoxP3^+$ subpopulation.

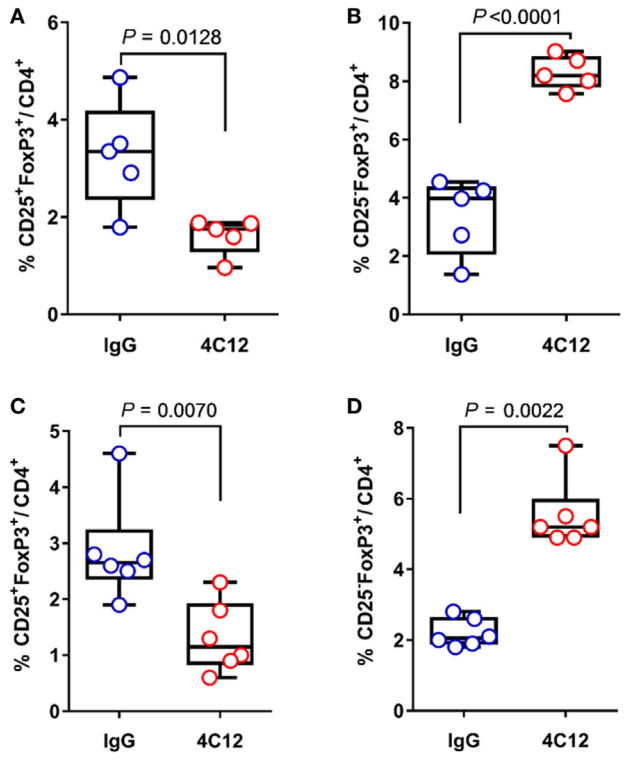

FIGURE 3 | DR3 stimulation correlates to the expansion of $CD25^-FoxP3^+$ cells during chronic ileitis. **(A,B)** Flow-cytometric analysis of mesenteric lymph node and **(C,D)** lamina propria cells from IgG- and 4C12-treated SAMP mice (10-week-old, $n = 6–5$) after staining with specific anti-CD25 and anti-FoxP3 Abs. The frequency of $CD25^+FoxP3^+$ and $CD25^-FoxP3^+$ cells is indicated. All data are presented as median ± interquartile range. Data in graphs A, B, and C were analyzed by two-tailed unpaired *t*-test. Data in graph D were analyzed by Mann–Whitney test. Data are representative of three independent experiments.

DR3 Deficiency Ameliorating Ileitis Severity and Expanding $CD25^+FoxP3^+$ Cells

In the current work, we demonstrated that DR3 signaling controls the balance between T_{reg} subsets favoring the enrichment of $CD25^-FoxP3^+$ over $CD25^+FoxP3^+$ cells in SAMP mice. Next, we tested whether the lack of DR3 in SAMP mice ($DR3_{KO}$) modulated the frequency of $FoxP3^+$ subpopulations in comparison to wild-type counterparts ($DR3_{WT}$), possibly promoting $CD25^+FoxP3^+$ cell expansion. As shown in **Figure 4A**, the ileum of $DR3_{KO}$ mice featured diminished villous distortion, transmural inflammation, and lymphocyte infiltration in the tissue in comparison to controls, resulting in a substantial decrease in ileitis severity in the former group (2.2-fold, 5.1 ± 1.5 vs. 11.1 ± 2.1, $P = 0.0002$, **Figure 4B**). The percentage of the area affected by cobblestones per cm of ileum was significantly reduced (2.8-fold) in $DR3_{KO}$ compared with $DR3_{WT}$ mice (5.4 ± 2.0 vs. 15.4 ± 2.6, $P = 0.0009$, **Figures 4C,D**). Interestingly, even though MLN and LP cells from $DR3_{KO}$ mice presented lower distribution of $FoxP3^+$ T_{regs} compared with those from $DR3_{WT}$ mice (1.6-fold, 4.8 ± 1.5 vs. 7.8 ± 1.5, $P = 0.0065$, **Figure 4E**; 1.6-fold, 3.0 ± 1.0 vs. 5.0 ± 1.2, $P = 0.0135$, **Figure 4F**), these organs were enriched in $CD25^+FoxP3^+$ cells (twofold, 2.5 ± 0.4 vs. 1.3 ± 0.3, $P = 0.0009$, **Figure 4G**; 1.8 ± 0.2 vs. 1.1 ± 0.3, $P = 0.0073$, **Figure 4I**), at the expense of $CD25^-FoxP3^+$ cells (2.5-fold, 2.7 ± 1.3 vs. 6.7 ± 1.0, $P = 0.0002$, **Figure 4H**; 1.6 ± 0.7 vs. 4.0 ± 1.2, $P = 0.0015$, **Figure 4J**). These findings indicate that genetic deletion of DR3 improves CD-like inflammation in a susceptible host, and it is associated with the contraction of $CD25^-FoxP3^+$ in favor of the expansion of $CD25^+FoxP3^+$ cells.

DR3 Signaling Correlates with Increased T-Helper Type-1 (T_H1) and T-Helper Type-2 (T_H2) Responses and Mitigates Anti-inflammatory Processes during Chronic Ileitis

In an effort to investigate T-cell functions, we stimulated MLN cells with anti-CD3/CD28 Abs and measured protein levels secreted in cell supernatants. Lymphocytes from 4C12-treated SAMP mice produced higher levels of T_H1 and T_H2 cytokines compared with those from the IgG-treated group (respectively,

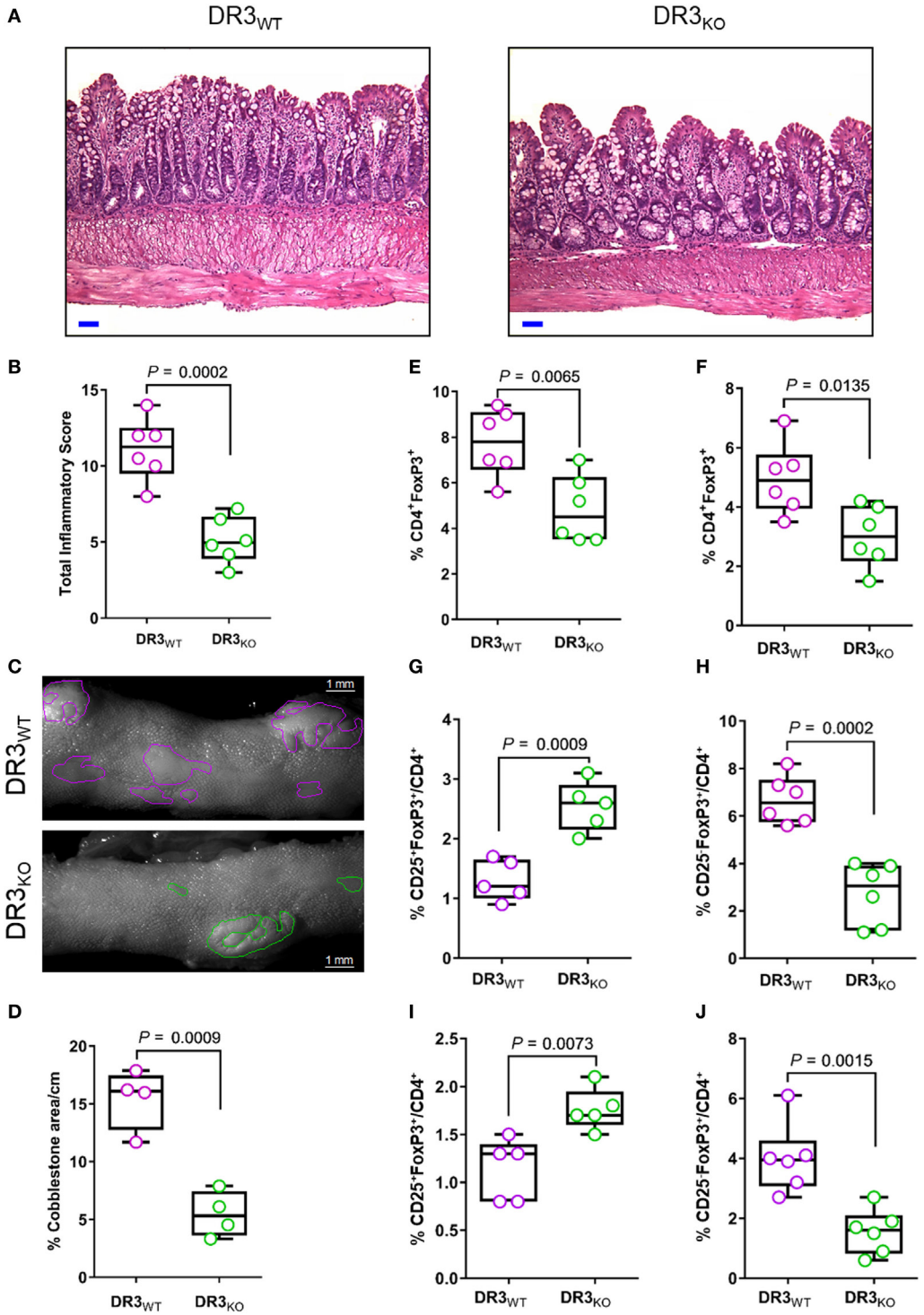

FIGURE 4 | DR3 deletion ameliorates ileitis severity and expands CD25$^+$FoxP3$^+$ cells in SAMP mice. **(A)** Representative photomicrographs of ileal sections of SAMP mice wild-type ($DR3_{WT}$) and lacking DR3 ($DR3_{KO}$). Scale bar is 50 μm. **(B)** Total histologic score presented as median ± interquartile range and analyzed by two-tailed unpaired *t*-test, $n = 6$. **(C)** Fixed postmortem ileal specimens collected from $DR3_{WT}$ and $DR3_{KO}$ mice (10-week-old, $n = 6$) and analyzed by stereomicroscopy to assess area of abnormal (i.e., cobblestone lesions) and normal mucosa. **(D)** Cobblestone area expressed as percentage of total specimen calculated in the ileum of $DR3_{WT}$ and $DR3_{KO}$ mice (10-week-old, $n = 6$). Data presented as median ± interquartile range and analyzed by two-tailed unpaired *t*-test, $n = 4$. **(E)** Flow-cytometric analysis of mesenteric lymph node (MLN) and **(F)** lamina propria (LP) cells from $DR3_{WT}$ and $DR3_{KO}$ mice (10-week-old, $n = 6$) after staining with specific anti-CD4 and anti-FoxP3. Data presented as median ± interquartile range and analyzed by two-tailed unpaired *t*-test, $n = 6$. **(G,H)** Flow-cytometric analysis of MLN and **(I,J)** LP cells from $DR3_{WT}$ and $DR3_{KO}$ mice (10-week-old, $n = 6$) after staining with specific anti-CD25 and anti-FoxP3 Abs. Data presented as median ± interquartile range and analyzed by two-tailed unpaired *t*-test. Data are representative of three independent experiments.

IFN-γ, 2.5-fold increase with 1072.3 ± 179.5 vs. 435.1 ± 88.9, $P < 0.0001$, **Figure 5B**; IL-13, 1.9-fold increase with 16.1 ± 4.7 vs. 8.4 ± 1.2, $P < 0.0001$, **Figure 5C**). Additionally, TNF-α level was also increased upon DR3 stimulation (82.2 ± 46.2 vs. 307.0 ± 35.9, $P = 0.0018$, Figure S4 in Supplementary Material). In contrast, IL-10 secretion was found dramatically reduced (3.7-fold) in MLN cells from 4C12-treated SAMP mice compared with controls (82.2 ± 46.2 vs. 307.0 ± 35.9, $P < 0.0001$, **Figure 5A**). We observed higher secretion (1.3-fold) of IL-17A protein by MLN cells from SAMP mice compare to those from AKR, indicating that *de novo* T_H17 response is enhanced under these experimental conditions, independently of the treatment administered here (9.4 ± 1.5 vs. 7.0 ± 2.9, $P = 0.0028$, **Figure 5D**).

DR3 Deficiency is Associated with Increased Anti-inflammatory Response and Concomitant Ablation of T_H1, T_H2, and T_H17 Mediators in SAMP Mice

With the intent of assessing the contribution of DR3 to T-cell function, we stimulated MLN cells from DR3-deficient SAMP ($DR3_{KO}$) mice with anti-CD3/CD28 Abs revealing a significant reduction of T_H1, T_H2, and T_H17 cytokines, including IFN-γ (2.5-fold increase, 1004.3 ± 374.8 vs. 394.5 ± 44.0, $P = 0.0027$, **Figure 6C**), IL-13 (6.7-fold increase, 24.3 ± 5.2 vs. 3.6 ± 4.5, $P = 0.0010$, **Figure 6B**), and IL-17A (1.5-fold increase, 7.7 ± 2.6 vs. 5.2 ± 1.1, $P = 0.0821$, **Figure 6D**), compared with those from wild-type counterparts ($DR3_{WT}$). In contrast, MLN cells from $DR3_{KO}$ mice secreted higher IL-10 protein level than those from littermate controls (2.0-fold increase, 143.7 ± 41.1 vs. 71.0 ± 16.4, $P = 0.0024$, **Figure 6A**). Taken together, these results suggest that the activity of DR3 signaling is mainly pro-inflammatory in a susceptible host.

DR3 Signaling Expands Innate Lymphoid Cell Group 1 (ILC1s) and Reduces ILC3s during Chronic Ileitis

Several reports have recently shown that DR3 is expressed on ILCs, inferring a role of this protein in ILC functionality. Furthermore, a large amount of data obtained from both human and mouse studies indicate a role for these cells in IBD,

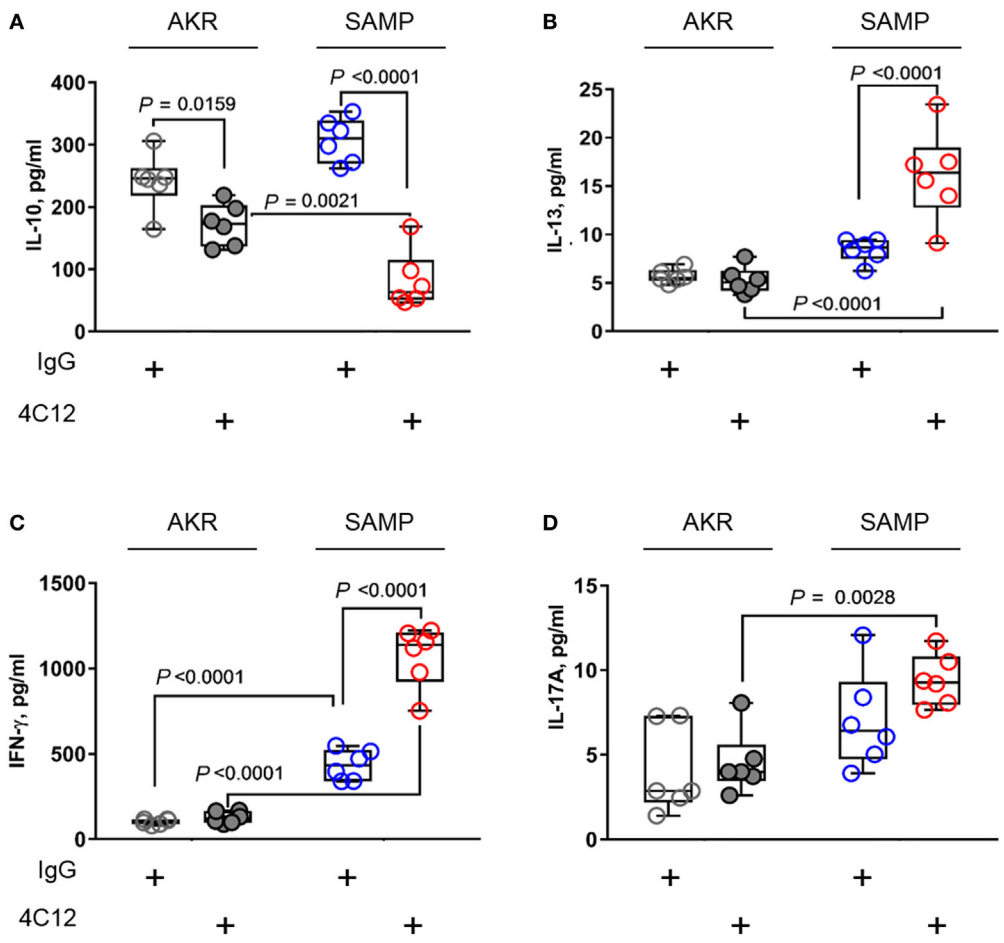

FIGURE 5 | DR3 stimulation triggers T_H1 and T_H2 mediators and reduces anti-inflammatory response during chronic ileitis. Mesenteric lymph node cells from IgG- or 4C12-treated SAMP and AKR mice (10-week-old, $n = 6$) were cultured in RPMI medium supplemented with anti-CD3/CD28 Abs. After 72 h, the secretion of indicated cytokines was quantified in cell supernatants [IL-10 **(A)**, IL-13 **(B)**, IFN-γ **(C)**, IL-17A **(D)**]. Data presented as median ± interquartile range and analyzed by two-way ANOVA, with Bonferroni's *post hoc* test. Data are representative of three independent experiments.

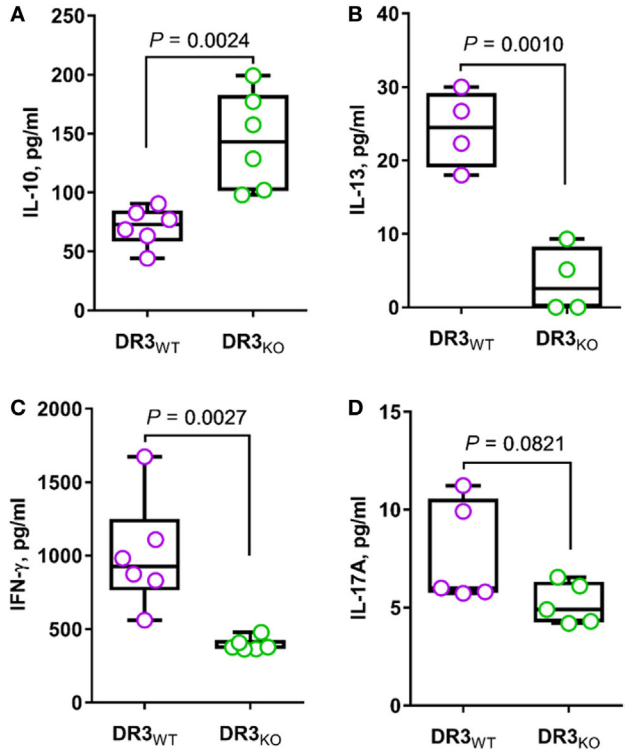

FIGURE 6 | Genetic deletion of DR3 enhances anti-inflammatory response and reduces T_H1, T_H2, and T_H17 cytokines in SAMP mice. Mesenteric lymph node cells from $DR3_{WT}$ and $DR3_{KO}$ mice (10-week-old, $n = 6$) were cultured in RPMI medium supplemented with anti-CD3/CD28 Abs. After 72 h, the secretion of indicated cytokines was quantified in cell supernatants [IL-10 **(A)**, IL-13 **(B)**, IFN-γ **(C)**, IL-17A **(D)**]. Data presented as median ± interquartile range and analyzed by two-tailed unpaired *t*-test. Data are representative of three independent experiments.

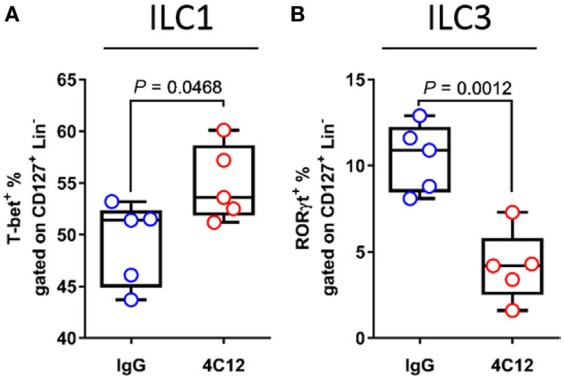

FIGURE 7 | Upon death receptor 3 stimulation, increased innate lymphoid cell group 1 (ILC1) and decreased ILC3 frequencies are associated with intestinal inflammation. Flow-cytometric analysis of mesenteric lymph node cells from IgG- or 4C12-treated SAMP mice (10-week-old, $n = 5$) after staining with specific Abs for detection of ILC populations, including **(A)** T-bet$^+$ ILC1s and **(B)** receptor-related orphan receptor-γt (ROR-γt$^+$) ILC3s. Cell frequencies presented as median ± interquartile range and analyzed by Mann–Whitney test. Data are representative of three independent experiments.

considering that some ILC subsets have regulatory functions in the healthy intestine (8, 10, 11). Hence, we used flow cytometry to measure the distribution of ILC subsets in MLNs collected from 4C12- and IgG-treated SAMP mice. Treatment with 4C12 expanded ILC1s compared with controls (1.1-fold increase, 54.9 ± 3.7 vs. 49.2 ± 4.1, $P = 0.0468$, **Figure 7A**). ILC3 frequency was found reduced (2.5-fold) in MLN cells from 4C12-treated mice compared with controls (4.2 ± 2.1 vs. 10.5 ± 2.0, $P = 0.0012$, **Figure 7B**). In contrast, the distribution of ILC2 subset was unaltered upon treatment (Figure S5A in Supplementary Material). Interestingly, the frequency of ILC3 cells expressing DR3 was significantly higher than that of ILC1s ($p < 0.0012$, Figure S5B in Supplementary Material), suggesting a higher dependency on DR3 signals of ILC3s compared with ILC1s. Hence, these data suggest that DR3 stimulation may promote the conversion of ILC3 into ILC1 cells in SAMP mice.

Considering that lack of DR3 ameliorates ileitis in SAMP mice, we anticipated a positive change in the frequency of ILC3s in $DR3_{KO}$ mice. Remarkably, the distribution of all ILC subtypes in MLNs from $DR3_{KO}$ mice was equal to that in the wild-type counterparts. To further investigate this result, we went on measuring the magnitude of ILC groups in our system. We found that the absolute cell number of ILCs, along with that of CD45$^+$ and CD127$^+$Lin$^-$ cells, was significantly decreased in MLNs from $DR3_{KO}$ compared with $DR3_{WT}$ mice (Figure S6 in Supplementary Material). This outcome may be ascribed to the fact that $DR3_{KO}$ mice harbor MLN organs of smaller size compared with $DR3_{WT}$ mice (Cominelli *et al.*, unpublished data) and, therefore, they carry a lower number of cell without affecting immune cell frequency.

DISCUSSION

In the present study, we investigated the role of DR3 signaling in the modulation of the balance between regulatory and effector lymphocytes during chronic experimental ileitis. To this end, we exploited a well-characterized mouse model for CD, i.e., SAMP1/YitFc (SAMP), in which the main pathology is a spontaneous ileitis that is strikingly similar to human CD, with skip lesions, transmural inflammation, and scarring that can lead to stricture formation (47, 48). The current understanding is that, upon TL1A binding, DR3 triggers a signaling cascade that increases the sensitivity of T cells to endogenous IL-2 *via* the IL-2 receptor (IL-2R), and enhances T-cell proliferation at the site of inflammation (54). Consistently with patients who suffer from CD, high expression levels of DR3 found in SAMP mice during the chronic phase of the disease support the concept that the TL1A/DR3 system contributes to pathogenic inflammation in this model (5–7). In line with this finding, our recent studies demonstrated that DR3 deletion in SAMP mice restores the mucosal immunostat, normalizes intestinal inflammatory gene expression, and prevents the development of inflammation-induced intestinal fibrosis, thereby affecting the functions of effector lymphocytes and their capacity to adoptively transfer ileitis (Cominelli *et al.*, unpublished data). To help unraveling the complexity of the TL1A/DR3 signaling pathway in the course of IBD, in the present study, we demonstrated that DR3

deletion ameliorates disease development by promoting anti-inflammatory processes, which were associated with enrichment of $CD25^+FoxP3^+$ cells in SAMP mice. Taken together, these findings suggest that the DR3 is required on T cells for local effector T-cell expansion and effector responses in a susceptible host.

A recent innovative approach developed by Podack et al. provided proof of concept for a regulatory role of DR3 in an allergic disease model (26). With the administration of a single injection of a stimulating DR3 antibody (4C12) to immunocompetent mice, Podack et al. demonstrated its highly efficacy at reducing pathology when used prophylactically (26). Other research groups applied successfully this methodology reporting improved organ allograft survival after treatment, attributable to a systemic over-proliferation of pre-existing $CD25^+FoxP3^+$ cells *in vivo* (27, 28). Therefore, the knowledge that activation of DR3 signaling modulates T_{reg} expansion in a healthy host leads to the appealing idea of using this mechanism as a potential therapeutic target in human inflammatory disease, such as CD. Promising findings recently gathered in CD patients treated with T_{regs} have reinvigorated the enthusiasm for this therapeutic approach (55–57). Therefore, we postulated the possibility of delaying inflammation or eradicating ileitis in SAMP mice by promoting T_{reg} proliferation prior to disease manifestation. This hypothesis was addressed in the current study through the administration of 4C12 to SAMP mice prior to disease initiation. Our results indicated that, in a susceptible host, DR3 stimulation increased the frequency of $Foxp3^+$ T_{regs}; however, contrary to expectations, this phenomenon was associated with significant exacerbated ileitis.

When the contribution of CD25 to the T_{reg} pool was investigated in SAMP mice, it became apparent that DR3 stimulation induced the reduction of $CD25^+FoxP3^+$ cells, in favor of $CD25^-FoxP3^+$ cells with increased T_H1/T_H2 responses. Although it is known that $CD25^-FoxP3^+$ cells contain some regulatory activity (58–60), there is a wide consensus on the fact that adequate suppression functions require expression of CD25 (61). This finding is in agreement with previous results from our laboratory, which identified the same $CD25^-FoxP3^+$ subtype in SAMP mice upon anti-CD25 Ab treatment as dysfunctional T_{regs} overrepresented during spontaneous ileitis, which acquired a T_H1/T_H2 effector phenotype (60). Studies in human systemic lupus erythematosus have confirmed these findings by reporting increased $CD25^-FoxP3^+$ cells with T_H1/T_H2 effector phenotype (62–65). Some evidence have demonstrated that T_{regs} may exert a milieu-dependent plasticity by readily switching to an effector phenotype in inflamed sites, accelerating inflammatory processes, thereby aggravating the underlying disease (66–68). Disease pathogenesis in SAMP mice is the result of a dual mechanism of inflammation consisting in an early inductive phase (4–7 weeks of age), driven by T_H1 responses, and a later chronic inflammatory phase (9–16 weeks of age), primarily mediated by T_H2 effector pathways (48). In this model, intestinal inflammation can be histologically assessed around 10 weeks of age, following CD-like lesions development (43, 47). Nonetheless, similar to human CD patients in preclinical phase, even though younger mice do not show histological features of disease, inflammatory processes have already initiated in the inductive phase (48). Hence, in the current work, administration of 4C12 to SAMP mice occurred in this phase, and not prophylactically. Earlier studies showed that 4C12 treatment during active disease aggravated the pathology (69), whereas the same antibody administered prophylactically to a healthy individual protected the host from future insults (26). Of note, the intense T_H1 responses typical of the inductive phase give rise to an inflammatory milieu (48), even before ileal tissue manifestations, that may support the dynamic conversion of regulatory cells into effector $FoxP3^+$ cells, which accelerate disease development. Moreover, mice lacking functional peripheral-derived T_{regs} develop T_H2 pathologies in the intestine and lungs, and dysbiosis (70). Additionally, extensive immunophenotyping of $CD25^+FoxP3^+$ and $CD25^-FoxP3^+$ cells in unmanipulated mice revealed that the former expresses specific markers considered to be critical for T_{reg} immunoregulatory properties, that instead are missing in $CD25^-FoxP3^+$ cells (Tables S1 and S2 in Supplementary Material). For instance, the frequency of cells expressing markers correlated to T_{reg} suppressive activity and stability, such as Nrp-1 and Helios, was dramatically reduced in the $CD25^-FoxP3^+$ subset, suggesting that this is an unstable and non-regulatory population. Moreover, the expression of DR3 was increased on $CD25^+FoxP3^+$ cells, suggesting a higher dependency of this subset on DR3 signals. Therefore, considering collectively all these data, we propose that, upon inflammatory environmental cues, DR3 stimulation targets $FoxP3^+$cells and converts regulatory cells to $CD25^-FoxP3^+$cells, which are dysfunctional lymphocytes that secrete effector mediators and accelerate disease manifestations in SAMP mice. Conversely, it cannot be excluded that the apparently opposite dynamics of $CD25^+FoxP3^+$ and $CD25^-FoxP3^+$ cells may not necessarily imply a conversion from the former to the latter. In fact, based on the evidence reported by some human and mouse studies, another hypothesis may be that $CD25^-FoxP3^+$ cells are indeed activated effector T cells, which transiently upregulate FoxP3, without exerting any regulatory activity (71, 72). Finally, we propose an alternative view consisting in the possibility that $CD25^-FoxP3^+$ cells may be T_{regs} which have transiently downregulated CD25 due to the local inflammatory milieu (59, 73). Hence, future work will be focused on dissecting the functional role of $CD25^+FoxP3^+$ and $CD25^-FoxP3^+$ cells in intestinal immunity-microbiota interactions, and in controlling adaptive immunity to restrain inflammation at mucosal surfaces. Additionally, considering the interesting approach suggested by Rouse group in collaboration with Podack, where 4C12 was combined with galectin-9, a protein able to selectively inhibit effector T-cell functions during chronic stromal keratitis (74), future experiments will investigate the efficacy of this combination therapy on the balance between regulatory and effector lymphocyte in our system.

Another novel finding described here is that DR3 stimulation in SAMP mice expanded ILC1s, which produce IFN-γ, at the expense of ILC3s. In agreement with our data, the frequency of the ILC1 subset was found elevated in inflamed intestine of CD patients, underlying a role for these cells in the pathogenesis of gut mucosal inflammation (32, 37, 38). Hence, the skewed

frequencies of ILCs in MLNs of 4C12-treated SAMP mice compared with controls could be explained by specific recruitment of cells to the periphery, according to environmental cues. Based on the remarkable plasticity of ILCs, a more intriguing explanation argues that ILC3s may switch to ILC1s in 4C12-treated SAMP mice. Data collected from human and mouse studies revealed that a fraction of ILC3s can downregulate ROR-γt, lose the ability to produce IL-22, and acquire the capacity to secrete IFN-γ in response to IL-12, diverting to an ILC1 phenotype (32, 75). Moreover, we showed in the current study that ILC3s from SAMP mice expressed higher levels of DR3, suggesting that this subset may be more sensitive and may represent a preferential target following DR3 manipulation. Of note, we previously demonstrated that TL1A synergizes with IL-12 to promote IFN-γ production by murine lymphocytes in SAMP mice (7). Therefore, we can infer that DR3 stimulation with 4C12 may generate a similar signaling cascade, resulting in this phenotype switch.

In conclusion, DR3 stimulation in SAMP mice aggravated the severity of ileitis possibly due to the expansion of dysfunctional $CD25^{-}FoxP3^{+}$ cells and ILC1s, both expressing an effector phenotype. The functional role of these cell subtypes during chronic inflammation needs to be further investigated in our mouse model to give better insights into the functional balance between protective and inflammatory lymphocytes. Altogether, our data suggest a model in which modulation of DR3 signaling in T_{regs}, T effectors and ILCs converge in a regulatory network that controls disease development and progression. Finally, dissecting the cellular mechanisms that govern lymphocyte functions following DR3 engagement and manipulating the resulting signaling cascade may provide a novel targeted therapy for CD.

ETHICS STATEMENT

All experimental procedures were approved by the Institutional Animal Care and Use Committee of CWRU and were in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care guidelines.

AUTHOR CONTRIBUTIONS

ZL and LB shared the first authorship. FC contributed to the design of the study. ZL, LB, ML, L-GJ, and JDW performed the experiments. LB and ZL analyzed the data. LB and ZL drafted the manuscript. K-AB conducted ILC isolation and immunophenotyping. FC, TP, and ML analyzed and interpreted the data. All authors approved the final version of the manuscript and agreed to be accountable to all aspects of this work.

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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.2018.00362/full#supplementary-material.

FIGURE S1 | DR3 stimulation does not affect the phenotype of AKR mice. Representative photomicrographs of ileal sections of AKR mice treated with control IgG isotype or with 4C12s. Scale bar is 50 μm. Data are representative of three independent experiments.

FIGURE S2 | Gating strategy for detection of regulatory T-cell subsets by flow cytometry. After duplet exclusion and identification of $CD4^{+}$ lymphocytes within mesenteric lymph node cells, T cells were further separated based on the cell surface markers CD25 and the intracellular protein FoxP3.

FIGURE S3 | Gating strategy for detection of innate lymphoid cell (ILC) populations by flow cytometry. Gating strategy exploited to identify ILC subsets in mesenteric lymph node cells, including ILC1 ($CD45^{+}Lineage^{-}CD127^{+}Gata\text{-}3^{-}ROR\text{-}\gamma t^{-}T\text{-}bet^{+}$), ILC2 ($CD45^{+}Lineage^{-}CD127^{+}Gata\text{-}3^{+}ROR\text{-}\gamma t^{-}$), and ILC3 ($CD45^{+}Lineage^{-}CD127^{+}Gata\text{-}3^{-}ROR\text{-}\gamma t^{+}$).

FIGURE S4 | DR3 stimulation triggers TNF-α response in SAMP mice. Mesenteric lymph node cells were collected from IgG- and 4C12-treated SAMP or AKR mice, and stimulated with anti-CD3/CD28 antibodies for 72 h. TNF-α level was quantified in cell supernatants by ELISA. Data presented as median ± interquartile range and analyzed by two-way ANOVA, with Bonferroni's *post hoc* test. Data are representative of three independent experiments.

FIGURE S5 | DR3 stimulation does not alter innate lymphoid cell group 2 (ILC2s) in SAMP mice. **(A)** Flow-cytometric analysis of mesenteric lymph node (MLN) cells from IgG- or 4C12-treated SAMP mice (10-week-old, $n = 5$) after staining with specific Abs for detection of $gata\text{-}3^{+}$ ILC2s. Cell frequency presented as median ± interquartile range and analyzed by Mann–Whitney test. **(B)** Frequency of DR3-expressing ILCs in MLN cells from SAMP mice (10-week-old, $n = 5$). Data presented as median ± interquartile range and analyzed by two-way ANOVA, with Bonferroni's *post hoc* test. Data are representative of three independent experiments.

FIGURE S6 | DR3 deficiency is associated with constitutive reduced innate lymphoid cell (ILC) number. Flow-cytometric analysis of mesenteric lymph node cell $DR3_{WT}$ and $DR3_{KO}$ mice (10-week-old, $n = 6$) after staining with specific Abs for detection of ILC populations, including $T\text{-}bet^{+}$ ILC1, $gata\text{-}3^{+}$ ILC2, and $ROR\text{-}\gamma t^{+}$ ILC3. **(A,C,E,G)** Cell frequencies and **(B,D,F,H)** absolute cell numbers presented as median ± interquartile range and analyzed by two-tailed unpaired *t*-test. Data are representative of three independent experiments.

TABLE S1 | Characterization of CD25+/–FoxP3+ cell subsets through selected markers in MLNs of unmanipulated AKR, SAMP and DR3KO mice. The results are expressed as the mean percentage ± one SD, and analyzed by 1-way ANOVA with Bonferroni post-hoc test. The number of samples is indicated in parentheses.

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Targeting Inflammatory T Helper Cells *via* Retinoic Acid-Related Orphan Receptor Gamma t Is Ineffective to Prevent Allo-Response-Driven Colitis

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Intestinal graft-versus-host disease (GvHD) is a life-threatening, inflammatory donor T cell-mediated complication of allogeneic hematopoietic stem cell transplantation (allo-HSCT). In the light of the reported efficacy of interleukin-23 (IL-23)-blockade to mitigate syngeneic intestinal inflammation in inflammatory bowel disease patients, targeting IL-23 and thereby interleukin-17a (IL-17a) producing T helper (Th17) cells as the T cell subset assumed to be mostly regulated by IL-23, has emerged as a putatively general concept to harness immune-mediated mucosal inflammation irrespective of the underlying trigger. However, the role of Th17 cells during allo-response driven colitis remains ambiguous due to a series of studies with inconclusive results. Interestingly, we recently identified granulocyte-macrophage colony-stimulating factor (GM-CSF$^+$) T cells to be promoted by interleukin-7 (IL-7) signaling and controlled by the activating protein-1 transcription factor family member basic leucine zipper transcription factor ATF-like (BATF) as critical mediators of intestinal GvHD in mice. Given the dual role of BATF, the contribution of IL-23-mediated signaling within donor T cells and bona fide Th17 cells remains to be delineated from the regulation of GM-CSF$^+$ T cells in the absence of BATF. Here, we found in a complete MHC class I-mismatched model that genetic inactivation of the IL-23 receptor (IL-23R) or the transcription factor retinoic acid-related orphan receptor gamma t (RORγt) within donor T cells similarly ablated Th17 cell formation *in vivo* but preserved the T cells' ability to induce intestinal GvHD in a compared to wild-type controls indistinguishable manner. Importantly, RORγt-independent manifestation of intestinal GvHD was completely dependent on BATF-regulated GM-CSF$^+$ T cells as BATF/RORγt double-deficient T cells failed to induce colitis and the antibody-mediated blockage of IL-7/IL-7R interaction and GM-CSF significantly diminished signs of intestinal GvHD elicited by RORγt-deficient donor T cells. Finally, in analogy to our murine studies, colonic

RORC expression levels inversely correlated with the presence of GvHD in allo-HSCT patients. Together, this study provides a crucial example of a BATF-dependent, however, IL-23R signaling- and RORγt-, i.e., Th17 fate-independent regulation of a colitogenic T cell population critically impacting the current understanding of intestinal GvHD.

Keywords: retinoic acid-related orphan receptor gamma t, interleukin-23 receptor, T helper 17 cells, granulocyte-macrophage colony-stimulating factor, basic leucine zipper transcription factor ATF-like, intestinal graft-versus-host disease, colitis

INTRODUCTION

Despite exerting crucial barrier-protective functions under homeostatic conditions, interleukin-17a (IL-17a) producing T helper (Th17) cells are the most-abundant subpopulation within intestinal inflammatory lesions found in patients with inflammatory bowel diseases (IBDs) and acute manifestation of gastrointestinal graft-versus-host disease (GvHD) after allogeneic hematopoietic stem cell transplantation (allo-HSCT) (1, 2). Hence, targeting Th17 cells appears to represent a master-switch to turn off unwanted inflammation in the gut (3–6). Indeed, we and others have previously demonstrated that T cells with a genetic inactivation of Th17 cell regulating transcription factors retinoic acid-related orphan receptor gamma t (RORγt), interferon regulatory factor-4, signal transducer and activator of transcription 3 (STAT3), or basic leucine zipper transcription factor ATF-like (BATF) uniformly failed to induce colitis in a series of syngeneic murine IBD models supporting the concept that harnessing unleashed intestinal inflammation can be achieved by targeting Th17 cell biology (7–11). In line within this assumption, genetic inactivation or blockage of interleukin-23 (IL-23) knowingly absolutely required for the occurrence of Th17 cells *in vivo* were shown to mitigate colitis in preclinical model systems and be effective in treating IBD (5, 12, 13). Overall, these data suggest that IL-23-driven T-cell responses are critically contributing to the manifestation of intestinal inflammation both in murine syngeneic colitis models and in human IBD and hence Th17-centered concepts are highly promising to provide progress for the therapy of IBD in the future.

However, in particular in respect to intestinal GvHD following allo-HSCT, the issue of the selective pathogenic contribution of bona fide Th17 cells to the manifestation of mucosal inflammation has continued to remain essentially unresolved in the light of a series of reports with inconclusive and in part diametrically opposed outcomes resulting in various interpretations of its role by the scientific community (14–16). Interestingly, we recently described that donor T cells lacking the expression of the Th17 lineage regulating transcription factor BATF indeed conferred protection against GvHD-associated colitis both in a major and minor histocompatibility mismatched model of allo-HSCT in mice (17). Importantly, besides the known role in Th17 cell differentiation (18), we found the development of interleukin-7 receptor (IL-7R)-responsive, granulocyte-macrophage colony-stimulating factor (GM-CSF) expressing donor T cells, also termed Th_{GM} cells (19–21), to be hampered in the absence of BATF in these model systems. More importantly, selective blockade of IL-7R^{hi} GM-CSF$^+$ T cells alone largely recapitulated the protection that we observed upon the transplantation of BATF-deficient donor lymphocytes (17). Given the dual role of BATF in regulating both Th17 cells and GM-CSF$^+$ T cells, these data urged us to further study a number of issues raised by these findings with the goal to ultimately disclose the functional relevance of Th17 cells compared to GM-CSF-expressing T cells in gastrointestinal GvHD. In the light of the notion provided by recent studies showing in experimental autoimmune encephalomyelitis, a murine model of multiple sclerosis, that GM-CSF-expressing T cells are driven by IL-23, express the master regulator of Th17 development RORγt and hence putatively represent a Th17 cell subset (22, 23), our current study was intended to characterize (1) the developmental relationship between Th17 and GM-CSF$^+$ T cells based on the dependency on upstream and transcriptional signals and (2) the subset-specific, functional contribution to the manifestation of acute GvHD-associated colitis *in vivo*.

MATERIALS AND METHODS

Mice

Female BALB/cJRj, and C57Bl/6 mice were purchased from Janvier Labs, and congenic CD45.1/Ly5.1 B6.SJL-PtrprcaPepcb/BoyCrl mice were purchased from Charles River Laboratories. 129S-*Batf*$^{tm1.1Kmm}$/J (termed *Batf*$^{-/-}$ mice) and B6.129P2(Cg)-*Rorc*^{tm2Litt}/J (termed *Rorc*$^{-/-}$ mice) were purchased from the Jackson Laboratory and intercrossed to generate *Rorc*$^{-/-}$ *Batf*$^{-/-}$ mice. *Il23r*$^{-/-}$ mice [B6NTac;B6N-A<tm1Brd> Il23r<tm2a (EUCOMM)Wtsi/Wtsi] were provided by the Trust Sanger Institute Mouse Genetics Project (Sanger MGP) (24). Mice were maintained under specific pathogen-free conditions. Mice older than 7 weeks were used. This study was carried out in accordance with the recommendations of the government of Mittelfranken in Bavaria, Germany. The protocol was approved by the government of Mittelfranken in Bavaria, Germany.

Allogeneic Bone Marrow (BM) Transplantation and GvHD Scoring

Graft-versus-host disease was induced and scored as described before (17). Briefly, 10- to 12-week-old female H-2d$^+$ Balb/c recipient mice received a single dose of 8 Gy X-ray *via* total body irradiation (day 0). At day 1 after irradiation, BM cells of allogeneic CD45.1/Ly5.1 B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyCrl mice were isolated and T cells of the BM were depleted by magnetic bead separation using anti-CD90.2 microbeads (Miltenyi Biotec) according to the manufacturer's instructions routinely resulting in a depletion of more than 98% of the pre-existing T cell fraction as confirmed

by flow cytometry. Then, 5×10^6 T cell depleted BM cells were i.v. injected into recipient mice. At day 2 after irradiation, mice received i.v. 0.7×10^6 allo-reactive $CD3^+$ T cells that were isolated from total splenocytes of C57Bl/6 wild type (termed WT mice), *Rorc*$^{-/-}$, *Il23r*$^{-/-}$ and *Rorc*$^{-/-}$ *Batf*$^{-/-}$ mice, respectively, by magnetic separation using the Mouse Pan T cell isolation kit II (Miltenyi Biotec) according to the manufacturer's instructions. Mice were assessed 3×/week for the presence of clinical GvHD symptoms. Indicated GvHD-associated symptoms (body weight, posture, activity, skin and fur texture, and stool consistency) were individually scored from 0 (no manifestation) to 2 (severe manifestation) and the sum of all individual scores per mouse added up to the clinical GvHD score.

Mouse Colonoscopy

Macroscopic mucosal inflammation was assessed by colonoscopy using an image 1™ S3 mini-endoscope (Karl Storz) as previously described (25). Therefore, mice were anesthetized by inhalation and inflammation of the colon was estimated using a modified murine endoscopic index of colitis severity based on the following parameters: thickening of the colon bowel wall, changes of the vascularity, granularity of the mucosal surface, and stool consistency. Every parameter was scored from 0 for no colitis to 3 for massive inflammation adding up to a maximum score of 12 as previously described (26).

In Vivo Antibody Treatment of Mice

In studies with *in vivo* antibody treatment, mice received 3×/week 300 µg anti-mouse IL-7R antibody (clone A7R34) starting on the day of BM transplantation (day 1) until day 15 and 300 µg anti-mouse GM-CSF antibody (MP1-22E9) throughout the experiment by i.p. injection. As a control, a group of mice was treated with 300 µg isotype rat IgG2a antibody (clone 2A3) 3×/week over the entire course of the experiment also by i.p. injection. All antibodies were purchased from BioXcell.

Histopathological Analysis

After euthanizing allo-HSCT recipient mice, the distal segment of the colon was removed, flushed with PBS, and fixed in 4.5% formaldehyde overnight. Sections (3 µm) of paraffin-embedded colon tissue were stained with hematoxylin and eosin and used for histopathological analysis. Inflammation was scored by a to the experimental groups blinded pathologist. GvHD-associated inflammation was scored semiquantitatively as previously described (27): 0 (none or minimal signs); 1 (mild signs); 2 (moderate signs); or 3 (severe signs) (17).

Colonic Lamina Propria (LP) Cell Isolation

Colonic LP cells were isolated as described before (17). Briefly, the colon was removed from euthanized mice, flushed with PBS to remove intestinal content, and cut into small pieces. After washing colonic pieces twice with HBSS supplemented with 1% EDTA (0.5 mM), intestinal tissue was digested with a solution containing DNase I (0.5 mg/ml), Collagenase D (1 mg/ml), Dispase II (6 U/ml) (all enzymes purchased by Roche), and 5% FCS. Digested tissue was filtered and washed with PBS. LP cells were enriched by density gradient centrifugation where 80% Easycoll was overlaid with cells resuspended in 40% Easycoll (Biochrom). Following centrifugation cells were washed with RPMI supplemented with 10% FCS and stored on ice until analysis.

Flow Cytometry

For the analysis of cell surface markers, LP cells were stained with fluorochrome-conjugated antibodies for 20 min at 4°C in the dark. After washing with FACS buffer (3% FCS in PBS), cells were analyzed on a FACSFortessa™ II (BD Biosciences) flow cytometer. For intracellular cytokine staining, isolated LP cells (1×10^6/ml) were cultured in supplemented DMEM (containing 10% FCS, 1% penicillin–streptomycin, 1% non-essential amino acids, 1% L-glutamine, and 0.1% β-mercaptoethanol) alone or in the presence of 50 ng/ml phorbol 12-myristate 13-acetate and 1 µM ionomycin for 4 h at 37°C. 1 µg/ml brefeldin A was added for the last 3 h of culture (all purchased from Sigma Aldrich). Thereafter, cells were stained for surface markers. Intracellular cytokine staining was performed as described previously (18). In brief, cells were fixed with 2% paraformaldehyde in PBS for 15 min at room temperature. After washing with FACS buffer, cells were permeabilized with 0.05% saponin (Sigma Aldrich, Germany) in FACS buffer followed by intracellular cytokine staining for 30 min at 4°C in the dark using fluorochrome-labeled antibodies dissolved 0.5% saponin in FACS buffer. After washing cells with 0.05% saponin in FACS buffer, cells were resuspended in FACS buffer cells and analyzed on a FACSFortessa™ II (BD Biosciences) flow cytometer. Data were analyzed using FlowJo 7.6.5 and 10.2 software (Tree Star Inc.). The following antibodies were used: α-CD3ε (17A2, BioLegend), α-CD4 (GK1.5, BioLegend), α-CD8a (53-6.7, BioLegend), α-CD45.1 (A20, BioLegend), α-CD45.2 (104, BioLegend), α-GM-CSF (MP1-22E9, BioLegend), α-IL-17a (TC11-18H10.1, BioLegend), and α-IFNγ (XMG1.2, BioLegend).

In Vitro T Cell Culture

For *in vitro* $CD4^+$ T cell cultures, splenic $CD4^+$ T cells were isolated by negative selection using magnetic microbeads of the $CD4^+$ T Cell Isolation Kit II (Miltenyi Biotec) according to the manufacturer's instructions. In the case of Th_{GM} cultures, enriched $CD4^+CD25^-$ T cells were further sort-purified using a FACS Aria cell sorter (Becton Dickinson) at the Cell Sorting and Immunomonitoring Core Unit of the University Hospital Erlangen, Friedrich Alexander University Erlangen-Nuremberg. T Cells (1×10^6/ml) were cultured at 37°C in a 24-well-plate in supplemented RPMI (containing 10% FCS, 1% penicillin–streptomycin, 1% non-essential amino acids, 1% L-glutamine, and 0.1% β-mercaptoethanol; Sigma Aldrich) for Th_{GM} differentiation (17) or in serum-free X-VIVO 15 medium (Lonza) supplemented with 1% penicillin–streptomycin for Th17 differentiation (26). T cells were stimulated with 10 µg/ml plate-bound anti-CD3 antibody (clone 145-2C11; BioXcell) and 1 µg/ml soluble anti-CD28 antibody (clone 37.51; BioXcell). For T cell differentiation, T cells were cultured in the presence of 10 µg/ml soluble anti-IFNγ (clone XMG1.2; BioXcell) alone (drift condition) or for Th_{GM} polarizing conditions together with 2 ng/ml recombinant IL-7 (R&D Systems) while for Th17 polarizing conditions 20 ng/ml recombinant IL-1β (Peprotech), 20 ng/ml recombinant IL-6 (Peprotech), and 50 ng/ml recombinant IL-23 (Milenyi Biotec)

were added to the cultures. Th_{GM} polarized T cells were harvested at day 3 while Th17 differentiated T cells underwent RNA isolation after 5 days of culture.

Quantitative Real-Time PCR (qPCR)

RNA was isolated from whole colon tissue using the NucleoSpin™ RNA isolation kit (Macherey Nagel). RNA of *in vitro* differentiated T cells was isolated using the RNeasy Micro Kit (Qiagen). 1 µg of RNA was reversed transcribed into cDNA using iScript™ cDNA Synthesis Kit (Bio-Rad) according to manufacturer's instructions. qPCR reactions were performed on a CFX Connect and CFX96 Real-Time PCR detection system (Bio-Rad) using iQ™ SYBR® Green Supermix (Bio-Rad). Data were analyzed with CFX Manager v3.1 (Bio-Rad). Expression levels of target genes for each sample were normalized relative to the housekeeping gene HPRT. Relative gene expression levels were calculated with the ΔΔCt method. For the analysis of gene expression within murine colon tissue samples, gene expression levels detected in no T cells (noT) controls were arbitrarily set to 1 and all other gene expression levels were calculated and displayed in relation to the normalized noT controls.

Following primers were used synthesized by MWG Eurofines (Germany): *Hprt* forward 5′-TGG ATA CAG GCC AGA CTT TGT T-3′, reverse 5′-CAG ATT CAA CTT GCG CTC ATC-3′, *Ifng* forward 5′-ATC TGG AGG AAC TGG CAA AA-3′, reverse 5′-TGA GCT CAT TGA ATG CTT GG-3′, *Csf2* forward 5′-ATC AAA GAA GCC CTG AAC CT-3′, reverse 5′-GTG TTT CAC AGT CCG TTT CC-3′, *Tnfa* forward 5′-CTT GTG GCA GGG GCC ACC AC-3′, reverse 5′-CCA TGC CGT TGG CCA GGA GG-3′, *Il-17a* forward 5′-GCT CCA GAA GGC CCT CAG A-3′, reverse 5′-AGC TTT CCC TCC GCA TTG A-3′, *Il1b* forward 5′-GTG ACG TTC CCA TTA GAC AA-3′, reverse 5′-TAT TTT GTC GTT GCT TGG TT-3′, *Rorc* forward 5′-CCG CTG AGA GGG CTT CAC-3′, reverse 5′-TGC AGG AGT AGG CCA CAT TAC A-3′, *Il23r* forward 5′-CAC AAC AAC TAC ACG TCC AT-3′, reverse 5′-TAC CAG TTT CTT GAC ATC GC-3′, *Batf* forward 5′-GGA AGA TTA GAA CCA TGC CTC-3′, reverse 5′-CCA GGT GAA GGG TGT CGG-3′.

Human Studies

This study was carried out in accordance with the recommendations of the ethics committee of the University Hospital Regensburg. The protocol was approved by the ethics committee of the University Hospital Regensburg. All subjects gave written informed consent in accordance with the Declaration of Helsinki. Colon tissue biopsies of allo-HSCT patients ($n = 52$) were collected during colonoscopy at the Department of Hematology & Oncology and Gastroenterology at the University Hospital Regensburg. Total RNA of human tissue was isolated with the RNeasy Mini Kit (Qiagen) followed by cDNA synthesis using moloney murine leukemia virus reverse transcriptase (Promega) according to the manufacturer's instructions. Gene expression of RORC and 18S ribosomal RNA was analyzed by quantitative PCR reactions that were performed on a Mastercyler Ep Realplex (Eppendorf) using a QuantiFast SYBR Green PCR Kit (Qiagen). The following primer sets were used: *RORc* forward, 5′-GCA GCG CTC CAA CAT CTT CTC-3′, reverse, 5′-GCA CAC CGT TCC CAC ATC TC-3′; *18SrRNA* forward, 5′-ACC GAT TGG ATG GTT TAG TGA G-3′, reverse, 5′-CCT ACG GAA ACC TTG TTA CGA C-3′. Expression of *RORC* was normalized to *18SrRNA*. The expression levels represent relative units and were calculated from a standard curve plotting three different concentrations of log dilutions against the PCR cycle number as previously described (28). Concurrently colonic tissue sections of the same patients were analyzed for the presence or absence of histopathological signs of intestinal GvHD by a pathologist experienced in GvHD-associated histopathology of the gut during clinical routine. Histopathological scoring of the colitis activity and the presence of GvHD-associated apoptotic events was performed as previously published (29). Apoptotic cells per high power field (hpf) were counted and apoptosis was semiquantitatively scored by two experienced pathologists: 0–1 apoptotic cell/hpf = 0; 2–4 apoptotic cell/hpf = 1; 5–7 apoptotic cell/hpf = 2; >7 apoptotic cell/hpf = 3. Samples with a sore of at least 1 were considered to be positive for apoptosis.

Statistical Analysis

Unpaired two-tailed Student's *t*-test was used for comparison of means of two datasets. Clinical GvHD scores were analyzed by two-way ANOVA and Bonferroni posttest. For comparison of gene expression data or colonoscopy scores with more than two datasets, one-way ANOVA and Bonferroni posttest was used. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$ was considered to be significant. Statistical analysis was performed with Graphpad Prism 5 software.

RESULTS

IL-23 Receptor Expression on Donor T Cells Is Dispensable for the Manifestation of GvHD-Associated Colitis

Previously, we reported that acute inflammatory tissue manifestation of GvHD in the lower gastrointestinal tract depends on the ability to express BATF within donor T cells (17). Surprisingly, BATF-deficient T cells were severely compromised in their ability to differentiate into IL-7R responsive GM-CSF-expressing T cells. Prompted by this finding, we assessed the functional contribution of these T cells and found that indeed combined blockade of IL-7R/IL-7 interaction and GM-CSF proved to be very effective in harnessing GvHD driven colonic inflammation. However, given the dichotomic role of BATF in governing both the differentiation of IL-17-producing Th17 cells and GM-CSF-producing T cells and the published concept on the IL-23 dependency of GM-CSF expressing Th17 cells as crucial drivers of tissue inflammation, e.g., in the central nervous system (17, 18, 22, 23), we reasoned that additional studies are required to clarify the precise, subset-specific functional contribution of bona fide Th17 cells to the manifestation of acute GvHD-associated colitis upon allo-HSCT. To achieve this goal, we first sought to assess whether the ability of the donor T cell to directly respond to IL-23 *via* IL-23/IL-23 receptor (IL-23R)-interaction on donor T cells is functionally relevant for the manifestation of intestinal GvHD. Importantly, employing a full MHC class I mismatch

model, we failed to observe any protective effects on the clinical course of GvHD upon transplantation of IL-23R-deficient compared to WT CD3+ donor lymphocytes (**Figure 1A**). Moreover, intestinal GvHD as assessed by both endoscopic and histopathological evaluations of matching colonic tissue specimen was indistinguishably detectable in mice receiving either WT or IL-23R-deficient donor T cells (**Figures 1B,C**).

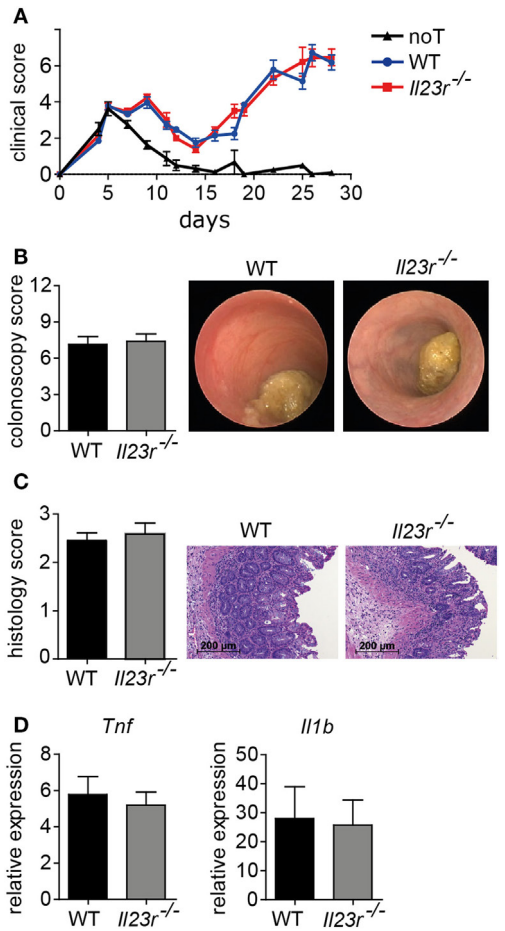

FIGURE 1 | Interleukin-23 receptor (IL-23R) expression by donor T cells is not required for acute graft-versus-host disease (GvHD)-associated colitis manifestation. To induce GvHD, BALB/c mice were irradiated (day 0), received intravenously T cell-depleted bone marrow (BM) (day 1) and were subsequently injected with allogeneic splenic CD3+ T cells of C57BL/6 [wild type (WT); $n = 14$] or *Il23r*$^{-/-}$ ($n = 15$) mice (day 2). As a control, mice received T cell-depleted BM alone (noT; $n = 8$). **(A)** Mice were observed three times a week and clinical symptoms of GvHD were scored. Data were analyzed by two-way ANOVA followed by Bonferroni's multiple comparisons posttest. **(B)** At day 28, colitis severity was assessed by colonoscopy and representative endoscopic images are shown. Mice were sacrificed between day 28 and day 30. **(C)** Histopathological scoring of the inflammation present in the distal colon (WT $n = 11$; *Il23r*$^{-/-}$ $n = 12$). One representative hematoxylin and eosin-stained histopathological cross-section of the distal colon per group is shown. Scale bars: 200 µm. **(D)** Transcript levels of *Tnf* and *Il1b* in colonic tissue of WT ($n = 14$) or *Il23r*$^{-/-}$ ($n = 15$) T cell receiving mice as described above were analyzed by quantitative real-time PCR. Data are combined from two individual experiments. Data were analyzed by Student's *t*-test and are shown as mean ± SEM.

Also on a molecular level, gene expression profiling analyses of proinflammatory cytokines known to promote inflammatory tissue damage in the colon demonstrated that *Il1b* and *Tnf* levels are comparably upregulated independent of the T cell's ability to express IL-23R (**Figure 1D**). Overall, these data demonstrate that direct effects of IL-23 *via* IL-23R expression on the intestinal GvHD-mediating donor T cell population are largely dispensable for disease manifestation.

Donor T Cell-Intrinsic RORγt Expression Does Not Drive Acute Colonic Inflammation Upon Allo-HSCT

Interleukin-1β/IL-6/IL-23-induced inflammatory Th17 cells consecutively upregulate IL-23R expression which renders these T cells progressively responsive to IL-23, thereby further promoting this differentiation pathway (**Figure 2A**) (30). Importantly, we and others have demonstrated that the induction of IL-23R expression is regulated by and dependent on bona fide Th17 cell differentiation-regulating transcription factors as, e.g., BATF and RORγt (**Figure 2A**) (26, 30). However, IL-23 responsiveness due to IL-23R expression is only one facet of Th17 cells (3). Therefore, we assessed whether RORγt-deficient T cells conferred protection against intestinal GvHD manifestation given the fact that RORγt represents a Th17 defining transcription factor that is highly related to BATF. More importantly, under syngeneic conditions, RORγt- and BATF-deficient T cells comparably failed to induce colitis upon transfer into lymphopenic *Rag1*$^{-/-}$ mice (9, 11). Strikingly, however, and in clear delineation from the protection conferred by BATF-deficient T cells, upon allo-HSCT WT and RORγt-deficient T cells induced virtually identical signs of systemic GvHD. Moreover, endoscopic assessment of GvHD-associated colitis revealed indistinguishable intestinal GvHD phenotypes between both groups of donor T cell receiving mice (**Figures 2B,C**). Histopathological evaluation of matching tissue specimen confirmed that colonic inflammation was comparably detectable in both groups (**Figure 2D**). Similar to our results obtained in the studies on the role of IL-23R expression on T cells, proinflammatory cytokine gene expression analyses (*Il1b* and *Tnf*) within GvHD-affected colon tissues samples yielded similar results between mice transplanted with WT and RORγt-deficient donor T cells (**Figure 2E**). Together, these series of studies demonstrate that both IL-23R and RORγt expression within the donor T cell fraction are seemingly dispensable for intestinal GvHD manifestation.

Intestinal GvHD Develops Despite Hampered Th17 Differentiation due to RORγt-Independent Th1 and GM-CSF-Expressing T Cell Formation

Further studies showed that both frequencies and absolute numbers of colonic LP-infiltrating CD4+ and CD8+ donor T cells were indistinguishable between WT and either IL-23R-deficient or RORγt-deficient donor T cells (**Figures 3A,B**). Hence, given the virtually identical intestinal GvHD phenotypes, we reasoned that also the quality of colonic GvHD-mediating T cell subsets might be unaffected by IL-23R- and RORγt-, i.e., Th17 deficiency,

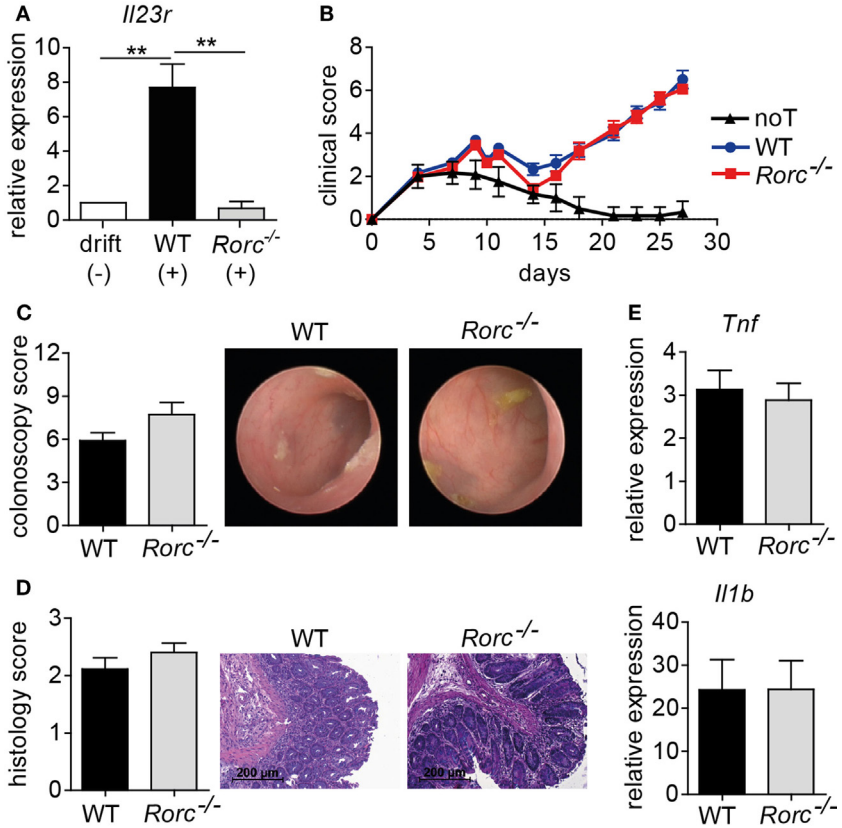

FIGURE 2 | Retinoic acid-related orphan receptor gamma t (RORγt)-dependent T helper (Th17) cells are dispensable for acute intestinal graft-versus-host disease (GvHD). **(A)** Naïve CD4+ T cells were isolated from the spleen by negative selection using magnetic microbeads and *in vitro* polarized into inflammatory Th17 cells by co-culturing anti-CD3/anti-CD28 activated T cells in the presence of anti-IFNγ antibodies and either without (–) or with (+) recombinant interleukin (IL)-1β, IL-6, and IL-23. After 5 days, T cells were harvested, RNA was isolated, and transcribed into cDNA followed by quantitative real-time PCR (qPCR) analyses of *Il23r* transcript levels. Gene expression levels detected within T cells cultured under drift conditions were arbitrarily set down to 1 and all other gene expression levels were normalized to the expression level of this control. Data are combined from two individual experiments and were analyzed by one-way ANOVA followed by Bonferroni's multiple comparisons posttest. $^{**}p < 0.01$ was considered significant. Data are shown as mean ± SEM. **(B–E)** For GvHD induction, BALB/c mice were irradiated (day 0), transplanted with T cell-depleted bone marrow (BM) (day 1) and subsequently injected with allogeneic splenic CD3+ T cells of wild-type (WT) ($n = 18$) or *Rorc*$^{-/-}$ ($n = 23$) mice (day 2). As a control, one group of mice received T cell-depleted BM alone [no T cells (noT); $n = 6$]. **(B)** Clinical symptoms of GvHD mice were scored three times a week. Pooled data from three independent experiments are shown and were analyzed by two-way ANOVA followed by Bonferroni's multiple comparisons posttest. **(C)** Between day 28 and day 30, colonic inflammation of WT ($n = 12$) or *Rorc*$^{-/-}$ ($n = 11$) T cell receiving mice was assessed by colonoscopy and representative endoscopic images are shown. Mice were sacrificed the day after colonoscopy. **(D)** Histopathological scoring of the colitis activity within the distal colon of WT ($n = 9$) or *Rorc*$^{-/-}$ ($n = 10$) T cell receiving mice was performed. One representative hematoxylin and eosin-stained histological cross-section of the distal colon per group is shown. Scale bars: 200 µm. Shown results represent pooled data from two individual experiments. **(E)** Gene expression levels detected within colonic tissue of WT or *Rorc*$^{-/-}$ T cell receiving mice of *Tnf* (WT $n = 16$; *Rorc*$^{-/-}$ $n = 12$) and *Il1b* (WT $n = 17$; *Rorc*$^{-/-}$ $n = 14$) around day 30 were analyzed by qPCR. Data display pooled data from at least three individual experiments. Data were analyzed by Student's *t*-test and are shown as mean ± SEM.

within the donor T cell compartment. To address this point, we first quantitated preferentially T cell-derived cytokine gene expression levels in total colon tissue samples of GvHD-affected and control mice (**Figures 4A,B**). Interestingly, *Il17a* is largely regulated in a GvHD-independent manner given our finding that *Il17a* gene expression was barely induced in colon tissues of GvHD-affected mice irrespective of the genotype of the GvHD-inducing donor T cells when compared to mice receiving T cell-depleted BM alone (**Figure 4A**). In contrast to *Il17a*, *Ifng*, and *Csf2* gene expression were significantly upregulated in the presence of intestinal GvHD. However, we failed to detect any negative regulatory effect on the expression of proinflammatory cytokine genes resulting from the usage of either IL-23R- or RORγt-deficient compared to WT donor T cells (**Figures 4A,B**). To further functionally characterize colonic donor-derived CD4+ T cell subsets, we performed flow cytometry and intracellular cytokine expression profiling experiments of LP-derived T cells *ex vivo*. Expectedly, these studies confirmed that both donor T cell-restricted genetic inactivation of IL-23R and RORγt, respectively, largely abrogated the ability of donor T cells to differentiate into Th17 cells (**Figures 4C,D**). By contrast, despite a slightly, however, significantly reduced frequency of GM-CSF-expressing

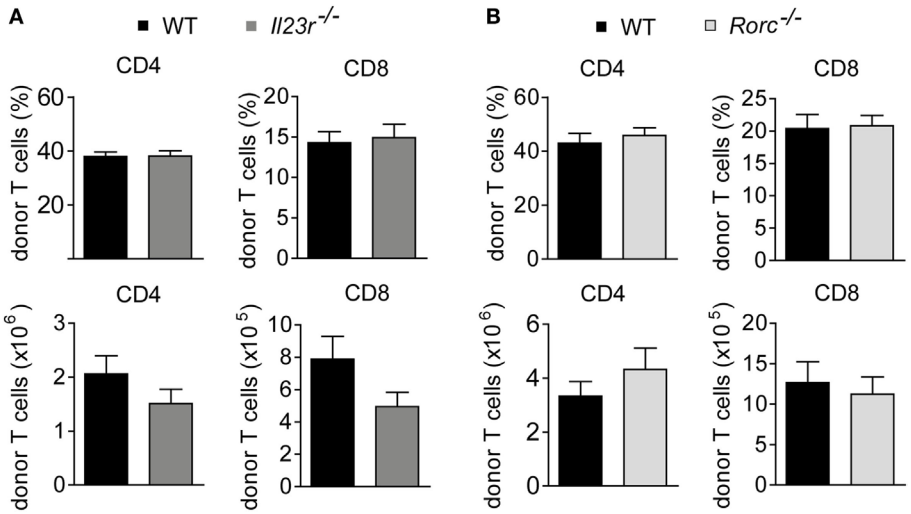

FIGURE 3 | Colonic donor T cell expansion occurs in an interleukin-23 receptor (IL-23R)- and retinoic acid-related orphan receptor gamma t-independent manner. At day 28–31, colonic lamina propria mononuclear cells of allogeneic hematopoietic stem cell transplantation BALB/c mice with fully established clinical signs of intestinal graft-versus-host disease due to the transfer of allogeneic T cells derived from wild-type (WT) (n = 14) and *Il23r*$^{-/-}$ (n = 15) mice **(A)** or WT (n = 12) and *Rorc*$^{-/-}$ (n = 11) mice **(B)** were analyzed by flow cytometry. The frequency and absolute number of CD4$^+$ and CD8$^+$ donor T cells are displayed. Data represent pooled results from two independent experiments and were analyzed by Student's t-test. Data display mean ± SEM.

IL-23R-deficient T cells, the absolute numbers of IFNγ-expressing Th1 cells and GM-CSF-expressing LP donor T cells remained unaffected in the absence of either IL-23R or RORγt expression within donor T cells (**Figures 4C,D**). Interestingly, quantification of indicated donor T cell subsets demonstrated that Th1 and GM-CSF-producing T cells combined absolutely outnumbered IL-17a-producing Th17 cells within the colonic LP compartment of GvHD-affected mice by about 10- to 15-fold. Overall, these data provide substantial experimental evidence for the conclusion that the donor T cells ability to directly respond to IL-23, i.e., express a functional IL-23R, and to express the master regulator of Th17 cell differentiation RORγt is dispensable for the colonic manifestation of intestinal GVHD.

BATF Deficiency Abrogates RORγt-Independent Donor T Cell-Mediated Colonic GvHD

T cells polarized under Th17 conditions upregulate both *Rorc* and *Batf* with the former being shown to occur downstream of *Batf* (18). However, given our findings that acute GvHD-associated colitis depends on donor T cell-intrinsic BATF but not RORγt expression, we sought to further explore how *Batf* expression in the inflamed colon relates to the presence of RORγt- and BATF-expressing donor T cells, respectively. For this, we transferred either RORγt- or BATF-deficient donor T cells and assessed colonic *Batf* expression levels around onset (day 15) and during fully established intestinal GvHD (day 30) (**Figures 5A,B**). Strikingly, despite abrogated Th17 potential, the transfer of RORγt-deficient T cells resulted in a significant upregulation of colonic *Batf* expression while upregulation of *Batf* was not observed upon transplantation of BATF-deficient donor T cells lacking intestinal GvHD-inducing potential (**Figure 5A**). Interestingly, we conversely found that *Rorc* expression levels in the colon were comparable between mice displaying severe signs of intestinal GvHD and GvHD-free noT controls due to receiving T cell depleted BM alone (**Figure 5B**). Importantly, quantification of RORC gene expression within human samples derived from allo-HSCT patients with or without GvHD revealed significantly downregulated colonic *RORC* levels in the presence of GvHD, thereby mirroring results of our murine studies (**Figure 5C**). These data collectively suggest that elevated *Batf* colon tissue expression levels reflect the presence of BATF-expressing, intestinal GvHD-mediating donor T cells while T cell-intrinsic RORγt expression is completely dispensable in this matter. Therefore, we hypothesized that while RORγt and BATF commonly regulate Th17 cell formation critically affecting, e.g., syngeneic colitis development (9, 11, 18, 31), in intestinal GvHD pathogenesis, however, RORγt is not involved in the formation of a second, previously as BATF-dependent described colitogenic T cell subset. To functionally test whether RORγt-independent manifestation of intestinal GvHD is indeed dependent on BATF, we generated RORγt-deficient T cells additionally lacking BATF by intercrossing BATF- and RORγt-deficient mice. As shown in **Figures 5D,E**, RORγt/BATF double-deficient donor T cells failed to induce allo-response-driven systemic signs of GvHD and endoscopically assessed manifestations of acute colitis while both systemic and intestinal GvHD phenotypes were again virtually indistinguishable between WT and RORγt-deficient T cells. In line with hampered GvHD induction, gene expression profiling analyses of whole colon tissue samples revealed that RORγt/BATF double-deficiency within the donor T cell compartment resulted in reduced expression of proinflammatory mediators (*Ifng*, *Csf2*) compared to RORγt-deficient T cell receiving mice

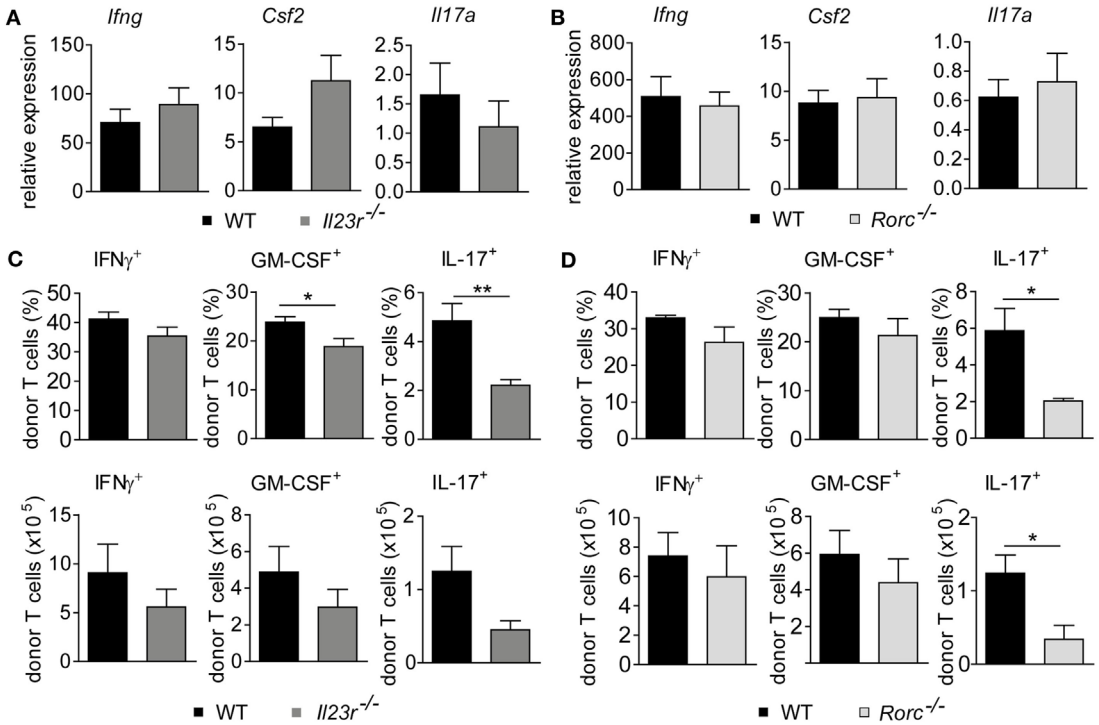

FIGURE 4 | Genetic depletion of retinoic acid-related orphan receptor gamma t-dependent T helper cells preserves the granulocyte-macrophage colony-stimulating factor (GM-CSF) expressing donor T cell pool. Experimental graft-versus-host disease (GvHD) induction was performed as described in **Figures 1** and **2**. **(A)** Gene expression levels in total colonic tissue of wild-type (WT) and *Il23r*$^{-/-}$ donor T cell receiving mice of *Ifng*, *Csf2* (WT $n = 14$; *Il23r*$^{-/-}$ $n = 15$) and *Il17a* (WT $n = 9$; *Il23r*$^{-/-}$ $n = 5$) transcripts were analyzed by quantitative real-time PCR (qPCR). **(B)** Gene expression levels in total colonic tissue of WT and *Rorc*$^{-/-}$ donor T cell receiving mice of *Ifng*, *Csf2* (WT $n = 17$; *Rorc*$^{-/-}$ $n = 14$) and *Il17a* (WT $n = 7$; *Rorc*$^{-/-}$ $n = 8$) transcripts were analyzed and quantitated by qPCR around day 30, i.e., when allogeneic hematopoietic stem cell transplantation mice displayed fully established signs of intestinal GvHD. Data represent pooled data from two **(A)** and at least three **(B)** individual experiments, respectively. **(C,D)** In addition, lamina propria mononuclear cells of WT ($n = 6$) and *Il23r*$^{-/-}$ ($n = 6$) T cell receiving mice **(C)** or WT ($n = 4$) and *Rorc*$^{-/-}$ ($n = 3$) T cell receiving mice **(D)** were assessed by flow cytometry for the frequencies and absolute numbers of GM-CSF-, IFNγ- or interleukin 17a (IL-17a)-producing CD4$^+$ donor T cells, respectively, employing intracellular cytokine staining techniques. Displayed data are derived from one representative of two independent experiments and were analyzed by Student's *t*-test. $^*p < 0.05$ and $^{**}p < 0.01$ was considered significant. All data are shown as mean ± SEM.

displaying severe signs of intestinal GvHD (**Figure 5F**). Finally, while the pool of Th1-differentiated IFNγ$^+$ donor T cells was largely unaltered, GM-CSF-expressing T cells were diminished in the absence of both RORγt and BATF, thereby underscoring the recently described role for BATF in this pathway (**Figure 5G**). Together, these results indicate that RORγt- and hence Th17-independent manifestation of intestinal GvHD depends on the preserved BATF expression within donor T cells and correlates with its abilities to sufficiently mount a GM-CSF$^+$ donor T cell pool *in vivo*.

Donor RORγt/Th17-Independent Colonic GvHD Is Driven by IL-7/IL-7R Interaction and GM-CSF

Our results so far suggest that while BATF and RORγt commonly and sequentially control IL-23R-dependent Th17 cell formation, only BATF but not RORγt positively regulates the GM-CSF-expressing T cell pool recently reported to drive colonic inflammatory manifestations of GvHD. Interestingly, it was recently reported that IL-7 promotes the *in vitro*-formation of GM-CSF-expressing T cells (Th_{GM} cells) due to their dependence on the transcription factor STAT5 (19). In contrast to T cells cultured in the presence of IL-23 and hence under inflammatory Th17 cell-promoting conditions (**Figure 2A**), we interestingly found that IL-7 supplementation of T cell cultures favoring Th_{GM} polarization did not result in the upregulation of IL-23R expression compared to naïve T cells that were assessed prior culturing (Figure S1 in Supplementary Material). This result that at least *in vitro* differentiated Th_{GM} cells are unresponsive to direct effects exerted by IL-23 substantially extends our previous observation that GM-CSF$^+$ T cells develop *in vivo* in an IL-23R-independent manner (**Figure 4C**). However, our assumption that RORγt-independent formation of intestinal GvHD is mediated by BATF-dependent GM-CSF$^+$ T cells is based on results that we previously obtained in studies employing T cells with a selective inactivation of the *Batf* gene (17). However, by taking the approach of co-inactivating two highly potent transcriptional regulators, our results might be confounded by the introduction of additional functional defects in the donor T cell compartment. Hence, we sought to selectively assess the contribution of GM-CSF to the manifestation of RORγt-independent intestinal GvHD by treating

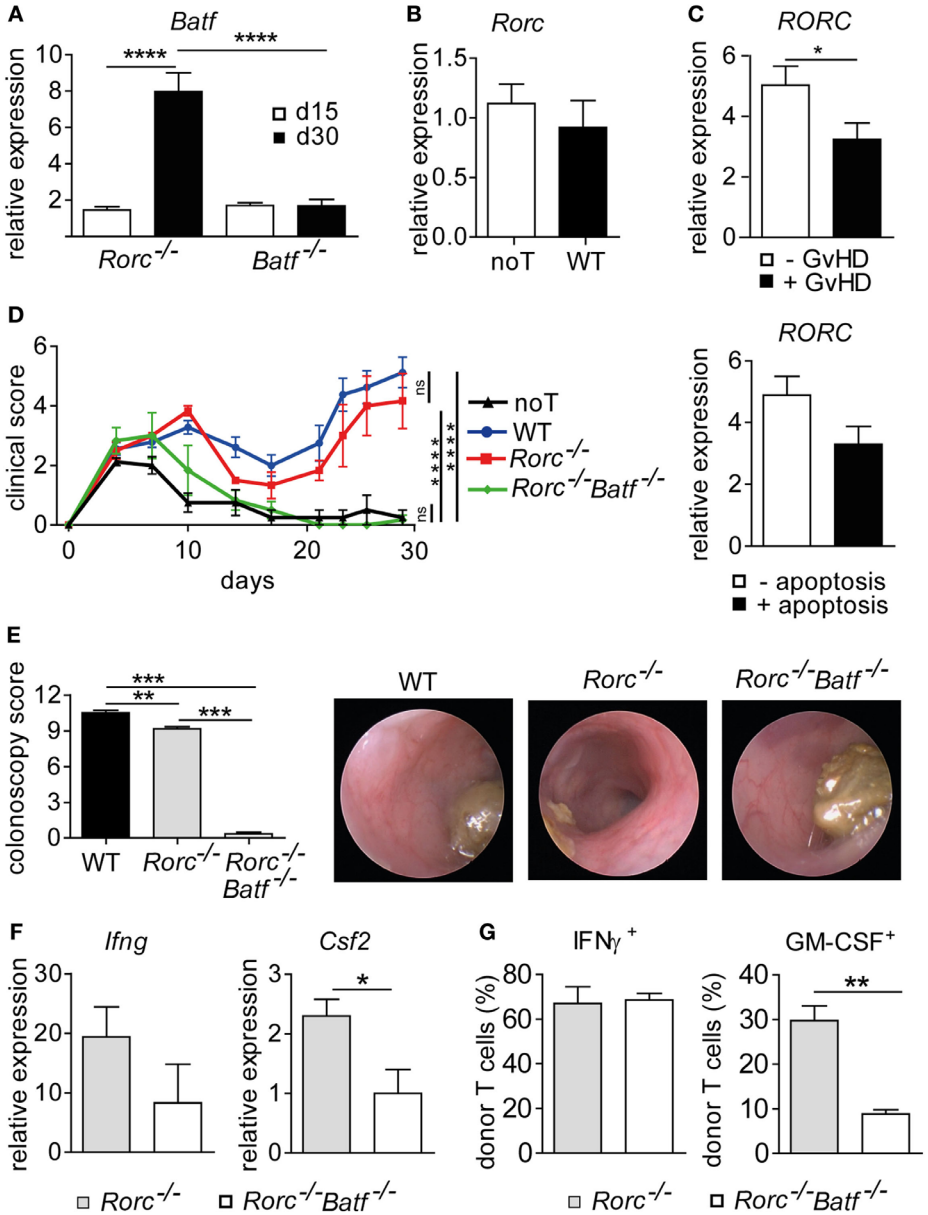

FIGURE 5 | Retinoic acid-related orphan receptor gamma t-independent intestinal graft-versus-host disease (GvHD) is controlled by basic leucine zipper transcription factor ATF-like (BATF). **(A)** Transcript levels of *Batf* in colonic tissue of allo-HSCT BALB/c mice with severe signs of intestinal GvHD following transplantation of allogeneic CD3+ T cells derived from of *Batf*$^{-/-}$ and *Rorc*$^{-/-}$ mice were measured by quantitative real-time PCR (qPCR) at day 15 (*Batf*$^{-/-}$ $n = 12$; *Rorc*$^{-/-}$ $n = 2$) and day 30 (*Batf*$^{-/-}$ $n = 12$; *Rorc*$^{-/-}$ $n = 4$). Data represent pooled data from two independent experiments. Gene expression levels detected in colonic tissues of no T cells (noT) controls at day 15 were arbitrarily set down to 1 and all other gene expression levels were normalized to the expression level detected within noT controls. **(B)** Transcript levels of *Rorc* in colonic tissue of irradiated BALB/c mice transplanted with T cell depleted bone marrow alone (noT, $n = 11$) or followed by injection of allogeneic CD3+ T cells of wild-type (WT) ($n = 12$) mice were measured by qPCR at day 30. **(C)** Transcript levels of *RORC* in colonic tissue biopsies from allo-HCT patients were measured by qPCR. Samples were grouped into indicated categories based on the absence (–GvHD; $n = 30$) or presence (+GvHD; $n = 22$) of GvHD-associated histopathological lesions or by the absence (–apoptosis; $n = 32$) or presence (+apoptosis; $n = 20$) of GvHD-related epithelial cell apoptosis. Data are shown as normalized relative *RORC* expression levels calculated from a standard curve. **(D–G)** Experimental GvHD induction was achieved as described in **Figures 1** and **2**. To this end, allogeneic hematopoietic stem cell transplantation BALB/c mice were transplanted with allogeneic CD3+ T cells derived from WT ($n = 4$), *Rorc*$^{-/-}$ ($n = 3$), or *Rorc*$^{-/-}$*Batf*$^{-/-}$ ($n = 3$) mice or received no T cells (noT) as a control ($n = 4$). **(D)** Mice were assessed three times a week for signs and severity of systemic GvHD. Data were analyzed by two-way ANOVA testing followed by Bonferroni's multiple comparisons posttest. ****$p < 0.0001$ was considered significant, whereas $p > 0.05$ indicates no significance (ns). **(E)** At day 28, colitis severity was determined by colonoscopy and representative endoscopic images are shown. **(F)** Expression levels of *Ifng* and *Csf2* transcripts within colonic tissues of *Rorc*$^{-/-}$ ($n = 7$) or *Rorc*$^{-/-}$*Batf*$^{-/-}$ ($n = 3$) T cell receiving mice were analyzed by qPCR. Data are shown from at least one experiment. **(G)** Colonic lamina propria mononuclear cells of indicated donor T cell receiving mice were isolated and the frequencies of granulocyte-macrophage colony-stimulating factor (GM-CSF+) or IFNγ+ CD4+ donor T cells were assessed by flow cytometry employing intracellular cytokine staining techniques. Data of panels **(A,E)** were analyzed by one-way ANOVA followed by Bonferroni's multiple comparisons posttest. **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$ was considered significant. Data of panels **(B,C,F,G)** were analyzed by Student's *t*-test. *$p < 0.05$ and **$p < 0.01$ was considered significant. All data are shown as mean ± SEM.

RORγt-deficient donor T cell receiving mice either with an IL-7R blocking antibody over the first half of the observation period together and anti-GM-CSF antibody throughout the experiment or with an isotype control antibody alone as described before (17). Strikingly, combined antibody-mediated blockade of IL-7R and GM-CSF but not continuous therapy with the control antibody alone resulted in a significant reduction of clinical signs of systemic GvHD elicited by RORγt-deficient T cells (**Figure 6A**). Moreover, colonoscopic evaluation of the intestinal GvHD phenotype revealed that RORγt-independent GvHD-associated colitis was mitigated upon dual antibody but not control antibody treatment (**Figure 6B**). The therapeutic effect on endoscopic signs of colitis was accompanied by diminished expression levels of proinflammatory mediators within colon tissues as we detected reduced amounts of *Il1b*, *Tnf*, *Csf2*, and *Ifng* transcripts in anti-IL-7R/anti-GM-CSF- compared to control antibody-treated mice (**Figure 6C**). Collectively, the results of our current study demonstrate that acute manifestation of intestinal GvHD occurs independent of IL-23R- and RORγt-expressing T cells with the latter being generally accepted to represent bona fide Th17 cells. Mechanistically, GM-CSF$^+$ T cells infiltrate the gut, expand in IL-7/IL-7R-interaction dependent, however IL-23R- and RORγt-independent manner and are major mediators of intestinal GvHD observed in the absence of T cell-intrinsic RORγt expression. Together, our study results strongly suggest that selective RORγt- and hence Th17-targeting represent an ineffective approach to limit allo-response driven intestinal inflammation.

DISCUSSION

Allo-response driven T cell-mediated mucosal inflammation is observed in about 50% of the patients undergoing allo-HSCT mostly because of an otherwise incurable hematopoietic malignancy and routinely represents a clinical challenge due to limited

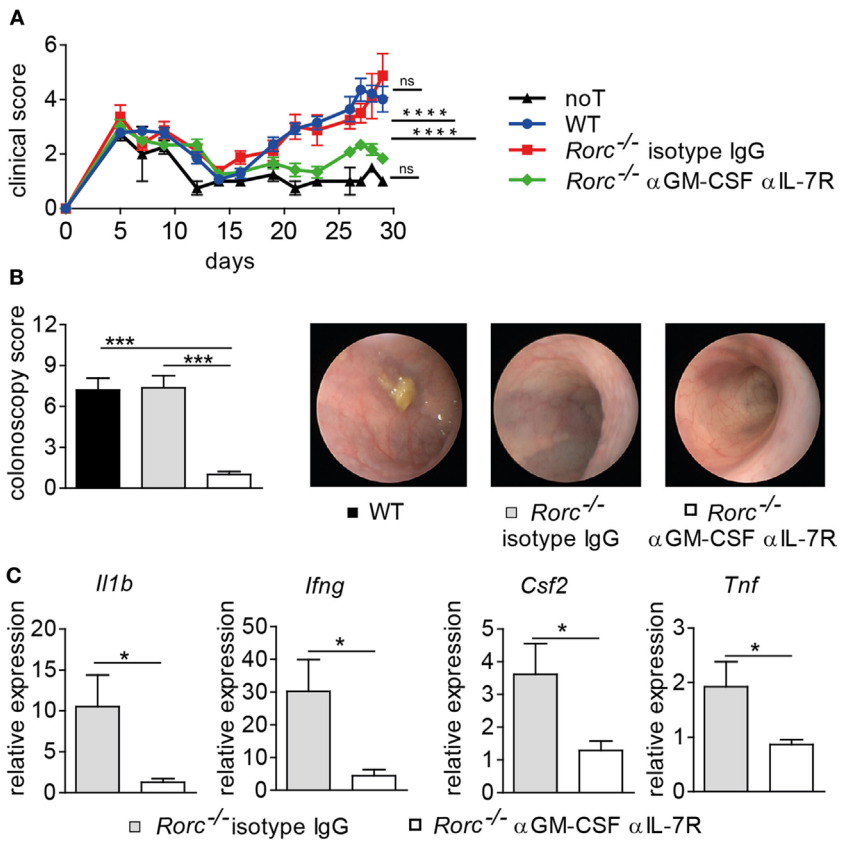

FIGURE 6 | Blockade of interleukin-7 receptor (IL-7R) and granulocyte-macrophage colony-stimulating factor (GM-CSF) mitigates graft-versus-host disease (GvHD)-associated colitis elicited by retinoic acid-related orphan receptor gamma t-deficient donor T cells. Irradiated BALB/c mice received T cell depleted bone marrow alone [no T cells (noT), $n = 2$] or were subsequently injected with allogeneic pan T cells of wild-type (WT) ($n = 6$) or *Rorc*$^{-/-}$ mice. One group of *Rorc*$^{-/-}$ T cell receiving mice was treated 3×/week with anti-GM-CSF antibody (αGM-CSF) throughout the experiment together with anti-IL7R (αIL-7R) until day 15 ($n = 5$) while another group was treated with isotype IgG antibody ($n = 4$) alone. Data are shown from one representative experiment. **(A)** Mice were evaluated 3×/week for the presence of clinical symptoms of GvHD and its severity was accordingly scored. Data were analyzed by two-way ANOVA testing followed by Bonferroni's multiple comparisons posttest. ****$p < 0.0001$ was considered significant, whereas $p > 0.05$ indicates no significance (ns). **(B)** At day 29, colitis activity was assessed by colonoscopy and representative endoscopic images are shown. Data were analyzed by two-way ANOVA testing followed by Bonferroni's multiple comparisons posttest. ***$p < 0.001$ was considered significant. Mice were sacrificed day 30. **(C)** Transcript levels of *Tnf*, *Il1b*, *Ifng*, and *Csf2* in colonic tissue of *Rorc*$^{-/-}$ T cell receiving mice treated with anti-GM-CSF together with anti-IL-7R or with isotype IgG antibody alone were analyzed by quantitative real-time PCR. Gene expression data were analyzed by Student's *t*-test. *$p < 0.05$ was considered significant. All data are shown as mean ± SEM.

therapeutic options and often therapy-refractory disease courses (32, 33). However, manifestations found in patients suffering from intestinal GvHD share a series of morphological, functional, and immunological characteristics with inflammatory lesions frequently observed in IBD patients (34). In respect to the immunological abnormalities most prominently found in both disease entities, T cells with a Th17 phenotype are similarly over-represented in patients displaying severe manifestations of acute intestinal GvHD (2). However, while there is both overwhelming preclinical and substantially increasing clinical evidence for a central pathogenetic role of IL-23 and bona fide Th17 cells in promoting intestinal inflammation in IBD (1), pathways dysregulated within donor T cells and hence contributing to the allo-response-driven manifestation of intestinal GvHD following allo-HSCT have been less well deciphered (1, 5, 13, 32, 33, 35, 36). In part due to the discordant usage of various preclinical model systems to induce experimental GvHD, genetic model systems to modulate donor T cell biology and overall non-overlapping experimental setups, it remains difficult to cross-compare results obtained by these studies to draw a clear conclusion (14–16). Furthermore, these studies were generally not designed to definitively discriminate between the functional contribution of bona fide IL-23-driven Th17 cells and of alternatively differentiated, however, Th17-related T cells as described, e.g., in the context of neuro-inflammation with the description of GM-CSF-producing T cells as the major mediator of tissue damage (22, 23, 37).

Recently, we elucidated the central role of BATF-expressing donor T cells in mediating acute intestinal GvHD (17). However, T cell-intrinsic BATF simultaneously controls both *in vitro* and *in vivo* the formation of IL-23-driven Th17 cells and IL-7-driven GM-CSF-expressing T cells, also called Th_{GM} cells (19), thereby excluding the possibility to selectively assess the role of Th17 cells. Hence, we sought to assess the functional contribution of IL-23R expression by donor T cells since IL-23R/IL-23 interaction is critically contributing to Th17 cell formation *in vivo*. Strikingly, employing the same model system and the same conditions under which BATF-deficient T cells—similarly devoid of IL-23R expression due to its BATF-dependent expression—conferred protection IL-23R-deficiency among donor T cells did not confer disease-preventive effects. However, this result essentially conflicts with data from another study that reported reduced mortality and subtle, however, significant reduction of colitis activity in the colon but no other GvHD-affected organs (38). We believe that a series of model-related aspects (e.g., conditioning regimen, composition of the resident intestinal microbiota within the recipient mice in the animal facility, employed genetic mouse models, and most importantly magnitude of the transferred donor T cell pool) may account for the observed differences in the GvHD phenotype best deduced from and exemplified by the parameter "survival": while in the other study two-thirds of the cohort of WT donor T cell receiving mice succumbed presumably due to rapid and exaggerated manifestation of systemic GvHD within the 30-day-interval after allo-HSCT (38), in our model system the kinetics of both systemic and intestinal GvHD manifestations is purposefully more moderate presumably mostly due to the transfer of a lower T cell dose to induce GvHD. Importantly, however, we reason at that point that our study reflects a presumably clinically more relevant setting since the manifestation of intestinal versus systemic GvHD displays a kinetics-wise more physiological pattern observed in GvHD-affected allo-HSCT patients.

Given the fact that IL-23R expression is only one, however, though both phenotypically and functionally critical hallmark of Th17 cells, we sought to assess whether RORγt-deficient T cells would exert comparably protective effects as previously observed in the absence of T cell-intrinsic BATF expression. RORγt was reported to represent the master regulator of Th17 cell differentiation (31), acts in concert with BATF in this pathway and cell type (18) and its absence provides full protection in syngeneic colitis models in a manner comparable to BATF deficiency (8, 9, 11). Strikingly, however, we failed to detect any GvHD-alleviating effects in regard to both systemic and intestinal inflammation following the transplantation of RORγt-deficient T cells when compared to WT T cell controls. Furthermore, in contrast to BATF (17), we were unable to detect any RORγt upregulation within both murine and human tissues irrespective whether GvHD was present or absent in allo-HSCT mice or men. However, the absence of IL-23R expression as well as RORγt expression within donor T cells expectedly led to a significant reduction of IL-17a-expressing T cells. Moreover, colonic T cells expressing the Th1 hallmark signature cytokine IFNγ were present in an IL-23R- and RORγt-independent manner. Strikingly and in clear delineation from the results obtained when we employed BATF-deficient T cells (17), RORγt-deficient T cells retained the ability to differentiate into allo-reactive GM-CSF-producing T cells. However, both GvHD-inducing capacity and GM-CSF expression of RORγt-deficient T cells were completely dependent on preserved T cell-intrinsic BATF expression since combined ablation of RORγt and BATF abrogated both GvHD manifestation and the GM-CSF expressing T cells. Furthermore, combined antibody-mediated blockade of the IL-7R and GM-CSF completely abrogated signs of systemic and intestinal GvHD elicited by RORγt-deficient T cells. Interestingly, in support of our hypothesis that IL-23R signaling-dependent Th17 cells and IL-7 promoted $GM\text{-}CSF^+$ T cells represent functionally non-overlapping T cell subsets, we found that IL-23R expression was not induced upon IL-7-mediated Th_{GM} polarization *in vitro*. This result fits well to the established model that IL-23R expression is regulated downstream of STAT3 signaling, e.g., in response to IL-6 and IL-23 itself while IL-7-induced STAT5 signaling was not reported to impact IL-23R expression regulation (30). Furthermore, this finding provides mechanistic insight complementing our functional data showing that while IL-23R-expressing T cell subsets (i.e., Th17 cells) are critically promoting intestinal inflammation in syngeneic models of intestinal inflammation and in human IBD, allo-response driven colitis is mediated by IL-23R-, RORγt- and hence Th17 cell-independent effector T cells that require signals (i.e., IL-7/IL-7R interaction) clearly distinguishable from signals controlling Th17 cells (i.e., IL-23R/IL-23 signaling) and are characterized by the exertion of effector mechanisms (GM-CSF) clearly distinct from those employed by Th17 cells (i.e., IL-17a, etc.).

The overall observation that syngeneic colitis preferentially results in the formation of functionally central Th17 cells while Th_{GM} cells predominately develop and promote intestinal GvHD in the context of allo-reactivity is intriguing but the mechanisms underlying both the quantitative and qualitative differences remain elusive. However, following disease-related aspects might contribute to this

phenomenon: first, although both syngeneic and allo-response-driven colitis formation depend on co-stimulatory signals that are provided by microbial components from colonizing microbiota (e.g., residing in the gut) and are also called pathogen-associated molecular pattern signals, host conditioning through whole body irradiation, etc., prior allo-HSCT results in a wide-spread tissue injury (39). In the course of the tissue response, a massive release of mediators like ATP or uric acid, also called danger-associated molecular pattern signals, takes place that additionally may account for the qualitative differences within colitogenic T cell pools observed during syngeneic versus allo-response driven colitis (40–43). Second, in addition to differential provision of co-stimulatory signals, setting-specific engagement of selective antigen-presenting cell subsets crucially impacts T cell differentiation outcomes. Accordingly it was shown that adoptively transferred T cells respond to microbial antigens presented by DCs (44) and $CD64^{+}CD11b^{hi}$ myeloid cells (45) and that their secretion of proinflammatory cytokines as IL-23 and IL-1β is critically contributing to the formation of colitogenic Th17 cells in the syngeneic colitis setting (46–48). By contrast, the expansion and differentiation of allo-reactive donor T cells were shown to be preferentially governed by donor-derived IL-12- and IL-6-secreting $CD103^{+}CD11b^{-}$ DCs (49). Since these studies did not investigate the impact on Th_{GM} cell formation, future studies need to investigate whether these DC subsets and DC-derived signals as suggested are acting in concert with IL-7 to drive the formation of intestinal GvHD-mediating GM-CSF expressing T cells.

Collectively, based on the large body of evidence in the literature RORγt-dependent Th17 cells are indispensable for the formation of a colitogenic T cell pool under syngeneic conditions (50). However, in the light of our current study results, we conclude that allo-response driven mucosal inflammation occurs in an IL-23R/Th17-independent, however, IL-7R/BATF-dependent manner and is mediated by GM-CSF rather than the classic Th17 signature cytokine IL-17a. Hence, we suggest that future attempts to limit GvHD-mediated mucosal inflammation especially affecting the colon should re-focus and evaluate the therapeutic efficacy of strategies designed to interfere with the development and/or functionality of GM-CSF expressing T cell subsets. However, one important question has not been answered yet: why and by what effector mechanisms exert Th17 cells colitogenecity in the syngeneic setting (11) but Th_{GM} cells (19) do not while as shown in this study Th_{GM} cells are highly pathogenic under allogeneic conditions whereas Th17 cells seem to be functionally dispensable? Hence, the solution of the major mechanistic conundrum overtly separating syngeneic from allogeneic colitis pathogenesis is reserved for future studies.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the government of Mittelfranken in Bavaria, Germany. The protocol was approved by the government of Mittelfranken in Bavaria, Germany. This study was carried out in accordance with the recommendations of the ethics committee of the University Hospital Regensburg. The protocol was approved by the ethics committee of the University Hospital Regensburg. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

VB, BA, and JR performed experiments with the help of TV, analyzed and interpreted data. EH and SG provided human colon tissue samples, performed analyses related to human colon biopsies and helped with the interpretation and critical discussion of the results. MB-H performed histopathological analyses. MN, EU, and EH gave critical advice and helped with the interpretation and critical discussion of the results. KH directed the study and wrote the manuscript together with VB.

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SUPPLEMENTARY MATERIAL

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REVIEW
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Cytokine Tuning of Intestinal Epithelial Function

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The intestine serves as both our largest single barrier to the external environment and the host of more immune cells than any other location in our bodies. Separating these potential combatants is a single layer of dynamic epithelium composed of heterogeneous epithelial subtypes, each uniquely adapted to carry out a subset of the intestine's diverse functions. In addition to its obvious role in digestion, the intestinal epithelium is responsible for a wide array of critical tasks, including maintaining barrier integrity, preventing invasion by microbial commensals and pathogens, and modulating the intestinal immune system. Communication between these epithelial cells and resident immune cells is crucial for maintaining homeostasis and coordinating appropriate responses to disease and can occur through cell-to-cell contact or by the release or recognition of soluble mediators. The objective of this review is to highlight recent literature illuminating how cytokines and chemokines, both those made by and acting on the intestinal epithelium, orchestrate many of the diverse functions of the intestinal epithelium and its interactions with immune cells in health and disease. Areas of focus include cytokine control of intestinal epithelial proliferation, cell death, and barrier permeability. In addition, the modulation of epithelial-derived cytokines and chemokines by factors such as interactions with stromal and immune cells, pathogen and commensal exposure, and diet will be discussed.

Keywords: chemokine, cytokine, epithelium, intestine, mucosal immunology

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INTRODUCTION

The intestinal epithelium separates the diverse and ubiquitous members of the intestinal luminal microbiome, virome, and mycobiome from the largest population of resident immune cells anywhere in the body, forming our largest single barrier to the external environment (1–4). As such, in addition to its critical role in digestion, the gut epithelium is also charged with mediating much of the interaction between luminal organisms and immune cells to ensure appropriate defensive reactions to pathogens versus tolerance of commensal microorganisms (1).

The physical intestinal barrier consists of a continuous single layer of columnar epithelial cells overlain by a variably thick layer of mucus. This mucus layer is embedded with antibodies and antimicrobial peptides and physically separates the epithelium from direct contact with much of the luminal microbiota (2). The majority of intestinal epithelial cells are absorptive enterocytes, but the epithelium also contains a number of more specialized cell types, including Paneth cells (in the small intestine only), goblet cells, hormone-secreting enteroendocrine cells, microfold (M) cells, and tuft cells (2, 5). Indeed, even these subtypes are too generalized to fully reflect the diversity

of intestinal epithelial cells. Recent single-cell sequencing data identified two subtypes of tuft cells and subclassified enteroendocrine cells beyond the eight subclasses previously reported (6).

The gut epithelium is continuously renewed by *Lgr5*$^{+}$ stem cells located in the base of the intestinal crypts. Newly formed precursor cells differentiate as they migrate away from the crypt toward the villus tip in the small intestine or luminal surface in the large intestine, where they are expelled into the lumen approximately every 4–5 days. The exception to this is Paneth cells, which are long-lived and instead move toward the crypt base (2, 5). Each cell type plays critical and distinct roles in intestinal function. Mucus-secreting goblet cells are crucial for maintenance of the luminal mucus layer and increase in frequency moving distally along the intestine, peaking at a frequency of approximately 25% of total epithelial cells in the distal colon (2). Small intestinal Paneth cells produce antimicrobial peptides and also contribute to stem cell maintenance and function through the production of Wnt3, pro-epidermal growth factor, and Notch ligands (2). M cells overlie gut-associated lymphoid tissues and facilitate the transport of luminal antigens to lymphoid cells, while tuft cells coordinate type 2 immune responses to parasites (5, 7, 8). Much of intestinal epithelial research, including a portion of that presented herein, has focused on the use of colorectal cancer cell lines to elucidate gut epithelial function. However, due to the heterogeneity of the intestinal epithelium *in vivo*, observations made from cell lines, which are not representative of all gut epithelial cell types, may be misleading. Recent advances in three dimensional intestinal epithelial organoid cultures, which differentiate into the various epithelial cell subtypes seen *in vivo*, are improving our ability to more effectively characterize intestinal epithelial function, and many of these studies will be highlighted in this review (9).

The gut-associated lymphoid tissues, including Peyer's patches and isolated lymphoid follicles, are likely the most well-recognized portion of the intestinal immune system. However, the entire gut is armed with a diverse repertoire of immune cells, which vary in location and frequency throughout the length of the intestine (2). The majority of these cells function in the lamina propria or within the epithelium of the intestinal mucosa. The epithelium predominantly hosts T cells, while the lamina propria is home to cells of both the adaptive and innate arms of the immune system, including T cells, B cells, innate lymphoid cells (ILCs), macrophages, dendritic cells, mast cells, and eosinophils (2). Immune cells may sense luminal antigens directly when the epithelial barrier is breached or by the extension of transepithelial dendrites, as has been observed in macrophages and dendritic cells. The intestinal epithelium is uniquely positioned and equipped with a cadre of pattern recognition receptors to sense luminal antigens and danger signals and relay this information to immune cells (2).

The intestinal epithelium faces the difficult challenge of permitting nutrient absorption and ion movement while maintaining an impermeable barrier to microorganisms and antigens in the gut lumen. The integrity of the intestinal mucosal barrier is critical for health; dysfunction of this barrier has been proposed to contribute to both intestinal and systemic disease, including inflammatory bowel disease (IBD) and multiple organ dysfunction syndrome (10, 11). Intestinal epithelial cells are linked by three types of specialized junctional complexes that attach adjacent cells and permit the selective paracellular movement of solutes and ions: desmosomes, adherens junctions, and tight junctions (10, 11). Desmosomes and adherens junctions predominantly serve as physical attachments between cells, while the more apically located tight junctions act as selective semipermeable barriers to intercellular spaces (12). Tight junctions are composed of four types of transmembrane proteins: junctional adhesion molecules, claudins, occludin, and tricellulin. Claudins are a family of proteins that are differentially expressed between tissues and exert different effects on paracellular permeability. Claudins critically regulate the selectivity of the epithelial barrier by forming charge- and size-specific channels between epithelial cells (12). The types of claudin proteins within tight junctions determine the permeability of these paracellular channels. For example, claudin-2 and claudin-6 have been shown to increase tight junction permeability. Intracellular zonula occludens proteins connect tight junction transmembrane proteins to cytoskeletal actin/myosin complexes, which facilitate opening of the tight junction under specific conditions (11, 12).

Cytokines and chemokines, soluble protein mediators critical for intercellular communication, support intestinal mucosal homeostasis but can also be key drivers of intestinal inflammation and inflammation-associated damage (1, 10, 13). For example, the genetic deletion of interleukin (IL)-10 or IL-2 precipitated spontaneous colitis in mice, suggesting that these cytokines are essential for colon homeostasis. However, a number of other cytokines, including IL-6, tumor necrosis factor (TNF), IL-18, IL-1β, and IL-17, are overexpressed in the inflamed intestine and have been implicated as contributors to intestinal damage (10). Despite these seemingly clear-cut observations, there is strong evidence that the traditional labels of pro- and anti-inflammatory are too simplistic and perhaps even deceiving when used to describe cytokine actions in the intestine. In support of this, clinical trials targeting cytokines thought to be predominantly pro-inflammatory in the intestine, such as IL-17, failed to induce remission in patients with IBD (10, 14). In addition, the literature contains conflicting and often equally convincing evidence for both pro- and anti-inflammatory actions of specific cytokines in the gut (10, 15). There are a number of potential explanations for these conflicting data, such as the timing of cytokine action, model system used, cytokine concentration, and the method of cytokine administration or removal (15–17). As such, cytokine actions should be interpreted on a situational basis to gain a more complete understanding of their diverse roles in health and disease.

Cytokines and chemokines can positively or negatively affect intestinal epithelial barrier integrity, and may be derived from resident innate or adaptive immune cells, infiltrating inflammatory cells, or from intestinal epithelial cells themselves (**Figure 1**) (10–12, 18–20). Intestinal epithelial proliferation and cell death can be induced or restricted by cytokines (21–23). Concordantly, various cytokines help heal the epithelial erosions and ulcerations characteristic of severe intestinal inflammation, while others exacerbate these lesions (10). Specific cytokines have also been shown to regulate opposing epithelial functions under different circumstances, for instance, proliferation or cell death (16, 22, 24–26). In addition, cytokines can directly alter intestinal epithelial

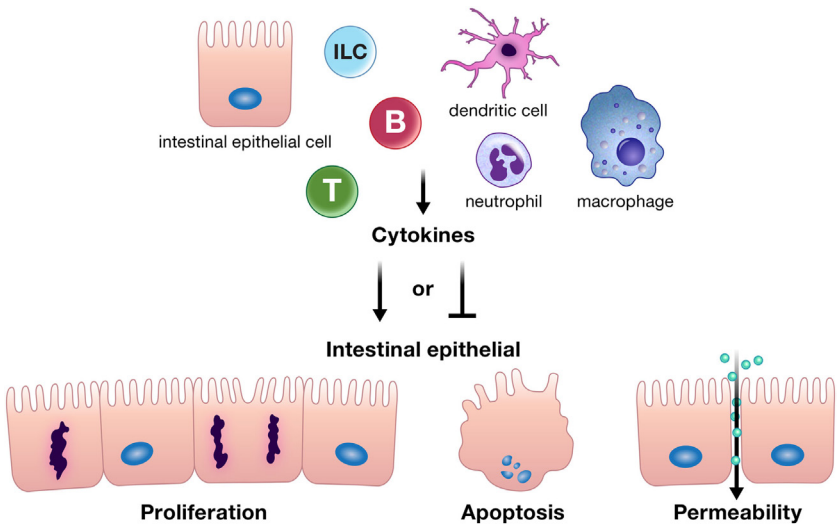

FIGURE 1 | Cytokines can positively or negatively affect intestinal epithelial barrier integrity by driving or inhibiting critical epithelial cell functions such as proliferation, apoptosis, and appropriate epithelial barrier permeability. These cytokines can be derived from resident innate or adaptive immune cells, infiltrating inflammatory cells, or from intestinal epithelial cells themselves. Abbreviations: T, T cell; B, B cell; ILC, innate lymphoid cell.

permeability (27, 28). The permeability of epithelial tight junctions may be increased or decreased by cytokine modification of the expression or localization of their protein components (11, 12, 27, 29, 30). Cytokines can also drive phosphorylation of myosin light chains, resulting in contraction and opening of tight junctions (11). Chemokine production by the intestinal epithelium recruits immune cells to areas of inflammation; however, whether this means epithelial suicide or survival depends on the inflammatory insult. Recruited immune cells may be crucial for defense against a pathogen but can perpetuate inflammation in conditions such as IBD (31–34). Regardless of mechanism, cytokines and chemokines are critical players in the integrity of the intestinal epithelial barrier. The purpose of this review is to highlight recent advances in our understanding of how cytokines and chemokines, both those made by and acting on the intestinal epithelium, orchestrate many of the diverse functions of the intestinal epithelium and its interactions with immune cells in health and disease.

CYTOKINE ACTIONS ON THE INTESTINAL EPITHELIUM

Cytokine Stimulation of Intestinal Epithelial Proliferation

Multiple cytokines regulate proliferation of the intestinal epithelium, a function that is crucial for both wound closure and replacing cells lost through homeostatic shedding (**Figure 2**) (7, 8, 16, 18, 35–50). Although generally thought to contribute to the pathology of IBD, recent studies have shown that TNF, IL-6, and IL-17 promote epithelial proliferation (14, 16, 18, 21, 44).

Tumor Necrosis Factor

In murine models of T cell activation and chronic chemically induced colitis, genetic ablation of either TNF or its receptor impaired Wnt/β-catenin signaling, resulting in reduced epithelial proliferation and delayed mucosal healing (16). This result may seem curious in light of the success of anti-TNF therapy in IBD patients; however, the authors offer an explanation for this perceived conflict by highlighting the mechanism of action of efficacious versus ineffective anti-TNF therapies. Therapeutic anti-TNF antibodies reduce inflammation in IBD patients by inducing apoptosis in inflammatory cells expressing membrane-bound TNF (51). By contrast, treatment with a soluble TNF receptor, which was ineffective in treating Crohn's disease, binds soluble TNF, which the authors propose blocks the ability of TNF to promote mucosal healing (16, 52).

Interleukin-6

Interleukin-6 increased proliferation and stem cell numbers in an *in vitro* model of murine small intestinal epithelial organoids, and the crypt epithelial cells also expressed IL-6, suggesting an autocrine signaling mechanism. Interestingly, the IL-6 receptor was only present on the basal membrane of crypt Paneth cells, making it unclear how IL-6 may affect epithelial cells in segments of the intestine lacking Paneth cells, such as the colon (18). However, Paneth cell metaplasia can be found in various types of colitis, in which case this mechanism of IL-6-facilitated epithelial repair could play a role (53). Furthermore, Kuhn et al. demonstrated that the early inhibition of IL-6 in murine models of bacterial colitis and wounding by biopsy impaired colon wound healing by limiting epithelial proliferation. They also demonstrated by *in situ* hybridization that IL-6 mRNA transcripts were enriched within the mucosa surrounding sites of intestinal perforation in human patients, suggesting that this IL-6-driven mechanism of wound healing may also be important in humans. These findings suggest that while Paneth cells may be crucial for IL-6-induced epithelial proliferation in the small

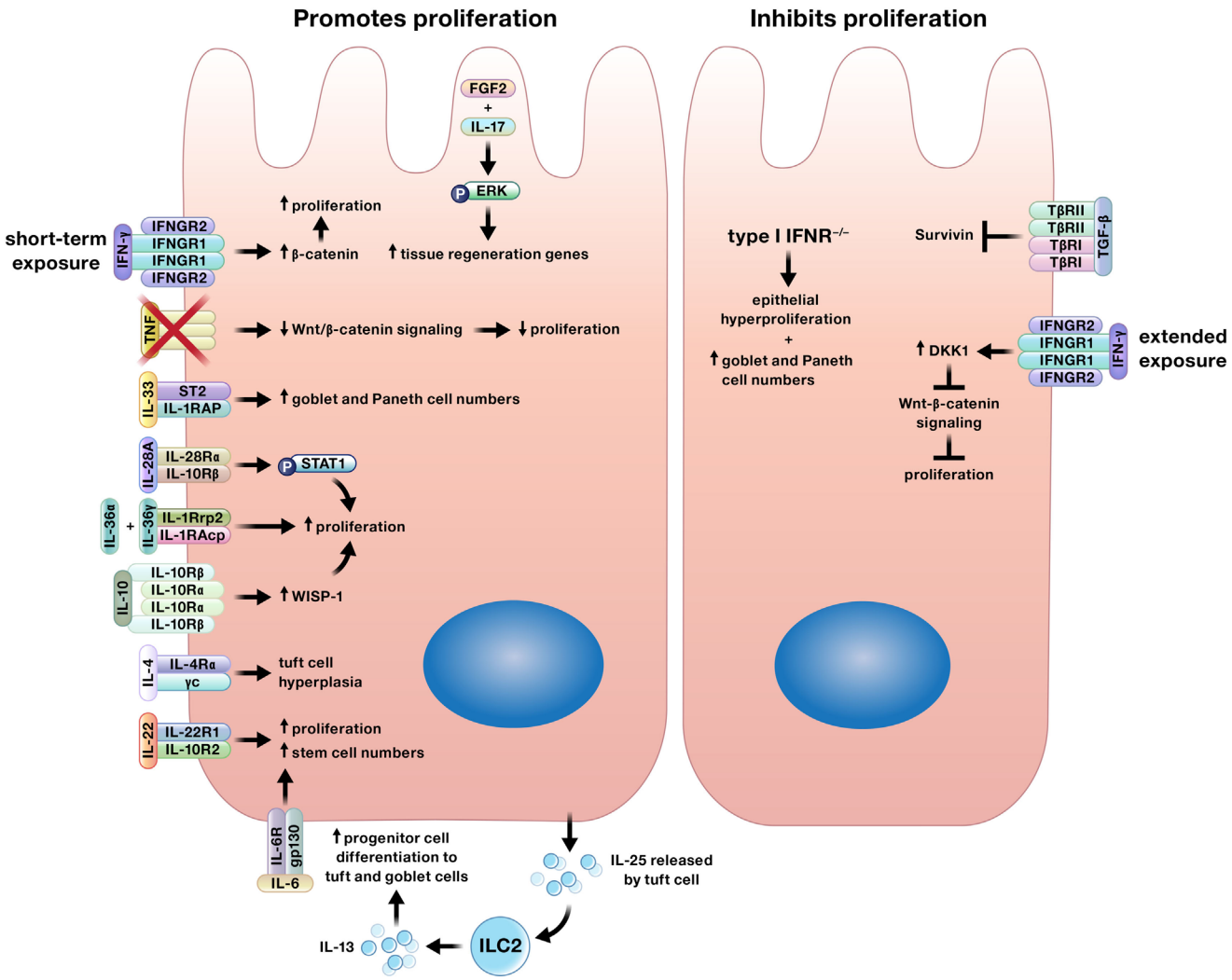

FIGURE 2 | Cytokines may promote or inhibit proliferation of intestinal epithelial cells. Interferon (IFN)-γ may induce or limit intestinal epithelial proliferation based on duration of exposure. In addition, specific cytokines may only induce proliferation of certain epithelial subtypes. For example, interleukin (IL)-4 increases tuft cell numbers, IL-13 signaling supports increases in tuft and goblet cells, and IL-33 stimulates the expansion of goblet and Paneth cells.

intestine, other mechanisms exist for IL-6 to drive epithelial repair in the colon (45).

Interleukin-17

Similarly, genetic ablation of IL-17 reduced intestinal epithelial cell proliferation and worsened dextran sulfate sodium (DSS)-induced murine colitis (44). Furthermore, IL-17 was shown to synergize with fibroblast growth factor 2 (FGF2) to promote intestinal healing in this study. FGF2 and IL-17 signaling synergistically activated ERK and induced genes related to tissue repair and regeneration in primary murine intestinal epithelial cells. The authors demonstrated that the mechanism of this synergy depended on Act1, an adaptor molecule that suppresses FGF2 signaling but is required for IL-17 signaling. When cells were co-stimulated with IL-17 and FGF2, Act1 was preferentially recruited to IL-17 receptors, preventing Act1-mediated suppression of FGF2 signaling (44). These findings may offer one explanation for the unexpected results of a clinical trial investigating the inhibition of the IL-17 receptor as a therapy for active Crohn's disease, in which a disproportionate number of patients actually experienced worsening disease with treatment (14).

Interleukin-22

Interleukin-22 increased growth in both human and murine intestinal organoids, both by inducing proliferation of the epithelial cells and facilitating stem cell expansion (46). IL-22 was also shown to be crucial for stem cell maintenance *in vivo* in the small intestine in a murine model of methotrexate-induced intestinal damage (54). During *Citrobacter rodentium* infection, IL-22 production by $CD4^+$ T cells was critical for colonic epithelial proliferation and resistance to infection-induced mucosal pathology (55).

Interleukin-36

Induction of IL-36 receptor signaling through any one of its ligands, IL-36α, IL-36β, or IL-36γ, induced proliferation of intestinal epithelial cells in *in vitro* organoid cultures, and mice with genetic deletion of the IL-36 receptor were more susceptible to chemically induced colitis, demonstrating higher disease activity, more severe colon pathology, greater bacterial translocation, and decreased survival. Furthermore, administration of a combination of IL-36α and IL-36γ accelerated wound healing in murine colons by increasing proliferation of epithelial cells adjacent to the experimental wounds (47).

Interleukin-28A

Similarly, IL-28A [also termed interferon (IFN) λ2] induced phosphorylation of signal transducer and activator of transcription 1 (STAT1) and proliferation in murine small and large intestinal epithelial organoid cultures (39). Mice with global knockout of the IL-28A receptor or intestinal epithelial cell-specific knockout of STAT1 developed more severe oxazolone and DSS-induced colitis, and the administration of IL-28A or genetic ablation of the IL-28A receptor in mice with induced colon wounds improved or delayed wound healing, respectively. The authors went further to link their murine models to human patients with IBD, demonstrating that both IBD patients and mice with colitis showed increased expression of the IL-28A receptor on the colon epithelium, as well as higher expression of IL-28A by cells within the lamina propria of the colon mucosa. Co-labeling of lamina propria cells in IBD patients identified dendritic cells as a major source of IL-28A (39).

Interleukin-10

A separate study also highlighted innate immune cells as a crucial cytokine source for mucosal healing. In a murine model of biopsy-induced colon injury, macrophage-derived IL-10 was crucial for optimal wound healing (48). IL-10 mRNA and protein were increased at wound sites within 1 day of wounding, and IL-10 induced epithelial proliferation by stimulating synthesis of Wnt1-inducible signaling protein-1. Interestingly, the absence of T and B cells in *Rag*1$^{-/-}$ mice also used in this study did not impair wound closure, further highlighting macrophages as the primary source of IL-10 in this model and suggesting that adaptive immune cells do not play a crucial role in this mechanism of wound healing (48).

IL-13, IL-4, and IL-33 Support the Differentiation of Specialized Epithelial Cells

Expansion of tuft cells, a specialized taste-chemosensory subtype of the intestinal epithelium, can also be induced by innate immune cells. During helminth infection, IL-25 secreted by tuft cells activates type 2 ILCs to produce IL-13, which induces the differentiation of increased numbers of tuft and goblet cells from epithelial progenitor cells (7, 8). IL-4, which shares the common receptor subunit IL-4 receptor α with IL-13, can also induce tuft cell hyperplasia (49). Mahapatro et al. demonstrated that IL-33 also directly affected the differentiation of epithelial progenitor cells. The constitutive expression of IL-33 in the small intestine of mice increased goblet and Paneth cell numbers but did not promote the proliferation/differentiation of absorptive enterocytes. Challenge of IL-33$^{-/-}$ mice with *Salmonella* Typhimurium demonstrated that IL-33 was critical for microbial defense, as mice lacking IL-33 had more severe intestinal damage and a greater *Salmonella* burden associated with decreased numbers of goblet and Paneth cells and reduced antimicrobial peptide production (50). Similarly, mice with genetic deletion of IL-33 or its receptor had decreased numbers of goblet cells and more severe colitis in a model of oxazolone-induced intestinal inflammation (17).

Cytokine-Induced Proliferation and Carcinogenesis

In the absence of wound closure, cytokine-induced intestinal epithelial proliferation may prove to be more deleterious than healing. In fact, a number of studies have suggested that cytokines, including IL-17, IL-6, IL-22, TNF-α, IL-4, and IL-13, either alone or in combination, may promote carcinogenesis in intestinal epithelial cells (56–60). Wang et al. demonstrated that IL-17 receptor type A (IL-17RA) signaling promoted proliferation of transformed colon enterocytes. IL-17RA signaling also induced IL-6 expression, a cytokine previously associated with colitis-associated cancer development (56). The concurrent neutralization of either IL-6 and IL-22 or TNF-α and IL-17A inhibited NF-κB or STAT3 signaling, respectively, and reduced the mitogenic effects of these cytokines on human colorectal cancer cells (57). Multiple studies have also shown that IL-22 alone can promote colorectal cancer progression (58, 59).

Furthermore, both IL-4 and IL-13 may contribute to colon cancer progression. IL-4 and IL-13 increased the expression of NADPH oxidase 1 in human colon cancer cell lines, which led to the production of reactive oxygen species and cellular proliferation. When examined in resected tissues from patients with colon cancer, the authors found increased active NADPH oxidase 1 in the tumor tissue relative to the adjacent normal colon tissue, leading them to suggest that IL-4/IL-13-driven NADPH oxidase 1 expression may drive colon carcinogenesis (60).

Cytokine Inhibition of Intestinal Epithelial Proliferation

In complement to the plethora of proliferation-inducing cytokines detailed earlier, a smaller number of cytokines limit intestinal epithelial proliferation (**Figure 2**) (24, 61–64).

Transforming Growth Factor-β (TGF-β)

Transforming growth factor-β suppressed expression of Survivin, a molecule critical for functional cell division in intestinal epithelial progenitor cells (61). Consistent with this finding, genetic disruption of TGF-β signaling in intestinal epithelial cells was sufficient for the development of invasive colon cancer in the face of chronic inflammation in mice (62).

Interferons

In a model of constitutive β-catenin signaling, Katlinskaya et al. demonstrated that type I IFNs limit intestinal epithelial proliferation (63). Concordantly, Tschurtschenthaler et al. characterized mice with intestinal epithelial-specific genetic deletion of the type I IFN receptor as having increased numbers of small

intestinal goblet and Paneth cells, epithelial hyperproliferation, and increased tumor burden following tumor induction with azoxymethane and DSS (64). Remarkably, the authors were able to eliminate the epithelial hyperproliferation and increase in tumors by cohousing the type I IFN receptor knockout mice with wild-type mice, demonstrating that these knockout-induced phenotypes were dependent on the gut microbiota (64).

The effects of the type II IFN, IFN-γ, on the intestinal epithelium vary with length of exposure. The short-term incubation of the intestinal epithelial cell line T84 with IFN-γ activated β-catenin signaling and induced proliferation of the T84 cells, peaking at 24 h. However, extended exposure of the T84 cells to IFN-γ induced expression of DKK1, which inhibited Wnt-β-catenin signaling and reduced proliferation. Interestingly, the addition of both TNF-α and IFN-γ enhanced these effects (24).

Damage Control: Cytokine Regulation of Apoptosis

While well-regulated apoptosis is essential for the homeostatic shedding of enterocytes, any perturbations to this process could quickly compromise the intestinal epithelial barrier. Indeed, increased apoptosis has been detected in the intestinal epithelium of IBD patients, although it is unclear if this is an initiating event in the disease, an effect of inflammation, or some combination of both (5). Increased intestinal epithelial apoptosis is also a consistent feature in critically ill humans and animal models of critical illness, such as sepsis. This increase in apoptosis contributes to intestinal epithelial barrier compromise in critical illness, which has been implicated as a critical driver of multiple organ dysfunction syndrome (11). Cytokines can induce or inhibit intestinal epithelial apoptosis (**Figure 3**) (16, 22–26, 65–67).

Interferons

Interferons have been shown to induce apoptosis of intestinal epithelial cells. Using human colon explant cultures, Jarry et al. demonstrated that administration of IFN-α-2a rapidly induced IFN-γ production by lamina propria resident T cells and IFN-γ-dependent epithelial apoptosis, a direct effect of IFN-γ on the intestinal epithelium that has been reported previously (24, 65, 66). Katlinskaya et al. also demonstrated a role for type I IFN in promoting apoptosis of the intestinal epithelium in a model of constitutive β-catenin signaling (63).

Tumor Necrosis Factor

In contrast to its ability to promote intestinal epithelial proliferation, one of the most well-characterized actions of TNF in the intestine is its ability to induce epithelial cell death. Injection of

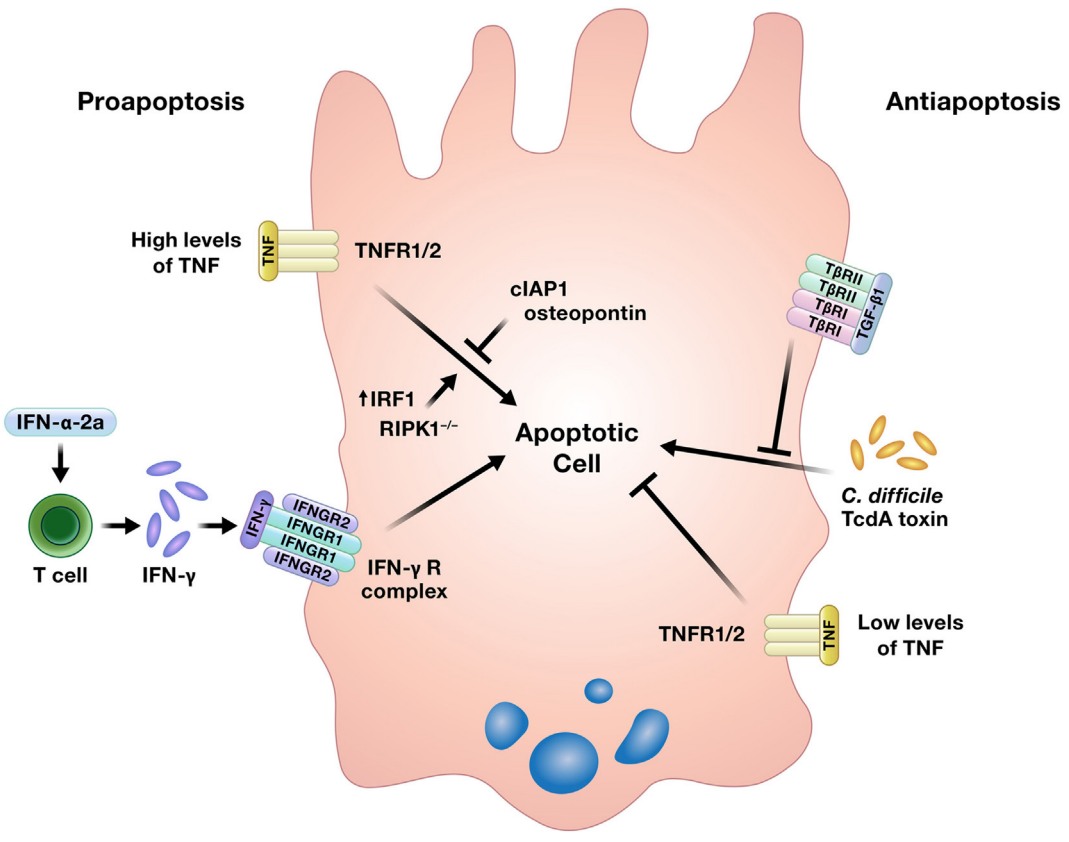

FIGURE 3 | Cytokines can induce or prevent apoptosis in intestinal epithelial cells. TNF has been shown to either promote or inhibit intestinal epithelial cell apoptosis under different conditions. Abbreviations: IAP, inhibitor of apoptosis protein; IRF1, interferon regulatory factor 1; RIPK1, receptor interacting protein kinase 1; TNF, tumor necrosis factor.

mice with TNF results in increased apoptosis of both small and large intestinal epithelial cells within 6 h, with a concentration of apoptotic cells in the intestinal crypts. Exposure of intestinal epithelial organoids derived from mice with genetic deletion of TNF receptors 1 and 2 revealed that while both receptors participated in TNF-mediated epithelial apoptosis, TNF receptor 1 signaling was predominantly involved. The authors further demonstrated that TNF-induced intestinal epithelial apoptosis is regulated by the inhibitor of apoptosis protein cIAP1. Inhibition of cIAP1 by second mitochondrial activator of caspases-mimetic compounds, tumor necrosis factor-related weak inducer of apoptosis (TWEAK), or genetic deletion sensitized mice to TNF-induced intestinal epithelial apoptosis (22). A separate *in vitro* study using cancerous and non-cancerous colon epithelial cell lines demonstrated that osteopontin reduced TNF-induced apoptosis, while the overexpression of IFN regulatory factor 1 increased TNF-mediated apoptosis (25). TNF was also implicated as contributing to the pathogenesis of intestinal inflammation in mice with conditional knockout of receptor interacting protein kinase 1 (RIPK1). Full RIPK1 knockout mice die perinatally, but the conditional RIPK1 knockout in intestinal epithelial cells in mice used in this study resulted in intestinal inflammation and early death associated with epithelial cell apoptosis. However, this phenotype was rescued by a deficiency in TNF receptor 1, and the lack of RIPK1 in *in vitro* cultured intestinal epithelial organoids sensitized the cultures to TNF-induced apoptosis (26).

In lieu of apoptosis, under certain circumstances, cells may undergo the pro-inflammatory process of regulated necrosis termed necroptosis (68). In addition to its ability to drive apoptosis, TNF can also initiate necroptosis of intestinal epithelial cells under specific conditions. In a model of conditional knockout of caspase 8 in intestinal epithelial cells, Günther et al. demonstrated that necroptosis in gut epithelial cells was triggered by TNF-α produced by other cells upon bacterial lipopolysaccharide (LPS) stimulation, not direct LPS-induced toll-like receptor 4 (TLR4) signaling in the epithelium. By contrast, gut epithelial necroptosis due to TLR3 ligation in the same model was cytokine-independent and directly initiated by TLR3 signaling (69).

In light of the strong evidence for a pro-apoptotic function of TNF in the gut, Bradford et al. curiously demonstrated an anti-apoptotic effect of TNF in the intestinal epithelium. In the murine model of T cell activation induced by anti-CD3 antibody injection used in this study, intestinal epithelial apoptosis is expected both acutely at the villus tips and later in the crypts around 24 h post-injection. Interestingly, and perhaps counterintuitive to the evidence presented herein thus far, administration of anti-CD3 antibody in $TNF^{-/-}$ mice resulted in a sevenfold increase in crypt epithelial apoptosis, suggesting that TNF works to limit epithelial apoptosis in this model (16). Other studies have also characterized an anti-apoptotic role for TNF in the intestinal epithelium, and the authors suggest that the level of TNF may determine whether it acts to promote or prevent apoptosis, with higher levels of TNF proposed to be pro-apoptotic (16, 67).

Transforming Growth Factor-β1

Transforming Growth Factor-β1 can also inhibit intestinal epithelial cell death. TGF-β1 reduced apoptosis and prevented necrosis in rat jejunal crypt epithelial cells exposed to the TcdA toxin of *Clostridium difficile* (23).

Cytokine Reinforcement of Intestinal Epithelial Barrier Integrity

Appropriate permeability of the intestinal epithelium is crucial for the balance between nutrient absorption and pathogen exclusion, and a number of cytokines positively affect this epithelial function (**Figure 4**) (12, 17, 27, 42, 70–72).

Interleukin-17

Inhibition of IL-17 receptor A by antibody neutralization worsened disease in the multidrug resistance-1a-ablated (*Abcb1a*$^{-/-}$) murine model of colitis and was associated with increased epithelial permeability as detected by increased serum concentrations of soluble CD14 and LPS binding protein and increased plasma concentrations of orally administered sucralose, lactulose, and mannitol (70). Lee et al. also demonstrated that a loss of IL-17 signaling increased intestinal epithelial permeability by showing increased amounts of orally administered fluorescein isothiocyanate (FITC)–dextran in the serum of mice with both chemically induced and T cell transfer-induced colitis in which IL-17 was removed by antibody neutralization or genetic deletion (27). The authors attributed the increase in gut epithelial permeability in the absence of IL-17 to disruptions in the structure of tight junctions, junctional complexes which are critical to the selectivity inherent in appropriate gut barrier permeability. The absence of IL-17 resulted in the intracellular mislocalization of the tight junction complex protein occludin and a loss of co-localization of occludin with F-actin. To provide more support for this mechanism, the authors applied TNF-α, a cytokine previously reported to disrupt tight junctions and increase epithelial barrier permeability, to cultured Caco-2 cells with or without co-stimulation with IL-17A (27, 28). Consistent with their observations *in vivo*, TNF-α altered the intracellular localization of occludin; however, co-stimulation with IL-17A reduced the TNF-induced occludin mislocalization (27). Along with the previously described ability of IL-17 to induce intestinal epithelial regeneration, the ability of IL-17 to reinforce the intestinal epithelial barrier offers an additional potential explanation for the worsening of Crohn's disease observed in clinical trial patients treated with an antibody to inhibit IL-17 receptor signaling (14).

Interleukin-10

Multiple studies have shown the positive effects of IL-10 signaling in the gut epithelium for maintenance of appropriate epithelial permeability (42, 73, 74). Stimulation of T84 cell monolayers with IL-10 restored transepithelial electrical resistance disrupted by compromise of the monolayers by incubation with IFN-γ. In addition, knockdown of the IL-10 receptor 1 in human intestinal epithelial cell lines impaired barrier formation as assessed by transepithelial electrical resistance and increased paracellular flux (42). These changes suggest alterations in the function of intercellular tight junctions owing to the lack of IL-10 signaling; however, this potential mechanism was not explored in this study. In the same study, mice with intestinal epithelial cell-specific

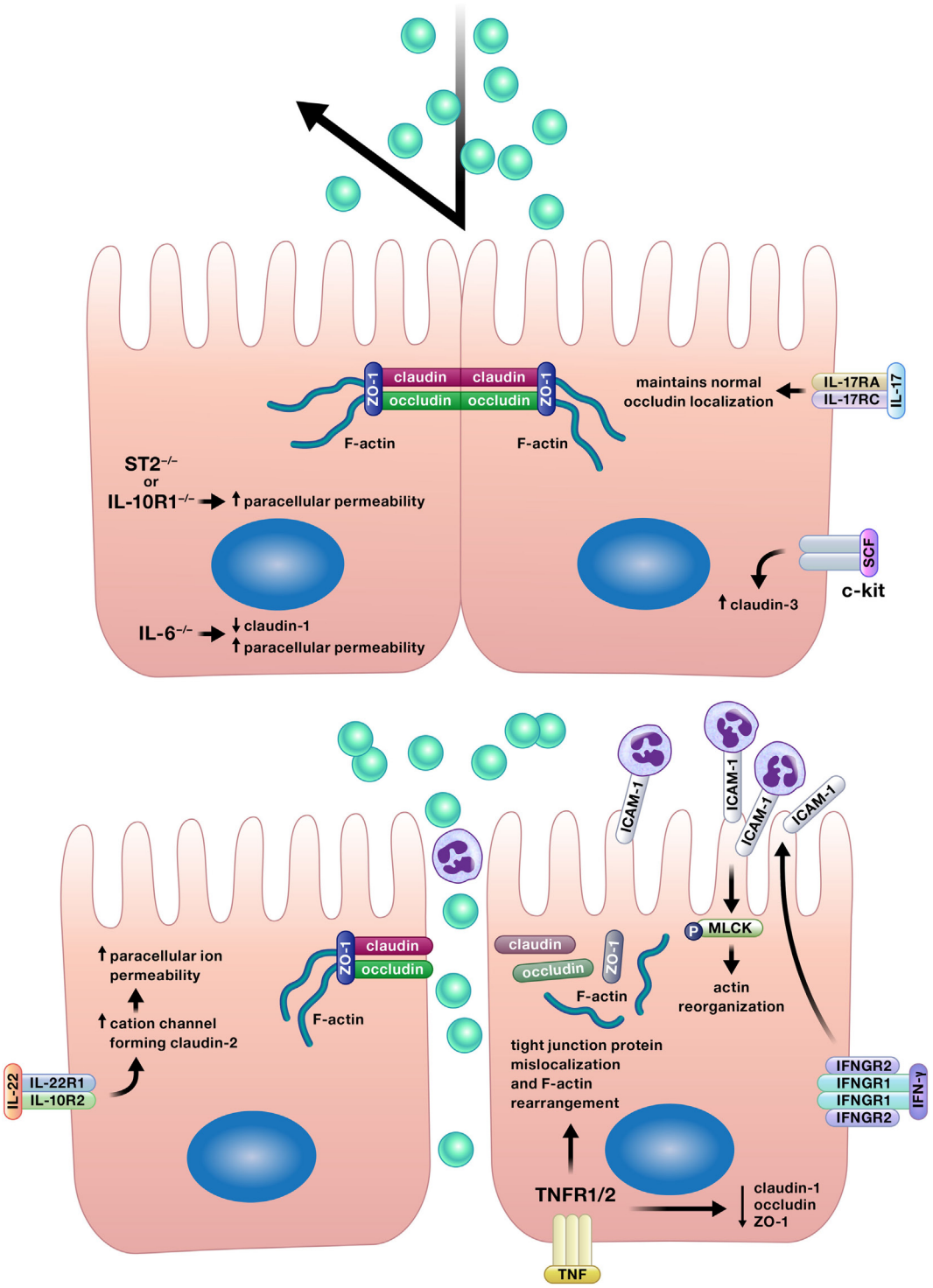

FIGURE 4 | Appropriate permeability of the intestinal epithelium maintains balance between nutrient absorption and pathogen exclusion. Cytokines may reinforce or impair the intestinal barrier by altering permeability of the epithelium. Epithelial tight junction permeability may be increased or decreased by cytokine modification of the expression or localization of tight junction protein components, such as various claudins, occludin, or zonula occludens protein-1 (ZO-1). Cytokines can also drive phosphorylation of myosin light chains, resulting in contraction and opening of tight junctions. Interferon (IFN)-γ increases intercellular adhesion molecule-1 (ICAM-1) expression, and subsequently, ICAM-1-mediated adherence of neutrophils to gut epithelial apical membranes. Neutrophil ligation of ICAM-1 drives the phosphorylation of myosin light-chain kinase (MLCK), resulting in actin reorganization leading to increased paracellular permeability and neutrophil transepithelial migration.

knockout of the IL-10 receptor 1 developed more severe chemically induced colitis with increased epithelial permeability to FITC–dextran (42). The authors concluded that the more severe colitis in these mice was driven by increased barrier permeability due to a lack of IL-10 signaling in epithelial cells. However, as previously discussed, IL-10 can induce proliferation in intestinal epithelial cells (48). As such, the inhibition of IL-10-induced epithelial restitution could have also contributed to the more severe colitis demonstrated in mice lacking intestinal epithelial expression of the IL-10 receptor 1 in this study.

In a separate study, Zheng et al. demonstrated how a cytokine, in this case IL-10, can interact with the intestinal microbiota to regulate epithelial function (73). Butyrate, a short chain fatty acid made by the intestinal microbiota *in vivo*, induced the expression of both IL-10 receptor α subunit mRNA and protein in T84 and Caco-2 cells. Treatment of T84 cells with butyrate and IL-10 increased epithelial barrier integrity more than butyrate alone as determined by increased transepithelial electrical resistance. Based on the increased expression of the IL-10 receptor α subunit in the epithelial cells due to butyrate treatment, the mechanism for this increase in barrier integrity owing to butyrate and IL-10 could be hypothesized to be an increase in IL-10 signaling due to increased IL-10 receptor expression. However, the authors did not compare these data with the transepithelial electrical resistance induced by IL-10 in the absence of butyrate. As a result, it is unclear from these data whether butyrate and IL-10 synergistically increase transepithelial electrical resistance in intestinal epithelial cells, or if the level reported in this study could have been induced by IL-10 alone. The authors went further to demonstrate that butyrate reduced both the mRNA and protein expression of the pro-permeability tight junction protein claudin-2 in T84 cells in an IL-10 receptor α-dependent manner, providing a potential mechanism for the observed increases in epithelial barrier integrity in the presence of butyrate (73). Interestingly, reductions in butyrate-producing bacteria have been reported in the microbiota of ulcerative colitis patients, suggesting a potential mechanism of epithelial barrier compromise due to dysbiosis as a contributing factor in this disease (75).

A study by Lorén et al. demonstrated how IL-10 can increase the effectiveness of other therapies (74). Previous work by this group correlated low IL-10 mRNA levels with poor glucocorticoid response in active Crohn's disease. In a later study, the authors discovered a possible mechanism for this observation, as treatment with a combination of IL-10 and glucocorticoids, but neither treatment alone, restored the transepithelial electrical resistance of Caco-2 cell monolayers following their disruption with TNF-α (74).

Interleukin-6

A study by Kuhn et al. provided more evidence for the crucial relationship between the microbiota, immune system, and intestinal epithelial barrier (71). Bacteria in the order Bacteroidales were sufficient to induce localization of intraepithelial lymphocytes in the colons of mice, and these cells were an important source of IL-6. IL-6 supported epithelial barrier function, as IL-$6^{-/-}$ mice displayed reduced expression of the tight junction protein claudin-1, a thinner mucus gel layer, and augmented paracellular permeability, all defects which were resolved by the transfer of IL-$6^{+/+}$ intraepithelial lymphocytes to affected mice (71).

Stem Cell Factor

C-kit signaling has also been shown to promote intestinal epithelial barrier integrity through the regulation of a tight junction protein. The overexpression of c-kit or administration of its ligand stem cell factor increased expression of the tight junction protein claudin-3 in colorectal cancer cells *in vitro*, and decreased claudin-3 expression was observed in the colon epithelium of mice lacking functional c-kit (72).

Interleukin-33

Rectal biopsies from adult and pediatric patients with ulcerative colitis have increased IL-33 expression relative to specimens lacking inflammation (17). To determine if this implicates IL-33 as a contributor to inflammation or an anti-inflammatory response in these patients, Waddell et al. investigated the role of IL-33 in chemically induced colitis in mice (17). Mice with genetic deletion of ST2, the receptor for IL-33, had decreased colon transepithelial electrical resistance and increased permeability to FITC–dextran, suggesting that IL-33 promotes colon epithelial barrier function. In support of these data, genetic deletion of either ST2 or IL-33 precipitated more severe chemically induced colitis in these mice (17). However, the authors did not fully characterize the mechanism by which IL-33 promoted epithelial barrier integrity in these studies. The authors reported that intestinal epithelial proliferation and apoptosis were unaffected by the absence of IL-33 or ST2 in this model of colitis, but that goblet cell numbers and *Muc2* expression were decreased in these mice. This suggests that alterations in the mucus layer could have influenced epithelial barrier permeability in these mice, but the mucus layer itself was not evaluated. In addition, potential effects of IL-33 on interepithelial junctional complexes were not assessed; however, the authors did demonstrate that IL-33-induced augmentation of transepithelial electrical resistance in T84 cell monolayers was dependent on ERK1/2 signaling (17). This is particularly curious in light of a recent paper that reported reduced transepithelial electrical resistance and claudin-1 expression induced by IL-33-stimulated ERK signaling in human keratinocytes (76). This discrepancy could be explained by the different cell types investigated; however, conflicting roles for IL-33 in intestinal inflammation have been reported. Other investigators have demonstrated exacerbation of multiple models of murine colitis and decreased intestinal epithelial barrier integrity due to the administration of IL-33 (77, 78). Waddell et al. suggest that these inconsistencies could be due to differences in IL-33 concentrations among studies or the differing characteristics of inflammation in each colitis model, two reasonable explanations that warrant further investigation (17). In support of the data reported by Waddell et al., Sattler et al. demonstrated the induction of protective IL-10-producing regulatory B cells by IL-33 (78). The administration of IL-33 accelerated spontaneous colitis in IL-10-deficient mice but did not induce intestinal inflammation in wild-type mice. In addition, the transfer of IL-33-induced, IL-10-producing regulatory B cells to IL-10-deficient mice reduced colitis severity and delayed disease onset (78). As previously discussed, IL-10 promotes epithelial barrier integrity (42, 73). As such, reduced IL-10 production owing to genetic ablation of IL-33 signaling is a potential mechanism for the increased intestinal epithelial permeability observed by Waddell et al. (17, 42, 73).

Falling Through the Cracks: Cytokine Promotion of Intestinal Epithelial Permeability

In contrast to the barrier reinforcing properties of the cytokines described earlier, a handful of cytokines can also disrupt the intestinal epithelium and promote barrier permeability (**Figure 4**) (29, 30, 79, 80).

Tumor Necrosis Factor

Various effects of TNF-α on the intestinal epithelium discussed herein could disrupt the epithelial barrier; however, TNF-α stimulation of intestinal epithelial cells has also been specifically demonstrated to decrease the protein expression of the tight junction proteins claudin-1, occludin, and zonula occludens protein-1 (ZO-1), as well as to induce cytoskeletal F-actin rearrangement and the mislocalization of occludin and ZO-1 (29, 30). Multiple studies have identified mechanisms to reduce TNF-α-induced epithelial barrier compromise, including the overexpression of anterior gradient protein 2 homolog, rebeccamycin treatment, and the stimulation of muscarinic cholinoceptor-mediated signaling (29, 30, 81).

Interleukin-22

Interleukin-22 also increases gut epithelial permeability *via* manipulation of tight junction protein expression. IL-22 stimulation of Caco-2 cells *in vitro* and murine colon epithelial cells *in vivo* increased the expression of the tight junction protein claudin-2, which forms cation channels. Caco-2 monolayers treated with IL-22 displayed decreased transepithelial electrical resistance, indicating increased paracellular ion permeability, but no change in movement of uncharged macromolecules across the monolayers was observed (79).

Interferon-γ

The increase in intestinal epithelial permeability induced by IFN-γ described by Sumagin et al. provides an elegant example of the intricate relationships between cytokines, the epithelium, and immune cells (80). Using the T84 intestinal epithelial cell line for an *in vitro* model of transepithelial migration of neutrophils, the authors demonstrated that IFN-γ induced expression of the intercellular adhesion molecule-1 (ICAM-1) on the apical membrane of T84 cells and increased the number of neutrophils adherent to the apical epithelial membranes *via* ICAM-1 post-migration. The ligation of ICAM-1 by neutrophils resulted in the phosphorylation of myosin light-chain kinase and a subsequent increase in epithelial permeability characterized by actin cytoskeletal reorganization, paracellular FITC–dextran flux, and a decrease in transepithelial electrical resistance. Notably in this model, this increase in epithelial permeability facilitated neutrophil transepithelial migration (80).

Additional Cytokine Effects on Intestinal Epithelial Function

In addition to those detailed earlier, cytokines modulate a wide array of other intestinal epithelial functions. While endogenous type III IFN produced by intestinal epithelial cells does not restrict human rotavirus replication due to viral antagonism of the type III IFN response, treatment of human rotavirus-infected small intestinal organoid cultures with exogenous type I IFN, and to a lesser extent exogenous type III IFN, limits rotaviral replication (82). However, other studies in mice have found that IFN-λ, a type III IFN, is more effective than type I IFNs in limiting viral replication in the intestinal epithelium in models of reovirus and rotavirus infection (83, 84).

In a somewhat unexpected role, IL-22 production by neutrophils in chemically induced murine colitis induced the expression of antimicrobial peptides by the colon epithelium and protected the epithelium from chemically induced damage (85). Epithelial signaling of the IL-17 receptor regulates colonization of the murine intestine with segmented filamentous bacteria through the epithelial expression of the apical NADPH oxidase *Nox1*, *polymeric immunoglobulin receptor* (*Pigr*), and α-defensins (86). In addition to the functions previously discussed, TNF stimulation of the intestinal epithelium has also been shown to reduce expression of the Cl^- / HCO_3 exchanging solute carrier family 26 member 3, which may represent a therapeutic target in IBD-associated diarrhea (87). TNF also augmented receptor activator of NF-κB ligand-induced M cell differentiation (88).

TALKING BACK: INTESTINAL EPITHELIAL-DERIVED CYTOKINES AND CHEMOKINES

Pro- and Anti-Inflammatory Functions of Intestinal Epithelial-Derived Cytokines

The intestinal epithelium is not simply beholden to respond to immune cell-derived cytokines but is a rich source of cytokines and chemokines, which may ameliorate or promote inflammation. The colonic epithelium was found to be a larger source of trefoil factor 2 (TFF2) than colon leukocytes, and TFF2 was protective in both acute and chronic models of DSS-induced colitis (19). In models of helminth infection, production of IL-25 by intestinal epithelial tuft cells regulated the helminth-induced type 2 immune response and facilitated worm expulsion (7, 49).

Intestinal epithelial cells also produce the anti-inflammatory cytokine IL-10, which likely contributes to tolerance to commensal bacteria. TLR4 ligation induced intestinal epithelial cell expression of IL-10, and this expression was enhanced by co-culture with macrophages (20). As previously discussed, IL-10 has been shown to stimulate intestinal epithelial cell proliferation and reinforce the integrity of the epithelial barrier (42, 48, 73). Thus, the microbiota may contribute to intestinal epithelial integrity through epithelial TLR4 ligation and the subsequent autocrine action of epithelial-derived IL-10. If so, IL-10 would not be the only cytokine with an autocrine mechanism for promoting epithelial homeostasis. IL-6 production by the intestinal epithelium has also been detected, which was shown to act in an autocrine manner to regulate crypt homeostasis (18).

In contrast to these anti-inflammatory and homeostatic effects, intestinal epithelial products may also promote inflammation. The accumulation of visceral fat has been associated with chronic intestinal inflammation, and in support of this, coculture of intestinal epithelial cells with differentiated adipocytes induced

epithelial expression of TNF and matrix metalloproteinase-9 (89). IL-1α release by necrotic intestinal epithelial cells in a murine model of chemically induced colitis induced cytokine production by mesenchymal cells and reactivated colon inflammation post-recovery when delivered *via* enema (90). The findings of Bersudsky et al. support these data, as genetic ablation of IL-1α ameliorated murine DSS-induced colitis (91).

Intestinal epithelial cells also secrete IL-33; however, there is conflicting evidence in the literature regarding its role in both IBD [reviewed by Griesenauer et al. (40)] and intestinal carcinogenesis. IL-33 expression was found to be increased in epithelial cells of both murine and human intestinal tumors, and IL-33 promoted tumor development in $Apc^{Min/+}$ mice (92, 93). Similarly, the expression of IL-33 by intestinal epithelial cells was increased in the murine azoxymethane/DSS model of colon cancer, and the authors went further to demonstrate that the epithelial expression of IL-33 was driven by epidermal growth factor (94). By contrast, knockdown of the IL-33 receptor, ST2, in colon cancer cells from mice enhanced tumor growth, suggesting a potential antitumorigenic role for IL-33 (95).

Calling in the Troops: Intestinal Epithelial Chemokine Production

Intestinal epithelial-derived chemokines can contribute to both cellular defense and pathology. *Listeria monocytogenes* infection of an intestinal epithelial cell line induced expression of the chemokines IL-8, CCL1, and CCL20. Consistent with the epithelial invasiveness of *L. monocytogenes*, the high levels of CCL20 and IL-8 were likely induced by intracellular TLR10 signaling, the knockdown of which reduced chemokine levels more than silencing of TLR1 or TLR2 (31). IL-8, CCL1, and CCL20 are responsible for neutrophil, Th2 and regulatory T cell, and Th17 and dendritic cell trafficking, respectively, and would promote the infiltration of these cell types in the infected mucosa (96). Interestingly, a separate study identified a non-chemotactic role for IL-8 in the intestine. Apically secreted intestinal epithelial cell-derived IL-8 in response to TLR2 and TLR5 ligation was shown to act in an autocrine manner to promote gene expression related to cellular differentiation (97).

Chemokines likely play a critical role in the perpetuation of intestinal inflammation in IBD patients. Dent et al. reported that cocultured eosinophils and intestinal epithelial cells synergized to increase neutrophil chemotactic activity and CXCL5 production; however, the authors did not quantify the individual contributions of each cell type to this increase (33). As evidence of activated eosinophils has been detected in acute flares of IBD, this could contribute to excessive neutrophil recruitment to the intestine and increased tissue damage in active IBD (33). Production of the cytokine IL-34 is increased in the intestine of patients with active IBD, and Franzè et al. demonstrated that production of the chemokine CCL20 was associated with IL-34 signaling in both the DLD-1 colon epithelial cell line and in mucosal explants from IBD patients (34). CCL20 production could fuel the inflammatory response in active IBD patients through the recruitment of Th17 and dendritic cells. However, the potential consequences of increased CCL20 production are not so clear-cut. In fact, these cells could aid in restitution of the epithelial barrier in IBD patients. As noted previously, IL-17 can increase intestinal epithelial cell proliferation and reduce barrier permeability, and dendritic cells are a critical source of IL-28A in the gut, another cytokine shown to induce intestinal epithelial proliferation (27, 39, 44, 70). Conversely, this hypothesized cytokine-induced proliferation could be too much of a good thing. IL-17 has been shown to both induce the proliferation of transformed enterocytes and stimulate IL-6 production, a cytokine implicated in colitis-associated carcinogenesis (56). The neutrophil chemokine CXCL1 has also been shown to promote carcinogenesis. The upregulation of CXCL1 by colon tumor epithelium was dependent on hypoxia-inducible factor 2α and contributed to colon carcinogenesis through neutrophil recruitment (32).

Intestinal Epithelial Responses to Pathogens and Commensals

The intestinal epithelium is uniquely located to be the ideal first line of defense or communication with intraluminal bacteria and viruses. A number of bacteria alter cytokine production by the gut epithelium (**Figure 5**) (98–103). Exposure of the colon epithelial cell line HCT-8 to Shiga toxin 2 produced by Shiga-toxigenic *Escherichia coli* increased protein expression of IL-8 and TNF-α. However, HCT-8 exposure to subtilase cytoxin produced by the same bacterium decreased protein expression of IL-8 and monocyte chemoattractant protein-1 relative to unstimulated control cells, suggesting that these bacteria may use specific toxin production to differentially modulate host defenses (98). Infection of Caco-2 monolayers with *Shigella flexneri* 2a or *Shigella dysenteriae* 1 induced IL-8 secretion, which was predominantly released from the basolateral aspect of the epithelial cells, and *Salmonella enterica* serovar Typhimurium activated non-canonical inflammasome activity in murine and human intestinal epithelial cells, facilitating IL-18 secretion and bacterial clearance (99, 100).

In contrast to these predominantly pro-inflammatory responses, stimulation of Caco-2 cells with commensal bacteria increased thymic stromal lymphopoietin (TSLP), IL-8, and TGF-β1 secretion, which resulted in the promotion of a tolerogenic dendritic cell phenotype by TSLP and TGF-β1 (101). In addition, probiotic bacterial strains have been shown to reduce gut epithelial production of IL-8 (102, 103).

Intestinal epithelial cytokine release prompted by viral infection can help clear infection or create pathology. Simian immunodeficiency virus infection of the intestinal epithelium of rhesus macaques induced IL-1β expression by Paneth cells before the induction of an antiviral IFN response. IL-1β expression was correlated with epithelial disruption characterized by the mislocalization and reduced expression of tight junction proteins, although these changes did not correspond to any aberrant responses to bacteria (104).

Multiple studies have documented the production of IFN-λ by virus-infected intestinal epithelial cells, although the ability of this cytokine to limit viral infection varied between studies (82, 84, 105). A possible explanation for these discrepancies may be found in the work of Hernández et al., which demonstrated that group 3 ILC-derived IL-22 amplified IFN-λ signaling in intestinal epithelial cells, and synergistic signaling by the two cytokines was necessary for a reduction in viral replication and optimal stimulation of IFN-induced gene expression (105).

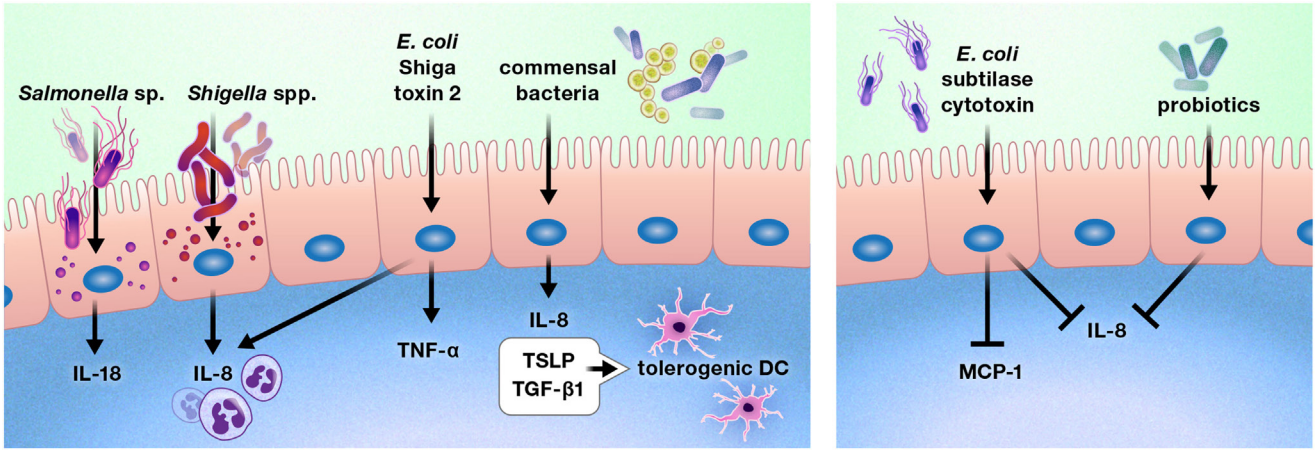

FIGURE 5 | Pathogens, commensal bacteria, and probiotics can increase or diminish the production of cytokines and chemokines by the intestinal epithelium. These interactions may promote or deter immune cell infiltration of the gut, such as by increasing or reducing the production of chemokines, including interleukin (IL)-8 and monocyte chemoattractant protein-1 (MCP-1). In some cases, bacterial interactions with the gut epithelium may instruct the intestinal immune system. For example, intestinal epithelial cells produce thymic stromal lymphopoietin (TSLP) and transforming growth factor-β (TGF-β) 1 in response to commensal bacteria, inducing a tolerogenic phenotype in dendritic cells.

Dietary Modulation of Intestinal Epithelial Mediator Release

Diet has been implicated as a possible contributing factor to IBD; however, research has failed to identify the "ideal" anti-inflammatory diet for IBD patients (106). Nevertheless, recent studies have identified anti-inflammatory effects of specific dietary components on the intestinal epithelium. Pretreatment of Caco-2 cells with the plant-derived flavonoid cyanidin-3-glucoside (C3G) reduced TNF-α-induced gene expression of IL-8 and TNF-α. C3G also inhibited endothelial cell activation and subsequent leukocyte adhesion stimulated by coculture with TNF-α-stimulated Caco-2 cells (107). Similarly, treatment of Caco-2 cells with the dietary fiber guar gum increased expression of the suppressor of cytokine signaling-1 (SOCS-1) and reduced TNF-α-induced IL-8 expression. In addition, guar gum administration to mice with chemically induced enteritis reduced disease activity and pro-inflammatory cytokine expression in the small intestine concurrent with an increase in SOCS-1 protein (108).

CONCLUDING REMARKS

Cytokines and chemokines are critical for intestinal epithelial homeostasis and responses to disease. The ability of cytokines to directly facilitate or restrict intestinal epithelial proliferation, apoptosis, and permeability makes them key players in the maintenance, or at times destruction, of the intestinal epithelial barrier. Furthermore, the release of cytokines and chemokines by the intestinal epithelium in response to pathogens, commensal organisms, interactions with other cell types, and dietary compounds allows these cells to have critical input into their microenvironment. Despite our frequent tendency to classify cytokines as either pro- or anti-inflammatory, we must realize that these labels fail to acknowledge the incredible diversity and situational basis of cytokine functions. While undoubtedly complex, the cytokine biology of intestinal mucosal immunology is a fascinating opportunity for investigations into both intestinal immunophysiology and potential translational approaches to modulate this physiology for much-needed novel therapies for intestinal disease.

AUTHOR CONTRIBUTIONS

CA, MM, and SD contributed to the development of the review topic. CA wrote the initial draft of the manuscript, and MM and SD critically reviewed and edited the manuscript for content.

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REVIEW
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Transforming Growth Factor-β1/Smad7 in Intestinal Immunity, Inflammation, and Cancer

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In physiological conditions, the activity of the intestinal immune system is tightly regulated to prevent tissue-damaging reactions directed against components of the luminal flora. Various factors contribute to maintain immune homeostasis and diminished production and/or function of such molecules trigger and/or propagate detrimental signals, which can eventually lead to chronic colitis and colon cancer. One such a molecule is transforming growth factor-β1 (TGF-β1), a cytokine produced by many inflammatory and non-inflammatory cells and targeting virtually all the intestinal mucosal cell types, with the down-stream effect of activating intracellular Smad2/3 proteins and suppressing immune reactions. In patients with inflammatory bowel diseases (IBD), there is defective TGF-β1/Smad signaling due to high Smad7, an inhibitor of TGF-β1 activity. Indeed, knockdown of Smad7 with a specific antisense oligonucleotide restores endogenous TGF-β1 activity, thereby inhibiting inflammatory pathways in patients with IBD and colitic mice. Consistently, mice over-expressing Smad7 in T cells develop severe intestinal inflammation in various experimental models. Smad7 expression is also upregulated in colon cancer cells, in which such a protein controls positively intracellular pathways that sustain neoplastic cell growth and survival. We here review the role of TGF-β1 and Smad7 in intestinal immunity, inflammation, and cancer.

Keywords: mucosal immunity, cytokines, inflammatory bowel diseases, colon cancer, antisense oligonucleotides

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INTRODUCTION

The gastrointestinal tract harbors a large number of commensal bacterial, viral, and fungal species, which trigger maturation of the mucosal immune system. There are over 300 Peyer's patches and more than 30,000 isolated lymphoid follicles in the small and large intestines, and the lamina propria compartment is infiltrated with many CD4+ and CD8+ T cells, B cells, plasma cells, dendritic cells (DCs), and macrophages; T cells and various subsets of innate lymphoid cells (ILCs) are also present in the gut epithelium (1, 2). This state of "physiological inflammation" contributes to provide resistance to invading pathogens, while preserving barrier integrity and allowing normal absorptive and digestive functions. The maintenance of gut integrity and intestinal homeostasis depends also on the barrier effect of the epithelium, which limits translocation of luminal antigens and promotes immune regulation (3). Elegant studies in animal models of inflammation and observations in patients with chronic colitis strongly support this notion. Indeed, defects in epithelial barrier and/or lack of expression/function of counter-regulatory molecules can break the immune tolerance toward the luminal flora thus resulting in colitis and colorectal cancer (CRC) (2, 4–9). Data emerging from such studies indicate clearly that transforming growth factor (TGF)-β1 is one of the key

molecules involved in the regulation of the epithelial cell biology and immunity in the gut (10, 11). TGF-β1 is a member of the TGF-β superfamily, which includes also TGF-β2, TGF-β3, bone morphogenetic proteins, and several growth and differentiation factors (12). In the gut, many immune and non-immune cells produce TGF-β1 and almost all the mucosal cells are targeted by this cytokine. TGF-β1 is secreted as part of a latent complex, which comprises latency-associated peptide (LAP) and latent TGF-β binding protein. In this form, TGF-β1 cannot bind to its receptor (13). TGF-β1 can be activated upon being released from the complex due to the proteolytic action of a number of proteinases or upon the interaction between the tripeptide integrin-binding motif on LAP and the correspondent binding sequence on αvβ3, αvβ5, αvβ6, or αvβ8 integrins expressed on the surface of epithelial cells, myofibroblasts, and DCs (14, 15). TGF-β1 signals through two transmembrane receptors with serine/threonine kinase activity, named TGF-β1 type 1 receptor (TβR1) and TGF-β1 type 2 receptor (TβR2) (13). Binding of TGF-β1 to TβR2 promotes auto-phosphorylation of the receptor and subsequent recruitment of TβR1 to form a transmembrane heterodimer. Then, the kinase activity of TβR2 determines phosphorylation of the regulatory glycine/serine-rich domain of TβR1 and, hence, the activated TβR1–TβR2 complex promotes phosphorylation of Smad2 and Smad3. Phosphorylated Smad2/3 proteins heterodimerize with Smad4 to generate a complex that moves to the nucleus, where it regulates the expression of target genes (13, 16) (**Figure 1**). TGF-β1-associated Smad signaling is a tightly controlled phenomenon, and another intracellular Smad protein, termed Smad7, acts as a negative regulator of such a pathway through various mechanisms. Smad7 can bind to TβR1 and compete with Smad2/3 for the catalytic site of phosphorylation, thus preventing the phosphorylation of Smad2/3 (**Figure 1**)

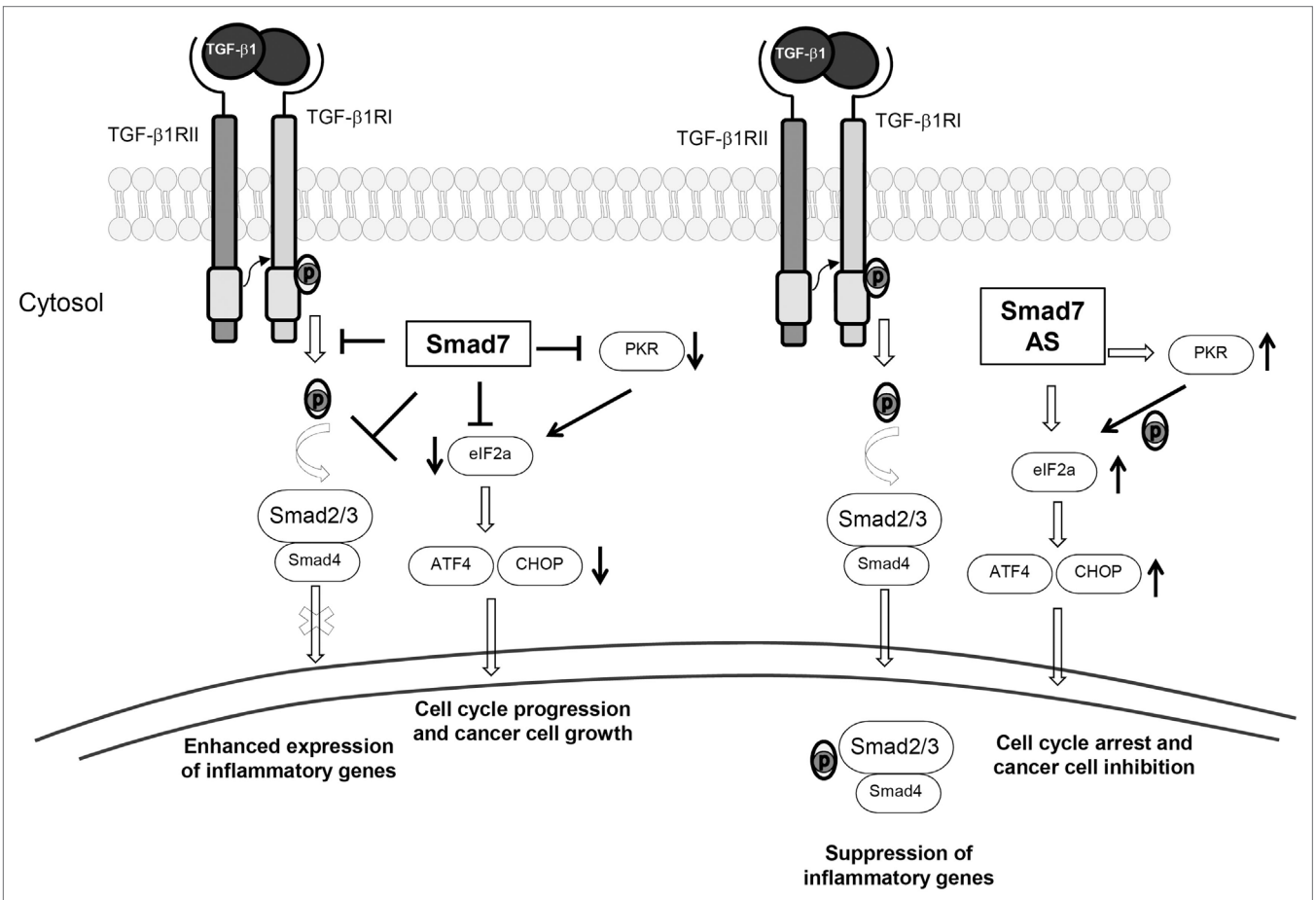

FIGURE 1 | Smad7-induced biological effects. The left side of the figure shows the inhibitory effect of Smad7 on transforming growth factor (TGF)-β1 intracellular signaling. Smad7 binds to TGF-β receptor type I and prevents TGF-β1-driven Smad2/3 phosphorylation (p), thus sustaining inflammatory gene expression. High Smad7 prevents also eukaryotic translation initiation factor-2α (eIF2α) phosphorylation, either directly or through the inhibition of protein kinase RNA (PKR), thereby leading to downregulation of transcription factor 4 (ATF4) and CCAAT/enhancer binding protein homology protein (CHOP) with the down-stream effect of stimulating cell cycle progression and cancer cell growth. The right side of the figure shows the effect of Smad7 inhibition with a specific antisense oligonucleotide (AS). Smad7 inhibition restores TGF-β1-induced Smad2/3 phosphorylation, subsequent interaction of these two proteins with Smad4 and translocation of Smad2/3/4 complex to the nucleus, thereby suppressing inflammatory gene expression. Moreover, Smad7 knockdown causes TGF-β1-independent eIF2α phosphorylation, upregulation of ATF4 and CHOP with the down-stream effect of cell cycle arrest.

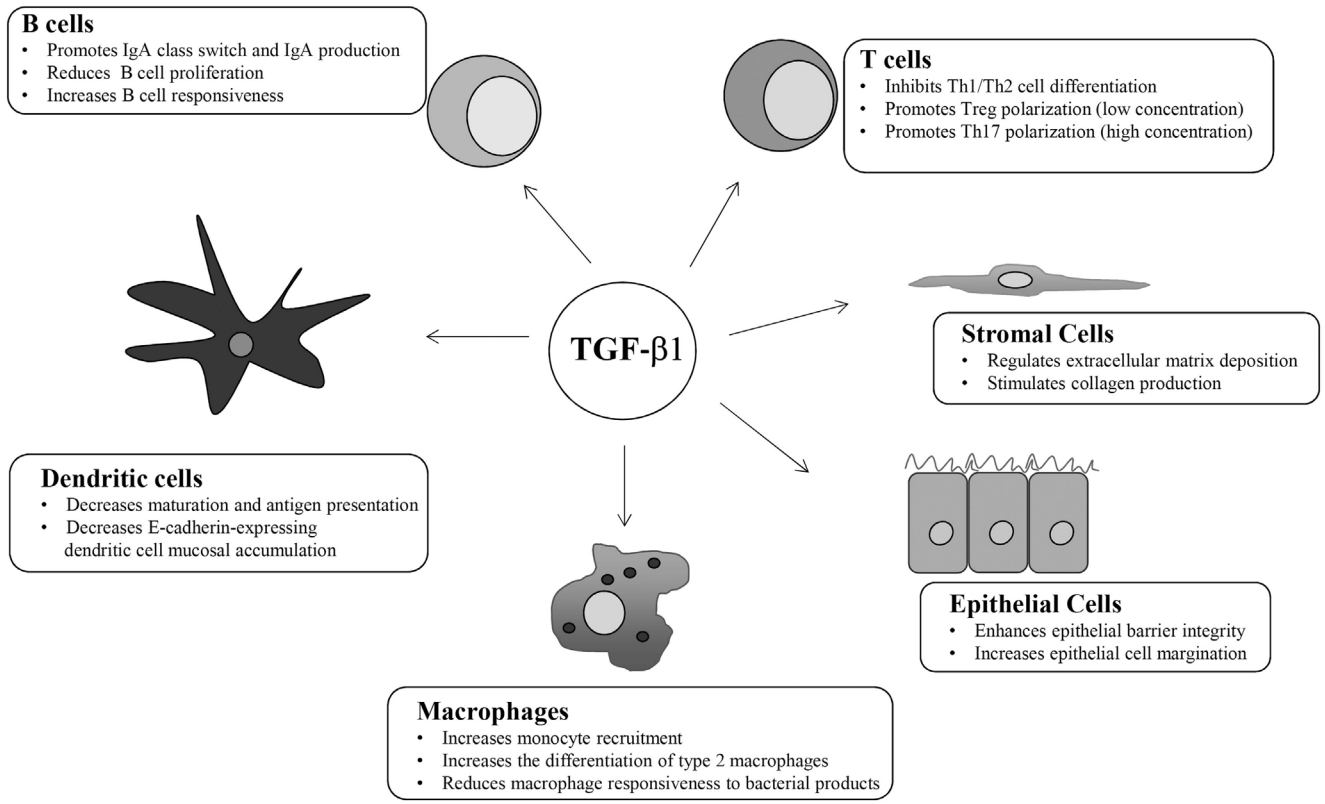

FIGURE 2 | Schematic view of the main cell targets and biological function of transforming growth factor (TGF)-β1 in the gut.

(17, 18). Smad7 can recruit phosphatases to TβR1 thereby promoting de-phosphorylation and inactivation of the site (19) and can promote ubiquitination-driven proteasome-mediated degradation of TβR1 in association with E3 ubiquitin ligases SMURF1/2 (20, 21). Moreover, Smad7 can localize into the nucleus and inhibit the association of Smad2-3/Smad4 complex with target genes (22). Besides its inhibitory effect on TGFβ1 signaling, Smad7 regulates the expression and function of several molecules involved in the control of inflammation and carcinogenesis in a TGF-β1-independent manner (**Figure 1**).

We here review the role of TGF-β1 and Smad7 in intestinal immunity, inflammation, and cancer.

TGF-β1 AND INTESTINAL HOMEOSTASIS

TGF-β1 regulates the function of many mucosal cell types in an autocrine and paracrine manner (**Figure 2**). For instance, TGF-β1 suppresses proliferation and activation of CD4+ T helper (Th) lymphocytes (23). Mice with T-cell targeted deletion of TβR2 or transgenic mice expressing a dominant-negative of TβR2 are unable to respond to TGF-β1 and show a phenotype characterized by systemic autoimmunity and severe colitis (11). In both strains, activated T cells accumulate in multiple organs, including the gut, highlighting the role of TGF-β1 in blocking T cell activation, and maintaining intestinal immune tolerance. CD4+ T lymphocytes have a high grade of plasticity and can differentiate in various subsets depending on the specific cytokine milieu at the induction and effector sites (24). TGF-β1 strongly inhibits Th1 and Th2 differentiation. TGF-β1-induced inhibition of Th1 cell differentiation is mediated by a direct downregulation of the transcription factor Tbet, a master regulator of Th1 cell polarization (25). Moreover, TGF-β1 downregulates the expression of IL-12Rβ2 and prevents the expansion of Th1 responses driven by IL-12, a key cytokine in the induction of Th1-type immunity in humans (26). TGF-β1 can also directly downregulate GATA3, a transcription factor involved in Th2 cell differentiation (27, 28). In contrast, TGF-β1 promotes directly and indirectly T cells polarization toward a regulatory (T regulatory cells, Tregs) phenotype (29). Tregs express the transcription factor Foxp3 and exert regulatory functions by acting mainly on effector T cells (30). Mice with loss of TGF-β1 signaling have reduced numbers of circulating CD4+Foxp3+ Tregs, raising the possibility that TGF-β1 contributes to intestinal immune homeostasis in part by inducing Tregs differentiation (31, 32). This hypothesis is supported by studies demonstrating that TGF-β1 promotes generation of naturally occurring Tregs, a subset of Tregs, which are generated in the thymus early after birth, as well as differentiation of peripherally induced Tregs from naïve T cells (33–35). Interestingly, the number of CD4+Foxp3+ Tregs remains unaltered in mice lacking TGF-β1 in CD4+ T-cells, while TGF-β1-null mice have reduced numbers of Tregs. These findings suggest that peripheral differentiation of Tregs depends

on TGF-β1 produced by other cell types rather than T cells. In the gut, CD103-expressing DCs are a major source of TGF-β1. These cells produce also elevated levels of retinoic acid (RA), which potentiates TGF-β1-induced expansion of Tregs because of a direct effect on Foxp3 promoter (36, 37). TGF-β1 and RA promote the *in vitro* differentiation of naïve T cells in another group of Foxp3-expressing Tregs, termed induced Tregs (38). TGF-β1 plays also a role in the interaction between intestinal immune system and gut microbiota. *Clostridium butyricum* promotes Tregs generation through the induction of TGF-β1 by colonic lamina propria DCs (39).

TGF-β1, along with IL-6, IL-21, and IL-1β, contributes also to the differentiation of Th17 cells, a CD4+ T cell subset characterized by the expression and activity of the master regulator retinoid acid-related orphan receptor-γt and producing various cytokines, including IL-17A (40). It has also been demonstrated that commitment of naïve CD4+ T cells along the Th17 or Tregs phenotype depends on TGF-β1 concentration, given that low concentration of the cytokine promotes Tregs differentiation through downregulation of IL-23 receptor, while high concentration of TGF-β1, in conjunction with IL-6 and IL-21, upregulates IL-23 receptor and promotes Th17 polarization (41).

TGF-β1 controls memory CD8+ T cells. Mice with deletion of TβR2 show a reduced number of antigen-specific memory CD8+ T cells in the gut, and this phenomenon seems to be, at least in part, secondary to a reduced expression of integrins (42).

TGF-β1 is also a relevant regulator of B cell and plasm-cell biology. TGF-β1 in B cells mediates IgA class-switch and promotes IgA production (43, 44). Mice lacking TGF-β signaling in B cells do not develop intestinal inflammation, but deletion of TβR2 in CD19-expressing B cells associates with B cell hyperplasia in Peyer's patches, modifications in B cell responsiveness and serum IgA deficiency (44, 45). Secretory IgAs control the bacterial composition, as they neutralize luminal bacteria by enhancing phagocytosis and improving the ability of DCs to present antigens. Moreover, IgAs block surface epitopes of luminal bacteria, thus inhibiting bacterial adhesion to the intestinal epithelium (46). In Peyer's patches, B cells and DCs interact through TGF-β1-activated integrin αvβ8, and this interaction promotes IgA production (47). TGF-β1 regulates IgA production through the canonical Smad-mediated pathway as the absence of Smad2 results in IgA deficiency, while overexpression of Smad3 and 4 determines an increased IgA production (48, 49).

Innate lymphoid cells are a family of hematopoietic cells involved in host defense, immune homeostasis, and tissue remodeling. These cells belong to the innate immune system and are abundantly present at mucosal sites where they act as a first line of defense against pathogens (50). Although there is no clear evidence that TGF-β1 regulates the function of ILCs in the gut, it is known that TGF-β1 guides the differentiation of type 1 ILCs in salivary glands through a Smad4-independent pathway (51).

TGF-β1 is involved in the control of intestinal DC function. Mice with deletion of TGF-β signaling in DCs due to a selective lack of TβR2 develop systemic autoimmunity and colitis, the later being characterized by loss of goblet cells and marked mucosal lymphocytic infiltration with altered Tregs differentiation, T cells and B cells with an activated phenotype, and increased expression of pro-inflammatory cytokines (52, 53). As mentioned above, DCs produce TGF-β1 and at the same time contribute to TGF-β1 activation. Mice lacking integrin β8 in DCs fail to activate TGF-β1 and spontaneously develop colitis (54). TGF-β1 negatively regulates the mucosal accumulation of other DC subtypes, such as those expressing the adhesion molecule, E-cadherin. Interestingly, adoptive transfer of E-cadherin-expressing bone marrow DCs into T-cell-restored Rag1-deficient mice enhances Th17 cell responses and exacerbates colitis (55).

TGF-β1 is an important regulator of macrophage function. TGF-β1 produced by intestinal epithelial cells can act as a chemokine and stimulate recruitment of blood monocytes to the intestinal mucosa (56). Moreover, TGF-β1 promotes differentiation of type 2 macrophages, a subset of anti-inflammatory cells, and reduces the macrophage responsiveness to bacterial products, thus promoting an anergic state that is crucial to maintain intestinal homeostasis (57). Mice with selective knockout of TβR2 in macrophages do not show spontaneous inflammation in the gut, but develop a more severe colitis after DSS administration, with reduced levels of IL-10, further confirming the ability of the cytokine to trigger anti-inflammatory signals in macrophages (58).

TGF-β1 targets also non-immune cells, including epithelial cells and stromal cells. Both these cell types produce elevated amounts of the cytokine. TGF-β1 promotes the expression of tight junction protein (i.e., Claudin-1) and adhesion molecules, thus enhancing epithelial barrier integrity, and is a powerful inducer of intestinal epithelial cell margination, a phenomenon that facilitates wound healing (**Figure 2**) (59). Mice with selective deletion of TGFβ1 signaling in the intestinal epithelium do not develop inflammation but are more susceptible to DSS-colitis (60). TGF-β1 stimulates stromal cells (i.e., myofibroblasts) to produce collagen and is a crucial regulator of extra-cellular matrix deposition (**Figure 2**), a phenomenon that is relevant for wound healing processes (61). A poorly controlled TGF-β1-induced extra-cellular matrix deposition has been involved in the development of intestinal strictures, such as those complicating Crohn's disease (CD) natural history (62, 63). In the gut, fibrogenesis is a complex and multifactorial process that involves several mediators, and TGF-β1 is supposed to be the most relevant pro-fibrogenic cytokine (64). TGF-β1 promotes fibronectin, type I collagen, and connective tissue growth factor production in fibroblasts isolated from CD strictures and enhances fibroblasts contractile activity (65).

TGF-β/Smad7 IN INTESTINAL INFLAMMATION

CD and ulcerative colitis (UC), are chronic, relapsing inflammatory disorders of the gastrointestinal tract and represent the two most relevant forms of IBD in humans (66). The etiology of IBD is unknown but accumulating evidence suggests that IBD are multifactorial diseases in which environmental and genetic factors trigger an abnormal immune response against component of intestinal microflora (67). In both disorders, defects of

counter-regulatory factors/mechanisms contribute to amplify mucosal inflammatory signals. One such defect involves the TGF-β1/Smad pathway. In the intestine of healthy individuals, TGF-β1 functions are properly suggested by the constitutive elevated levels of phosphorylated Smad3. Moreover, *in vitro* treatment of normal intestinal mucosal samples with an anti-TGF-β1 antibody increases expression of pro-inflammatory molecules such as T-bet and IFN-γ and stimulation of normal lamina propria mononuclear cells (LPMC) with exogenous TGF-β1 inhibits NF-kB activity and reduces IL-8 production (68–70). In contrast, stimulation of IBD LPMC with TGF-β1 neither inhibits NF-kB activation nor reduces the production of pro-inflammatory mediators, highlighting the possibility that IBD cells are resistant to TGF-β1-mediated immune suppression (70). This is consistent with the demonstration that in inflamed intestine of IBD patients, there is reduced Smad2/3 phosphorylation (69). This finding associates with enhanced expression of Smad7 (69). Interestingly, analysis of Smad7 content in mucosal samples of IBD patients revealed that Smad7 is upregulated at protein but not RNA level, suggesting a post-transcriptional regulation of Smad7 (71). Indeed, we have previously shown that, in both CD and UC mucosa, Smad7 protein stability is sustained by post-translational mechanisms, which enhance Smad7 acetylation thereby inhibiting ubiquitination-driven proteasomal-mediated degradation. Such modifications are partly due to p300, as silencing of this transcription coactivator, which is over-produced in CD mucosa, reduces Smad7 acetylation thereby stimulating ubiquitination-driven proteasomal-mediated degradation (71). Additional factors are supposed to stabilize Smad7 expression in IBD. In this context, our data indicate that, in IBD tissue, cells over-expressing Smad7 have reduced levels of SIRT1, a component of the mammalian Sirtuin family proteins that deacetylates the lysine residues of Smad7 with the down-stream effect of reducing Smad7 expression (72).

THE PATHOGENIC ROLE OF Smad7 IN THE GUT

Various approaches have been used to assess the role of Smad7 in IBD. Initially, IBD LPMC and mucosal explants were treated with a specific Smad7 antisense oligonucleotide (AS). Inhibition of Smad7 associated with enhanced Smad3 phosphorylation and reduced production of inflammatory cytokines (**Figure 1**) (69). Pre-incubation of IBD LPMC with a blocking TGF-β1 antibody abrogated the Smad7 AS-mediated effects, indicating that the anti-inflammatory function of Smad7 AS is mediated by TGF-β1. As pointed out above, TGF-β1 promotes differentiation of Foxp3-expressing Tregs and the activity of the cytokine is required for the function of such cells (73, 74). Mucosal IBD CD4+ T cells show resistance to Foxp3-expressing Tregs-mediated suppression and this has been associated with Smad7, as knockdown of Smad7 restores the responsiveness of effector CD4+ T cells to Foxp3-expressing Tregs (75). Like IBD patients, mice with trinitrobenzene sulfonic acid (TNBS-) and oxazolone-mediated colitis, two mouse models of colitis, which resemble CD and UC, respectively, express elevated levels of TGF-β1 in the inflamed colons (76, 77). In both these models, Smad7 expression is upregulated and p-Smad3 is reduced. Colitic mice given oral Smad7 AS exhibit enhanced p-Smad3 expression, reduced expression of inflammatory cytokines and a less severe colitis (78). We generated a T cell-specific Smad7 transgenic (Tg) mouse on C57B6 genetic background, which does not develop spontaneously colitis (75). However, using the T cell-transfer model of colitis, we showed that adoptive transfer of Smad7 Tg naïve CD4+ T cells into immunodeficient mice produced a more severe intestinal inflammation than that documented in mice reconstituted with wild-type cells, and colitis induced by Smad7 Tg cells was only partly inhibited by co-transfer of Tregs (75). Finally, we showed that Smad7 Tg mice develop a more severe colitis in comparison to wild-type mice after DSS administration (79).

T cells of Smad7 transgenic mice have reduced levels of aryl hydrocarbon receptor (AhR), a transcription factor that stimulates IL-22 production and promotes regulatory mechanisms in the gut (80). In the T-cell transfer colitis model, AhR activation significantly ameliorates the course of colitis driven by wild-type T cells, but does not influence colitis induced by Smad7 transgenic T cells. Consistently, in normal but not in IBD LPMC, TGF-β1 enhances AhR expression (81). Altogether, these data show an inverse correlation between AhR and Smad7 expression in the gut.

THERAPEUTIC BENEFIT OF Smad7 INHIBITION IN PATIENTS WITH INFLAMMATORY BOWEL DISEASES

The demonstration that Smad7 is upregulated in IBD mucosa and inhibition of this protein allows endogenous TGF-β1 to dampen the ongoing mucosal inflammation paved the way for the development of an oral Smad7 AS-containing pharmaceutical compound. This drug, initially named GED0301 and later on mongersen, was formulated in order to facilitate the deliver of the active molecule in the terminal ileum and right colon, which are the primarily affected sites in CD (82). A phase 1, open label, dose-escalating clinical trial conducted in 15 patients with active, steroid-dependent/resistant CD showed that 7-day treatment was safe and well-tolerated by the patients and associated with clinical benefit (82). Patients enrolled in the trial were monitored for the development of intestinal strictures. Six months after the end of the treatment, no patient developed strictures (83). To further assess the impact of Smad7 inhibition on the development of intestinal fibrosis, we used a mouse model of TNBS-mediated colitis-driven intestinal fibrosis. Interestingly, treatment of colitic mice with mongersen reduced the degree of intestinal inflammation and limited the development of intestinal fibrosis (84).

A subsequent phase 2, multicenter double-blind, placebo-controlled clinical trial was conducted in steroid-resistant/dependent CD patients with inflammatory lesions confined to the terminal ileum and/or right colon. The study confirmed the safety profile of mongersen and showed that 55 and 65% of the patients treated with the highest doses of the drug (i.e., 40 or 160 mg/day for 2 weeks) achieved clinical remission (primary end-point) as compared to 10% in the placebo group (85). Responders to

mongersen exhibited reduction in the serum levels of CCL20, a chemokine that contributes to recruit immune cells to the intestine ad is over-produced in the epithelium of CD patients (86). Next, an exploratory, phase 2, multicenter study confirmed the clinical efficacy of the drug and documented an endoscopic improvement in nearly one-third of the patients treated with mongersen for 4–12 weeks (87). Next, a phase III clinical trial was conducted in steroid-resistant/dependent CD patients with inflammatory lesions of the terminal ileum and/or colon and endoscopic evidence of active inflammation. The trial was discontinued in October 2017 as an interim analysis documented a lack of efficacy of the drug.

SMAD7 IN CRC

Colorectal cancer represents a leading cause of cancer-related morbidity and mortality, with 1.65 million new cases and almost 835,000 deaths estimated worldwide in 2015 (88). In 70% of cases, CRC arises as sporadic disease, with several environmental and genetic factors involved in the pathogenesis, most of which are still unknown (89). Instead, in 2% of cases, CRC arises in patients with long-standing UC or extensive CD (colitis-associated cancer, CAC), with a cumulative risk that has been related with disease duration, extension, and severity of inflammation (90, 91). TGF-β1 seems to play both pro-tumorigenic and anti-tumorigenic roles in CRC depending on the tumor stage and probably reflecting the complexity of TGF-β1 function and the large number of biological processes in which the cytokine is involved. While at early stages of tumorigenesis, TGF-β1 contributes to maintain cell differentiation and restricts epithelial cell growth, thus acting as a tumor suppressor, at later stages, it promotes epithelial–mesenchymal transition, neo-angiogenesis, cancer progression, and metastasis (12, 92). Similarly, a dual role of Smad7 has been described in various types of cancer with pro-tumorigenic or anti-tumorigenic effects according to the cancer site and biology (93). Single nucleotide polymorphisms of Smad7 gene associate with CRC (i.e., rs4939827, rs12953717) (93, 94). Moreover, in a study from Boulay and colleagues, Smad7 mutations were analyzed in 264 CRC biopsies, and amplification of Smad7 gene was associated with a poor prognosis, with a possible dose effect, while deletion of Smad7 gene associated with a better outcome (95). Our studies have recently evidenced a link between Smad7 expression in immune cells and CAC. In particular, a reduced number of Smad7-expressing CD4+ T lymphocytes were documented in the colonic mucosa of IBD patients who developed CAC compared to IBD patients with uncomplicated disease (79). In line with this observation, in an experimental model of CAC, Tg mice that overexpress Smad7 in T cells developed a more severe intestinal inflammation, characterized by an abundant infiltrate of cytotoxic CD8+ T cells and NKT cells, compared to control mice, and were largely protected from tumors. The anti-tumorigenic effect of Smad7 over-expression in T cells appeared related to the action of IFN-γ, as genetic deletion of such a cytokine abolished the protective effect of Smad7 on colon carcinogenesis (79). Consistent with these observations is the demonstration that Smad7 Tg mice develop less tumors than wild-type littermates following the subcutaneous injection of syngenic MC38 colon carcinoma cells (96). Altogether, these data suggest that high Smad7 in immune cells promotes the amplification of Th1-cytokine responses, which protect against colon carcinogenesis. However, in line with the above-referenced genetic studies, a different scenario emerges when the role of Smad7 is analyzed in sporadic CRC. Indeed, CRC cells produce huge amounts of Smad7 and knockdown of Smad7 with a specific AS inhibits the *in vitro* and *in vivo* growth of CRC cells (97). This effect of Smad7 AS relies on the modulation of cell cycle-related proteins and ultimately results in S phase arrest and cell death. Since these findings are seen in CRC cells unresponsive to TGFβ1 and the anti-proliferative effect of Smad7 AS is not affected by stimulation of CRC cells with TGF-β1 or anti-TGF-β1, it is highly likely that Smad7 exerts pro-tumorigenic effects in a TGFβ1-independent manner (**Figure 1**) (97). Analysis of the basic mechanisms underlying the mitogenic effect of Smad7 in CRC cells showed that Smad7 knockdown causes phosphorylation of eukaryotic translation initiation factor 2α (eIF2α), a transcription factor that regulates cell cycle arrest, and consequent upregulation of activating transcription factor 4 (ATF4) and CCAAT/enhancer binding protein homology protein (CHOP) (98). Silencing of the serine–threonine protein kinase RNA abrogates Smad7 AS induced eIF2α phosphorylation and ATF4/CHOP induction, thus preventing cell death (**Figure 1**) (98). In contrast, an anti-tumorigenic role for Smad7 has been recently reported by Wang and colleagues, who showed that nuclear reporter subfamily 2, group F, and member 2 (NR2F2), a molecule involved in many cancers, induces a TGFβ1-dependent epithelial–mesenchymal transition by inhibiting Smad7, thus promoting CRC metastasizing process (99).

Altogether, these data highlight the complex role of Smad7/TGFβ1 signaling in colon carcinogenesis.

CONCLUSION

The findings discussed in this review underline the crucial role of TGF-β1 in the maintenance of intestinal homeostasis and suggest that defective function of this cytokine can contribute to trigger and/or amplify detrimental signals in the gut. There is also discussion on the expression of Smad7 in patients with IBD and the crucial role played by this protein in inhibiting TGF-β1 function and sustaining IBD-related inflammation. Knockdown of Smad7 allows endogenous TGF-β to suppress effector responses. In line with this, CD patients treated with oral compound containing Smad7 AS showed clinical and endoscopic improvement during phase 1 and phase 2 clinical trials. However, a recent phase 3 clinical trial has been discontinued apparently due to the lack of efficacy of the drug, but the reasons for the discrepancy between phase 2 and phase 3 studies remain unknown. Similarly, some issues regarding the expression/function of Smad7 in IBD and CRC remain to be addressed. For instance, we still do not known whether, in IBD, Smad7 is regulated in a cell-specific manner and which factors/mechanisms contribute to maintain the elevated levels of Smad7. It would also be relevant to know whether molecular profiling of IBD patients can help to identify better candidates to treatment

with Smad7 inhibitors. Further experimentation is also needed to clarify whether and which regulatory effects of Smad7 on the ongoing mucosal inflammation and colon carcinogenesis are in part independent on TGF-β1, as it is known that Smad7 controls some biological functions in a TGF-β1-independent manner.

AUTHOR CONTRIBUTIONS

ET and IM wrote the manuscript. CS supervised parts of the project. GM designed the paper, supervised the project, and wrote the manuscript.

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Conflict of Interest Statement: GM has filed a patent related to the treatment of inflammatory bowel diseases with Smad7 antisense oligonucleotides, while the remaining authors have no conflict of interest.

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Effects of Anti-Integrin Treatment With Vedolizumab on Immune Pathways and Cytokines in Inflammatory Bowel Diseases

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Background and aims: Despite proven clinical efficacy of vedolizumab (VDZ) for inducing and maintaining remission in patients with Crohn's disease (CD) and ulcerative colitis (UC), subgroups of patients have no therapeutic benefit from anti-α4β7 integrin therapy with VDZ. Within this study, we aimed to identify genetic, cellular, and immunological mechanisms that define response and failure to VDZ treatment.

Methods: Intestinal RNA sequencing was performed in UC and CD patients before and at week 14 of VDZ therapy. α4β7 expression on peripheral and mucosal immune cells was assessed by flow cytometry and immunohistochemistry. Cellular modes of VDZ-mediated action were analyzed *ex vivo* and in VDZ-treated inflammatory bowel disease patients.

Results: Transcriptome analysis showed an impairment of signaling cascades associated with adhesion, diapedesis, and migration of granulocytes and agranulocytes upon VDZ therapy. In non-remitters to VDZ therapy, a tissue destructive and leukocyte-mediated inflammatory activity with activation of TNF-dependent pathways was present, all of which were inhibited in remitters to VDZ. Clinical remission was associated with a significant reduction of α4β7 expression on Th2 and Th17 polarized mucosal $CD4^+$ T cells at week 14 of VDZ therapy and with significantly higher numbers of α4β7-expressing mucosal cells prior to the initiation of VDZ therapy compared with non-remitters.

Conclusion: Intestinal α4β7 expression prior to VDZ therapy might represent a biomarker that predicts therapeutic response to subsequent VDZ treatment. Due to high activation of TNF signaling in VDZ non-remitters, anti-TNF treatment might represent a promising therapeutic strategy in VDZ refractory patients.

Keywords: inflammatory bowel diseases, ulcerative colitis, Crohn's disease, vedolizumab, RNA sequencing, integrin, cytokines

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INTRODUCTION

Rapid recruitment of leukocytes from the blood stream into the intestinal lamina propria is a key process for the homeostatic immune surveillance and the exaggerated mucosal immune response that is observed in inflammatory bowel diseases (IBD) such as Crohn's disease (CD) and ulcerative colitis (UC). The process of leukocyte migration into the mucosa is a multistep cascade involving

tethering, rolling, adhesion, and finally diapedesis of leukocytes, all of which is orchestrated by a complex network of cell-intrinsic and -extrinsic factors as well as regulatory molecules on T cells and endothelial cells, such as PSGL-1, ICAM-1, and LFA-1 and in addition by chemokines and their specific receptors (1, 2). Since marked lymphocyte accumulation within the lamina propria is one of the pathogenic and histologic hallmarks observed in IBD patients, targeting T cell homing to the gut has emerged as a novel and promising therapeutic option in IBD patients.

This is particularly relevant for the integrin α4β7 that is expressed on the surface of gut-tropic effector lymphocytes. Homing of α4β7-expressing T cells is mediated by the specific interaction between α4β7 and its ligand mucosal addressin cell adhesion molecule-1 (MAdCAM-1), which is expressed under steady-state conditions by gut endothelial cells (3). Experimental models of chronic intestinal inflammation using a murine antibody against the α4β7 integrin have shown that the inhibition of the interaction between MAdCAM-1 and α4β7 can successfully inhibit the entry of $CD4^+$ T cells to the intestinal mucosa system and efficiently suppress intestinal inflammation (4, 5). Based on this experimental evidence, vedolizumab (VDZ) was developed as a humanized antibody that binds to a conformational epitope that is unique to the heterodimerization of the human α4 with the β7 chain (6, 7), thereby conferring specificity toward the α4β7 integrin. Subsequently, VDZ was shown to suppress migration and accumulation of α4β7-bearing effector T cells from UC patients to the inflamed colon *in vivo* suggesting that this antibody may suppress lymphocyte trafficking in IBD (8, 9). Clinical studies revealed that VDZ has statistically significant therapeutic efficacy in placebo controlled phase 3 clinical trials of patients with moderately-to-severe active UC (10) or CD leading to the approval of VDZ in the US and Europe for the treatment of both IBD entities (11, 12).

Nevertheless, only a distinct proportion of 40–60% of IBD patients will achieve clinical response under VDZ therapy (11, 12), and only 15% of CD patients will achieve clinical remission at week 10 of treatment (12, 13). Furthermore, in light of the increasing diversification of biological therapy modalities (14, 15) and to allow a targeted, timely, effective, and economic treatment, factors that define or predict therapeutic response are needed in daily practice. Based on these considerations, we aimed to define genetic and immunological differences between responders and non-responders to VDZ therapy and to identify factors that predict therapeutic response prior to the initiation of anti-adhesion molecule therapy with VDZ.

MATERIALS AND METHODS

Clinical Disease Activity and Definition of Response and Remission

During VDZ induction therapy, patients were seen at week 0, 2, 6, 10, and 14 in the IBD outpatient department. At each visit, clinical scoring was assessed using the Harvey–Bradshaw index (HBI) for CD (16) and the Mayo Clinic Score for UC (17, 18). At week 14, therapeutic response toward VDZ was assessed. Clinical remission was defined as a HBI < 5 for CD (19) and as a Mayo Clinic score of 2 or lower and no subscore higher than 1 for UC (11). Clinical response was defined as a decrease from baseline in Mayo score by $\geq$30% and $\geq$3 points, with a decrease in rectal bleeding subscore ≥ 1 or rectal bleeding subscore of 0 or 1 (UC) or a decrease in HBI > 4 points from baseline (CD).

IBD Patients and Flow Cytometric Analysis of Human Blood and Lamina Propria Mononuclear Cells

To determine the α4β7 expression on subsets of T cells in humans, peripheral blood and colonic samples from IBD patients were analyzed. Human intestinal lamina propria cells and peripheral blood mononuclear cells (PBMCs) from IBD and control patients were isolated as previously described (20, 21). After gating on $CD3^+CD4^+$ T cells, the following signature cytokines and transcription factors were used with the following antibodies to analyze differentially polarized T cells: Th1: IFNγ (anti-human IFNγ APC, eBiosciences, Cat. 17-7319-82); Tbet (anti-human/mouse T-bet PerCPCy5.5, eBiosciences, Cat. 45-5825-82), Th2: IL-4 (anti-human IL-4 APC, eBiosciences, Cat. 17-7049-82), IL-13 (anti-human IL-13 PerCP/Cy5.5, BioLegend, Cat. 501911), GATA3 (anti-human/mouse Gata-3 PE, eBiosciences, Cat. 12-9966-42); Th17: IL-17 (anti-human IL-17A PerCP-Cy5.5, Cat. 45-7179-42).

α4β7 was visualized using commercially available VDZ (Takeda, Tokyo, Japan), which was stably labeled with fluorescein isothiocyanate (FITC). For analysis of α4β7 expression on peripheral blood lymphocytes under VDZ therapy, 12 patients with CD and 10 patients with UC were included. From all patients, blood samples were obtained before the initiation of anti-integrin therapy with VDZ and directly before the second, third, and fourth administration of 300 mg VDZ by intravenous infusion at weeks 0, 2, 6, and 14 respectively (11). For analysis of α4β7 expression on lamina propria mononuclear cells, a total of 11 patients with UC and 12 patients with CD were included. Of the 11 patients with UC, 5 had a clinical remission while 6 UC patients were refractory to VDZ therapy. Of the twelve patients with CD, six patients were remitters while six CD patients did not have remission to VDZ therapy and α4β7 expression was comparatively assessed between remitters and non-remitters.

The study was carried out in accordance with the recommendations of the ethical committee of the University Hospital, Friedrich-Alexander-Universität Erlangen-Nürnberg, Germany. The protocol was approved by the ethical committee of the University Hospital, Friedrich-Alexander-Universität Erlangen-Nürnberg, Germany. Each patient gave written informed consent in accordance with the Declaration of Helsinki before inclusion into the study.

Expression of Th1, Th2, and Th17 Polarizing Cytokines From PBMCs and Regulation of Cell Death in the Presence of VDZ

Peripheral blood mononuclear cells from IBD patients were isolated and cultured in RPMI medium 1640 (Gibco) containing

10% FCS (Pan Biotech), and 1% penicillin/streptomycin. Cells were stimulated with anti-human CD3 (eBioscience, clone OKT3) and anti-human CD28 (BD Pharmingen, clone CD28.2) at a final concentration of 1 µg/mL in the presence of 40 µg/mL VDZ or IgG1 isotype control. After 24 and 48 h, supernatant was collected, and quantification of cytokine production was performed by ELISA for IFN-γ (human IFNγ ELISA Ready-SET-Go, eBiosciences, Cat. 88-7316), IL-4 (Human IL-4 ELISA Ready-SET-Go, eBiosciences, Cat. 88-7046), IL-17a (human IL-17A ELISA Ready-SET-Go, eBiosciences, Cat. 88-7176), and IL-10 (BD OptEIA Human IL-10 ELISA Kit II, BD Pharmingen, Cat. 550613). In separate experiments, PBMCs from IBD patients (UC: $n = 7$; CD: $n = 7$) were cultured for 24 and 48 h and induction of cell death in the presence of 40 µg/mL VDZ or IgG1 isotype control was assessed using the cell death detection plus Kit (Roche Diagnostics, Mannheim, Germany).

Effects of VDZ and Isotype Control on the Adhesion of PBMCs to MAdCAM-1 Expressing HeLa Cells

Expression of MAdCAM-1 on HeLa cells was verified by immunohistochemistry using rabbit anti-human MAdCAM-1 (anti-MAdCAM, Abcam, Cat. ab178549) or an isotpye control antibody at 1 µg/mL as primary antibodies and anti-rabbit Alexa-647 as a secondary antibody. For adhesion assays, MAdCAM-1 expressing HeLa cells were grown to confluence for 48 to 72 h in µ-Slides (µ-Slide 8 Well, Ibidi, Cat. 80826) Subsequently, 5×10^5 PBMCs labeled with a vital dye from healthy controls ($n = 4$) were preincubated with 40 µg/mL VDZ or IgG1 isotype control for 60 min and then added to HeLa cells. After 60 min, non-attaching PBMCs were washed of by three vigorous washes with TBS. Cells were fixed with 4% paraformaldehyde and HeLa cells were stained for MAdCAM-1 as described above followed by nuclear counterstaining with DAPI before final analyses by fluorescence microscopy (Keyence, Osaka, Japan).

In separate experiments, cryopreserved PBMCs from VDZ naïve IBD patients (UC: $n = 3$; CD: $n = 3$) and matched PBMC controls from the same patients after VDZ induction therapy were thawn, stained with vital dye, and added to confluently grown HeLa cells for 60 min. Afterward, non-attaching cells were similarly washed off and were fixed with 4% paraformaldehyde. HeLa cells were stained for MAdCAM-1 as described above followed by nuclear counterstaining with DAPI before final analyses by fluorescence microscopy (Keyence Corp., BZ-X710).

Internalization of α4β7 Over Time in the Presence of VDZ

1×10^6 PBMCs from each of seven IBD patients were isolated and cultured for the indicated time points in the presence of 40 µg/mL VDZ or IgG1 isotype control. Afterward, cells were stained for surface expression of $CD3^+$, $CD4^+$, and $CD8^+$ for 15 min at room temperature. Subsequently, half of the cells were permeabilized before α4β7 staining with FITC-labeled VDZ, while in the other α4β7 staining with FITC-VDZ was performed prior to permeabilization of the cells. Mean fluorescence intensity (MFI) of the FITC signal in the respective CD3, CD4, and CD8 gates were analyzed by flow cytometry.

In another set of experiments, PBMCs or peripheral $CD4^+$ T cells isolated from IBD patients using magnetic sorting (CD4 Microbeads, MACS, Miltenyi, Cat. No 130-045-101) were cultured in the presence of 40 µg/mL FITC-labeled VDZ or FITC-labeled IgG1 isotype control for 24 h. Cells were then fixed in 4% paraformaldehyde and after nuclear counterstaining with DAPI, internalization of FITC-VDZ or FITC-IgG1 was assessed by confocal microscopy (Leica TCS SP8, Leica, Germany).

Immunohistochemistry of Mucosal α4β7 Expression in IBD Patients

Cryosections from colonic biopsies of IBD patients were used for immunohistochemistry. Tissue sections were fixed in 4% paraformaldehyde, followed by sequential incubation with avidin/biotin- (Vector Laboratories), and protein-blocking reagent (Roth) to suppress unspecific background staining. Sections were incubated with primary antibodies specific for human CD3 (rat anti-human CD3, Bio-Rad, Cat. MCA1477), human CD4 (rat anti-human CD4, Bio-Rad, Cat. MCA484G), and FITC-labeled vedolizumab (Entyvio®, Takeda). Furthermore, sections were incubated with an isotype matched control antibody as negative controls. Subsequently, samples were incubated with Alexa 647 conjugated secondary antibodies. Nuclei were counterstained with DAPI before final analysis by confocal microscopy (Leica SP8 Microscope). Positive cells in 6–10 high power fields (HPFs) were subsequently counted in all patients. In some images, an inset of a higher magnification was included to better identify the stained nuclei.

RNA Sequencing Analysis

Total RNA was extracted from biopsy specimens using the RNeasy Mini Kit according to the manufacturer's instructions. Quality of RNA was analyzed using a 2100 Bioanalyzer system (Agilent Technologies). RNA sequencing was performed in the Next-Generation-Sequencing Core Unit of the University of Erlangen-Nürnberg. Library preparation, sequencing on a HighSeq-2500 platform (Illumina, San Diego, CA, USA), as well as mapping and counting of reads were performed as previously described (22).

Sequencing data analyses were performed using R version 3.2.1 (R Foundation for Statistical Computing, Vienna, Austria) and Bioconductor (23), separately for CD and UC samples. In particular, differential expression analysis of genes with non-zero median count was performed with the DESeq2 package v.1.8.1 (24). First, expression before vs. after therapy was compared, controlling for patient effects. Afterward, the response to therapy was included in the design to analyze expression in responders and non-responders. Genes with p-value <0.01 were considered to be differentially expressed. Heatmaps of differentially expressed genes were obtained using for each gene regularized log-transformed data standardized across samples. Functional annotation analysis was performed using Ingenuity Pathway Analysis (IPA, QIAGEN Redwood City). A network-level visualization of the relationships between enriched pathways identified by IPA was obtained relying on Cytoscape (25). Similarity between a pair of pathways was taken equal to the Jaccard similarity coefficient calculated

between the associated gene sets. Only pathways with p-value <0.025 and similarity >0.3 with at least one other pathway were visualized.

Statistical Analysis

Statistics were processed using SPSS (SPSS Inc., Chicago, IL, USA) and GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Values presented in this manuscript represent mean ± SEM. For statistical analyses, after testing for normal distribution, significant differences between samples were calculated using non-parametric tests (Mann–Whitney U test). A two-tailed $p \leq 0.05$ was considered statistical significant: $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$.

RESULTS

RNA Sequencing of Responders and Non-Responders Before and After VDZ Induction Therapy

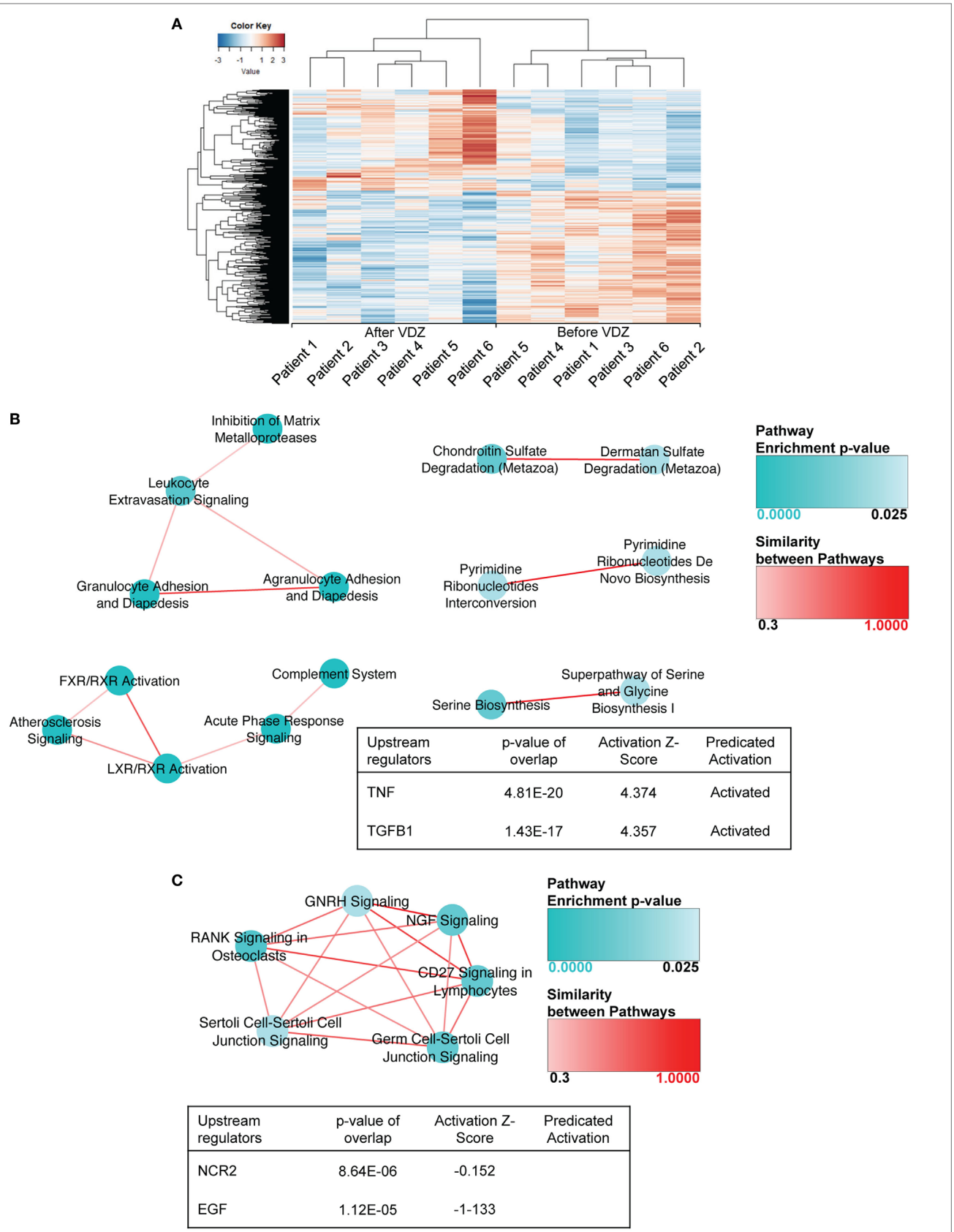
To gain large-scale insights into underlying differences between remitters and non-remitters to anti-integrin therapy with VDZ, we initially performed whole transcriptome expression analysis using an RNA sequencing approach. For this purpose, we analyzed mucosal gene expression in 10 IBD patients both before and at week 14 of VDZ therapy. Sequencing yielded a median of 47 M reads per sample, with 58% median counted reads. The analyzed cohorts were comprised of three CD and two UC patients with remission upon VDZ therapy (remitters), as well as three CD and two UC patients in which VDZ failed to induce clinical remission (non-remitters). For all patients, biopsies obtained before commencement of VDZ and at week 14 of VDZ therapy were analyzed, thereby allowing control for patient-specific variability.

Given the high differences expected between CD and UC, biopsy samples for the two diseases were analyzed separately. In a first set of analyses, differences in the composition of the transcriptome before and after VDZ induction therapy in CD and UC patients were analyzed. To identify which genes were differentially expressed, we relied on the DESeq2 package within the R/Bioconductor environment. This analysis identified a total of 883 differentially expressed genes in CD (p-value <0.01), with 382 genes showing an increased expression and 501 genes showing a decreased expression at week 14 of VDZ therapy when compared with before initiation of VDZ therapy (Table S1 in Supplementary Material). As shown in **Figure 1A**, unsupervised hierarchical clustering of the expression profiles of differentially expressed genes showed clear discrimination between CD patients before and after completion of VDZ induction therapy. Interestingly, when analyzing underlying signaling pathways in the differentially expressed genes in CD with IPA program, both granulocyte adhesion and diapedesis as well as agranulocyte adhesion and diapedesis were among the top three canonical pathways identified (Table S2 in Supplementary Material), thereby not only corroborating the proclaimed mode of action of VDZ at the transcriptional level but also extending these mechanisms to agranulocytic cells.

To better understand differences in the composition of the transcriptome between remitters and non-remitters to VDZ therapy on RNA level, we comparatively assessed canonical pathways as well as upstream regulators that were differentially regulated between CD patients with remission and those without remission upon VDZ therapy, with disease remission defined *via* clinical disease activity scoring (HBI < 5) (19). Baseline disease and clinical characteristics before and after VDZ induction therapy are comparatively shown for CD patients with remission to VDZ induction therapy and those exhibiting no remission toward VDZ in **Table 1**. No significant confounder regarding therapeutic response could be found in this comparative listing.

When analyzing the transcriptional expression profile in three CD patients with no remission upon VDZ therapy, we found a total of 1,014 genes to be differentially regulated ($p < 0.01$) in biopsies obtained at week 14 of VDZ therapy compared with matched biopsies form the same colonic segment obtained prior to VDZ induction (Table S3 in Supplementary Material). Importantly, among those genes significantly upregulated by at least 1.5-fold, many contained pro-inflammatory properties such as members of the TNF signaling cascade (*TNI3P*, *TNFAIP6*, *TNFRSF10C*, *TNFSF8*, *TNFSF15*, and *TNFSF13B)* and various pro-inflammatory cyto- and chemokines or its corresponding receptors (*IL31RA*, *IL23A*, *IL7R*, *IRAK2*, *IL33*, and *CXCL6*). Of note, many of the chemo- and cytokines found to be upregulated in CD patients with no remission upon VDZ have been previously shown to be involved in the generation of pathogenic Th2 and Th17 responses such as IL-33 and IL-31 (26–29) and furthermore, the IL-33/ST2 axis (30–35), IL-31 (36), as well as IL-23 (37–39) all of which have been previously implicated in the pathogenesis of T cell-mediated intestinal inflammation. Furthermore, several members of the metalloproteinase family were upregulated (*MME*, *MMP3*, *MMP10*, *ADAM12*, *MMP2*, *MMP7*, and *MMP12*), indicative of the presence of a tissue destructive milieu in CD patients with no remission to VDZ. When analyzing underlying signaling pathways of the differentially expressed genes in non-remitters to therapy, granulocyte adhesion and diapedesis were the most significantly regulated canonical pathway and TNF as well as TGFβ1 were the most significantly regulated upstream regulators with a predicted activation of both cytokines (**Figure 1B**). Altogether, these analyses were suggestive of a persistent level of leukocyte-mediated inflammatory activity that is driven by TNF as a major pro-inflammatory cytokine and TGFβ1, the latter of which has been associated with stricturing CD (40).

When analyzing the transcriptome in CD patients with remission to VDZ therapy, 263 genes were differentially expressed ($p < 0.01$) and the pro-inflammatory signature was considerably less present: among those genes significantly upregulated by at least 1.5-fold, only three contained immunogenic and pro-inflammatory properties (*CXCL13*, *TGFβ2*, and *TNFSF11*) and no tissue destructive signature was observed in CD patients with remission to VDZ therapy (Table S4 in Supplementary Material). On IPA, RANK signaling and CD27 signaling in lymphocytes were among the most significantly regulated pathways and NCR2 and EGF were the most significantly regulated upstream regulators (**Figure 1C**). Hence, while TNF-driven inflammatory



Upstream regulators	p-value of overlap	Activation Z-Score	Predicated Activation
TNF	4.81E-20	4.374	Activated
TGFB1	1.43E-17	4.357	Activated

Upstream regulators	p-value of overlap	Activation Z-Score	Predicated Activation
NCR2	8.64E-06	-0.152	
EGF	1.12E-05	-1-133	

FIGURE 1 | Continued

FIGURE 1 | RNA sequencing in patients with Crohn's disease under treatment with anti-α4β7 therapy with vedolizumab (VDZ). **(A)** Unsupervised hierarchical clustering of the expression profiles of differentially expressed genes showed two distinct clusters of expression, discriminating Crohn's disease (CD) patients before and after VDZ therapy. **(B)** Canonical pathways and upstream regulators associated with differentially expressed genes in CD patients with no remission to VDZ therapy. In non-remitters, among the 1,014 genes that were differentially regulated in biopsies obtained after completed VDZ therapy compared with matched biopsies obtained prior to VDZ induction, many contained pro-inflammatory properties such as members of the TNF signaling cascade, various pro-inflammatory cyto- and chemokines or its receptors, and several members of the metalloproteinase family. In CD patients with no remission to VDZ therapy, granulocyte adhesion and diapedesis were the most significantly regulated canonical pathway and TNF as well as TGFβ1 were the most significantly regulated upstream regulators and both were predicted to be activated. **(C)** Canonical pathways and upstream regulators associated with differentially expressed genes in CD patients with remission to VDZ therapy. In VDZ remitters, RANK signaling and CD27 signaling in lymphocytes were among the most significantly regulated pathways and NCR2 and EGF were the most significantly regulated upstream regulators. Compared with non-remitters the pro-inflammatory signature was considerably less present, and among all genes significantly regulated by at least 1.5-fold, only three contained immunogenic and pro-inflammatory properties. Turquoise dots represent pathway enrichment *p*-values; similarity of the pathways is represented by red lines.

TABLE 1 | Clinical data of Crohn's disease (CD) patients included for RNA sequencing.

	CD	
	Remitters (*n* = 3)	**Non-remitters (*n* = 3)**
Baseline characteristics		
Sex (m/f)	0/3	2/1
Age (years)		
Mean ± SD	38.6 ± 19.5	44.9 ± 5.2
Disease manifestation		
Ileum		
Ileum + colon	1	2
Ileum + colon + upper GI tract	2	1
Disease duration		
Mean ± SD (years)	6.7 ± 1.6	7.3 ± 4.5
Prior anti-TNF treatment	3	3
Concomittant medication		
Glucocorticoids only	1	1
Immunosuppressants only[a]		1
No glucocorticoids and immunosuppressants	2	1

	Before vedolizumab (VDZ)	**VDZ week 14**	**Before VDZ**	**VDZ week 14**
Disease activity, CD				
HBI, mean ± SD[b]	14 ± 1.2	2.3 ± 1	14.3 ± 1.5	12 ± 6
SES-CD, mean ± SD[c]	15.6 ± 3.2	7.6 ± 2.1	5.6 ± 4.9	6.3 ± 5.5
Leukocyte count ± SD ($\times 10^9$/L)	9.5 ± 2.4	10.6 ± 6.4	7.3 ± 1	7.2 ± 1.1
CrP ± SD (mg/L, reference < 5)	8.3 ± 10.9	10.3 ± 11	6.2 ± 9.8	4.5 ± 4.9

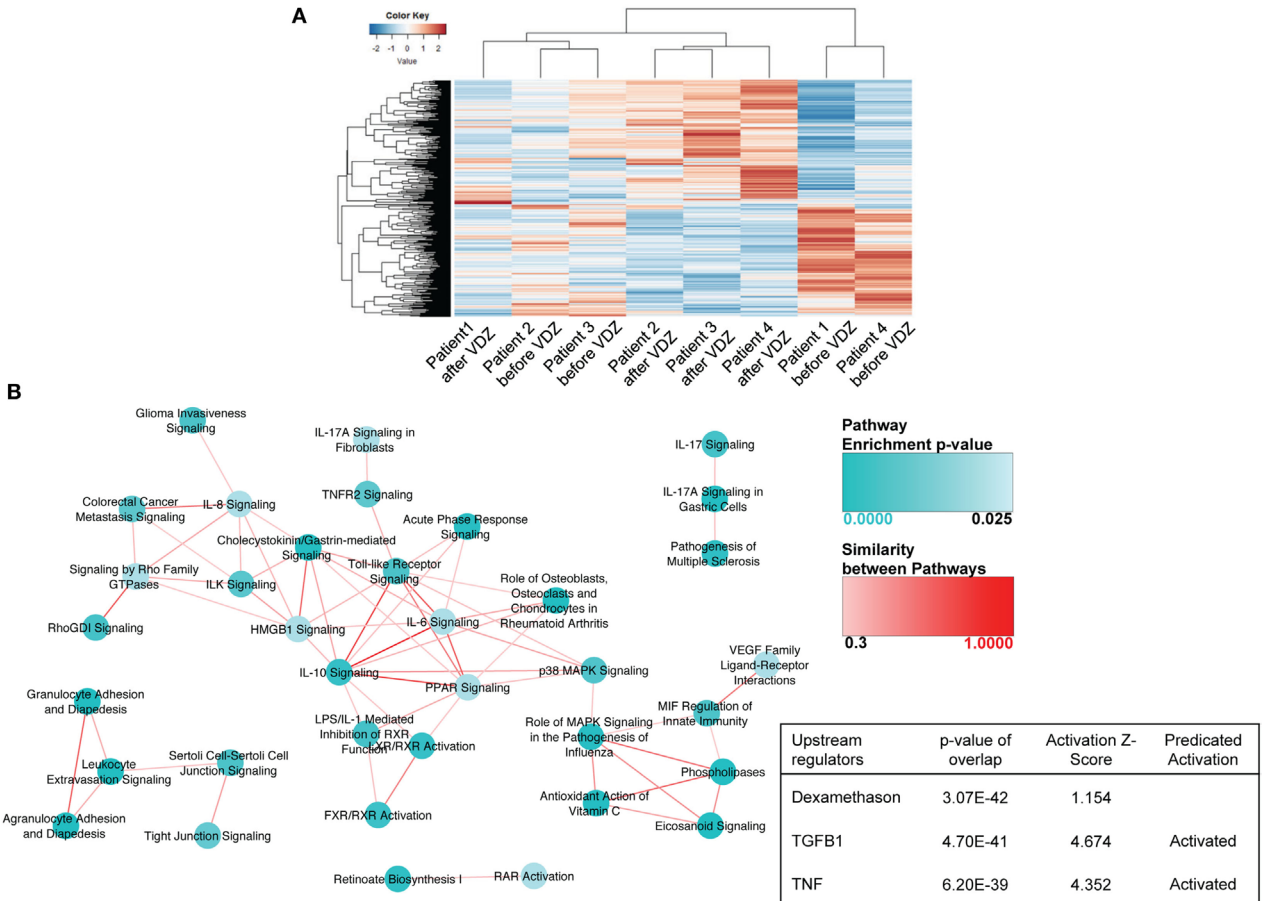
[a]*Immunosuppressants included azathioprine, methotrexate, and mercaptopurine.*
[b]*The Harvey–Bradshaw index (HBI) ranges from 0 to 26, with higher scores indicating more active disease. Disease remission was defined as a HBI < 5.*
[c]*The Simple Endoscopic Score for Crohn's Disease (SES-CD) assesses ulcer size, ulcerated surface, surface affected from disease and the presence of stenosis and ranges from 0 to 56, with higher scores indicating more severe disease.*

activity was observed in non-remitters to VDZ, remitters to therapy showed no clear pro-immunogenic milieu consistent with the idea that non-remitters are characterized by TNF-mediated mucosal inflammation. Our findings thus suggest that these patients may benefit from therapeutic strategies that target alternative pathways to leukocyte migration, especially anti-TNF targeted treatment.

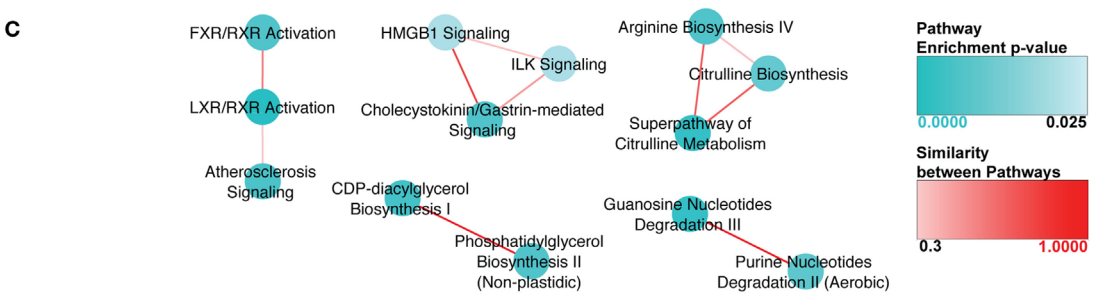
When comparing expression profiles in UC patients before and after VDZ therapy, a total of 565 genes were differently expressed, with 297 genes showing an increased expression and 268 genes showing a decreased expression upon VZD therapy (Table S5 in Supplementary Material). Hierarchical clustering revealed more variability across patients than observed for CD (**Figure 2A**). As already observed for CD, IPA of the differentially regulated genes in UC upon VDZ therapy revealed agranulocyte adhesion and diapedesis to be significantly regulated (Table S6 in Supplementary Material).

When performing similar comparative analyses according to therapeutic response in UC patients with disease remission defined *via* clinical disease activity scoring (Mayo Clinic score of 2 or lower and no subscore higher than 1) (11), we found a total of 788 genes to be differentially regulated in mucosal biopsies from non-remitters obtained at week 14 of VDZ therapy compared with matched samples obtained prior to VDZ induction in the same patients. Baseline disease and clinical characteristics before and at week 14 of VDZ therapy are comparatively shown for UC patients with remission to VDZ induction therapy and those exhibiting no remission toward VDZ in **Table 2**. No significant confounder regarding therapeutic response could be found in this comparative listing.

Importantly, as observed for CD, among those genes significantly upregulated by at least 1.5-fold in UC patients with no remission to VDZ therapy, many were involved in the recruitment of T cells and the T cell polarization such as the CXCR3 binding cytokines CXCL9, CXCL10, and CXCL11 (41–46), the Th2-dependent cytokine CCL26 (47), or the receptor of the Th2-dependent cytokine IL-13RA2 (48, 49) and the p40 containing cytokine IL23A, which has previously been shown to be essential for T cell-mediated colitis (39) (Table S7 in Supplementary Material). Furthermore, several members of the matrix metalloproteinase (MMP) family were upregulated such as MMP-1, MMP-3, MMP7, ADAM12, ADAMTS12, and ADAMTS2 consistent with the known role of MMPs for tissue degradation, the persistence of the inflammatory state, and fibrosis associated with IBD (50). When analyzing underlying signaling pathways in the differentially expressed genes in UC patients with no remission to therapy, both granulocyte adhesion and diapedesis and agranulocyte adhesion and diapedesis were among the three most significantly regulated pathways, and both IL1β and TNF were among the most significantly modulated upstream regulators, with a predicted activation of both upstream regulators on IPA (**Figure 2B**).



Upstream regulators	p-value of overlap	Activation Z-Score	Predicated Activation
Dexamethason	3.07E-42	1.154	
TGFB1	4.70E-41	4.674	Activated
TNF	6.20E-39	4.352	Activated
IL-1ß	2.39E-35	4.198	Activated



Upstream regulators	p-value of overlap	Activation Z-Score	Predicated Activation
TNF	8.10E-25	-2.653	Inhibited
IFNG	7.75E-17	-2.279	Inhibited
Lipopoly-saccharide	1.05E-15	-1.164	

FIGURE 2 | RNA sequencing in patients with ulcerative colitis (UC) under treatment with anti- α4β7 therapy with vedolizumab (VDZ). **(A)** Unsupervised hierarchical clustering of the expression profiles of differentially expressed genes upon VDZ therapy. **(B)** Canonical pathways and upstream regulators in UC patients with no remission to VDZ therapy. Ingenuity Pathway Analysis (IPA) of the differentially regulate genes in UC before and after VDZ therapy revealed granulocyte and agranulocyte adhesion and diapedesis to be among the three most significantly regulated pathways and TGFβ1 and TNF were among the three most significantly regulated upstream regulators with a predicted level of activation. **(C)** Canonical pathways and upstream regulators in UC patients with remission to VDZ therapy. In VDZ remitters, LXR/RXR activation and guanosine nucleotides degradation III were the most significantly regulated pathways on IPA and TNF and IFNγ as pro-inflammatory cytokines and upstream regulators were predicted to be inhibited in UC patients with remission to VDZ therapy. Turquoise dots represent pathway enrichment *p*-values; similarity of the pathways is represented by red lines.

TABLE 2 | Clinical data of ulcerative colitis (UC) patients included for RNA sequencing.

	UC			
	Remitters (*n* = 2)		**Non-remitters (*n* = 2)**	
Baseline characteristics				
Sex (m/f)	2/0		2/0	
Age (years)				
Mean ± SD	27.9 ± 7.2		38.5 ± 22	
Disease manifestation				
Rectum and sigmoid colon only				
Left-sided Pancolitis	2		2	
Disease duration				
Mean ± SD (years)	4 ± 0		5.5 ± 2.1	
Prior anti-TNF treatment	2		2	
Concomittant medication				
Glucocorticoids only			2	
Immunosuppressants only[a]				
No glucocorticoids and immunosuppressants	2			
	Before vedolizumab (VDZ)	**VDZ week 14**	**Before VDZ**	**VDZ week 14**
Disease activity[b]				
Total Mayo Score, mean ± SD	7.5 ± 3.5	3 ± 1.4	6 ± 1.4	6.5 ± 2.1
Mayo Endoscopic Score, mean ± SD	2 ± 1.4	1 ± 1.4	5.6 ± 4.9	6.3 ± 5.5
Leukocyte count ± SD ($\times 10^9$/L)	9.75 ± 2.1	10.3 ± 0.2	10.3 ± 1.9	10.8 ± 2.2
CrP ± SD (mg/L, reference < 5)	3.2 ± 3.2	8.2 ± 3.8	1.9 ± 0.4	3.9 ± 5.3

[a]*Immunosuppressants included azathioprine, methotrexate, and mercaptopurine.*
[b]*The partial Mayo Score ranges from 0 to 9, with higher scores indicating more active disease. The total Mayo Score consists of partial Mayo Score + Mayo Endoscopic Score and ranges from 0 to 12. Disease remission was defined as a Mayo Clinic score of 2 or lower and no subscore higher than 1.*

By contrast, as already observed for CD, the pro-inflammatory signature was considerably less present in UC patients with remission to VDZ therapy. In VDZ remitters, a total of 504 genes were differentially regulated before and at week 14 of VDZ therapy, and the only cyto- or chemokines with broad immunogenic properties that were upregulated by at least 1.5-fold were TNFSF9, IL-11, CXCL6, and IL-34 (Table S8 in Supplementary Material). Conversely, when performing IPA in these patients, the two most significantly modulated upstream regulators were TNF and IFNγ with a predicted level of inhibition instead of activation as observed in UC patients who showed no remission to VDZ treatment (**Figure 2C**).

Taken together, these data substantiate the concept that distinct gene signatures characterize remitters and non-remitters to VDZ treatment. In fact, IBD patients with no remission to VDZ therapy are characterized by a persistent activation of TNF-dependent signaling pathways and genes that mediate mucosal inflammation and tissue destruction while induction of remission with VDZ was associated with a decreased inflammatory signature and downregulated TNF signaling.

α4β7 Expression on Lymphocyte Subsets in Patients With IBD

Based on the above findings, we hypothesized that systemic and mucosal leukocytes within the lamina propria of the gut might also show certain immunologic properties that define or indicate therapeutic response to VDZ therapy. In a first set of experiments, we thus quantified α4β7 expression on different subsets of T cells in IBD patients before as well as after induction therapy with VDZ. For this purpose, we labeled commercially available VDZ and a human IgG1 isotype with FITC. Staining with FITC-VDZ allowed a clear discrimination of α4β7 expression on peripheral blood $CD3^+$, $CD4^+$, and $CD8^+$ positive T cells, on B cells ($CD19^+$), macrophages ($CD14^+$), and on natural killer (NK) cells ($CD56^+$) (Figure S1A in Supplementary Material) as well as on Th1, Th2, and Th17 polarized $CD4^+$ T cells, as defined by the expression of IFNγ or T-bet and IL-4 or IL-13 or GATA3 and IL-17a, respectively (**Figure 3A**). Prior to VDZ therapy, highest expression of α4β7 was observed in B cells (34%), followed by $CD8^+$ T cells (27%), $CD3^+$ T cells (18%), and $CD4^+$ T cells (20%) (Figure S1B in Supplementary Material) from IBD patients. Importantly, two applications of 300 mg VDZ led to a significant decline of α4β7 expression on peripheral $CD3^+$, $CD4^+$, and $CD8^+$ T cells as well as in B cells and NK cells (Figure S1C in Supplementary Material). Consistent with the robust decline of α4β7 expression on overall peripheral $CD4^+$ T cells, anti-α4β7 therapy with VDZ led to a progressive decline of α4β7 expression in Th1, Th2, and Th17 polarized $CD4^+$ T cells from patients with CD (**Figure 3B**) and in Th17 polarized $CD4^+$ T cells from UC patients under anti-α4β7 therapy with VDZ (**Figure 3C**).

We then turned our attention to α4β7 expression on gut immune cells of IBD patients. Of note, when quantifying α4β7 expression in LPMCs from seven IBD patients prior to initiation of VDZ therapy, we observed similar expression levels compared with those seen in peripheral blood lymphocytes with highest expression in mucosal $CD19^+$ B cells (32%) and $CD3^+$ (20%), $CD4^+$ (11%), and $CD8^+$ (25%) T cells (Figure S1D in Supplementary Material). To address the potential relevance of altered intestinal α4β7 expression for driving and perpetuating mucosal inflammation in IBD, we comparatively analyzed differences of α4β7 expression in lamina propria T cell subsets in remitters and non-remitters to VDZ treatment (11 UC, 5 remitters, 6 non-remitters; 12 CD, 6 remitters, 6 non-remitters), with disease remission defined *via* clinical disease activity scoring [UC: Mayo Clinic score of 2 or lower and no subscore higher than 1 (11); CD: HBI < 5 (19)]. Importantly, in UC patients with clinical remission upon anti-integrin therapy with VDZ, we observed a significantly decreased α4β7 expression in Th1 polarized lamina propria $CD4^+$ T cells, as defined by expression of IFNγ or the transcription factor T-bet, in Th2 polarized lamina propria $CD4^+$ T cells expressing either IL-4 or the transcription factor GATA3, as well as in Th17 cells expressing IL-17a (**Figure 3D**). In CD patients, a significant reduction of α4β7 expression on Th2 and Th17 lamina propria $CD4^+$ T cells was likewise observed in VDZ

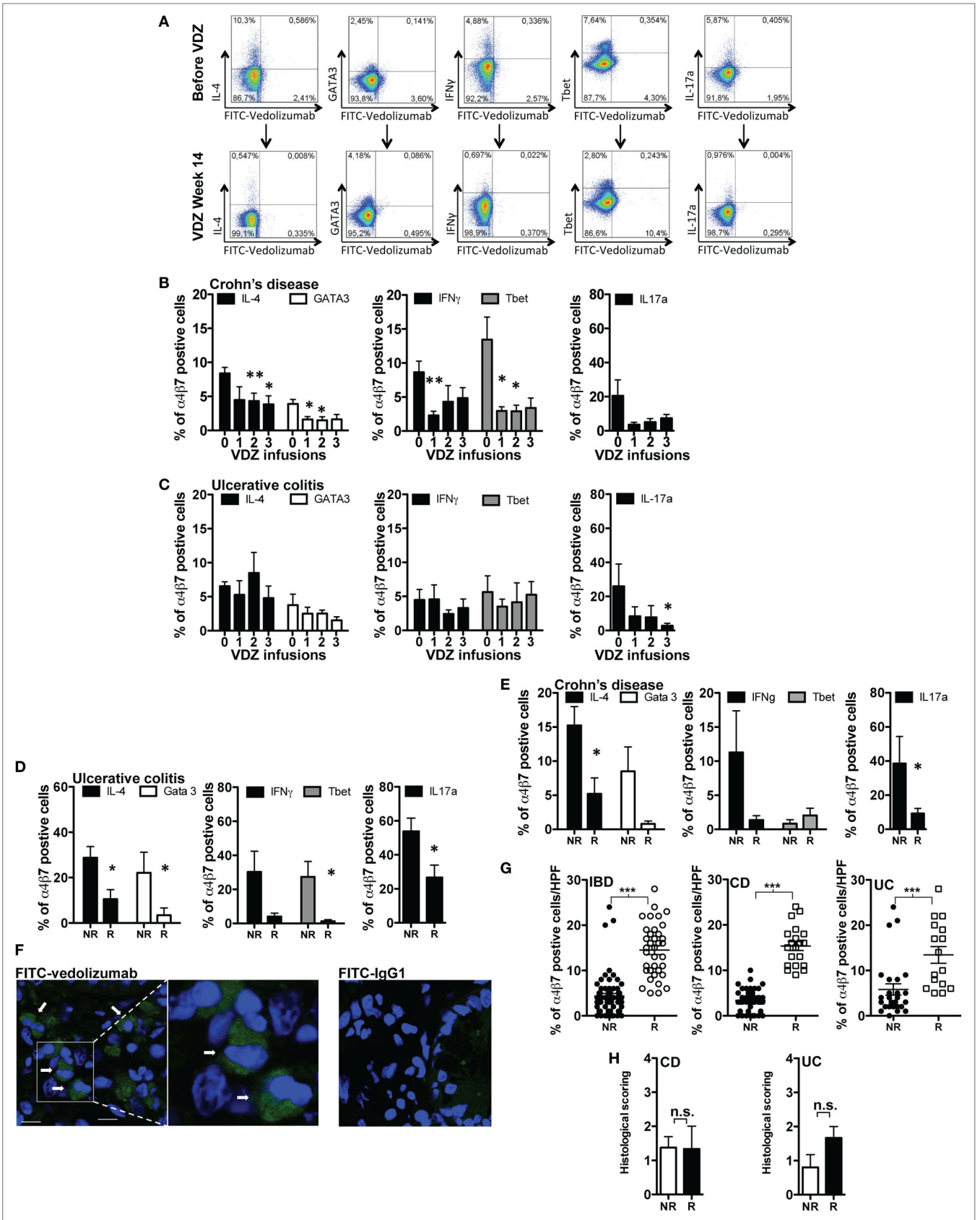


FIGURE 3 | Continued

FIGURE 3 | α4β7 expression on lymphocyte subsets in inflammatory bowel disease (IBD) patients. **(A)** Representative flow cytometric stainings of α4β7 expression on Th1, Th2, and Th17 polarized CD4+ T cells (previously gated on CD3 and CD4) before and after induction therapy with vedolizumab (VDZ) (week 14). Gray line: isotype control. **(B,C)** Quantification of α4β7 expression in different subsets of peripheral T cells (previously gated on CD3, CD4, and T-bet, IFNγ, IL-4, GATA-3, or IL-17, respectively) from patients with Crohn's disease (CD) [**(B)**, $n = 12$] and ulcerative colitis (UC) [**(C)**, $n = 10$] before initiation of anti-integrin therapy with VDZ (indicated by "0") (week 0) and directly before the second (week 2), third (week 6), and fourth (week 14) administration of 300 mg VDZ (indicated by "1," "2," and "3," respectively). **(D,E)** Quantitative comparison of α4β7 expression in different subsets of lamina propria T cells in patients with UC [**(D)**, $n = 11$] and CD [**(E)**, $n = 12$]. α4β7 expression was compared between remitters and non-remitters for each disease after completed induction therapy with VDZ (UC: $n = 5$ remitters, $n = 6$ non-remitters; CD: $n = 6$ remitters, $n = 6$ non-remitters). **(F)** Cryosections of colonic biopsies from IBD patients were stained with fluorescein isothiocyanate (FITC)-VDZ or FITC-IgG1. After counterstaining with DAPI (Thermo Fischer Scientific, Cat. D1306), cryosections were analyzed with confocal microscopy. The arrows indicate α4β7-expressing mucosal cells. **(G)** The amount of α4β7-expressing cells within the lamina propria was assessed in 15 IBD patients (8 CD and 7 UC) patients prior to the commencement of VDZ therapy. For each patient a mean of 5 high power field (HPF) were analyzed by fluorescence microscopy and the amount of α4β7-expressing cells prior to VDZ therapy was quantified. UC and CD patients with clinical remission toward subsequent VDZ exhibited significantly higher levels of α4β7-expressing cells within the lamina propria compared with non-remitters. **(H)** Histopathological scoring from the same remitters and non-remitters [as shown in **(G)**] prior to VDZ therapy. Results are presented as mean ± SEM. Differences between samples are compared with the Mann–Whitney *U* test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

remitters compared with CD non-remitters, whereas the reduction of α4β7 expression on Th1 polarized CD4+ T cells did not reach statistical significance (**Figure 3E**).

To further substantiate these findings, we hypothesized that the amount of α4β7-expressing mucosal cells prior to the initiation of VDZ therapy might be directly related to the outcome and therapeutic efficacy of subsequent anti-α4β7 VDZ therapy. To address this question, we first verified that FITC-VDZ allowed immunohistochemical identification of α4β7-expressing cells in the lamina propria in intestinal cryosections of IBD patients compared with staining with a FITC-IgG1 isotpye control (**Figure 3F**). To analyze whether the initial amount of α4β7-expressing cells in the lamina propria of IBD patients correlated to the outcome of anti-α4β7 therapy with VDZ, we assessed α4β7 expression in gut immune cells in cryosections from 15 IBD patients (8 CD and 7 UC) prior to the commencement of VDZ therapy. Of these 15 IBD patients, 7 patients (3 UC and 4 CD) exhibited remission at week 14 of VDZ therapy as defined by clinical scoring. Importantly, these remitters were characterized by significantly higher levels of α4β7 expression on mucosal immune cells prior to VDZ therapy initiation compared with non-remitters (**Figure 3G**). As shown in **Figure 3G**, in the cohort of IBD patients, remitters were characterized by a mean number of 14.5 α4β7+ cells per HPF in the lamina propria prior to the initiation of VDZ therapy compared with 4.4 α4β7-expressing cells per HPF in patients which showed no remission to subsequent VDZ therapy ($p < 0.0001$). Similar observations were made when UC and CD patients were analyzed separately. CD patients with remission upon VDZ were characterized by a mean number of 15.4 α4β7+ cells/HPF prior to commencement of VDZ therapy compared with only 3.4 α4β7-expressing cells/HPF in non-remitters ($p < 0.0001$). Similarly, UC patients with remission to subsequent VDZ therapy exhibited significantly higher amounts of α4β7+ cells in the lamina propria before VDZ therapy compared with non-remitters (13.4 vs. 5.8, $p = 0.0003$). Importantly, for both CD and UC, there were no differences in histopathologic scoring prior to VDZ therapy between remitters and those without remission toward subsequent anti-integrin therapy with VDZ (**Figure 3H**). This observation excludes differences in histological baseline disease activity in remitters and non-remitters before initiation of VDZ therapy as a potential confounder of therapeutic efficacy and thus confirms that the amount of α4β7+ cells within the lamina propria might represent a determinant for subsequent outcome of anti-integrin with VDZ. Furthermore, UC and CD patients with remission toward VDZ did not exhibit differences in leukocyte counts, serum CrP levels, clinical disease activity, concomitant use of glucocorticosteroids and immunosuppressant, or prior exposure to anti-TNF therapy compared with non-remitters (**Table 3**), thereby corroborating that remitters and non-remitters to VDZ exhibited similar clinical parameters of disease activity and severity as well. Individual phenotypical and clinical characteristics of every patient included in each experiment are shown in Table S9 in Supplementary Material.

Taken together, our findings suggest that the amount of mucosal α4β7-expressing cells prior to the initiation of VDZ therapy might well function as an *a priori* indicator or predictive biomarker for therapeutic efficacy of subsequent VDZ therapy. Hence, the quantification of α4β7 expression within the lamina propria might allow for stratification of IBD patients according to their individual expression of the therapeutic target and the associated likelihood to achieve subsequent therapeutic remission to the VDZ treatment directed against it.

Cellular Modes of Action of VDZ

Having shown that the adhesion and diapedesis of granulocytes and agranulocytes is among the most significantly regulated pathways under VDZ therapy and that α4β7 expression shows a progressive decline on effector T cells upon VDZ therapy, we hypothesized that surface α4β7 is internalized under VDZ therapy, leading to an impaired adhesion of lymphocytes. At the same time, we aimed to rule out other major cellular and immunological modes of action of VDZ. Since the induction of cytokine release by therapeutic antibodies is a known side effect that can have deleterious clinical consequences (51), we first analyzed whether VDZ alters or induces the expression of various cytokines. For this purpose, peripheral blood leukocytes from IBD patients were incubated with VDZ or IgG1 Isotype control for 24 and 48 h. Based on pharmacokinetic data that IBD patients responding to VDZ therapy exhibit therapeutic serum trough levels from 11 to 38 μg/mL (11, 12), we chose a concentration of 40 μg/mL to study *in vitro* effects of VDZ. Incubation of peripheral leukocytes with VDZ did not elicit release of the Th1 cytokine IFNγ, the Th2 cytokine IL-4, the Th17 cytokine IL17a, or IL-10 in cultivated leukocytes from patients with CD (**Figure 4A**)

TABLE 3 | Clinical data of inflammatory bowel disease patients with α4β7 quantification prior to anti-integrin therapy with vedolizumab.

	Ulcerative colitis (UC)		Crohn's disease (CD)	
	Remitter (*n* = 3)	**Non-Remitter (*n* = 4)**	**Remitter (*n* = 4)**	**Non-Remitter (*n* = 4)**
Baseline characteristics				
Sex (m/f)	2/1	3/1	1/3	1/3
Age (years)				
Mean ± SD	46.6 ± 12.9	35.6 ± 13.6	38.9 ± 15.8	27.9 ± 7.4
Leukocyte count ± SD ($\times 10^9$/L)	10 ± 2.1	9.8 ± 1.6	9 ± 4.9	11.1 ± 6.2
CrP ± SD (mg/L, reference < 5)	10 ± 2.1	9.8 ± 1.6	9.3 ± 7.6	18.1 ± 29
Disease characteristics				
Extend, UC				
Rectum and sigmoid colon only	1	1		
Left-sided				
Pancolitis		1		
Extend, CD	2	2		
Ileum				
Ileum + colon			2	3
Ileum + colon + upper GI tract			2	1
Disease duration				
Mean ± SD (years)	8.3 ± 3.2	6.75 ± 5.3	9 ± 4.9	11.3 ± 8
Disease activity, UC[a]				
Partial Mayo Score, mean ± SD	5.6 ± 2.1	6 ± 2.2		
Mayo Endoscopic Score, mean ± SD	2.3 ± 1.2	2 ± 0.8		
Disease activity, CD				
HBI, mean ± SD[b]			13 ± 2.6	13.2 ± 7.8
SES-CD, mean ± SD[c]			13.6 ± 6.7	16.3 ± 2.5
Prior anti-TNF treatment	3	3	4	4
Concomittant medication				
Glucocorticoids only	1	1	1	4
Immunosuppressants only[d]			1	
No glucocorticoids and immunosuppressants	2	3	2	
Prednisone-equivalent dose (mg)				
Mean ± SD	7.5 ± 0	20 ± 0	20 ± 0	22.5 ± 13.4

[a]*The partial Mayo Score ranges from 0 to 9, with higher scores indicating more active disease. The total Mayo Score consists of partial Mayo Score + Mayo Endoscopic Score and ranges from 0 to 12. Disease remission was defined as a Mayo Clinic score of 2 or lower and no subscore higher than 1.*

[b]*The Harvey–Bradshaw index (HBI) ranges from 0 to 26, with higher scores indicating more active disease. Disease remission was defined as a HBI < 5.*

[c]*The Simple Endoscopic Score for Crohn's Disease (SES-CD) assesses ulcer size, ulcerated surface, surface affected from disease and the presence of stenosis and ranges from 0 to 56, with higher scores indicating more severe disease.*

[d]*Immunosuppressants included azathioprine, methotrexate, and mercaptopurine.*

or UC (**Figure 4B**) compared with isotype control treated cells. To rule out induction of programmed cell death by VDZ, we then followed a similar approach and measured the release of cytoplasmic histone-associated DNA fragments as an established marker of induced cell death. Importantly, there was no difference in the quantity of histone-associated DNA fragments in leukocytes that have been incubated with VDZ, isotype control, or media only (**Figure 4C**).

To gain first insights into the potential internalization of α4β7 after binding to VDZ, we co-incubated peripheral blood leukocytes with fluorescent labeled VDZ and visualized cells with confocal microscopy. As shown in **Figure 4D**, after 24 h of incubation at 37°C, we observed a punctate perinuclear staining pattern indicative of a successfully cellular internalized α4β7 receptor complex. Furthermore, a clear intracellular FITC signal was present in sorted peripheral $CD4^+$ T cells from IBD patients after 24 h of incubation with FITC-VDZ (**Figure 4D**). Based on these observations, we then quantified internalization of α4β7 after binding to VDZ in peripheral leukocytes from seven IBD patients over a specified time period in flow cytometric analyses (**Figure 4E**). After incubation of peripheral blood leukocytes for different periods of time with VDZ, half of the cells from each patient were first permeabilized to make the intracellular compartment accessible for staining, followed by staining with fluorescent labeled VDZ (upstream permeabilization) while the other half of the cells were first stained with FITC-VDZ and then permeabilized (downstream permeabilization). As shown in **Figure 4E**, there was no difference in the FITC MFI between upstream and downstream permeabilized cells at baseline. However, over time we observed a progressive increase in the FITC MFI in $CD3^+$, $CD4^+$, and $CD8^+$ T cells, in which the intracellular compartment has been made accessible prior to staining with FITC-VDZ and upstream permeabilized cells exhibited a significantly higher FITC MFI after 12 and 24 h compared with downstream permeabilized cells. These results therefore indicate that after addition of VDZ, surface α4β7 is internalized over time with a peak of the receptor internalization after 12 and 24 h.

To demonstrate that α4β7 internalization by VDZ directly impairs the interaction with MAdCAM-1, we established *in vitro* adhesion assays using MAdCAM-1 expressing HeLa cells. First, strong MAdCAM-1 expression in HeLa cells was verified by immunohistochemistry (**Figure 4F**). HeLa cells were then grown to confluence and after 2 days, peripheral leukocytes from healthy donors were labeled with a vital dye and preincubated with VDZ or IgG1 for 60 min. Subsequently, leukocytes were co-incubated with HeLa cells, and after washing away non-attaching cells, fixed cells were analyzed with fluorescence microscopy (**Figure 4G**). Importantly, leukocytes preincubated with VDZ were significantly impaired to adhere to MAdCAM-1 expressing HeLa cells when compared with IgG1 treated cells (**Figure 4H**).

In a similar set of experiments, adherence of peripheral leukocytes from IBD patients before and after completion of VDZ induction therapy was assessed. For this purpose, peripheral leukocytes from CD ($n = 3$) and UC ($n = 3$) patients were obtained and for each patient, leukocytes before and after completion of VDZ induction therapy were available, thereby directly allowing assessment of VDZ treatment on the adherence of leukocytes for each individual patient. As shown in **Figure 4I**, after washing away non-attaching cells, leukocytes derived from CD and UC patients after completed VDZ induction therapy were

significantly impaired to adhere to MAdCAM-1 expressing HeLa cells when compared with matched leukocytes derived from the same patients prior to the initiation of VDZ therapy.

In their totality, these data provide evidence that the main mode of action by which VDZ acts on the cellular levels is indeed the internalization of surface α4β7, leading to the biological effect of impaired interaction with MAdCAM-1.

DISCUSSION

Vedolizumab is the first anti-integrin antibody therapy that has been approved for the treatment of UC and CD patients. Despite clinically proven therapeutic efficacy in up to 50% of treated IBD patients showing clinical response upon VDZ induction therapy and a favorable safety profile, data from large clinical studies show

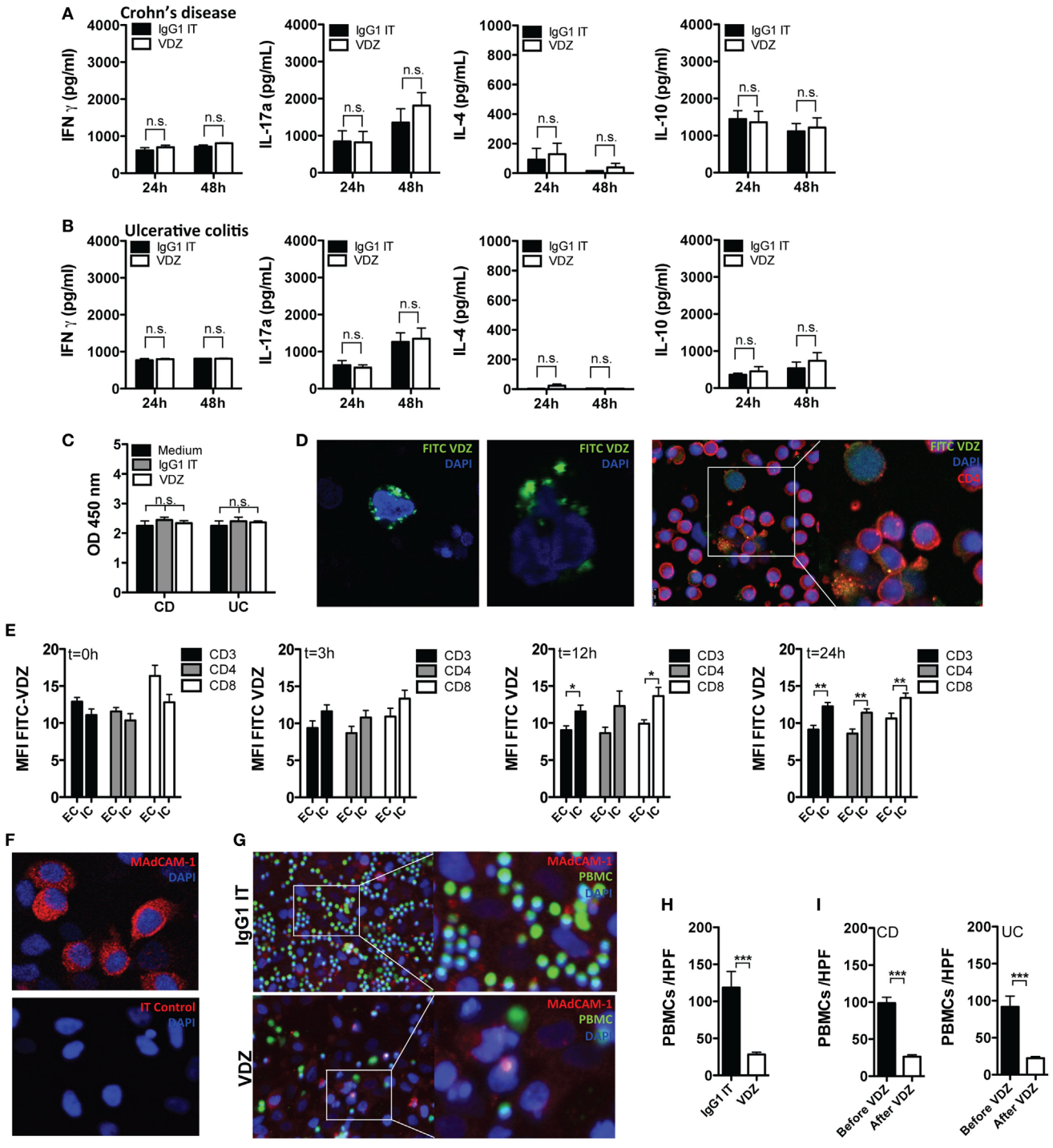


FIGURE 4 | Continued

FIGURE 4 | Cellular mechanism of action of vedolizumab (VDZ). **(A,B)** Peripheral blood leukocytes from patients with Crohn's disease (CD) [**(A)**, $n = 7$] and ulcerative colitis (UC) [**(B)**, $n = 7$] were incubated with VDZ or isotype (IT) control for 24 and 48 h. Afterward, concentration of IFNγ, IL-4, IL17a, and IL-10 in the supernatant was quantified by ELISA. **(C)** Peripheral blood leukocytes from patients with CD ($n = 7$) and UC ($n = 7$) were incubated with VDZ, IT, or media only for 24 h. Afterward, release of cytoplasmic histone-associated DNA fragments as a marker of induced cell death was quantified by ELISA. **(D)** Confocal microscopy of peripheral blood leukocytes (left panels) and sorted $CD4^+$ T cells (right panels) incubated for 24 h with fluorescein isothiocyanate (FITC)-labeled VDZ. **(E)** Internalization of α4β7 after binding to VDZ over time. Peripheral leukocytes from seven inflammatory bowel disease patients were incubated for the indicated time points with VDZ. From each patient, 5×10^5 peripheral leukocytes were first permeabilized to make the intracellular compartment accessible for staining followed by staining with fluorescent labeled VDZ. Another 5×10^5 cells were first stained with FITC-VDZ and were then permeabilized. Afterward, α4β7 expression was quantified in $CD3^+$, $CD4^+$, and $CD8^+$ T cells. **(F)** Mucosal addressin cell adhesion molecule-1 (MAdCAM-1) expression in HeLa cells. Upper panel: staining of MAdCAM-1. Lower panel: isotype control. **(G,H)** Peripheral blood leukocytes from healthy controls ($n = 4$) were labeled with a vital dye (green) and co-incubated with IgG1 and VDZ for 60 min on confluent grown MAdCAM-1 expressing HeLa cells (red). Afterward, non-attaching cells were removed by repeated washing. Adhering cells were visualized with fluorescence microscopy and attaching leukocytes per high power field (HPF) were counted. **(I)** Peripheral leukocytes from CD ($n = 3$) and UC ($n = 3$) patients before and after completion of VDZ induction therapy from the same patient were labeled with a vital dye and co-incubated with IgG1 and VDZ for 60 min on confluent grown MAdCAM-1 expressing HeLa cells. Afterward, non-attaching cells were removed by repeated washing. Adhering cells were visualized with fluorescence microscopy and attaching leukocytes per HPF were counted. Results are presented as mean ± SEM. Differences between samples are compared with the Mann–Whitney *U* test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

that VDZ is not effective in subgroups of IBD patients. Given these considerations, it is important not only to understand the biologic mode of action but also to characterize differences between responders and non-responders to anti-integrin therapy with VDZ on the cellular and molecular level. In this study, we therefore set off to explore factors that define clinical response toward VDZ and to delineate the immunologic differences between responders and non-responders to anti-integrin therapy with VDZ.

In an initial approach, we performed whole transcriptome analysis using RNA Sequencing in UC and CD patients responding to VDZ compared with non-responders. For both diseases, we found that failure of VDZ to induce clinical remission was associated with an upregulation of several pro-inflammatory and immunogenic genes such as several pro-inflammatory chemo- and cytokines or its receptors and a tissue destructive signature as indicated by the upregulation of a large panel of genes encoding for various proteases. Furthermore, IPA revealed a strong activation of TNF-dependent signaling in non-remitters to VDZ treatment. By contrast, in UC and CD patients with remission to VDZ treatment, no clear pro-inflammatory or immunogenic signature was present. Instead, in UC patients with remission to VDZ, TNF, and IFNγ as broad and potent pro-inflammatory cytokines were predicted to be inhibited instead of activated on Ingenuity analyses.

Importantly, endoscopic and clinical parameters of disease severity such as leukocyte counts, serum CrP levels, clinical disease activity, concomitant use of glucocorticosteroids and immunosuppressant, or prior exposure to anti-TNF therapy were not different between remitters and non-remitters before initiation of VDZ therapy. Thus, it can be ruled out that the observed regulations of the above-mentioned genes and related pathways are due to pre-existent differences in disease severity between remitters and non-remitters but can be rather related to VDZ treatment itself.

To further delineate the effects of VDZ treatment on the expression of α4β7 on systemic and gut immune cells, we comparatively quantified the amount of surface α4β7 on various systemic and intestinal effector T cells. Interestingly, α4β7 expression showed a progressive decline on systemic Th1, Th2, and Th17 polarized $CD4^+$ T cells from patients with CD and on Th17 cells from patients with UC. These observations fit well to our mechanistic studies on the mode of action of VDZ therapy. Using flow cytometric analyses and confocal microscopy, we were able to show that treatment with VDZ leads to internalization of the α4β7 receptor which results in significantly impaired interaction with and adherence to MAdCAM-1 as shown *in vitro* in this report, while cellular effects such as cytokine production or the induction of programmed cell death remained unaltered. These data are consistent with observations made in clinical trials in which no changes in serum levels of cytokines such as TNF, IL-1, or IFNγ were observed under VDZ treatment (10–12, 52, 53). Furthermore, during the antibody engineering of VDZ, point mutations were made to the Fc receptor (FcR) binding motif ELLGGP. As such, Leu239 and Gly241 were mutated to Ala to reduce FcR binding, and the unaltered cytokine production and apoptosis induction very well fit to the lack of elicitation of FcR effector functions from VDZ and results of a previous study which has shown that VDZ does not induce complement-dependent cytotoxicity and antibody-dependent cytotoxicity (54). Consistent with the internalization observed *in vitro* and the progressive decline of surface α4β7 expression during VDZ induction therapy, we were able to show in functional studies that cells exposed to exogenously added VDZ as well as cells taken from patients after completed VDZ induction therapy are significantly impaired in their attachment to MAdCAM-1 expressing cells. Hence, the observed progressive decrease of surface α4β7 on peripheral effector T cells during VDZ induction therapy might well reflect VDZ-induced internalization of α4β7. One might argue that receptor internalization and re-surfacing of unbound and biologically active receptor is a continuous process, leading to the re-appearance of α4β7 on the surface. However, in this regard, it is important to note that the mean half-life of VDZ is approximately 15–22 days and it has been shown in clinical trials that, after administration of VDZ at weeks 0 and 2, followed by dosing at every fourth or eighth week, greater than 95% of α4β7 receptors were saturated (11, 12). Hence, it appears plausible that steady-state serum concentrations of VDZ lead to immediate binding and subsequent internalization of re-surfacing or newly synthesized α4β7 receptor, so that the majority of α4β7 is internalized at steady-state serum levels of VDZ.

Based on the decline of α4β7 on the surface of effector T cells during VDZ therapy, we expected that also a decrease of α4β7-expressing cells within the lamina propria of IBD patients would be noted after completed VDZ induction therapy. Indeed, both

UC and CD patients with remission to VDZ therapy exhibited a robust decrease of α4β7 on Th1, Th2, and Th17 polarized $CD4^+$ T cells after completed VDZ induction therapy compared with non-remitters.

To allow a timely, targeted, and economic treatment regimen with biological agents, the identification of factors that predict therapeutic responses to treatment is of central importance for the management of IBD patients. Recently, our group demonstrated that the amount of mTNF-expressing cells in the lamina propria of CD patients prior to the initiation of anti-TNF therapy is directly related to the outcome of subsequent anti-TNF therapy (55). Specifically, patients with high numbers of $mTNF^+$ cells showed significantly higher short-term response rates at week 12 upon subsequent anti-TNF treatment when compared with patients with low amounts of $mTNF^+$ cells (55). Furthermore, the clinical response in patients with high $mTNF^+$ cells was sustained over a follow-up period and was associated with mucosal healing (55). In addition, exploratory studies from an UC clinical phase II trial with the anti-adhesion molecule antibody etrolizumab, which binds to the β7 subunit of the α4β7 and αEβ7 integrins, strengthen the approach of assessing the expression of the target molecule of anti-adhesion molecule antibody treatment. In this regard, it was shown *via* immunofluorescence staining that high expression of αE-positive cells in colonic biopsies at baseline was directly related to the therapeutic response upon etrolizumab treatment (56).

In this report, we followed a similar approach and quantified α4β7 expression in colonic biopsies from UC and CD patients before the commencement of anti-integrin therapy with VDZ. Importantly, UC and CD patients with remission to subsequent VDZ therapy exhibited significantly higher amounts of α4β7-expressing cells at baseline compared with non-remitters. These data further substantiate the concept that the expression of the target molecule can be used as a prognostic marker for therapeutic response, and to the best of our knowledge, our report is the first to demonstrate that the expression of α4β7 at baseline might well be used for prediction of therapeutic responses toward subsequent anti-integrin therapy with VDZ.

Limitations of the study should also be discussed, one of which is the low number of patients included in RNA sequencing, thereby rendering these results exploratory in nature. However, our approach of comparatively including both UC and CD remitters and non-remitters before and at week 14 of VDZ therapy allowed to analyze drug effects and difference between remitters and non-remitters while controlling for patient-specific factors. Furthermore, we used colonic biopsies for RNA sequencing so that changes in the transcriptome cannot be related to a certain cell type. Given these considerations, it seems clears that results should be corroborated in larger patient cohort using more specific sequencing analyses such as single-cell sequencing.

In summary, we have shown that remitters and non-remitters to VDZ therapy exhibit distinct genetic signatures, which are characterized by the upregulation of genes that mediate mucosal inflammation and tissue destruction in IBD patients with remission to VDZ treatment. Furthermore, RNA sequencing revealed a strong activation of TNF-dependent signaling pathways in non-remitters to VDZ treatment, thereby providing first evidence that anti-TNF therapy might be therapeutically effective in IBD patients with VDZ failure. Treatment with VDZ was associated with a progressive decline of α4β7 expression on the surface of peripheral leukocytes while therapeutic remission was characterized by significantly higher amounts of α4β7-expressing cells in the lamina propria of CD and UC patients at baseline compared with non-responders. Hence, quantification of α4β7-expressing cells within the lamina propria might represent a novel approach which may allow prediction of therapeutic success prior to the initiation of anti-integrin therapy with VDZ, thereby opening up new avenues for personalized medicine in IBD patients.

AVAILABILITY OF DATA

All data generated or analyzed during this study are included in this published article (and its supplementary files). RNA sequencing data have been submitted to the Sequence Read Archive (study accession number SRP151738).

ETHICS STATEMENT

The study was carried out in accordance with the recommendations of the ethical committee of the University Hospital, Friedrich-Alexander-Universität Erlangen-Nürnberg, Germany. The protocol was approved by the ethical committee of the University Hospital, Friedrich-Alexander-Universität Erlangen-Nürnberg, Germany. Each patient gave written informed consent in accordance with the Declaration of Helsinki before inclusion into the study.

AUTHOR CONTRIBUTIONS

All the authors made substantial contributions to the conception and design of the study (or the acquisition of data, or analysis and interpretation of data), the drafting the article (or critically revising it for important intellectual content), and to the final approval of the version to be submitted. TR performed the experiments and wrote the manuscript and together with MN and RA designed the study. UB performed experiments, helped in study design, and critically revised the manuscript for important intellectual content. FF and AE performed RNA sequencing and analyzed sequencing data. MV performed histopathological scoring and critically revised the manuscript. MN and RA designed the study and critically revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01700/full#supplementary-material.

FIGURE S1 | α4β7 expression on lymphocyte subsets in inflammatory bowel disease (IBD) patients. **(A)** Staining of α4β7 expression on $CD3^+$, $CD4^+$, and $CD8^+$ positive T cells, on B-cells ($CD19^+$), macrophages ($CD14^+$), and on natural killer (NK) cells ($CD56^+$) with fluorescein isothiocyanate (FITC)-labeled vedolizumab (VDZ) on lamina propria lymphocytes. Gray line: isotype control. **(B)** Quantification of α4β7 expression by flow cytometry in different subsets of peripheral leukocytes from 11 IBD patients prior to VDZ therapy. **(C)** α4β7 expression in peripheral blood lymphocytes in 13 IBD patients before VDZ therapy (indicated by "0") (week 0) and directly before the second (week 2) and third (week 6) administration of 300 mg VDZ (indicated by "1" and "2," respectively). **(D)** Quantification of α4β7 expression by flow cytometry in different subsets of lamina propria lymphocytes from five IBD patients. Results are presented as mean ± SEM. Differences between samples are compared with the Mann–Whitney U test.

TABLE S1 | CD Genes before and after VDZ.

TABLE S2 | CD pathways before and after VDZ.

TABLE S3 | CD Non-Remitter Genes before and after VDZ.

TABLE S4 | CD Remitter Genes before and after VDZ.

TABLE S5 | UC Genes before and after VDZ.

TABLE S6 | UC pathways before and after VDZ.

TABLE S7 | UC Non-Remitter Genes before and after VDZ.

TABLE S8 | UC Remitter Genes before and after VDZ.

TABLE S9 | Individual phenotypical and clinical characteristics of patients included in each experiment.

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Adipokines and Their Role in Intestinal Inflammation

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Fat tissue was initially described for its endocrine and metabolic function. Over the last two decades increasing evidence indicated a close interaction with the immune system. Partly responsible for this immune modulatory function are soluble factors released by the fat tissue, most prominently the so-called adipokines. These discoveries led to the question how adipokines influence inflammatory diseases. Linking inflammation and adipose tissue, Crohn's disease, a chronic inflammatory bowel disease, is of particular interest for studying the immune modulatory properties of adipokines since it is characterized by a hyperplasia of the mesenteric fat that subsequently is creeping around the inflamed segments of the small intestine. Thus, the role of several adipokines in the creeping fat as well as in intestinal inflammation was recently explored. The present review selected the four adipokines adiponectin, apelin, chemerin, and leptin and provides a working model based on the available literature how these factors participate in the maintenance of intestinal immune homeostasis.

Keywords: adipokines, inflammatory bowel disease, Crohn's disease, creeping fat, immune modulation

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INTRODUCTION

Adipokines represent a group of mediators primarily released by adipocytes that modulate a variety of metabolic functions within the fat tissue and additional organs such as liver, brain and muscle. In addition to the metabolic functions, a regulatory role within the immune system was identified early on for a number of adipokines. This includes adiponectin which in first reports was shown to suppress mature macrophage function (1) as well as leptin that was identified as a T cell stimulatory factor (2). These initial publications induced a plethora of studies exploring the impact of various adipokines in several inflammatory diseases. Why is intestinal inflammation of particular interest for the regulatory function of adipokines?

In Crohn's disease, one subtype of inflammatory bowel disease (IBD), characteristic changes of the mesenteric fat suggest that the mesenteric fat might play a central immune modulatory role in the pathogenesis of Crohn's diseases (3). In this disease, the mesenteric fat becomes hyperplastic and is creeping around the inflamed segments of the small intestine, suggesting a direct role of this fat tissue compartment in Crohn's disease. In order to provide a concise overview how adipokines regulate intestinal inflammation, we here selected adipokines for which experimental data in intestinal inflammation were available. Thus, we chose apelin, adiponectin, chemerin, and leptin. In order to include the relevant literature, we chose the names of the respective adipokine in combination with either colitis, IBD, Crohn's disease, or ulcerative colitis as search terms in PubMed. This review will first focus on the selected adipokines (summary provided in **Table 1**), namely apelin, adiponectin, chemerin and leptin, and then provide a disease-relevant working model.

TABLE 1 | Adipokines and the regulatory effect on intestinal homeostasis.

Adipokine	Effect on mucosal homeostasis	References
Adiponectin	*Function* Adiponectin plays a key role in regulating insulin sensitivity. In addition, an anti-inflammatory effect has been demonstrated for atherosclerosis.	(4)
	in vitro Globular adiponectin has been shown to enhance intestinal epithelial cell proliferation and prevented these cells from apoptosis. In line, *adiponectin*$^{-/-}$ mice developed more severe DSS-induced colitis accompanied by a decreased epithelial proliferation, increased apoptosis and cellular stress. This could be reversed *in vitro* in the presence of adiponectin.	(5, 6)
	in vivo models The data on the *in vivo* role are controversial. Fayad et al. provided evidence suggesting a pro-inflammatory role for adiponectin in DSS- as well as TNBS-induced colitis. Conversely, Nishihara et al. found the opposite. In addition, work by Sideri et al. demonstrated that silencing of AdipoR1 was followed by deterioration of TNBS-induced colitis. These obvious discrepancies might be explained by differences in knockout mice.	(7–10)
	Human data Adiponectin has been shown to be upregulated in the creeping fat of Crohn's disease as compared to non-creeping fat of Crohn's disease, ulcerative colitis and healthy controls.	(11)
Apelin	*Function* Apelin induces proliferation of intestinal epithelial cells. Apelin plays a role in the development and stabilization of lymphatic vessels.	(12)
	in vivo models Apelin is upregulated in the intestinal epithelial cells of colitic mice. Apelin administration ameliorated colitis in *Il10*$^{-/-}$ mice by enhancing intestinal lymphatic function.	(13, 14)
	Human data Apelin is upregulated in the intestinal epithelial cells of IBD patients. Apelin is highly expressed in the mesenteric adipose tissue of Crohn's disease patients.	(12, 15)
Chemerin	*Function* Chemerin has been shown to serve as chemoattractant for cells of the innate immune system.	(16)
	in vivo models Administration of chemerin aggravated DSS-induced colitis and was associated with a decrease in anti-inflammatory macrophages. Accordingly, mice deficient in the chemerin receptor, chemokine-like receptor 1 (CMKLR1), develop colitis in a delayed course, ultimately resulting in similar disease severity.	(17, 18)
	Human data Chemerin was elevated in the serum of IBD patients.	(19)
Leptin	*Function* Leptin was initially identified as a hormone and satiety factor. Early work revealed an enhancing effect on T cell proliferation and polarization.	(2, 20)
	in vitro Leptin has been shown to induce an increased intestinal epithelial cell proliferation. In addition, intestinal epithelial cells produce leptin and luminal leptin *vice versa* activates the NF- κB pathway in intestinal epithelial cells. In line, rectal application *in vivo* results in intestinal inflammation. Leptin signaling is required for *in vitro* polarization of T_{h17} cells as leptin receptor (Lepr) deficient T cells display decreased STAT3 signaling and consecutively less RORγt expression and impaired IL-17 and IFNγ production.	(21–23)
	in vivo models Leptin-deficient *ob/ob* mice are protected from experimental colitis. Colitis induction depends on the activation of T cells by leptin as proven in the T cell transfer model of colitis. Leptin produced by T cells does not contribute to colitis development.	(24–27)
	Human data Leptin is upregulated in the mesenteric fat of Crohn's disease patients. The presence of leptin in the mesenteric fat favors the polarization of tissue macrophages toward an anti-inflammatory phenotype.	(28, 29)

AdipoR1, adiponectin receptor type 1; DSS, dextran sodium sulfate; IBD, inflammatory bowel diseases; NF- κB, nuclear factor "kappa-light-chain-enhancer" of activated B-cells; TNBS, trinitrobenzene sulphonic acid.

ADIPOKINES

Apelin

Early on, apelin has been shown to be elevated in the colonic tissue of mice suffering from colitis as well as in humans with IBD. Apelin was mainly expressed by epithelial cells and addition of synthetic apelin in cell culture increased epithelial cell proliferation (12). More recently, apelin was described to play a significant role in the development as well as the stabilization of lymphatic vessels (13, 14). Remarkably, defects in the lymphatic transport of the mesentery have previously been suggested to be involved in the pathogenesis of Crohn's disease. This is reflected by an increased density of lymphatic vessels as well as the presence of tertiary lymphoid organs in the mesentery

of Crohn's disease patients (30, 31). Accordingly, apelin was shown to be highly expressed in the mesenteric adipose tissue of Crohn's disease patients. In addition, the administration of apelin to *Il10*$^{-/-}$ mice with established colitis resulted not only in an amelioration of disease by lowering the production of pro-inflammatory cytokines such as TNFα, IL-6, and IL-1β but furthermore enhanced intestinal lymphatic function. This was shown by an increased lymphatic vessel density. In addition, lymphangiography indicated an augmented lymphatic drainage (15). Based on these findings, one can propose a regenerative function of apelin on intestinal epithelial cells as well as a supportive role with regard to intestinal lymphatic drainage.

Adiponectin

The adipokine adiponectin plays a key role in regulating insulin sensitivity and had previously been shown to play an anti-inflammatory role in atherosclerosis (4). The study by Yamamoto et al. analyzed the adiponectin expression in the creeping fat of Crohn's disease patients, that revealed an upregulation of adiponectin expression in creeping fat of Crohn's disease patients when compared to non-creeping fat of Crohn's disease patients as well as fat from ulcerative colitis patients or controls (11).

A study by Fayad et al. explored the function of adiponectin in intestinal inflammation by subjecting adiponectin-deficient animals to two models of experimental colitis, the model of dextran sulfate sodium (DSS)- and the model of trinitrobenzene sulphonic acid (TNBS)-induced colitis. Adiponectin was shown to rather induce the production of pro-inflammatory cytokines in the colon as adiponectin stimulation of colon organ cultures of DSS-treated mice resulted in increased production of IL-6 and MIP-2, whereas adiponectin-deficient APN knock out mice were protected from experimental colitis. Thus, these data were somewhat contrary to the findings described for atherosclerosis (8). A second study by Nishihara et al. explored again adiponectin-deficient mice in the DSS and TNBS model of colitis. In contrast to the previous study, adiponectin protected from inflammation, possibly mediated by a direct anti-inflammatory effect on the colonic epithelial cells (7). These discrepancies between the two studies as well as in several *in vitro* studies are most likely due to different types of knockout mice or adiponectin used (9).

In line with the first study, Ogunwobi and colleagues provide data analyzing the effect of adiponectin on colon epithelial cells by using the colonic epithelial cell line HT-29 and by carefully distinguishing globular adiponectin from full-length adiponectin. Remarkably, in particular globular adiponectin mediated pro-proliferative as well as pro-inflammatory effects through activation of extracellular-signal regulated kinase (ERK), p38 mitogen-activated protein kinase (MAPK) NF-κB signaling on colonic epithelial cells (5). When the human NCM60 epithelial cell line was exposed to fat-conditioned media obtained from IBD patients, cells showed a reduced expression of adiponectin receptor 1 (AdipoR1). Silencing of AdipoR1 in mice resulted in an exacerbation of TNBS-induced colitis (10).

In a recent study, adiponectin-deficient mice treated with DSS exhibited more severe colitis accompanied by an increased presence of activated B cells, pro-inflammatory cytokines such as IL-1β, IL-4, and IL-6 and increased STAT3 signaling in the colon. The epithelium of the knockout animals revealed a decrease in cell proliferation as well as increased apoptosis and cellular stress. In *in vitro* experiments these effects could be reversed by adiponectin. These data are supporting the concept that adiponectin maintains intestinal homeostasis (6).

Chemerin

Chemerin has been shown to serve as chemo-attractant for cells of the innate immune system (16). Serum from Crohn's disease ($n = 230$), ulcerative colitis patients ($n = 80$), and healthy controls ($n = 80$) was recently compared for the expression of chemerin and adiponectin. Chemerin was elevated in IBD patients, whereas adiponectin was decreased (19). An *in vivo* study furthermore indicated that the administration of chemerin resulted in aggravation of DSS-induced colitis in mice by augmenting TNFα and IL-6 production, whereas a decrease in IL-10−producing anti-inflammatory macrophages could be detected. This could be confirmed *in vitro*, where the presence of chemerin prevented macrophages from polarizing into an anti-inflammatory phenotype resulting in impaired expression of Arginase-1 and IL-10 (17). The chemokine-like receptor 1 (CMKLR1) is the receptor for chemerin. Mice deficient in CMKLR1 developed DSS-induced colitis in a delayed time course, albeit ultimately presenting with a similar disease activity (18).

Leptin

Leptin was initially identified as a satiety factor (20, 32). Work by Lord and colleagues revealed that the adipokine leptin supports T cell proliferation and results in increased T helper cell type 1 (T_{h1}) and in suppressed T_{h2} cytokine production. In addition, administration of leptin to starved mice resulted in an abrogation of starvation-induced immunosuppression (2). The additional characterization of three children with congenital leptin deficiency indicated that leptin substitution not only reversed their metabolic dysfunction including insulin resistance and fatty liver degeneration, but furthermore increased the number of circulating $CD4^+$ T cells as well as their proliferative capacity (33). These findings raised the question whether leptin exerts a modulating effect on autoimmune diseases. First evidence was provided from the model of experimental autoimmune encephalomyelitis. Here, leptin-deficient (*ob/ob*) mice were protected from T cell-mediated neuronal damage and leptin substitution augmented the disease susceptibility in mice. Additional work on the same model revealed that CNS-infiltrating T cells themselves are able to produce leptin and that this effect could be blocked by an anti-leptin receptor antibody (34, 35).

In an early study, systemic leptin concentrations were determined during the course of acute experimental colitis. In this study, the concentration of leptin in the plasma increased in TNBS-induced colitis and indomethacin-induced ileitis and correlated with disease severity. However, this observed increase was only transient and returned to control concentrations over time (36). The association of a distinct disease to plasma levels has proven difficult for several disease entities in the past. However, independent studies revealed a strong up-regulation of leptin

expression in the mesenteric fat of Crohn's disease patients (28, 37).

In our own work, we were able to demonstrate that leptin-deficient *ob/ob* mice are protected from DSS-induced colitis and that leptin administration reverses disease susceptibility in mice (24). To confirm the previously established concept that leptin mediates pro-inflammatory effects, at least partly, via T cells, we performed a T cell transfer model of colitis and transferred naive $CD4^+$ T cells lacking the signaling Ob-Rb-isoform of the leptin receptor. In fact, the development of colitis was significantly delayed using this approach, indicating that the stimulatory effect of leptin plays a crucial role in this model (25). Additional data showing that *ob/ob* mice are protected in models driven by either T_{h1} (TNBS) or T_{h2} (oxazolone) cells underline that the T cell-stimulating capacity of leptin is important for the observed effects (27). However, this enhancing factor does not apply for all T cell subpopulations, since leptin has been shown to inhibit the proliferation of regulatory T (T_{reg}, $FoxP3^+CD4^+CD25^+$) cells. Accordingly, the absence of leptin, as demonstrated for *ob/ob* and *db/db* mice (mice deficient for the signaling Ob-Rb isoform of the leptin receptor), resulted in an increased proliferation of functional T_{reg} cells (38). Likewise, Reis and colleagues could demonstrate that $Lepr^{fl/fl-}$*CD4-Cre* mice harboring a conditional knock out of the leptin receptor in their $CD4^+$ T cell compartment also showed higher frequencies of $FOXP3^+$ T_{reg} cells under steady state conditions. Importantly, *Lepr*-deficient T cells displayed a severe defect in T_{h17} differentiation due to a decreased activation of the STAT3 signaling cascade, resulting in impaired cytokine production of IL-17 and IFNγ, which protected recipient *Rag*-$^{-/-}$ mice receiving *Lepr*-deficient T cells from transfer colitis (23).

Data from the experimental autoimmune encephalomyelitis model have previously indicated that leptin produced by T cells might play a key role in mediating disease severity (23, 35). Thus, we performed again the T cell transfer model of colitis and chose this time naive T cells isolated from *ob/ob* mice as disease-inducing cell population in comparison to wild-type cells. In contrast to the data cited above, no differences were observed with regard to disease severity or cytokine production, implying that leptin produced by T cells is irrelevant for intestinal inflammation (26).

Recent data indicate that the administration of a pegylated leptin antagonist (PG-MLA) was sufficient to protect from chronic experimental colitis. Amelioration of colitis was here associated with a decrease in the expression of mucosal pro-inflammatory cytokines and an increase of mucosal T_{reg} cells (39).

Having shown that leptin modulates intestinal inflammation via the T cell compartment, the question arose whether there is an additional effect on epithelial cells. The leptin receptor (Ob-Rb) is in fact expressed in human colonic tissue as well as on the HT-29 colon cancer cell line. Stimulation of HT-29 cells with leptin was followed by activation of p42/44 MAPK as well as increased proliferation *in vitro* and *in vivo* (21). An interesting aspect was added by the study of Sitaraman and colleagues that were able to demonstrate that inflamed colonic epithelial cells produce and release leptin into the intestinal lumen. The presence of luminal leptin resulted in the activation of the NF- κB pathway in intestinal epithelial cells. Rectal application of leptin was followed by intestinal inflammation and epithelial wall damage (22). Following up on these luminal effects, Hoda et al. analyzed the effect of luminal leptin on ion transport capacities under inflammatory conditions. Human intestinal epithelial cells (T84) as well as intestinal tissue from a rat model of chemotherapy-induced enterocolitis were analyzed in Ussing chambers. These served to determine the transepithelial short-circuit current I(sc). The presence of leptin resulted in an increase of basal I(sc) of T84 cells, which was mediated by activation of the MAPK pathway. In line, in the enterocolits model luminal leptin equally induced an increase in I(sc), that was more prominent in the proximal colon (40).

A recent study was able to link leptin, cell metabolism and intestinal epithelial function. The authors provided evidence indicating that leptin is able to induce lipid droplet formation in intestinal epithelial cells (IEC-6). This was accompanied by an increased production of CXCL1/CINC-1, CCL2/MCP-1, and TGFβ. In line with previous data, leptin induced cell proliferation. The process of lipid droplet induction and the associated effects depended on the mammalian target of rapamycin (mTOR) pathway, since it was completely abrogated in the presence of rapamycin (41). Thus, one can conclude that leptin, very similar to adiponectin, exerts direct effects on intestinal epithelial cells and consequently plays a role in intestinal homeostasis.

HOW GETS THE MESENTERIC FAT TISSUE STIMULATED AND ADIPOKINES RELEASED?

The question arises how the mesenteric fat gets involved in the first place. Courageous proposals suggest that changes in the mesenteric fat tissue might in fact be the initiating event of Crohn's disease. However, this is not only difficult to proof but it also seems to be more likely that the transmural inflammation and consecutive chronic bacterial translocation is the initiating factor for these changes. Crohn's disease, in contrast to ulcerative colitis, presents with a transmural inflammation and can also affect the small intestine. Several experimental models as well as studies in humans provided evidence of an increased translocation of bacteria into the mesenteric fat (42, 43). Previous data had already proven that adipocytes as well as preadipocytes express functional receptors of the innate immune system including toll-like receptors (Tlr) and nucleotide oligomerization domains 1 (NOD1) and 2 (NOD2) (44, 45). Accordingly, while wild type mice featured an up-regulation of pro-inflammatory mediators in chronic DSS colitis, *Myd88*$^{-/-}$ mice, thus mice with a dysfunctional innate immune system, failed to respond to these translocalizing bacteria and showed a significantly increased mortality, suggesting that the mesenteric fat serves as a potential second barrier (42, 46). In line, wild-type mice revealed an up-regulation of leptin in the mesenteric fat, whereas this was not observed in *Tlr9*$^{-/-}$ mice. However, IL-6 production was unaffected by the absence of Tlr9 (47), indicating that other innate receptors contribute to the effects described before (42).

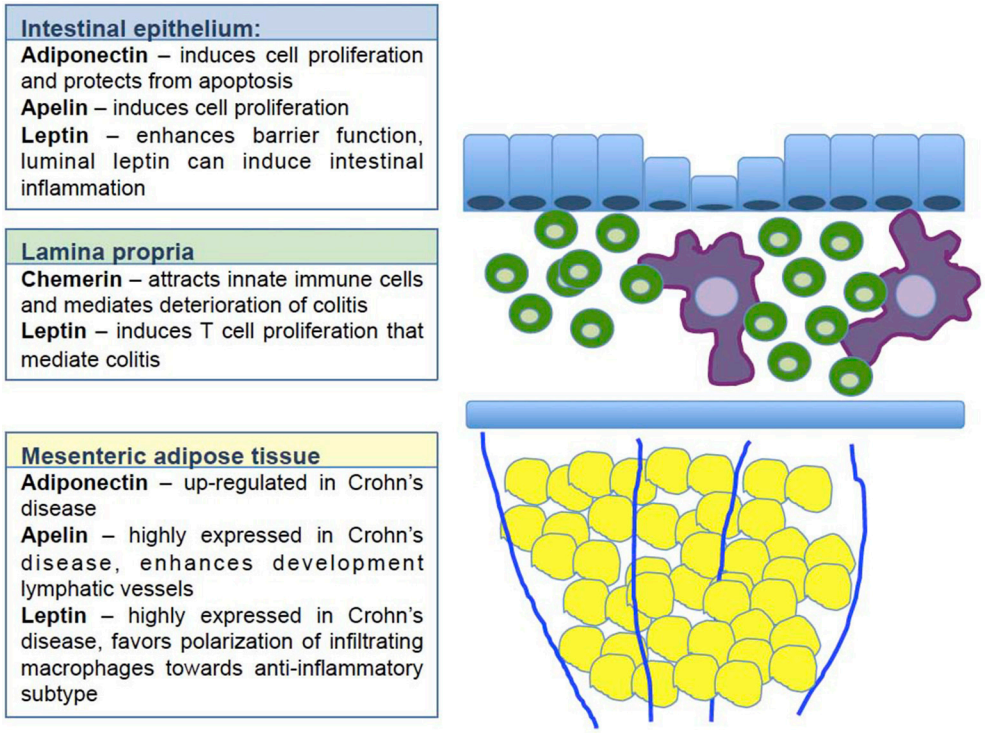

FIGURE 1 | Regulatory effect of selected adipokines on intestinal homeostasis. The figure illustrates at which layer of the intestinal wall the selected adipokines exert their respective regulatory function. Blue indicates the intestinal epithelial layer. Adiponectin, apelin and leptin have been shown to enhance cell proliferation and enhance barrier function. The majority of these data derive from *in vitro* data, thus it remains open how these adipokines contribute to the complex crosstalk between epithelial cells and immune cells *in vivo*. Green indicates the lamina propria with all cell populations included. Chemerin has been shown here to attract innate immune cells resulting in a deterioration of colitis. Leptin induces T cell proliferation thus enhancing inflammation. Yellow indicates the mesenteric fat tissue. Adiponectin, apelin and leptin are all up-regulated in this compartment. Apelin enhances the function of the lymphatic vessels, that are known to be dysfunctional in Crohn's disease. Leptin strongly influences the polarization of infiltrating monocytes toward rather anti-inflammatory macrophages.

What is the subsequent effect of adipokines produced within the mesenteric adipose tissue? Leptin has been shown to not only modulate the polarization of the T helper cell compartment but to equally affect together with adiponectin the myeloid compartment. Leptin as well as adiponectin favored the polarization of anti-inflammatory macrophages, the dominant macrophage population detectable in creeping fat of Crohn's disease patients (29). In addition, as described above for mice (42) and patients with Crohn's disease (43), the mesenteric fat develops an effector response to the translocalizing antigens. Remarkably, a dysfunction of this effector response, as discussed above for the *Myd88*$^{-/-}$ mice, results in an increased mortality, thus suggesting a primarily protective function. Together this fits into the concept that the mesenteric fat represents a second barrier and thereby prevents the Crohn's disease patient from continuous bacteremia (48, 49).

A summary of the effects mediated by adipokines is provided in **Figure 1**.

CONCLUSIONS

Continuous work over the last two decades has substantially provided insights into the regulation of adipokines and intestinal inflammation. The data discussed for adiponectin, apelin, chemerin, and leptin within this review indicate a complex specific function for each individual adipokine in the regulation of intestinal inflammation. However, the summary also reveals the shortcomings of our current understanding. Thus, the complex interplay between the adipose tissue and the immune system presents a hot topic for current research. In our view, it is rather unlikely that adipokines will serve as therapeutic option for patients with IBD. Nevertheless, the overall metabolic condition of a patient, e.g., body mass index, presence of diabetes, might strongly influence the adipokine response on one hand, on the other hand, anti-inflammatory treatment in IBD patients might result in direct changes of the adipokine and thus metabolic profile. Therefore, this particular cross-regulation will become even more important in the foreseeable future.

AUTHORS CONTRIBUTIONS

JZ, ML, and FS contributed distinct parts of the manuscript. CW participated in writing and finalizing the manuscript. BS wrote the first draft and finalized the manuscript.

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The Tumor Necrosis Factor Superfamily Members TNFSF14 (LIGHT), Lymphotoxin β and Lymphotoxin β Receptor Interact to Regulate Intestinal Inflammation

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Over 1.5 million individuals in the United States are afflicted with inflammatory bowel disease (IBD). While the progression of IBD is multifactorial, chronic, unresolved inflammation certainly plays a key role. Additionally, while multiple immune mediators have been shown to affect pathogenesis, a comprehensive understanding of disease progression is lacking. Previous work has demonstrated that a member of the TNF superfamily, TNFSF14 (LIGHT), which is pro-inflammatory in several contexts, surprisingly plays an important role in protection from inflammation in mouse models of colitis, with LIGHT deficient mice having more severe disease pathogenesis. However, LIGHT is a single member of a complex signaling network. It signals through multiple receptors, including herpes virus entry mediator (HVEM) and lymphotoxin beta receptor (LTβR); these two receptors in turn can bind to other ligands. It remains unknown which receptors and competing ligands can mediate or counteract the outcome of LIGHT-signaling during colitis. Here we demonstrate that LIGHT signaling through LTβR, rather than HVEM, plays a critical role in the progression of DSS-induced colitis, as LTβR deficient mice exhibit a more severe disease phenotype. Further, mice deficient in LTαβ do not exhibit differential colitis progression compared to WT mice. However, deletion of both LIGHT and LTαβ, but not deletion of both LTαβ and LTβR, resulted in a reversal of the adverse effects associated with the loss of LIGHT. In sum, the LIGHT/LTαβ/LTβR signaling network contributes to DSS colitis, but there may be additional receptors or indirect effects, and therefore, the relationships between these receptors and ligands remains enigmatic.

Keywords: TNF superfamily, Colitis, Lymphotoxin (LT), Light, DSS (dextran sulfate sodium)

INTRODUCTION

Inflammatory bowel disease is an immune-mediated disease in which, among other components, the microbiome, genetics and immune system all contribute to disease (1). Multitudes of bacteria and other microbes reside in the intestine, and at steady state homeostasis is maintained by a controlled and balanced intestinal mucosal immune system (2).

This immune system includes various types of epithelial cells, myeloid cells and lymphocytes, along with a plethora of antimicrobial peptides and inflammatory and regulatory mediators that these cells actively produce (3). While the initial driving forces may vary, an imbalance in this immune response can lead to the development of IBD (4). Of interest, different mediators of the mucosal immune system can either protect from, or exacerbate disease (1). Thus, our understanding of the role of different mediators during IBD is evolving.

The tumor necrosis factor (TNF) superfamily of cytokines and receptors have a diverse, but not fully defined, function in mucosal immunity and IBD pathogenesis (5–7). In fact, antibodies blocking TNF are commonly used as therapeutic agents for IBD patients (8). Over expression of TNF superfamily member 14 (TNFSF14, or LIGHT [homologous to **l**ymphotoxins, exhibits **i**nducible expression, and competes with HSV **g**lycoprotein D for **H**VEM, a receptor expressed by **T** cells]) in transgenic mice leads to colitis (9, 10). Also, LIGHT expression by T cells is increased in Crohn's disease patients (11), and LIGHT promotes inflammation in the skin and lung (12, 13). On the other hand, our previous work has shown that LIGHT, surprisingly, exhibits a protective effect in colitis induced by dextran sulfate sodium (DSS) and by transfer of naïve $CD4^+$ T cells to immune deficient mice (5). This was most thoroughly studied in the DSS model, in which mice deficient in LIGHT had decreased colon length, increased pathology scores and increased immune cell infiltration to the colonic lamina propria in a chronic DSS model, in which at least two rounds of the chemical were administered. However, whether other members of LIGHT's signaling network affect the progression of DSS-induced colitis remains to be determined.

LIGHT can bind two receptors, the lymphotoxin beta receptor (LTβR or TNFRSF3) and the herpes virus entry mediator (HVEM or TNFRSF14) (14). Both receptors are members of the TNF receptor superfamily. Accordingly, stimulation of either receptor has been previously shown to drive an inflammatory response (14). However, in the context of DSS-induced colitis, these two receptors may have different effects. While LIGHT appears to be protective during DSS-induced colitis (5), HVEM deficient mice exhibit disease pathology similar to wild type (WT) mice (6). Conversely, antibody mediated blockade of LTβR results in worsened colitis (5). The results from these experiments suggest that LTβR is the critical receptor in maintaining the protective effect of LIGHT during DSS-induced colitis. Interestingly, in addition to LIGHT, LTβR can also be activated by surface lymphotoxin (LTαβ), a heterotrimer comprised of two TNF superfamily members, a single unit of TNFSF1 (LTα) and two units TNFSF3 (LTβ) (15). Signaling of LTβR by LTαβ is required for the formation of lymph nodes (16). Additionally, activation of LTβR by LTαβ has also been shown to play a role in a variety of inflammatory disorders (17), but whether LTαβ signaling through LTβR plays a role in intestinal inflammation remains undefined.

Here, we report the generation and analysis of a variety of double-mutant mice to delineate the complex interplay of LIGHT/LTαβ with LTβR/HVEM signaling during DSS-induced colitis. We demonstrate that LIGHT signaling through LTβR is indispensable for protection from exacerbated DSS-induced colitis. Additionally, HVEM activation does not seem to contribute to DSS-induced colitis, even in the absence of LTβR. While LTαβ signaling by itself is not critical for altering the severity of colitis, LTαβ deletion rescued the pathogenic effect of LIGHT deletion, but not of LTβR deletion. This shows that the role of specific ligands becomes difficult to predict when multiple members of the TNF superfamily are depleted, and suggests the possibility that LTαβ has effects that extend beyond its interaction with LTβR, or alternatively, that LTβR integrates additional signals to affect the outcome in DSS-colitis.

RESULTS

Lymphotoxin Beta Receptor Activation Prevents Exacerbated Colitis

Previous results demonstrated that LIGHT signaling protects from DSS-induced colitis (5). In the absence of LIGHT, the innate immune response was augmented, especially in the chronic DSS model, with increased IL-6, IL-1β, and oncostatin M (5). Further, one of LIGHT's receptors, HVEM, was not found to contribute to colitis, when mice deficient for HVEM expression were tested. Additionally, an antibody that blocks LIGHT-LTβR but not LTαβ-LTβR binding led to more severe DSS-induced colitis, strongly implicating a role for the LTβR (6). However, the antibody epitope and how it selectively blocked one TNFSF ligand and not the other remains undefined, and it is possible that the antibody has mixed agonist-antagonist properties. Therefore, to more definitively address whether LTβR contributes to LIGHT mediated protection from DSS-induced colitis, we administered DSS in drinking water to LTβR deficient mice and controls. Of note, gene knockout mice were created by crossing $Ltbr^{fl/fl}$ mice to CMV-cre for ubiquitous depletion of LTβR (18, 19). DSS administration resulted in increased weight loss in $Ltbr^{fl/fl}$-CMV-cre mice, compared to controls, indicative of increased disease (**Figure 1A**) (20). Additionally, $Ltbr^{fl/fl}$-CMV-cre mice displayed a decreased colon length (**Figure 1B**), typically indicative of fibrosis and a more severe colitis phenotype (20). Indeed, histological analysis of both the colon and cecum of $Ltbr^{fl/fl}$-CMV-cre mice revealed an increased histological score (**Figures 1C–E**) (5), indicating that DSS-induced colitis is more severe in these mice. Similar to mice deficient for LIGHT protein, mice lacking LTβR had increased inflammatory cell infiltrates, epithelial disruption and evidence for intestinal edema. Additionally, these mice had increased mRNA encoding IL-1β, similar to mice lacking LIGHT (data not shown). Together, these results demonstrate that LTβR activation is necessary for protection from exacerbated DSS-induced colitis, with a phenotype similar to the absence of LIGHT.

Lymphotoxin Signaling Does Not Contribute to Colitis Progression

In addition to activation via LIGHT, LTβR can also be activated by a second ligand, LTαβ (15). While LIGHT can be both soluble and a cell surface protein, LTαβ is exclusively a cell-surface

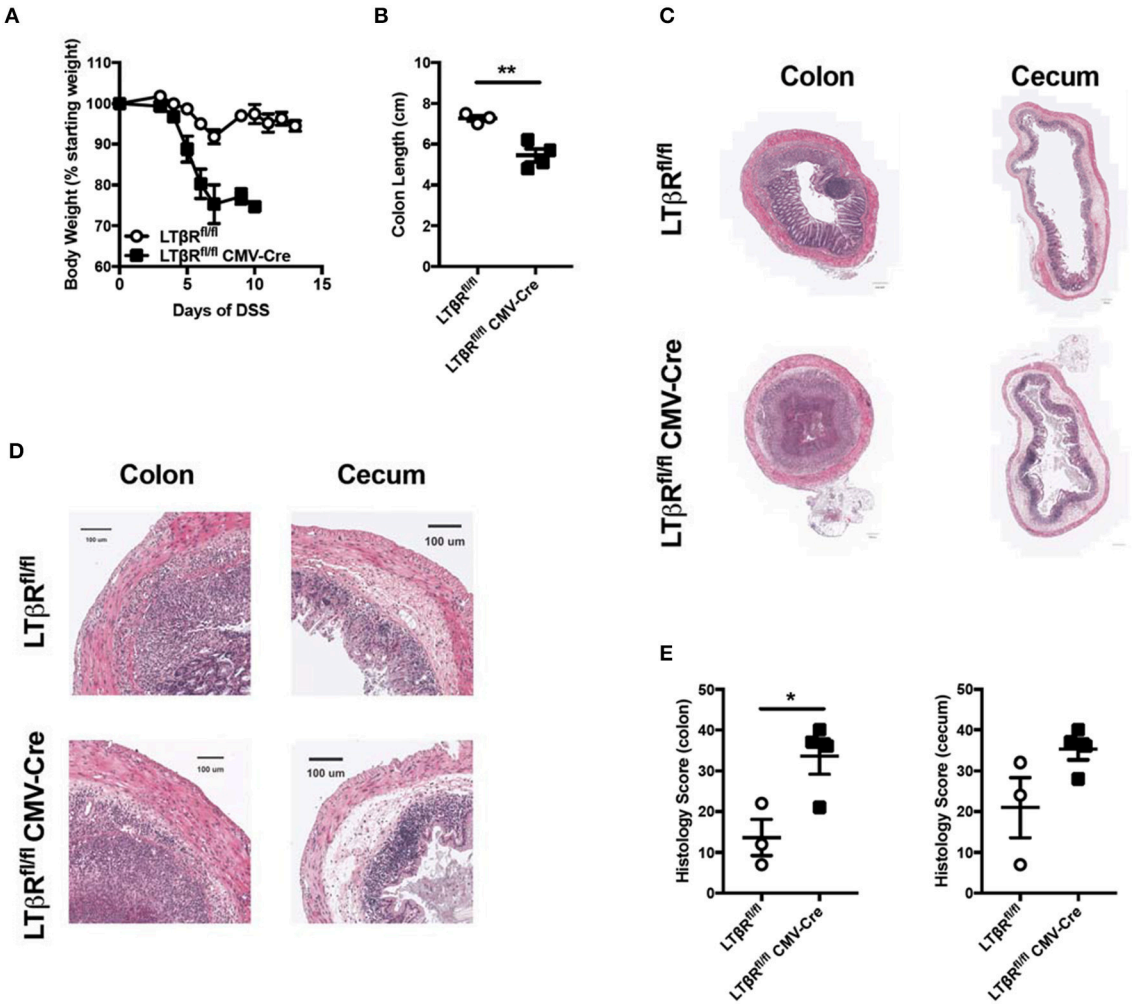

FIGURE 1 | Loss of LTβR leads to exacerbated DSS-induced colitis. Eight week-old *Ltbr*$^{fl/fl}$ CMV-cre ($n = 4$) and *Ltbr*$^{fl/fl}$ ($n = 3$) female mice received 3% DSS in the drinking water. **(A)** Weight loss was monitored daily. **(B)** Following termination of experiment, colon lengths were measured. **(C)** Representative H&E staining of cecum and distal colon cross-section (scale bar = 200 μM). **(D)** Magnified section of cecum and distal colon cross-section from **C** (scale bar = 100 μM). **(E)** Histologic scoring of ceca and distal colons. Data are representative of one of three individual experiments. Data represent mean ± S.E.M. Student's *t*-test, $^{*}p < 0.05$, $^{**}p < 0.01$.

heterotrimer comprised of one LTα unit two LTβ units. Notably, LTβ is required for LTα to bind LTβR, so in LTβ deficient mice the only available signaling through LTβR is via LIGHT (15). Thus, to determine whether LTαβ contributes to LTβR protective effects in in DSS-induced colitis, LTβ deficient mice were treated with DSS. Unlike LTβR deficient and LIGHT deficient mice, *Ltb*$^{-/-}$ mice exhibited weight loss and colon lengths similar to WT controls after DSS treatment (**Figures 2A,B**). Further, histological analysis revealed that *Ltb*$^{-/-}$ mice also exhibited a phenotype similar to WT controls (**Figures 2C–E**). These data demonstrate that LTαβ signaling through LTβR does not contribute to preventing severe DSS-induced colitis, consistent with the hypothesis that LIGHT-LTβR binding is essential.

Mice Deficient in Both Light and LTβ Are Protected From Exacerbated Colitis

Given that LIGHT protects from exacerbated DSS-induced colitis and that LTαβ does not contribute to enhanced colitis progression, we hypothesized that mice deficient for both LIGHT and LTβ would develop augmented colitis. To test whether the absence of both TNFSF cytokines would affect colitis progression, DSS was administered to *Light*$^{-/-}$*Ltb*$^{-/-}$ mice and WT controls. After 12 days, the effects of DSS administration were evaluated. Unlike *Light*$^{-/-}$ mice, which displayed a more rapid weight loss, *Light*$^{-/-}$*Ltb*$^{-/-}$ mice exhibited little weight loss and had colon lengths similar to controls (**Figures 3A,B**). Further, *Light*$^{-/-}$*Ltb*$^{-/-}$ colons and cecal tissue appeared similar to those of DSS-treated WT controls, while the colon

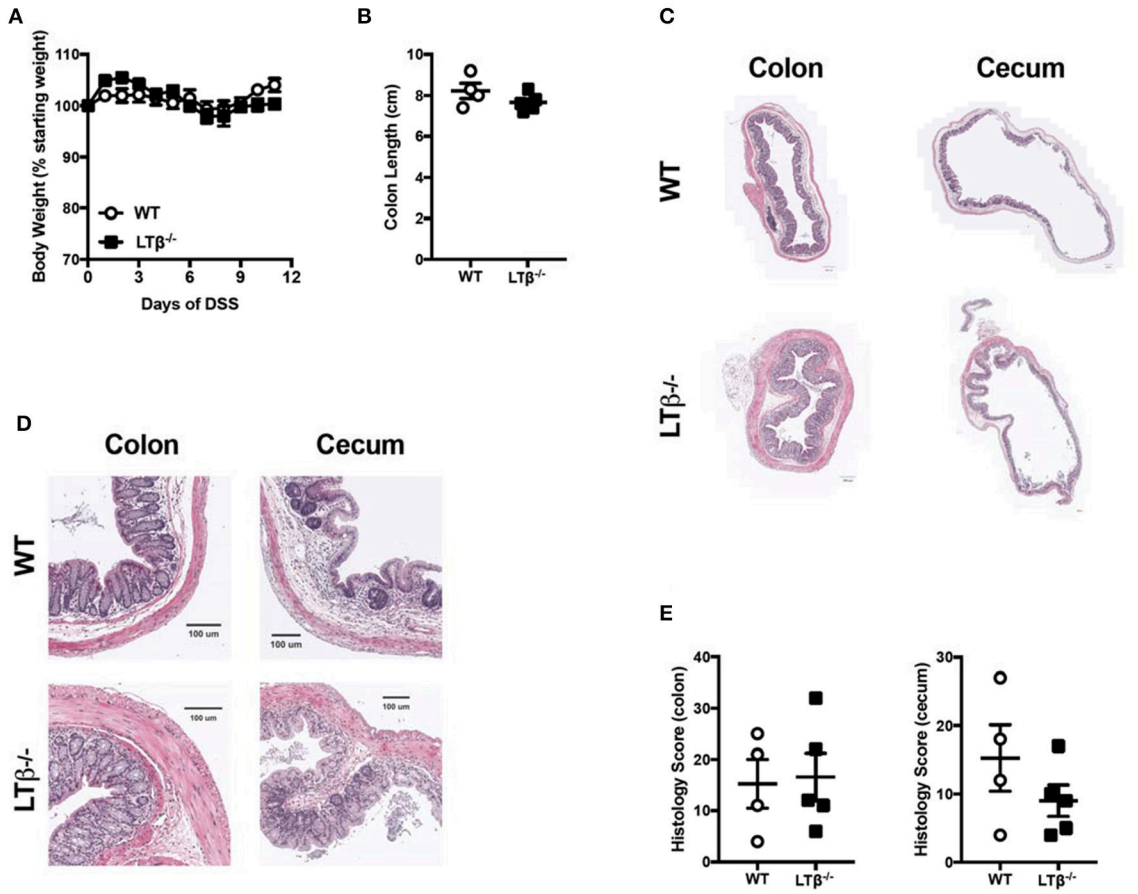

FIGURE 2 | Loss of LTβ does not affect the pathogenesis of DSS-induced colitis. Eight week-old $Ltb^{-/-}$ ($n = 5$) and WT ($n = 4$) female mice received 2% DSS in the drinking water. **(A)** Weight loss was monitored daily. **(B)** Following termination of experiment, colon lengths were measured. **(C)** Representative H&E staining of cecum and distal colon cross-section (scale bar = 200 μM). **(D)** Magnified section of cecum and distal colon cross-section from **C** (scale bar = 100 μM). **(E)** Histologic scoring of ceca and distal colons. Data are representative of one of three individual experiments. Data represent mean ± S.E.M. Student's *t*-test.

and cecum $Light^{-/-}$ mice displayed increased inflammation, quantified by an increased histological score (**Figures 3C–E**). These results indicate that deficiency of LIGHT is not sufficient to exacerbate DSS-induced colitis when LTβR signaling by LTαβ is also impaired. Additionally, these observations confound our understanding of this signaling network in DSS and suggest that other mechanisms may be contributing. One possible explanation is that in the absence of LIGHT signaling, LTαβ binds to LTβR and drives increased inflammation.

Mice Deficient in Both LTβ and LTβR Exhibit Exacerbated Colitis

To directly test the hypothesis that LTβ-LTβR signals drive severe colitis, we crossed two strains to generate double knock out (DKO) mice deficient for LTαβ and LTβR and determined if these mice exhibited augmented DSS-induced colitis progression. In these DKO mice, LIGHT-HVEM interactions occur independently of a possible HVEM competition with LTβR for binding to this ligand. Administration of DSS to $Ltb^{-/-}Ltbr^{-/-}$ mice resulted increased weight loss compared to WT controls (**Figure 4A**). This increased weight loss correlated with decreased colon length in $Ltb^{-/-}Ltbr^{-/-}$ mice (**Figure 4B**). Further, histological analysis of the colon and cecum revealed that $Ltb^{-/-}Ltbr^{-/-}$ mice exhibited an increased histology score, indicative of increased inflammation in the tissue (**Figures 4C–E**). The exacerbation of colitis in the combined absence of LTαβ and LTβR disproves the hypothesis that in the absence of LIGHT increased binding of LTβ to LTβR drives disease.

Mice Deficient in HVEM and LTβR Exhibit Exacerbated Colitis

It is possible that LTβR and HVEM compete for LIGHT and that in the absence of LTβR, LIGHT binding to HVEM drives inflammation. In this proposed mechanism, LTβR acts in part as a sink for LIGHT protein, preventing it from binding HVEM to the fullest extent. To test this mechanism, we analyzed DSS colitis in $Ltbr^{-/-}Hvem^{-/-}$ DKO mice. Notably, if HVEM signals drive severe inflammation in the absence of LTβR, mice deficient in both receptors should be protected from

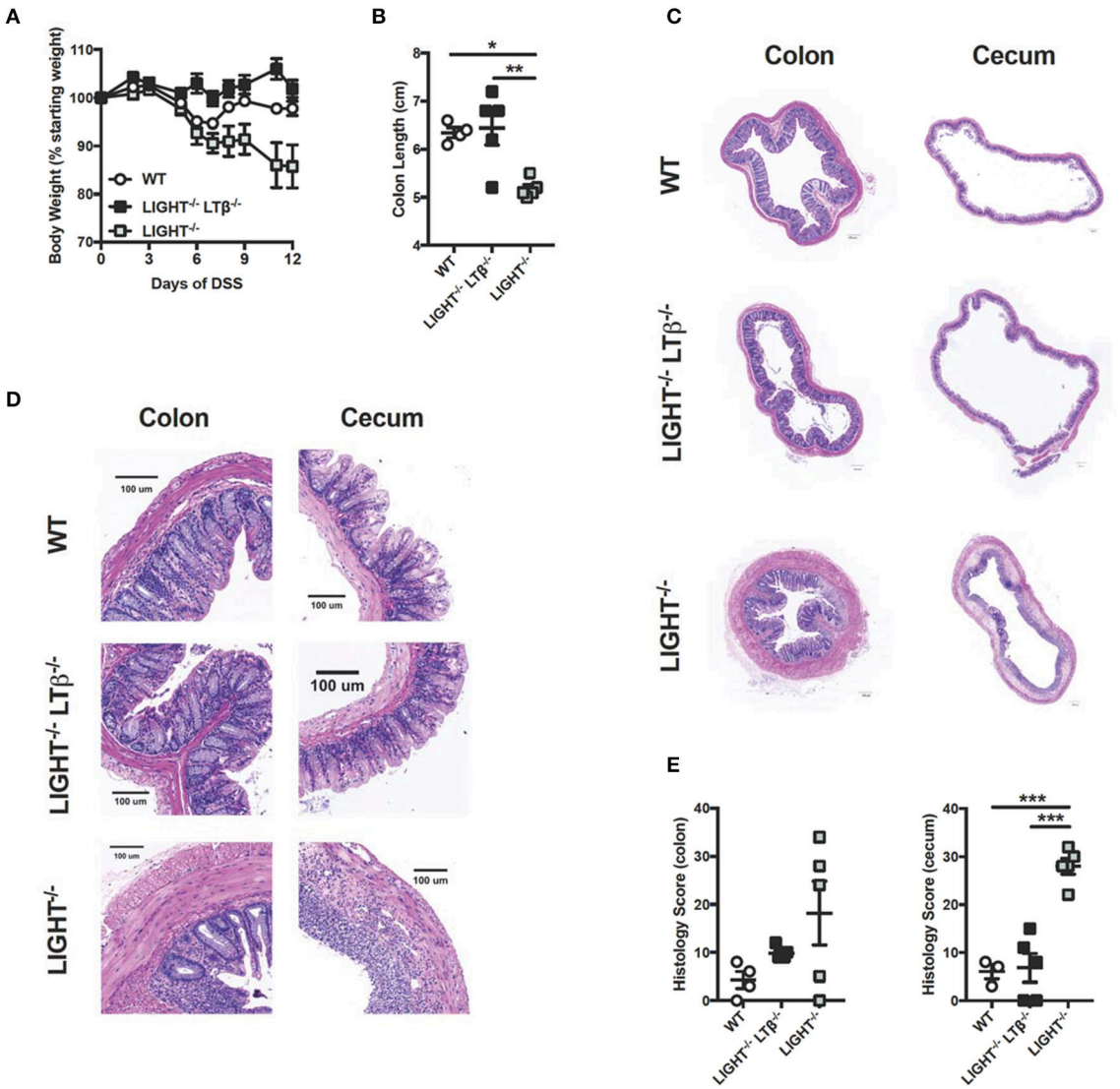

FIGURE 3 | Combined deficiency of LTβ and LIGHT rescues from severe colitis. Eight week-old *Light*$^{-/-}$*Ltb*$^{-/-}$ ($n = 5$), *Light*$^{-/-}$ ($n = 5$) and WT ($n = 4$) male mice received 3% DSS in the drinking water. **(A)** Weight loss was monitored daily. **(B)** Following termination of experiment, colon lengths were measured. **(C)** Representative H&E staining of cecum and distal colon cross-section (scale bar = 200 μM). **(D)** Magnified section of cecum and distal colon cross-section from **C** (scale bar = 100 μM). **(E)** Histologic scoring of ceca and distal colons. Data represent mean ± S.E.M. One way ANOVA with Tukey's correction, $^{*}p < 0.05$, $^{***}p < 0.001$.

exacerbated DSS-induced colitis. However, after administration of DSS, *Ltbr*$^{-/-}$*Hvem*$^{-/-}$ mice exhibited increased weight loss after DSS treatment, similar to *Ltbr*$^{-/-}$*Hvem*$^{het(+/-)}$ mice (**Figure 5A**). *Ltbr*^{het}*Hvem*$^{-/-}$ mice displayed weight loss similar to *Ltbr*^{het}*Hvem*^{het} mice. In addition to increased weight loss compared to LTβR^{het}HVEM^{het} mice, subsequent studies found that *Ltbr*$^{-/-}$*Hvem*$^{-/-}$ mice also exhibited increased weight loss compared to WT mice (**Figure 5B**). Additionally, this increased weight loss correlated with shorter colon lengths (**Figure 5C**). In sum, these data suggest that LIGHT does not signal through HVEM to drive severe inflammation in the absence of LTβR.

DISCUSSION

Inflammatory bowel disease affects over 1.5 million Americans, and effective treatment options for this debilitating autoimmune disorder are lacking (21). While anti-TNF therapies have proved efficacious in certain patient populations, more therapeutic approaches are clearly needed (22). Given that other members of the TNF superfamily have been shown to play a role in a mouse model of colitis, we aimed to interrogate the LIGHT/LTαβ/LTβR/HVEM signaling network to determine if one or more of the involved components displays an important role in DSS-induced colitis pathogenesis. Analysis of this

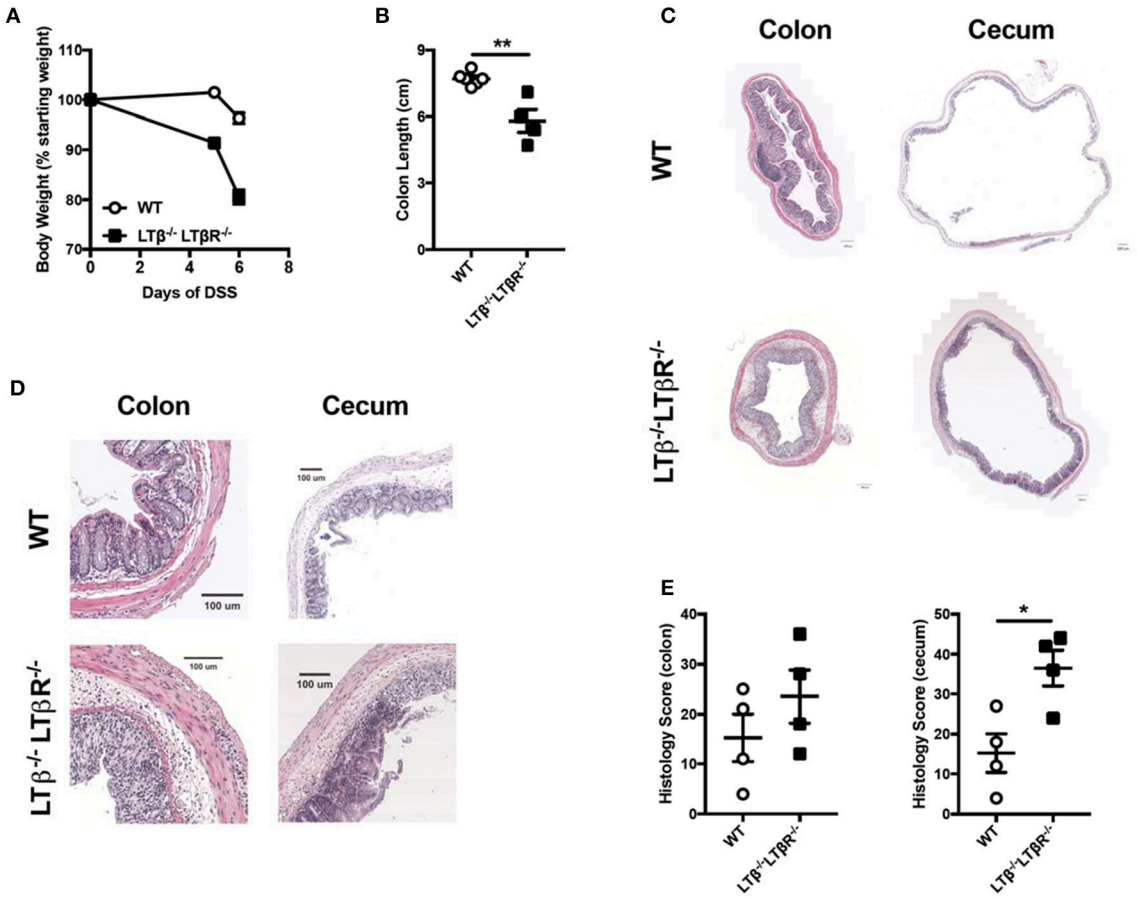

FIGURE 4 | Combined deficiency of LTβ and LTβR causes exacerbated DSS-induced colitis. Eight week-old $Ltb^{-/-}Ltbr^{-/-}$ ($n = 4$) and WT ($n = 4$) female mice received 2% DSS in the drinking water. **(A)** Weight loss was monitored daily. **(B)** Following termination of experiment, colon lengths were measured. **(C)** Representative H&E staining of cecum and distal colon cross-section (scale bar = 200 μM). **(D)** Magnified section of cecum and distal colon cross-scetion from **C** (scale bar = 100 μM). **(E)** Histological scoring of ceca and distal colons. Data are representative of one of two individual experiments. Data represent mean ± S.E.M. Student's *t*-test, $^{*}p < 0.05$, $^{**}p < 0.001$.

signaling cascade revealed a complex interaction between ligands and receptors that is influenced by which members are present.

Our previous work demonstrated that deficiency of LIGHT leads to exacerbated DSS-driven colitis (5). This suggested that LIGHT plays a protective role, directly or indirectly, in the context of colitis pathogenesis. Herein, we demonstrate that the likely LIGHT binding receptor for this phenomenon is LTβR. Similar to LIGHT deficient mice, genetic ablation of LTβR resulted in exacerbated colitis with a similar overall phenotype, consistent with previous reports (23, 24). Conversely, removal of the other LIGHT receptor, HVEM, had no effect on DSS-induced colitis, even in the absence of LTβR (**Figure 5**). It remains to be determined which cells in the colon are critical for LIGHT and LTβR expression. Determining the critical LIGHT expressing cell type in disease models has proven difficult given that antibodies reactive for mouse LIGHT are of insufficient quality. On the other hand, it is well known that epithelial, stromal and myeloid cells express LTβR, but lymphocytes do not (5, 18, 25). We found that LTβR mRNA is expressed by fibroblasts, neutrophils and other $CD11b^{+}$ cells at steady-state and during DSS-induced colitis (5). Examining cell type specific LTβR knockouts in the context of disease could aid in identifying which cell type(s) is most important and help to improve our understanding of the mechanisms underlying severe disease. Thus, although the full mechanism remains elusive, our data strongly suggest that signaling of LTβR via LIGHT is necessary for protection from exacerbated DSS-induced colitis.

On the other hand, $Ltb^{-/-}$ and $Light^{-/-}Ltb^{-/-}$ mice are protected from exacerbated colitis pathogenesis. The fact that $Ltb^{-/-}$ mice do not display differential colitis pathogenesis led to our initial belief that LTαβ does not contribute to DSS-induced colitis. However, if this were the case then $Light^{-/-}Ltb^{-/-}$ mice should show a similar phenotype to $Light^{-/-}$mice, which is not the case. This could indicate that in the absence of LIGHT, LTαβ drives inflammation through LTβR. However, as demonstrated in **Figure 4**, $Ltb^{-/-}Ltbr^{-/-}$ mice exhibited exacerbated colitis, which opposes this hypothesis.

The fundamental conundrum is that mice deficient for LTβR expression have a different phenotype in DSS colitis from mice deficient for both of its known ligands, LIGHT and LTβ. This

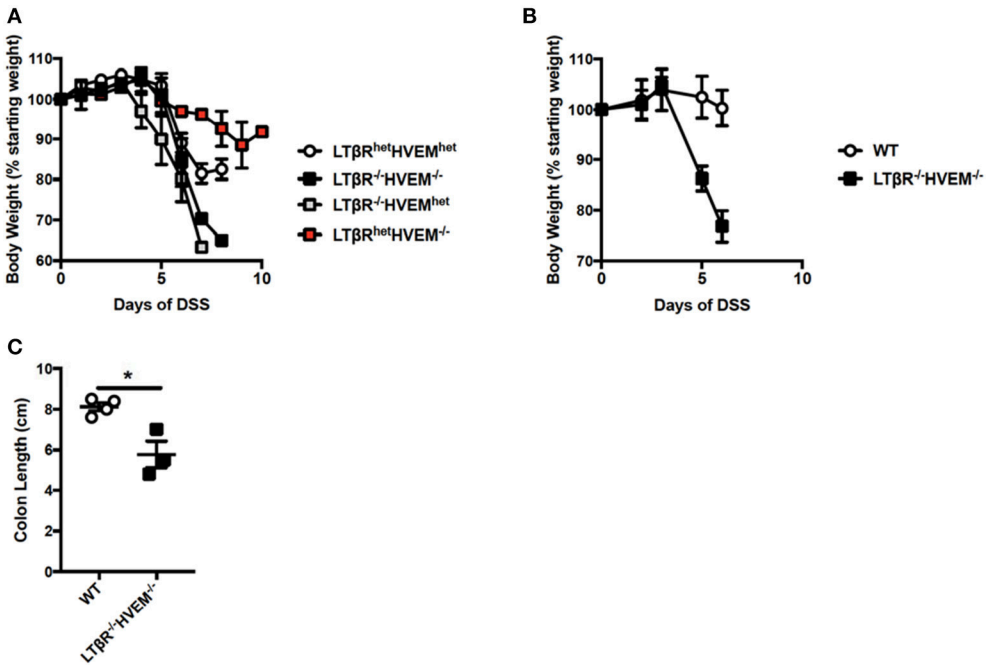

FIGURE 5 | Severe colitis in mice deficient for LTβR and HVEM. **(A)** Eight week-old $Ltbr^{-/-}Hvem^{-/-}$ ($n = 3$), $Ltbr^{het}Hvem^{-/-}$ ($n = 3$), $Ltbr^{-/-}Hvem^{het}$ ($n = 3$) and $Ltbr^{he-}Hvem^{het}$ ($n = 4$) male mice received 3% DSS in the drinking water. Weight loss was monitored daily. **(B,C)** Eight week-old $Ltbr^{-/-}Hvem^{-/-}$ ($n = 3$) and WT ($n = 4$) control female mice received 2.5% DSS in the drinking water. **(B)** Weight loss was monitored daily. **(C)** Following termination of experiment, colon lengths were measured. Data represent mean ± S.E.M. Student's *t*-test, $^*p < 0.05$.

cannot be explained by a compensating effect of increased signaling by LIGHT-HVEM when LTβR is missing (**Figure 5**). We cannot rule out the possibility of an indirect effect, such that when *Ltb* is deleted there is increased LTα3 expression. This cytokine can signal through both TNFR1 and TNFR2, and the increased signaling could be protective. It is uncertain, however, why increased LTα3 would be protective in the context of LIGHT deficiency but not LTβR deficiency. It is also possible that there is another player in this signaling network, either an additional receptor for LTβ or another ligand for LTβR. Recent findings have indicated that some TNFSF receptors have multiple ligands, including HVEM and 4-1BB (26), including binding partners for these receptors that are not TNFSF proteins. In this regard, it is of interest that LTβR deficiency has a greater effect on lymph node genesis than either LTβ deficiency or LIGHT deficiency, suggesting LTβR might integrate other signals. We note that the absence of LIGHT has little or no effect on lymph node genesis, in the absence of LTβ caudal and mesenteric lymph nodes are still present, while all lymph nodes require LTβR (14). Additionally, we cannot rule out a technical issue in comparing different gene deficient strains, such as an effect of a gene deletion on a nearby gene or the presence of a few non-C57BL/6 genes remaining in one of the strains not created on the C57BL/6 background, despite extensive back crossing. In sum, the LIGHT/LTβR signaling critically contributes to DSS-induced colitis, but is subject to a degree of opposing regulation in the absence of LTαβ. Further work is needed to fully delineate this signaling network and how it affects intestinal disease in a cell-type specific manner.

METHODS

Animals

All mice were bred and housed under specific pathogen-free conditions at the La Jolla Institute for Allergy and Immunology (La Jolla, CA). All mice were on the C57BL/6J background. C57BL/6J were originally purchased from the Jackson Laboratory. HVEM mice were bred and described previously (27). $Ltbr^{-/-}$ mice were generated by crossing mice with a CMV-cre construct (Jackson Laboratories; Bar Harbor, ME) to $Ltrb^{fl/fl}$ mice, that were previously described (18). LIGHT deficient mice ($Tnfsf14^{-/-}$) and $Ltb^{-/-}$ mice were provided by Dr. Klaus Pfeffer (University of Düsseldorf, Germany) (28). Double mutants were created by inter-crossing of the above strains. All procedures were approved by the La Jolla Institute for Allergy and Immunology Animal Care and Use Committee.

Chronic Dextran Sulfate Sodium-Induced Colitis

Mice received 2.5% DSS (Affymetrix) in the drinking water for a maximum of two cycles. As previously described, 1 cycle is comprised of 5 days of water plus DSS and 2 days with regular drinking water without DSS (29). Given

that both male and female mice develop robust colitis after DSS administration (30), both sexes were used for separate experiments but never mixed, as noted in the figure legends. Body weight and appearance were monitored daily. Mice were euthanized in compliance with our animal protocols within 24 h of losing more than 20% of their starting body weight.

Histology

Upon termination of an experiment, cecum and colon were isolated. Following measurement of colon length, a piece of distal colon and cecum were fixed in zinc formalin (Medical Chemical Corporation). Following paraffin embedding, fixed tissue was stained with hematoxylin and eosin. Resulting slides were then blinded and scored according to previously described criteria (5). Representative images were selected from 5 or more sections per organ, generated on an Axioscan Z1 platform (Zeiss) with a 40x objective in automatic scan mode and Zeiss Zen 2.3 software. Scale bars represent 200 μm for cross-sections and 100 μm for magnified images.

Statistical Methods

All data were analyzed using GraphPad Prism 7 software. Statistical significance was determined by unpaired Student's *t*-test for direct comparisons when there were two groups. For determination of statistical significance for three or more groups, one-way ANOVA was employed with Tukey's *post hoc* test to assess differences between specific groups. All data are displayed as mean with standard error of the mean (S.E.M.).

AUTHOR CONTRIBUTIONS

MK and AT contributed to the design of the study. DG, SZ, PK, EV, TR, and VM performed the experiments. DG, SZ, PK, and MK analyzed the data. DG and MK drafted the manuscript.

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Divergent Innate and Epithelial Functions of the RNA-Binding Protein HuR in Intestinal Inflammation

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HuR is an abundant RNA-binding protein acting as a post-transcriptional regulator of many RNAs including mRNAs encoding inflammatory mediators, cytokines, death signalers and cell cycle regulators. In the context of intestinal pathologies, elevated HuR is considered to enhance the stability and the translation of pro-tumorigenic mRNAs providing the rationale for its pharmacological targeting. However, HuR also possesses specific regulatory functions for innate immunity and cytokine mRNA control which can oppose intestinal inflammation and tumor promotion. Here, we aim to identify contexts of intestinal inflammation where the innate immune and the epithelial functions of HuR converge or diverge. To address this, we use a disease-oriented phenotypic approach using mice lacking HuR either in intestinal epithelia or myeloid-derived immune compartments. These mice were compared for their responses to (a) Chemically induced Colitis; (b) Colitis- associated Cancer (CAC); (c) T-cell mediated enterotoxicity; (d) *Citrobacter rodentium*-induced colitis; and (e) TNF-driven inflammatory bowel disease. Convergent functions of epithelial and myeloid HuR included their requirement for suppressing inflammation in chemically induced colitis and their redundancies in chronic TNF-driven IBD and microbiota control. In the other contexts however, their functions diversified. Epithelial HuR was required to protect the epithelial barrier from acute inflammatory or infectious degeneration but also to promote tumor growth. In contrast, myeloid HuR was required to suppress the beneficial inflammation for pathogen clearance and tumor suppression. This cellular dichotomy in HuR's functions was validated further in mice engineered to express ubiquitously higher levels of HuR which displayed diminished pathologic and beneficial inflammatory responses, resistance to epithelial damage yet a heightened susceptibility to CAC. Our study demonstrates that epithelial and myeloid HuR affect different cellular dynamics in the intestine that need to be carefully considered for its pharmacological exploitation and points toward potential windows for harnessing HuR functions in intestinal inflammation.

Keywords: cytokine regulation, intestinal inflammation and cancer, post-transcriptional regulation, inflammatory bowel disease, animal models of human disease

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INTRODUCTION

The effective post-transcriptional control of mRNAs involved in mucosal responses and immunity by specific RNA-binding proteins is emerging as important for the homeostasis of intestinal epithelial barrier (1). In the context of intestinal inflammation, cytokine and other inflammatory mRNAs are actively controlled by degradation or translation regulation within the immune repertoires that act to safeguard the intestinal epithelium and to provide tolerance to commensal microbiota (2, 3). Similarly, the ever-changing life-cycle of resident intestinal epithelial cells and their response to commensal and threatening signals may be affected by selective post-transcriptional programs altering the fate of mRNAs involved in differentiation, proliferation and death (1). The lack of prudent post-transcriptional controls in either mucosal immune cells or constituent epithelia can alter their cross-talk to provide the impetus for uncontrollable inflammation, intestinal degeneration and cancer.

A prototypical system of post-transcriptional control that is seemingly active in immune and epithelial layers of the intestine concerns mRNAs containing U-and AU-rich elements in their untranslated termini. As exemplified by the paradigm of the ARE-containing TNF mRNA (2–5), such mRNAs encode for a variety of factors relevant to the responses of the intestinal mucosa and are targeted by a class of RNA binding proteins (RBPs) such as the decay promoting family of Zfp36 proteins and hnRNPD/AUF1; translational inhibitors like TIA1 and TIAR; several associated factors and non-coding RNAs; and the pleiotropic factor Elavl1/HuR. Although most of these ARE-associated factors have gained attention as post-transcriptional modifiers in immunopathology (2), Elavl1/HuR is a well-studied example of clinical relevance to intestinal diseases. HuR is a ubiquitous and conserved RBP that binds to U- and AU- rich RNA motifs and shuttles between nucleus and cytoplasm via signal-induced interactions (6, 7) with nuclear export/import adaptors. In the nucleus, HuR may act to modify the maturation and/or processing of coding and non-coding RNAs (8). In the cytoplasm, HuR may promote the synthesis of inflammatory mediators and tumor-associated proteins by stabilizing the corresponding mRNAs thus acting as ***a pro-inflammatory and pro-tumorigenic factor*** (9). However, it may also alter ARE-mediated mRNA translation and turnover differentially, through synergies and antagonisms with other RBPs, miRNAs and long non-coding RNAs (10–12). HuR binds to an extensive list of RNAs (13, 14), and as such may appear as non-discriminatory in terms of pathologic and homeostatic functions. However, the expanding list of tissue restricted mouse mutations (15–24) reveal that HuR can have-sometimes unpredictable—tissue and signal restricted functions.

With respect to intestinal epithelia, a pathologic role for HuR is supported by the clinical connection of its –total or cytoplasmic—elevation to intestinal cancers. These elevations correlated: (a) positively to the degree of transformation, malignancy and tumor angiogenesis; and (b) negatively to the overall survival of patients with rectal and colonic tumor (25–32). A multitude of cellular studies connected HuR to the stabilization of mRNAs promoting cancer traits like tumor cell proliferation, survival, tumor angiogenesis, and metastasis (33, 34). Most experimental data stem from such cellular studies or from the xenotransplantation of tumor epithelia, and point toward the regulation of cell cycle and proliferation as *the* major function regulated by HuR in intestinal epithelia (29). A set of genetic studies supported this notion; when HuR was deleted inducibly post-birth in intestinal epithelia, its loss hampered epithelial regeneration under several conditions whereas models of colitis associated cancer (CAC) and APC driven cancers showed signs of remission (16). A pathologic profile of elevated total HuR has been detected in histological samples from active IBD-namely Crohn's disease and Ulcerative Colitis. Collectively, these observations rendered HuR as target of clinical relevance in intestinal disease and colon cancer; and culminated the search for specific pharmacological modulators inhibiting HuR's translocation or binding (35).

However, disparate data did point toward a differential role for HuR in both the intestinal epithelium and mucosal immunity. When HuR is deleted acutely post-birth, its loss leads either to barrier degeneration and progenitor loss (if deletion is systemic) or villus shortening (if deletion is IEC-restricted) connecting to developmental changes in cell survival and death (15, 16). However, when deleted earlier and continuously in IECs, its loss induces a partial shortening of jejunum villi but does not affect intestinal ontogeny and barrier function (18); however and depending on the challenge, the latter group of mice reveal problems either in regeneration or cadherin-mediated junctions (18, 36). These studies provide support for HuR functions in IEC ontogeny, survival, and barrier integrity. In the context of IBD, and although HuR appears elevated in inflamed epithelia, its expression in transitory dysplastic epithelia connecting IBD to CAC seems to reduce to normal levels (37). With regard to its function in inflammatory cells, HuR's sole function as an RNA activator has been revisited, primarily because genetic studies on innate immune effector cells did not fully support this notion. In mice rendered deficient for HuR in myeloid cells and the immune derivatives, inflammation was not suppressed but rather enhanced to a pathologic extent (20, 38). With respect to mucosal responses, these mice displayed an exacerbated response to the model of chemical colitis and –most profoundly- to CAC (20). The opposite experiment was even more revealing with elevated macrophage HuR suppressing pro-inflammatory reactions including chemical colitis and CAC (17, 20).

The consideration of HuR inhibition as a therapeutic strategy against intestinal inflammation and cancer was most profoundly challenged during the pre-clinical testing of one of its pharmacological inhibitors (37). In models of familial CRC, HuR inhibition appeared effective in suppressing tumor growth and progression. In the context of inflammatory CAC, HuR inhibition not only failed but also exacerbated tumor progression.

As such the current data as to whether HuR plays a pathologic or protective role in intestinal inflammation appear ambiguous possibly due to divergent cell type specific effects. Here we focus on two prototypical subsets located in the intestinal mucosa, involved in inflammatory disease—myeloid-derived immune cells vs. intestinal epithelia- and dissect the

functions of HuR in several models of pathologic and beneficial inflammation. Our data reveal that the cell-restricted functions of HuR drive divergent, non-overlapping and context-dependent inflammatory responses in the intestinal mucosa, altering the clinical outcome of intestinal disease that need to be considered for clinical intervention.

MATERIALS AND METHODS

Mice and Study Approvals

Elavl1$^{fl/fl}$, *VillinCre*$^{+}$, *LysozymeCre*$^{+}$, *LysozymeCre*$^{+}$ *HuR*$^{fl/fl}$, and *Tnf*$^{\Delta ARE/+}$ mice have been previously described (4, 17, 20, 39). To generate *VillinCre*$^{+}$ *Elavl1*$^{fl/fl}$ mice, *Elavl1*$^{fl/fl}$ mice were crossed with *VillinCre*$^{+}$. For *TgATFHuR*$^{+}$ mice the complete cDNA sequence for human HuR was obtained from the IMAGE clone number 2901220 (GenBank accession number BC003376) and was subcloned in-frame to an AviTEVFLAG (ATF) sequence for the expression of an N-terminally tagged form. Fusion was verified by sequencing. Subsequently, the ATF-HuR cDNA was used for the generation of the transgenic construct. The device was removed from the carrier vector via the digestion of flanking PmeI sites. For the production of transgenic mice, fertilized C57Bl/6J zygotes were coinjected with the transgenic device procedures employed by the INFRAFRONTIER-GR/Trangenesis Unit of BSRC "Al. Fleming" (http://www.infrafrontier.gr/). To identify and maintain transgenic founder mice, tail DNA was used for Southern blot hybridizations and PCR with specific probes and primers to detect the transgene. Five founder lines were identified; line Tg6105 is the one employed in this study. All mouse lines were maintained in a C57BL/6J background and in the animal facilities of the BSRC "Alexander Fleming" under specific-pathogen free conditions. All experiments were performed with mice aged between 8 and 16 weeks, with a minimum of 3 mice per genotype and a maximum of 30, according to the experiment, as indicated in the figure legends. Littermates were used as controls. Animal experiments were approved by the Prefecture of Attica (licenses #5995/2012, 4371-4376/2014, #6198/2017, #3547/2018, #2824/2018) in accordance to national legislation and the European Union Directive 63/2010.

DSS Colitis and CAC

Mice 6–8 week old were fed *ad libitum* for up to 2 cycles with water containing 1.5–2% (wt/vol) DSS (MW 40,000 kDa; MP Biomedicals Inc.) for 6 days, followed by intervals of 15 days on regular water. For induction of CAC, 6–8-week-old mice were injected i.p. with 20 mg/kg mouse DMH (SIGMA-Aldrich). After 5 days, 1.5–2% (wt/vol) DSS was provided in drinking water for 6 days, followed by 15 days of regular water. This cycle was repeated twice. During the course of the experiment of acute inflammation or tumorigenesis, mice were monitored daily for body weight, diarrhea, and rectal bleeding. Values were used for calculation of DAI (40, 41). Mice were sacrificed at indicated time points or at the end of the protocol (15 weeks) for the isolation of colonic tissue. Tumor sizes were measured using an electronic Vernier caliper.

Colon Explant Cultures

Colon explant cultures were performed as previously described (20). Briefly, whole colons were opened longitudinally, washed with PBS supplemented with 20 mg/ml gentamycin to remove residual intestinal bacteria and cut in 1.5-cm pieces into a 48-well plate containing 500 μl RPMI-1640 per well. Tissues were incubated at 37°C, 5% CO_2 for 24 h, and supernatants were collected for cytokine/chemokine ELISA measurements.

Immunoblotting and ELISAs

For Western blots, lysates were analyzed on SDS-polyacrylamide gels (7–14%), along with protein molecular weight markers (SM0431; Nippon MWP03; Fermentas, Thermo Scientific) and blotted onto nitrocellulose membrane (GE Healthcare). After blocking with 5% milk or 4% BSA in TBS-Tween 20 buffer, membranes were incubated with primary and HRP-conjugated secondary antibodies; signals were visualized by enhanced chemiluminescence (ECL; GE Healthcare) using films or a ChemiDocTMXRS+ System with Image LabTM software. Antibody used: HuR (Santa Cruz, sc-5261). Supernatants were analyzed via specific ELISAs (Peprotech; eBioscience) or Cytometric Bead Arrays (BD Biosciences).

Histology and Immunohistochemistry

Dissected intestines were mounted onto a solid surface and fixed in formalin 10% (pH = 6.9–7.1) O/N at 4°C before processing for paraffin embedding. At least 2 serial sections of 5 μm were stained with: hematoxylin and eosin (H&E) for general histology. Periodic Acid Schiff and Nuclear Fast Red stains were carried out using standard protocols. For Immunohistochemistry of paraffin embedded tissues sections were deparaffinized, hydrated, and treated with boiling Citrate buffer pH 6.0 under microwaves for 20 min. Sections were blocked in diluent (0.1% gelatin, 0.5% Triton-X, 0.05% Tween-20 in TBS) supplemented with 1%BSA, 3% FBS, and 3% H2O2 for 1 h. Primary (HuR 3A2, SantaCruz, sc-5261; Lysozyme, DAKO Cytomation, A0099; Ki67, Abcam, ab15580 (*TgATFHuR*$^{+}$ characterization) or Thermo, MA5-14520 (*C. rodentium* experiment) and HRP-conjugated secondary antibodies (Southern Biotech) were incubated in diluent for 1–24 h. Visualization was performed with DAB (Vector) and counterstained with hematoxylin; Photomicrographs were acquired using a Nikon ECLIPSE E200 microscope equipped with a Nikon Digital Sight DS-5M digital camera.

T Cell Mediated Enteropathy

Mice received a single i.p. injection of 50 μg hamster anti-CD3 antibody (2C11; LEAF grade-Biolegend) in saline and were fasted 16 h before their sacrifice. Mice were sacrificed at different time points for histology and luminal exudate collection. For the latter, the whole intestine was removed, the small intestine was cut in two halves and its luminal exudate was collected with two flushes of 5 ml PBS, 5% FBS for each half. Fecal pellets were gently removed from the colon using forceps before collecting the luminal exudate in 5 ml of the same buffer. For the flow cytometric detection of cell death, cells were stained with PI or 7AAD (Sigma) and Annexin-V (eBioscience) and were analyzed

with a FACSCanto II flow cytometer. Analysis of luminal cells was performed as in Piguet et al. (42).

Citrobacter Rodentium Infections

C. rodentium strain DBS100 (ATCC 51459) was cultured to exponential phase ($1 < OD_{600} < 1.4$) in Luria-Bertani Broth overnight at 37°C with shaking at 200 rpm. Culture was spun 10 min at 3,000 g at 4°C the pellet was washed twice in ice-cold PBS and cells were resuspended in ice-cold PBS. Eight to twelve weeks old mice received an oral gavage of 2×10^9 CFUs in 200 μl suspension in PBS after 8 h of fasting. To reduce mouse-to-mouse transmission, mice were caged single or in duplet. For CFU determination in feces, 20–60 mg of fecal matter was collected, weighed and homogenized in 1 ml PBS, serial dilutions were prepared in PBS in 96-well plates and single drops of 10 μl from each dilution were plated in duplicate on McConkey-agar plates before overnight incubation at 37°C. CFUs were counted from the same dilution for every sample and were normalized with respective fecal weight. *C. rodentium* colonies were identified as pink with a narrow white trim. Experiments with *C. rodentium* were carried out in the Biosafaty Level 2 facility of "Alexander Fleming."

Microbiome Analysis

Fecal samples were collected fresh, weighed, and homogenized in 1 ml PBS at room temperature before transfer to −80°C until use. Samples were thawed and centrifuged at 100 g for 3 min. The supernatant was mixed 1:1 with 2x lysis buffer (8% SDS, 0.2 M DTT, 0.2 M Tris-HCl pH 7.5) and subjected to heating at 95°C for 5 min and pulsed probed sonication 30 s × 2 with 45 s interval. The sample was finally cleared from insoluble material by centrifugation at 14,000 g for 20 min. The protein extract was subjected to trypsin digestion using the sp3 procedure (single pot protocol) (43). The resulting peptides mix were analyzed with LC-MS/MS using a 4 h gradient as described in Elkouris et al. (44). Methods for data processing is provided in **Supplementary Material**.

Histopathological Scoring of DSS Colitis and CAC, T-Cell Mediated Enteropathy, and *C. rodentium* Infection

The histological scoring system of DSS-induced colitis and CAC was described in Yiakouvaki et al. (20). For DSS-colitis it included severity and extent of inflammation (ranging from 0 to 3), crypt damage (from 0 to 4), and percentage of organ affected (from 0 to 4). For *C. rodentium* colitis, inflammation scoring system was as for DSS and crypt damage was scored in a scale of 0–4 (none, rare, occasional, frequent, extensive) for frequency of observed damaged crypts. For T-cell mediated enteropathy, the histological score was calculated as for DSS-colitis. For colonic crypt length measurements, using the 10x objective lens the whole distal colon was divided in optic fields, photos were taken from every field that had at least one well-oriented crypt (visible from base to apical opening) and the length of at least 12 well-oriented crypts was measured, using the ImageJ image processing software, and was averaged for each mouse. Same strategy was followed for the measurement of small intestinal villi and crypts at the ileum. Well-oriented villi with their associated crypts were measured. All histological assessments were performed in a blinded fashion.

Crypt Proliferation

Paraffin sections were processed for staining with anti-Ki67 antibody. Labeling index was defined as the frequency of $Ki67^+$ cells in each crypt and/or villus compartment for each position, from position 1 (base of crypt) to position 40. Fifteen well-oriented crypts were examined for each mouse and positive counts for each position were summed and expressed as percentage of the total cells counted.

RNA Extraction and qRT-PCR

Tissues were dissected, transferred in 0.5 ml Tri Reagent (MRC, USA), snap frozen in liquid nitrogen and stored at −80°C until RNA extraction. Total RNA was extracted after homogenization using an Ultra-Turrax homogenizer (IKA, Germany) according to manufacturer's instructions. RNA integrity was verified with agarose gel electrophoresis and concentration was measured with a Nano-Drop device. One to two μg of total RNA was subjected to DNase treatment (RQ1 RNase-Free DNase, Promega, Madison, WI, USA) and reverse transcribed with M-MLV Reverse Transcriptase (Promega, Madison, WI, USA) using an oligo(dT) primer. qPCR was performed using EvaGreen SsoFast mix (Bio-Rad, Hercules, CA, USA) on a RotorGene 6,000 machine (Corbett Research, Qiagen, Venlo, Netherlands). Expression was normalized to β2-microglobulin. The relative mRNA expression in the test samples was calculated as the difference from the control values that were assigned an arbitrary expression value of 1, using Bio-Rad RelQuant (Bio-Rad). Primers used are provided in **Supplementary Material**.

Intestinal Epithelial Cell and Immune Cell Isolation

For IECs, intestines were opened longitudinally, washed with PBS and cut into 1.5 cm pieces that were washed with inversion in tubes containing HBSS with 2% FBS and 1 mM HEPES. Pieces were transferred to 20 ml HBSS supplemented with 2% FBS, 2 mM HEPES, 5 mM EDTA, and 1 mM DTT and placed at a rotary and at 37°C for 1 h. After setting for 15 min, suspensions were filtered through sterile gauze and centrifuged at 1,200 rpm for 5 min. Epithelial cells were isolated via centrifugation through 25–40% discontinuous Percoll gradient at 600 g for 15 min. The isolated fractions were then used for lysis and immunoblotting. The isolation of bone marrow derived macrophages (BMDMs) and non-adherent splenocytes were performed as in Papadaki et al. and Yiakouvaki et al. (19, 20). Briefly, for BMDMs, bone marrow was isolated from tibias and femurs by flushing, treated with Gey's solution to remove erythrocytes and cultured for 8 days in RPMI (5% FBS) supplemented with antibiotics, glutamine and supernatant from L929 cell culture. These were used for immunoblots or were stimulated with LPS (100 ng/ml) for 24 h for measurement of cytokines. For non-adherent splenocytes, cells were collected using standard procedures and were stimulated with PMA/ionomycin (10/500 ng/ml) for 24 h.

Ribonucleoprotein Immunoprecipitation (R-IP)

Four colonic tumors, of similar size, per mouse from day 60 DMH/DSS treated mice were pooled and snap frozen to create a sample. Three samples per genotype were used. R-IP experiments were performed as described previously (45). Briefly, cells were lysed in buffer containing 100 mM KCl, 25 mM EDTA, 5 mM $MgCl_2$, 10 mM HEPES, 0.5% Nonidet P40, 2 mM DTT, 0.2% vanadyl ribonucleoside complex (Invitrogen) and 100 U/ml RNAse OUT (Invitrogen). Antibody coated beads (agarose or protein A-sepharose) were washed and maintained in 750 μl of NT-2 buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM MgCl2, and 0.05% Nonidet P40). For IP, 200 μl of lysate were loaded onto the beads and incubated for 4 h on a rotary at 4°C. Subsequently, beads were washed five times in NT-2 buffer plus two times in NT-2+1M urea and finally resuspended in NT-2. Ten microliters of samples were removed for immunoblot verification and the remaining were used for RNA isolation via proteinase K treatment, phenol/chloroform extraction and precipitation. Antibodies included unmodified anti-FLAG (M2; Sigma) and mouse immunoglobulin antibodies (Santa Cruz and R&D). Methods for Microarray (Chip) Profile Analysis is provided in **Supplementary Material**.

Statistical Analysis

All data were analyzed with Graphpad Prism 6.01 (Graphpad Software). Appropriate statistical tests for each experiment are indicated within the figure legends except for R-IP-Chip data analysis where statistical analysis is provided in **Supplementary Material**.

RESULTS

A Similar Role for Intestinal and Myeloid HuR in the Control of Chemically Induced Colitis but a Differential Role in CAC

To dissect the functions of HuR in intestinal inflammation, we compared the effects of its deletion in intestinal epithelium to those incurred by its deletion in myeloid-derived immune compartments. This entailed the comparative analyses of mice harboring a loxP flanked $Elavl1^{fl/fl}$ allele (46) rendered as inactive either (a) in intestinal epithelial cells by means of a *Cre* gene driven by a Villin promoter ($VillinCre^{+}Elavl1^{fl/fl}$ named hereafter as IEC-HuRko mice); or (b) in myeloid-derived cells by means of a *Cre* driven by a Lysozyme M promoter ($LysMCre^{+}Elavl^{fl/fl}$ named hereafter as M-HuRko mice;) (20).

First we analyzed the responses of IEC-HuRko mice to a combined mouse model of colitis and CAC, compared to those we previously published for M-HuRko mice conducted under the same experimental conditions. In this model, disease is induced via a first challenge with a pro-carcinogen (dimethylhydrazine-DMH), and then fed with an inflammatory agent (dextran sodium sulfate-DSS) in repetitive 6-day cycles intermitted by water cycles (47). In control mice, the initial DSS/water cycle led to colitis between days 4–10, which regressed by day 12 (**Figures 1A–C**). A second DSS/water cycle from day 20 led to a more chronic response between days 23 and 35. IEC-HuRko mice appeared more vulnerable to this challenge; during the initial DSS cycle they manifested partial mortality and an earlier macroscopic disease onset relative to controls (**Figures 1A–C**). The fast clinical activity of IEC-HuRko mice correlated with aggressive histological features, including the rapid recruitment of mucosal infiltrates and extensive epithelial ulceration even on the 3rd day of the challenge and a delay in epithelial regeneration/restitution (**Figure 1C**). Similarly, during the chronic phase, IEC-HuRko mice showed persistent disease activity, inflammation and extensive tissue damage (**Figures 1B,C**). Analysis of afflicted colons revealed the heightened accumulation of selective inflammatory mediators in IEC-HuRko mice, namely TNF and IL-6 at the acute phase (day 6), which dropped to control levels during remission (day 12) (**Figure 1D**). Interestingly, other known inflammatory HuR targets like CCL2 and IL10 remained unaltered. mRNAs of inflammatory lymphokines and cytokine effectors, such as *ifnγ*, and of *il4*, *Ptgs-2*/*Cox2*, and *Tgfβ* known to be associated with chronic responses (day 28) were significantly upregulated in the colons of IEC-HuRko mice compared to controls, reflecting an exacerbated chronic inflammatory response (**Figure 1E**). The clinical response of the IEC-HuRko mice to DSS-driven intestinal inflammation phenocopied in part that previously reported for M-HuRko mice [**Figure S1** and (20)]. Differences included the much faster degeneration of the colonic epithelial layer during the third day of the protocol, associated with signs of early mortality in the IEC-HuRko mice, as opposed to the heightened and prolonged responses in M-HuRko mice (**Figures 1B,C** and **Figure S1**). Qualitatively however, our data indicate that epithelial and myeloid HuR share the same end effect toward the control of inflammation and the protection of the epithelial barrier from degeneration during the DSS challenge.

In contrast, the responses of the cell restricted, HuR mutant mice to the tumor phases of the protocol diverged. In the DMH/DSS model, inflammation enhances dysplasia toward CAC after the 40th day of challenge. As published, the increased inflammatory response of M-HuRko mice to the DSS/AOM challenge correlated with increased tumor formation, growth and progression [**Figure S1**, and (20)]. Although the increased inflammation in IEC-HuRko colons would point toward a respective progression in tumorigenesis, we found a decrease in the number of tumors per mouse and a profound decrease in the size of these HuR-deficient tumors (**Figures 1F,G**). Taken together, these data show that in the context of DSS-colitis, and although epithelial and myeloid HuR have a protective role in suppressing inflammation, they have divergent and exclusive roles in CAC signifying their contributions in different inflammatory processes.

A possible explanation for the different tumorigenic response could be that the loss of myeloid HuR elicits different chronic inflammatory responses than those driven by the loss of HuR in IECs. In contrast to the first acute challenge elicited by DSS which drives mainly innate immune responses, the chronic and tumor phases of the model require the additional contribution of the adaptive arm of immunity. To reciprocate such a complex chronic response, we switched to a genetic

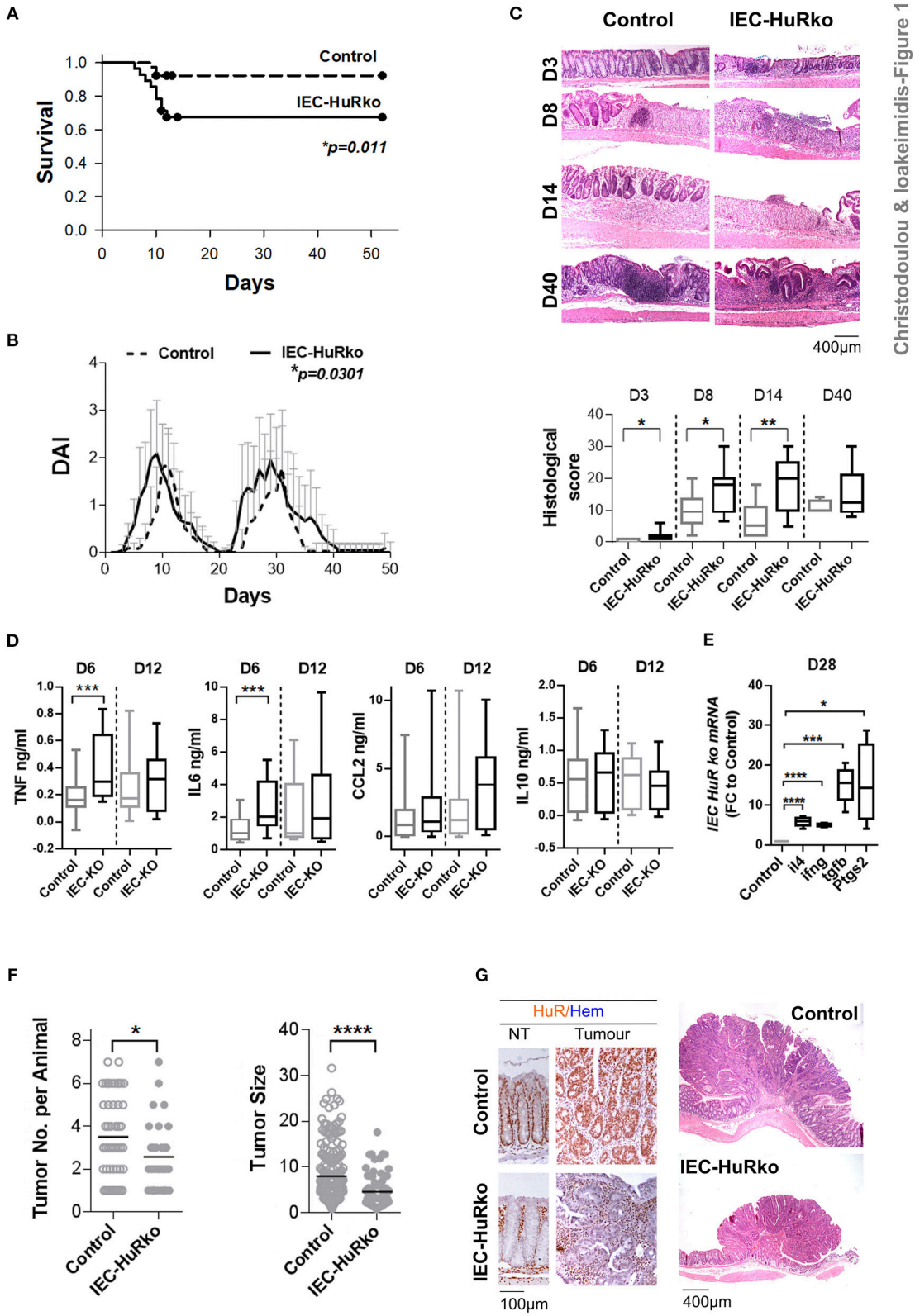

FIGURE 1 | Loss of IEC HuR exacerbates DSS-colitis but attenuates CAC. **(A)** Kaplan-Meier survival analysis following treatment with two rounds of DSS (Days 0 and 20). $n = 30$/group. p-value denotes result of Log-rank test. **(B)** Macroscopic Disease Activity Index (DAI) of the same experimental group as in **(A)**. Line graphs depict

(Continued)

FIGURE 1 | mean values (± SD). *p*-value denotes result from Kolmogorov-Smirnov test. $n = 20$–30 live mice/group. **(C)** Representative histology (H&E) and histological scores from control and IEC-HuRko mice treated with DSS indicating differences in acute inflammation and ulceration (Days 3–8), epithelial restitution (Day 14) and chronic inflammation (Day 40). $n = 8$–12 mice/group. **(D)** ELISA quantification of inflammatory mediators in colon cultures isolated from DSS-treated mice at the indicated time points. Horizontal lines of box plots represent medians. $n = 3$ cultures/group/time point. Student unpaired *t*-test. **(E)** qPCR detection of mRNAs from DSS-treated colons of IEC-HuRko mice on day 28 after treatment expressed as fold change (FC) relative to control mice. *il4*, *interleukin 4*, *ifnγ*, *interferon gamma*, tgfβ, *transforming growth factor beta*, *Ptgs2*, *Prostaglandin-Endoperoxide Synthase 2*. $n = 5$/group. Student unpaired *t*-test. **(F)** Graphs depicting tumor number (left) and tumor size (right) in control and IEC-HuRko mice 60 days after challenge with DMH/DSS. Dots represent individual measurements and horizontal lines represent means. Student unpaired *t*-test. **(G)** (Left panel) Immunohistochemical detection of HuR in colon tumors and adjacent normal tissue (NT). (Right panel) Representative histology (H&E) of colonic tumors in control and IEC-HuRko mice. All box plots show range and median. In all graphs, *,**,***, ****, denote *p*-values < 0.05, 0.01, 0.001, and 0.0001 respectively. Please compare the response of IEC-HuRko mice to the one presented in **Figure S1** for M-HuRko mice.

model of Crohn's like chronic inflammatory bowel disease driven by chronic TNF synthesis, namely the $Tnf^{\Delta ARE/+}$ mouse (4). Due to an induced deletion in the 3′ARE elements of the murine *Tnf* mRNA, the biosynthesis and functioning of TNF in these mice can no longer be modulated at the post-transcriptional level by ARE-binding proteins (including HuR). The uncontrolled production of TNF supports the spontaneous development of inflammatory ileitis in $Tnf^{\Delta ARE/+}$ mice, which becomes histologically evident by the age of 2 months and it progresses continually leading to transmural infiltrates and loss of mucosal architecture by the age of 6 months. The progression of chronic IBD in these mice requires an interplay between innate, adaptive and tissue resident cells (4, 48–50) and as such it approximates the complexities of chronic intestinal inflammation which could be affected differently by the loss of myeloid or IEC HuR. Strikingly however, disease initiation and progression in IEC-HuRko $Tnf^{\Delta ARE/+}$ mice and M-HuRko $Tnf^{\Delta ARE/+}$ mice appeared similar to that in control $Tnf^{\Delta ARE/+}$ mice (**Figure S2**). We did note however a small delay in the damaging effects in transmural inflammation in M-HuRko mice past the 4th month of age. Still, histological hallmarks of IBD were comparable in all mouse cohorts suggesting that chronic inflammatory processes were not affected by the cell-restricted loss of HuR.

An Exclusive Role for Intestinal Epithelial HuR in the Death Response Elicited via Acute Inflammatory Signals

Next, we assessed whether HuR is required to alter susceptibility to inflammatory damage either through enhanced pro-inflammatory innate signals or altered epithelial responses to such signals. Given the well-established role of HuR in proliferative regeneration of the intestine, we sought an acute model of inflammatory enteropathy that cannot be easily compensated by changes in regeneration. The systemic administration of agonistic antibodies targeting the CD3/T-cell receptor complex activates mucosal and submucosal T-cells to mount an aggressive local immune response that targets the intestinal epithelia for apoptosis via Fas, TNF, perforin and p53 signals (51). In control mice, disease is rapidly manifested in the small intestine and proceeds via the detachment of the apical epithelia, villus shortening, inflammation and crypt loss, within a period of 24 h, whereas the colon is only marginally affected (**Figure 2A**). IEC-HuRko mice were more susceptible to this model and displayed such symptoms even at 6 h post challenge whereas at 24 h they developed aggressive ulcerations (small intestine) and—surprisingly—apical degenerations in the large intestine (**Figures 2A,B**). Villus/crypt ratio and colonic crypt length were markedly reduced in IEC-HuRko mice at 24 h and at 6 h, respectively (**Figure 2B**). Enumeration of detached cells in lumen washes and annexin V/PI staining verified the increased shedding of apoptotic enterocytes in the intestines of IEC-HuRko mice (**Figure 2C**). On the other hand, the response of M-HuRko mice in this particular model was indistinguishable from that of the controls (**Figures 2A–C**). These data suggest that HuR in IECs exclusively desensitizes differentiated enterocytes to the death-promoting effects of inflammation thus revealing a first qualitative difference between IEC and myeloid HuR.

Differential Effects of Myeloid vs. Intestinal Epithelial HuR in the Control of *Citrobacter rodentium* Induced Colitis

To gain further insight into the possible role of intestinal and myeloid HuR in the elicitation of inflammation we employed a model of infectious colitis and pathogen control that also serves as a model of IBD observed during the invasion of enteropathic bacteria (52). *Citrobacter rodentium* is a natural pathogen of the intestinal mucosa that causes Transmissible Murine Colonic Hyperplasia and closely resembles the enteropathogenic and enterohaemorrhagic *Escherichia coli* strains (53). After oral administration, the bacterium colonizes the colon enforcing a series of interactive events in the resident tissue and the underlying immune compartments which—if failed—allow the infection to breach the barrier and become systemic. On the one hand, adhesion of the bacterium drives in part apical intestinal epithelial cell death to release infected cells, and this is counteracted by a rapid proliferative response that results in the lengthening of colonic crypts through propagation of transient amplifying cells (52). Underlying, this response, *C. rodentium* is initially phagocytosed by myeloid-derived immune compartments and recognized primarily via NLRP-3 inflammasome pathway leading to the release of pro-inflammatory interleukins. This cascade leads to a complex inflammatory response involving both innate and adaptive immune subsets, thus aiding the clearance of the pathogen and the production of antimicrobial proteins by colonocytes (52).

For our analyses, mutant and control mice were infected with *C. rodentium* and monitored via the estimation of the pathogen load in fecal matter for a period of 25 days; whereas histology was monitored at days 12 and 25 that correspond to peak disease and

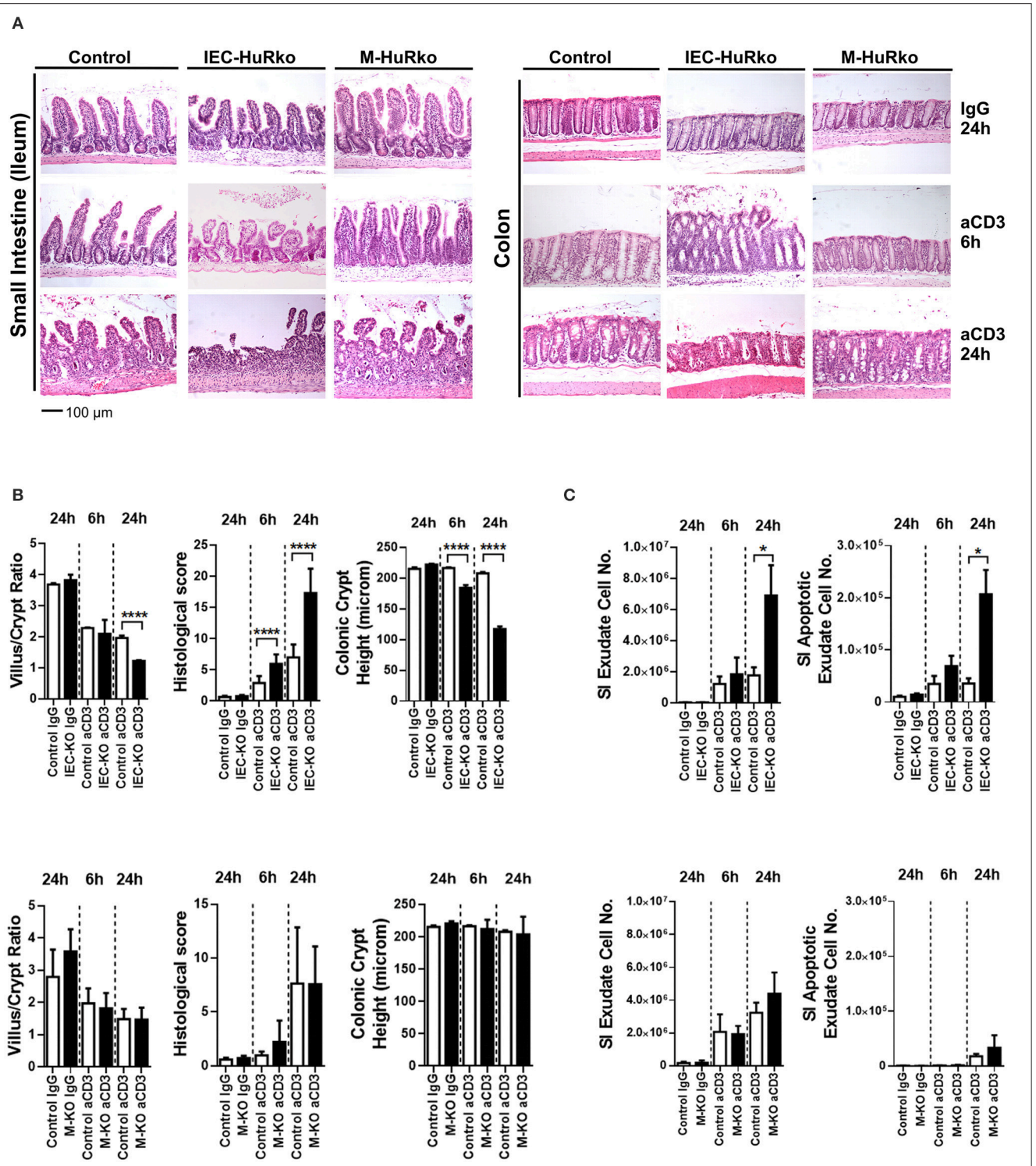

FIGURE 2 | Loss of HuR in IECs –but not in myeloid cells- sensitizes the epithelium to acute inflammatory death signals. **(A)** Representative H&E histology of paraffin embedded intestines from control and HuR mutant mice exposed either to aCD3 or isotype IgG for the indicated times. **(B)** Quantification of villus/crypt length ratio, colonic crypt height and histological score of IEC-HuRko treated mice (upper panel) and M-HuRko mice (lower panel). $n = 3$–6/group/time point. Student unpaired t-test. **(C)** (left panels) Enumeration of cells expelled in lumen washes from the intestines of mice exposed to aCD3 or IgG control. (right panels) Flow cytometric determination of apoptotic cell numbers of the same exudates, analyzed 6 and 24 h post injections in IEC-HuRko (upper panel), M-HuRko (lower panel) mice and respective controls. $n = 3$–6/genotype/time point. Student unpaired t-test. All bar graphs show means (±SE). In all graphs, *,****, denote p-values < 0.05 and 0.0001 respectively.

recovery phases, respectively. In control mice, the fecal bacterial load reached a plateau of 10^9–10^{10} colony forming units (cfu) per gram of feces by day 9 post infection and dropped to a negligible minimum by day 25 (**Figure 3A**). The response correlated with detectable histological alterations in crypt damage, hyperplasia, inflammation, and goblet cell depletion during the peak phase of the disease, and also with the local synthesis of pro-inflammatory *il1β* and *il18* mRNAs and antimicrobial *RegIIIβ* and *RegIIIγ* mRNAs (**Figures 3A–C**).

In IEC-HuRko mice, the kinetics of fecal bacterial loads was indistinguishable to that in controls, suggesting that these mice are capable of responding and clearing the infection (**Figure 3A**). However, and at the histological level, HuR-deficient epithelia displayed enhanced symptoms of degeneration, (e.g., crypt loss and apical epithelial detachment **Figures 3A,C**), relating to our findings in the T-cell mediated enteropathy model. Contrary however to the ascribed role of IEC-HuR in intestinal regeneration (16, 18), IEC-HuRko mice mounted a stronger crypt hyperplastic counter-response maintained even till day 25, which was associated with a near-significant upregulation in crypt proliferation as assessed via Ki67 immunostainings during the peak of the disease at day 12 (**Figures 3A,C,D**). Notably, the histological analysis of inflammatory infiltrates and the local synthesis of pro-inflammatory *il1β* and *il18* mRNAs did not reveal any significant changes (**Figures 3A,B**). However, HuR-deficient epithelia possessed higher levels of antimicrobial *RegIIIβ* and *RegIIIγ* mRNAs (**Figure 3B**). Thus, the loss of HuR in IECs alters the resistance and antimicrobial properties of the epithelium to combat the infection independently of the underlying inflammatory process.

The response of the M-HuRko mice to *C. rodentium* was strikingly different. In these mice, bacterial loads did follow the proper kinetics but were substantially reduced and the bacterium was cleared more efficiently (**Figure 3A**). All histopathological hallmarks of the disease appeared significantly attenuated with a negligible effect in tissue damage and a near lack in the proliferative hyperplastic response of the infected crypts (**Figures 3A,C,D**). The clinical image of the infected M-HuRko mice correlated with an extensively heightened response in *il1β* – but not *il18*- mRNA suggestive of an increased activation of innate immunity (**Figure 3B**). Notably, the inducible synthesis of epithelial antimicrobial mRNAs in M-HuRko epithelia appeared similar to that in the infected controls. From these data collectively, we deduce that the loss of M-HuR aggravates the initial response of myeloid-derived immunity early in the process leading to the rapid elimination of *C. rodentium*. This suggests that similar to cases of pathologic inflammation, and in contrast to HuR in IECs, myeloid HuR acts to inhibit rather than promote beneficial inflammatory responses contributing in pathogen clearance.

The virulence and host resistance against *C. rodentium* may also depend upon the composition of the gut microbiota (52, 54); in turn *C. rodentium* itself is known to cause dysbiosis (55) with unpredictable effects in intestinal inflammation. Moreover, the pharmacologic inhibition of HuR has been shown to affect the composition of the intestinal microbiome (37). To examine whether the observed shifts in the susceptibility of our mutants could be accounted for by a shift in commensal microbiota we analyzed its composition in fecal matter derived from uninfected and infected mice using a proteomic approach. We restricted our analyses to the percentile representation of the bacterial phyla of Firmicutes and Becteroidetes since these are the most predominant in the mouse intestine (56–58), and correlated our measurements with the representation of *C. rodentium* itself since its colonizing properties alters the ratio of Firmicutes to Bacteroidetes (F/B ratio; **Figure 3E**). In our analysis we could not identify any changes in F/B ratio either in IEC-HuRko or in M-HuRko mice; this suggests that their cell-restricted deletion does not alter significantly the composition of the commensal microbiota. Changes in the presence of *C. rodentium* were further validated via our proteomics approach which verified its presence in IEC-HuRko mice and its reduced propagation in M-HuRko mice (**Figure 3E**). Still, the response of the commensal microbiota during the infection appeared indistinguishable to that in the controls (**Figure 3E**). Thus, the changes in the susceptibility to *C. rodentium* were not due to changes in the colonic microbiota but rather to cell intrinsic changes in IECs or myeloid cells, respectively.

The Combinatorial Elevation of HuR Discriminates the Inflammatory From Tumorigenic Phases in CAC

The data from the intestinal and myeloid HuR deficient mice indicate the differential requirement for HuR in controlling excessive inflammation from myeloid-derived immune cells, and in providing a balance between inflammatory damage of the epithelium and regenerative proliferation. Although informative, our data do not reveal what actually happens when HuR is elevated in conditions of IBD or cancer where myeloid and IEC functions co-exist. To gain such information we performed the opposite experiment by elevating HuR via the additional transgenic integration of an avidin/FLAG tagged-human HuR protein (*TgATFHuR*$^+$ mice) driven by a promoter resistant to silencing (59) (**Figure 4A**). Five transgenic lines were originally generated; however only two expressed sufficient levels of HuR mRNA and from those we selected the one having the maximal expression pattern. In general, the expression of the transgene in this line (Tg6105; named hereafter as *TgATFHuR*$^+$) was ubiquitous and yielded an increase in 30–40% in HuR protein across tissues (**Figure 4A**). At the cell subset level of lymphocytes, Bone Marrow Derived Macrophages (BMDMs) and IECs, the elevation was consistently around 20% (**Figure 4A**). Flow cytometric analysis of major immune cell subpopulations in bone marrow, thymus and spleen of *TgATFHuR*$^+$ mice did not show any aberration in immune cell composition (**Figure S3**). Supernatants from activated macrophages and non-adherent splenocytes (lymphocytes) coming from *TgATFHuR*$^+$ mice, revealed decreases in proinflammatory targets of HuR, TNF, and CCL2 in macrophages, as previously described for high expressing macrophage HuR transgenic lines (20), however effects upon other targets reported in other transgenic lines, were either not observed (like a reduction in IL6) or did not reach significance (like an increase in IL10) suggesting that the

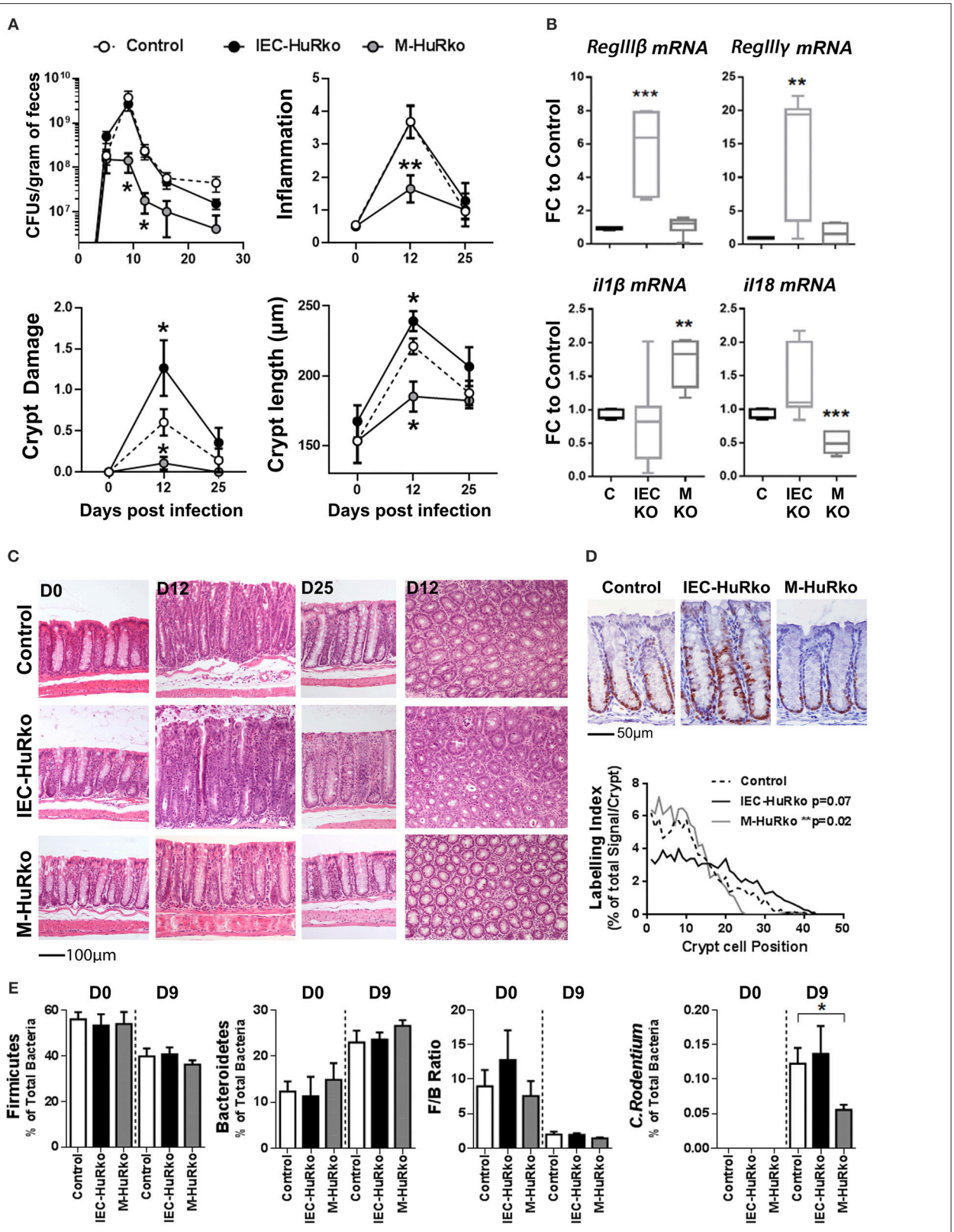

FIGURE 3 | Differential susceptibility of IEC-HuRko and M-HuRko mice to *C. rodentium* infection. **(A)** (upper left) Fecal bacterial counts in mice orally infected with *C. rodentium*. (n = 16/group). Histological score of inflammation (upper right) and crypt damage (lower left) of uninfected (day 0) and infected mice (days 12 and 25). day

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FIGURE 3 | 0, $n = 5$ per/group; day 12–16, $n = 10$–16 per/group. (Lower right) Crypt length measurements from H&E slides. Values are means (±SEM). **(B)** qPCR detection of mRNAs from distal colons on day 6 post infection expressed as fold change (FC) relative to control mice. $n = 5$–6 per/group. **(C)** Representative H&E histology of paraffin embedded distal colon sections of uninfected (day 0) and infected mice (day 12 and day 25 post infection). **(D)** Representative Ki67 immunohistochemical staining of distal colons from *C. rodentium* infected mice 12 days post infection (upper panel) and histograms of Ki67 labeling indices of proliferating epithelial cells in control and mutant mice measured from photomicrographs as in (upper panel). $n = 6$–7 mice/group. Wilcoxon Rank Sum test. **(E)** Relative abundance of Firmicutes, Bacteroidetes, *C. rodentium* taxa and Firmicutes to Bacteroidetes ratio (F/B Ratio) in the feces of uninfected (day 0) and infected (day 9) mice. Total intensity values for each taxon were used (see **Supplementary Methods**). Day 0, $n = 7$–14; day 9 $n = 12$–15. Student unpaired *t*-test. In all graphs *, **, *** denote *p*-values < 0.05, 0.01, and 0.001 respectively.

elevation of HuR is rather moderate (**Figure 4B**). This was also revealed when $TgATFHuR^+$ mice were tested for sensitivity to systemic effects occurring in LPS-induced endotoxemia where these mice displayed only a mild trend of resistance to lethality (**Figure 4C**). With respect to intestinal epithelia, we noted a significant increase in the villus/crypt ratio in the small intestine of aged (>10 months) $TgATFHuR^+$ mice, relating to an increase in crypt proliferation based on Ki67 staining but with no significant effects in cellularity (**Figures 4D–F)**. We do note however that no changes were observed for colonic $TgATFHuR^+$ crypts, which were indistinguishable from control crypts.

Collectively, $TgATFHuR^+$ mice appear as a system that could reciprocate –in part- the functions of elevated HuR in the different subsets as they appear in cases of IBD and CAC (37). To test this, first we challenged $TgATFHuR^-$ and $TgATFHuR^+$ mice with the DMH/DSS protocol. During the inflammatory phases of the response, $TgATFHuR^+$ mice displayed lower clinical inflammatory disease activity and histological scores than their controls and faster signs of epithelial restitution (**Figures 5A–C**). Analysis of the acute inflammatory profile of afflicted colons showed significant reductions in TNF and CCL2 production, an increase in anti-inflammatory IL10 and a variable response in IL-6 (**Figure 5D**), consistent with the partial loss of a pro-inflammatory character and the attenuated disease activity of $TgATFHuR^+$ mice. This was also reflected at the chronic phase (day 28), were the mRNA of pro-inflammatory *ifnγ* was downregulated as opposed to transcripts of immunomodulatory *il4, ptgs2,* and *tgfβ* that were upregulated in $TgATFHuR^+$ mice (**Figure 5D**). In sharp contrast to their diminished pro-inflammatory response, $TgATFHuR^+$ mice appeared more heavily susceptible to tumorigenesis, developing a significantly greater number of tumors of increased size per mouse (**Figures 5E,F**) which was different from what occurs when HuR is exclusively overexpressed in macrophages, where the reduction in inflammation correlated with a reduction in tumorigenesis (20). This suggests that during conditions of overexpression, the anti-inflammatory functions of HuR in immune cells segregate from its pro-tumorigenic effects in epithelia.

Finally, to assess whether the functions of TgATFHuR correlate with its cognitive characteristics we selected size-matched CAC-derived tumors from control and transgenic mice and performed Ribonucleoprotein Immunoprecipitation (RIP), with an anti-FLAG antibody, followed by Microarray Profile Analysis (**Figure S4A,B**). Approximately 2,000 genes were found bound by ATFHuR above control levels (**Table S2**). Gene Ontology enrichment analysis of these genes revealed groups involved in the processes of inflammation, cell cycle/apoptosis, development, epithelial function and others, consistent with previously published processes where HuR is involved **(Figure S4C)**. Moreover, known target transcripts of HuR such as *Tnf*, *Ccr2*, *Cdc42* were also identified to be bound by ATFHuR in our RIP setting. Thus, the contrasting effects of ATFHuR relate to the true cognitive functions of HuR in the different cellular settings.

The Combinatorial Elevation of HuR Diversifies Enterotoxic Responses From Inflammatory Responses

Next, we used a T-cell dependent model of inflammation to assess the role of overexpressed HuR in inflammatory progression vs. inflammatory epithelial damage following the same rationale as in the cell specific knockout mutants. First, the lack of a clear effect mediated by HuR in chronic inflammation was also conferred from our analyses of $TgATFHuR^+$ $TNF^{\Delta ARE/+}$ mice that did not display any significant differences from their $TgATFHuR^-$ $TNF^{\Delta ARE/+}$ controls (**Figure S2**). In sharp contrast, $TgATFHuR^+$ mice were markedly more resistant to anti-CD3 mediated enterotoxicity with reduced symptoms of inflammatory degeneration and a near lack of a response in terms of exudate epithelial cells in the lumen (**Figure 6A**). As such, these mice displayed the opposite epithelial sensitivity of IEC-HuRko mice to inflammatory damage, which could connect also to their increased susceptibility to CAC. Finally, $TgATFHuR^+$ mice displayed a significant delay in the early control of *C. rodentium* as indicated by fecal bacterial counts on the 9th day of the infection and the proteomic detection of representative peptides (**Figures 6B,C**). This indicated that the elevation of HuR compromised in part the efficiency of *C. rodentium* clearance. However, the remaining response was unaltered both in terms of the histological features as well as in the consistency of commensal microbiota (**Figures 6B–E**) suggesting a new balance in epithelial responses compensates to allow for the subsequent mechanism of defense against the bacterium.

By comparison, the response of $TgATFHuR^+$ mice appears as exactly the opposite of what we could expect from the sum of differences in the debilitating mutations of HuR in myeloid-derived immune vs. epithelial compartments, thus verifying that HuR is acting as an anti-inflammatory regulator in myeloid-derived immunity and as a pro-survival and pro-tumorigenic factor in intestinal epithelia, with differential consequences in different contexts of intestinal inflammation.

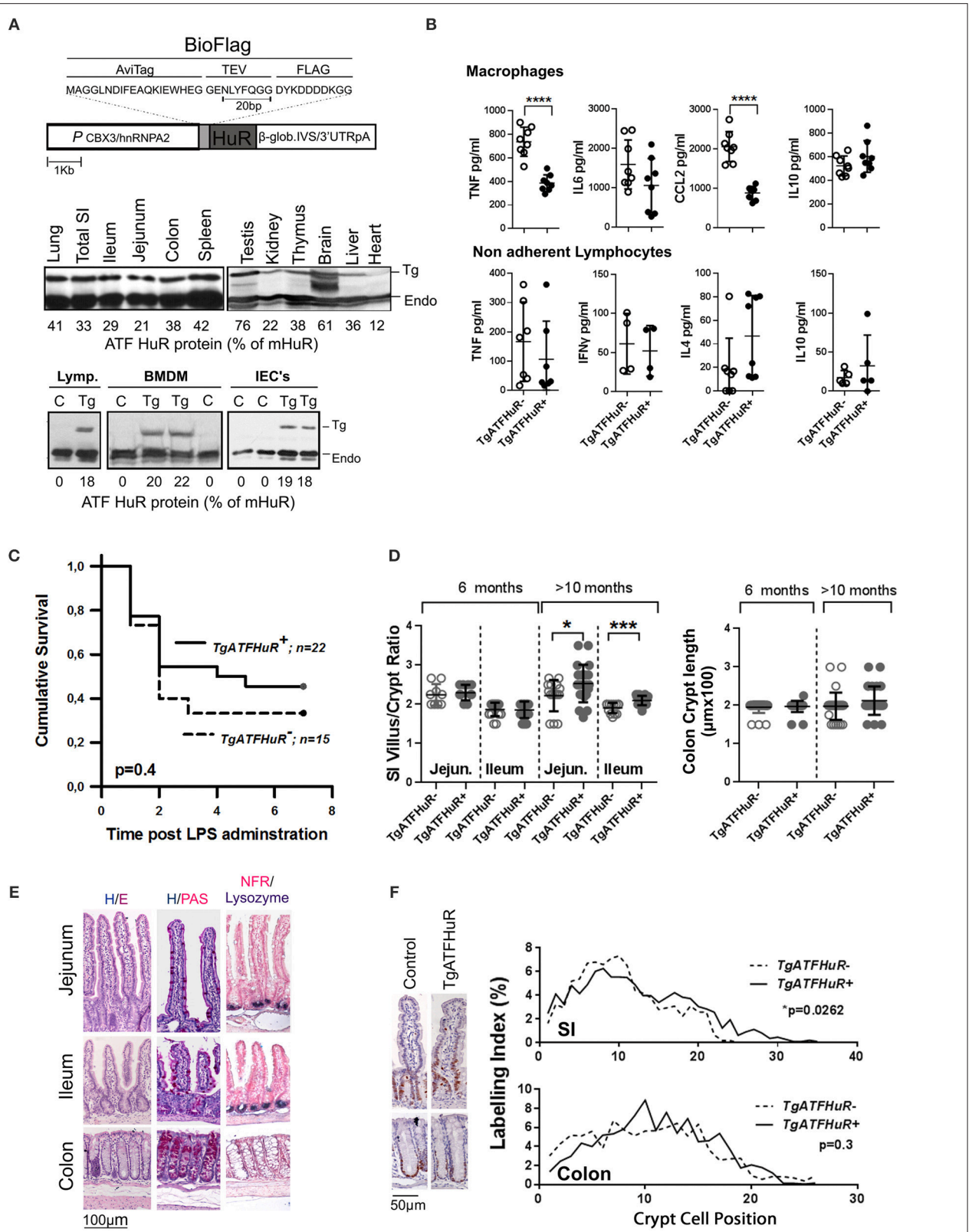

FIGURE 4 | Generation and characterization of HuR overexpressing *TgATFHuR*$^{+}$ mice. **(A)** (upper panel) Diagrammatic representation of the construct used for the generation of the TgATFHuR^{+} mice indicating the presence of: the CBX3/hnRNPA2 promoter; the tagged version of the human HuR with aminoacid composition of

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FIGURE 4 | the Avidin and FLAG tags; the presence of β-globin intron/3′UTR that ensures a gene like structure and a 3′UTR and polyadenylation signal. (Middle panel) Representative immunoblots for the detection of the transgenic ATF-HuR protein in mouse tissues and quantitation to endogenous mouse HuR (mHuR). Membranes blotted with an anti-HuR antibody (3A2). (Bottom panel) Representative immunoblots for the detection of the transgenic ATF-HuR and endogenous mHuR proteins in extracts from *TgATFHuR*$^{+}$ lymphocytes (lymp.), macrophages (BMDMs) and IECs. **(B)** ELISA detection of elicited inflammatory mediators in supernatants from cultured BMDMs (Upper panel), in the presence of Lipopolysaccharide (LPS; 100 ng/ml) for 24 h, and from non-adherent splenocytes (lymphocytes) in the presence of PMA/ionomycin. Data represent individual values/culture and mean (±SD). **(C)** Kaplan-Meier analysis following endotoxemia induction. Two experiments were performed with intraperitoneal administration of low (150 μg/25 g) and high (400 μg/25 g) doses of LPS, with similar results. Combined data from the two experiments are shown. Total group numbers are shown. *p*-value denotes result of Log-rank test. **(D)** Quantification of villus/crypt (jejunum and ileum) length ratio and colonic crypt height of younger (6 months old) and older (10–12 months old) mice. Data represent values of individual mice and mean (±SD). Student unpaired *t*-test. **(E)** Representative histology of sections from paraffin embedded intestinal tissue stained for general tissue architecture (hematoxylin/eosin), for Goblet cells (hematoxylin/periodic acid-Schiff), and for Paneth cells (anti-Lysozyme, blue stain, counterstained with Nuclear Fast Red). **(F)** (Left) Representative immunohistochemical staining of Ki67 (brown) counterstained with hematoxylin for detection of proliferating epithelial cells in resting TgATFHuR^{-} and TgATFHuR^{+} mice at the age of 2 months. (Right) Histograms of Ki67 labeling indices measured from photomicrographs as in (Left). Data compiled from $n = 3$–5 mice per group. *P*-values from Wilcoxon Rank Sum test. In all graphs *, ***, **** denote *p*-values < 0.05, 0.001, and 0.0001 respectively.

DISCUSSION

In this study we followed a disease-oriented approach to examine whether HuR's independent functions in two distinct compartments co-existing in the intestinal mucosa (i.e., myeloid-derived immune cells and intestinal epithelial cells) contribute similarly or differentially in the same contexts of inflammatory intestinal disease. We chose to focus on the phenotypic effects of HuR deletion in these cell types, rather than on the molecular mechanisms that underlie them, in an effort to (a) highlight the specificity of HuR's functions thus providing a contextual roadmap for future molecular analyses; and (b) address the issue of HuR's exploitation in the clinical management of intestinal inflammation and degeneration. In doing so, we identified that the independent cellular responses guided by HuR's post-transcriptional functions have differential outcomes in intestinal inflammation.

In myeloid-derived immune compartments, HuR has primarily a regulatory role acting to maintain intestinal inflammation within physiological thresholds. This is supported by the differential susceptibility of our M-HuRko mice (augmented) to HuR overexpressing *TgATFHuR*$^{+}$ mice (suppressed) in chemically-induced colitis; and is in line to previous observations in other settings of pathologic inflammation (17, 20, 38, 60). In molecular terms, this connects to HuR's capability of reducing the translation of several pro-inflammatory cytokine mRNAs (e.g., *Tnf, il6, Ccl2, Ccr2*) in innate immune cells, as opposed to their stability. Herein, we extend the regulatory functions of HuR toward beneficial myeloid-derived immunity functions required for host defense. This was exemplified by our data from the model of *C. rodentium* invasion where the loss of HuR enhanced pathogen clearance and limited inflammatory damage. This could connect to the capacity of HuR-deficient phagocytosing cells to uptake and clear the invading bacteria rapidly due to rapid CCL2/CCR2 mediated recruitment which seem to be connected to *C. rodentium* clearance (61) and whose synthesis is augmented in HuR-null macrophages supporting their enhanced homing at sites of inflammation (20). Alternatively, and though not addressed, HuR-null macrophages may display an enhanced inflammasome activity which is required for the clearance of this pathogen. This is indicated by several circumstantial evidence. In M-HuRko mice commensal bacteria respond properly to the invasion of *C. rodentium* suggesting that the bacterium can colonize the mucosa in these mice during the initial stages of the infection. On the other hand, the synthesis of antimicrobial RNAs known to be induced in IECs during the infection were not altered. The production of these RNAs in IECs is under the direct control of adaptive immune cells recruited as a result of the activation of myeloid-derived immune cells by the bacterium; as such, it signifies that adaptive immune response was not altered in M-HuRko mice despite their enhanced capability to control the infection. In contrast, we detected heightened levels of IL1b mRNA expression which could signify an enhanced activation of the inflammasome pathway that needs to be further examined at the molecular level.

The effects of myeloid HuR in the control of chronic inflammation appear less defined. In the TNF-mediated model of IBD, the loss of HuR did not alter the quality of the pathologic response-despite the signs for a delay in disease progression. This could be due to the complex hierarchy of pathologic responses supporting disease in this model which, besides myeloid-derived immune compartments, it is heavily dependent on the adaptive arm of immunity as well as on several stromal tissues (48–50). It is well-recognized that epithelial and stromal cells in the intestine express numerous receptors commonly found in immune cells and in this way they actively participate in the coordination of the immune response (62, 63). Alternatively, this could be due to the fact that TNF cannot be regulated by HuR since its mRNA is missing the HuR binding site. However, evidence that myeloid HuR can control chronic inflammation stem from the response of these mice during CAC. During CAC, a variety of toxic and regenerative inflammatory responses drive dysplasia and adenoma formation whereas subsequent tumor-associated inflammation (which includes Tumor Associated Macrophages) support tumor growth and invasion. The enhanced tumorigenesis and tumor growth observed in M-HuRko mice suggest that these responses may be either uncontrolled or qualitatively different.

The intestinal epithelial functions of HuR appear equally diverse. Our data are in line with previous suppositions on HuR acting positively toward intestinal epithelial programs of proliferation and regeneration. This is supported by the delayed restitution of DSS-challenged or irradiated epithelia when HuR is lost [this study and (18)] and its rapid occurrence when HuR is elevated; or by the diminishing effects of HuR's loss in tumor

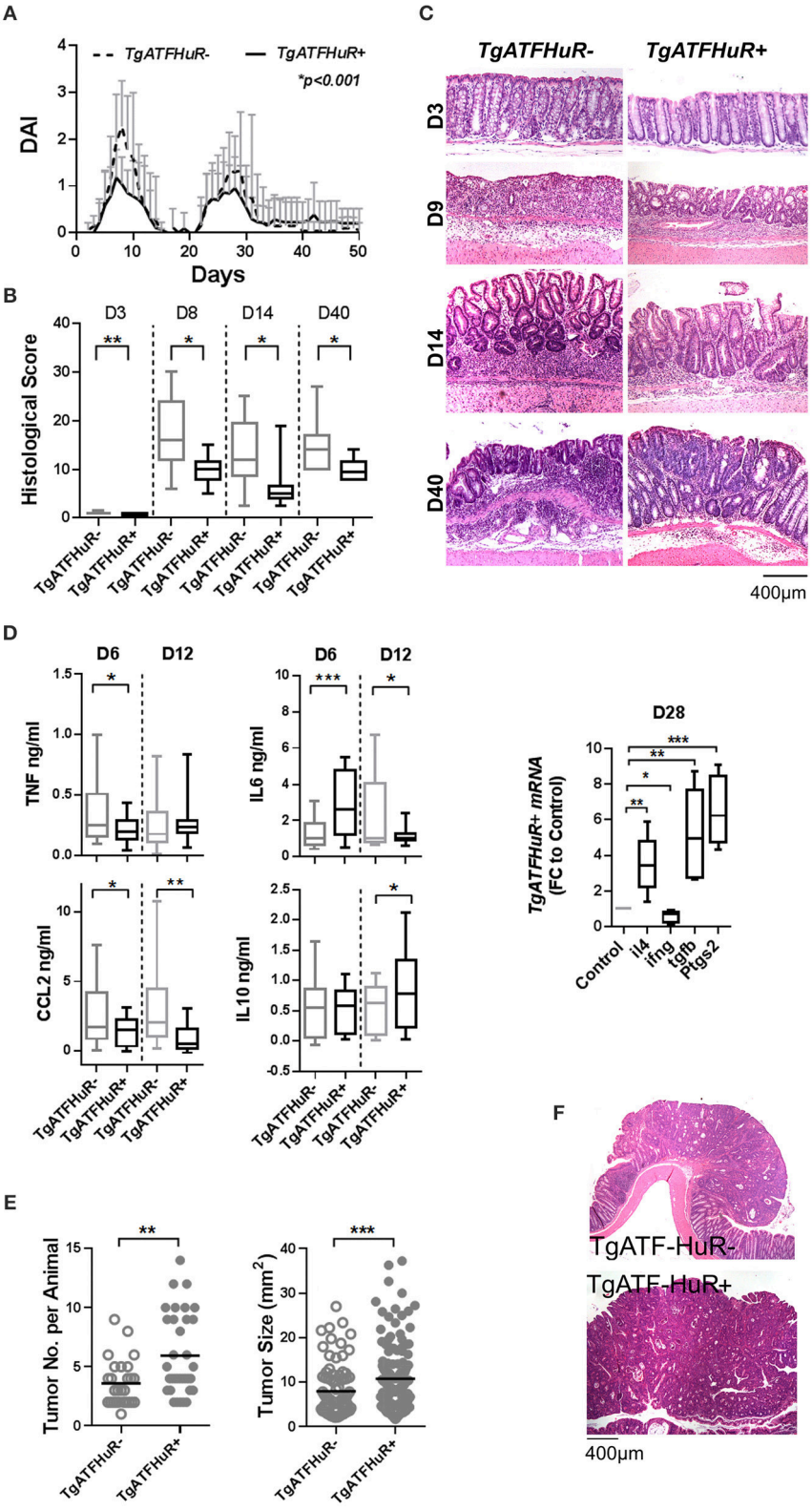

FIGURE 5 | The combinatorial elevation of HuR attenuates chemically-induced colitis but augments CAC. **(A)** Macroscopic Disease Activity Index (DAI) during the 2 rounds of DSS administration (Days 0 and 20) in TgATFHuR^{-} and TgATFHuR^{+} mice. Line graphs depict mean values (±SD). n = 20–30/group. Kolmogorov-Smirnov

(Continued)

FIGURE 5 | test. **(B)** Histological scores of colitis at the indicated time points. Box plots show range and median. $n = 8$–12 mice/group. **(C)** Representative histology (H&E) of slides scored in **(B)**. **(D)** (Left and middle columns) ELISA quantification of inflammatory mediators in colon cultures isolated from DSS-treated mice at the indicated time points. Box plots show range and mean values. $n = 3$ cultures/group/time point. Student unpaired *t*-test. (Right) qPCR analysis of RNAs from DSS-treated colons of IEC-HuRko mice on day 28 after treatment expressed as fold change (FC) relative to control mice. *il4*, interleukin 4, *ifng*, interferon gamma, *tgfb*, transforming growth factor beta, *Ptgs2*, Prostaglandin-Endoperoxide Synthase 2. **(E)** Graphs depicting tumor number per mouse (left) and tumor size (right) in TgATF$^-$ and TgATFHuR$^+$ mice 60 days after challenge with DMH/DSS. Dots represent individual mice (left) and tumors (right); horizontal lines represent means. Student unpaired *t*-test. **(F)** Representative histology (H&E) of paraffin embedded colonic tumors measured in **(E)**. In all graphs, *, **, ***, denote *p*-value < 0.05, 0.01, and 0.001 respectively.

growth during CAC as opposed to its enhancing effect when HuR is overexpressed. Based on the extensive literature, this could be due to HuR's control over the Frizzled co-receptor Lrp6 (18), E-cadherin (36) and β-catenin (12) promoting Wnt-induced progenitor expansion; or the pleiotropic Rho GTPAse Cdc42 affecting proliferation, actin organization and migration (64). Although these mechanisms are now well-accepted, they reflect HuR's functions in stem and transient amplifying cells involved in regeneration. Our data indicate that HuR has a more predominant role in the homeostasis of differentiated enterocytes during inflammation for the preservation of intestinal barrier integrity under inflammatory stress via different means. This is heavily exemplified by the augmented response of IEC-HuRko mice in the model of T-cell mediated enterotoxicity which measures solely the response of enterocytes to inflammatory death signals. This connects to several post-transcriptional effects of HuR affecting directly or indirectly death responses. Directly, HuR may oppose enterocyte death, due to its positive regulation of prosurvival signals as in the cases of *Bcl2, Mcl1,ProT alpha, Sirt1,* and *PGC1a* as well as effectors of mitochondrial resistance (65, 66). Indirectly, this could also connect HuR's functioning in controlling the adherens junctions of enterocytes via its control over the stabilization of E-cadherin mRNA (36). To that end IEC-HuRko mice phenocopy those lacking E-cadherin, which display shedding and apoptosis of enterocytes, villus shortening and augmented colitis (67, 68). Conversely, *TgATFHuR*$^+$ mice resemble mice with augmented E-cadherin/β-catenin junctions that are resistant to colitis (69, 70).

Arguably however, enterocyte cell death can be an inevitable side effect of a lack of continuous proliferation. However, our data on *C. rodentium* argue that in the physiological mucosa the prosurvival functions of HuR in enterocytes are more dominant than its effects in stem cell programs driving regeneration. In contrast to M-HuRko, IEC-HuRko mice possessed a normotypic response toward the clearance of the infection. However, HuR-deficient epithelia displayed a heightened level of tissue damage accompanied by a heightened proliferative response to compensate for that damage. Conversely, the elevation of HuR in *TgATFHuR*$^+$ epithelia did not alter their hyperplastic response despite their resistance in enterotoxicity suggesting that the elevated HuR provided a new balance between inflammatory death and regeneration. Definitely however, and as indicated by the response of *TgATFHuR*$^+$ mice to CAC, this balance is tilted toward the proliferative end providing benefits for the conversion of progenitors to dysplastic tissue, adenomas and carcinomas.

Our studies with *C. rodentium* suggest that HuR may also control the defensive properties of the epithelia. This is suggested by the augmented response in RNAs promoting antimicrobial defense (like RegIII) correlating with the proper resolution of the infection as opposed to the enhanced tissue damage. Whether this is directly regulated by HuR in IECs or via an indirect control over signals promoting antimicrobial gene expression in these cells remains to be determined.

Collectively, our data provide several indications for the clinical use of HuR inhibitors in combating intestinal inflammatory diseases. Several lead compounds have been reported in the literature. Their clinical use in intestinal inflammatory diseases is based on the assumption that they could act as selective and efficient means for inflammation control by blocking the activation of several pro-inflammatory, ARE-containing, cytokine mRNAs driving intestinal inflammation. In that sense they could provide more specific therapeutic means than generic immunosuppressants (e.g., corticosteroids, aminosalicylates, methotrexate, cyclosporine, azathioprine, and mercaptopurine) or more effective than single cytokine inhibitors (e.g., anti-TNF, anti-IL12/23 or NSAIDs for Cox-2) currently used to treat patients suffering from, for example, IBD.

Our findings indicate that these suppositions need to be reconsidered since HuR appears to have potent activities in regulating the acute responses of myeloid-derived mucosal subsets and the protection of the intestinal barrier from these responses. Similar considerations should be made for the applicability of HuR inhibitors in combating intestinal transformation and CRC. The main issue is the inflammatory context that either supports or impedes tumor initiation and progression. Our study provides a clear rationale as to why such inhibitors seem to fail in preclinical models of CAC since they could aggravate the early pro-inflammatory responses of myeloid-derived immune subsets driving IBD and enhance the genotoxic damage of the intestinal epithelial barrier thus enhancing the transformation process. We postulate that monotherapeutic schemes blocking HuR functions should be avoided in CRC arising in the context of IBD, where anti-cytokine therapies are also envisaged for applicability (e.g., anti-IL6).

Despite these issues, both our study and other studies on animal models support that HuR blockade could be of benefit in specific cases –or windows- of intestinal disease (37). For example, HuR inhibitors may be applicable in CRC's arising due to genetic mutations—like in the case of FAP and models of APC mutations- where inflammation may have a secondary role. In that context, HuR inhibition can clearly hinder the proliferative expansion of transformed cells, promote tumor death and aggravate an immune response targeting tumor cells for clearance. As such it could act as a more selective and efficient therapeutic strategy than current chemotherapeutics and surgical procedures for FAP.

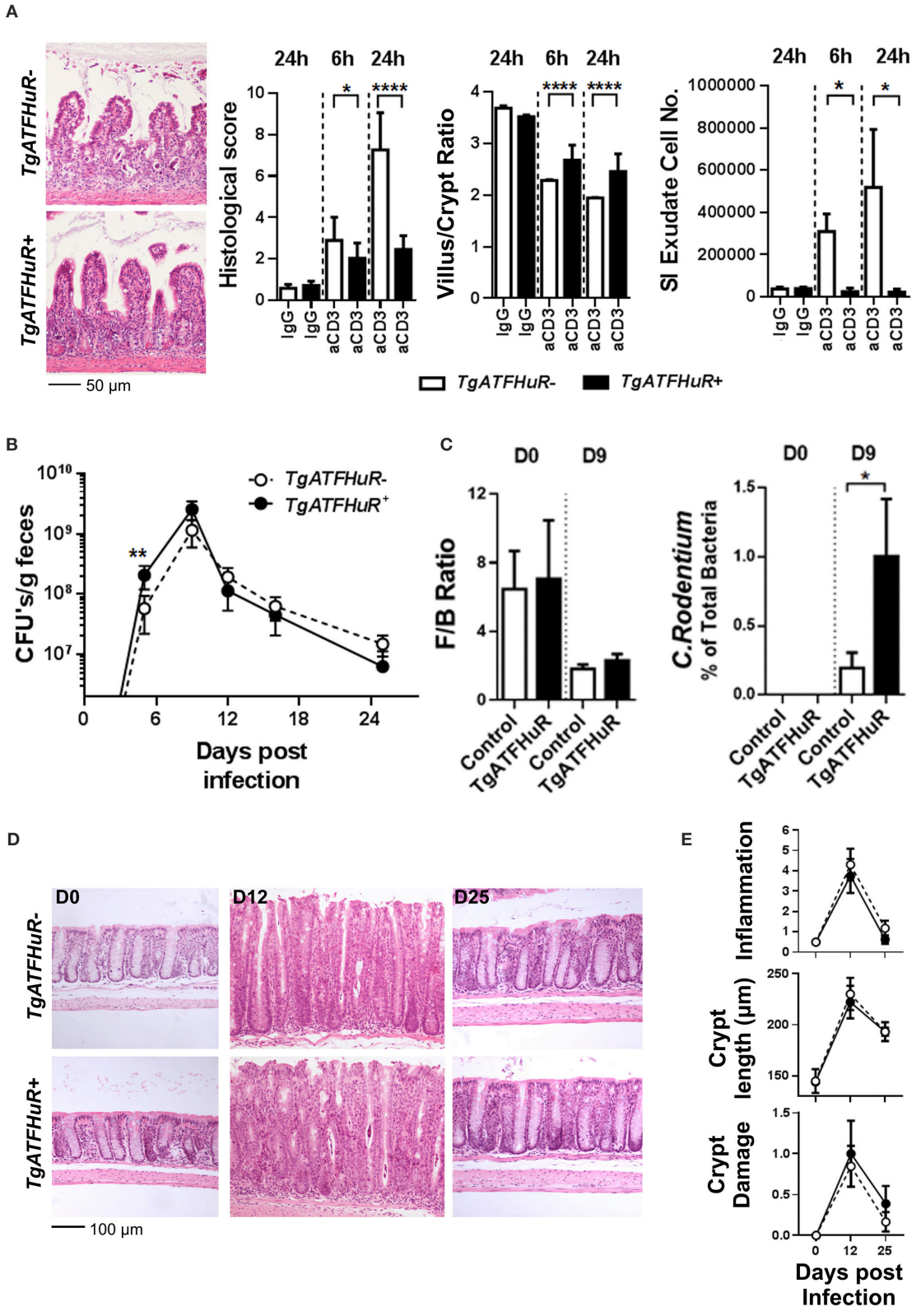

FIGURE 6 | The combinatorial elevation of HuR protects the epithelium from acute inflammatory death signals but compromises inflammatory pathogen clearance. **(A)** Representative histology (H&E), histological scores, villus/crypt ratio quantification and determination of total cell number in the exudate of the small intestine at

(Continued)

FIGURE 6 | indicated time points post anti-CD3 antibody injections. Photomicrographs of ilea 24 h post injections are shown. Histological score and morphometry, $n = 10$/genotype/time point, Student unpaired *t*-test; Exudate cell number, $n = 6$–12/group, Mann-Whitney test. **(B)** Fecal bacterial counts in mice orally infected with *C. rodentium*. $n = 10$–25/group/time point. Student unpaired *t*-test. **(C)** Firmicutes to Bacteroidetes (F/B) ratio (left) and relative abundance of *C. rodentium* (right) in the feces of uninfected (day 0) and infected (day 9) mice. $n = 8$–12 mice/group/time point. Mann-Whitney test. **(D)** Representative histology of paraffin embedded distal colons from mice orally infected with *C. rodentium* at indicated time points post infection. **(E)** Histological parameters of *C. rodentium* colitis at the indicated time points post infection. Graphs show means (±SEM). $N = 8$–13/group/time point. In all graphs, *,**, ****, denote *p*-value < 0.05, 0.01, and 0.0001, respectively.

Perhaps, the anti-tumorigenic capabilities of HuR blockade could also be harnessed for CAC through the combinatorial-yet carefully controlled—use of anti-inflammatory drugs or drugs inhibiting the recruitment of myeloid-derived cells in the mucosa, alongside HuR inhibition regimes; or devise strategies for the selective uptake of HuR inhibitors by cancer cells thus bypassing the adverse targeting of myeloid-derived compartments. Finally, our data do support the beneficial use of HuR blockade in combating infections with enteropathogenic and enterohaemorrhagic bacteria since they could boost up beneficial immune and epithelial responses driving pathogen clearance. As such they could be used in combination with antibiotic or other antimicrobial regimes.

In general however, the ambiguity in the clinical effects of HuR blockade reflects the ambiguity in HuR functions both in cell intrinsic events and cellular interactions. As it stands, HuR drives both beneficial and adverse reactions in myeloid-derived and epithelial compartments. The molecular details for HuR functions in discriminating the post-transcriptional fate of tissue specific programs remain to be elucidated. To provide more definitive strategies on HuR blockade would require the dissection of HuR's protein and RNA partners that differentiate the beneficial from pathologic post-transcriptional programs and selectively target only the pathologic ones. To that end our study provides a framework to seek for such partnerships in the context of intestinal disease.

DATA AVAILABILITY STATEMENT

R-IP-Chip raw files that were generated for this study can be found on the Array-Express repository (https://www.ebi.ac.uk/arrayexpress/) with accession number E-MTAB-4018.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Institutional Committee of Protocol Evaluation in conjunction with the Veterinary Service Management of the Hellenic Republic Prefecture of Attika and in accordance to national legislation and the European Union Directive 63/2010. Protocols were approved by Prefecture of Attika (licenses #5995/2012, 4371-4376/2014, #6198/2017, #3547/2018, #2824/2018).

AUTHOR CONTRIBUTIONS

In vivo experiments were performed by EC-V, FI aided by MA; $TgATFHuR^{+}$ mice generated by IK; histology and evaluation by AP (histopathologist), EC-V, FI, and MA; RIP experiments and analyses by GG, EC-V, and MR; microbiota and proteomic analyses by GS and MS; manuscript written by EC-V, FI, and DK.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02732/full#supplementary-material

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Combined IL-2 Immunocomplex and Anti-IL-5 mAb Treatment Expands Foxp3$^+$ Treg Cells in the Absence of Eosinophilia and Ameliorates Experimental Colitis

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Interleukin (IL)-2 is expressed during T cell activation and induces the proliferation and differentiation of T cells. CD4$^+$Foxp3$^+$ regulatory T cells (Tregs) constitutively express the high affinity IL-2 receptor (CD25/IL-2Rα) and rapidly respond to IL-2 to elaborate numerous suppressive mechanisms that limit immune-mediated pathologies. Accumulating evidence supports the concept that an aberrant balance between Tregs and Teff contribute to the pathology of intestinal inflammation and that the IL-2/Treg axis is a potential pathway to exploit for the treatment of inflammatory bowel disease (IBD). Here, we show that treatment of mice with IL-2/IL-2 antibody (JES6-1) immunocomplex during DSS-induced colitis induced Foxp3$^+$ Treg expansion, but also potently stimulated GATA3$^+$ type 2 innate lymphoid cell (ILC2) proliferation and high-level expression of IL-5. Furthermore, IL-2/JES6-1 treatment resulted in massive eosinophil accumulation and activation in the inflamed colon, and afforded only modest protection from colitis. In light of these findings, we observed that combined IL-2/JES6-1 and anti-IL-5 mAb treatment was most effective at ameliorating DSS-induced colitis compared to either treatment alone and that this regimen allowed for Foxp3$^+$ Treg expansion without concomitant eosinophilia. Collectively, our findings provide insight into how blockade of IL-5 may aid in optimizing IL-2 immunotherapy for the treatment of intestinal inflammation.

Keywords: IL-2, IL-5, Treg, eosinophil, ILC2, IBD

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INTRODUCTION

Interleukin (IL)-2 is a T cell growth factor that is essential for the proliferation and differentiation of T cells into effector and memory populations (1). These critical functions have led to the use of IL-2 in stimulating immune responses *in vivo*, particularly for anti-tumor immunotherapy and for boosting T cell numbers in AIDS patients (2, 3). However, the relatively high-doses of IL-2 required to induce beneficial responses *in vivo* are often accompanied by untoward side effects including vascular leak syndrome and hepatic and renal dysfunction, which have limited clinical use of high-dose IL-2 (4, 5). In addition to delivering IL-2 to augment immune activation, blockade of the high-affinity α chain of the IL-2 receptor (CD25) using the monoclonal antibody basiliximab, has also been employed to suppress organ transplantation rejection associated with IL-2 signaling

(6). These clinical uses of IL-2 delivery or IL-2 receptor blockade to amplify or inhibit immune responses, respectively, fit with the well-defined immune stimulatory roles of IL-2.

Interestingly, IL-2 also plays a major role in the development, survival, expansion, and suppressive functions of a unique population of regulatory $CD4^+$ T cells (Treg) that constitutively express high levels of CD25 and Foxp3 (7–18). Tregs play a vital role in negative regulation of immune-mediated inflammation in autoimmune and autoinflammatory disorders, cancer, infections, allergy, and intestinal inflammation. These cells also play key roles in suppressing metabolic inflammation and promoting tissue repair processes. Based on these suppressive functions the *in vivo* expansion of $Foxp3^+$ Tregs has been explored as an avenue for treatment of inflammatory conditions. One method to expand $Foxp3^+$ Tregs *in vivo* that has been pursued is the delivery of low-dose IL-2 either alone or complexed with antibodies. Low-dose IL-2 has been shown to preferentially expand $Foxp3^+$ Tregs in part due to their constitutive CD25 expression as well as other cell-intrinsic factors (19). Treatment with low-dose IL-2 has shown promise in numerous inflammatory disorders including chronic graft vs.-host-disease (GVHD), allograft survival, systemic lupus erythematosus, and type I diabetes, among others (20, 21). Further, IL-2 can be complexed to antibodies that permit targeting to either $Foxp3^+$ Tregs or effector T cells and some innate immune cell populations depending on the specific region of IL-2 the antibodies bind. For example, the anti-IL-2 antibody JES6-1 binding to IL-2 induces allosteric changes that permit preferential activation of CD25-expressing cells, such as Tregs, while a different anti-IL-2 antibody, S4B6, induces distinct conformational changes in IL-2 allowing for selective interaction with IL-2 receptor beta (IL-2Rβ/CD122) and expansion of $CD8^+$ T cells and NK cells (22, 23). Consistent with the ability of IL-2/JES6-1 immunocomplexes to expand $Foxp3^+$ Tregs *in vivo*, they have shown beneficial results in the treatment of several autoimmune and inflammatory diseases in mice (21). IL-2/JES6-1 immunocomplexes and low-dose IL-2 therapy are also stimulatory for group 2 innate lymphoid cells (ILC2s) that express CD25, which can contribute to IL-5 production and eosinophilia (24). Thus, the favorable effects of IL-2-mediated $Foxp3^+$ Treg expansion and immune suppression may be tempered by simultaneous activation of immune stimulatory effector cells such as eosinophils.

Given the vast number of microbes in the intestine, tightly regulated immune responses are instrumental in the maintenance of gut homeostasis, and IL-2 and $Foxp3^+$ Tregs play a vital function in this process (25–27). Upon bacterial colonization of the intestine in early life or upon colonization of germ-free mice with microbiota, IL-2 is rapidly induced and consequently drives the expansion of $Foxp3^+$ Tregs that aids in establishing tolerance toward the microbiota (28, 29). The importance of IL-2 signaling and $Foxp3^+$ Tregs in maintaining intestinal homeostasis is most evident in mice lacking IL-2 or Foxp3, which develop spontaneous intestinal inflammation (30, 31). Further, naive $CD4^+$ T cells induce chronic colitis when transferred into immunodeficient mice in the absence of Foxp3+ Tregs (32). In this T cell transfer model, delivery of $Foxp3^+$ Tregs can both prevent and treat disease (25). Based on these observations, *in vivo* expansion of $Foxp3^+$ Tregs using IL-2 and other methods has been explored as a potential therapeutic for human inflammatory bowel disease (IBD) (33, 34).

In this report, we show that treatment of mice with IL-2/JES6-1 immunocomplex during DSS-induced colitis promoted $Foxp3^+$ Treg expansion, but also potently stimulated $GATA3^+$ group 2 innate lymphoid cell (ILC2) proliferation and high-level expression of IL-5 in the colon. Furthermore, IL-2/JES6-1 treatment resulted in massive eosinophil accumulation and activation in the inflamed colon, and afforded only modest protection from colitis. In light of these findings, we further demonstrated that combined IL-2/JES6-1 immunocomplex and anti-IL-5 mAb treatment was most effective at ameliorating DSS-induced colitis compared to either treatment alone and that this regimen allowed for $Foxp3^+$ Treg expansion without concomitant eosinophilia. Collectively, our findings provide insight into how blockade of IL-5 may aid in optimizing IL-2 immunotherapy for the treatment of intestinal inflammation.

RESULTS

IL-2/JES6-1 Immunocomplexes Induce Colonic Foxp3+ Treg Accumulation During DSS-Induced Intestinal Inflammation

IL-2/JES6-1 immunocomplexes have been shown to induce rapid $CD25^+Foxp3^+$ Treg expansion in the spleen of mice and afford protection from dextran sodium sulfate (DSS)-induced colitis when delivered for one week prior to the initiation of DSS (22). In order to explore if IL-2 delivery can induce the accumulation of $Foxp3^+$ Treg in the colons of mice during experimental colitis, we treated wild-type C57BL/6J mice with IL-2/JES6-1 immunocomplexes beginning at the time of DSS administration (day 0) and continuing every 2 days. At day 6, DSS was discontinued and colons were harvested at day 10. IL-2/JES6-1 immunocomplex treatment led to a significant increase in $Foxp3^+$ Treg frequency and absolute cell number (**Figures 1A,B**). Additionally, the expanded $Foxp3^+$ Treg population in IL-2/JES6-1 immunocomplex-treated mice coincided with increased colonic Foxp3, Il2ra, and Ctla4 mRNA expression (**Figure 1C**). Collectively, these data demonstrate that IL-2/JES6-1 immunocomplexes are capable to inducing robust $Foxp3^+$ Treg accumulation in the colons of DSS-treated mice even when initiated at the same time as DSS dosing.

Delivery of IL-2/JES6-1 Immunocomplexes to DSS-Treated Mice Drives Colonic Eosinophil Accumulation And Activation

Previous studies have reported IL-5-induced eosinophilia associated with IL-2/JES6-1 immunocomplex treatment in a murine model of dermatitis as well as in cancer patients that received high- or low-dose IL-2 immunotherapy (24). Therefore, we next explored whether IL-2/JES6-1 immunocomplexes affect colonic eosinophils during DSS-induced colitis. As shown in **Figures 2A,B**, IL-2/JES6-1 immunocomplex treatment led to a significant increase in $Siglec\text{-}F^+$ eosinophil frequency and absolute cell number in the colons of DSS-treated mice. We

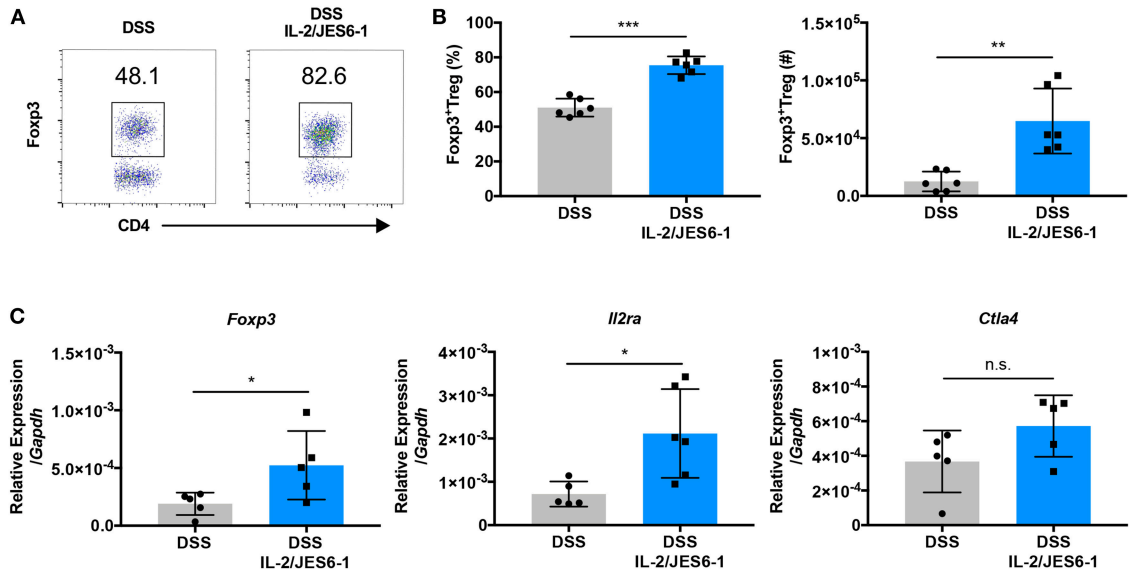

FIGURE 1 | IL-2/JES6-1 immunocomplexes induce colonic Foxp3+ Treg accumulation during DSS-induced intestinal inflammation. WT mice were treated with 3% DSS in drinking water. At day 6, DSS water was replaced with normal water. IL-2/JES6-1 immunocomplexes were delivered every 2 days. $CD4^+Foxp3^+$Treg cells were analyzed by flow cytometry. Representative dot plots are shown in **(A)**. Frequency of $CD4^+Foxp3^+$ T cells are shown in **(A)** and total cell numbers of CD4+Foxp3+T cells are shown in **(B)**. **(C)** Expression of the Treg-related genes, *Foxp3*, *Cd25*, and *Ctla4* were analyzed by qPCR using total colonic tissue. Data are representative of two independent experiments with 4–6 mice/group. All data are presented as mean ± SEM; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, one-way ANOVA with Tukey's multiple comparison test.

next assessed whether IL-2/JES6-1 immunocomplex treatment led to eosinophil activation by analyzing Gr-1 expression, since previous studies have shown that eosinophils in the inflamed intestine show increased expression of intermediate, but not high, levels of Gr-1 (35). The Gr-1 antibody reacts with both Ly6C and Ly6G antigens that are also expressed by monocytes and neutrophils, respectively. Using both Gr-1 expression and side scatter (SSC) properties, these populations can be distinguished into $Gr\text{-}1^{lo-neg}SSC^{hi}$ eosinophils, $Gr\text{-}1^{hi}SSC^{int}$ neutrophils, and $Gr\text{-}1^{lo-int}SSC^{lo}$ monocytes (36). Upon activation, eosinophils can become $Gr\text{-}1^{int}SSC^{hi}$, and still be clearly distinguished from neutrophils and monocytes. Indeed, we observed that Siglec-F^+ eosinophils in IL-2/JES6-1 immunocomplex-treated mice showed significantly higher mean fluorescence intensity (MFI) of Gr-1 when compared to non-treated controls (**Figure 2C**). We also investigated eosinophil cationic protein 2 (encoded by the *Ear2* gene) expression as another eosinophil activation marker (37). Consistent with enhanced Gr-1 expression, ear2 mRNA expression was increased by IL-2/JES6-1 immunocomplex administration to DSS-treated mice (**Figure 2D**). These data indicate that IL-2/JES6-1 immunocomplexes can potently induce eosinophil accumulation and activation in the inflamed intestine during experimental colitis, which may be an unwanted side effect of IL-2-based immunotherapy forintestinal inflammation.

IL-2/JES6-1 Immunocomplex Administration Promotes Intestinal ILC2 Expansion and IL-5 Expression

To determine the mechanism of colonic eosinophil accumulation induced by IL-2/JES6-1 immunocomplex treatment in DSS-treated mice, we next investigated group 2 innate lymphoid cells (ILC2s). ILC2s are defined as $lin^-CD127^+CD90^+$ and express the transcription factor GATA3 (38). Several studies have reported that ILC2s express CD25 and respond to IL-2 stimulation by proliferating and elaborating type 2 cytokine expression (39, 40). Additionally, a recent report showed that IL-5-producing ILC2s control eosinophilia induced by IL-2 therapy in humans and mice (24). Upon analysis of DSS-treated mice, we observed that administration of IL-2/JES6-1 immunocomplexes induced a significant increase in IL-5 mRNA expression in total colonic tissue (**Figure 3A**). Furthermore, IL-2/JES6-1 immunocomplexes significantly increased $lin^-CD90.2^+GATA3^+$ ILC2 frequency and absolute cell number (**Figures 3B,C**). IL-5 expressing ILC2s were also significantly increased in IL-2/JES6-1 immunocomplex-treated mice (**Figure 3D**), as was IL-13 mRNA expression (**Supplementary Figure 1A**) and IL-13 expressing ILC2s (**Supplementary Figure 1B**). While Ccl11 mRNA expression was significantly induced following DSS, IL-2/JES6-1 immunocomplex treatment did not significantly augment expression levels (**Supplementary Figure 1C**). Together, these observations suggest that eosinophil accumulation and activation induced by IL-2/JES6-1 immunocomplex treatment was strongly associated with IL-5- and IL-13-producing ILC2 induction.

Combined Delivery of IL-2/JES6-1 Immunocomplexes and Anti-IL-5 Mab Ameliorates DSS-Induced Colitis

In light of our observations that IL-2/JES6-1 immunocomplexes induce $Foxp3^+$ Tregs, but also IL-5-producing ILC2s and activated eosinophils during DSS-induced colitis, we next attempted a combined approach to optimally ameliorate

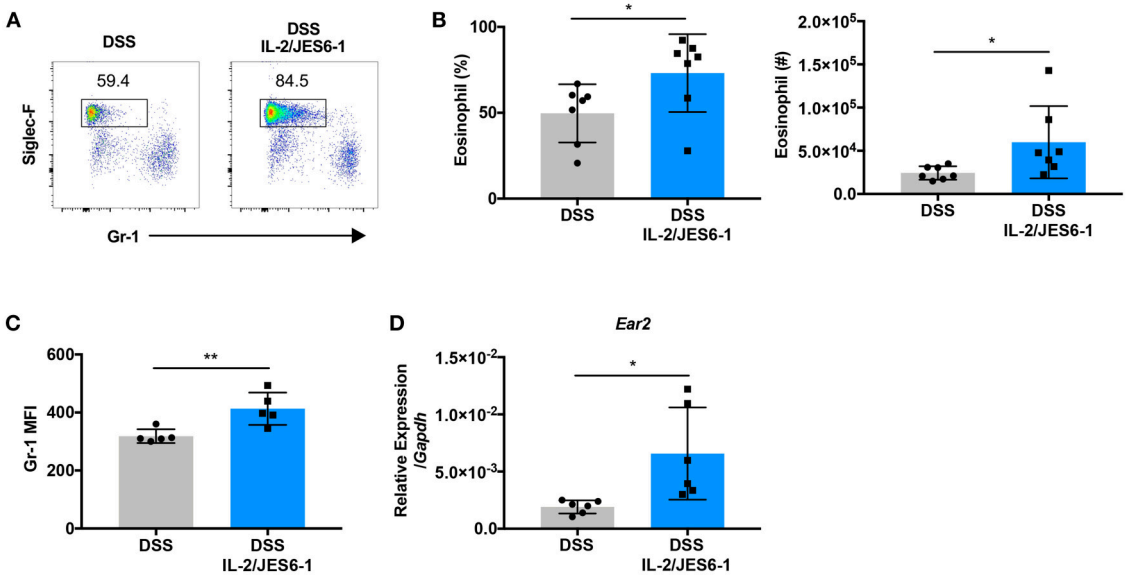

FIGURE 2 | Delivery of IL-2/JES6-1 immunocomplexes to DSS-treated mice drives colonic eosinophil accumulation and activation. WT mice were treated with 3% DSS for 6 days and received normal water thereafter. IL-2/JES6-1 immunocomplexes were delivered every 2 days. On day 10, eosinophils were analyzed by flow cytometry by pre-gating on live, $CD11b^+$ cells. Representative dot plots are shown in **(A)**, and the frequency and total cell numbers are shown in **(B)**. The mean fluorescence intensity (MFI) of Gr-1 staining in eosinophils is shown in **(C)** after pre-gating on live, $CD11b^+$, $Siglec\text{-}F^+$ cells. Expression of ECP (*Ear2*) was measured using qPCR **(D)**. Data are representative of two independent experiments with 5–7 mice/group. All data are presented as mean ± SEM; $^*P < 0.05$, $^{**}P < 0.01$, one-way ANOVA with Tukey's multiple comparison test.

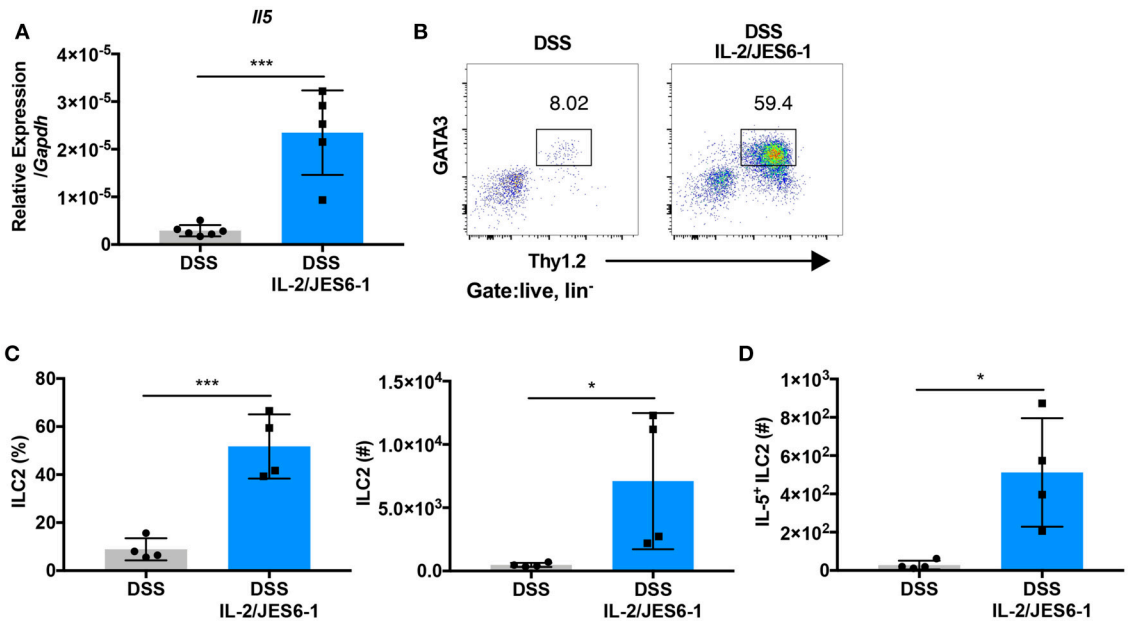

FIGURE 3 | IL-2/JES6-1 immunocomplex administration promotes intestinal ILC2 expansion and IL-5 expression. WT mice were treated with 3% DSS for 6 days and received normal water thereafter. IL-2/JES6-1 immunocomplexes were delivered every 2 days. IL-5 mRNA expression in total colon tissue was analyzed by quantitative real-time PCR **(A)**. LPL from each mouse were restimulated by PMA/ionomycin and ILC2 cells were analyzed by flow cytometry. Represent dot plots are shown in **(B)** and the frequency and total cell numbers are shown in **(C)** after pre-gating on live, lin- cells. IL-5+ILC2 were analyzed by flow cytometry **(D)**. Data are representative of two independent experiments with 4–6 mice/group. All data are presented as mean ± SEM; $^*P < 0.05$, $^{***}P < 0.001$, one-way ANOVA with Tukey's multiple comparison test.

colitis by expanding colonic Foxp3+ Tregs via delivery of IL-2/JES6-1 immunocomplexes while simultaneously blocking eosinophil accumulation and activation by using anti-IL-5 monoclonal antibodies (mAb). We first assessed whether anti-IL-5 mAb treatment was able to prevent colonic eosinophilia in our experimental model. Indeed, while IL-2/JES6-1 immunocomplexes increased colonic eosinophil frequency and absolute cell number in DSS-treated mice, this effect could be prevented by co-administration of anti-IL-5 mAb (**Figures 4A,B**). Of note, administration of anti-IL-5 mAb alone was also able to significantly reduce colonic eosinophils when compared to control IgG administration to DSS-treated mice. Similarly, IL-2/JES6-1 immunocomplexes increased colonic eosinophil activation as evidenced by increased frequencies and absolute cell numbers of cells expressing intermediate, but not high, levels of Gr-1, and this effect could be prevented by co-administration of anti-IL-5 mAb (**Supplementary Figures 2A,B**). We next examined the effects of these 4 different treatments (control IgG, IL-2/JES6-1 immunocomplexes alone, anti-IL-5 mAb alone, and IL-2/JES6-1 immunocomplexes + anti-IL-5 mAb) on DSS-induced colitis and recovery. While the IL-2/JES6-1 immunocomplex group and the anti-IL-5 mAb group each displayed improvement in disease outcome compared the control IgG treated group, combined delivery of IL-2/JES6-1 immunocomplexes + anti-IL-5 mAb afforded the most significant protection as measured by reduced colonic shortening (**Figure 4C**), weight loss (**Figure 4D**), and disease activity index (DAI; **Figure 4E**). Consistent with these observations, combined delivery of IL-2/JES6-1 immunocomplexes + anti-IL-5 mAb also provided optimal reduction in pathological tissue inflammation (**Figure 4F**) and histology score (**Figure 4G**). As summarized in **Supplementary Figure 3**, these data collectively demonstrate that combined administration of IL-2/JES6-1 immunocomplexes and anti-IL-5 mAb, which allows for Foxp3$^+$ Treg expansion in the absence of eosinophilia, is highly effective at ameliorating DSS-induced colitis compared to the use of IL-2/JES6-1 immunocomplexes or anti-IL-5 mAb alone.

DISCUSSION

In this study, we provide evidence demonstrating that combined delivery of IL-2 immunocomplexes and anti-IL-5 mAb is highly effective at expanding Foxp3$^+$ Treg cells in the absence of eosinophilia and ameliorating DSS-induced colitis in mice. While evidence strongly suggests that Tregs are instrumental in establishing and maintaining gut homeostasis, an effective approach to expand these cells *in vivo* for the treatment of intestinal inflammation, without undesired side effects, has proven challenging (21, 33, 34). A previous report elegantly demonstrated that IL-2/JES6-1 immunocomplexes are effective at expanding Foxp3+ T cells in the spleens of mice and when delivered for 1 week prior to the initiation of DSS were able to lessen colitis (22). Our data confirms and extends these findings by showing that administration of IL-2/JES6-1 immunocomplexes at the time of initiating DSS treatment is sufficient to expand colonic Foxp3$^+$ Tregs and ameliorate colitis.

Interestingly, we noted that IL-2/JES6-1 immunocomplex delivery to DSS-treated mice also potently expanded IL-5 producing ILC2s and eosinophils in the inflamed colon, which is consistent with observations in other tissues (24, 40). These data suggested that accumulation and activation of eosinophils may have been impairing the beneficial effects of Foxp3$^+$ Treg expansion induced by IL-2/JES6-1 immunocomplexes. Consistent with this concept, activated eosinophils have been associated with human IBD (41–44) and DSS-induced histopathology is attenuated in mice deficient in eosinophils (45–47), although eosinophils appear to play a dual role in the intestine and can provide beneficial effects (48, 49). Given the potent role for IL-5 in driving eosinophil accumulation, blockade of IL-5 has also been shown to reduce intestinal eosinophilia and modestly ameliorate experimental colitis in some models (35), yet not in others (50, 51). Indeed, two previous studies analyzing DSS-induced colitis using IL-5-deficient mice (50, 51) concluded that IL-5 alone plays a minor role in the DSS model, whereas we observed a modestly more robust effect in our study. These modest effects of IL-5 deficiency on DSS colitis may be due to eosinophils providing both pro- and anti-inflammatory functions during intestinal inflammation (52), as well as the use of IL-5-deficient mice in these studies and antibody-mediated IL-5 neutralization in our study. In response to IL-2/JES6-1 immunocomplex administration and increased levels of IL-5, the augmented number and activation status of eosinophils may tip the balance in the pro-inflammatory direction, which may explain our observed beneficial effect of combined IL-2/JES6-1 immunocomplex and IL-5 blockade in the DSS model of colitis. It is noteworthy that the acute DSS model of colonic damage and repair predominantly involves innate immune activation and whether this combined treatment strategy will also be effective in chronic, T-cell dependent models of intestinal inflammation remains to be investigated.

The expansion of ILC2s by IL-2 immunocomplexes has been well-documented and consistent with the high expression of CD25 on ILC2s (21, 24, 40). However, whether ILC2 activation is beneficial or deleterious during intestinal inflammation is still being actively unraveled. Recent reports have shown that these cells are involved in mounting type 2 immune responses to helminthic infections of the gastrointestinal tract and coordinate with tuft cells to elaborate an IL-25-ILC2-IL-13 immune circuit that promotes intestinal defense and remodeling (53–56). During DSS-induced colitis however, the function of ILC2s is less clear. Gut-associated ILC2s have been reported to secrete the growth factor amphiregulin which can limit intestinal inflammation and promote tissue repair processes (57). As with eosinophils, ILC2 expansion by IL-2 immunocomplexes may alter the normal function of these cells. Clearly, additional studies are warranted to fully understand how IL-2 delivery regulates ILC2s as well as eosinophils.

Overall, further understanding of how to exploit IL-2 signaling to enhance the development, function, and antigen specificity of Tregs, without unwelcome side effects, is an active area of investigation with strong translational potential for many

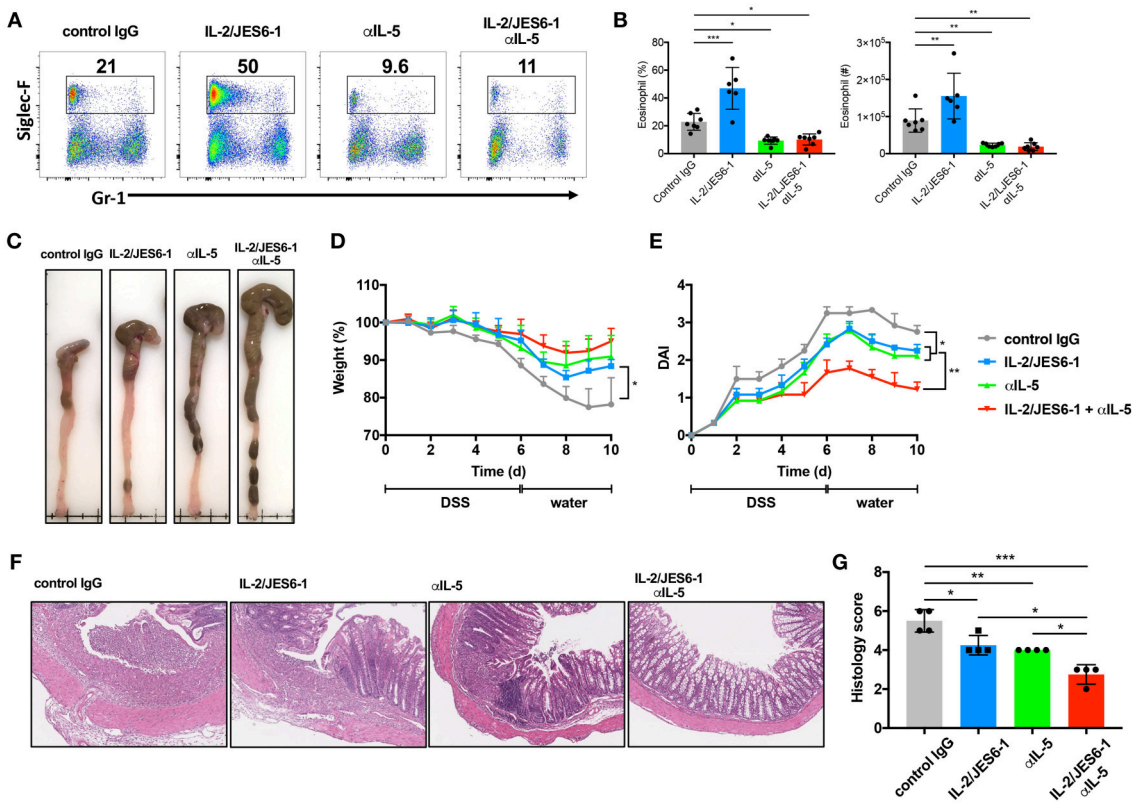

FIGURE 4 | Combination of IL-2/JES6-1 immunocomplexes and anti-IL-5 mAb effectively ameliorate DSS-induced colitis. WT mice were treated with 3% DSS for 6 days and received normal water thereafter. IL-2/JES6-1 immunocomplex were delivered every 2 days and anti-IL-5 mAb was delivered every day. Eosinophils (Siglec-F^{+}) were analyzed by flow cytometry **(A)** and the frequency and total cell numbers are shown in **(B)**. Representative images of colons from indicated conditions are shown in **(C)**. Weight loss **(D)** and disease activity index (DAI) **(E)** were monitored every day. Representative hematoxylin/eosin stained colon sections are shown in **(F)** and histology scoring of individual colon sections are shown in **(G)**. Data are representative of two independent experiments with 4 mice/group. All data are presented as mean ± SEM; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, one-way ANOVA with Tukey's multiple comparison test.

inflammatory diseases (34). IL-2/JES6-1 immunocomplexes have been successfully used to expand Foxp3^{+} Treg cells in mice and these findings have led to the development of an anti-human IL-2 mAbs that selectively expands Tregs in humans (21, 34, 58). Recently, a novel anti-IL-2 antibody, F5111.2, was developed that stabilizes IL-2 in a conformation that leads to the selective expansion of Tregs. Complexing of F5111.2 with human IL-2 led to the remission of type 1 diabetes and reduced disease severity in mouse models of graft-vs.-host disease and experimental autoimmune encephalomyelitis (59). Another clinically attractive approach for preferentially expanding Tregs *in vivo* is to directly modulate IL-2 itself to allow for optimal conformation for binding to CD25 (34). These so-called IL-2 "muteins," like IL-2 immunocomplexes, may also lead to ILC2 expansion, IL-5 production, and eosinophilia. The data presented here suggests that these approaches for IL-2 driven Treg expansion *in vivo* may be further optimized by blocking IL-5 and limiting eosinophil activation.

In the present study we assessed IL-5 induction as one unwarranted side-effect of IL-2 immunocomplex treatment, however, it is important to note that stimulation of ILC2s with IL-2 or IL-2 immunocomplexes also induces other type 2 cytokines that may contribute to intestinal inflammation, including IL-9 (60) and IL-13 (40). Interestingly, both IL-9 and IL-13 can contribute to the pathogenesis of experimental models of colitis and human inflammatory bowel disease (IBD) (61, 62) and are known inducers of the eosinophil chemokine Ccl11 (63, 64). These findings may thus provide a framework for future investigations into whether blockade of IL-5, IL-9, IL-13, and/or Ccl11 may enhance the effectiveness of IL-2 immunocomplex treatment during intestinal inflammation as well as other inflammatory disease settings.

MATERIALS AND METHODS

Mice

C57BL/6J mice were obtained from the Jackson Laboratory and maintained in specific pathogen-free conditions. In all experiments, sex-matched mice were used at 7–8 weeks of age. Animal procedures were approved by the Institutional Animal Care and Use Committee of Georgia State University.

DSS Model of Colitis

Mice were treated with 3% (wt/vol) DSS (MP Biomedicals; molecular weight: 36,000–50,000) in their drinking water for 6 days and then DSS was replaced with normal water. Mice receiving DSS were monitored daily for weight change and disease index activity (DAI).

Flow Cytometry

Fluorescent dye-labeled antibodies specific for CD4, CD25, CD11b, Gr-1 (clone RB6-8C5), Foxp3, GATA3, B220, NK1.1, CD19, CD3, IL-5, and IL-13 were purchased from eBioscience and Biolegend. Fc block (2.4G2) was purchased from BD. Dead cells were stained by fixable aqua dead cell staining kit. Intracellular staining for Foxp3 and GATA3 was performed by Intracellular Fixation and Permeabilization Buffer Set (eBioscience). Intracellular staining of IL-5 was performed after restimulation with PMA, ionomycin and brefeldin A for 5 h. Restimulated cells were treated with Intracellular Fixation and Permeabilization Buffer Set (eBioscience) and stained with IL-5 antibodies. Flow cytometric analysis was performed on a Beckman Coulter Cytoflex flow cytometer and analyzed by Flowjo software (Tree Star, Ashland, OR).

Isolation of Colonic Lamina Propria Cells From Large Intestine

Colon tissues were cut into 0.5 cm pieces and transferred into 50 mL conical tubes. Then tubes were shaken at 250 rpm for 20 min at 37°C in Hanks' balanced salt solution supplemented with 5% FBS with 5 mM EDTA. This process was repeated twice. Cell suspensions were passed through a cell strainer and remaining colon tissues were washed and minced, transferred to 50 mL conical tubes and shaken for 10 min at 37°C in Hanks' balanced-salt solution supplemented with 5% FBS and type VIII collagenase (1 mg/mL). Cell suspensions were passed through a cell strainer and pelleted by centrifugation at 300 g.

In vivo Administration of IL-2/JES6-1 Immunocomplexes and Anti-IL-5 mAb

IL-2/JES6-1 immunocomplexes were prepared by pre-incubating recombinant IL-2 (PeproTech) with anti-IL-2 mAb (JES6-1; Bioxcell) at a 2:1 cytokine:antibody molar ratio for 30 min at room temperature (23, 65). Mice were injected with anti-IL-5 antibodies (200 μg) and/or IL-2/JES6-1 immunocomplexes (1 mg every 2 days) by i.p injection.

RNA Isolation and Real-Time PCR

Total RNA was isolated from colon tissue using the Qiagen RNeasy Mini Kit, according to the manufacturer's protocols with on-column DNase digestion using the RNase Free DNase set. cDNA was generated using Hi-capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to manufacturer's protocols. qPCR was performed with SYBR Green Master Mix (BioRad) on Step One Plus real time PCR system (Applied Biosystems), and gene specific primers.

Il5 F: 5′-CGCTCACCGAGCTCTGTTG-3′
Il5 R: 5′-CCAATGCATAGCTGGTGATTTTT-3′
Foxp3 F: 5′-CACCCAGGAAAGACAGCAACC-3′
Foxp3 R: 5′-GCAAGAGCTCTTGTCCATTGA-3′
Gapdh F: 5′-TGGCAAAGTGGAGATTGTTGCC-3′
Gapdh R: 5′-AAGATGGTGATGGGCTTCCCG-3′
Ctla4 F: 5′-TGTTGACACGGGACTGTACCT-3′
Ctla4 R: 5′-CGGGCATGGTTCTGGATCA-3′
Il2ra F: 5′-CCACCACAGACTTCCCACAA-3′
Il2ra R: 5′-CCATCTGTGTTGCCAGGTGA-3′
Ear2 F: 5′-ACCAGTCGGAGGAGAACACC-3′
Ear2 R: 5′-CAAAGGTGCAAAGTGCTGGC-3′
Ccl11 F: 5′-AGAGCTCCACAGCGCTTCTATT-3′
Ccl11 R: 5′-GGTGCATCTGTTGTTGGTGATT-3′

Histology

Colon tissues were fixed in 10% neutral buffer formalin and embedded in paraffin. Paraffin embedded tissue sections were stained using hematoxylin/eosin. The score of inflammation and epithelial damage was graded in a blinded manner using a scale from 0 to 3 for each parameter.

Statistics

All statistical analyses were performed with GraphPad Prism software, version 7 (GraphPad Software). One-way ANOVA and Tukey's Multiple Comparison Test or Student's *t*-test were used to determine significance. $P < 0.05$ were considered significant. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the supplementary files.

AUTHOR CONTRIBUTIONS

HA and TD conceived the idea for this project and designed the experiments. HA performed all of the experiments and analyzed the data. KF and DG performed critical preliminary experiments. VN and AH provided technical assistance. HA and TD wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00459/full#supplementary-material

Supplementary Figure 1 | IL-2/JES6-1 immunocomplex administration induces expansion of IL-13-producing intestinal ILC2s. WT mice were treated with 3% DSS for 6 days and received normal water thereafter. IL-2/JES6-1

immunocomplexes were delivered every 2 days. IL-13 mRNA expression in total colon tissue was analyzed by quantitative real-time PCR **(A)**. LPL from each mouse were restimulated by PMA/ionomycin and IL-13+ ILC2s total cell numbers were analyzed by flow cytometry after pre-gating on live, lin- cells **(B)**. Ccl11 mRNA expression in total colon tissue was analyzed by quantitative real-time PCR **(C)**. All data are presented as mean ± SEM; $*P < 0.05$, $**P < 0.01$.

Supplementary Figure 2 | Anti-IL-5 mAb treatment efficiently prevents IL-2/JES6-1 immunocomplex-induced eosinophil activation during DSS-induced colitis. WT mice were treated with 3% DSS for 6 days and received normal water thereafter. IL-2/JES6-1 immunocomplexes were delivered every 2 days and anti-IL-5 mAb was delivered every day. Siglec-F^+ populations were gated as Gr-1 low, int, and hi. Representative dot plots are shown in **(A)**. Frequency and cell numbers of each population are shown in **(B)**. All data are presented as mean ± SEM; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Supplementary Figure 3 | Model for combined effects of IL-2/JES6-1 immunocomplex and anti-IL-5 treatment during DSS colitis. IL-2/JES6-1 immunocomplexes during DSS-induced colitis induce $Foxp3^+$ Treg expansion, but also potently stimulates $GATA3^+$ILC2 proliferation and expression of IL-5, leading to eosinophil accumulation and activation in the inflamed colon. Combined IL-2/JES6-1 and anti-IL-5 mAb treatment permits Foxp3+ Treg expansion in the absence of associated eosinophilia and is effective at ameliorating DSS-induced colitis.

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REVIEW
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TL1A (TNFSF15) and DR3 (TNFRSF25): A Co-stimulatory System of Cytokines With Diverse Functions in Gut Mucosal Immunity

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TL1A and its functional receptor DR3 are members of the TNF/TNFR superfamilies of proteins. Binding of APC-derived TL1A to lymphocytic DR3 provides co-stimulatory signals for activated lymphocytes. DR3 signaling affects the proliferative activity of and cytokine production by effector lymphocytes, but also critically influences the development and suppressive function of regulatory T-cells. DR3 was also found to be highly expressed by innate lymphoid cells (ILCS), which respond to stimulation by TL1A. Several recent studies with transgenic and knockout mice as well as neutralizing or agonistic antibodies for these two proteins, have clearly shown that TL1A/DR3 are important mediators of several chronic immunological disorders, including Inflammatory Bowel Disease (IBD). TL1A and DR3 are abundantly localized at inflamed intestinal areas of patients with IBD and mice with experimental ileitis or colitis and actively participate in the immunological pathways that underlie mucosal homeostasis and intestinal inflammation. DR3 signaling has demonstrated a dichotomous role in mucosal immunity. On the one hand, during acute mucosal injury it exerts protective functions by ameliorating the severity of acute inflammatory responses and facilitating tissue repair. On the other hand, it critically participates in the pro-inflammatory pathways that underlie chronic inflammatory responses, such as those that take place in IBD. These effects are mediated through modulation of the relative mucosal abundance and function of Th1, Th2, Th17, Th9, and Treg lymphocytes, but also of all types of ILCs. Recently, an important role was demonstrated for TL1A/DR3 as potential mediators of intestinal fibrosis that is associated with the presence of gut inflammation. These accumulating data have raised the possibility that TL1A/DR3 pathways may represent a valid therapeutic target for chronic immunological diseases. Nevertheless, applicability of such a therapeutic approach will greatly rely on the net result of TL1A/DR3 manipulation on the various cell populations that will be affected by this approach.

Keywords: TL1A, DR3, mucosal immunity, inflammatory bowel disease, co-stimulatory

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INTRODUCTION

Mucosal homeostasis at the gastrointestinal tract requires a delicate co-existence of gut microbiota with the gut-associated mucosal immune system, an interaction that is constantly challenged by environmental factors. Hence, mucosal "health" is depended on an intact genetic structure, preserved by the integrity of the epithelial barrier, and fine-tuned by immunoregulatory responses. Failure of one or more of these balancing elements leads to breakdown of homeostasis and predominance of pro-inflammatory immunological circuits. The latter are critically dependent on key cellular and/or soluble mediators, most prominent among which are cytokines and their receptors. The prototypical disorders that are signified by such dysregulated mucosal immunity are the Inflammatory Bowel Diseases (IBD), in particular Crohn's disease (CD) and ulcerative colitis (UC). Thus, it is of no surprise that cytokines have been the main targets of therapeutic interventions in IBD, which represents a rapidly expanding field in recent years.

TL1A (Tumor necrosis factor-like cytokine 1A) was first reported in 2002 (1). It is a member of the TNF superfamily of proteins (TNFSF) and it is encoded by the *Tnfsf15* gene that is located on chromosome 9q32 in humans and chromosome 4 in mice. TL1A is a type II transmembrane protein with a molecular weight of 28 kDa, which contains 251 amino acids. Similar to other members of the TNF-family, TL1A forms a stable trimer. It exists in a membrane-bound form (mTL1A), which may also be cleaved by matrix metalloproteinases and released as soluble, fully-functional 20-kDa protein (sTL1A) (1, 2).

The functional receptor for TL1A is DR3 (death domain receptor 3), which is encoded by the *Tnfrsf25* gene that is located at the 1p36.3 position in humans (3, 4). DR3 is a type I membrane protein with a 417 AA sequence and a molecular weight of 45 kDa that shares the highest homology to TNFR1 among all members of the TNFRSF. DR3 contains a death domain in its cytoplasmic region; thus it may participate in apoptotic processes. Nevertheless, DR3 signaling also mediates inflammatory/immunological responses. An important characteristic of human DR3 is the existence of several splice variants (13 in humans and 10 in mice). The functional implications of such variety are not fully understood, although encoded proteins may differ in their function (5). To date, the only proven ligand for DR3 is TL1A (including the short variant, TL1/vascular endothelial growth inhibitor).

There is now able evidence that interactions between TL1A and its functional receptor DR3 affect gut mucosal immunity both during homeostatic conditions and in various inflammatory states (**Figure 1**). In particular, their role in IBD is supported by a variety of genetic, immunological, experimental, and translational data. The current review aims to critically present existing literature on the role of TL1A and DR3 in mucosal immunity.

TNFSF15 POLYMORPHISMS AFFECT SUSCEPTIBILITY TO INTESTINAL DISEASES

A first line of evidence for the potential importance of TL1A in the pathogenesis of IBD is derived from studies that reported significant associations between genetic variations in the *Tnfsf15* gene and susceptibility to IBD (**Table 1**).

In 2005, Yamazaki et al. were the first to report that a specific genetic variant of *Tnfsf15*, tnfsf15_28, was strongly associated with susceptibility to IBD in Japanese patients, whereas the gene was monomorphic in a Caucasian population from the UK (19). Further investigation of these two ethnic groups revealed 5 different SNPs, tnfsf15_26, 31, 35, 36, and 41, that were polymorphic in both groups, forming three different haplotypes which affected susceptibility to IBD. In both ethnic groups, haplotype A was identified as a high-risk marker for susceptibility to IBD, whereas haplotype B was found to be a low-risk genetic factor. Haplotype C was not significantly associated with IBD risk in either population, despite its frequent detection. Two years later, Picornell et al. investigated the aforementioned three haplotypes in Jewish and non-Jewish IBD and control populations in Los Angeles, USA. In the non-Jewish population, similar to the previous study, haplotype B was less frequent in both CD and UC patients compared to controls, highlighting a possible protective role (20). On the other hand, no association of haplotype A with IBD was seen in either population, suggesting that *Tnfsf15* polymorphisms are ethnic-specific. These findings were further supported by independent studies from Asia and Europe.

Interestingly, haplotype B of the *Tnfsf15* gene has been found to affect protein production by monocytes and macrophages. Peripheral CD14+ monocytes and monocyte-derived macrophages from patients carrying Haplotype B or the rs6478109 A minor allele produce higher levels of TL1A in response to FcγR or LPS stimulation (21, 22). Interestingly, haplotype B has been reported to confer CD risk in Jewish patients but the rs6478109 A minor allele conferred protection to subjects of European descent. However, the studies on the effect of *TL1A* gene Haplotypes on membrane bound TL1A has provided conflicting results. Earlier studies have shown that membrane expression of TL1A was up-regulated on peripheral monocytes from Jewish but not from non-Jewish CD patients that carry the B haplotype but later studies have shown increase membrane bound TL1A in homozygotes of the protective rs6478109 A allele in European descent subject (21, 22).

A Korean study in pediatric CD patients and adult controls showed that six specific haplotypes of the *Tnfsf15* gene were more frequently reported and, of those, two were significantly different between the two groups; the haplotype including T-C-A-T-C SNPs (rs3810936-rs6478108-rs6478109-rs7848647-rs7865494) was more frequent in controls, whereas haplotype including C-T-G-C-C SNPs in CD patients (7). On the other hand, in a Chinese study consisting of 13 pediatric patients with Very-early-onset IBD (VEO-IBD), no association between *Tnfsf15* gene mutations and VEO-IBD was found and this finding may be

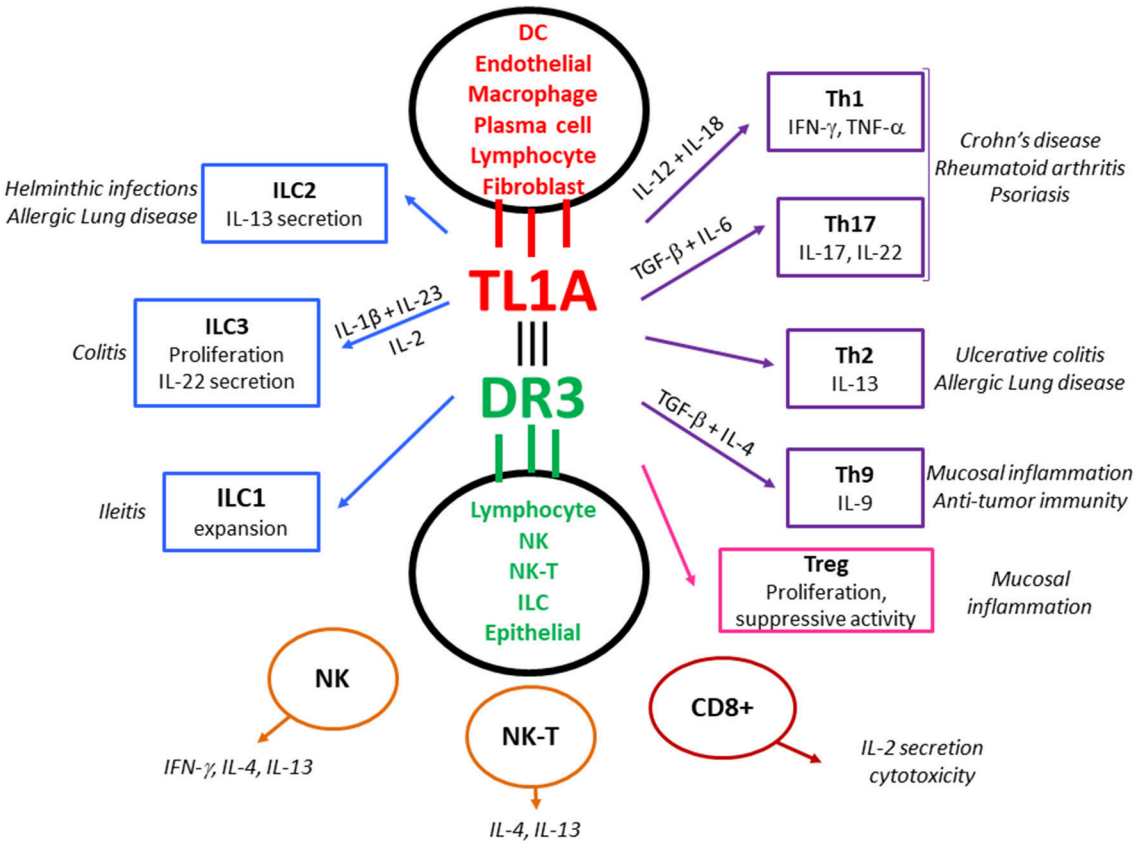

FIGURE 1 | The TL1A/DR3 system as a central regulator of mucosal immune responses, allergy and autoimmunity. TL1A is not constitutively expressed but is induced in mucosal APCs (and other types of immunocytes) following stimulation via microbial and non-microbial antigens. TL1A binds to the functional receptor, DR3, which is expressed by various lymphocytic populations upon activation. TL1A/DR3 signaling enhances proliferation and optimizes cytokine production by responding lymphocytes, acting as a co-stimulatory system that amplifies TCR or cytokine provided signals. This function is of particular importance under conditions of sub-optimal lymphocyte stimulation. All types of effector T cells (Teff: Th1, Th2, Th9, Th17) respond to stimulation with TL1A. DR3 is also expressed by regulatory lymphocytes (Tregs), which proliferate in response to TL1A, although this may be accompanied by a temporary halt of suppressive function, especially in the event of acute inflammation. DR3 expression has also been demonstrated in innate lymphoid cells (ILCs) and DR3 signaling affects their function. Finally, TL1A/DR3 signaling pathways have been reported in NK and NK-T cells, as well as CD8+ lymphocytes. This universal expression of DR3 by innate and adaptive effector and regulatory populations implies a key regulatory role of the TL1A/DR3 system in mucosal immunity. Alongside, experimental data from animal models and translational data from patients indicate an important contribution of the TL1A/DR3 system in allergic lung inflammation and autoimmune diseases such as Crohn's disease, Ulcerative colitis, Rheumatoid arthritis, and Psoriasis.

the result of either ethnic differences or the small number of patients included in the study (23). Contrary to the previous study, it was recently shown that the *Tnfsf15* rs4246905 SNP was associated with development of CD in children with chronic granulomatous disease (24).

The importance of the ethnic background for *Tnfsf15* polymorphisms was further highlighted in an Indian study showing that haplotype A was significantly more frequent in IBD cases than in healthy individuals, while the opposite was observed for haplotype C. Further investigation identified two additional SNPs (rs10114470 and rs4263839) and generated 7 different haplotypes, from which haplotype H had a possible protective role being more frequent in healthy individuals, whereas haplotypes I and J conferred susceptibility to IBD (25). Haplotypes of the *Tnfsf15* gene that are associated with either susceptibility or protection from CD have also been reported in the European population (26). Similar to Yamazaki, a Korean study showed that the allele T of the SNP rs6478108 is indeed a "risk" allele as it is more frequently found in patients with UC (8). In a recent study that included a large number of IBD patients and healthy individuals across Europe and Asia showed that, although allele frequencies are similar between the European and Asian population, *Tnfsf15* variants have a stronger association with IBD susceptibility in people originating from Asia (18).

Other studies have also proposed that certain *Tnfsf15* alleles may bear prognostic value for the severity of IBD. In the Chinese population, the T allele of SNP rs10114470 was associated with increased probability of developing stricturing, penetrating, or perianal complications (27). In the same notion, Pernat Dobrez et al. identified the SNP rs4263839 as a possible marker for disease progression. In their study, 72.2% of CD patients who were carriers of allele A had progressed from an inflammatory (B1)

TABLE 1 | Genetic associations of *Tnfsf15* gene with susceptibility and phenotype of IBD.

Polymorphism	Ethnicity	Susceptibility/Phenotype	References
rs3810936	Asian, Caucasian	CD	(6)
	Asian	UC	
rs6478108	Asian, Caucasian	CD	(6) (7)
	Asian, Caucasian	UC	(8)
	Asian	CD B2/B3	(9)
rs4979462	Asian	CD	(6)
rs6478109	Asian, Caucasian	CD	(6) (7) (10)
	Asian	UC	(6)
rs7848647	Asian, Caucasian	CD	(6)
	Asian	UC	
	Caucasian, Asian	CD early onset	(7, 11)
rs7869487	Asian, Caucasian	CD	(6)
rs4263839	Caucasian	Colonic CD location	(12)
	Caucasian	Bowel resection	(13)
	Asian	CD, B2/B3	(14)
rs4574921	Asian	CD B3p	(9)
rs11554257	Caucasian	MR-UC	(15)
rs3810936	Asian	Severe CD	(16)
	Asian	CD	(17)
	Asian	CD B3p	(7)
rs4246905	Asian	IBD	(18)

MR, Medically refractory; B2, B3, p, Montreal classification indicators for Crohn's disease.

to stricturing (B2) or penetrating (B3) phenotype, as compared to only 55% of those bearing the allele G (28). In another study, polymorphisms in the *Tnfsf15* gene were associated with medically refractory UC (15). The susceptibility loci for CD reported by Yamazaki et al. were also detected in CD patients from Ryukyu Islands, near Japan. Interestingly, CD patients, bearing the risk alleles, had increased bacterial abundances of *Bacteroidetes* and more specifically, of *Prevotella*, but whether the genetic background is the outcome of microbiome composition alteration or *vice versa*, remains unclear (29).

Interestingly, besides IBD, *Tnfsf15* genetic variants have also been associated with other GI diseases, such as diverticulitis and Irritable Bowel Syndrome (IBS). Connelly et al. discovered that the SNP rs7848647 is highly associated with diverticulitis and that haplotype carriage may predict the need for surgical intervention (30). Additional protective or risk-conferring haplotypes for diverticulitis were reported later by the same group (31) Regarding IBS, Zucchelli et al. reported a strong association between the SNP rs4263839 and patients suffering from IBS. In particular, the G allele of the SNP rs4263839 was identified as a high-risk marker and further investigation revealed that it could lead to higher expression levels of TNFSF15 in healthy individuals (32).

Taken together, various SNPs of the *Tnfsf15* gene have been associated with not only IBD, but also diverticulitis and IBS, and seem to be promising predictors for intestinal disease susceptibility and/or progression. Nonetheless, the interpretation of the role of *Tnfsf15* mutations seem to be influenced by ethnic background, as a stronger association with Asians has been reported. In addition, no definitive functional implications for existing, risk-associated polymorphisms have been reported. Nevertheless, a few studies have proposed that certain polymorphisms may be associated with specific functional effects, a finding that, interestingly, was also affected by the ethnic background in some cases (22, 33, 34).

THE COMPLEX ROLE OF TL1A/DR3 IN MUCOSAL IMMUNITY

Expression, Regulation, and Function of the TL1A/DR3 System in Immune Cells

Mononuclear Phagocytes

The cellular sources of TL1A and requirements for its expression indicate its important role in innate and adaptive immune responses especially at the intestinal mucosa. Besides the original description of its constitutive expression in endothelial cells (1), TL1A was also found to be expressed by mononuclear phagocytes at the intestinal lamina propria of both mice and humans under inflammatory conditions. In particular, early studies in murine models of ileitis and colitis have shown that TL1A was mainly expressed by $CD11c^{high}$/MHC-II^{+} mononuclear phagocytes of the lamina propria and the MLNs and $CD11c^{low}$/MHC-II^{-} mononuclear phagocytes of the lamina propria that probably represent dendritic cells and macrophage subsets (35, 36). In patients with CD or UC immunolocalization of TL1A was reported in tissue macrophages and lymphocytes, as well as in infiltrating plasma cells in UC (37). A recent study has further characterized lamina propria mononuclear phagocytes that primarily produce TL1A in mice, CD patients and healthy subjects and reported expression of CD14 and CX3CR1 surface markers, which classifies them as antigen sampling mucosal macrophages. TL1A production by $CD11c^{+}CX3CR1^{+}$ mononuclear phagocytes correlated with disease activity in CD in humans. In mice $CD11c^{+}CX3CR1^{+}$ mononuclear phagocytes produced TL1A, in a MyD88-dependent fashion, in response to mucosal-adherent bacteria (38).

The abundant cellular expression of TL1A in APCs, led to studies on the regulation of its expression in this particular population. These studies showed that TL1A expression by APCs is responsive to signaling through FcRγ receptors, TLRs or microbial antigens. In particular, in monocytes and monocyte-derived dendritic cells (DCs) TL1A mRNA and protein (both soluble and transmembrane forms) was highly induced by FcRγ stimulation through plate-bound, cross-linked human IgG (39). A potential clinical relevance of this association was implied in two studies in rheumatoid arthritis (RA). Cassatella et al. reported that mononuclear phagocytes strongly expressed TL1A in rheumatoid factor–positive but not rheumatoid factor–negative patients (40). *In vitro* stimulation of monocytes with various preparations of insoluble immune complexes led to significant upregulation of TL1A (40). Furthermore, Bamias et al., demonstrated that soluble TL1A concentrations were significantly higher in rheumatoid factor–positive than rheumatoid factor–negative patients (41).

In addition to stimulation through the FcRγ receptor, bacterial signals also upregulate the expression of TL1A. In the monocytic cell line U937, TL1A was induced by LPS. This pathway involved activation and binding of the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB) to a specific binding site in the 5′ flanking region of TL1A (42). LPS responsiveness was also confirmed in bone-marrow derived dendritic cells (43).

Shih et al. studied the effect of stimulation by cultured microorganisms on TL1A expression by monocytes or DCs (44). Cells were stimulated with a variety of cultured microorganisms (gram-negative [*Escherichia coli*, *E. coli* Nissle 1917, and *Salmonella typhimurium*], gram-positive [*Listeria monocytogenes* and *Staphylococcus epidermidis*], partial anaerobes [*Campylobacter jejuni*], and obligate anaerobes [*Bacteroides thetaiotaomicron*, *Bifidobacterium breve*, and *Clostridium* A4]). In all cases, upregulation of TL1A was seen through an NF-kB (as well as p38 MAPK)–dependent mechanism. TLR signaling could only partially substitute for the effect of whole bacteria stimulation. Moreover, TLR7/8 signaling demonstrated a negative effect by significantly decreasing IC-induced TL1A expression of monocytes (45). In another study, Kamada et al. also reported TL1A induction by heat-killed bacteria in CD-associated LP macrophages (46).

Effector Lymphocytes

TL1A is also expressed on activated lymphocytes, indicating the formation of positive regulatory loops during mucosal inflammation (47, 48). Especially in the small bowel, membrane bound TL1A is expressed by activated gut specific $CCR9^+$ lymphocytes (47) and it is possibly up-regulated by stimulation through the T-cell receptor (TCR) (i.e., by phytohemagglutinin) (49).

Expression of DR3, the cognate receptor of TL1A, is mainly detected in lymphocytic populations, mostly following activation. DR3 signaling enhances CD4+ lymphocyte proliferation by increasing both IL-2 production and expression of IL-2RA and IL-2RB (1). The effect of TL1A co-stimulation is particularly important in conditions of sub-optimal stimulation via CD3 or CD28 (43). TL1A preferentially enhances proliferation of murine memory CD4+ T cells (35), but can also induce mild proliferation and strong IL-2 and IFN-γ expression by naïve T cells (43). TL1A co-stimulation of CD4+ T cells results in production of multiple cytokines including IL-2, IL-4, IL-13, interferon-gamma (IFNγ), and IL-17 (1, 43, 47, 48, 50). Although, TL1A-DR3 interaction enhances T cell proliferation and is required for optimal cytokine production, it appears to be dispensable for differentiation of naive T cells into Th1, Th2, or Th17 effector cell subtypes (43).

TL1A-DR3 is proposed as an important system for enhancement of immune responses in the periphery. Indeed, TL1A acts in synergy with IL-12 and IL-18 to enhance adaptive Th1 and innate IFNγ responses by T cells (49, 51, 52). Specific T cell subsets that up-regulate IFNγ and TNFα production in response to TL1A and IL-12/18 co-stimulation have been characterized by the expression of CCR9 (47), CD161 (53), and IL-18Rα (52). Interestingly, TL1A responsive T cells were preferentially localized at the intestinal mucosa, which indicates a prominent role of TL1A in intestinal IFNγ-mediated immune responses. Consistent with its role as a non-specific co-stimulator, irrespective of T-cell lineage commitment, TL1A was shown to enhance secretion of Th2 cytokines by activated T cells and amplified IL-13 production by NKT cells in a murine model of allergic lung inflammation (43, 48). The role of TL1A in Th17 responses appears more complex. Pappu et al. found that $TL1A^{-/-}$ dendritic cells exhibited reduced ability to support differentiation of Th17 lymphocytes and TL1A co-stimulation was required for optimal antigen-independent proliferation of differentiated Th17 lymphocytes (50). In contrast, Jones et al. reported that TL1A-DR3 interactions inhibit polarization toward the Th17 lineage but support IL-17 production in fully committed Th17 cells (54). Differences of *in vitro* experimental conditions for Th17 polarization may account for the aforementioned discrepancies. Furthermore, TL1A has been found to induce IL-22 production by human peripheral memory $CD4^+$ T cells and committed Th17 cells, through up-regulation of IL-9 (55). Nevertheless, TL1A co-stimulation has been proven essential for both gut and cerebral immunopathology that depends on Th1 and Th17 in relevant mouse models (36, 43, 50).

Tregs

Control of proliferation and suppressive function of regulatory T cell (Treg) is another way through which the TL1A-DR3 system controls local immune responses. Tregs constitutively express DR3, and DR3 signaling has been found to enhance proliferation of Tregs partly by enhancing their responsiveness to IL-2 (56–58). The proliferative effect of TL1A on Tregs has been recently confirmed for human cells in *ex vivo* studies (59). However, TL1A inhibits Treg suppressive ability both directly and indirectly by rendering activated Teff cells resistant to Treg mediated suppression (56, 57). Removal of TL1A completely restores the suppressive ability of Tregs both *in vitro* and *in vivo* (56, 58). Given the transient nature of TL1A up-regulation by APCs, an accelerated effector T cell response could be accompanied by increased numbers of Tregs capable of controlling activated T cells when local concentrations of TL1A have decreased. These findings suggest the existence of an elegant operational system that promptly amplifies immune responses against invading pathogens and rigorously dampens immune activation once the pathogens is eliminated (60). Finally, the addition of TL1A in conventional T cells cultured under FoxP3-promoting conditions, inhibited iTreg differentiation (57, 61). Instead, forced overexpression of high levels TL1A by FoxP3-expressing T cells promoted proinflammatory characteristics such as the production of IL-4 and IL-13 (61). TL1A-overexpressing Tregs were unable to protect from colitis in the T cell transfer colitis model (61). However, transgenic Tregs expressing low levels of TL1A were able to suppress T cell transfer colitis, an effect dependent on DR3 signaling and associated with protective levels of IL-17 and TGFβ (61). The importance of this low level

expression of TL1A for the maintenance of Treg populations and functions in the periphery remains to be elucidated.

Th9 Cells

Although the TL1A-DR3 system had no effect on Th1/Th2 polarization and a debated effect on Th17 differentiation, it recently became evident that it plays an important role for the generation of Th9 cells that are involved in defense against helminthes and allergy. On the one hand, TL1A co-stimulation enhances generation of Th9 T cells in the presence of TGFβ and IL-4, conditions that favor Th9 differentiation (62). On the other hand, in the presence of TGFβ and IL-2, conditions that favor iTreg generation, TL1A diverts the differentiation of iTregs to Th9 cells (62). Interestingly, TL1A up-regulates IL-9 secretion though an alternative pathway that involves STAT5 activation by IL-2 instead of STAT6 activation by IL-4 (62). *In vitro* data have also been coupled by *in vivo* evidence of an increased pathogenicity of Th9 cells in the presence of TL1A in a model of Th9-dependent allergic ocular and lung inflammation (62). In addition to T cell dependent allergic lung inflammation, intact DR3 signaling has also been found important for ILC2 expansion and pathogenicity in innate models of allergic lung inflammation (63). Besides allergic immunopathology, TL1A-directed Th9 polarization, has been associated with $CD4^+$-dependent anti-tumor responses. Dectin-1-activated dendritic cells, acting partly though TL1A-DR3, have been found to induce Th9 cells that enhance tumor-specific CTL activity against OVA-expressing melanoma tumors (64).

Other Cell Populations

TL1A co-stimulation enhances proliferation, IL-2 production, and cytotoxicity of DR3 expressing CD8+ T cells (65). Moreover, natural killer (NK) cells are capable of expressing DR3 after stimulation with IL-12 and IL-18, which led to enhanced IFNγ production and anti-tumor responses following TL1A stimulation (51, 66). Innate lymphoid cells (ILCs) also express DR3 and increase cytokine production upon TL1A stimulation (63). These findings indicate a broader role of TL1A/DR3 system in protective immunity. DR3 is also highly expressed on NKT cells. In this population, unlike in T cells, TL1A appears to promote a more restricted set of cytokines, enhancing IL-4 and IL-13 but not IFNγ production (48). B cells also express DR3, especially after polyclonal stimulation through the B-cell receptor (67). Plasma cells (but not B cells) also expressed very high levels of DR3 in a mouse model of collagen-induced arthritis (68). However, the role of TL1A/DR3 in B cell functions is not yet clear.

Functional Roles of TL1A/DR3 in Mucosal Homeostasis and Inflammation

The original identification of TL1A in 2002 was followed by an abundance of studies that have largely brought about the significance of the TL1A/DR3 system in immunological responses, with particular emphasis in mucosal immunity pathways. Although originally presented as Th1 polarizing molecules, TL1A and DR3 were soon proved to display a vast array of multiple and even opposite immune functions that are critically dependent on the particular clinical or experimental scenario. In this process the contribution of genetically manipulated murine models and the application of neutralizing or stimulatory monoclonal antibodies have been of paramount importance (**Table 2**).

Protective Functions During Acute Injury and Repair: The Role of TL1A/DR3 in Innate Immunity

Recent studies have provided evidence for a protective role of TL1A/DR3 in host defense against acute harmful stimuli. Buchan et al. demonstrated expression of TL1A by $F4/80^+$ macrophages in the spleen of mice during *Salmonella enterica* Typhimurium infection (72). In the same model, DR3 signaling was essential for optimal expansion of activated/memory $CD4^+$ T cells that produced IFNγ and facilitated bacterial clearance (70, 72). Similarly, in the absence of DR3 signaling, early antiviral immunity against murine cytomegalovirus was impaired and fewer virus-specific CD4+ and CD8+ cells were generated, resulting in increased viral loads (73). A recent study by Pham et al. further showed that DR3 signaling is essential for non-cognate stimulation of Th1 cells and effective elimination of intracellular bacteria in mice (74). Studies on human memory T cells have also shown that TL1A and IL-15 synergize to enhance proinflammatory cytokine production independently of cognate TCR–MHC-II interactions (52). They describe a significant population of memory $CD4^+$ T cells characterized by the expression of IL-18R and DR3 and located preferentially in mucosal surfaces such as the small intestine, the colon, the nasal mucosa and the skin. Stimulation with TL1A/IL-15 induced strong IFNγ responses accompanied by production of IL-6, TNF-α, GM-CSF, IL-5, IL-13, and IL-22 with concomitant suppression of IL-10 production. These findings imply that TL1A/DR3 supports the innate activity of mucosal memory T cells. Whether these cells exert protective mucosal roles during acute inflammation, however, remains to be shown. On the other hand, identification of large numbers of these $IL\text{-}18R\alpha^+DR3^+$ T cells has been reported in the small bowel of CD patients. This rather indicates a primary pathogenetic/proinflammatory role for TL1A in chronic autoimmune intestinal inflammation (74).

Further support for an important role of TL1A/DR3 signaling in mucosal homeostasis arose from the recent discovery of the stimulatory effect of TL1A on innate lymphoid cells (ILCs). Type 2 cells (ILC2) at mucosal surfaces may depend on TL1A/DR3 signaling, as high DR3 expression was detected in both human and murine ILC2. Stimulation with TL1A resulted in enhanced expansion, survival, and function of ILC2 (75). More importantly, this effect was independent of the critical ILC2 regulators IL-25 and IL-33. The biological significance of these experimental findings were substantiated by the increased susceptibility of $DR3^{-/-}$ lymphopenic mice to gut helminthic infections (75) and their failure to develop lung responses to nasal challenge with papain (75). Finally, Meylan et al. reported that TL1A-dependent co-stimulation of ILC2 was involved in experimental allergic lung disease (63).

Group 3 ILCs are defined by their expression of $ROR\gamma t^+$ and ability to produce IL-17 and IL-22 (76). They reside mostly at the intestinal mucosa to enhance intestinal barrier integrity and epithelial repair primarily through the production of their

TABLE 2 | Effects of genetic or immunological manipulation of TL1A/DR3 expression.

Model	TL1A Tx	TL1A-Tg	TL1A ko	DR3 ko	Anti-TL1A	References
Spontaneous phenotype		Ileitis, TH2				(56, 57, 69)
DSS			Worsening	Worsening	⇓ Chronic	(36, 70)
TNBS					⇓ Weight loss ⇓ Histology	(57)
SAMP ileitis				Protected		
Gai2 ko transfer					⇓ Weight loss ⇓ Histology	(36)
Experimental allergic encephalomyelitis (EAE)			⇓ Clinical score	⇓ Clinical score		(43, 50)
Collagen-induced arthritis (CIA)	Worsening				Protected	(71)
Antigen-induced arthritis (AIA)	Worsening			⇓ Chronic arthritis	Protected	(71)
Ovalbumin (Ova) lung hypersensitivity pneumonitis				Protected	Protected	(43, 62)

signature cytokine IL-22 (77). ILC3-derived IL-22 is critical for constraining commensal bacteria and protecting against pathogenic bacteria and viruses via the regulation of intestinal anti-microbial peptides and B cell responses (78–84). TL1A is one of the intermediate messengers of innate immune responses produced by ILC3s that rescue mice from *C. rodentium*–induced colitis. Specifically, bacterial sensing CX3CR1$^+$ mononuclear phagocytes in the intestinal lamina propria produce TL1A, IL-1β, and IL-23 that upregulate production of IL-22 from intestinal ILC3s and protect against infectious colitis and dextran sodium sulfate (DSS)-mediated acute colitis (38, 85). Up-regulation of IL-22 production was depended on DR3 signaling in both mouse and human ILC3 that constitutively express this receptor (85, 86). Furthermore, the combination of IL-1β, IL-23 and TL1A, induced the expression of CD25 on human ILC3 cells to enhance IL-2-mediated proliferation (86). Collectively, the above findings indicate that TL1A/DR3 have a major role in orchestrating innate immunity pathways in the intestine by the regulation of the local ILC3 pool and cytokine production.

The role of TL1A/DR3 in preserving mucosal homeostasis was shown with the use of TL1A- and DR3-deficient mice (70). DSS-colitis was more severe in the absence of either TL1A or DR3, indicating protective roles for these proteins during acute mucosal injury and repair. This was associated with a compromised ability to maintain adequate numbers of Foxp3$^+$ regulatory T cells in the periphery and inability to restrain Th17 immune responses. Similar results have been observed in the T cell transfer model, where intact DR3 signaling and a low level of TL1A expression on Foxp3$^+$ regulatory T cells was required to maintain their suppressive function and rescue from colitis (61). It should be noted, however, that in the DSS model the effects of TL1A and DR3 deficiency were not identical as there were differences between the two cases in regards to mortality and kinetics of inflammatory responses (70). However, Castellanos et al have failed to detect significant differences in Foxp3$^+$ T regulatory or IL-17-producing RORγt$^+$ (Th17) cells during acute colitis in DR3-deficient mice (38). Using mice with ILC3 specific DR3 deletion (DR3$^{\Delta ILC3}$) they showed that exacerbation of DSS colitis was due to the downregulation of IL-22 production by ILC3s, whereas treatment with recombinant IL-22 rescued survival of DR3$^{\Delta ILC3}$ mice (38).

Pro-inflammatory Functions During Chronic Inflammation: The Role of TL1A/DR3 in Adaptive Immunity

Many studies during the last decade have demonstrated that the TL1A/DR3 system is up-regulated in patients with IBD and chronic intestinal inflammation. Gut tissue specimens from CD and UC patients exhibit increased TL1A transcripts and protein expression, which correlated with the severity of tissue inflammation. Paired samples of macroscopically uninvolved intestine from the same patients had intermediate levels of TL1A, and minimal or un-detectable amounts were observed in healthy controls (37, 49). Recent studies have correlated the increase of TL1A transcripts at the colonic mucosa of IBD patients to the levels of IL-17A expression (87). Immunohistochemical studies and cytometric analysis on isolated cells from the lamina propria further specified that the increased amounts of TL1A detected in IBD tissue were derived from infiltrating lymphocytes and intestinal macrophages in CD and plasma cells in UC. A similar pattern of expression, which correlated with disease activity, was observed for DR3 (37, 49). Furthermore, systemic levels of TL1A and its decoy receptor DcR3 parallel disease activity in colonic CD and UC. Recently, a significant correlation of the expression of DR3 on peripheral blood mononuclear cells with CRP levels was observed in newly diagnosed children and adults with IBD (88). Both local expression and systemic levels were found to decrease following effective treatment (88–90).

Studies in animal models with transgenic expression of TL1A provided more mechanistic insights about the possible involvement of TL1A in IBD. Mice with forced constitutive expression of TNFSF15 (TL1A-tg) in either the lymphoid or myeloid cell compartments demonstrated a stable phenotype of mild ileitis (56, 57, 69). All transgenic mice developed inflammatory changes in the terminal ileum that included disrupted villi architecture, infiltration of the lamina propria with inflammatory cells, goblet cell hyperplasia and thickening of the muscularis propria. Inflammatory changes were accompanied by lengthening of the small intestine and failure to gain weight.

The patchy distribution of the inflammatory lesions and the development of intestinal fibrosis are two characteristics shared with human CD. Small bowel pathology was associated with increase in activated T cells and regulatory $Foxp3^+CD4^+$ T cells. The most prominent feature was a predominant Th2 mucosal response. Indeed, TL1A-Tg mice displayed elevations in mucosal IL-13 and IL-5 mRNA content, whereas, blockade of IL-13 ameliorated the severity of ileitis (56, 57, 69). The striking resemblance of the TL1A–induced pathology to intestinal anti-parasitic responses led the investigators to further study the role of TL1A in this field. They subsequently observed that TL1A can stimulate IL-13 production by Group 2 Innate lymphoid cells, an effect mediated via DR3 (63). However, the TL1A-dependent innate pathway they identified was not required for effective intestinal anti-parasitic responses but mostly played a major role in allergic lung pathology in relevant murine models (63).

It is now widely accepted that Th2 predominant immunity is a characteristic of the late maintenance stages of clinical and experimental IBD, as it was shown in great detail in CD-like ileitis in SAMP1/YitFc mice (91–94). Interestingly, both TL1A-Tg and SAMP1/YitFc mice develop small intestinal inflammation, marked hypertrophy of the muscular layer, and high mucosal expression of IL-13 and IL-5. Additionally, mucosal TL1A mRNA expression is also upregulated in the chronic phase of SAMP1/YitFc ileitis (35). These studies raise the possibility that the proinflammatory function of TL1A may be partially mediated through induction of Th2/IL-13 dependent mucosal responses, which are now recognized as central pathogenetic factors in IBD. The importance of TL1A/DR3 in murine colitis has also been investigated in the TNBS, DSS and the G-protein ai2 deficient models (36, 57). Development of colitis was associated with mucosal upregulation of TL1A and DR3 and colitis was effectively prevented or attenuated by the administration of anti-TL1A neutralizing antibodies. Taken together, mucosal overexpression of TL1A (primary or secondary) may be implicated in the induction of pathogenetic effector proinflammatory pathways.

A functional dichotomy between membrane and soluble forms of TL1A may also occur, as it was shown recently. Using a membrane restricted TL1A transgenic mouse Ferdinand et al., have shown that increased production of soluble TL1A, possibly by APCs, was required to produce maximal small bowel pathology. In contrast membrane bound TL1A, mostly of T-cell localization, was required to elicit inflammatory responses including IL-13, IL-17, and IL-9 production in murine lungs (95).

DR3 and TL1Ako mice do not develop any gross abnormalities. Nevertheless, immunological characterization of these strains detected specific defects that may be of interest. It was shown that DCs from $TL1A^{-/-}$ mice fail to support the differentiation and proliferation of Th17 lymphocytes (50). Consistently, these mice were protected from Th17-mediated inflammation in a model of Experimental Autoimmune Encephalomyelitis (43, 50). Similarly DR3 signaling was required for Th2-mediated lung immunopathology in an Ova model of allergic lung inflammation (43). TL1A deficiency has also been reported to induce broader changes on the gut immune microenvironment, such as marked decrease of intraepithelial $TCR\gamma\delta^+$ and $CD8^+$ lymphocytes and reduced expression of the activating receptor NKG2D (96). Quite unexpectedly, there were also significant changes in gut microbial composition with significantly suppressed cecal *Clostridium* cluster *IV*, altered cecal *Firmicutes*/*Bacteroidetes* ratio, and reduction in ileal *Lactobacillus* spp. This was also associated with reduced body weight, and decreased size of adipose tissue and adipokine expression (96). The latter raise the possibility that TL1A may affect microbiota-related metabolic pathways that regulate adipose tissue development. Collectively, the above data support an important role for the TL1A/DR3 system in the maintenance of mucosal homeostasis and a significant contribution, when unrestrained, to late IBD-related immunopathology.

Mechanistic evidence for the implication of TL1A/DR3 signaling in the effector pathways that mediate chronic inflammation was recently presented by Li et al. (97) who tested the effect of DR3 stimulation of DR3 deletion in the ileitis-prone SAMP1/YitFc mouse model of CD. They, first, showed that administration of an agonistic antibody against DR3 (4C12) prior to disease development markedly worsened the severity of ileitis in SAMP mice. The immunological effects of DR3 stimulation included overproduction of T_H1 and T_H2 cytokines, expansion of dysfunctional $CD25^-FoxP3^+$ and ILC1 cells, and concomitant reduction of $CD25^+FoxP3^+$ and ILC3 cells. By comparison, genetic deletion of DR3 effectively reversed the inflammatory phenotype in SAMP mice. This was associated with selective expansion of $CD25^+FoxP3^+$ over $CD25^-FoxP3^+$ cells and upregulation of IL-10. These data demonstrate a central, multicellular modulation of adaptive immunity by DR3, via the regulation of the relative abundance of T_{regs}, T effectors, and ILCs, which, subsequently, dictates the progression of CD-like ileitis in SAMP mice. More recently, Castellanos et al., have shown that TL1A induced ILC3 expression of OX40L in $MHCII^+$ ILC3s that supports pathogenic T cell responses in the T-cell depended colitis transfer model. ILC3-spesific deletion of DR3 protected mice from the development of colitis (38). It follows that modification of DR3 signaling holds promise toward being an effective means for restoring the immunological balance between protective and inflammatory lymphocytes at the intestinal mucosa.

TL1A/DR3 as Mediators of Fibrosis

Fibrosis refers to the process of excessive accumulation of extracellular matrix due to increased connective tissue assembly and ineffective matrix remodeling. Mostly, but not always, it represents the end result of repeated cycles of tissue inflammation, ulceration, and repair that ultimately lead to scarring and decline in organ function (98). Despite the wealth of knowledge of inflammatory pathways that have resulted in largely effective anti-inflammatory biologic treatment for various autoimmune diseases, including IBD, the fibrogenetic cascades that result to tissue scarring remain relatively understudied and effective therapies to prevent or, more importantly, reverse fibrotic processes are currently lacking (99).

TL1A amplifies multiple immunological pathways that, when sustained, could be associated with the development of fibrosis. Interleukin 17A favors the development of fibrosis in experimental models of lung and skin fibrosis and has been found to be overexpressed in intestinal strictures of CD patients (100, 101). IL-13 has been implicated in murine experimental intestinal fibrosis, acting mostly through TGFβ, and also is upregulated in strictures of patients with CD (102, 103). Finally, TL1A-induced expansion of Treg populations with altered function in the periphery may theoretically promote local saturation with IL-13 and, most importantly, TGFβ, which is the key regulator of pro-fibrotic pathways and a major activator of mesenchymal cells (104). Interestingly, elevated TL1A and DR3 expression has been found in the SAMP1/YitFc model of murine ileitis that is phenotypically associated with the development of overt intestinal strictures (35).

TL1A transgenic mice develop IL-13-dependent inflammation of the small bowel (57). Phenotypically, TL1A-tg mice are characterized by small bowel wall thickening, especially at the terminal ileum, which is the usual site of stricture development in human CD. Pathology was characterized by enhanced infiltration of the lamina propria with inflammatory cells, increase of the numbers and size of goblet and Paneth cells and hypertrophy of the muscularis propria (56, 57). These changes were accompanied by increased expression of IL-5, IL-13, and IL-17 by intestinal tissue and mesenteric lymph nodes.

Another set of studies by a different group on transgenic mice that constitutively expressed TL1A on the lymphoid or myeloid compartment have demonstrated a similar phenotype characterized by ileitis and Paneth cell hyperplasia (69). However, they further describe increased accumulation of collagen in intestinal tissue. Examination of the same transgenic mice under colitogenic conditions, in the context of the DSS and the adoptive T cell transfer model, revealed the development of overt intestinal strictures at the small and the proximal large bowel (105). The fibrotic phenotype was associated with increased local expression of TGFβ and IGF in colitic mice. Interestingly, constitutive production of TL1A was associated with relative expansion of $CD4^{+}IL17^{+}$ effector T cells in the mesenteric lymph nodes in the DSS by not in the adoptive T cell transfer model. This may indicate that diverse, TL1A-mediated profibrotic immunological pathways may dominate depending on the colitogenic conditions.

Further studies by the same group demonstrated that treatment with antibodies against TL1A was capable to reduce inflammation and to reverse fibrosis in both DSS and adoptive transfer model even when treatment was administrated late in the course of disease, that is after inflammation and fibrosis had been established (106). Anti-TL1A treatment decreased expression of pro-fibrotic molecules such as IGF1, CTGF, and TIMP1 in the inflamed intestinal tissue. Furthermore, mucosal expression of DR3 was associated with fibrotic changes in the bowel wall and $DR3^{-/-}$ mice exhibited reduced numbers of intestinal fibroblasts and myofibroblasts. Intestinal myofibroblasts responded to TL1A with increased expression of Col1a2 and IL-31Ra, a myofibroblast activation marker. Finally, neutralization of TL1A reduced expression of a-SMA and vimentin, activation markers of colonic fibroblasts, and expression of TGFβ1 and Smad3 in the colonic tissue of adoptively T cell transferred colitic mice (107). However, only a subset (25%) of intestinal myofibroblasts expressed DR3, and the relative contribution of the TL1A/DR3 system on fibrosis independent of its anti-inflammatory effects was not explored. Despite this, these studies demonstrated for the first time that an anti-inflammatory therapy, in this case TL1A neutralization, can not only prevent but also potentially reverse established intestinal fibrosis. This is especially important for human CD which often has a long indolent course resulting in both inflammatory and fibrostenotic segments of the small bowel on initial patient presentation and disease diagnosis.

A recent study by Jacob et al., suggests that the pro-fibrotic effects of TL1A on bowel mucosa may depend on the composition of the intestinal microflora (108). TL1A-Tg mice raised under germ-free condition were protected from spontaneous ileitis and cecal collagen deposition. A direct effect of the host microflora was demonstrated on colonic fibroblasts exhibiting enhanced migration/proliferation and collagen production when derived from specific pathogen free as opposed to germ free littermates, and on wild type fibroblasts exposed to specific pathogen free microflora. Interestingly, gnotobiotic TL1A-Tg mice colonized with human gut microflora were protected from both ileitis and cecal fibrosis. Through 16S rRNA sequencing characterization of ileal and cecal microbiome the authors were able to depict possible bacterial genera and species that differentially promote fibrosis in the respective localizations in the context of TL1A overexpression (108).

In human fibrotic conditions, including IBD, the immunological and profibrotic cascades driven by TL1A remain underexplored. The potential implication of TL1A in such pathways was recently highlighted by the report of TL1A expression by human intestinal myofibroblasts that were isolated from IBD patients (109). TL1A expression by intestinal myofibroblasts was up-regulated by pro-inflammatory cytokines (IFN-γ, TNF-α, IL-1α) or supernatants of intestinal tissue cultures from IBD patients (109). The same pro-inflammatory cytokines induced expression of DR3 and DcR3 on cultured epithelial cells, whereas, supernatants from cultures of stimulated epithelial cells were capable to induce upregulation of TL1A in intestinal myofibroblasts. Similar findings have been reported for lung fibrosis and indicate the existence of additional TL1A-dependent possibly pro-fibrotic cascades mediated by epithelial/stromal cell interactions (110). These results point to the existence of a mucosal amplification loop that is initiated by the local pro-inflammatory milieu and then perpetuates itself through reciprocal stimulation of epithelial cell and intestinal myofibroblasts. Nevertheless, whether such interaction leads to increased collagen accumulation and fibrosis remains to be shown. An approach toward the elucidation of the relative contribution of TL1A/DR3 signaling in intestinal fibrosis independently from its role in inflammatory cascades would be the generation and study of conditional DR3 and TL1A knockout mice with tissue specific TL1A and DR3 deficiency on intestinal fibroblasts or epithelial cells (106).

TL1A/DR3 IN THE CROSSROADS OF SYSTEMIC INFLAMMATION: ASSOCIATIONS WITH EXTRAINTESTINAL INFLAMMATION

The TL1A/DR3 pathway is considered one of the common denominators in various pathologies associated with inflammation in different tissues that develops through aberrant immune responses. Genetic studies support its implication in autoimmune diseases and mycobacterial infections. Case-control and GWA studies have identified *Tnfsf15* gene variants, such as rs6478108 alleles, that associate with increased susceptibility to psoriasis and psoriatic arthritis in populations of European descent (111, 112). Furthermore, alleles rs6478108 (T) and rs4979462 (T), have been found to increase susceptibility to both Primary Billiary Cholangitis (PBC) and CD, while protecting from leprosy (6, 113). Moreover, the rs6478108 (G) allele has been associated with increased risk for CD and the pathological inflammatory host response in leprosy known as Type 1 reaction (114, 115).

Further genetic evidence links the TL1A/DR3 pathway to major extraintestinal manifestations of IBD. Indeed, genetic variation in the *Tnfsf15* gene has been associated with increased susceptibility in spondyloarthropathies, ankylosing spondylitis, and anterior uveitis (116–118).

Similarly to intestinal inflammation, increased TL1A was found in the serum of patients with rheumatoid arthritis and ankylosing spondylitis and also correlated with disease activity in both conditions (41, 119). More importantly, TL1A levels are decreased following treatment with anti-TNF. Local levels of TL1A have also been found increased in rheumatoid arthritis with mononuclear phagocytes being the major source of TL1A in the synovial tissue and synovial fluid of rheumatoid factor positive patients (40). In murine models of antigen induced arthritis and collagen induced arthritis TL1A administration promoted osteclastogenesis and exacerbated disease, whereas anti-TL1A ameliorated pathology (71). TL1A effects were found to be DR3-dependent as $DR3^{-/-}$ mice were protected from cartilage depletion and joint destruction. This was partly attributed to decreased production of CXCL1 in the joints of $DR3^{-/-}$ mice and the relative reduction of infiltrating neutrophil numbers which correlated with decreased local levels of MMP-9 (120).

Psoriasis is a common concomitant immune-mediated disorder in patients with IBD. Psoriasis and IBD share pathogenetic pathways such as the IL-23/IL-17 pathway, have common genetic risk alleles and are associated with abnormalities in intestinal and skin microflora and both respond to anti-TNF and anti-p40 treatment (121). There is evidence that TL1A/DR3 may represent an additional pro-inflammatory pathway that is shared between the two diseases. TL1A and DR3 have been found to be increased in psoriatic skin lesions and specifically in macrophages and keratinocytes (122). TL1A seems to synergize with IL-23 to stimulate PMBCs from patients with psoriasis to increase production of IL-17 (123). Uveitis is another extraintestinal manifestation of IBD that has been associated with the TL1A/DR3 pathway. Specifically, experimental murine autoimmune uveoretinitis was dependent on DR3 as $DR3^{-/-}$ mice are protected from disease development (124). Although a direct association of TL1A/DR3 with the extraintestinal manifestations of IBD has not been established yet, there is indirect evidence that such an association may exist making TL1A/DR3 a possible common denominator of the gut-skin-joint-eye autoimmune inflammation axis.

CONCLUDING REMARKS

Since its initial description, TL1A has arisen as an important mucosal factor that is implicated in homeostasis and inflammation through its association with DR3. In the light of translational medicine, it is important that this role of TL1A/DR3 is supported by converging lines of evidence. In particular, polymorphisms in *Tnfsf15* significantly affect susceptibility to IBD and may be associated with altered function of the respective protein. In addition, there is significant upregulation and abundant expression of TL1A and DR3 in inflammatory conditions that affect the intestines, mainly IBD. So far, functional properties of the TL1A/DR3 system involve several immunological pathways that are considered important in the pathogenesis of IBD. Finally, the proof of concept for the therapeutic application of TL1A/DR3 modification has been fulfilled in animal models of intestinal inflammation. In all, it could be said that the system of TL1A/DR3 may represent a desirable therapeutic target for a subset of IBD patients.

Nevertheless, caution is also required as important questions remain unanswered still. One area of concern is the potential effect on Tregs. As TL1A/DR3 have positive effects on Treg function, their neutralization may compromise this population and jeopardize its important anti-inflammatory function in intestinal immunity. A second question is whether blockade of TL1A or DR3 should be the preferable approach in clinical practice. This is of importance as recent evidence from animal models of inflammation reported similar but not identical effects of genetic or immunologic deletion of the two molecules. Finally, antibodies against DR3 have shown agonistic effects which depend on the target population. All these parameters should be taken into account for the design of clinical trials that aim to disrupt TL1A/DR3 signaling.

AUTHOR CONTRIBUTIONS

VV, GK, and GB all contributed to the development of the concept of the review, performed the literature review and analyzed existing data, and wrote and edited the manuscript.

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Cytokine-Mediated Regulation of Innate Lymphoid Cell Plasticity in Gut Mucosal Immunity

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Mucosal barriers are active sites that encounter a bombardment of antigenic stimuli derived from both the commensal flora and a variety of pathogens, as well as from environmental insults. As such, the ability to mount appropriate innate immune responses is an important first line of defense that confers protection to the host. Central to innate immunity are innate lymphoid cells (ILCs), which were first described a decade ago, and represent a family of heterogeneous cells driven by specific transcription factors and exhibit distinct cytokine profiles that are shared with their $CD4^+$ T-helper cell counterparts. ILCs are particularly enriched at mucosal surfaces, and the tissue microenvironment and cytokine milieu in which ILCs reside are critical factors that drive the behavior and overall function of these cells. In fact, ILCs situated at mucosal barriers must be able to temper their response to a constant exposure of environmental antigens, but also promptly react to pathogens or signals that are potentially harmful to the host. In this context, the ability of ILCs to readily transdifferentiate in response to their dynamic surroundings has become a vigorous area of research, and defining specific mechanism(s) of ILC plasticity is at the advent of discovery. This review will summarize what is currently known regarding the network of cytokines and regulatory elements that enable ILCs to readily transform, based on the range of diverse signals and signal gradients they encounter that lead to either protective or pathogenic function(s), with focus on the gut mucosal immune system.

Keywords: innate lymphoid cells (ILCs), cytokines, plasticity, gut mucosal immunity, innate immunity

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INTRODUCTION

Innate lymphoid cells (ILCs) are a diverse family of developmentally-related immune cells that are heterogeneous in their tissue location, cytokine secretion, and effector functions. The term "ILC" has been widely used since 2010, with distinct subsets formally proposed in 2013, based on the transcription factors and specific cytokines regulating their development and function (1). Initially, three groups of ILCs were described, representing innate counterparts that functionally mirror

Abbreviations: c-Maf, musculoaponeurotic fibrosarcoma oncogene homolog; Eomes, Eomesodermin; GATA3, GATA binding protein-3; GFP, green fluorescent protein; GM-CSF, granulocyte macrophage colony stimulating factor; IFNγ, interferon-γ; IL-, interleukin-; ILC, innate lymphoid cell; ILCreg, regulatory innate lymphoid cell; LTi, lymphoid tissue inducer; NCR, natural cytotoxicity receptor; NK, natural killer; TGFβ, transforming growth factor-beta; TSLP, thymic stromal lymphopoietin; Th, T-helper; Treg, T-regulatory cell.

$CD4^+$ T-helper cell subsets. More recently, NK and lymphoid tissue inducer (LTi) cells have expanded this family, thereby representing five different ILC groups (2). Group 1 ILCs (ILC1s) express IFNγ and rely on the transcription factor, T-bet, but unlike closely-related NK cells, they do not depend on Eomesodermin (Eomes) and are, in general, non-cytotoxic; however, a phenotypic hybrid of ILC1s and NK cells also exists (3). ILC2s produce the Th2 cytokines, IL-4, IL-5, and IL-13, as well as IL-9 and amphiregulin, and are functionally influenced by the transcription factors, GATA3 and RORα. ILC3s utilize RORγt to drive production of IL-22, but also IL-17, and are distinguished into further subsets based on expression of the natural cytotoxicity receptors (NCRs), NKp46 and NKp44. LTi cells are also dependent on RORγt, but produce lymphotoxin and are critical for secondary lymphoid organ formation, including Peyer's patches. Although organizing ILCs into these five subgroups provides a basic infrastructure to understand the ILC family in regards to development and function, it is also important to consider whether this diversity is discrete, or reflects a given subset's adaptability to the changing tissue microenvironment, such as what occurs during disease pathogenesis, and for these cells to undergo transformation.

In this context, several significant questions remain unanswered regarding the mechanisms underlying ILC development, divergence, and differentiation. What kind of environmental cues and other regulatory factors are necessary to determine cell fate? How do these processes relate to that of the $CD4^+$ T cell helper population? Which ILC subgroups are terminally-differentiated, or like their corresponding $CD4^+$ T helper cells, can convert from one ILC type to another? Although investigation of ILC plasticity is still in its infancy, these questions are now being answered, particularly with the emergence of more advanced techniques, such as single-cell transcriptomics, which facilitates a better understanding of the complexity and heterogeneity of ILCs (4–7).

In general, ILC2s and ILC3s have the ability to transdifferentiate into ILC1s, which is a reversible process, and highly dependent on the cytokine milieu and tissue microenvironment in which these cells reside. Transformation of ILC2s to ILC3s has also been reported, while plasticity between "natural" and "inflammatory" ILC2s, as well as of NK cells to ILC1s, may represent transient stages or terminally-differentiated events during disease pathogenesis, respectively. The following sections will summarize current findings regarding ILC plasticity and what signals (*e.g.*, cytokines) control these processes, with particular focus on the gut mucosal immune system. We will also discuss the more recently coined regulatory ILC (ILCreg) subset and its contribution to mucosal immunity.

CYTOKINES THAT REGULATE PLASTICITY OF LTI AND ILC3S

Plasticity within ILC subgroups was first observed in LTi cells, in which a gradient of RORγt expression is stabilized by IL-7 and the gut microbiome, while IL-12 and IL-15 accelerate its loss. Specifically, $ROR\gamma t^+$ LTi cells produce IL-22 and are functionally protective, whereas $ROR\gamma t^-$ LTi cells secrete IFNγ and can induce colitis (8, 9). Similarly, ILC3s that are $NKp46^-CCR6^{-/low}$ are able to differentiate into $NKp46^+$ ILC3s, depending on upregulation of the prototypic ILC1 transcription factor, T-bet, which stimulates IFNγ and IL-22 production that are important for protection against *Salmonella* infection (10). In addition, Notch signaling mediated by T-bet guides the development of $NKp46^+$ ILC3s (11). Interestingly, this upregulation of T-bet in a subpopulation of ILC3s is concurrent with loss of RORγt expression (8, 12).

How these "ex-ILC3s" are generated is of great interest and the focus of recent investigation. Although different mechanisms have been implicated, the tissue-specific microenvironment appears to be essential to skew ILC3 identity, as the frequency of these subsets differs depending on their residence. Most abundant within the intestines are ILC3s, wherein $CCR6^+$ ILC3s and/or LTi-like cells are most prominently found in cryptopatches, whereas $NKp46^{+/-}CCR6^{-/low}$ ILC3s are located within the lamina propria. Interestingly, Pearson et al., recently demonstrated that ILC3s can mobilize from intestinal cryptopatches to the lamina propria, and this migration is dependent on GM-CSF (13). Another mechanism for ILC3 switching was revealed through the use of RORγt-GFP reporter mice (14), which have been instrumental for lineage-tracing experiments (15); these mice retain GFP expression in their $ROR\gamma t^+$ cells, even after loss of RORγt expression. Studies show that within the ILC1 subset, some cells have the ability to produce IFNγ, a typical ILC1 cytokine, and still have traceable GFP expression, suggesting that they were likely once $ROR\gamma t^+$ ILC3s (*i.e.*, ex-ILC3s) (8–10, 16–18). Although ILC3s rely on IL-7 signaling for proper development and maintenance, ex-ILC3s downregulate CD127 and c-Kit, and are more responsive to IL-15; in contrast, all other ILC3 subsets are not entirely dependent on IL-15 signaling (18, 19). Furthermore, upon IL-23 stimulation, STAT4/T-bet-dependent regulation of NCR^+ ILC3s can promote IFNγ production and plasticity towards type 1 fate (20).

More recently, an intermediate population has been identified in human tonsils and small intestines, with characteristics of both ILC3s and intraepithelial $CD103^+$ ILC1s (21). This population expresses CD103, CCR6, and CD300LF to different degrees, with scRNA-seq analysis providing evidence that ILC3s are able to convert *in vivo* into $CD103^+$ ILC1s. Furthermore, Aiolos (encoded by *IKZF3*), a member of the Ikaros family of transcription factors, and expressed mainly by ILC1s and NK cells, is critical for skewing ILC3s into $CD103^-$ (22) and intraepithelial $CD103^+$ ILC1s (21). The transcription factor, c-Maf, is also implicated in controlling the homeostatic balance of ILC3s by directly inhibiting T-bet, and therefore transdifferentiation to ILC1s (23, 24); in the absence of c-Maf, $CD196^-$ ILC3s transdifferentiate to an ILC1 phenotype (23). Another factor, BCL6, regulates ILC3-to-ILC1 plasticity by repressing ILC3-promoting pathways, such as IL-23-induced signaling (24), resulting in reduced ILC1 frequency.

In humans, the transition from ILC3s to ILC1s is contingent on downstream signals directed by IL-12 that induce the

expression of T-bet (12). This is the case in inflamed intestinal tissues from Crohn's disease patients, wherein increased ILC1s, at the expense of ILC3s, is observed, highlighting the prevalence of ILC3s during homeostasis, possibly by supporting T regulatory cell (Treg) activity *via* IL-2 (25), and ILC1s during (pathogenic) inflammatory events (26). Interestingly, differentiation from NKp46$^-$ to NKp46$^+$ ILC3s, and then to NKp46$^+$ ex-ILC3s (*i.e.*, "ILC1s"), modulated by low-to-high T-bet expression, is reversible both *in vivo* and *in vitro*, and is dependent on IL-23, IL-2, and IL-1β, and is further enhanced by retinoic acid (12). This ILC3-to-ILC1 polarization also depends on the presence of CD14$^+$ and CD14$^-$ dendritic cells, wherein an increase in the former promotes ILC1 differentiation, and an increase in the latter, induces ILC3 skewing. These findings indicate that, as much as the cytokine milieu and activation of transcription factors can affect ILC subset composition, so can environmental signals from local immune cells. In other studies, fate-mapping experiments have established that NKp46$^+$RORγt$^+$ ILC3s can downregulate *in vivo* expression of NKp46, generating NKp46$^-$RORγt$^+$ ILC3s (11, 27).

As mentioned earlier, T-bet itself can direct the development of NKp46$^+$ ILC3s, which is mediated through Notch signaling (10). Confounding this finding, however, Notch is also reported to regulate plasticity within ILC3 subsets by controlling the fate of NKp46$^+$ ILC3s (28). Furthermore, interconversion within the ILC3 subset occurs in response to TGFβ (29). Transcripts for the two subunits of the TGFβ receptor, TGFβ receptor I and II, can be detected in ILCs (30). Deletion of TGFβ receptor II leads to expansion of NKp46$^+$ ILC3s, indicating that TGFβ impairs the development of NKp46$^+$ ILC3s. Additionally, TGFβ antagonizes Notch signaling, implying that the ILC3 phenotype depends on fine-tuning the divergent effects of both TGFβ and Notch signaling that may be required to preserve homeostasis, *in vivo* (29).

Taken together, these studies indicate that ILC3s exhibit bi-directional differentiation that can be modulated by T-bet and RORγt gradients within the ILC3 lineage (**Figure 1A**). Are ex-ILC3s generated from NCR$^+$ ILC3s, and are they all dependent on Notch and TGF-β signaling? How does T-bet and RORγt detect the extent of inflammation and deliver equivalent immune responses? To determine how these transcription factors readily impose various ILC3 effector programs, it is imperative to recognize the underlying molecular mechanisms for ILC3 plasticity. How this balance is maintained could reflect blunted inflammatory responses (*i.e.*, in presence of commensal flora during homeostasis), while being poised to mount a vigorous immune reaction when challenge or insult occurs, particularly at mucosal barriers. Various ILC3 subsets may be actively modulated by the temporal degree of inflammation that then directs T-bet and RORγt expression.

CYTOKINES THAT REGULATE PLASTICITY OF ILC2S

ILC2s have also been reported to alter their functional and transcriptional programs. ILC2s are capable of converting to IFNγ-producing ILC1s in both mice and humans (31–33). Specifically, ILC2s derived from human blood proliferate *in vitro* in the presence of IL-2 and IL-7, upregulate T-bet, and secrete IFNγ *via* IL-12 (34). Other studies show that, in the presence of IL-1β, human ILC2s not only express T-bet, but also the IL-12 receptor subunits, IL-12RB1 and IL-12RB2, enabling ILC2s to respond to IL-12 (32). Interestingly, patients with defects in IL-12Rβ1 suffer from a syndrome referred to as Mendelian susceptibility to mycobacterial disease; these individuals are not capable of generating ILC2s that potentially can convert to ILC1s (34). IL-12 is also important in inducing genomic modifications in the *IFNγ* locus, allowing for IFNγ secretion; these IFNγ-expressing "ex-ILC2s" are also able to secrete IL-13 (32). Similarly, Bal et al. showed in an inflammatory environment (*i.e.*, lung tissues of patients with chronic obstructive pulmonary disease) that ILC2s can convert into IFNγ-producing ILC1s by exposure to either combination IL-1β and IL-12, IL-33, or TSLP (thymic stromal lymphopoietin) and IL-12 (31). After adoptive transfer into humanized mice, these ILC2s downregulate chemoattractant receptor-homologous molecule expressed on T-helper type 2 cells (CRTH2) and c-Kit expression, which are typical markers for ILC2s (31). These CRTH2$^-$Kit$^-$ ex-ILC2s also express increased Tbx21 (T-bet) compared to CRTH2$^+$Kit$^+$ ILC2s. In humans, two subsets of CRTH2$^+$ ILC2s were identified in peripheral blood, based on CD117 expression: CD117$^-$ ILC2s, indicating mature ILC2s, and CD117$^+$ ILC2s, showing some features of ILC3s, such as RORγt expression (35, 36). Upon IL-33 stimulation, CD117$^+$ ILC2s produce Th2 cytokines, whereas IL1β and IL-23 induce these cells to produce IL-17 (35, 36) and CCR6, which is also expressed by IL-17-producing ILC3s (37). ILC2s have also been reported to display phenotypic plasticity in response to infectious agents, such as influenza virus, respiratory syncytial virus, *Staphylococcus aureus*, and interestingly, cigarette smoke (33). GATA3 expression is downregulated in ILC2s in response to exposure to these agents, with a subsequent increase in IL-12Rβ2, IL-18Rα, and T-bet. This effect was confirmed *in vivo* by adoptive transfer of ILC2s from ST2/IL-33R reporter mice into recipients lacking T cells and ILCs (*Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice) that were then infected with influenza. Although donor ILC2s downregulate GATA3 and upregulate IL-18Rα and IL-12Rβ2, they do not express T-bet; however, upon stimulation with IL-12 and IL-18, a portion of these ex-ILC2s are capable of IFNγ secretion, suggesting skewing towards ILC1s (33).

Interestingly, similar to ILC3-to-ILC1 conversion, ILC2-to-ILC1 transdifferentiation is also reversible. Although the mechanism for this process is not entirely clear, eosinophil-derived IL-4 appears to prevent IL-12-mediated ILC2 differentiation to ILC1 in an inflammatory milieu, such as within nasal polyps of patients with chronic rhinosinusitis (31). Furthermore, expression of the receptors for IL-1β, IL-12, IL-18, and IL-33 influence ILC2 expansion and T-bet induction, facilitating ILC2 conversion towards an ILC1 phenotype (31–33).

TGFβ from pulmonary epithelial cells has been observed to promote allergic immune responses by expanding IL-13–secreting

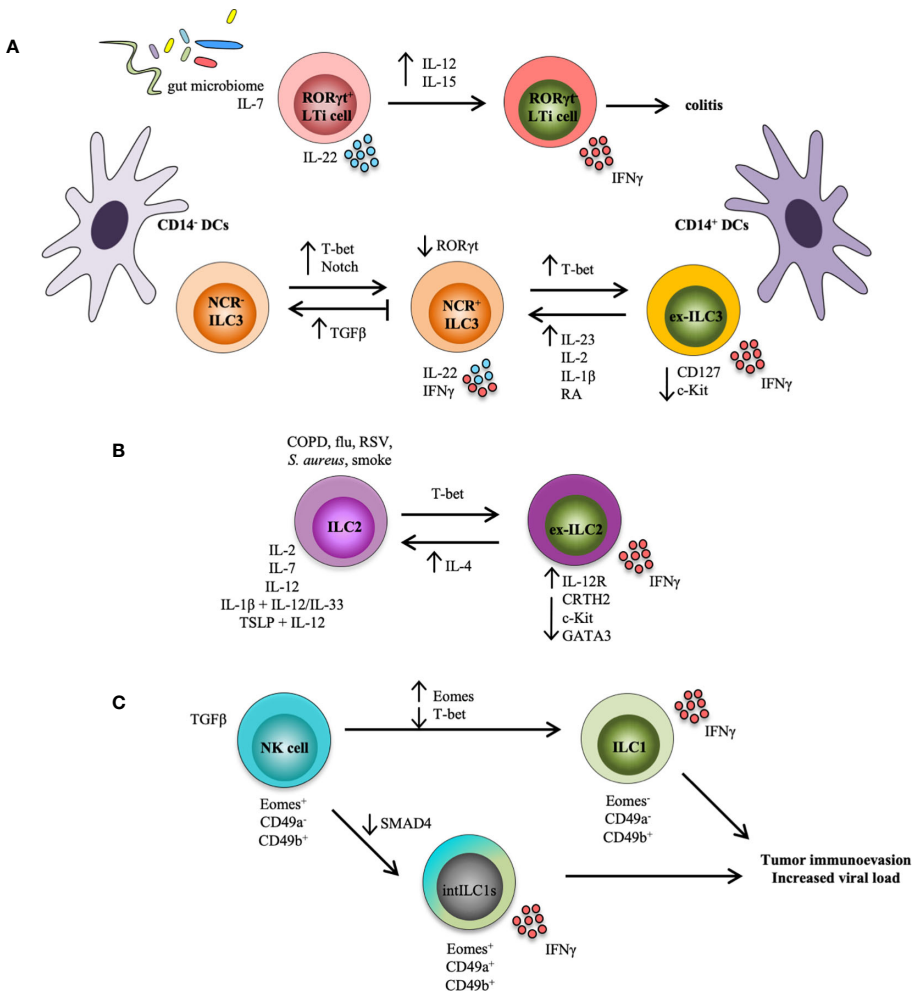

FIGURE 1 | ILC transdifferentiation occurs in response to cytokine and/or pathogen stimulation. Schematic representation summarizing plasticity among ILC populations reported both in mouse models and in human settings. **(A)** Plasticity within the LTi and ILC3 populations. RORγt in LTi cells is stabilized by IL-7 and the gut microbiome; these cells produce IL-22 and are functionally protective. Conversely, IL-12 and IL-15 accelerate loss of RORγt in LTi cells, which secrete IFNγ and induce colitis. NCR^- ILC3s can convert into NCR^+ ILC3s under the influence of T-bet and Notch. T-bet upregulation of NCR^+ ILC3s can transform ILC3s into ILC1-like cells (*i.e.*, ex-ILC3s) that downregulate expression of CD127 and c-Kit, and secrete IFNγ. This process is reversed by IL-23, IL-1β, IL-2, and retinoic acid (RA), while TGFβ inhibits NCR^+ ILC3 development from NCR^- ILC3s and possibly drives reversion of these cells. Increased presence of $CD14^+$ dendritic cells (DCs) drives an ex-ILC3 phenotype, while $CD14^-$ DCs promote the generation of NCR^- ILC3s. **(B)** Plasticity within the ILC2 population. In the presence of an inflammatory environment typical of COPD (chronic obstructive pulmonary disease), smoke, and bacterial or viral infection, or after stimulation with IL-2, IL-7, IL-12, IL-1β, and IL-12, as well as TSLP and IL-12, ILC2s can assume an ILC1-like phenotype (*i.e.*, ex-ILC2) that upregulates expression of the IL-12 receptor, downregulates ILC2-associated proteins (CRTH2, c-Kit, GATA3), and produces IFNγ. GATA3, a critical transcription factor for maintaining ILC2 identity, is repressed by T-bet. This route can also be reversed by eosinophil-derived IL-4. **(C)** Plasticity within the NK and ILC1 populations. In the context of a tumor microenvironment, NK cells ($Eomes^+CD49a^-CD49b^+$) can convert to intermediate ILC1s ($Eomes^+CD49a^+CD49b^+$), or intILC1s, upon stimulation with TGFβ. Similarly, NK cells can also transform to real ILC1s ($Eomes^-CD49a^-CD49b^+$). SMAD4 promotes TGFβ signaling, but the lack of SMAD4 in NK cells transforms them into intILC1s. These ex-NK cells are not functional however, and provide a mechanism for tumors to escape surveillance, and may allow for increased viral burden in infection.

ILC2s (30). IL-33 induces proliferation of ILC2s and stimulates TGFβ secretion in lung airways to activate ILC2 function and migration (30). The possibility therefore exists that TGFβ, in line with other inflammatory mediators, acts as a modulator of ILC2 and ILC3 fate. In fact, it has been proposed that murine ILC2s can be categorized into two subsets: a transient "inflammatory" subpopulation whose fate and function is dependent on the transcription factor, BATF, and expresses more IL-25R (thereby responding preferentially to IL-25), and a tissue resident "natural" $ST2^+$ ILC2 subpopulation induced by IL-33 (38, 39). Inflammatory (i)ILC2s are not responsive to IL-33, but can convert into natural (n)ILC2s, both *in vivo* and *in vitro*, and express low quantities of RORγt and upon stimulation, IL-17 and IL-13, indicating plasticity between iILC2s and ILC3-like cells (38).

Although this phenomenon has not been recapitulated in humans, human clones of ILC2s and ILC3s that secrete both IL-22 and IL-13 have been characterized (40, 41). Similarly, ILC2s

can respond to inflammation in skin and lungs by transdifferentiating into IL-17-producing ILC3s; specifically, ILC2s co-cultured with dermal cells and the fungus, *Candida albicans*, produce IL-17 and acquire a phenotype similar to that of NKp44$^-$ ILC3s (35). Finally, patients with more severe non-allergic asthma, with blunted Th2 responses, display both IL-5- and IL-13-producing ILC2s, and IL-17-producing ILC3s and ILC2s (42).

Together, these studies provide support for the existence of ILC2 phenotypic flexibility (**Figure 1B**). Future studies will benefit from experiments that can trace genetic lineages to clarify issues related to ILC2 plasticity. Discriminating between *in vivo* expansion of already low numbers of ILC subsets vs. transdifferentiation is challenging. Identifying extracellular influences and why lineage flexibility is necessary, will facilitate knowledge as to how pathogenic responses can be controlled and skewed toward protective function(s), leading to potential therapeutic interventions (8, 43).

CYTOKINES THAT REGULATE PLASTICITY OF ILC1S AND NK CELLS

Previous sections of this review have highlighted interconversion of ILC3s and ILC2s into ILC1s, and vice-versa. This section will examine ILC plasticity of the closely-related subgroups, NK cells and ILC1s. Although both subsets possess identical cell surface markers (NKp46, NKG2D), parallel transcriptional programming, and similar cytokine profiles (IFNγ, TNF), they are now considered distinct populations. For instance, NK cells developmentally do not need GATA3 (44), but some ILC1s do (45–48). The proposed cellular basis of this divergence is due to the transcription factors, inhibitor of DNA binding protein-2 (ID2) and promyelocytic leukemia zinc finger protein (PLZF), which are expressed in ILC precursors, but not in NK cells (18, 49). Recent studies, however, using polychromic reporter mice, show that ILC precursors actually have considerable NK precursor activity, challenging the existing paradigm that considers ILC1 and NK cells as two different subsets (50, 51). Moreover, conversion of NK cells into ILC1s is observed in mice with non-alcoholic fatty liver disease, and likely mediated, in part, by TGFβ (52). NK cells also express Eomes that, together with T-bet, modulate production of lysis-associated granules containing perforin and granzyme (53). Therefore, conceptually, if ILCs are innate counterparts of CD4$^+$ T helper cells, then NK cells represent the innate version of CD8$^+$ T cells.

Current investigation, however, suggests that mature NK cells and ILC1s can also undergo plasticity between themselves. Forced Eomes expression in T-bet$^+$ ILC1s is, in fact, sufficient for transformation to CD49b$^+$ NK cells (54). Conversely, IL-12 can convert Eomes$^+$ NK cells into Eomes$^-$ ILC1-like cells following infection with *Toxoplasma gondii* (55). Intriguingly, in humans, an Eomes$^+$Tbet^{lo} liver-resident NK cell subset has been identified (56, 57). More recently, two reports show the requirement for TGFβ in driving NK cell-to-ILC1 conversion in a tumor microenvironment (58, 59) and during virus infection (59). Gao et al., recently identified NK cells that convert into intermediate ILC1s (intILC1s), also *via* TGFβ (58). Surprisingly, even though it is well-established that SMAD4 promotes signaling by TGFβ family members, and TGFβ encourages skewing towards the ILC1 lineage, Cortez, et al. demonstrated that SMAD4 deficiency in ILC1s does not affect their differentiation, but instead converts SMAD4-deficient NK cells into ILC1-like cells (59). This suggests that SMAD4 acts as a negative regulator of TGFβ signaling in NK cells, and inhibits typical TGFβ imprinting that is characteristic of ILC1s. More importantly, these intILC1s possess gene expression profiles that are intermediate between ILC1s and NK cells, and are incapable of limiting tumor burden or viral load. Whether these ILC1-like cells can actually revert back to NK cells is uncertain, but these studies reveal a mechanism by which the ILC continuum is controlled by a rheostat that includes a cytokine milieu driven by the local tissue microenvironment (**Figure 1C**).

NEW ILC ON THE BLOCK: ILCREGS

Like Tregs, ILCs are reported to have a corresponding population, aptly-called ILCregs (60). Wang, et al. identified ILCregs in mouse and human intestines that are induced upon inflammatory stimuli, such as DSS, anti-CD40 antibody, as well as *Salmonella typhimurium* and *Citrobacter rodentium* infection (60). ILCregs purportedly originate from common helper ILC precursors (CHILP), and not ILC precursors, and express the transcription factors, ID3 and Sox4. Although ILCregs do not express CD4 or Foxp3, they possess a gene identity distinct from other ILC subsets and Tregs. Importantly, ILCregs produce IL-10 and TGFβ, and suppress activation of ILC1s and ILC3s, but not ILC2s, in an IL-10-dependent manner. TGFβ is necessary for maintenance of ILCregs and autocrine TGFβ is required for its expansion during intestinal inflammation. The role of ILCregs in colorectal cancer (CRC) show that these cells transdifferentiate from ILC3s during CRC progression *via* TGFβ, indicating potential pro-tumorigenic function during ILC3-to-ILCreg plasticity (61). Furthermore, retinoic acid is reported to induce transdifferentiation of ILC2s into IL-10-producing ILCregs during airway inflammation (62), while ILC2s provide a predominant and inducible source of IL-10 in the GI tract (63). The existence of this novel ILC subset is, however, controversial and the topic of current investigation that elicits several open-ended questions. How do the transcription factors, ID3 and Sox4, synergize to modulate ILCreg development? Since ILCregs constitutively express *Tgfbr1, Tgfbr2, Il2rb*, and *Il2rg*, can they be stimulated directly by IL-2 and TGFβ? Why are ILC2s unresponsive to the effects of IL-10-producing ILCregs? It is interesting to note that a subset of IL-10-secreting ILC2s, detected in murine lungs after IL-33 treatment or papain stimulation, associates with reduced lung eosinophilia (64). Comparable to ILCregs, these IL-10$^+$ ILC2s also express the anti-inflammatory gene, *Retnla*, thereby suggesting anti-inflammatory properties, yet warrants further investigation in other pathogenic inflammatory states.

CONCLUSIONS AND FUTURE DIRECTIONS

ILCs are particularly enriched at mucosal surfaces, and studies over the last decade highlight various functions that are important at the intestinal barrier (65), both in maintaining gut homeostasis, but also during chronic inflammation. ILC plasticity allows adaptability in response to changes in the local tissue microenvironment that are critical to appropriately respond to pathogenic challenge, without the need for *de novo* ILC generation and recruitment. Investigation into the precise mechanisms that control plasticity of specific ILC subsets, particularly at mucosal surfaces, is in its infancy and will aid in further understanding disease pathogenesis and designing targeted therapies in the future.

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CS and K-AB contributed equally to researching, writing of initial drafts, and assembling manuscript. TP conceptualized, edited, and assembled the final submitted manuscript. All authors contributed to the article and approved the submitted version.

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