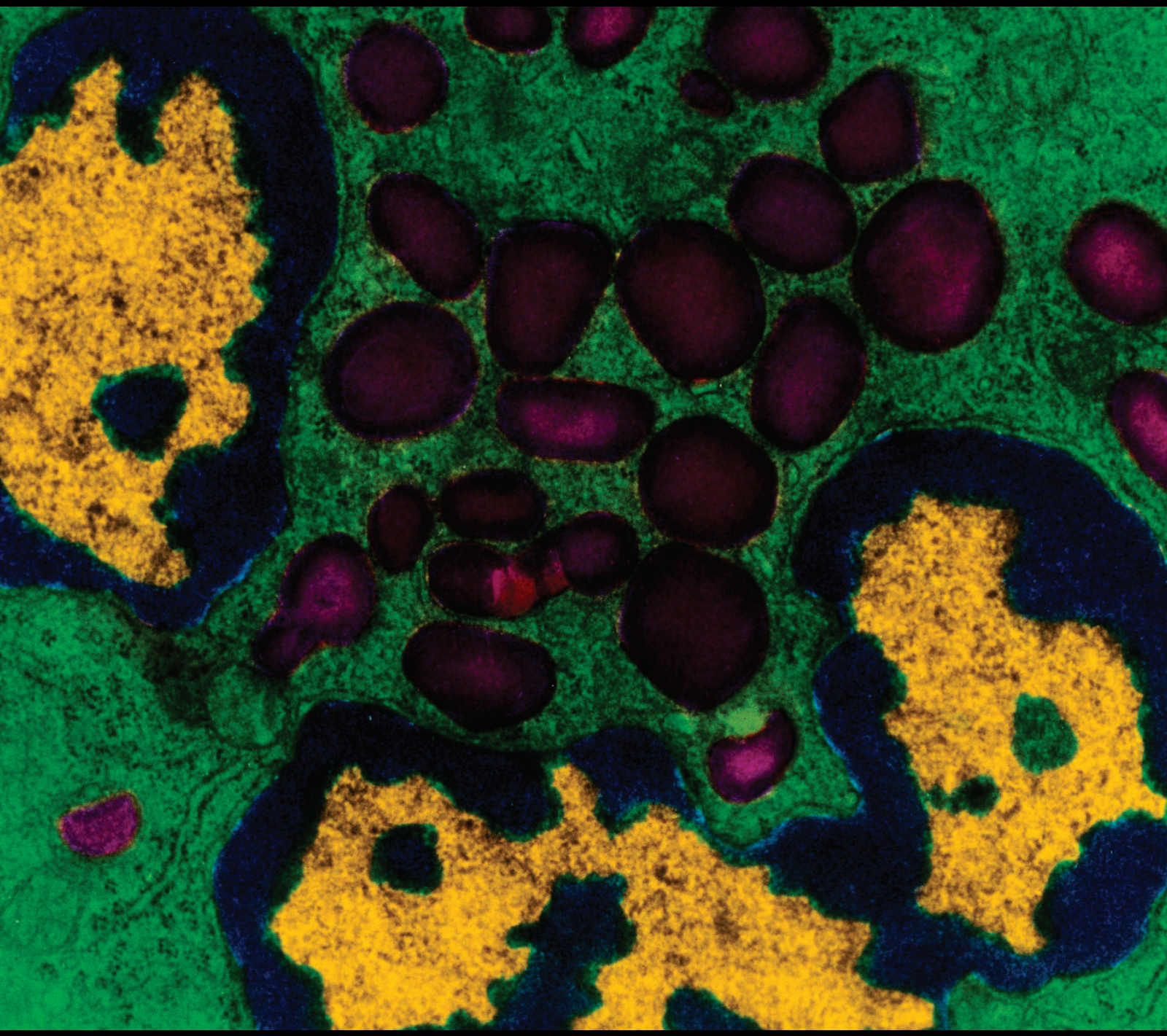
Mediators of Inflammation

Mediators of Gut Mucosal Immunity and Inflammation

Guest Editors: Ishak Ozel Tekin, H. Barbaros Oral, and Ronit Shiri-Sverdlov



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Editorial Board

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Editorial

Mediators of Gut Mucosal Immunity and Inflammation

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The gastrointestinal tract is continuously exposed to foreign antigens such as those derived from food and microbiota of gut. Gut flora is an important entity of human body that plays crucial role in healthy immune system and various immunological disorders. Exploration of interactions between gut flora and immune system is an important area of current investigations. Cytokines and chemokines are the integral component of the adaptive and innate immune response in the gastrointestinal system. Cytokines are involved in a variety of immunological, inflammatory, and infectious diseases. Chemokines are a family of small cytokines or proteins secreted by numerous cells. The major role of chemokines is to guide the migration of particular cells. The mediators of gut mucosal immunity and inflammation are not limited to chemokines and cytokines. There are many inflammatory and anti-inflammatory molecules derived from gut mucosal endothelial cells and leucocytes.

In this special issue, we report findings regarding the mediators of gut mucosal immunity and inflammation. The papers have been contributed by a number of experts in the field and include both review articles that provide an overview of the work conducted to date and original articles reporting recent developments. This special issue contains 19 papers, representing original research articles, a clinical research article, and reviews. In order to highlight the translational relevance, several papers are focused on colitis mechanisms as well as clinical evidence related to inflammatory bowel diseases. Some papers offer important relationship between microbiota, probiotics, functional foods, and gut mucosal immunity and inflammation.

We hope that these papers will be beneficial for clinicians and researchers in understanding of gut mucosal immunity and inflammation on health or diseases. Each of the papers in this series is briefly highlighted as follows.

Y.-C. Hou et al. in "Glutamine Supplementation Attenuates Expressions of Adhesion Molecules and Chemokine Receptors on T Cells in a Murine Model of Acute Colitis" describe an animal study that investigates the effect of glutamine on the expression of some adhesion molecules and chemokine receptors on T cells. According to this paper, glutamine may ameliorate the inflammation of colitis via suppression of T-cell migration.

H. Rajkumar et al.'s "Effect of Probiotic (VSL#3) and Omega-3 on Lipid Profile, Insulin Sensitivity, Inflammatory Markers and Gut Colonization in Overweight Adults: A Randomized, Controlled Trial" is a clinical study that examined the effect of VSL#3 and omega-3 on some biochemical and inflammatory markers and gut colonization in overweight human adults. In this study VSL#3 alone improves atherogenic biochemical profile and insulin sensitivity and changes gut microbiota. Omega-3 has a similar effect with probiotics but it has no effect on gut microbiota. The combination of VSL#3 and omega-3 has more pronounced effect on HDL, insulin sensitivity, and hsCRP.

"Intestinal Mucosal Barrier Is Injured by BMP2/4 via Activation of NF-κB Signals after Ischemic Reperfusion" by K. Chen et al. is a research article reporting the effect of bone morphogenic proteins (BMP 2 and BMP 4) on intestinal mucosal barrier for an ischemia-reperfusion model. In this study, BMP2 and BMP4 can directly activate NF-κB, induce

the expression of some inflammatory cytokines in the intestinal epithelial cells, and decrease the expression of the tight junction protein occludin, which could result in disruption of the intestinal barrier.

"An Overview of the Role of Innate Lymphoid Cells in Gut Infections and Inflammation" by S. Sedda et al. is a review about innate lymphoid cells in the gut. In this review, the authors summarized the current knowledge on the distribution of ILCs in the intestinal mucosa, with particular focus on their role in the control of both infections and effector cytokine response in immune-mediated pathologies.

The important role of IL-35 and IL-37 in IBD patients is presented by Y. Li et al. in the research article "The Possible Role of the Novel Cytokines IL-35 and IL-37 in Inflammatory Bowel Disease." The study focuses on IL-35, IL-37, and IBD. According to the authors, serum IL-35 and IL-37 might be potentially novel biomarkers for IBD. And the upregulation of encoding genes to intestinal IL-35 and IL-37 proteins may provide a new possible target for the treatment of IBD.

A. K. Kumawat et al. presented a paper entitled "An In Vitro Model to Evaluate the Impact of the Soluble Factors from the Colonic Mucosa of Collagenous Colitis Patients on T Cells: Enhanced Production of IL-17A and IL-10 from Peripheral CD4+ T Cells." This model reveals implications of soluble factors from collagenous colitis mucosa on peripheral T cells, enhancing their production of both pro- and anti-inflammatory cytokines.

K. S. Brown et al.'s "Tumor Necrosis Factor Induces Developmental Stage-Dependent Structural Changes in the Immature Small Intestine" is a research article reporting the effects of TNF on structural changes in the immature small intestine. In this study, the researchers examine acute, brief, or chronic exposures of TNF in neonatal and juvenile mice. In this model, TNF-induced blunting caused by feeding-induced or other chronic inflammation could subsequently decrease the distance between the luminal contents of the intestine and the lamina propria. This shortening of distance would greatly increase the ability of bacteria to reach the crypt and infiltrate the intestine. The authors observed also the other effects of TNF such as depletion of the mucous layer and degranulation of Paneth cells, which may allow for easier bacterial penetration into the intestinal lamina propria, leading to the inflammatory response and coagulation necrosis characteristic of NEC.

"The Impact of ATRA on Shaping Human Myeloid Cell Responses to Epithelial Cell Derived Stimuli and on T-Lymphocyte Polarization" by A. Chatterjee et al. is a research article about the importance of ATRA on triangle of myeloid cell, epithelial cell, and T cell.

Various immune cell infiltrations in the epithelium and lamina propria are seen in microscopic colitis immunopathology. "Enhanced Levels of Chemokines and Their Receptors in the Colon of Microscopic Colitis Patients Indicate Mixed Immune Cell Recruitment" by S. Gunaltay et al. is a research article. The study focuses on the chemokines and their receptor levels in the colon of microscopic colitis patients. The results of this study expand the current understanding of the involvement of various immune cells in MC immunopathology and endorse chemokines as potential diagnostic markers as well as therapeutic candidates. Moreover, this study further supports the hypothesis that CC and LC are two different entities due to differences in their immunoregulatory responses.

The review entitled "Transcriptional Regulators of Claudins in Epithelial Tight Junctions" by N. Khan and A. R. Asif focuses on the transcriptional regulators of claudins. This review indicates that altered expression of claudins family proteins in tight junctions plays a key role in numerous abnormalities like cancers, IBDs, and leaky diarrhea and a better understanding of their regulatory mechanism could help in designing innovative therapeutic strategies.

J. Michalkiewicz et al. in "Innate Immunity Components and Cytokines in Gastric Mucosa in Children with *Helicobacter pylori* Infection" evaluate innate immunity in gastric mucosa in children with HP infection. This study showed that *H. pylori* infection in children resulted in mRNA upregulation of IL-6, IL-10, TNF-α, IFN-γ, and CD163 and unchanged expression of MyD88, TLR2, and TLR4 mRNA in the gastric mucosa. According to the authors, these findings are associated with *H. pylori* driven immune manipulation.

"Moderate Exercise Training Attenuates the Severity of Experimental Rodent Colitis: The Importance of Crosstalk between Adipose Tissue and Skeletal Muscles" is a research article about rodent experimental colitis realized by J. Bilski et al. In this study, diet induced obesity delays the healing of experimental colitis. Release of myokines in trained animals contributes to improvement of intestinal healing. The authors recommend an exercise program for IBD patients.

S. O'Sullivan et al. in "Matrix Metalloproteinases in Inflammatory Bowel Disease: An Update" reviewed the effects of matrix metalloproteinases in IBD. This review describes new roles of MMPs in the pathophysiology of IBD and suggests future directions for the development of treatment strategies in this condition.

"*Enterococcus faecium* NCIMB 10415 Modulates Epithelial Integrity, Heat Shock Protein, and Proinflammatory Cytokine Response in Intestinal Cells" by S. Klingspor is a research article about relationship between *Enterococcus faecium* NCIMB 10415 and intestinal cells. The effects of *E. faecium* observed in this study indicate a protective effect of this probiotic in acute intestinal inflammation induced by ETEC.

M. Endale et al. in "Central Role of Gimap5 in Maintaining Peripheral Tolerance and T Cell Homeostasis in the Gut" reviewed the role of GTPase of immunity-associated protein 5 (Gimap5) in maintaining peripheral T-cell tolerance in the gut. The authors discuss how defects in Gimap5 function impair immunological tolerance and lymphocyte survival and ultimately drive the development of CD4+ T cell-mediated early-onset colitis in this paper.

"Oxidative Stress in Patients with Alzheimer's Disease: Effect of Extracts of Fermented Papaya Powder" is a review article written by M. Barbagallo et al. This review focuses on the effects of fermented papaya in the patients with Alzheimer diseases.

Y. Kurashima et al. in "Pathophysiological Role of Extracellular Purinergic Mediators in the Control of Intestinal Inflammation" reviewed the recent findings regarding the

pathophysiological role of purinergic mediators in the development of intestinal inflammation.

"Gut Inflammation and Immunity: What Is the Role of the Human Gut Virome?" by A. Focà is a review article about the role of human gut virome on gut inflammation and immunity. The authors reviewed recent evidence on the viruses found in the gastrointestinal tract, discussing their interactions with the resident bacterial microbiota and the host immune system, in order to explore the potential impact of the virome on human health.

Finally, "Claudin-4 Undergoes Age-Dependent Change in Cellular Localization on Pig Jejunal Villous Epithelial Cells, Independent of Bacterial Colonization" by J. A. Pasternak et al. showed that *FcRn* gene (FCGRT) was minimally expressed in 6-week-old gut and newborn 24 jejuna but it was expressed at significantly higher levels in the ileum of newborn piglets. pIgR was highly 25 expressed in the jejunum and ileum of 6-week-old animals but only minimally in neonatal gut. According to the authors, CLDN4 transcript abundance and CLDN5 transcript abundance were conserved in jejunum and ileum in age-matched animals and that striking differences in CLDN4 expression did not occur in either region of the gut with age. CLDN5 showed significantly higher expression in the jejunum and ileum from the 24-hour-old animals relative to the older animals.

We sincerely hope that the present special issue may provide useful information to understand the mediators of gut mucosal immunity and inflammation. We hope that the reader will find some novel input for future researches.

Acknowledgments

We would like to acknowledge the authors for their excellent contributions and constructive work. We would also like to express our gratitude to all the reviewers on these articles, for their kind assistance and helpful insights.

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Research Article

Enhanced Levels of Chemokines and Their Receptors in the Colon of Microscopic Colitis Patients Indicate Mixed Immune Cell Recruitment

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Microscopic colitis (MC), comprising collagenous colitis (CC) and lymphocytic colitis (LC), is a common cause of chronic diarrhea. Various immune cell infiltrations in the epithelium and lamina propria are seen in MC immunopathology. We compared gene and protein expressions of different immune cell attracting chemokines and their receptors in colon biopsies from MC patients in active disease or histopathological remission (CC/LC-HR) with controls, using qRT-PCR and Luminex, respectively. CC and LC patients with active disease demonstrated a mixed chemokine profile with significantly enhanced gene and/or protein expressions of the chemokines CCL2, CCL3, CCL4, CCL5, CCL7, CCL22, CXCL8, CXCL9, CXCL10, CXCL11, and CX_3CL1 and the receptors CCR2, CCR3, CCR4, CXCR1, CXCR2, and CX_3CR1. Enhanced chemokine/chemokine receptor gene and protein levels in LC-HR patients were similar to LC patients, whereas CC-HR patients demonstrated almost normalized levels. These findings expand the current understanding of the involvement of various immune cells in MC immunopathology and endorse chemokines as potential diagnostic markers as well as therapeutic candidates. Moreover, this study further supports the hypothesis that CC and LC are two different entities due to differences in their immunoregulatory responses.

1. Introduction

Microscopic colitis (MC), comprising collagenous colitis (CC) and lymphocytic colitis (LC), is characterized clinically by chronic watery diarrhea, abdominal pain, and/or weight loss. The diagnosis relies on typical histopathological features that are observed upon microscopic examination: lymphocytic infiltration of the epithelium and lamina propria as well as a damaged, flattened, and detached epithelial layer and in CC a characteristic thickened subepithelial collagen layer [1–4]. Biopsies from both LC and CC patients reveal a mixed inflammatory cell infiltrate in lamina propria, including T and B lymphocytes, plasma cells, eosinophils, neutrophils, mast cells, and macrophages [5–9]. Although the etiology of MC remains unclear, barrier dysfunction, increased numbers of immune cells, and/or immune response to luminal agents have all been suggested to be part of the pathogenesis [1].

Chemokines are small (~8–14 kDa) secreted proteins that orchestrate leukocyte migration by chemotaxis in homeostasis and inflammation [10]. Specificity in chemotaxis depends on both differential expressions of chemokines and their corresponding receptors expressed by leukocyte subsets [11]. Hence, dysregulated expression of chemokines and/or receptors may contribute to pathogenesis in different chronic inflammatory disorders [12]. Therefore, our aim in this study was to compare gene and protein expressions of a number of

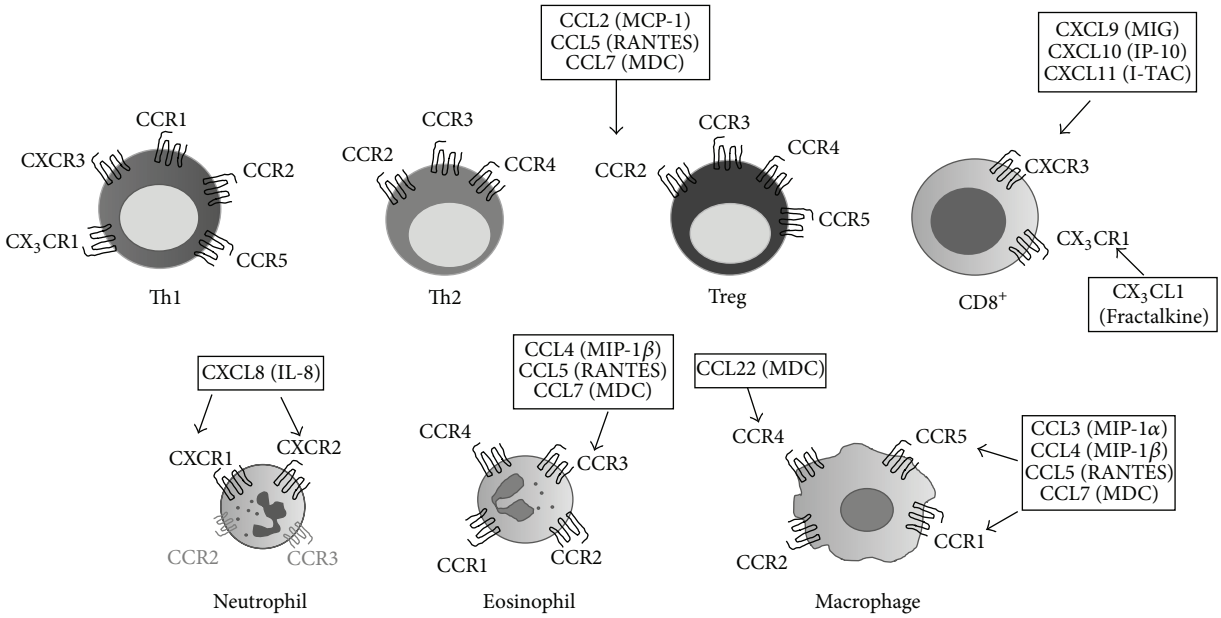

Figure 1: Summary of chemokines and their receptors investigated in this study. The chemokines interacting with their corresponding receptors are indicated once for each receptor but are valid for all cell types expressing these receptors.

chemokines and their receptors, summarized in Figure 1, in colon biopsies from MC patients with active disease (CC/LC) or those clinically active but in histopathological remission (CC/LC-HR) with controls. To the best of our knowledge, this is the first comprehensive study demonstrating increased mucosal gene and protein expressions of immune cell attracting chemokines and their receptors in CC and LC. LC-HR patients showed similarities with LC patients in terms of enhanced chemokine and receptor expression levels, whereas CC-HR patients had normalized expressions. These results contribute to the knowledge of MC immunopathology, a subtler type of inflammatory bowel disease (IBD).

2. Materials and Methods

2.1. Patients. The MC patients underwent colonoscopy because of watery diarrhea, abdominal pain, and/or weight loss. Routine biopsy specimens were obtained from the proximal, transverse, and distal colon for confirmation of diagnosis through histopathological examination of paraffin embedded slides by an experienced gastropathologist. Histopathological criteria for CC were a diffusely distributed and thickened subepithelial collagen layer (≥10 μm), epithelial damage such as flattening and detachment, inflammation in the lamina propria with mainly mononuclear cells, and increased numbers of intraepithelial lymphocytes (IELs). Histopathological criteria for LC were, in addition to epithelial damage and inflammation in the lamina propria, ≥20 IELs per 100 surface epithelial cells but with a normal collagen layer [1]. The clinical characteristics of the patients and controls were summarized in Table 1.

Four patients with an established diagnosis of CC and six patients with LC no longer fulfilled the histopathological criteria for MC despite clinical symptoms of the disease. These patients were therefore categorized as clinically active but histopathologically in remission (CC-HR/LC-HR) [13] and were analyzed separately (Table 1).

In gene and protein expression analyses, two patients with CC and two patients with LC were treated with budesonide at the time of colonoscopy, including only one patient having budesonide treatment 3 days before the colonoscopy. In protein expression analysis we included one more patient with CC, who was on budesonide treatment. These patients were identified in the graphs as circled symbols. We were unable to detect any effects of budesonide on the parameters tested.

Fourteen control individuals underwent colonoscopy due to changes in bowel habits ($n = 2$), iron deficiency anemia ($n = 3$), rectal bleeding ($n = 2$), follow-up after diverticulitis ($n = 1$), hemorrhoids ($n = 1$), irritable bowel syndrome ($n = 1$), colon cancer screening ($n = 3$), or abdominal pain ($n = 1$). The colonoscopy was macroscopically normal except for occasional diverticula in the left colon, and routine biopsy specimens from ascending, transverse, and distal colon revealed no pathological alterations.

Biopsy specimens for this study were obtained from the proximal colon with standard biopsy forceps and were immediately immersed in RNA*later* (Ambicon, Life Technologies, Foster City, CA, USA) and then stored at −80°C for later analysis.

2.2. RNA Isolation. Total RNA was isolated with miRNeasy Kits (Qiagen, GmbH, Hilden, Germany) according to the manufacturer's protocol and was quantified using a NanoDrop ND 1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA).

Table 1: Clinical characteristics of patients and controls.

	CC[a]		CC-HR[b]		LC[c]		LC-HR[d]		Control	
	mRNA	Protein	mRNA	Protein	mRNA	Protein	mRNA	Protein	mRNA	Protein
Number of patients	9	13	3	4	8	5	6	6	9	10
Male/female	1/8	2/11	0/3	0/4	0/8	0/5	0/6	0/6	6/3	5/5
Age (y)	66.7[e] (35–84)	62.5 (35–84)	55 (50–64)	57.3 (50–64)	69.1 (49–86)	74 (65–86)	61 (24–80)	57.8 (24–80)	61.1 (29–88)	53.4 (28–78)
Duration of disease (y)	6.7 (0–17)	6.9 (0–17)	5 (1–9)	6 (1–9)	2.4 (0–7)	1.8 (0–6)	1.2 (0–3)	2.2 (0–7)	n/a	n/a

[a]CC: specimens from collagenous colitis; [b]CC-HR: specimens from clinically active CC patients in histopathological remission; [c]LC: specimens from lymphocytic colitis; [d]LC-HR: specimens from clinically active LC patients in histopathological remission. [e]Data are shown as mean (range).

2.3. Reverse Transcription and Quantitative Real Time Reverse Transcription Polymerase Chain Reaction (qRT-PCR). All products used in the reverse transcription and qRT-PCRs were ordered from Applied Biosystems, Life Technologies (Austin, TX, USA). cDNA transcription of 500 ng/μL total RNA was performed by High-Capacity cDNA Reverse Transcription Kits according to the manufacturer's protocol. The following TaqMan primer-probe sets were used: CCL2/MCP-1 (Hs00234140_m1), CCL3/MIP-1α (Hs00234142_m1), CCL4/MIP-1β (Hs99999148_m1), CCL5/RANTES (Hs00174575_m1), CCL7/MCP-3 (Hs00171147_m1), CCL22/MDC (Hs01574247_m1), CXCL8/IL-8 (Hs00174103), CXCL9/MIG (Hs00171065_m1), CXCL10/IP-10 (Hs01124251_g1), CXCL11/I-TAC (Hs04187682_g1), CX_3CL1/Fractalkine (Hs00171086_m1), CCR1 (Hs00928897_s1), CCR2 (Hs00704702_s1), CCR3 (Hs00266213_s1), CCR4 (Hs00747615_s1), CCR5 (Hs99999149_s1), CXCR1 (Hs01921207_s1), CXCR2 (Hs01891184_s1), CXCR3 (Hs01847760_s1), and CX_3CR1 (Hs01922583_s1). Normalization of qRT-PCR results was performed using the mean of three housekeeping genes GAPDH (Hs99999905_m1), GUSB (Hs99999908_m1), and 18S (Hs99999901_s1). For gene expression assays, TaqMan Fast Universal Master Mix was used with the thermal cycling parameters suggested in the manufacturer's protocol. The samples were run in the GeneBio-rad CFX96 Touch Real-Time PCR Detection System (Bio-rad Laboratories Inc., Hercules, CA, USA). Gene expressions were expressed relative to the average of the housekeeping genes. The comparative threshold cycle method was used to compare control and patient results [14].

2.4. Protein Extraction and Chemokine Analysis. The mean (±SEM) weight of biopsy specimens used for chemokine quantification was 5.6 ± 1.5 mg. The biopsies stored in RNA*later* were homogenized using Tissuelyser II (Qiagen, GmbH, Hilden, Germany) at 25 Hz for 5 times 1 minute in RIPA buffer (Sigma Aldrich, Steinheim, Germany) containing proteinase inhibitor cocktail (catalog number P8340, Sigma Aldrich). The homogenization mixture was centrifuged for 5 min at 10,000 rpm, and the supernatant was divided into aliquots and stored at −80°C until further processing. Tissue protein levels of CCL2, CCL3, CCL4, CCL7, CXCL8, CXCL10, and CX_3CL1 were analyzed in duplicate by xMAP technology developed by Luminex (Austin, TX, USA). The concentrations were determined using the Milliplex Map Kit (catalog number SPR217) according to the manufacturer's instructions (Millipore, MA, USA). The levels of different chemokines from MC and controls were expressed as pg/mg tissue, according to a standard curve with known amounts of each analyte (Millipore).

2.5. Statistical Analysis. Data values were compared according to the nonparametric Mann-Whitney test with statistical significance set at $P < 0.05$ (GraphPad Prism 4, San Diego, CA, USA). Statistical outliers were any points found below first quartile (Q_1) − 1.5 × interquartile range (IQR) and above Q_3 + 1.5 × IQR. When present they are marked as crosses (X) in the graphs and excluded from the statistical analysis. The different patient groups including CC and LC and those in histopathological remission (CC-HR/LC-HR) were compared to noninflamed control tissues. Also, patients with active disease or with the same disease in histopathological remission were compared to each other.

3. Results

3.1. Increased Expressions of the Th1 and $CD8^+$ T Cell-Associated Chemokines CXCL9, CXCL10, CXCL11, and CX_3CL1 in MC Patients. CXCL9, CXCL10, CXCL11, and CX_3CL1 are important chemokines in Th1 and $CD8^+$ T cell recruitment [15, 16]. Significantly increased gene expressions of CXCL9, CXCL10, CXCL11, and CX_3CL1 were detected in CC patients compared to both controls and CC-HR patients (Figures 2(a)–2(d)). CC-HR patients also had decreased CXCL10 gene expression compared to controls (Figure 2(b)). Likewise, LC patients showed significantly enhanced CXCL9, CXCL10, CXCL11, and CX_3CL1 gene expressions compared to controls (Figures 2(a)–2(d)). The enhanced gene expression levels of CXCL9, CXCL11, and CX_3CL1 in LC-HR patients compared to controls indicate similarities with LC patients (Figures 2(a), 2(c), and 2(d)).

Protein expression of CXCL10 was enhanced in CC patients compared to controls and CC-HR patients (Figure 2(e)), in line with CXCL10 gene expression (Figure 2(b)). Likewise, LC patients had enhanced CXCL10 protein levels in comparison with both controls and LC-HR patients (Figure 2(e)). CX_3CL1 protein level was significantly increased in LC patients only compared to controls (Figure 2(f)).

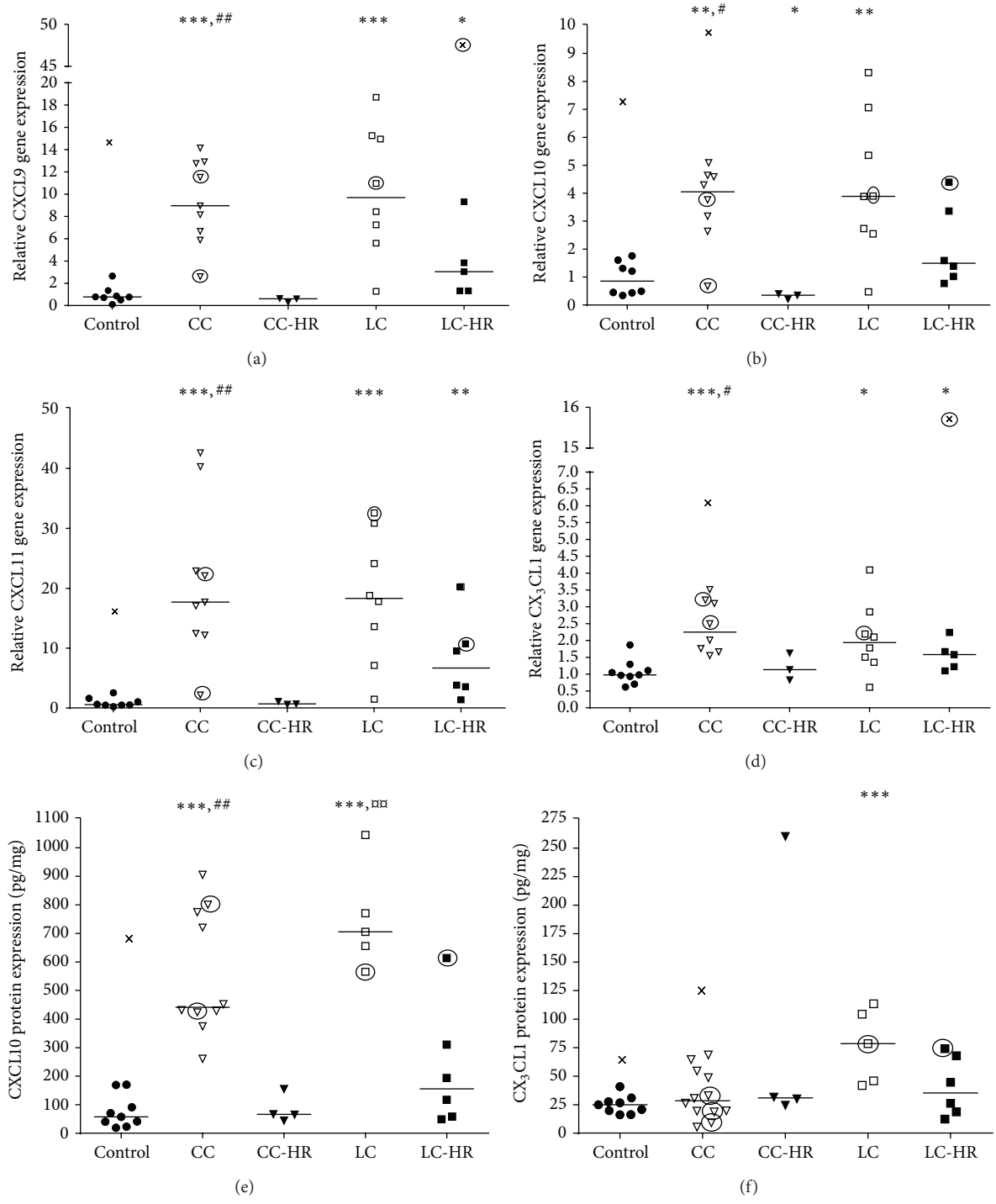

FIGURE 2: Gene and protein expressions of the Th1 and $CD8^+$ T cell recruiting chemokines CXCL9, CXCL10, CXCL11, and CX_3CL1. Each symbol represents one patient and the medians of the values are depicted as a line. Statistical outliers are marked as crosses (X) and budesonide treated patients are encircled. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P \leq 0.001$ versus controls, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus CC-HR, and $^{¤}P < 0.05$, $^{¤¤}P < 0.01$ versus LC-HR.

3.2. Gene Expression of CX_3CR1 but not CXCR3 Was Upregulated in MC Patients. CXCL9, CXCL10, and CXCL11 bind to a common receptor, CXCR3 [15], whereas CX_3CL1 only interacts with CX_3CR1 [16]. CX_3CR1 gene expression was significantly increased in CC, CC-HR, and LC-HR patients compared to controls (Figure 3(a)). The only significant alteration in CXCR3 gene expression was diminished expression in CC-HR patients compared to CC patients (Figure 3(b)) as well as a trend towards decreased expression compared to controls ($P = 0.06$, Figure 3(b)).

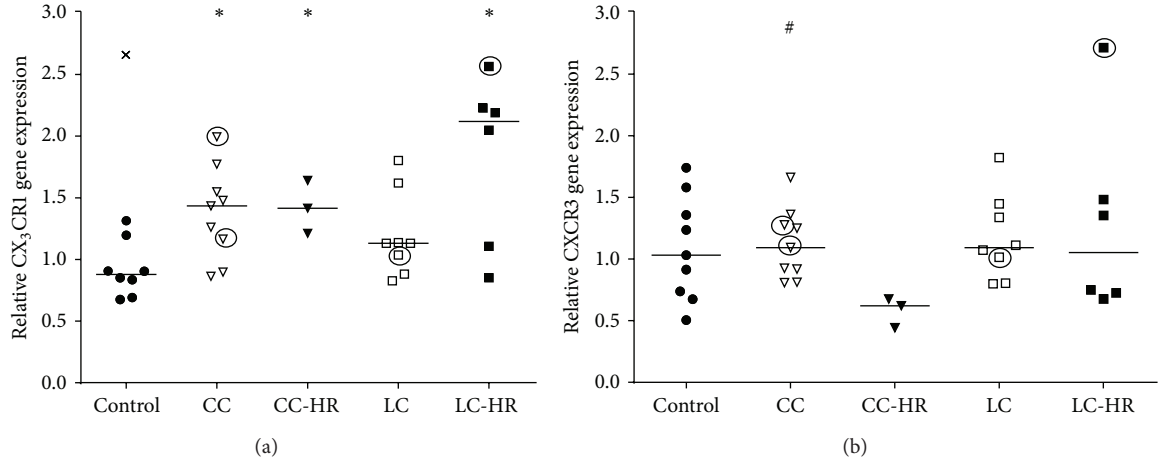

Figure 3: Relative gene expressions of the chemokine receptors CX_3CR1 and CXCR3. Each symbol represents one patient, and the medians are depicted as a line. Statistical outliers are marked as crosses (X) and budesonide treated patients are encircled. $^{*}P < 0.05$ versus controls and $^{\#}P < 0.05$ versus CC-HR.

3.3. The Neutrophil Recruiting CXCL8 and Its Receptors CXCR1 and CXCR2 Showed Enhanced Gene and Protein Levels in MC Patients. Gene and protein expressions of CXCL8 were significantly increased in both CC and LC patients compared to controls, with the highest expressions recorded in CC patients (Figures 4(a) and 4(b)). In addition, CC patients showed significantly increased CXCL8 gene expression compared to CC-HR patients (Figure 4(a)) as well as a trend towards increased protein levels ($P = 0.06$, Figure 4(b)). The two receptors, CXCR1 and CXCR2, showed significantly increased gene expressions in all MC patient groups compared to controls (Figures 4(c) and 4(d)).

3.4. Enhanced Gene and Protein Levels of CCL2, CCL3, CCL4, CCL7, and CCL22 in MC Patients. CCL2, CCL3, CCL4, CCL5, CCL7, and CCL22 are pleiotropic chemokines attracting Th1, Th2, regulatory T (Treg) cells, neutrophils, eosinophils, and/or macrophages [16–20].

CCL2 showed increased gene as well as protein expression in CC, LC, and LC-HR patients compared to controls (Figures 5(a) and 5(b)). CC-HR patients showed intermediate levels of CCL2 protein, being significantly increased compared to controls but significantly decreased compared to CC patients (Figure 5(b)).

CCL3 showed a trend towards increased gene expression ($P = 0.06$, Figure 5(c)) and significantly increased protein levels (Figure 5(d)) in CC patients compared to controls. CC-HR patients demonstrated diminished CCL3 gene expression compared to controls (Figure 5(c)) but no change in protein levels (Figure 5(d)). Both gene and protein expressions of CCL3 in LC patients were increased compared to controls (Figures 5(c) and 5(d)). CCL3 protein levels were also enhanced in LC-HR patients compared to controls (Figure 5(d)).

CCL4 also showed enhanced gene and protein expressions in both CC and LC patients compared to controls (Figures 5(e) and 5(f)), whereas CC and LC patients in histopathological remission (CC/LC-HR) had normalized CCL4 gene and protein expressions (Figures 5(e) and 5(f)).

Eosinophils, Th1, and Th2 cells are recruited by CCL5 [18–20], which was significantly increased in LC patients compared to both controls and LC-HR patients (Figure 6(a)).

CCL7 did not show significant changes in gene expression in any group of MC patients (data was not shown), whereas significantly increased protein expression was detected in LC-HR patients but not in any other patient group compared to controls (Figure 6(b)).

As opposed to CCL7, CCL22 gene expression was significantly increased in CC, LC, and LC-HR patients compared to controls (Figure 6(c)). In contrast, CC-HR patients had significantly decreased gene expression compared to CC patients, which was not different from the levels in controls (Figure 6(c)).

3.5. Increased Gene Expressions of Chemokine Receptors CCR2, CCR3, and CCR4 in MC. CCR2 binds CCL2, CCL5, and CCL7 and is expressed on neutrophils, eosinophils, macrophages, Th1, Th2, and Treg cells [21, 22]. It showed increased gene expression in CC patients only compared to controls (Figure 7(a)). In contrast, CCR3, binding CCL4, CCL5, and CCL7 and being expressed on neutrophils, eosinophils, Treg, Th1, and Th2 cells [18, 20, 23], had increased gene expression in all MC patient subgroups compared to controls (Figure 7(b)).

CCR4, binding to CCL22 and being found on macrophages, eosinophils, Th2, and Treg cells [18, 20], was significantly upregulated in CC, LC, and LC-HR patients compared to controls (Figure 7(c)). In CC patients CCL22 gene expression was also significantly upregulated compared to CC-HR patients (Figure 7(c)).

In contrast, neither CCR1 nor CCR5 showed any significant changes in gene expression (data was not shown).

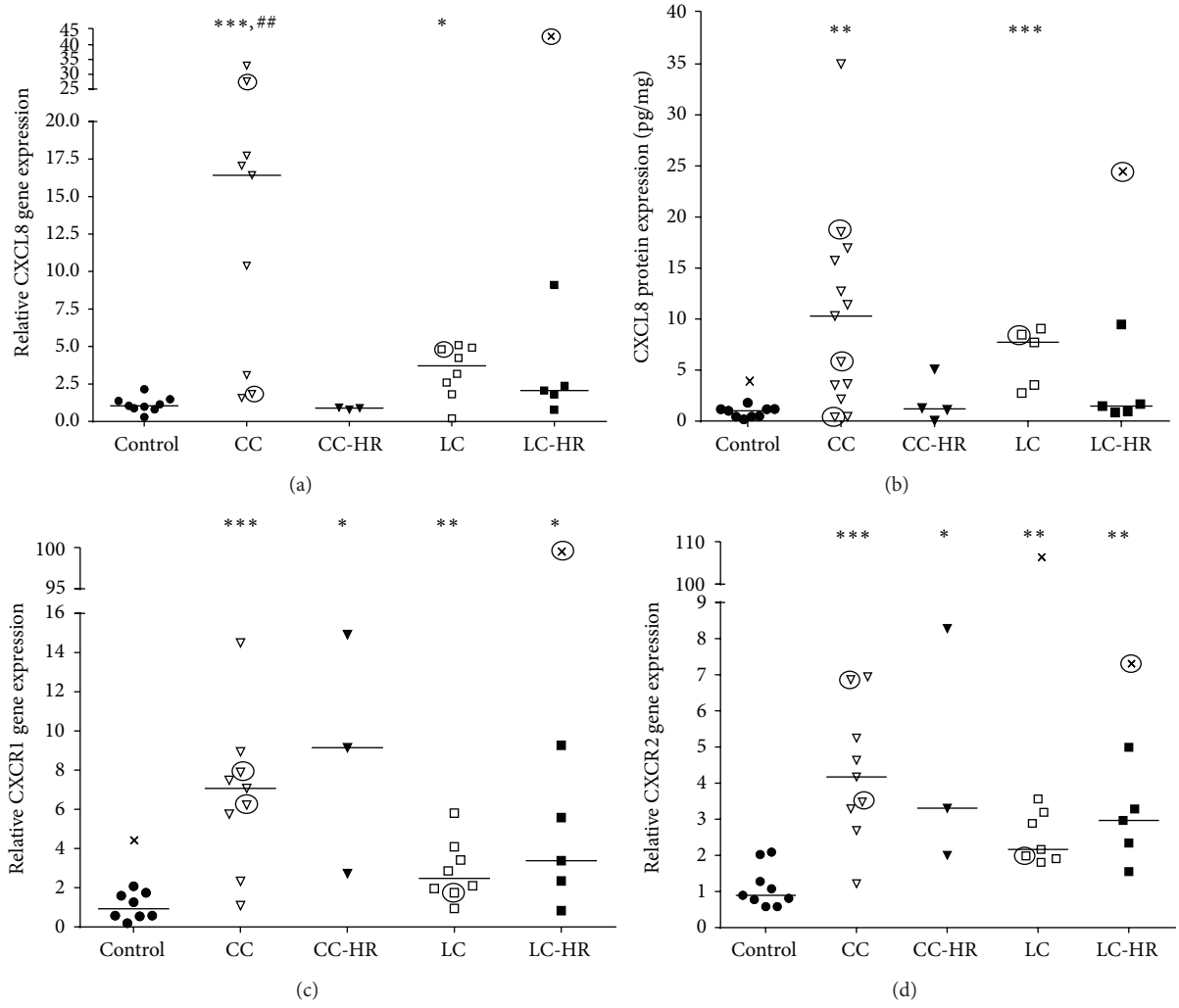

Figure 4: Relative gene and protein expressions of the neutrophil recruiting chemokine CXCL8, as well as gene expression of its receptors CXCR1 and CXCR2. Each symbol represents one patient and the medians of the values are depicted as a line. Statistical outliers are marked as crosses (X) and budesonide treated patients are encircled. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P \leq 0.001$ versus controls and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus CC-HR.

4. Discussion

Although clinical and epidemiological data on MC are emerging the pathophysiology is still unclear and the searches for triggering factors and underlying dysfunctions in the mucosal immune system are still in an early stage [1–3]. Both CC and LC show infiltration primarily of T cells but also plasma cells, eosinophils, mast cells, macrophages, and neutrophils [5–8], which may be recruited by different chemokines and their receptors. The chemokines investigated in this study are all secreted from different cell types: CCL2, CCL3, CCL4, CCL5, CCL7, CXCL8, CXCL9, CXCL10, CXCL11, and CX_3CL1 are secreted by colon epithelial cells [11, 21, 24, 25], whereas CCL22 is expressed by macrophages, mast cells, and dendritic cells [26, 27]. In addition, these chemokines can also be secreted by immune cells in the lamina propria, for example, macrophages (CCL2, CCL5, CCL22, CXCL8, and CXCL10), mast cells (CCL2, CCL5, and CCL22), eosinophils (CCL2, CCL5, and CCL7), and neutrophils (CXCL8 and CXCL10) [18, 21, 26, 28–32]. The gene and/or protein expressions of all these chemokines have been demonstrated to be increased in Crohn's disease (CD) and ulcerative colitis (UC) patients [21, 24, 33, 34]. However, to the best of our knowledge, there is only one study analyzing chemokine expression in MC patients, and that study was limited to observations of CXCL9 and CXCL10 in three LC patients compared to controls [35]. Therefore, we focused on chemokines and their receptors possibly involved in immune cell infiltration in MC immunopathology in order to increase our understanding of the disease mechanism(s) and eventually reveal possible therapeutic candidates.

Enhanced CXCL9, CXCL10, CXCL11, CX_3CL1, and CX_3CR1 gene and protein levels are likely involved in the $CD8^+$ T cell infiltration [16, 33, 36] in the intraepithelial compartment and lamina propria of both CC and LC patients previously reported by us [9, 13]. These chemokines are also

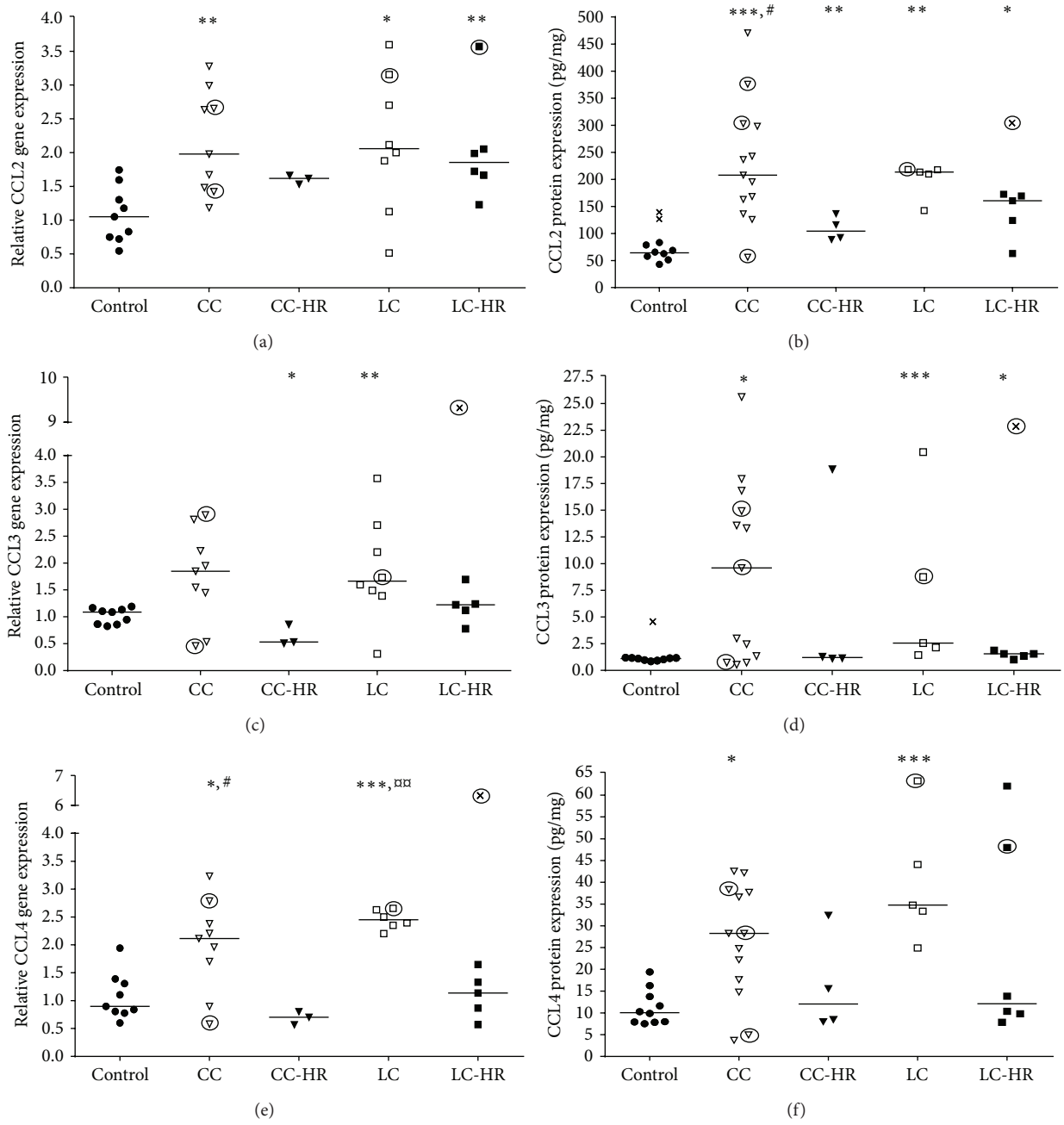

Figure 5: Relative gene and protein expressions of chemokines mediating recruitment of eosinophils, neutrophils, macrophages, Treg, Th1, and/or Th2 cells. Each symbol represents one patient and the medians are depicted as a line. Statistical outliers are marked as crosses (X) and budesonide treated patients are encircled. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P \leq 0.001$ versus controls, $^{\#}P < 0.05$ versus CC-HR, and $^{¤}P < 0.05$, $^{¤¤}P < 0.01$ versus LC-HR.

associated with Th1 cell recruitment. However, we observed increased numbers of $CD8^+$ T cells only in MC patients and decreased numbers of $CD4^+$ T cells. Therefore, these chemokines are more likely involved in $CD8^+$ T cell recruitment in MC. IFN-γ is inducing CXCL9, CXCL10, and CXCL11 expressions, and we previously reported upregulated IFN-γ mRNA levels in CC, LC, and LC-HR patients, with normal levels in CC-HR patients [37]. These results corroborate our present results demonstrating enhanced expression of these chemokines in CC, LC, and LC-HR patients with normalized levels in CC-HR patients.

Enhanced gene and protein expressions of CXCL8 and its receptors CXCR1 and CXCR2 in MC patients suggest an important role for neutrophils, previously observed in the lamina propria of MC patients [3, 16, 19]. The significant upregulation of CXCR1 and CXCR2 gene expressions also in CC-HR and LC-HR patients could be related to neutrophil recruitment due to ongoing contact with the gut microbiota,

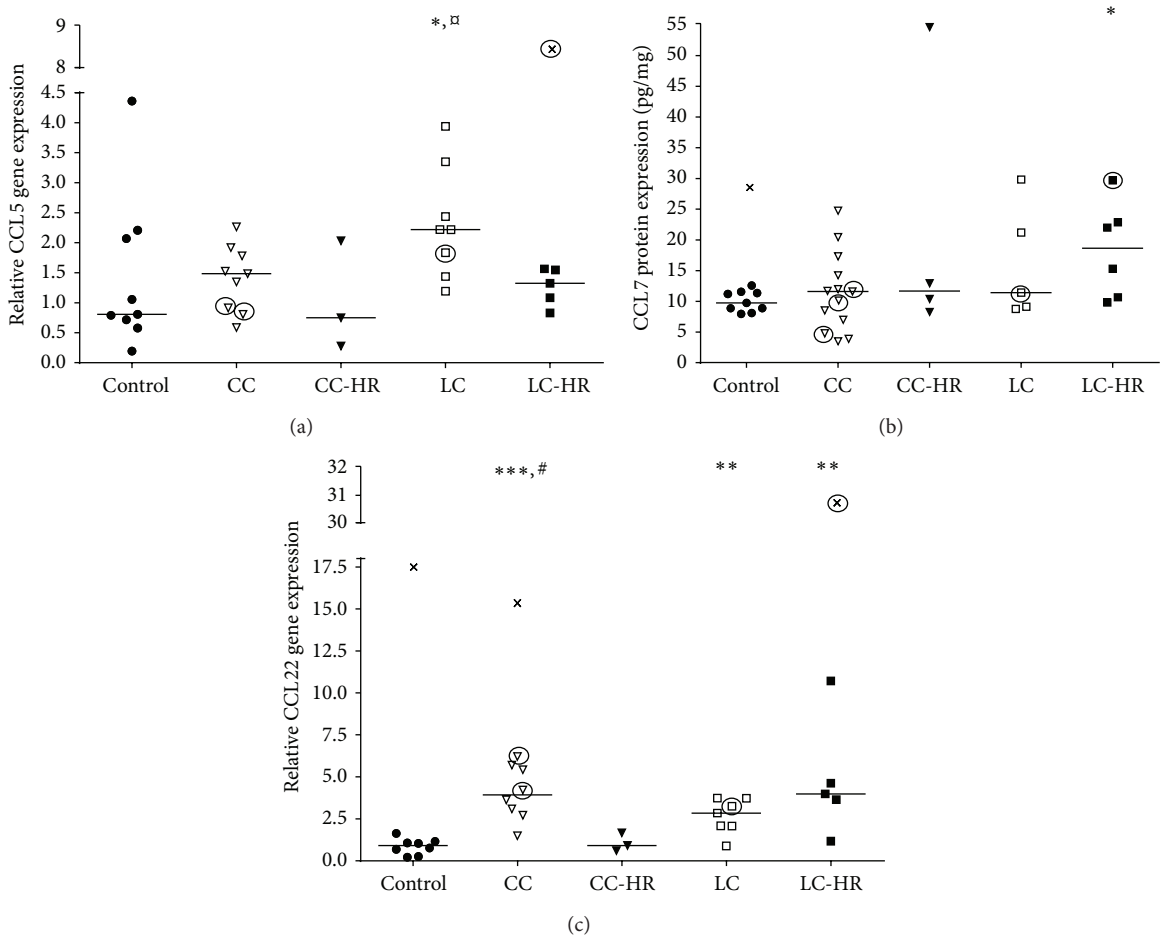

Figure 6: Relative gene and protein expressions of CC chemokines involved in recruitment of eosinophils, neutrophils, macrophages, Treg, Th1, and/or Th2 cells. Each symbol represents one patient and the medians are depicted as a line. Statistical outliers are marked as crosses (X) and budesonide treated patients are encircled. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P \leq 0.001$ versus controls, $^{\#}P < 0.05$ versus CC-HR, and $^{¤}P < 0.05$ versus LC-HR.

as these patients still have histopathological evidence of inflammation, although not fulfilling the criteria for CC/LC diagnosis (gastropathologist Agnes Hegedus, personal communication).

In IBD patients increased CCL2 expression has been correlated with disease activity, mainly in areas of epithelial cell damage [24]. Our findings of enhanced CCL2 gene and protein expressions in both active and histopathological remission patients may likewise correlate with epithelial cell damage in MC, similar to IBD immunopathology [3].

Although CCL3, CCL4, CCL5, CCL7, and CCL22 attract many different cell types such as eosinophils, neutrophils, macrophages, Treg, Th1, and/or Th2 cells [16, 18, 19], decreased numbers of $CD4^{+}$ T cells detected by us in MC patients [9, 13], these chemokines are likely involved mainly in eosinophil, neutrophil, and macrophage recruitment. Eosinophil infiltration in CC patients has previously been demonstrated [7, 38, 39]. Because of high levels of these chemokines also in LC and LC-HR patients, eosinophils may be involved also in LC immunopathology.

Infiltration of inflammatory macrophages, with their high IL-23 production [40], has been suggested as part of IBD immunopathology [41–43]. The present data, together with our previously reported enhanced gene expression of IL-23, may also suggest an important role of macrophages in MC immunopathology [37]. Accordingly, CCR2, CCR3, and CCR4 are predominantly expressed on eosinophils and macrophages [16, 20, 44]. However, Treg cells, found in increased amounts in lamina propria of MC patients [45], may be another source of these chemokine receptors [16, 20].

A potential limitation of this study is the small cohort of MC patients collected. However, as the majority of parameters investigated show statistically significant changes, we believe that the small cohort is not an obstacle in this study. In addition, we chose to analyze patients with clinical symptoms but not fulfilling the histological criteria for CC/LC separately, increasing our knowledge about these subgroups. MC can only be diagnosed upon histopathological examination and patients with an MC diagnosis usually do not undergo repeated colonoscopies. Nevertheless, as there is still

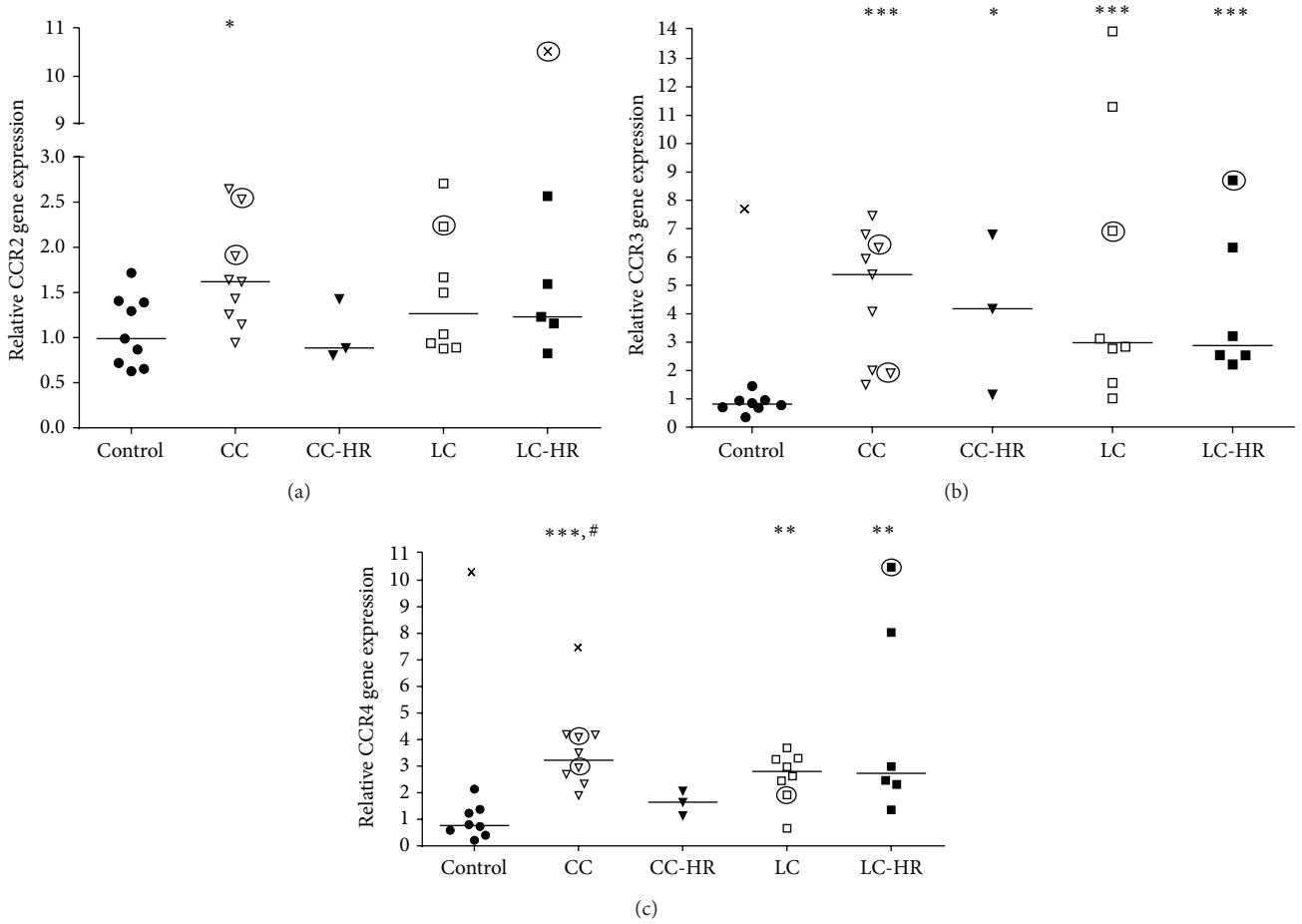

Figure 7: Relative gene expression of the chemokine receptors CCR2, CCR3, and CCR4. Each symbol represents one patient and the medians are depicted as a line. Statistical outliers are marked as crosses (X) and budesonide treated patients are encircled. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P \leq 0.001$ versus controls and $^{\#}P < 0.05$ versus CC-HR.

no cure for MC and the medications only relieve disease symptoms, studies like this one are necessary to increase the understanding of the immunopathogenesis and to find new avenues for treatment. This study also further supports the legitimacy of MC as a "model" to study the role of changes in immune regulation and basic pathophysiology of IBD, where MC patients may reveal important immunoregulatory mechanisms.

5. Conclusion

One of the diagnostic criteria of CC and LC is increased numbers of lymphocytes in both the epithelium and the lamina propria, but infiltration of additional immune cells such as neutrophils, eosinophils, and macrophages is also observed. We found enhanced mRNA and protein expressions of a mixed profile of chemokines and their receptors in CC and LC patients. Interestingly, LC-HR showed similarities with LC patients, whereas CC-HR patients had almost normalized expression patterns. The contrasting expression patterns in CC and LC patients in histopathological remission (CC/LC-HR) further support the hypothesis of CC and LC being different entities. These results contribute to the knowledge of MC immunopathology by suggesting important immunoregulatory roles of chemokines and their receptors involved in recruitment of $CD8^{+}$ T cells, Treg cells, neutrophils, eosinophils, and macrophages. The parameters investigated in this study might be important possible future therapeutic targets to interfere with cell recruitments in MC patients.

Ethical Approval

The study was ethically approved by the Ethical Committee of Örebro-Uppsala County (no. 2008/278). Both patients and controls were informed of the study protocol before colonoscopy and gave their written consent to donate tissue samples for research purposes.

Conflict of Interests

Nils Nyhlin has served as a speaker for MSD. Johan Bohr has served as a speaker for Dr Falk Pharma. Curt Tysk has served as a speaker for Dr Falk Pharma, Tillotts Pharma, Ferring, MSD, and AstraZeneca.

Acknowledgments

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Research Article

Enterococcus faecium NCIMB 10415 Modulates Epithelial Integrity, Heat Shock Protein, and Proinflammatory Cytokine Response in Intestinal Cells

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Probiotics have shown positive effects on gastrointestinal diseases; they have barrier-modulating effects and change the inflammatory response towards pathogens in studies *in vitro*. The aim of this investigation has been to examine the response of intestinal epithelial cells to *Enterococcus faecium* NCIMB 10415 (*E. faecium*), a probiotic positively affecting diarrhea incidence in piglets, and two pathogenic *Escherichia coli* (*E. coli*) strains, with specific focus on the probiotic modulation of the response to the pathogenic challenge. Porcine (IPEC-J2) and human (Caco-2) intestinal cells were incubated without bacteria (control), with *E. faecium*, with enteropathogenic (EPEC) or enterotoxigenic *E. coli* (ETEC) each alone or in combination with *E. faecium*. The ETEC strain decreased transepithelial resistance (TER) and increased IL-8 mRNA and protein expression in both cell lines compared with control cells, an effect that could be prevented by pre- and coincubation with *E. faecium*. Similar effects were observed for the increased expression of heat shock protein 70 in Caco-2 cells. When the cells were challenged by the EPEC strain, no such pattern of changes could be observed. The reduced decrease in TER and the reduction of the proinflammatory and stress response of enterocytes following pathogenic challenge indicate the protective effect of the probiotic.

1. Introduction

Probiotic bacteria have been shown to have positive effects on hosts with intestinal diseases such as *Clostridium difficile*-, Rotavirus-, and *Escherichia coli*-induced diarrhea and also in the prevention of antibiotic-associated diarrhea and nosocomial infections [1–5]. According to the WHO, probiotics are defined as live organisms that, when ingested in sufficient amounts, have a beneficial effect on the overall health of the host [6]. In animal nutrition, probiotics are used as feed additives with positive effects regarding health and growth performance traits [7–9].

The probiotic *Enterococcus faecium* NCIMB 10415 (*E. faecium*) is licensed as a feed additive for sows and piglets and has been demonstrated to reduce diarrhea incidence and severity in weaning piglets [10, 11]. In human medicine, *E. faecium* is used successfully in the treatment of acute diarrheal diseases and in the prevention of antibiotic-associated diarrhea [12, 13]. However, the crosstalk between the probiotic and other microorganisms and between the probiotic

and epithelial and immune cells of the intestinal wall is extremely complex [14, 15], and many of the underlying signaling mechanisms are still only partially understood.

In vivo models of gastrointestinal infections have demonstrated a positive effect of various probiotic feed additives on the functional barrier of the intestine [16–18]. These results are supported by data from *in vitro* cell culture infection models in which probiotic strains prevent or ameliorate damage to epithelial integrity by a pathogenic challenge [19, 20]. Furthermore, the immunological response of the mucosa can be influenced by probiotic strains, which modulate the release of cytokines, amongst other effects [21, 22]. For example, culture supernatant of *Lactobacillus plantarum* 2142 had a suppressive effect on the interleukin-8 (IL-8) and tumor necrosis factor-α (TNF-α) levels induced by oxidative stress in IPEC-J2 cells [23].

The expression of heat shock proteins (HSPs), a set of proteins that are involved in many regulatory pathways and that serve as chaperones for preserving cellular proteins, can be influenced by the intestinal microbiota and by probiotics [24–26]. Some of these HSPs, such as the inducible form of HSP70, are upregulated by several noxious conditions and can therefore be considered as cellular stress indicators [27]. HSPs and cytokines, in turn, are able to regulate barrier properties by influencing tight junction proteins and the structure and function of the cytoskeleton or transport properties of the epithelium [24, 28].

In previous studies, feed supplementation with *E. faecium* has been demonstrated to increase the absorptive and secretory capacity and improve barrier function of the small intestinal epithelium of piglets [29, 30]. Furthermore the proinflammatory cytokine IL-1α, which is expressed in higher amounts in Peyer's patches of *E. faecium*-treated piglets [31], increases chloride secretion in the small intestine of pigs [29].

The hypothesis of the current study is that intestinal epithelial cells play an important role in innate immune responses during enteric infections and that *E. faecium*, in turn, modulates the severity of enteric infections *via* the altered generation of proinflammatory cytokines and HSPs in intestinal cells. Thus, the aim of the present study has been to investigate the influence of the probiotic *E. faecium* and two different pathogenic *E. coli* strains on the HSP and proinflammatory cytokine responses and the epithelial integrity of two intestinal epithelial cell lines. We have further tested whether pre- and coincubation with *E. faecium* change the epithelial cell response to pathogenic *E. coli* strains.

2. Materials and Methods

2.1. Cells and Culture Conditions. The cell cultivation is described in detail in Lodemann et al. [32]. The human epithelial intestinal cell line from colorectal adenocarcinoma, Caco-2 (ATCC Catalog number HTB-37, ATCC, Manassas, USA; passages 37–45), was used as a model for the human small intestine. The porcine intestinal epithelial cell line (IPEC-J2; passages 73–79) was used as a model for the pig small intestine. This cell line was established from the jejunum of a newborn pig [33] and kindly provided by Professor Dr. Anthony Blikslager (North Carolina State University, USA). The cells routinely tested negative for *mycoplasma* contamination.

Cells for the experiments were allowed to differentiate for 14 days (IPEC-J2) or 21 days (Caco-2). On the day prior to experiments, the cells were fed with serum- and antibiotic-free media.

2.2. Bacterial Strains. Three different bacterial strains were used for the experiments: (1) the probiotic strain *Enterococcus faecium* NCIMB 10415 (cultivated from Cylactin, DSM, Heerlen, the Netherlands), (2) the enterotoxigenic *E. coli* IMT4818 (ETEC, isolated from a two-week-old piglet with enteritis, O149:K91:K88 (F4), and found to be positive for the presence of virulence genes est-1a, est-2 (genes coding for heat stable enterotoxins I and II) and elt-1a/b (gene coding for heat labile enterotoxin I) by the polymerase chain reaction (PCR)), and (3) the human enteropathogenic *E. coli* E2348/69 (EPEC, serotype O127:H6, positive for the eae gene coding for the *E. coli* attaching-effacing factor).

The *E. faecium* NCIMB 10415 strain was cultivated in brain-heart infusion (BHI) broth (OXOID GmbH, Wesel, Germany) and the *E. coli* strains in LB medium according to Miller, containing 10 g/L tryptone (OXOID GmbH, Wesel, Germany), 5 g/L yeast extract (OXOID GmbH, Wesel, Germany), and 10 g/L NaCl, at a pH of 7.0.

After overnight incubation of the cells at 37°C, subcultures of bacteria were grown for 3 to 4 h until mid-log phase and then centrifuged. Cell pellets were washed twice in phosphate-buffered saline (PBS, Biochrom, Berlin, Germany). The bacteria were resuspended in antibiotic- and serum-free Caco-2 or IPEC-J2 cell culture medium to reach a concentration of $\approx 10^8$ colony-forming units (CFU)/mL.

The optical density was measured to determine the concentration of bacterial cells. The measurement was confirmed by serial dilution on agar plates. The intestinal cells were infected with 10^6 bacteria per cell culture insert (1.12 cm^2) or per well (1.91 cm^2), corresponding to a multiplicity of infection (MOI) of about 10 bacteria per seeded cell. The bacteria were added to the apical pole of the cells.

2.3. Experimental Setup and Procedure. For each experiment, the cell monolayers for the real-time quantitative PCRs (RT-qPCR), for enzyme-linked immunosorbent assay (ELISA), and for the transepithelial electrical resistance (TER) measurements were incubated for 2 h with the respective bacterial strains (ETEC, EPEC, or *E. faecium*) at a concentration of 10^6 bacteria per cell culture insert or well (see Figure 1). The control cells received the equivalent amount of bacteria-free medium. Two hours after the bacterial incubation, the cells were washed twice with gentamicin-supplemented media to wash out and kill the bacteria, and gentamicin-supplemented media were added. Gentamicin was added to the media at a concentration of 50 μg/mL (Biochrom, Berlin, Germany). The cells were incubated for various periods of time with regard to the different parameters measured, the exact time periods being given in Figure 1 under the specific headings. When the cells were incubated with the probiotic and either

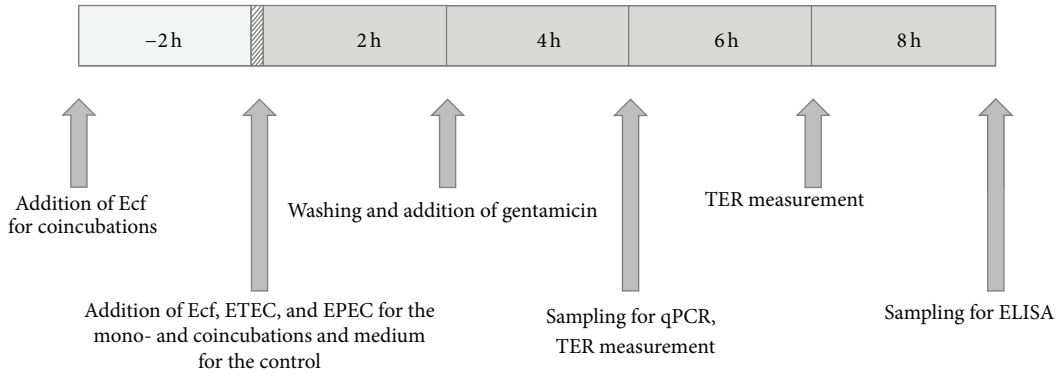

Figure 1: Timeline for the experimental setup.

the ETEC or the EPEC together, the cells were preincubated with the *E. faecium* for 2 h, and then the pathogens were added. The cells were in contact with the pathogens for the same amount of time as in the monoincubation with the ETEC or EPEC. In the following, this experimental setup will be called "coincubation" and the incubation time will be given as the time that the cells were incubated with the pathogens.

A total of six independent experiments were performed for each cell line.

2.4. Transepithelial Electrical Resistance (TER) Measurements. For TER measurements, the cells were seeded at a density of 10^5 cells on cell culture inserts (Transwell, clear polyester membrane, 12 mm diameter, 1.12 cm^2 area, 0.4 μm pore size; Corning B.V., Schiphol-Rijk, the Netherlands). The inserts for IPEC-J2 cells were coated with rat tail collagen type I (Serva Electrophoresis GmbH, Heidelberg, Germany). TER was measured by a Millicell-ERS (Electrical Resistance System; Millipore GmbH, Schwalbach, Germany). TER values were measured every two hours and corrected for the resistance of blank filters and for the membrane area.

2.5. Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR). For determining mRNA expression, the cells were seeded on 24-well cell culture plates (TPP, Biochrom, Berlin, Germany) at a density of 10^5 cells/1.91 cm^2. Confluent cell monolayers were incubated with the bacterial strains as described in the section "Experimental Setup." After 4 h, cells were washed two times with PBS, stored in RNA*later* RNA Stabilization Reagent (Qiagen GmbH, Hilden, Germany), and frozen at −20°C.

The isolation of the total RNA of the harvested cells, the assessment of the RNA quality, and the cDNA synthesis are described in detail in other publications [29, 32]. The samples had to have a RNA integrity number above 7 to be used for RT-qPCR. For the synthesis of cDNA, 100 ng total RNA for the IPEC-J2 cells and 500 ng total RNA for the Caco-2 cells were used. Primer information is given in Table 1. Three reference genes were selected for normalization (ACTB, TBP, and UBP for the Caco-2 cells and GAPDH, TBP, and YWHAZ for the IPEC-J2 cells). The primer information for the reference genes can be found in Lodemann et al. [32].

RT-qPCR was performed and the relative amount of the target genes in the experimental groups was calculated by iQ5 software (Bio-Rad Laboratories GmbH, München, Germany) as described in Klingspor et al. [29].

2.6. Enzyme-Linked Immunosorbent Assay (ELISA). For the ELISA experiments, the cells were seeded on 24-well cell culture plates (TPP, Biochrom, Berlin, Germany) at a density of 10^5 cells/1.91 cm^2, and incubation with bacterial strains was as described above. After 8 h, the supernatants of the cells were harvested, and species-specific IL-8 ELISAs were performed on cell culture supernatants according to the manufacturers' instructions (Invitrogen ELISA Kit, Swine IL-8, Invitrogen Life Technologies GmbH, Darmstadt, Germany, for IPEC-J2 supernatants; Thermo scientific human IL-8 ELISA Kit, Pierce Biotechnology, Rockford, USA, for Caco-2 supernatants). Assays were performed in duplicate.

For the HSP70 ELISA, protein was extracted from the cells. For this purpose, the Complete Lysis-M reagent set (Roche Diagnostics Deutschland GmbH Mannheim, Germany) was used, and the lysis buffer containing a mild detergent in bicine buffer and protease inhibitors was prepared following the manufacturers' instructions. The cells were washed with PBS, and 200 μL lysis buffer was added to each well. After 5 min of incubation at room temperature and gentle shaking, the cell lysate was collected. HSP70 ELISA of the extracted protein was performed according to the manufacturers' instructions (Porcine HSP70 ELISA Kit, Bio-Medical Assay for IPEC-J2 cell extracts and HSP70 ELISA Kit, StressMARQ Biosciences for Caco-2 cell extracts). Assays were performed in duplicate.

2.7. Statistical Analysis. Statistical evaluations were carried out by means of the IBM SPSS-Statistics program for Windows, version 22 (International Business Machines Corp., Armonk, United States of America). Graphs were plotted with SPSS and Microsoft Excel 2010. Results are given as means ± SEM. N refers to the number of experiments.

Statistical significance of differences was assessed by variance analysis. The fixed factor was "treatment of the cells" (incubation with medium (control), *E. faecium*, ETEC, EPEC, ETEC or EPEC in coincubation with *E. faecium*). An overall

Table 1: Oligonucleotide primers and amplicon length of PCR products.

Gene information	Primer sequence	Amplicon length	Accession number	Reference
HSP70 (heat shock protein 70, *Homo sapiens*)	(S) 5′-ACT GCC CTG ATC AAG CGC-3′ (AS) 5′-CGG GTT GGT TGT CGG AGT AG-3′	81 bp	NM_005346	[34]
IL8 (*CXCL8*) (interleukin-8 (chemokine (C-X-C motif) ligand 8), *Homo sapiens*)	(S) 5′-ATG ACT TCC AAG CTG GC-3′ (AS) 5′-ACT TCT CCA CAA CCC T-3′	274 bp	NM_000584.3	[35]
HSP70 (heat shock protein 70, *Sus scrofa)*	(S) 5′-GTG GCT CTA CCC GCA TCC C-3′ (AS) 5′-GCA CAG CAG CAC CAT AGG C-3′	114 bp	M29506	[36]
IL8 (*CXCL8*) (interleukin-8, *Sus scrofa*)	(S) 5′-GGC AGT TTT CCT GCT TTC T-3′ (AS) 5′-CAG TGG GGT CCA CTC TCA AT-3′	154 bp	X61151	[37]

analysis of the data for each cell line and each parameter (TER, mRNA-, and protein expression) was performed. A P value of <0.05 was assumed to indicate statistically significant differences. If a statistical significant difference occurred in the overall analysis each time point (4 h, 6 h, 8 h) was analyzed separately. In the case of a significant difference between groups (treatment of the cells), the Fisher least significant difference post hoc test was performed.

3. Results

3.1. TER Measurements. The TER values of confluent cell monolayers were recorded as a measure of epithelial integrity.

3.1.1. Caco-2. The overall analysis revealed significant differences of TER between incubations with the various bacteria ($P < 0.001$). Monoincubation of the cells with the probiotic *E. faecium* caused an increase of TER compared with the control (at 4 h; Figure 2) indicating a positive effect of *E. faecium* on barrier function. Exposure of the cells to ETEC significantly decreased TER (at 4 h and 6 h), whereas the TER of EPEC-treated cells did not show significant alterations compared with the control. Cell monolayers coincubated with *E. faecium* and ETEC showed a significantly higher TER compared with cells incubated with ETEC alone.

3.1.2. IPEC-J2. In IPEC-J2 cells, significant differences between the various bacterial incubations could also be detected ($P = 0.01$). In this cell line, the TER values were not affected in cells that were incubated with *E. faecium* alone compared with the control (Figure 3). However, the ETEC strain, again, caused a significant decrease in TER (4 h, 6 h), whereas the TER of cells incubated with EPEC did not differ significantly from the control. Pre- and coincubation with *E. faecium* reversed the decreased TER of ETEC-treated IPEC-J2 cell monolayers at 4 h and, as a trend, at 6 h, while TER of EPEC-treated cells was not changed by pre- and coincubation with *E. faecium*.

3.2. HSP Expression. As a stress indicator for the pathogenic challenge, HSP70 expression was tested in both cell lines at the mRNA and protein levels.

3.2.1. Caco-2. In the overall analysis, the differences between the treatment groups were statistically significant for the mRNA expression of HSP70 ($P = 0.001$).

After four hours of incubation, no differences could be observed between control cells and cells incubated with EPEC or *E. faecium* (alone or in combination with ETEC and EPEC) (Figure 4(a)). However, the mRNA expression of HSP70 was significantly higher in the cells that were incubated with the ETEC strain alone compared with all other treatments. This implied that the coincubation of ETEC with *E. faecium* significantly reduced the increase in mRNA expression observed during incubation with ETEC alone.

These results were reflected by the changes in HSP70 protein expression (Figure 4(b)).

3.2.2. IPEC-J2. For IPEC-J2 cells, the mRNA and protein expression of HSP70 showed some numerical, but no statistically significant, differences between the treatment groups (Figure 5).

3.3. IL-8 Expression. The cytokine IL-8 was chosen as a measure of the proinflammatory response of the cells.

3.3.1. Caco-2. The bacterial incubation significantly affected the mRNA expression of IL-8 ($P = 0.008$). The mRNA expression of IL-8 did not differ between the cells incubated with *E. faecium* and the control cells. However, it was significantly increased 4 h after incubation with the ETEC strain compared with the control and the *E. faecium*-treated cells. Coincubation with *E. faecium* significantly reduced this increase (Figure 6(a)). Although a numerical increase was seen in all groups incubated with bacteria, the mRNA expression did not differ significantly between the other treatment groups and the control (Figure 6(a)).

The changes in mRNA expression were mirrored by similar changes in IL-8 protein expression (Figure 6(b)).

3.3.2. IPEC-J2. The results regarding IL-8 expression by the IPEC-J2 cell line were largely comparable with the results obtained for Caco-2 cells. In the overall analysis, significant differences for IL-8 mRNA ($P = 0.001$) and protein expression ($P = 0.001$) were observed, and statistical differences

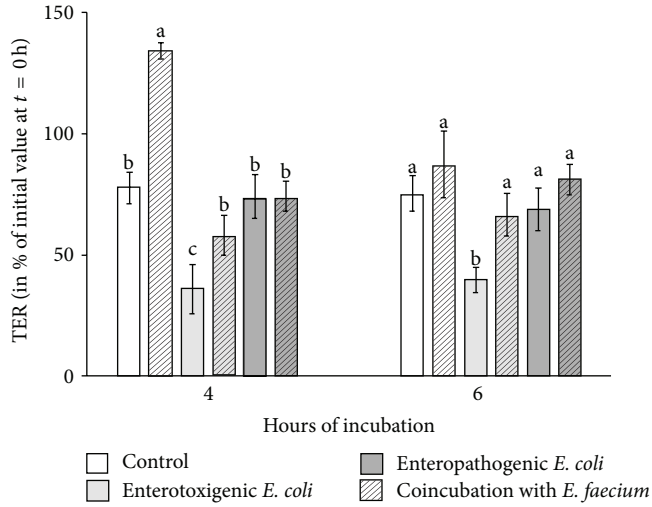

Figure 2: TER values of Caco-2 cells in percent of the initial value before the beginning of the experiment (t = 0 hours) (means ± SEM). Cell monolayers were treated with various bacterial strains for 4 h and 6 h. N = 6 independent experiments; different letters indicate significant differences between the differently treated cells ($P \leq 0.05$).

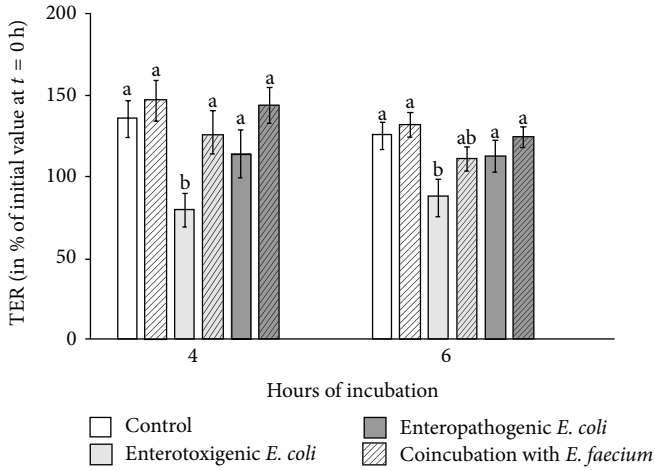

Figure 3: TER values of the IPEC-J2 cells in percent of the initial value before the beginning of the experiment (t = 0 hours) (means ± SEM). Cell monolayers were treated with various bacterial strains for 4 h and 6 h. N = 6 independent experiments; different letters indicate significant differences between the differently treated cells ($P \leq 0.05$).

between individual groups were as described for Caco-2 cells (Figure 7).

4. Discussion

The aim of this study was to elucidate the effects of the probiotic *E. faecium* on barrier function and the inflammatory response of the intestinal epithelium, the latter effect being, in addition to other functions, an important part of the immune system of the gut. To examine whether the probiotic strain could modify the epithelial response to a pathogenic challenge, epithelial cell monolayers were incubated with two selected pathogenic *E. coli* strains. Our hypothesis was that epithelial integrity might be improved, whereas the expression of HSP70 and proinflammatory cytokines might be reduced because of the action of *E. faecium*.

Such "challenge" experiments can be carried out in various ways. One approach is to treat the confluent cell monolayers directly with the pathogenic bacteria for the whole duration of the experiment. The second is to incubate the epithelial cells with supernatants of the bacterial cultures [23, 38]. The advantage of using supernatants is the avoidance of pH decreases in the medium or nutrient competition by the rapidly growing bacteria [39]. However, supernatants or dead bacteria cannot mimic the conditions of living bacteria and exclude potential direct interactions of living bacteria with the epithelial cells. To include such bacteria-epithelial interactions, the experimental design of the present

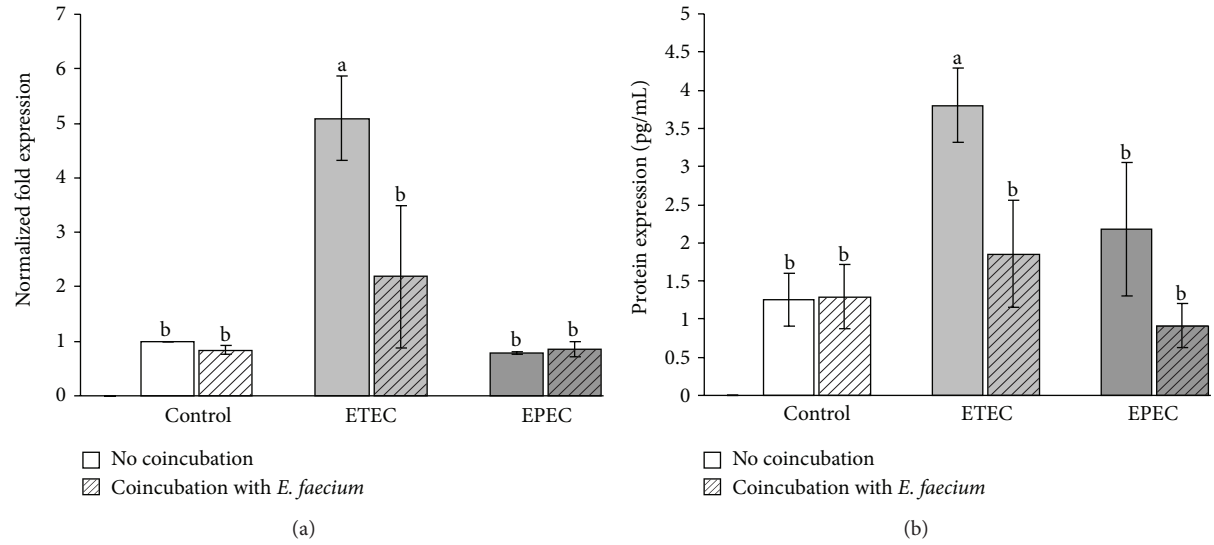

Figure 4: HSP70 expression in Caco-2 cells after treatment with various bacterial strains (means ± SEM). (a) mRNA expression, $N = 5$ independent experiments and (b) protein expression, $N = 4$ independent experiments; different letters indicate significant differences between the differently treated cells ($P \leq 0.05$).

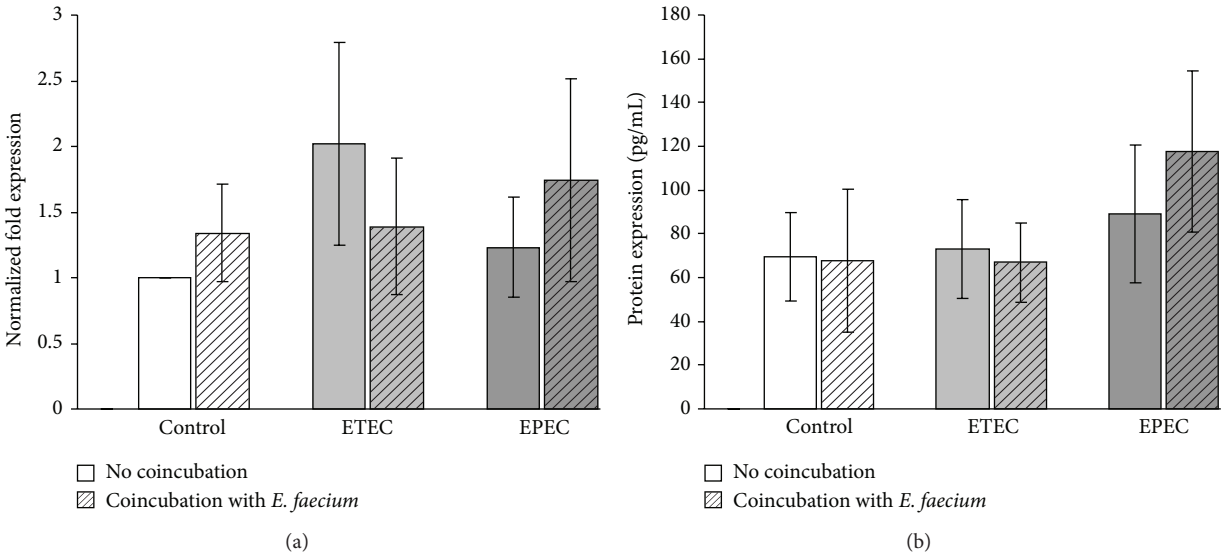

Figure 5: HSP70 expression in IPEC-J2 cells after treatment with various bacterial strains (means ± SEM). (a) mRNA expression, $N = 6$ independent experiments and (b) protein expression, $N = 4$ independent experiments.

study included an initial incubation with living bacteria for 2 h. Thereafter, bacteria were killed by gentamicin, with the intention of diminishing secondary effects of the bacterial incubation. Two intestinal epithelial cell lines were used: porcine IPEC-J2 and human Caco-2 cells.

The Caco-2 cell line is well established and has been used for many years to investigate specific aspects of small intestinal function, including investigations on the effects of probiotic bacteria [40, 41]. Nonetheless, it has limitations because of its cancerogenic origin from the colon. The porcine IPEC-J2 cells seem to be a suitable model to mimic *in vivo* conditions of the small intestine, as they have no tumorous origin and were originally isolated from the small intestine [30, 42]; they have been recently used to study other probiotic microorganisms [23, 39]. The second aim of this study has therefore been to compare Caco-2 and IPEC-J2 cells under the same experimental conditions to provide further evidence for the use of IPEC-J2 cells as a model for small intestinal simulation.

4.1. TER. TER is a compound measure of paracellular and transcellular resistance and has been used to assess epithelial integrity in probiotic studies [43, 44]. In the present study, the TER of both cell lines was slightly increased after 4 h

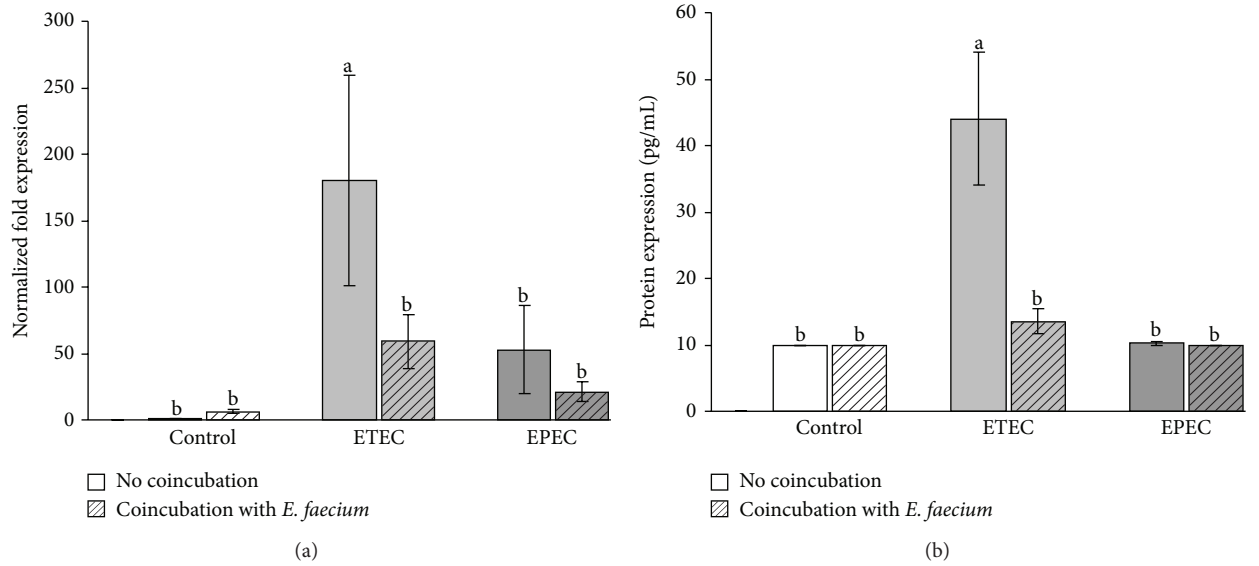

Figure 6: IL-8 expression in Caco-2 cells after treatment with various bacterial strains (means ± SEM). (a) mRNA expression, $N = 5$ independent experiments and (b) protein expression, $N = 5$ independent experiments, different letters indicate significant differences between the differently treated cells ($P \leq 0.05$).

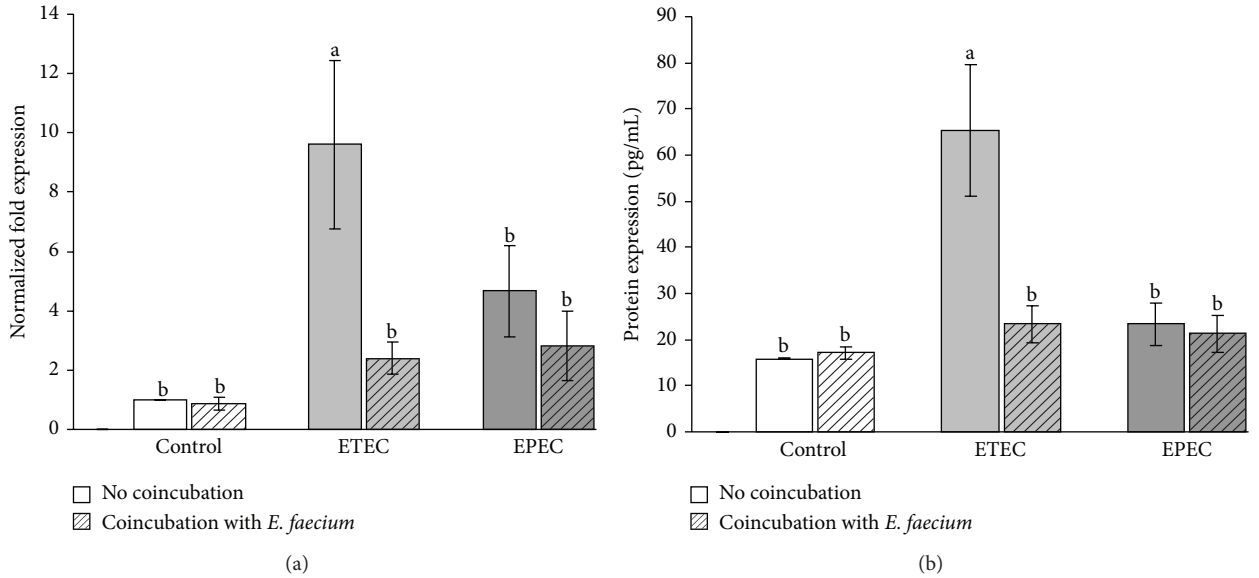

Figure 7: IL-8 expression in IPEC-J2 cells after treatment with various bacterial strains (means ± SEM). (a) mRNA expression, $N = 6$ independent experiments and (b) protein expression, $N = 6$ independent experiments; different letters indicate significant differences between the differently treated cells ($P \leq 0.05$).

of incubation with *E. faecium* alone, but significantly only in Caco-2 cells. These results are in agreement with data of earlier studies showing that various probiotic bacterial strains had either no or an enhancing effect on the TER of intestinal epithelial cells of various origins [45–47]. In porcine IPEC-J2 cells, no enhancing effect has been reported so far [23]. The ETEC strain but not the EPEC strain significantly reduced TER in both cell lines compared with the control; this indicates a change in the epithelial integrity as shown previously by other authors [48, 49]. This decrease was inhibited when cell monolayers were (pre- and) coincubated with the *E. faecium* strain. Similar results have been obtained for other probiotic strains in *in vitro* infection studies with various intestinal cell lines of human origin, such as T84, HT29, or Caco-2 cells [43, 44, 50]. In a porcine cell model (IPEC-1) *Lactobacillus sobrius* DSM 16698 also inhibited the decrease of TER caused by an ETEC strain [51].

In former studies, a probiotic action to prevent the TER decrease induced by ETEC has been correlated with the inhibition of *E. coli* adhesion, which is the first step of

ETEC infection [52]. Another possibility by which *E. faecium* could prevent the decrease of TER after ETEC infection might be related to tight junction (TJ) protein expression and localization or to the cytoskeletal organization [51].

These parameters have not been assessed here but will be the target of further studies.

4.2. HSP70. HSPs protect cells, tissues, and organs against various types of stress factors by reducing or avoiding the stress-induced denaturation of proteins. Gene expression of inducible HSP is triggered by many different stressors and is mainly regulated at the transcriptional level [53–55]. In the gastrointestinal tract, the expression of inducible HSP is markedly influenced by the intestinal microbiota [56, 57].

The inducible form of HSP70, which was assessed in the present study, is often considered to be cytoprotective as its induction by minor stressors can protect the tissue from major challenges [58, 59]. Some probiotic bacteria obviously activate this cytoprotective mechanism as part of their mode of action [23, 60, 61]. *E. faecium* alone, however, did not alter expression of HSP70 in the present study, indicating that different probiotics rely on different mechanisms and pathways to exert their effects on the host [15, 60].

However, in the Caco-2 cells, the expression of HSP70 was significantly increased in the cells incubated with the ETEC. This increase could be prevented by probiotic pre- and coincubation. In the IPEC-J2 cells, a similar tendency could be observed.

Although, as stated above, the upregulation of HSP70 is often interpreted as a positive effect because of its cytoprotective effects, evidence has also been presented suggesting that HSP70 expression is an indicator of pathological stress [27].

In conjunction with the effects on TER and the proinflammatory cytokine response, the increased expression of HSP70 after pathogenic challenge indicates a stress reaction of the intestinal cells. As such, the reduced increase in HSP70 after pre- and coincubation with *E. faecium* in the present study suggests that cells are less damaged by ETEC.

4.3. IL-8. The proinflammatory cytokine response in the gastrointestinal tract is influenced by the gut microbiota. Among others, the cytokine expression of epithelial cells can be modulated by probiotic strains [21, 40, 62]. Altered cytokine release, in turn, can regulate the structure and function of TJ and cytoskeleton [63, 64] and the transport properties of epithelial cells [65, 66].

In the present study, IL-8 has been chosen as a representative cytokine of the epithelial proinflammatory response. IL-8 belongs to the proinflammatory "chemokine" family and is reported to induce neutrophil and T-lymphocyte chemotaxis, neutrophil activation, and the enhanced expression of neutrophil adhesion molecules [67, 68]. One of the responses of intestinal epithelial cells as part of the innate immune system to various inflammatory stimuli is the production of IL-8 [69].

In the present study, IL-8 expression was considerably increased when the cells were incubated with ETEC; this increase was abrogated by concomitant incubation with *E. faecium*. Similar reductions of proinflammatory responses to a pathogen by probiotic strains have been observed in other *in vivo* and *in vitro* models [23, 41, 51, 70]. In some cases, this was accompanied by the maintenance of epithelial barrier function [70]. In porcine intestinal epithelial cells, various *lactobacilli* and bacilli strains counteract the increase in IL-8 and other proinflammatory cytokines elicited by stimulation with ETEC, *S. typhimurium*, oxidative stress, or lipopolysaccharide. In some cases, this is associated with the protection of the epithelial barrier [23, 39, 71].

Although IL-8 secretion is part of the innate immune response aimed at the elimination of pathogens, the persistent production of IL-8 accompanied by the constant infiltration of neutrophils leads to massive epithelial cell damage [72], which is one cause of diarrhea. Several studies have demonstrated that epithelial damage can be prevented by interventions that suppress the IL-8 levels in IBD [73, 74]. This could also be an approach for reducing epithelial damage and diarrhea in acute infections such as ETEC and, here, we consider it to be one of the positive effects of healthy microbiota [61]. Taking this into account, the effects of *E. faecium* observed in the present study indicate a protective effect of this probiotic in acute intestinal inflammation induced by ETEC.

The exact mechanisms by which *E. faecium* exerts its influence on cytokine secretion have to be further investigated.

5. Conclusion

Preincubation with the probiotic *E. faecium* abrogates or reduces all examined effects induced by the ETEC such as the HSP70 stress response, the elevated expression of the proinflammatory cytokine IL-8, and the decrease in TER as a measure of epithelial integrity.

A key feature of the intestinal immune system is its ability to protect against pathogens while avoiding a destructive inflammatory response. An exaggerated proinflammatory cytokine secretion such as IL-8 leads to disease states and, in this case, a reduction of proinflammatory cytokines together with the reduction of HSP70 expression and the prevention of potential epithelial damage might alleviate symptoms, indicating a positive effect by the probiotic. The underlying mechanisms will be the subject of further studies.

Both cell lines react in a similar manner to incubation with pathogens and the probiotic. Therefore, the IPEC-J2 cell line can be considered as a reliable model for studying the effects of probiotics on the protection of the intestinal epithelium from stressful conditions and inflammation. Furthermore, the parallel response in the two cell lines underlines the general transferability of the effects of *E. faecium* seen in the present study.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Review Article

Matrix Metalloproteinases in Inflammatory Bowel Disease: An Update

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Matrix metalloproteinases (MMPs) are known to be upregulated in inflammatory bowel disease (IBD) and other inflammatory conditions, but while their involvement is clear, their role in many settings has yet to be determined. Studies of the involvement of MMPs in IBD since 2006 have revealed an array of immune and stromal cells which release the proteases in response to inflammatory cytokines and growth factors. Through digestion of the extracellular matrix and cleavage of bioactive proteins, a huge diversity of roles have been revealed for the MMPs in IBD, where they have been shown to regulate epithelial barrier function, immune response, angiogenesis, fibrosis, and wound healing. For this reason, MMPs have been recognised as potential biomarkers for disease activity in IBD and inhibition remains a huge area of interest. This review describes new roles of MMPs in the pathophysiology of IBD and suggests future directions for the development of treatment strategies in this condition.

1. Introduction

Inflammatory bowel disease (IBD) which includes both ulcerative colitis (UC) and Crohn's disease (CD) is a chronic and relapsing autoimmune disease characterised by inflammation of the gastrointestinal tract. The estimated mean prevalence of IBD in western countries is 1 in 1,000 [1, 2] and although data are less available for the developing world, incidence of the disease is rising globally [3, 4]. Both idiopathic forms of the disease share common symptoms of abdominal pain, diarrhoea, rectal bleeding, and fever. Ulcerative colitis is characterised by continuous inflammation involving the rectum and colon which extends proximally. Crypt abscesses from infiltration of neutrophils and ulceration of the mucosa is observed. Crohn's disease may affect any region of the gastrointestinal tract intermittently with the terminal ileum being the most common. The inflammatory process may extend through the intestinal wall narrowing the intestinal lumen and is histologically characterized by the formation of granulomas, fibrosis, and fistulae [5, 6].

The human matrix metalloproteinases (MMPs) are a family of 24 zinc dependent endopeptidases. They are grouped by domain structure and substrate preference into collagenases, gelatinases, stromelysins, and membrane type MMPs (MT-MMPs) [7]. The subgroups of MMPs have distinct structural domains but all possess a conserved catalytic domain with a Zn^{2+} at the active site and a prodomain which confers latency. The family of proteases were first studied for their ability to degrade the extracellular matrix and basement membrane to facilitate cell migration, infiltration, and tissue remodelling. As our understanding of MMPs has grown, they have been recognised as key regulators of cell function through their ability to cleave a vast range of cytokines, chemokines, receptors, proteases, and adhesion molecules to alter their function [8, 9]. MMPs are regulated at several levels from transcription, translation, secretion, and activation. There is also a large list of physiological inhibitors of MMPs which serve to regulate MMP activity and proteolysis. The four tissue inhibitors of MMPs (TIMPs) are specific inhibitors of MMPs that reversibly inhibit the MMPs in a 1 : 1 stoichiometric fashion.

These enzymes have long been linked with IBD and their role in intestinal inflammation was reviewed by Medina and

Radomski in 2006 [5]. Our current understanding of the aetiology of IBD is that genetic susceptibilities in gut barrier integrity and innate and adaptive immune response can lead to an inappropriate inflammatory reaction in response to bacteria in the gut or other environmental factors [10, 11]. It is in this context that we review the recent evidence for the role of MMPs in the disease.

2. Association between MMPs and IBD: Enzymes Involved and Cellular Source

Most MMPs are transcriptionally upregulated in response to proinflammatory cytokines, cell-cell, or cell-ECM interactions [12]. The collagenases (MMP-1, -8, and -13), gelatinases (MMP-2 and -9), stromelysins (MMP-3 and -10), matrilysin (MMP-7), and macrophage elastase (MMP-12) are the most studied in the context of IBD. The wide range of cellular sources, which has been known to include epithelial cells, mesenchymal cells, and leukocytes, has been reinforced in recent studies. Myofibroblasts are now recognised as playing an active role in intestinal inflammation and the pathogenesis of IBD. These stromal cells have been shown to secrete MMP-2 and, upon stimulation, MMP-1, -3 and -9 [13–15]. Human colonic epithelium was shown to produce increased amounts of MMP-1, -3, -7, -9, -10, and -12 in IBD patients [16] and mucosal biopsies from UC patients identified vascular endothelial cells and infiltrating leukocytes as the major sources of MMP-7 and -13 [17]. Infiltrating macrophages were seen to be a major source of MMP-8, -9, and -10 in human IBD and a mouse model of colitis [18, 19] and isolated IgG plasma cells from IBD patients were shown to produce high and sustained amounts of MMP-3 [20]. Neutrophils are also major contributors of MMP-9 in intestinal inflammation where it is stored in granules and can be released upon stimulation [18, 21].

The range of cell types that secrete MMPs during intestinal inflammation reflects their integral involvement in the pathogenesis of IBD. Several studies from the previous decade have suggested a role for MMPs in IBD by showing their transcriptional upregulation and increased activity during active inflammation in the gut. The evidence for the involvement of MMPs in human IBD is unequivocal and recent reports further describe instances, pattern of expression, and cellular sources of the MMPs.

Transcripts or protein levels of MMP-1, -2, -3, -7, -9, -10, -12, and -13 are demonstrated to be upregulated in inflamed IBD mucosa or serum of IBD patients and MMP proteolytic activity was increased in cells from inflamed IBD epithelium [16, 22–25]. Gene expression profiling showed that MMP-1 was upregulated in UC and CD and linked to HIF-1 mediated inflammation. MMP-3 and -7 were upregulated in UC and MMP-7 was associated with genes known to regulate angiogenesis [26, 27]. Other studies have focused on the involvement of groups or individual MMPs in IBD or models of colitis.

2.1. Gelatinases (MMP-2, -9). MMP-9 mucosal expression and protein levels, as well as serum antigen levels were significantly higher in UC patients compared to controls and these levels corresponded to the severity of the disease. Interestingly, these trends were not replicated in lymphocytic colitis or collagenous colitis where MMP-9 does not seem to contribute to the severity of the disease [28]. Gene expression profiling combined with qPCR has shown the upregulation of MMP-2 in paediatric CD [29]. This enzyme was also shown to be upregulated in a rat TNBS-induced colitis model and corresponded with the severity of the disease [30] which is in agreement with earlier studies [31, 32]. A murine DSS induced colitis model showed increased gelatinase mRNA levels in the colon [33]. Furthermore, patients with ischaemic colitis show increased gelatinase expression in inflamed areas compared with noninflamed areas or control patients implicating them in inflammation [34] and gelatinase-double knockout mice were protected from DSS, TNBS, or *Salmonella typhimurium* induced colitis [35].

2.2. Stromelysins (MMP-3, -10). The expression of MMP-3 has been shown to be significantly upregulated in inflamed areas of colons of IBD patients compared to uninflamed areas implicating its involvement in the inflammatory process [36]. In addition, increased expression of epithelial MMP-10 and stromal TIMP-3 has been found in both UC and CD paediatric patients compared to non-IBD patients [37]. The stromelysins were deemed to be the greatest contributors to DSS induced colitis in one study and their inhibition with siRNA or blocking of the signalling pathways leading to their upregulation resulted in an amelioration of colitis [38].

2.3. Collagenases (MMP-1, -8, -13). MMP-1 has been shown to be upregulated in ulcerated and inflamed areas of colon mucosa of UC patients and its expression correlates with severity of inflammation [39]. MMP-1 and TIMP-1 plasma and colonic mRNA levels are increased in UC correlate with disease severity [40]. Expression is also greater in the inflamed areas of colons in UC patients and MMP-1/TIMP-1 ratio is a measure of inflammation [41]. A group studying the Na^+/H^+ exchanger (NHE3) discovered that NHE3$^{-/-}$ mice developed spontaneous colitis restricted to the mucosa of the distal colon with a concomitant 15-fold increase in MMP-8 expression [42]. MMP-13 has also shown to be present in the inflamed areas of colon in IBD patients but absent in noninflamed colons or in acute diverticulitis and MMP-13 expression correlated with histological measures of disease [43].

2.4. Macrophage Elastase (MMP-12). MMP-12 was also shown to be upregulated in IBD patients as well as T-cell mediated model of colitis and contribute to epithelial degradation and MMP-12$^{-/-}$ mice were protected against TNBS induced colitis [44]. Epithelial and stromal MMP-12 along with MMP-3 and -7 have been also upregulated in pouch mucosa of paediatric onset UC, suggesting that the expression of MMPs paediatric UC pouch in the long-term shares characteristics with IBD [45].

3. Genetic Basis of IBD: MMP Polymorphisms

Susceptibility to the development of IBD is associated with polymorphisms in genes coding for elements of the immune system or epithelial barrier. First degree relatives of IBD patients have an increased relative risk of up to tenfold compared with background population [46–48]. NOD2 (CARD15/IBD1) has been identified as a susceptibility gene for CD which can affect host interaction with LPS and trigger NF-κB signalling [49–51]. Several large genome wide studies have identified loci that are linked to IBD; 16q12 (IBD1), 12q13 (IBD2), 6p21 (IBD3), 14q11 (IBD4), 19p13 (IBD5), 5q31-q33 (IBD6), and Xq21.3 [52–57] and polymorphisms of genes other than NOD2 have been identified as conferring susceptibility including IL23R and ATG16L1 [58–60]. Given the recognised involvement of MMPs in IBD and the strong genetic component of the disease, several groups have investigated the associations between known MMP polymorphisms and IBD phenotypes.

An extensive study of MMP-1, -2, -3, -7, -8, -9, -10, -12, -13, and -14 and TIMP-1, -3, and -4 single nucleotide polymorphisms (SNPs) in UC, carried out in a New Zealand cohort, found that SNPs in MMP-3, MMP-8, MMP-10, and MMP-14 were associated with the disease [61]. The study was able to make associations with some of the SNPs and disease phenotype but the associations made with UC were not replicated in a Dutch cohort. Primary sclerosing cholangitis (PSC) is a cholestatic liver disease characterised by chronic inflammation and fibrosis. It is believed to share pathologies with IBD and 50–80% of PSC patients also suffer from IBD [62]. Polymorphisms of the MMP-3 gene have been associated with PSC and with UC where the mechanisms are likely to be the same but have yet to be determined [63–65]. An MMP-3 SNP has also been associated with increased risk of stenosing behaviour in CD [66]. Preliminary studies have shown associations between collagenous colitis and an MMP-9 SNP but not MMP-1 or MMP-7 SNPs [67]. Two different TIMP-1 SNPs were associated with increased susceptibility to CD [66].

4. Role of Bacteria in IBD: Interactions with MMPs

There is a body of evidence to suggest that gut microbes are the key to the initiation and development of IBD. It is likely that the disease is triggered by interaction of the gut microflora with host defences following impaired barrier function. Antibiotics or some probiotics have shown to be of benefit in treating IBD [68, 69]. Further evidence for the role of bacteria in triggering the disease is that gnotobiotic mice do not develop colitis but it rapidly emerges when normal luminal flora are reintroduced [70, 71] and that experimental colitis can be induced in mice in response to adherent-invasive *E. coli*, strains of *Salmonella* [72] or *Helicobacter* [73, 74].

Much interest has been generated over the years in specific species of bacteria which may be causative agents for IBD. Mycobacterium avium paratuberculosis (MAP) has sparked much recent debate regarding its involvement in Crohn's disease and is the subject of numerous reviews and meta-analyses [75–84]. The subspecies is the causative agent of Johne's disease, an inflammatory disease mainly in ruminants with similarities to CD such as diarrhoea, leukocyte infiltration to the intestinal wall, and intestinal lesions. Indeed, MAP has been shown to be present in a higher percentage of IBD patients than healthy controls; however, the associations have not proven to be conclusive [77, 80]. MAP infection is associated with an upregulation of MMPs in cattle and it upregulates MMPs in cultured murine macrophages [85–87]. A recent study examined the expression of MMPs in UC patients who tested positive for MAP DNA but found it no different to patients without MAP DNA [88]. In contrast to this, another group showed that mice given oral MAP had increased colonic expression of MMP-2, -9, -13, and -14 as well as TIMP-1 in response to the bacteria [89].

A combination of the antibiotic minocycline and the probiotic *E. coli Nissle 1917* was shown to improve recovery form DSS induced colitis in mice including improved ratio of beneficial/harmful bacteria and reduced MMP-9 expression [90]. However, no experiments were carried out to discern the antibiotic effects of minocycline from its MMP inhibitory and immunomodulatory effects in reference to the protective effect observed. A group studying *Citrobacter rodentium*-induced colitis in mice found that MMP-9 was upregulated in the model. While epithelial barrier integrity and histopathological observations were unchanged between MMP-$9^{-/-}$ and wild type mice, increased IL-17 expression was observed in the MMP-$9^{-/-}$ mice. Interestingly, the gut microbiome was altered in wild type mice following infection but not in MMP-$9^{-/-}$ mice, implicating a role for MMP-9 in the depletion of microbial diversity in the gut, after infection [91]. MMP-7 can also modulate the gut microbiome where it has been shown to cleave the inactive alpha-defensin, procryptdins, to their active form [92]. Cryptdin-4 is mostly active against noncommensal bacteria; however, its reduced form, which is inactivated by MMP-7, demonstrates greater bacteriocidal activity against commensal gut bacteria [93].

Interestingly, direct antibacterial effects for MMP-12 have been demonstrated as it can disrupt bacterial cell membranes in the macrophage phagosome [94].

5. Recent Pathways Regulating MMPs in IBD

MMPs are regulated at several levels from transcription to enzyme activation. The interconnectedness of inflammatory networks including activation of signal transduction pathways, where release of a cytokine can trigger an inflammatory cascade, or indeed the protease web where activation of a proenzyme can in turn lead to the activation of a host of other enzymes and their targets, makes delineation difficult. Despite decades of study, the place and role of MMPs in this network is still under investigation and here we summarize the recent studies of the place of MMPs in this network in IBD (Figure 1).

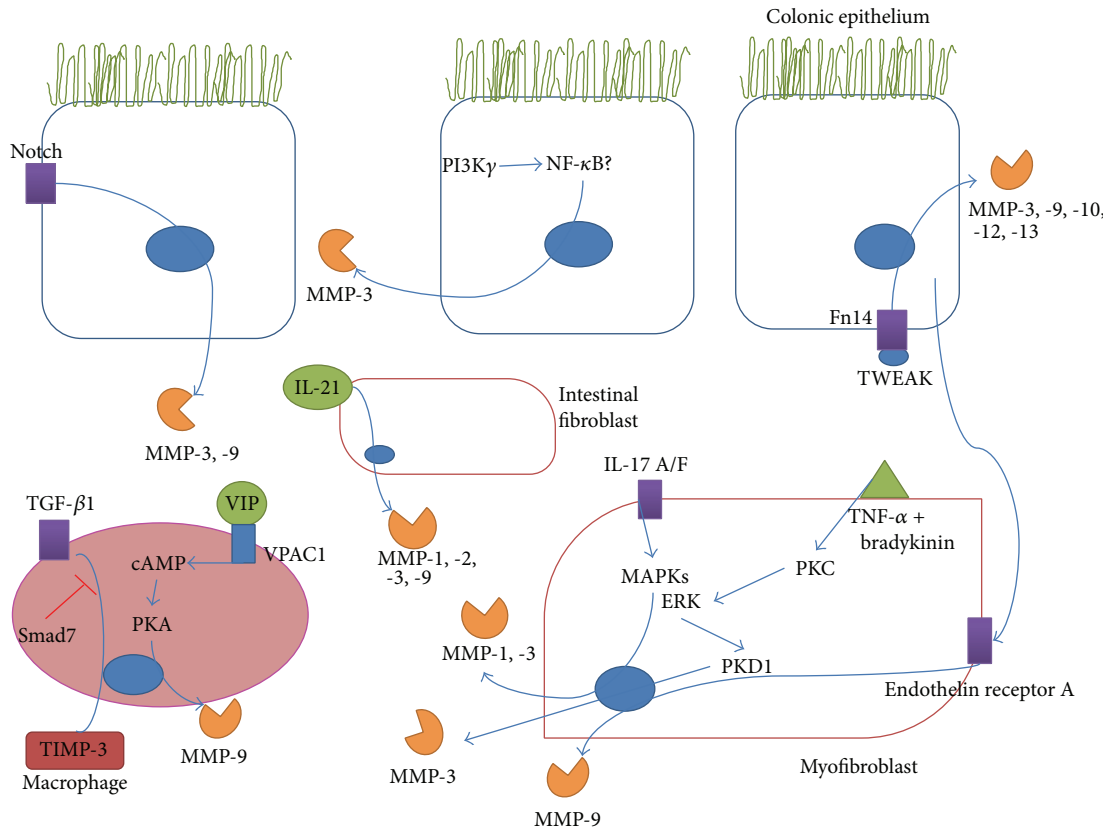

Figure 1: Recently described signalling pathways in the gut leading to the upregulation of MMPs in IBD or models of colitis. The various mediators whose interaction with receptors on colonic epithelial cells, intestinal fibroblasts, myofibroblasts, and macrophages can trigger signal transduction pathways leading to increased expression of MMPs or TIMPs are shown. PI3Kγ (phosphatidylinositol-3 kinase γ), NF-κB (nuclear factor κB), TWEAK (TNF-related weak inducer of apoptosis), TGF-β (tissue growth factor β), cAMP (cyclic adenosine monophosphate), PKA (protein kinase A), VIP (vasoactive intestinal peptide), VPAC1 (vasoactive intestinal peptide receptor 1), MAPKs (mitogen activated protein kinases), ERK (extracellular signal-regulated kinase), PKC (protein kinase C), PKD (protein kinase D), and IL (interleukin).

Some of these studies have shown new inducers of MMPs in IBD. IL-17A and IL-17F can increase secretion of MMP-1 and -3 in subepithelial myofibroblasts and also enhance the actions of IL-1β and TNF-α on these MMPs in a MAPK mediated manner [95]. IL-21 was also shown to play a part in the upregulation of MMP-1, -2, -3, and -9 in intestinal fibroblasts without an increase in TIMPs [96]. TNF-like weak inducer of apoptosis (TWEAK) is a member of the TNF-family of cytokines that signals through its receptor, fibroblast growth factor-inducible molecule 14 (Fn14). Inhibition of the TWEAK pathway resulted in reduced severity of TNBS induced colitis in mice resulting in reduced expression of MMP-3, -9, -10, -12, and -13 along with other inflammatory mediators [97].

Other recent studies have characterised various new signalling pathways involved in the transcriptional upregulation of MMPs in intestinal inflammation. For example, inhibition of Notch signalling reduces MMP-3 and -9 expression in DSS induced colitis [98]. A recent study showed that inhibition of PI3Kγ had anti-inflammatory effects in TNBS induced colitis which resulted in increased Treg response and decreased NF-κB mediated expression of MMP-9 and other inflammatory mediators [99]. VPAC1, a receptor for vasoactive intestinal peptide (VIP), enhances DSS induced colitis through activation of PKA and increased MMP-9 expression, among other mediators [100]. Colonic myofibroblasts were shown to produce MMP-3 in response to bradykinin and TNF-α through a pathway that involved activation of PKC and ERK, establishing a critical role for the downstream PKD1 [15]. A study investigating the crosstalk between subepithelial myofibroblasts and colonic epithelial cells found that the myofibroblasts produced MMP-9 in response to the cytokines TNF-α, IL-1β, and TGF-β but was inhibited by IFN-γ. Interestingly, incubation of the myofibroblasts with media containing the releasate of cytokine stimulated epithelial cells resulted in an upregulation of MMP-9 which was mediated by endothelin receptor A signalling [14]. TGF-β1 was shown to protect against TNBS induced colitis in mice through upregulation of TIMP-3 in lamina propria mononuclear cells. Knock-down of Smad7, the TGF-β1 receptor antagonist, resulted in increased TIMP-3 expression [101]. Chymase has been shown to be a relevant activator of pro-MMP-9 in DSS induced colitis [102]. In a study on the effects of mast cell tryptase in IBD, MMP-3, -9, and -13 were downregulated in DSS treated mast cell protease (MCP) $6/7^{-/-}$ mice compared to wild type DSS treated showing a regulation of these MMPs

Table 1: Recently described roles for MMPs in IBD.

MMP	Role in IBD	Reference
MMP-1	Prevention of fibrosis	[145]
MMP-2	Generation of antiangiogenic factors, maintenance of epithelial barrier function, and prevention of fibrosis	[119, 135, 145]
MMP-3	Generation of endostatin	[118]
MMP-7	α-defensin activation, chemokine expression, wound healing, and generation of endostatin	[105, 106, 118]
MMP-8	Neutrophil infiltration	[18]
MMP-9	Chemokine expression, neutrophil infiltration, generation of anti-angiogenic factors, VEGF-A processing, decreased goblet cell differentiation, and prevention of fibrosis	[18, 107, 118, 119, 125, 134, 145]
MMP-10	Wound healing,	[19]
MMP-13	Activation of TNF-α and generation of endostatin	[111, 118]
MMP-20	Generation of endostatin	[118]

by the proteases [103]. All these studies contribute to our understanding of the signalling pathways involved in MMP regulation in IBD.

6. New Evidence for Functions of MMPs in IBD

As stated previously, the view of MMPs as simple ECM proteases is vastly oversimplified. MMPs can activate or inhibit a wide range of cytokines, chemokines, receptors, adhesion molecules and signalling molecules in order to regulate local inflammation in the gut and new roles are being discovered continually (Figure 2). Here we review the recent literature concerning MMP action in intestinal inflammation beyond ECM degradation. A summary is provided in Table 1.

6.1. MMPs in the Immune Response. The α-defensins, which modulate IL-1β, are cleaved and activated by MMP-7 [104]. MMP-$7^{-/-}$ mice were more susceptible to DSS induced colitis. MMP-7 is postulated to reduce IL-1β release through activation of α-defensins [105]. Another study highlights the dual effect of the protease in colitis where it mediates both tissue injury and also healing in DSS induced colitis. MMP-$7^{-/-}$ mice had a lower mortality rate and the increased inflammation in the wild type animals was ascribed to increased neutrophil migration through increased expression of the chemokines KC and MIP-2 [106]. Transgenic mice overexpressing epithelial MMP-9 developed worse DSS and ST induced colitis which correlated with an increased expression of KC [107]. An interesting recent study described how MMP-$10^{-/-}$ mice developed a more severe colitis in response to DSS and that MMP-10 derived from macrophages was required for gut healing. Although the mechanisms of this protection were not described, depleted macrophage numbers in the MMP-$10^{-/-}$ mice may have prevented colonic healing [19]. Proline-glycine-proline (PGP) is a product of collagen breakdown by propyl endopeptidase (PE) and MMPs and is known to be a chemoattractant for neutrophils [108]. It was recently demonstrated that this is a novel mechanism for MMP induced neutrophil infiltration in IBD where MMP-8, -9, and PE were upregulated in the inflamed intestines of IBD patients and in mice with DSS induced colitis. Generation of these enzymes resulted in PGP and increased infiltration of neutrophils. PGP neutralisation resulted in decreased neutrophil infiltration and lessened the severity of the colitis [18].

TNF-α is a proinflammatory cytokine whose levels are increased in the blood, colonic mucosa, and stools of IBD patients. It contributes to the pathogenesis of the disease by increasing inflammation through MAPK and NF-κB activation, increasing cell proliferation and altering epithelial barrier permeability [109]. Anti-TNF therapy has been a major breakthrough in recent years for the treatment of moderate to severe CD and UC refractory to traditional therapies. Upon synthesis, homotrimeric TNF-α migrates to the cell membrane where it is cleaved into the soluble and biologically active form. Until recently, TACE/ADAM17 was believed to be the only relevant in vivo activator of TNF-α [110]; however, MMP-13 has now been demonstrated to perform the same function. Vandenbroucke et al. discovered that the observed effects of MMP-13 on epithelial integrity in DSS induced colitis, such as mucus depletion, intestinal inflammation, and loss of tight junction function were mediated through activation of TNF-α [111]. The significance of this discovery in development of a therapy will remain to be seen.

6.2. MMPs in Angiogenesis. Angiogenesis is now believed to play a major role in the process of chronic inflammation and has been suggested to contribute to the pathology of IBD. Early cytokine, chemokine, and growth factor release facilitate the process which promotes increased leucocyte infiltration. The understanding of angiogenesis in IBD was summarised previously [112]; here we limit ourselves to the more recent studies where MMPs are implicated.

Endothelial cell-produced MMP-1, -3, and -9 are upregulated in human IBD and experimental colitis. These enzymes are potentially involved in different aspects of angiogenesis. MMPs could have dual roles in angiogenesis acting as proangiogenic mediators during tissue remodelling and then as antiangiogenic mediators through generation of angiostatin preventing vessel maturation. At a simplified level, MMPs

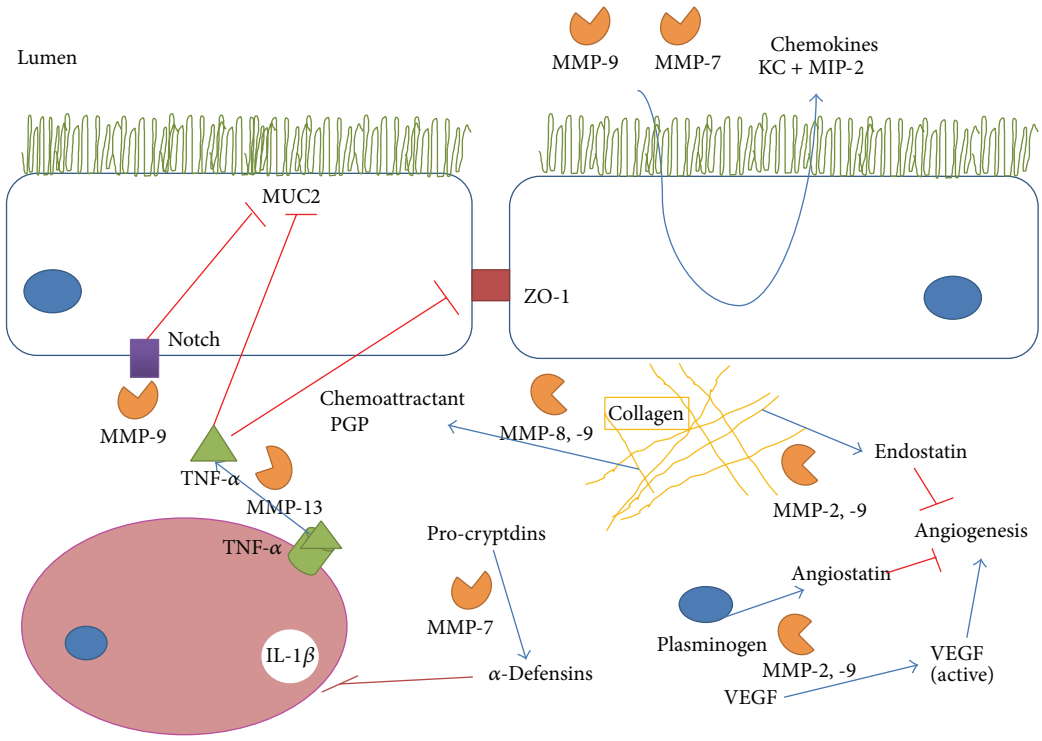

Figure 2: Recently described functions for MMPs in IBD. A summary of some of the roles of MMPs in IBD is shown including regulation of epithelial barrier through MUC2 expression and activation of immune response through cleavage of procryptdins, chemokines, and cytokines. MMPs also have a role to play in angiogenesis through allowing migration of endothelial cells and alteration of VEGF but also release of endostatin and angiostatin. MUC2 (mucin 2), KC (CXCL1), MIP-2 (macrophage inflammatory protein 2), ZO-1 (zona occludens protein 1), VEGF (vascular endothelial growth factor), and PGP (proline-glycine-proline).

facilitate angiogenesis through remodelling of the ECM, permitting incorporation of migrating endothelial cells which then form new vessels [113]. While this may be accurate, regulation of angiogenic factors in the gut by MMPs is also likely to contribute. It is known that MMPs can release bound forms of VEGF-A from the ECM, cell membrane, heparin affinity regulatory peptide (HARP), and connective tissue growth factor (CTGF), although the functional relevance of this has been assessed in cancer models and not in IBD [8]. MMP-1 and -3 can cleave heparin-sulfate proteoglycan in endothelial cells to release basic fibroblast growth factor (bFGF) [114] and transforming growth factor-β (TGF-β) can also be released and activated by MMPs [115–117]. Further evidence for the roles of MMPs in angiogenesis is reviewed by Rodríguez et al. [8].

Cleavage of collagen XVIII and plasminogen by several MMPs can generate endostatin and angiostatin, respectively, which are both antiangiogenic factors [118]. These antiangiogenic factor levels were upregulated in a rat model of UC. Administration of mesalamine was able to restore the proangiogenic balance by inhibiting gelatinases and thus generation of these fragments [119].

Others argue that increasing VEGF can lead to excessive and pathological angiogenesis in IBD [120–122] and that angiogenesis blockade may even be viable as a therapeutic strategy for reducing disease severity in IBD [123]. In this context, the increasing levels of MMP-9 lead to an increased endostatin concentration and treatment with endostatin in MMP-9$^{-/-}$ mice can reduce the severity of colitis [124] indicating a protective role for the enzyme. The ability of MMP-9 to release bound VEGF-A and alter its angiogenic outcome [125, 126] was not assessed in the context of IBD in the studies described; however, serum MMP-9 levels were found to correlate with serum VEGF in CD but not UC patients [127]. The studies of the regulation of angiogenesis by MMPs in IBD are limited in scope but provide evidence that many of the discoveries made in cancer and other diseases would also hold true for IBD. The net contribution of the MMPs will depend on the microenvironment and thus the generation of pro- or antiangiogenic factors.

6.3. MMPs and Epithelial Barrier Function. Epithelial barrier integrity is essential in maintaining intestinal homeostasis. Infiltration of luminal contents into the lamina propria triggers a local inflammatory response leading to release of proinflammatory mediators, release of MMPs, and further epithelial degradation and inflammation. Indeed, leaking of bacteria or alarmins into the bloodstream can trigger a systemic response, sepsis, or multiorgan failure [128]. MMPs have been implicated in modulation of the epithelial barrier elsewhere in the body [129–131] and owing to its importance in intestinal inflammation, also in the gut, as discussed below.

When investigating the effect of MMP-9 on the colonic epithelial barrier in a model of colitis, it was found that

MMP-9$^{-/-}$ mice had increased goblet cell numbers and increased MUC2 expression. Overexpression of MMP-9 resulted in a decrease in goblet cell differentiation [107] and thus decreased MUC2 expression. This reduction of MUC2 expression reduces the protective mucin barrier and was shown to affect the adherence of *Salmonella typhimurium* [132]. The tight junction protein claudin-1, which has been previously implicated in colitis associated cancer (CAC) [133], was shown to be involved in epithelial homeostasis. Upregulation of claudin-1 following DSS induced colitis results in an upregulation of MMP-9 which triggered Notch signalling resulting in decreased MUC2 expression through decreased goblet cell differentiation [134]. It was also shown that the gelatinases play opposing roles in intestinal inflammation where MMP-9 can potentiate colitis but MMP-2 participates in maintaining epithelial barrier function to prevent the initiation of colitis [135]. MMP-13 indirectly regulates epithelial barrier function through activation of TNF-α. Activation of the cytokine increases intestinal epithelial permeability by mediating the endocytosis of the tight junction protein ZO-1 and reducing MUC2 expression, effects that are absent in MMP-13$^{-/-}$ mice [111].

In a DSS induced model of chronic colitis described previously, wild type animals recovered more quickly and completely from the colitis than MMP-7$^{-/-}$ animals which may be due to decreased neutrophil infiltration to the mucosa [106]. MMP-7 was shown to be hugely upregulated in an intestinal epithelial wound healing model and resulted in faster resolution of the wound. Under inflammatory conditions, simulated by addition of TNF-α and IL-1β, the expression levels were increased further which delayed wound healing [136]. This observation is likely applicable to many of the MMPs, where physiological roles contribute to the pathology of IBD under inflammatory conditions. Another study showed the ability of MMP-7 to cleave galectin-3 and to reverse its wound healing abilities [137]. The contribution of this effect in vivo is undetermined but adds a further layer of complexity.

6.4. MMPs in Intestinal Inflammation-Induced Fibrosis. Fibrosis is a pathological accumulation of ECM which occurs in the intestine as a consequence of IBD and has been recently reviewed [138]. MMP expression, and the balance between their levels and those of the TIMPs or other inhibitors, is crucial for normal ECM homeostasis. A disruption of this balance may promote fibrosis in the intestine. Despite therapeutic advances in IBD, none prevent or reverse established strictures. In UC, fibrosis will normally affect the mucosa and submucosa whereas in CD, transmural thickening can lead to stricture and require surgery [139]. In humans, TGF-β/Smad pathway seems to be a major contributor to fibrosis in the gut where it can inhibit MMPs and increase the production of TIMPs in mucosa overlaying strictures and in cultured myofibroblasts [96, 140] We have also found that the TGF-β/ALK5/Smad pathway participates in the pathogenesis of experimental intestinal fibrosis. Indeed, upregulation of ALK5 and TIMP-1, phosphorylation of Smad2 and Smad3 proteins, and increased intestinal wall collagen deposition were found in anaerobic bacteria- and TNBS-induced colitis [141]. In addition, the antifibrotic effects of glutamine in TNBS induced colitis was partly attributed to abrogation of the overexpression of TGF-β, phosphorylated Smad3, and TIMP-1 [142].

IL-13 is also said to play a role in fibrosis elsewhere in the body partly through regulation of MMP-1 and TIMP-1 expression [143, 144]. A recent study showed that the cytokine can inhibit expression of MMP-1, -2, and -9 in cultured fibroblasts and that MMP-2 synthesis is not coordinately upregulated along with that of collagen in fibrotic CD colons [145]. In a DSS model of chronic colitis, the increased expression of gelatinases was said to protect against fibrosis through collagen degradation which is another protective role for these enzymes [33].

6.5. MMPs in Colitis Associated Cancer. Chronic inflammation plays a critical role in gastrointestinal carcinogenesis. As examples, chronic hepatitis, Barrett's oesophagus and IBD. Indeed, patients suffering from IBD are at higher risk for developing colonic neoplasia than normal population, particularly those with extensive colorectal inflammation (pancolitis) which continues for longer periods of time [146]. Indeed, colorectal cancer accounts for one sixth of all UC-related deaths [147]. The involvement of MMPs in colorectal cancer and metastasis has been extensively studied [148]. We have also found that MMP-9 upregulation is an early event in the adenoma-carcinoma sequence and, therefore, MMP-9 might be a molecular marker for early colorectal carcinogenesis [149]. However, colitis-associated colorectal cancer (CAC) does not follow an adenoma-carcinoma sequence which is initially associated with genomic instability and the concomitant loss of key tumour suppressor genes. In contrast, CAC shows an inflammation—dysplasia—carcinoma sequence, in which a p53 mutation plays a key role in the early stage and later the APC function is diminished [150]. The p53 functions as tumour suppressor; therefore, the loss or mutation of p53 could lead to neoplasia formation.

A summary of the role of MMPs in CAC is provided in Figure 3. It is interesting to note that while colitis is mediated by MMP-9, the same enzyme may play a protective role in CAC. In fact, MMP-9 could play dual roles in CAC. It has been shown that MMP-9$^{-/-}$ mice are more susceptible to CAC than wild type mice and the protective effect of MMP-9 is believed to be mediated through Notch-1 activation and a subsequent decrease in β-catenin [151]. The same group further investigated these effects and concluded that the upregulation of MMP-9 in colitis leads to activation of Notch-1, increased p53 expression leading to increased levels of p21$^{Waf/Cip1}$, and members of the Bax family proteins to resulting in cell cycle arrest and apoptosis [152].

In contrast to the protective effects of MMP-9, a mouse model of CAC found that activation of neutrophils by the chemokine CXCL2 induced MMP-9 expression which promoted neovascularization and possibly drove CAC [153]. Where integrin linked kinase (ILK) has been implicated in carcinogenesis, ILK-intestinal epithelial cell knockout mice showed reduced tumour growth and MMP-9 expression in

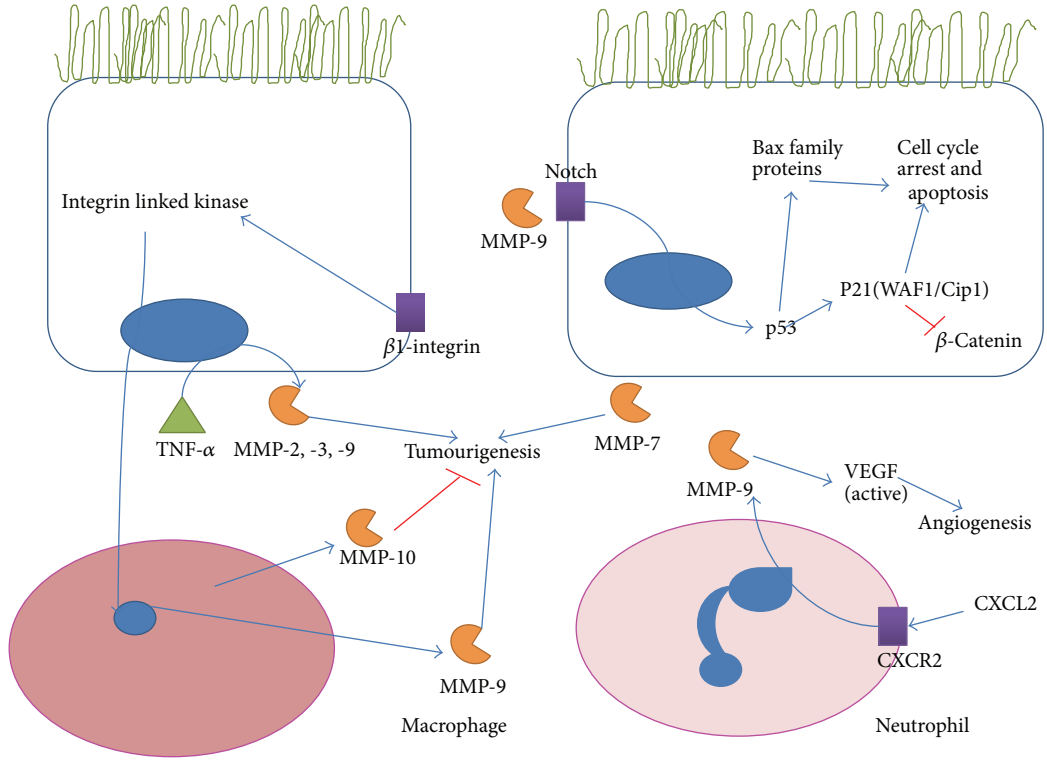

Figure 3: MMPs in colitis associated cancer. A summary of the reported involvement of MMPs in colitis associated cancer (CAC) including the dual roles of MMP-9 where it increases apoptosis and cell cycle arrest through notch cleavage and activation of p53 but also promotes tumour growth, partially through activation of vascular endothelial growth factor (VEGF). MMP-7 is upregulated in UC associated dysplasia and MMP-2 and -3 have also been reported to increase tumour growth. MMP-10 from macrophages is believed to inhibit colitis associated cancer based on a knock-out mouse study.

experimentally induced CAC [154]. In addition, it has been also shown that infliximab, an anti-TNF-alpha antibody, could prevent CAC in DSS-induced colitis, an effect which was accompanied by the reduction of MMP-9 and MMP-11 levels [155]. Similar results were observed when omeprazole exerted a proapoptotic effect in a model of CAC and inhibited MMP-9 and -11 and MT1-MMP [156] and celecoxib reduced gelatinase colon levels in models of CAC [157].

Examination of human UC biopsy samples revealed that there was a direct correlation between the expression of MMP-7 and the grade of UC-associated dysplasia or carcinoma [158]. Further study is required to uncover the precise role of the protease in the progression of the disease. On the other hand, MMP-10 seems to play a protective role from CAC development as it has been found that MMP-10$^{-/-}$ mice had significantly worse inflammatory scores and also higher propensity for development of dysplastic lesions after DSS exposure [19]. More research is required to discover the expression patterns of specific MMPs at the various stages of CAC, to reveal the roles that the enzymes play and the net contribution to tumorigenesis. This may then facilitate successful targeting of specific enzymes as a treatment.

7. MMP Inhibition

Failure of MMP inhibitors (MMPIs) in cancer trials led to a major rethink of the potential of these compounds in the clinic, but we can now reflect on how little was known about the functions of the specific MMPs in a given setting and the clinical effect of inhibition. Early phase I trials revealed unexplained musculoskeletal pain and inflammation which limited the dosage that could be administered. Phase II/III trials examining efficacy, were met with further problems. Owing to the fact that the MMPIs were cytostatic and not cytotoxic, conventional measures of efficacy such as reduction in tumour size were not appropriate. Chosen endpoint measures such as reduction in serum biomarkers were criticized for not necessarily reflecting any reduction in tumour growth and also for being unable to demonstrate MMP inhibition [159]. Ultimately, the trials failed to demonstrate efficacy and several were abandoned. Many researchers now agree that invasive or metastatic cancer may not have been appropriate diseases to trial of the drugs and that preclinical studies suggest the drugs may be more useful in treating earlier stage cancers or inflammatory conditions [160]. A review by Hu et al. covers the history and considers the future for MMP inhibition in the treatment of cancer and inflammation [12]. With continuous studies teasing the physiological from the pathological roles of MMPs in the setting of IBD, we are getting closer to being able to imagine clinical use of an MMPI in IBD. Here we summarise some of the reports of MMP inhibition in intestinal inflammation since 2006 (Table 2).

Table 2: Recently reported MMP inhibitors in models of intestinal inflammation. Novel MMP inhibitors, plant extracts tested in IBD models, and existing IBD therapies are included where MMP-9 expression or activity has been measured.

MMP inhibitor	MMPs inhibited	Model	Reference
RO28-2653	MMP-2, -9	DSS (mouse) (acute)	[163]
Ilomastat	MMP-1	TNBS (rat)	[173]
Minocycline	MMP-2, -3, -9, -13	DSS or TNBS (mouse)	[172]
Etiasa (mesalazine)	MMP-2	TNBS (rat)	[30]
Irsogladine maleate	MMP-2	DSS (mouse)	[209]
Infliximab	MMP-1, -2, -3, -9, -13	Human CD (serum and biopsy)	[174, 175]
CC-10004	MMP-3	Mononuclear cells from human CD	[165]
Nitrate-barbiturates	MMP-9	Cytokine stimulated Caco-2 cells	[161]
Auraptene	MMP-7, -2, -9	DSS (mouse)	[180]
coumarin 4-methylesculetin	MMP-9	TNBS (rat)	[181]
Tris(methoxymethoxy)chalcone	MMP-7	TNF-α stimulated HT-29 cells	[182]
Curcumin	MMP-3	Cultured colonic myofibroblasts from IBD patients	[183]
Phenylpropanoid glycosides: teupolioside and verbascoside	MMP-2, -9	DNBS (rat)	[184, 185]
Neovastat	MMP-9	TNBS (rat)	[188]
Alpha-lipoic	MMP-9	DSS (mouse)	[210]
Cordyceps militaris	MMP-3 and -9	DSS (mouse)	[189]
Calcium ($CaHPO_4$)	MMP-9, -10, -13	HLA-B27 transgenic rat	[190]

7.1. Synthetic MMP Inhibitors. Various studies have examined the potential of novel small molecule inhibitors of MMPs in animal models of colitis. Barbiturate-nitrate hybrids can inhibit MMP-9 activity partly through their nitric oxide mimetic properties [161]. NO mimetics may have the paradoxical effect of reducing NO/iNOS activity through negative feedback, therefore, reducing inflammation and associated MMP-9 release. Our group has recently reviewed the complex interactions of NO and MMP-9 and exploiting these interactions may be therapeutically beneficial in IBD [162]. Integration of a nitrate group as an NO donor may be a novel approach to enhancing efficacy and reducing side-effects of small molecule MMP inhibitors. The benefits of the MMPI RO28-2653 in the DSS model were attributed to its gelatinase selectivity. In particular, ability to spare MMP-1 and -7 reduced the side-effects observed with broad spectrum inhibition and efficacy was comparable to that of doxycycline [163]. Novel synthetic curcuminoid pyrazole derivatives inhibit MMP-9 activity in TNF-α and IL-1β stimulated Caco-2 cells [164]. Sequestration of MMP-2 by hydroxamate beads can inhibit MMP-2 activity and prevented disruption of the epithelial barrier in an in vitro model and is an interesting approach to MMP inhibition at the membrane [129]. The thalidomide analogue, CC-10004, was shown to reduce TNF-α and MMP-3 levels from mononuclear cells isolated from the lamina of CD patients [165].

Vitamin D has inherent immunomodulatory properties and synthetic analogues reduce hypercalcaemia. One such compound, ZK156979, has been shown to be effective in preventing TNBS induced colitis [166] and was shown to inhibit gelatinase activity in cultured peripheral mononuclear cells from healthy and IBD patients [167]. ZK191784 is an intestine specific vitamin D analogue which can exert an anti-inflammatory effect without causing hypercalcaemia [168–170]. This compound and calcitriol were tested in cultured colon biopsies of healthy and IBD patients and were found to reduce MMP-2, -3, and -9 levels as well as the adhesion molecules ICAM-1 and MAdCAM-1 [171].

Several studies have tested known MMPIs in models of human IBD or assessed the ability of therapeutics used in IBD to inhibit MMPs. The tetracycline antibiotics have long been known for their anti-inflammatory properties and for their ability to inhibit MMP expression. Minocycline was shown to reduced inflammation in DSS or TNBS models by reducing the expression of iNOS, proinflammatory cytokines, and MMP-2, -3, -9, and -13 [172] with greater benefit seen in DSS induced colitis when it is combined with the probiotic *E. coli Nissle 1917* [90]. The first generation hydroxamate MMPI ilomastat was shown to protect rats from TNBS induced colitis by inhibiting MMP-1 expression in the colon [173]. Slow release granules of mesalazine were able to reduce MMP-2 expression and inflammation [30]. A novel mechanism was described for 5-ASA in angiogenesis during UC where inhibition of TNF-α and gelatinase levels, reduced the levels of angiostatin and endostatin [119].

The effects of the clinically used infliximab on gelatinase levels in CD patients were examined and found that MMP-9 serum levels were consistently decreased following infliximab treatments and MMP-9 expressing polymorphonuclear

leukocytes were also reduced in biopsy samples [174]. MMP-3 and -12 were decreased following 10 weeks of infliximab treatment in CD patients that were responders to the treatment [175]. Using mucosal explants of IBD and control patients, infliximab was shown to downregulate MMP-1, -3, and -9 levels as well as reducing their increase in response to pokeweed mitogen in a genotype dependent manner following analysis of TNF, MMP, and TIMP SNPs [176]. It has also been shown that infliximab could reduce the incidence of tumour development through reduction of MMP-9 and -11 in DSS-induced colitis [155]. Treatment of CD patients with anti-TNF therapy (infliximab or adalimumab) or with corticosteroids and other immunosuppressives (methotrexate or azathioprine) resulted in a decrease of epithelial MMP-7 and stromal MMP-9 and -26 and TIMP-1 and -3 [177]. In a similar study, the same group showed results in paediatric IBD where glucocorticoid therapy reduced serum MMP-7, TIMP-1, and MMP-7/TIMP-2 and anti-TNF therapy reduced MMP-7 but to a lesser extent. Interestingly, MMP-8 and -9 levels were not statistically significantly altered [178]. A larger study used microarrays to measure the mucosal expression of 24 MMPs along with TIMPs, ADAM(T)s, and growth factors. Most MMP expression was increased in IBD patients with the exception of MMP-28 which was downregulated and responders to infliximab had a gene expression and gelatinase activity was restored to control levels following treatment [179].

7.2. Natural Products. Research is increasing in the field of natural products as medicines and many of these have been directed towards MMP inhibition in intestinal inflammation.

The coumarin, auraptene, is found in several citrus fruits and has been shown to inhibit MMP-7 activity following DSS induced colitis [180]. The naturally occurring coumarin, 4-methylesculetin, showed comparable effects to sulphasalazine and prednisolone in TNBS induced colitis where it was able to inhibit MMP-9 [181]. 2′,4′,6′-Tris (methoxymethoxy) chalcone (TMMC) protected against TNBS induced colitis and was able to inhibit MMP-7 upregulation induced by TNF-α in HT-29 cells [182]. Curcumin is a component of turmeric with known anti-inflammatory properties and one group investigating its use in IBD showed that it could reduce MMP-3 among other mediators in ex vivo cultured colonic myofibroblasts from IBD patients [183]. PPGs and verbascoside were shown to reduce inflammation, pro-inflammatory signalling and gelatinase expression in DNBS induced colitis [184, 185]. Neovastat, a product found in shark cartilage, is a known inhibitor of MMPs and angiogenesis and has undergone clinical trials for the treatment of renal carcinoma and plaque psoriasis [186, 187]. More recently, this agent has shown to inhibit intestinal inflammation in TNBS induced colitis through inhibition of gelatinase expression [188]. *Cordyceps militaris* is a traditional medicine widely used in East Asia to treat inflammatory conditions and has been shown to inhibit disease activity, along with iNOS and MMP-3 and -9 expression in a DSS induced model of colitis [189]. Calcium supplementation has shown benefit in reducing epithelial permeability and inflammation in the intestine through reduced expression of MMP-9, -10, and -13 in HLA-B27 transgenic rat model of colitis [190].

The pineal gland product melatonin is a known scavenger of free radicals and has anti-inflammatory effects in experimental colitis [191]. In certain instances, NO and peroxynitrite can modulate MMP-9 expression and activity [162] and it is likely that the antioxidant properties of melatonin could reduce colon gelatinase expression in DNBS induced colitis [192]. Further investigation shows that it can mediate NF-κB, STAT-3, IL-17, Cox-2, nuclear erythroid 2-related factor 2, connective tissue growth factor, and MMP-9 [193] and its contribution to IBD has been summarised recently [194].

Endogenous fatty acids are known gelatinase inhibitors [195] and polyunsaturated fatty acids were recently shown to be anti-inflammatory in the gut through decreased Cox-2 and MMP-9 expression [196]. Orally administered docosahexaenoic acid had comparable efficacy to sulfasalazine in DSS induced colitis but with stronger inhibition of MMP-3, -10, and -13 [197].

8. MMPs as Biomarkers for IBD

The association of MMPs with IBD is now widely accepted. MMP expression or protein levels are now a standard readout for inflammation in the experimental models described previously. There is an unmet need for additional biomarkers to assess the progression of the disease or identification of flares without the need for invasive, time consuming, or expensive imaging techniques. Here we review the recent studies regarding the potential and usefulness of MMPs as markers for inflammation in IBD.

Faecal MMP-9 levels were reported to correlate with the overall Mayo and endoscopic scores, serum CRP, and faecal calprotectin levels in UC patients [198]. As a biomarker, faecal MMP-9 also has potential in recognising severity of pouchitis and, to a lesser extent, CD where correlation with the SES (simple endoscopic score) CD was not statistically significant but overall correlations were better than calprotectin [199]. However, neither faecal calprotectin nor faecal MMP-9 can differentiate between *Clostridium difficile* induced and a natural relapse in IBD [200].

Serum MMP-9 correlates with disease activity in UC and CD and levels were found to be higher in UC. As a result, this may aid in differentiation between UC and CD where serum MMP-9 was more effective than CRP levels [127]. Neutrophil secreted MMP-9 is often complexed with NGAL and circulating levels of NGAL or NGAL/MMP-9 complex have been associated with breast cancer [21] and kidney disease [201]. This complex has recently been described as a potential biomarker for mucosal healing in UC where infliximab reduced serum levels, predictive of mucosal healing [202]. Immunohistochemical staining of colonic biopsy samples for paediatric onset UC patients showed that MMP-9 levels correlated with the histological measures of inflammation but not with any other marker of disease [203]. Urinary gelatinase levels have also been found to be independent predictors of IBD in paediatric patients

[204] and MMP-3 and MMP-9 serum levels were found to be potentially useful diagnostics of disease activity in children with UC [205].

A gene expression approach was used to assess differences between responders and nonresponders to corticosteroid treatment in severe paediatric UC. MMP-8 was strongly upregulated in nonresponders compared to those patients who had responded to treatment. This effect was believed to be due to the inhibitory effect of methylprednisolone on IL-8, a known inducer of MMP-8 [206].

Microarrays were used to assess the potential of biomarkers in peripheral blood as diagnostic indicators for IBD. MMP-9 was found to be upregulated in IBD patients which corresponded to the combined epithelial and lamina expression in biopsy samples. MMP-9 was identified in this study as one of the top 5 peripheral blood transcripts which could be used in combination to diagnose UC or CD [207]. A large study investigated useful and appropriate biomarkers in IBD and their correlation with the Mayo score in UC or with the ileocolonoscopy (ICO), computed tomography enterography (CTE), or combined ICO-CTE score in CD patients as measures of inflammation. The study concluded that combined faecal calprotectin and serum MMP-9 best predicted inflammation in UC and combination of faecal calprotectin, serum MMP-9, and serum IL-22 best correlated with ICO-CTE score in CD [208].

The expression pattern of the TIMPs often mirrors that of the MMPs and so the level of inflammation. Several studies have investigated the potential of also measuring TIMP levels as readouts of inflammation severity. MMP-1 and TIMP-1 are well known to be upregulated in UC and colonic expression was found to correlate with disease severity. The fact that plasma levels of these proteins correlated well with the mucosal expression was of potential clinical interest [40]. The intestinal expression of MMPs and TIMPs in CD patients was measured before and following immunosuppressive treatment and found that the histological score correlated positively with neutrophil MMP-9, MMP-26, and macrophage TIMP-1. Calprotectin levels followed a similar trend to expression of stromal MMP-26, TIMP-1, and -3. Crohn's disease endoscopic index of severity (CDEIS) value correlated positively with macrophage TIMP-1 and stromal TIMP-3 and negatively with epithelial TIMP-3 which also negatively correlated with C-reactive protein values (CRP) [177]. The levels of MMPs and TIMPs were measured by immunohistochemistry only, and neither serum nor plasma levels were assesed. In paediatric IBD, serum MMP-7 mirrors the disease activity and, together with TIMP-1 expression, is a measure of response to glucocorticoid therapy but to a lesser extent, anti-TNF therapy [178].

9. Concluding Remarks

Following recognition of the limited understanding of the MMPs in earlier trials, the field has grown enormously to discover more roles for the proteases. The fact that the expression and activity of the MMPs is upregulated in colitis and IBD has been confirmed through numerous animal model experiments and analysis of IBD patient biopsies. This has led to significant research into the use of MMPs as biomarkers for the severity of inflammation in the colon where results appear promising.

More studies continue to identify the primary cellular sources of the MMPs in the colon and triggers of their transcriptional upregulation or activation. A definitive understanding of cell types and signals involved in MMP upregulating will allow us to target the pathologically upregulated enzymes.

It is clear from the range of MMP substrates and degradomic experiments that the functions of MMPs in IBD go far beyond the simple digestion of ECM proteins. In recent years, more and more studies highlight the role of MMPs in regulation of the immune response through activation or inhibition of cytokines and chemokines, for example, the activation of TNF-α which is exciting given the established role of this cytokine in the pathogenesis of IBD [110]. MMPs have the ability to regulate both pro- and antiangiogenic factors which may contribute to the pathogenesis of IBD or mucosal healing. The proteases have a similar role in epithelial homeostasis through control of tight junction proteins, goblet cell differentiation and degradation of ECM proteins. These studies are vital to understand the contribution of specific MMPs to a range of processes that make up IBD. However, the potential to therapeutically exploit these discoveries raises several questions. Paramount to the discussion of MMP inhibition is the potential knock on effect of the inhibition. Blocking a single enzyme will affect activation of other proteases and its considerable list of substrates which is likely to disrupt physiological processes. Delineating these activation pathways, completing substrate lists, and understanding the contexts where upregulation of the protease is pathological will remain relevant questions before MMP inhibition becomes a therapeutic option in the clinic. Therefore, while new functions emerge for the MMPs, it will be crucial to understand the setting where an MMP is promoting or inhibiting wound healing, pro- or antiangiogenic, or inhibiting or amplifying the immune response. As answers to these questions emerge, we can be more confident that targeted inhibition of the MMPs could be of benefit in treating IBD.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Innate Immunity Components and Cytokines in Gastric Mucosa in Children with *Helicobacter pylori* Infection

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Purpose. To investigate the expression of innate immunity components and cytokines in the gastric mucosa among *H. pylori* infected and uninfected children. *Materials and Methods.* Biopsies of the antral gastric mucosa from children with dyspeptic symptoms were evaluated. Gene expressions of innate immunity receptors and cytokines were measured by quantitative real-time PCR. The protein expression of selected molecules was tested by immunohistochemistry. *Results. H. pylori* infection did not lead to a significant upregulation of *MyD88, TLR2, TLR4, CD14, TREM1,* and *TREM2* mRNA expression but instead resulted in high mRNA expression of *IL-6, IL-10, IFN-γ, TNF-α,* and *CD163. H. pylori cagA*(+) infection was associated with higher *IL-6* and *IL-10* mRNA expression, as compared to *cagA*(−) strains. *H. pylori* infected children showed increased IFN-γ and TNF-α protein levels. IFN-γ mRNA expression correlated with both *H. pylori* density of colonization and lymphocytic infiltration in the gastric mucosa, whereas TNF-α protein expression correlated with bacterial density. *Conclusion. H. pylori* infection in children was characterized by (a) Th1 expression profile, (b) lack of mRNA overexpression of natural immunity receptors, and (c) strong anti-inflammatory activities in the gastric mucosa, possibly resulting from increased activity of anti-inflammatory M2 macrophages. This may explain the mildly inflammatory gastric inflammation often observed among *H. pylori* infected children.

1. Introduction

Gastric mucosa epithelial cells and myeloid cells (monocytes, macrophages, and dendritic cells) form the first barrier to *Helicobacter pylori* (*H. pylori*) infection. They recognize bacteria through pattern recognition receptors (PRRs), which interact with conserved microbial structures called pathogen-associated molecular patterns (PAMPs).

One of the PRRs systems involved in *H. pylori* recognition is a family of Toll-like receptors (TLRs). TLRs are present

both on gastric epithelial cells and on immune cells infiltrating gastric mucosa. TLRs in the gastric mucosa involve 5 members of this family [1–3]. Studies on epithelial cell lines showed that *H. pylori* could induce proinflammatory gene expression *via* interaction with four of them, that is, TLR2, TLR4, TLR5, and TLR9 [1–4]. Expression of TLR2, TLR4, and TLR5 has also been detected in the gastric mucosa of *H. pylori* infected patients [1–4].

TLR signaling is mediated by two main pathways: MyD88 dependent (leading to the expression of proinflammatory cytokines) and MyD88 independent (responsible for interferon type I production). MyD88 is an adaptor protein that is used by all TLRs with the exception of TLR3, which utilizes exclusively the MyD88-independent pathway. TLR4 is unique, because it can induce both the MyD88-dependent and independent pathways [3, 5].

MyD88 expression in macrophages has been found to be essential for *H. pylori* induction of inflammatory cytokines (IL-6, IL-1β, IL-10, and IL-12) [2, 3, 5]. Both TLR2 and TLR4 proved to be crucial as signaling receptors for these responses in mouse macrophages [2, 5, 6]. MyD88 dependent signaling was also required for induction of protective immune responses (IL-17, antimicrobial peptides) against *H. felis* in animal models [6].

Another class of innate immunity molecules, which may be involved in the *H. pylori* mediated immune response, are triggering receptors expressed on myeloid cells (TREMs) [7]. TREM-1 is a 30-kDa glycoprotein of the Ig family which is expressed mainly on neutrophils and monocytes [7]. TREM-1 is engaged in amplification of TLR-dependent signals, as well as enhancement of NOD-like receptors (NLRs) mediated responses, including the NOD1 pathway involved in protection against *H. pylori* infection [8]. TREM-1 is also expressed in gastric mucosa epithelial cells, and its expression is elevated in the gastric mucosa of *H. pylori* infected adult patients [7]. TREM-2 is expressed mainly on macrophages and dendritic cells [9, 10]. Its activation results in induction of anti-inflammatory reactions [9, 11], but so far this receptor has not been studied in *H. pylori* infected patients.

CD163 is a cell-surface glycoprotein receptor that is highly expressed on most subsets of resident tissue macrophages [12]. The expression of CD163 is strongly induced by anti-inflammatory mediators, such as glucocorticoids and IL-10, and is inhibited by proinflammatory mediators such as IFN-γ, TNF-α, and others [13]. CD163 is a marker of anti-inflammatory M2 macrophages [14]. In contrast, M1 macrophages are associated with strong proinflammatory and cytotoxic responses induced by IFN-γ, TNF-α, and IL-6. *H. pylori* infected asymptomatic patients show mixed M1/M2 phenotype in their gastric mucosa [15]. M1 polarized macrophages can be identified by their contribution to high inflammatory responses, epithelial atrophy, and premalignant lesions, whereas CD163 plays a role in protective immunity against bacterial infection [16], so it may also be important in *H. pylori* infection.

The CD14 receptor is a cell surface molecule expressed on monocytes and macrophages and serves as a part of the LPS recognizing complex. Its presence is necessary for interaction with LPS and generation of signal transduction pathways leading to production of many proinflammatory cytokines. Interaction with LPS changes the CD14 expression [17]. However, interaction of *H. pylori* LPS with CD14 is rather weak, because of the structural features of *H. pylori* lipid A [17, 18]. Nevertheless, the expression level of CD14 may indicate an infiltration of the gastric mucosa by monocytes/macrophages, and it may change as a result of interaction with LPS.

The aim of this study was to examine the expression of innate immunity components (MyD88, TLR2, TLR4, CD14, TREM1, and TREM2) in relation to other mediators of the inflammation (IL-1β, IL-2, IL-6, IL-10, IL-12, TNF-α, and IFN-γ) in the gastric mucosa of *H. pylori* infected and uninfected children. The results were correlated with gastric inflammation scores and the density of *H. pylori* colonization.

2. Materials and Methods

2.1. Patients. The study was undertaken in accordance with the Helsinki declaration, with approval from the Ethics Committee of the Collegium Medicum at Nicolaus Copernicus University in Bydgoszcz, Poland. Informed consent was obtained from all the parents of patients and from patients older than 16 years.

Pediatric patients, from the Department of Pediatric Endoscopy and Gastrointestinal Function Testing, University Hospital in Bydgoszcz, Poland, displaying dyspeptic symptoms were eligible for inclusion. Exclusion criteria included (1) previous diagnosis of *H. pylori* infection and its treatment, (2) a history of antibiotic, antacid, H_2 blocker, proton pump inhibitor, bismuth compound, or nonsteroidal anti-inflammatory drug use during the previous 4 weeks, (3) previous diagnosis of other inflammatory diseases, such as coeliac disease, inflammatory bowel disease, or allergy, and (4) gastric perforation or hemorrhage, history of abdominal surgery, or evidence of other gastrointestinal pathology.

Each subject underwent a urea breath test and endoscopic examination of the upper gastrointestinal tract. Three antral biopsies were taken from each patient. One biopsy was submerged in RNA*later* solution and frozen for real-time PCR analysis. The other specimens were formalin-fixed and embedded in paraffin, sectioned, and stained with hematoxylin and eosin for histological analysis. Biopsy specimens were graded for gastritis by two independent pathologists, according to the updated Sydney system.

A patient was considered *H. pylori* infected when the urea breath test and either the microscopic evaluation or the PCR analysis of the gastric mucosa were positive for *H. pylori*. A patient was considered not infected when all three tests were negative.

2.2. Molecular Methods

2.2.1. Genotyping of H. pylori Obtained from Gastric Mucosa. The cagA status of *H. pylori* was determined by the PCR method, as described previously [19].

2.2.2. Expression of IL-1β, IL-2, IL-6, IL-10, IL-12, IFN-γ, TNF-α, TLR2, TLR4, TREM1, TREM2, MyD88, CD14, and CD163 mRNA in Gastric Mucosa. RNA was isolated from gastric mucosa by using GenElute Mammalian Total RNA Kit (Sigma Aldrich, St. Luis, MO), according to the manufacturer's instructions. Isolated RNA was subjected to DNase digestion to remove DNA traces. Synthesis of cDNA was performed using the TaqMan Reverse Transcription Reagents kit (Applied Biosystems) and mixed primers (hexamers). The expression of mRNA of IL-1β, IL-2, IL-6, IL-10, IL-12, IFN-γ, TNF-α, TLR2, TLR4, TREM1, TREM2, MyD88, CD14, CD163, and the reference gene (G3PDH) were assessed by real-time PCR using primers published elsewhere [10, 20–23]. The amplification reaction was conducted in a volume of 25 μL, using 1 μL of cDNA, 12.5 μL SYBR GreenPCR Master mix (Applied Biosys), and 250 nM of each primer in the thermocycler 7500 Real Time PCR System. All samples were run in duplicates and template negative controls were included in each run. The following reaction conditions were used: 95°C for 10 min (initial denaturation), followed by 40 cycles by denaturizing at 95°C for 15 sec and a 1 min extension at 60°C. Melting curve analysis was performed after each run, to control for product amplification and to ensure that no dimers interfered with the reaction. Cycle threshold (Ct) values were determined by SDS 1.2 software (Applied Biosystems). The expression levels of genes studied were calculated by the $2_{-\Delta\Delta Ct}$ method and results were expressed as relative values (fold change) in relation to the control group of *H. pylori*-negative or *cagA*-negative samples, after normalization to the expression levels of the endogenous control (G3PDH gene).

2.3. Expression of TNF-α and IFN-γ in Gastric Mucosa. Immunohistochemical staining of antral gastric mucosa biopsies was performed using primary mouse antibodies against TNF-α (1 : 100) and INF-γ (1 : 200) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Tissue sections were incubated with primary antibodies overnight at 4°C. The antigen-antibody complex was detected using Anti-Mouse EnVision HRP-Labeled Polymer (DakoCytomation, Glostrup, Denmark), a peroxidase detection system, and localized with DAB (3-3′diaminobenzidine) as a chromogen. Finally, the sections were counterstained with hematoxylin, dehydrated in increasing grades of ethyl alcohol (80, 90, 96, 99.8%), and mounted with Shandon Consul Mount (Thermo Scientific).

The expression of TNF-α and INF-γ was evaluated in epithelial cells and lamina propria of antral gastric mucosa, by using a light microscope, ECLIPSE E800 (Nikon Instruments Europe, Amsterdam, Netherlands).

The immunohistochemical expression of analyzed proteins was estimated as a percentage of positive cells multiplied by the intensity of staining, according to morphometric principles based on the modified Remmele-Stegner scale (IRS—Index Remmele-Stegner) [24]. The morphologic studies were performed at 20x original objective magnification. The final level of estimated protein expression was evaluated as the ratio of the expression intensity and the positively expressed number of cells/tissue area (total scale range 0–9). The number of positive immunoreactive area was categorized as 0, negative; 1 = 1 − 5 positive cells; 2 = 6 − 20 positive cells; 3 = ≥ 20 of positive cells. The intensity of staining was scored as follows: 0—negative, 1—low, 2—moderate, 3—strong.

During immunohistochemical staining, for determination of the appropriate antibody dilution, and elimination of false positive results, as well as for the reduction of the background reaction, a series of positive control reactions were performed on a model tissue selected according to the antibodies datasheet, and reference sources (The Human Protein Atlas http://www.proteinatlas.org). The positive control for TNF-α was performed on the kidney sections, and the representative expression was estimated in cells in tubules. The positive control for INF-γ was performed on the placenta, and the expression was estimated in trophoblastic cells. Moreover, negative immunohistochemical control reactions were performed, by substituting the primary antibody by a solution of diluted 1% BSA (bovine serum albumin) in PBS (phosphate buffered saline).

2.4. Statistical Analysis. Data were analyzed by the nonparametric Mann-Whitney U test. The relationship between histological parameters and the level of gene expression was evaluated using Spearman's correlation coefficient. Statistical calculations were made using STATISTICA 6.0 for Windows PL, with the level of statistical significance at $P < 0.05$.

3. Results

A total of 78 children were included in the study (55 girls; age range 7–18 years, mean 14.0). Infection of *H. pylori* was confirmed in 40 (51%) patients, 20 (50%) of whom were carriers of *cagA*-positive strains. None of the patients had peptic or duodenal ulcers.

3.1. Expression of Inflammatory Mediators in the Gastric Mucosa. Significantly higher (from 3.4- to 6.5-fold) expression of *TNF-α*, *INF-γ*, *IL-6*, *IL-10,* and *CD163* mRNA was found in the gastric mucosa of *H. pylori* infected patients as compared to uninfected individuals (all P values < 0.01). In contrast, mRNA expression of *TLR2*, *TLR4*, *TREM1*, *TREM2, CD14,* and *MyD88* did not differ between the two groups (Table 1). Higher expression of TNF-α and INF-γ in the gastric mucosa in *H. pylori*-positive patients was also detected by immunochemistry ($P = 0.02$; $P < 0.01$, Figure 1). INF-γ expression correlated with both the density of *H. pylori* colonization and lymphocytic infiltration in the gastric mucosa ($r = 0.41$, $P < 0.001$; $r = 0.42$, $P < 0.01$), whereas TNF-α expression correlated only with bacterial density ($r = 0.51$, $P = 0.02$). No correlation between expression of the remaining immunological markers and the intensity of inflammation or bacterial load in the gastric mucosa was noted.

Children infected with the *cagA*-positive strain had higher levels of IL-6 (2.5-fold, $P = 0.03$) and IL-10 (3.5-fold, $P < 0.01$) mRNA than those with the cagA-negative strain,

Table 1: mRNA expression of inflammatory mediators in the gastric mucosa in relation to *H. pylori* and *cagA* status.

Gene	Expression in *H. pylori* infected relative to noninfected patients	*P* value	Expression in *H. pylori cagA*(+) relative to *H. pylori cagA*(−) patients	*P* value
IL-1β	1.1	*0.06*	1.5	*0.13*
IL-2	1.4	*0.86*	1.4	*0.29*
IL-6	**4.6**	***<0.01***	**2.5**	***0.03***
IL-10	**6.5**	***<0.01***	**3.5**	***<0.01***
IL-12β	1.2	*0.56*	1.0	*0.95*
INF-γ	**3.4**	***<0.01***	1.6	*0.26*
TNF-α	**5.5**	***<0.01***	1.0	*0.25*
MyD88	1.4	*0.54*	0.8	*0.67*
TLR2	1.7	*0.30*	1.2	*0.58*
TLR4	1.5	*0.11*	1.2	*0.48*
TREM1	1.4	*0.39*	1.1	*0.4*
TREM2	1.4	*0.23*	1.2	*0.31*
CD14	1.5	*0.43*	1.0	*0.99*
CD163	**4.2**	***<0.01***	1.4	*0.44*

The expression of each gene is given relative to the expression in *H. pylori*-negative or *H. pylori cagA*-negative samples (fold change in $\log_{10}$ RQ).

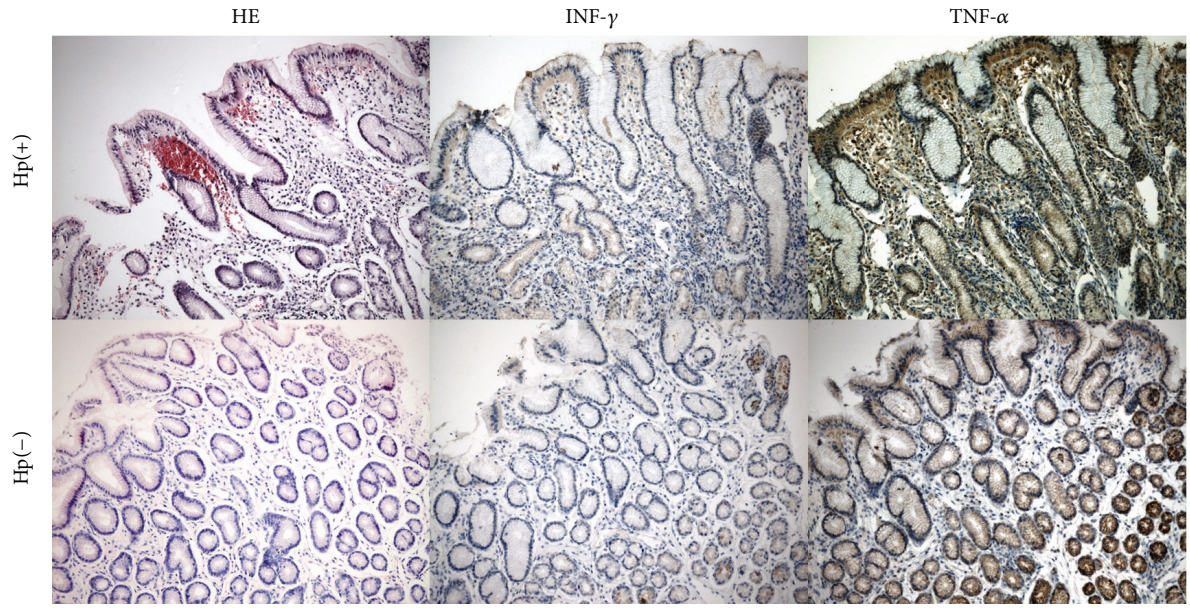

Figure 1: Hematoxylin-eosin (HE) staining and immunohistochemical staining with anti-IFN-γ and anti-TNF-α in *H. pylori* infected (Hp(+)) and noninfected children (Hp(−)). Magnification, ×20.

whereas no differences were found for other markers studied (Table 1).

4. Discussion

This study showed that *H. pylori* infection in children resulted in mRNA up-regulation of *IL-6*, *IL-10*, *TNF-α*, *IFN-γ*, and *CD163* and unchanged expression of *MyD88*, *TLR2*, and *TLR4* mRNA in the gastric mucosa. *H. pylori cagA*(+) infection was connected with an upregulation of *IL-10* and *IL-6* mRNA expression. These data confirm the results of other studies showing the Th1 profile of *H. pylori*-mediated inflammation [25, 26]. However, these changes occurred without induction of basic TLRs system components (MyD88, TLR2, and TLR4) and other innate immunity molecules (TREM1, TREM2, and CD14) [2, 4, 26, 27].

One of the key molecules is MyD88 intracellular adaptor protein, which is necessary for mediating signals from all TLRs except TLR3 [3]. MyD88 dependent signaling pathways are involved in induction of several inflammatory cytokines (IL-6, IL-1β, IL-12, and IL-10) in the bone marrow-derived macrophages, which enables the elimination of the pathogen and protects against tissue damage [2, 5, 28].

Our results suggest that *H. pylori* may modulate MyD88 expression. Lack of significant MyD88 induction by *H. pylori* may be responsible for infection persistence and induction of endotoxin tolerance, which may lead to a reduced inflammatory response after repeated challenge by LPS [28, 29].

In contrast to Enterobacteriaceae, LPS of *H. pylori* is less immunogenic and does not use the TLR4 pathway but induces mainly TLR2-mediated signaling [6, 30–32]. The same pathway is used by *P. gingivalis* LPS. It uses TLR-2 mediated activation signaling that is associated with impaired endotoxin tolerance, neutrophil-dominated chronic inflammation, elevated levels of IL-8 and MIP-2, but low production of IFN-β [18, 23]. It is unclear whether these observations can be applied to *H. pylori*-mediated inflammation.

The described lack of or poor TLR4 engagement in *H. pylori* recognition may be at least partially explained by unchanged gastric mucosa *CD14* mRNA expression level. The main CD14 function is its interaction with LPS and induction of TLR4/MD2 mediated signaling pathway engaged in activation of many proinflammatory reactions [33]. So the unchanged CD14 expression found here may result in its impaired engagement in *H. pylori*-LPS-mediated mucosal inflammation [34, 35].

These data might suggest that gastric mucosa *IL-6*, *TNF-α*, *IL-10,* and *CD163* mRNA upregulation found here possibly did not depend on LPS-mediated signaling because *H. pylori* infection did not change CD14 transcript levels. Gastric mucosa *CD14* mRNA expression depends on the level of mucosal infiltration by macrophages and neutrophils, and their activation status [33]. *H. pylori* infected adults showed increased CD14 expression in the gastric mucosa, especially in gastric tumor tissues [36].

On the other hand, our results contrast those of *H. pylori* infected adults, who showed an increased expression of TLR2 and TLR4 in the gastric mucosa [37]. Also, a recent study in a group of 50 children from Mexico City showed that *H. pylori* infection was associated with increased expression of TLR2, TLR4, TLR5, and TLR9 proteins in the gastric epithelium, as well as up-regulation of the cytokines IL-10, IL-8, and TNF-α [26]. These discrepancies may stem from ethnic characteristics or children involved in both studies. The Mexican group consisted mostly of the Mexican population, which expressed Amerindian genetic markers. These divergent results may also result from differences in the pathogenicity of infecting *Helicobacter pylori* strains and TLRs genetic polymorphisms [38].

We previously found that *H. pylori* infection in children is associated with systemic activation of circulating monocytes (upregulation of CD11b, CD11c, and CD18), which is downregulated following eradication therapy [39]. This observation is partially consistent with the elevation of CD163 and IL-10 mRNA expression in the gastric mucosa of *H. pylori*-infected children. CD163 is a cell surface molecule that is expressed exclusively on resident tissue macrophages [12], therefore high CD163 mRNA in the gastric mucosa of infected children may be associated with increased numbers of activated peripheral blood monocytes migrating into sites of inflammation in the gastric mucosa, where they may eventually turn into macrophages with high CD163 expression.

The role of CD163 in *H. pylori* infection is not known. As this molecule binds both Gram-positive and Gram-negative bacteria, it may contribute to the host defense against infection [13, 16]. On the other hand, CD163 is widely recognized as a marker of M2 macrophages [13, 14, 22, 40]. M2 macrophages exhibit anti-inflammatory and immune-modulating functions and induce mainly Th2 responses [16], which do not contribute to *H. pylori* elimination. Depending on the induction agent, M2 macrophages can be divided into at least three different subpopulations, with high IL-10 synthesis as a common feature. High expression of *IL-10* mRNA in the gastric mucosa, documented in this study, may, at least partially, originate from M2 macrophages, which are highly increased in the gastric mucosa of *H. pylori* infected subjects [15, 34]. Additionally, IL-10 upregulates expression of *CD163* and other monocyte anti-inflammatory genes like *IL-1 receptor antagonist* (*IL-1r*) [41] or *suppressors of cytokine signaling-3* (*SOCs-3*) [42], which downregulate immune responses. These findings confirm recent data indicating that *H. pylori*-mediated inflammation is related to the generation of tolerogenic macrophages and dendritic cells contributing to the formation of different types of suppressor T cells (Treg, Tr1, and Th3). The latter are especially numerous and active in children [43, 44] and in a mouse model system [45].

We found that the gastric expression of TREM 1 and TREM 2 mRNA was not affected by *H. pylori* infection [7]. In contrast, *H. pylori* infected adults had elevated expression of TREM1 in gastric epithelial cells. TREM1 expression in the gastric mucosa also reflects the extent of macrophages and neutrophils infiltration. TREM 2 acts antagonistically to TREM 1 and promotes anti-inflammatory response [9]. It can also negatively affect TLR-dependent response [11]. Lack of changes in TREM1 expression found in our study further confirms the tolerogenic status of leucocytes present in the gastric mucosa of *H. pylori*-infected children, since their activation leads to TREM 1 and TREM 2 upregulation [7–11]. Together, these results suggest that the TLRs system is poorly involved in *H. pylori*-induced inflammation in the gastric mucosa in children, and that other PRRs may be more engaged in pathogen recognition [3].

The positive correlation between the lymphocyte infiltration and IFN-γ found in this study indicates that lymphocytes (T and NK cells) infiltrating the gastric mucosa may produce IFN-γ and switch the response towards a Th1 profile, as has previously been described in other studies [3, 25, 30, 35]. Th1 response did not limit *H. pylori* colonization, because its load correlated with TNF-α and INF-γ expression in the gastric mucosa. These observations confirm previous studies in adults and children, showing that Th1 response is not protective [30].

5. Conclusions

To summarize, this study demonstrated that *H. pylori* infection in children was characterized by (a) nonprotective Th1 response associated with high *H. pylori* load and increased lymphocytic infiltration into gastric mucosa and (b) presence of anti-inflammatory activities (high expression

of CD163 and IL-10 mRNA). All of these activities were induced without significant activation of innate immunity components, such as TLRs system molecules (TLR2, TLR4, and MyD88), and inflammatory markers of macrophages (TREM1, TREM2). The ability of *H. pylori* to manipulate the immune response (activation or inactivation of TLR-dependent response) may be responsible for bacterial survival and a mild course of infection in children.

Conflict of Interests

No company had any input or influence into the design, analyses, interpretation, or content of this paper. There are no conflict of interests for any author.

Authors' Contribution

Jacek Michalkiewicz and Anna Helmin-Basa have equal participation in publication.

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Research Article

Claudin-4 Undergoes Age-Dependent Change in Cellular Localization on Pig Jejunal Villous Epithelial Cells, Independent of Bacterial Colonization

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Newborn piglets are immunologically naïve and must receive passive immunity via colostrum within 24 hours to survive. Mechanisms by which the newborn piglet gut facilitates uptake of colostral cells, antibodies, and proteins may include FcRn and pIgR receptor-mediated endocytosis and paracellular transport between tight junctions (TJs). In the present study, FcRn gene (FCGRT) was minimally expressed in 6-week-old gut and newborn jejunum but it was expressed at significantly higher levels in the ileum of newborn piglets. pIgR was highly expressed in the jejunum and ileum of 6-week-old animals but only minimally in neonatal gut. Immunohistochemical analysis showed that Claudin-5 localized to blood vessel endothelial cells. Claudin-4 was strongly localized to the apical aspect of jejunal epithelial cells for the first 2 days of life after which it was redistributed to the lateral surface between adjacent enterocytes. Claudin-4 was localized to ileal lateral surfaces within 24 hours after birth indicating regional and temporal differences. Tissue from gnotobiotic piglets showed that commensal microbiota did not influence Claudin-4 surface localization on jejunal or ileal enterocytes. Regulation of TJs by Claudin-4 surface localization requires further investigation. Understanding the factors that regulate gut barrier maturation may yield protective strategies against infectious diseases.

1. Introduction

In utero, the pig fetus does not share circulation with the sow and therefore piglets are born immunologically naïve (i.e., without a complement of maternal antibodies). As a result, piglets must ingest colostrum within the first day of life or they will die from infectious diseases. In addition, piglets are born hypoproteinemic and require rapid maturation of the serum protein profile [1]. Colostrum-derived immunoglobulins (Igs) and other macromolecules (such as albumin, cytokines, and antimicrobial peptides, as well as many other bioactive products) traverse the gut wall then enter into the vasculature where they play a variety of roles including passive protection against disease [1–3]. There are several proposed mechanisms by which Igs and other macromolecules are absorbed by the gut wall but the primary mechanism in the newborn piglet is through nonselective pinocytosis by fetal-derived enterocytes [4–6] until cellular replacement occurs (approximately 19 days after birth [7]). "Gut closure" is defined as the "time after which intestinal epithelial cells no longer take up or internalize macromolecules via pinocytosis" [4] but although the upper half of the small intestine undergoes "gut closure" much earlier than the lower half of the small intestine, both the upper and lower regions of the small intestines lose their capacity to transport macromolecules to the blood at approximately two days of age [4, 5, 8]. Lecce (1973) further speculated that dietary-management regimen had a profound effect on the capacity of the neonatal gut epithelium to absorb and transport macromolecules as piglets starved for three days after birth continued to transport internalized macromolecules into the blood in a manner similar to the one-day-old piglet, but fed piglets did not [4]. However, this effect may have been mediated by the stress and inflammation induced by starvation rather than

a direct impacted gut permeability [9]. Others showed that the capacity for the transmission of macromolecules was higher in low birth weight piglets (<1 kg) and that insulin may play a role in initiation of gut closure [10, 11].

Beyond pinocytosis, maternal IgG can be bound by the FcRn protein on the apical surface of the epithelial cells whereupon IgG-FcRn are endocytosed, trafficked to the basolateral surface, and released to the *lamina propria* in a pH-dependent manner [12]. Igs bound by FcRn are protected from proteolysis by being trafficked away from the lysosomal pathway [13] and back to the plasma membrane [14] where the elevated extracellular pH results in dissociation from FcRn. In pigs, humans, and nonhuman primates, FcRn is present on the intestine in adulthood and, therefore, may be a mechanism for antibody-mediated sampling of lumenal contents and uptake beyond the uptake of maternal antibodies in the neonate [15–17]. In contrast, rodent intestinal epithelial cells do not express FcRn after weaning [18]. Another mechanism of antigen uptake across the gut wall may occur via the pIgR receptor. Gut-derived plasma cells home to the mammary gland and secrete dimeric SIgA into the colostrum/milk, via pIgR transport [19, 20]. Upon ingestion of colostrum/milk by the neonate, SIgA may be absorbed by the gut through binding to pIgR possibly with an antigen in tow which can be transported to the circulation [21, 22]. Circulating or mucosal IgA may be subsequently transported from the basolateral to apical side of the gut mucosa via pIgR [21, 23]. Other mechanisms of macromolecular uptake across the gut wall include (1) *lamina propria* dendritic cell sampling of lumenal antigens by extension of their processes between epithelial cells whilst maintaining barrier integrity through the expression of tight junction (TJ) proteins [24] and (2) uptake across the characteristic follicle-associated epithelium (FAE) containing "microfold" (M) cells [25]. These specialized thin epithelial cells transfer effectively soluble, and especially particulate, antigens such as microorganisms from the lumen to dendritic cell [26]; (3) further, at least in mice, there is evidence that goblet cells deliver luminal antigen to dendritic cells in the small intestine [27]. Thus, there are multiple mechanisms by which antigen can traverse the gut wall.

A single layer of epithelial cells separates the apical and basolateral domains of the gut mucosa, and intercellular transport is regulated by complexes of TJ proteins, adherens, and desmosomes. Of these protein complexes, TJ proteins are located at the most apical side and play a central role in regulating permeability through the intercellular space within epithelial sheets [28–30]. TJs are composed of numerous structural and functional proteins including occludin and Claudin family members [31, 32] which together form a selectively permeable intercellular barrier [33]. Claudin family members have different expression pattern depending on cell type, location, and age, which may not be conserved across species [34–37]. Claudin-2, Claudin-3, and Claudin-4 have been detected in rat intestine [37, 38] and Claudin-1 to Claudin-4, Claudin-7 to Claudin-13, Claudin-15, and Claudin-18 have been detected in murine intestine [36]. Claudin-5 was initially attributed to be an endothelium-specific TJ protein [38, 39] but it has been specifically identified as an epithelial TJ protein as well [36, 37, 40]. Further, there are "tightening" Claudins (such as Claudin-1, Claudin-3, Claudin-4, and Claudin-5) [30, 40–42] as well as Claudins which meditate paracellular permeability for cations (such as Claudin-2 and Claudin-12) [37, 43]. Finally, mutations or changes in expression or surface localization of TJ proteins may lead to changes in intestinal permeability [30, 42, 44]. For instance, Bergmann et al. (2013) showed that mouse pups stressed for 12 hours showed increased intestinal permeability coincident with translocation of Claudin-4 from the region of the TJ on the surface of villous epithelial cells to the cytoplasm [42]. Age and environmental factors, at least in rodents, clearly impact epithelial cell surface localization of TJ proteins. Whether piglets, which are much more precocious at birth, also experience transitioning of TJ protein expression with age and region of the gut has not been elucidated.

We intend to establish whether there are regional and/or age-specific differences in the expression patterns of genes for FcRn, pIgR, Claudin-4, and Claudin-5. Florescent immunohistochemistry is used to establish patterns of Claudin-4 and Claudin-5 surface localization within distinct regions in the pig intestine over time to determine whether their surface localization changes are coincident with changes in intestinal permeability as the newborn gut matures. Claudin-4 and Claudin-5 were selected as a representative "tightening" TJ proteins found on intestinal epithelial cells and blood vessel endothelial cells, respectively. Because the gut of the newborn is "sterile" and microbiota contributes to maturation of the gut [45–48], we further investigated the role of commensal microbiota on tight junction protein surface localization.

2. Materials and Methods

2.1. Animal Use and Ethics and Description. This work was approved by the University of Saskatchewan's Animal Research Ethics Board and adhered to the Canadian Council on Animal Care Guidelines for humane animal use.

Conventionally raised Landrace cross piglets were obtained from the Prairie Swine Centre, Inc., Saskatoon, SK, Canada, and piglets within the 6 weeks of age group were weaned at 28 days of age.

Derivation of germ-free piglets, preparation of isolators, and experimental conditions for these piglets have been previously published [46]. Briefly, 16 piglets (>800 g of BW, Large White × White Duroc) were allocated to 4 treatment groups (n = 4/treatment) including piglets that remained germ-free (GF). Two groups were bottle-fed milk containing either 2 mL of 10^8 colony-forming units (CFU)/mL non-pathogenic *Escherichia coli* (EC) or 2 mL 10^9 CFU/mL with *Lactobacillus fermentum* (LF) (cultured from feces from a healthy sow as detailed in [46]) at 24 h and 30 h after birth. The bacterial inoculants were isolated from the cecum of a healthy adult sow, cultured for 18 hours at 37°C in a tryptic soy broth (BBL, Sparks, MD), and a subsample from each culture was taken for enumeration. Bacterial inoculants were typed to species level by sequencing of the chaperonin-60 universal target gene and query of cpnDB

Table 1: Primer information.

Gene	Source	Sequence (5′-3′)	Amplicon size	Annealing temperature
ACTB	Nygard et al., 2007 [49]	Forward: CACGCCATCCTGCGTCTGGA Reverse: AGCACCGTGTTGGCGTAGAG	108	60
CLDN4	NM_001161637.1 (NCBI)	Forward: CAACTGCGTGGATGATGAGA Reverse: CCAGGGGATTGTAGAAGTCG	140	60
CLDN5	NM_001161636.1 (NCBI)	Forward: CCTTCCTGGACCACAACATC Reverse: CACCGAGTCGTACACCTTGC	110	60
FCGRT	NM_214197.2 (NCBI)	Forward: GTCTGGGAAAGCCAGGTGT Reverse: CCTCCTTCCTCCAAGGTTTT	104	60
HPRT	Nygard et al., 2007 [49]	Forward: GGACTTGAATCATGTTTGTG Reverse: CAGATGTTTCCAAACTCAAC	91	60
OCLN	NM_001163647.2 (NCBI)	Forward: GAGTACATGGCTGCTGCTGA Reverse: TTTGCTCTTCAACTGCTTGC	102	60
PIGR	NM_214159.1 (NCBI)	Forward: GCCAAGGTCCTGGACAGATA Reverse: GTACACGGATTTCGGCTTCT	116	60
RPL19	AF_435591 (NCBI)	Forward: AACTCCCGTCAGCAGATCC Reverse: AGTACCCTTCCGCTTACCG	147	60

(http://www.cpndb.ca/cpnDB/home.php) [50]. Pigs in the conventional isolator were also fed at 24 hours and 30 hours after birth but their milk contained 2 mL of each of the monoassociated inoculants and 2 mL of fresh feces (obtained from a conventionally reared sow at Prairie Swine Centre Inc. mixed 1 : 1 with sterile phosphate-buffered saline (0.01 M phosphate, 0.15 M NaCl; pH 7.4)). This latter group is referred to as the sow feces (SF) group. Collectively, we will refer to the GF, EC, LF, and SF groups as the "gnotobiotic piglets." As described in Shirkey et al. (2006), all piglets were fed to satiety at 3-hour intervals for the first 24 hours after birth with sterile-filtered porcine serum (Gibco, Burlington, Canada) mixed 1 : 1 with Similac (Abbott Laboratories, Abbott Park, IL) [46]. On Day 1, pigs were trough-fed a mixture of 2 : 1 Similac water (4.7 g/100 mL protein; 12.2 g/100 mL lipid; 24.3 g/100 mL carbohydrate) *ad libitum*. Pigs were fed milk in troughs at eight-hour intervals for the remainder of the trial.

2.2. Tissue Collection. From conventionally raised piglets, piglets were humanely killed by captive bolt and exsanguinated. We obtained tissues from 24-hour-old ($n = 5$) and 6-week-old ($n = 5$) piglets for gene expression analysis. A 10-cm small segment of Peyer's patch-free jejunum and ileum was excised, sliced into smaller fractions, immediately snap-frozen in liquid nitrogen, and then stored at −80°C until RNA extraction. Immunohistochemistry (IHC) was performed on tissues from piglets that are 24 hours old ($n = 3$), 48 hours old ($n = 3$), 3 days old ($n = 3$), 5 days old ($n = 3$), and 6 weeks old ($n = 3$). Tissues were fixed in 10% buffered formalin (Sigma-Aldrich, Oakville, ON, Canada) for 48 hours and then processed and embedded in paraffin by Prairie Diagnostic Services, University of Saskatchewan.

From gnotobiotic piglets, as published in [46], piglets ($n = 4$ per group) were removed from the isolators at 14 days of age, weighed, and killed by submersion in CO_2 and exsanguinated. The small intestine was carefully dissected from the mesentery and its length was recorded. A 2 cm segment obtained at 50% (jejunum) and 95% (ileum) of the small intestinal length was placed in 10% buffered formalin for 24 hours before being transferred to 70% ethanol and embedded in paraffin.

2.3. Primer Design. Real-time primer sets for Claudin-4 gene (CLDN4), Claudin-5 gene (CLDN5), FcRn gene (FCGRT), pIgR gene (PIGR), and three stable reference genes (ACTB, HPRT, and RPL19) were designed using Primer3 software based on sequence data obtained from the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/) (Table 1). Where possible, primers were designed to span exon-exon junctions as identified by BLAST like alignment tool (BLAT) comparison with SusScrofa10.2 genomic build. The primer sets were further verified for dimer and hairpin formation using OligoAnalyser v3.1 (Integrated DNA Technologies (IDT); http://www.idtdna.com/pages/scitools/) and target specificity was confirmed using the basic logical alignment search tool (BLAST) against the NCBI nucleotide database. The PCR efficiency for the primer probe set was evaluated against a serial dilution of pooled samples and found to be greater than 95% for all genes. Data was normalized to the geometric mean of the reference genes and statistical analysis was carried out on ΔCt values.

2.4. RNA Extraction and Gene Expression Data Analysis Using Real-Time Quantitative PCR. Gastrointestinal jejunum and ileum ($n = 5$ biological replicates per age group) were ground using a mortar and pestle and total RNA was isolated from using Trizol Reagent (Life Technologies, Carlsbad, CA, USA) as per the manufacturer's instructions with the addition of a second isopropanol (Commercial Alcohols, Inc., Brampton, ON, Canada) precipitation to completely remove phenol and other contaminants. DNA contamination was removed using the DNA-free kit (Life Technologies) before RNA quantity

was determined on a NanoDrop spectrophotometer ND-1000 (NanoDrop, Wilmington, DE USA). RNA integrity was then evaluated on a 1.2% (w/v) denaturing agarose gel (Life Science Research Division, Bio-Rad Laboratories (Canada) Ltd., Mississauga, ON, Canada) to ensure that all samples had clear 28S and 18S ribosomal RNA banding patterns before they were carried forward. Reverse transcription (RT) was performed on 2 μg of total RNA using the High Capacity cDNA Reverse Transcription Kit (Life Technologies) as per manufacturer's instructions. Sample cDNA was then diluted in nuclease-free water to 10 ng/μL equivalent cDNA. Quantitative real-time polymerase chain reaction (qPCR) was performed in duplicate using 20 ng of equivalent cDNA, Kappa Fast Universal Mastermix (Kapa Biosystems, Wilmington, MA USA) with a primer concentration of 1 μM using the IQ5 qPCR system (Bio-Rad, Hercules, CA, USA). Data is presented at the log normalized $2^{-\Delta\Delta Ct}$ form held relative to average expression for corresponding tissues from the 6-week-old animals.

2.5. Immunohistochemistry. Tissue sections were deparaffinised in xylene (Sigma-Aldrich) and rehydrated to distilled water in decreasing concentrations of ethanol (Commercial Alcohols, Inc). Heat-induced antigen-retrieval (HIAR) was carried out in Tris-EDTA buffer (10 mM Tris, 1 mM EDTA Solution, 0.05% Tween 20, pH 9.0; Sigma-Aldrich) for 30 min at 90°C. Slides were blocked for 3 hrs at room temperature in 5% (w/v) skim milk (Bio-Rad) in PBSA and then incubated overnight at 4°C with a 1 : 250 dilution of rabbit anti-CLDN4 (ab53156, Abcam, Cambridge, MA, USA) or 1 : 100 dilution of rabbit anti-CLDN5 (ab53765, Abcam) in incubation buffer (1% BSA, 1% Donkey Serum, and 0.5% Triton X-100 in PBS; Sigma-Aldrich). Slides were then washed three time in PBS and incubated in a 1 : 500 dilution of FITC-labeled goat anti-rabbit IgG (4030-02, Southern Biosystems, Birmingham, AL, USA) for anti-CLDN4 or PE-labeled goat anti-rabbit IgG (ab97070; Abcam) in incubation buffer at 4°C for 4 hours. Slides were again washed three times in PBS before the cover slip was added with Prolong Gold antifade with DAPI (Life Technologies). Intestinal villi were imaged using an Axiovert 200 M with a 63X neoFluor objective (Zeiss, Oberkochen, Germany) under oil immersion. Finally, DAPI and FITC/PE images were background subtracted and merged using ImageJ software [51].

2.6. Statistical Analysis. All statistical analyses and graphing were performed using GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA). As outcome variables were found to not be distributed normally, differences among all groups were examined by using a Wilcoxon matched-pairs signed rank test. Differences were considered significant if $P < 0.05$. ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{****}P < 0.0001$).

3. Results and Discussion

3.1. Gene Expression Analysis on Jejunal and Ileal Tissues. To determine whether FCGRT, PIGR, CLDN4, and CLDN5 gene expression changed with age and/or showed differential expression depending on the region of the gut under investigation, qRT-PCR analysis was performed on segments of jejunum and ileal gut tissue from piglets that were 24 hours old ($n = 5$) or 6 weeks of age ($n = 5$). As expected, the gene expression patterns for the antibody binding receptors FcRn and pIgR were strikingly different. Tissues from 24-hour-old piglets and 6-week-old pigs showed equivalent expression levels of FCGRT in the jejunum while, in the ileum, 24-hour-old piglets showed a statistically significant induction of FCGRT over that of 6-week-old animals (Figure 1(a); $P < 0.01$). pIgR, on the other hand, was expressed at minimal levels in both the neonatal jejunum and ileum relative to that of jejunal and ileal-derived tissues from the 6-week-old pigs (Figure 1(b); $P < 0.0001$). Of course, it is not surprising that pIgR levels are high in 6-week-old piglets as this receptor would be required to translocate piglet-derived SIgA to the lumen. We simply point out that, because the PIGR is minimally expressed on newborn piglets' jejunal or ileal enterocytes, colostrum-derived SIgA would not be taken across the gut wall through binding and receptor-mediated endocytosis of the pIgR.

With regard to the expression transcripts for TJ proteins, CLDN4 was expressed at equivalent levels in the jejunum and ileum from 24-hour-old piglets relative to corresponding tissues in the 6-week-old animals with no statistically significant difference (Figure 1(c)). Statistically, less CLDN5 mRNA was expressed in the jejunum (Figure 1(d); $P < 0.05$) and ileum ($P < 0.01$) in the 6-week-old animals compared to the region-specific tissues obtained from animals that were 24 hours old (Figure 1(d)). As with CLDN4, the median values for CLDN5 expression from each region of the gut were highly conserved across both tissues for each age group.

3.2. Evaluation of Claudin-4 Surface Localization on Jejunal and Ileal Enterocytes. Different TJ family members localize to distinct regions (i.e., on the crypts or the villi) and, at the cellular level, they can be expressed along the lateral surface between adjacent cells or preferentially on the apical or basolateral surfaces. Tamagawa et al. (2003) showed that, in the mouse (age not specified), Claudin-2 was present within the crypts of the small and large intestine, Claudin-3 was present in both the villi and crypts, and Claudin-4 was only modestly associated with the villous tips of the small and large intestine [52]. Within these regions, Claudin-2 and Claudin-3 were localized to the apical surfaces of the intestinal epithelial cells but Claudin-4 appeared to be highly localized to FAE dome of the mouse intestine, which the authors speculate may regulate intercellular junctions to allow antigen sampling by dendritic cells [52]. Patel et al. (2012) showed that Claudin-3 localized to the TJs of crypt epithelium in 2- day-old and 2-week-old mice and transitioned to being also localized to the TJs in villous epithelium at three weeks of age when the murine gut is mature [53]. The kinetics of Claudin protein expression and surface localization may correlate with changes in gut permeability. Corroborating this hypothesis, Bergmann et al. (2013) showed that mouse pups stressed for 12 hours showed increased intestinal permeability coincident with translocation of Occludin and Claudin-4 from the region of the TJ on the surface of villous epithelial

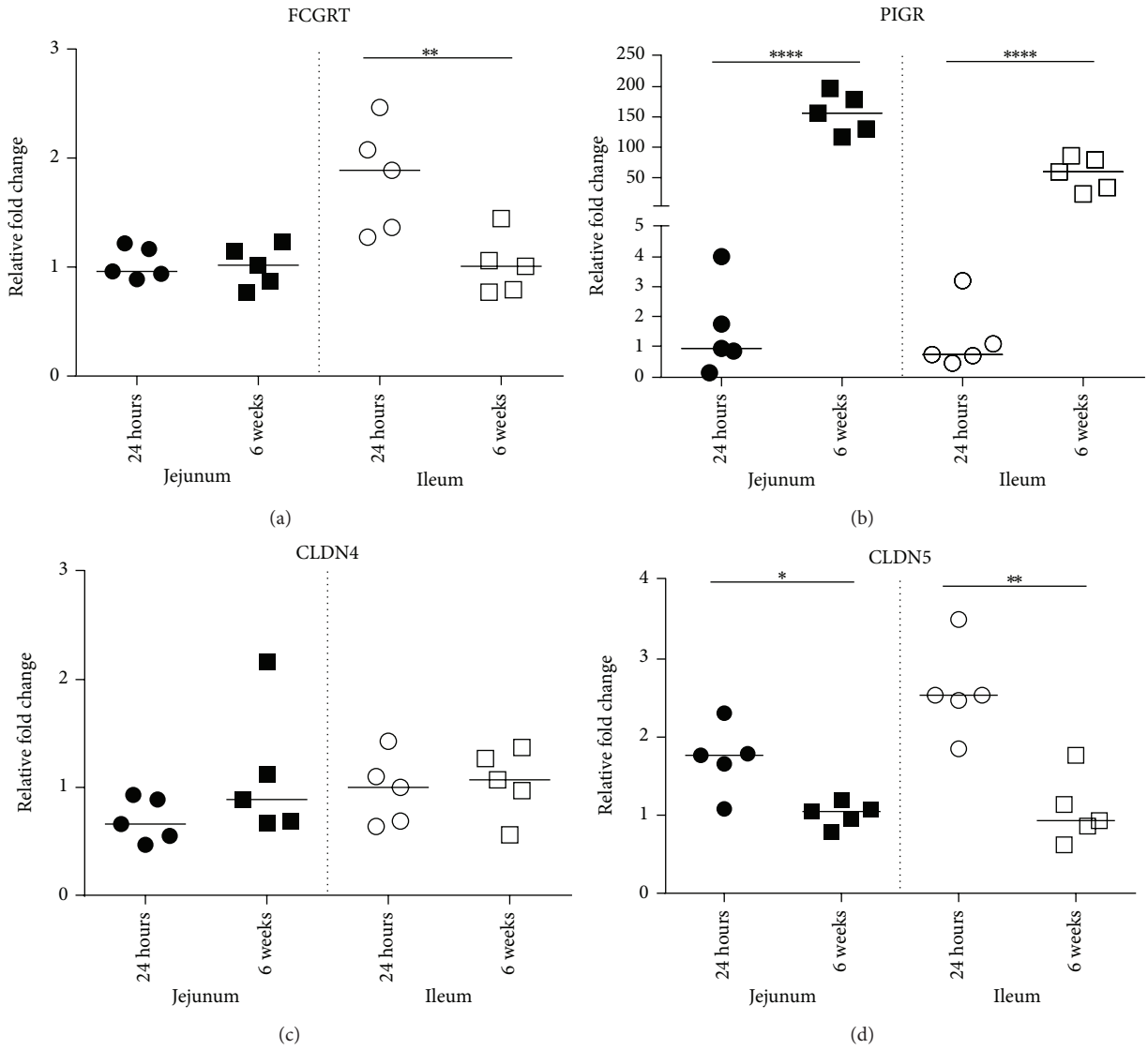
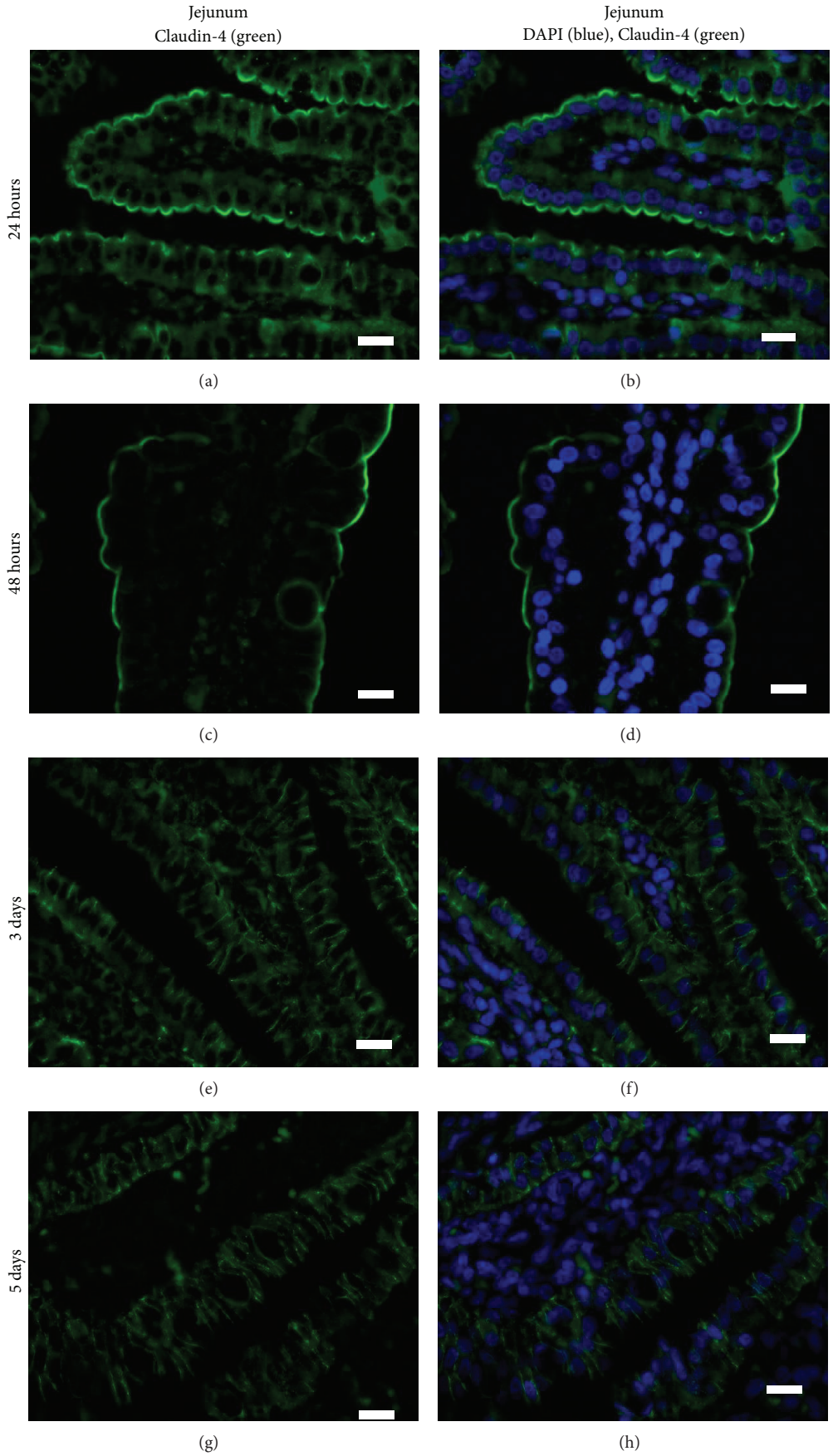


Figure 1: QPCR analysis of CLDN4 and CLDN5 expression in jejunal and ileal gut tissue. The mRNA expression levels of FCGRT, PIGR, CLDN4, and CLDN5 genes were normalized with the reference genes and were calculated with $2^{-\Delta\Delta Ct}$ relative quantification. Dots show the data for each biological replicate ($n = 5$ per group). Horizontal bars represent the median values ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{****}P < 0.0001$).

cells to the cytoplasm [42]. We used IHC to investigate whether there were age-specific or region-specific changes in Claudin-4 surface localization in pig intestinal epithelial cells. We observed that at 24–48 hours of age, Claudin-4 (green) was localized to the extreme apical aspect of the jejunal enterocytes along the villi and the crypts (only villi shown; Figures 2(a)–2(d)). During these times, very little if any Claudin-4 protein was present at the region of the cell where the TJs are formed. From tissues obtained from piglets that were 3 and 5 days of age, however, we clearly see that Claudin-4 was highly localized on the lateral surface of the cells in contact with adjacent enterocytes (Figures 2(e), 2(f), 2(g), and 2(h)). At 6 weeks of age, Claudin-4 level of expression appears even stronger than at 3 and 5 days of age and it is still localized along the cell surface adjacent to other enterocytes (Figures 2(i) and 2(j)). In stark contrast, when we investigated the surface localization pattern of Claudin-4 on ileal intestinal epithelial cells, we observed that, even at 24 hours of age (Figures 3(a) and 3(b)), Claudin-4 was localized along surface of the cells adjacent to other enterocytes (including the region of the TJs) and this pattern remained unchanged with age (Figures 3(c)–3(j)).

Thus, our data shows that, at 24 and 48 hours of age (Figures 2(a)–2(d)), Claudin-4 was localized to the apical surface of the jejunal enterocytes which coincides with the period of time in which the piglet jejunum is permeable to both maternal Ig and other macromolecules as well as maternal cells which, after ingestion, are reported to be quickly found in the circulation [4, 5]. Between 48 hours of age and 3 days of age, Claudin-4 relocated along the surface of the cells that are immediately adjacent to neighbouring epithelial cells which coincides with the time that jejunal



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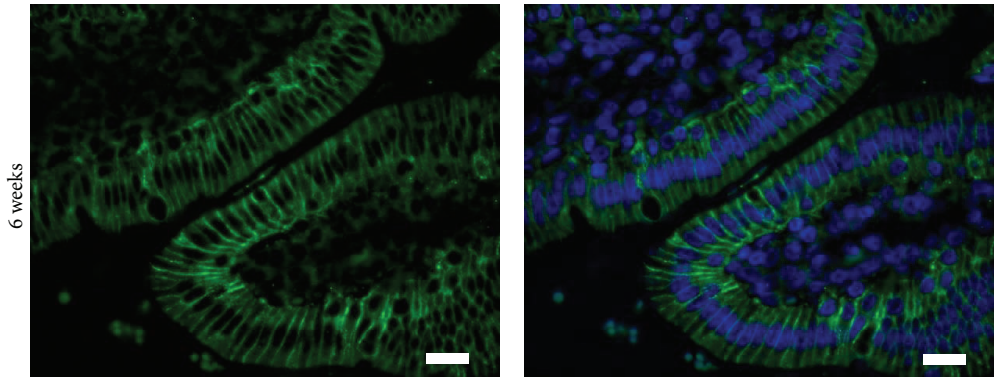
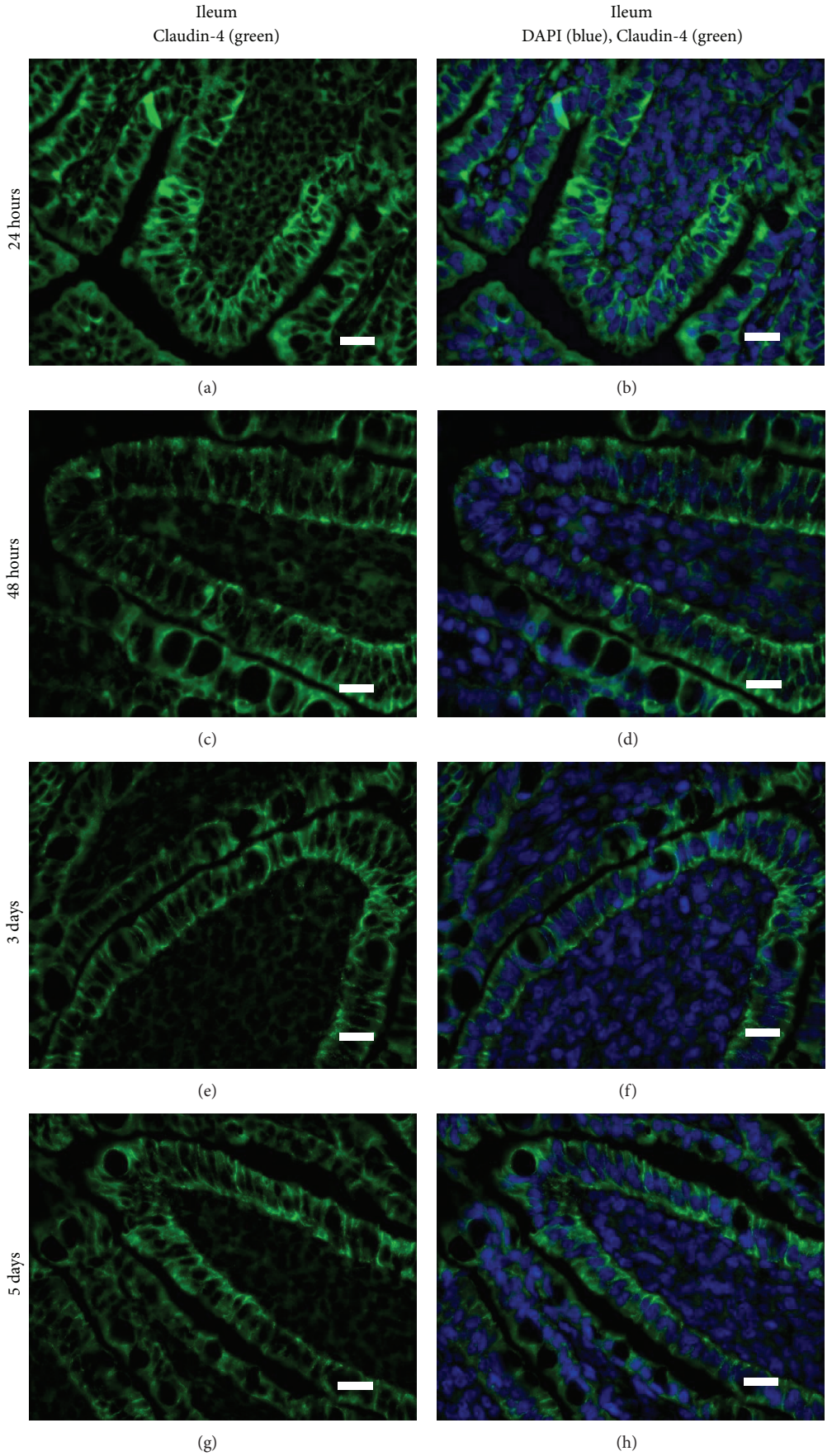


Figure 2: Claudin-4 surface localization changes over time in piglet jejunum. Claudin-4 is localized to the apical aspect of the surface of jejunal intestinal enterocytes at 24 hours of age ((a), (b)) and at 48 hours of age ((c), (d)). At 3 ((e), (f)) and 5 ((g), (h)) days of age, Claudin-4 is localized along the cellular surface between the adjacent cells and no longer at the apical aspect of the enterocytes. At 6 weeks of age, there is very strong expression at the lateral membranes between adjacent enterocytes ((i), (j)). These images are representatives of IHC performed on tissue from 3 animals per time point. Primary antibody: rabbit anti-Claudin-4. Secondary antibody: FITC-labeled goat anti-rabbit (green). Nuclear stain: DAPI (blue).

enterocytes cease to absorb macromolecules. We believe it is unlikely that jejunal enterocytes at 3 days of age represent "nonfetal" derived cells as the fetal enterocytes are reported to be replaced after 2-3 weeks [7]. However, more studies will need to be performed to verify that it is indeed fetal-derived enterocytes, which show a change in Claudin-4 surface localization. Claudin-4 is known to be a "tightening" Claudin [42] and the fact that it localizes to the tight junction region in the jejunum at the same time these cell lose their capacity to absorb macromolecules may reflect a mechanism of decreasing gut permeability [4]. Williams (1993) determined that, in colostrum-deprived piglets that were less than 4 hours old, ingested FITC-labeled colostral leucocytes penetrated the jejunal epithelium (in which our data indicates that Claudin-4 is not yet at the site of TJs at this time) but not the ileum (in which our data indicates that Claudin-4 is at the site of TJs within 24 hours after birth) [54]. However, at this time, we cannot say whether events are related or simply coincidental. In future studies, confocal microscopy will be used to establish whether jejunal or ileal enterocytes show a change in localization of Claudin-4 from the cytoplasm to the surface, which may contribute to changes in gut permeability. Further, we cannot find any studies which determine how long after birth maternal colostrum-derived cells can traverse the gut wall. If they can cross the gut wall after one week of age, for example, then it is unlikely that Claudin-4 surface localization to the region of tight junctions regulates maternal cell uptake. However, if maternal cells cannot traverse the gut wall after 2 days of age, then perhaps regulation of Claudin-4 surface localization may mediate cellular uptake. These studies may have important implications for pig husbandry as piglets are routinely cross-fostered and therefore do not have continuous access to colostrum from their dams.

3.3. Evaluation of Claudin-5 Surface Localization on Jejunal and Ileal Blood Vessel Endothelial Cells. CLDN5 is a TJ-regulating gene on epithelial cells and endothelial cells which plays a role in regulating intestinal and vascular permeability [36, 37, 40, 55, 56]. We next investigated whether Claudin-5 was expressed on piglet intestinal epithelial cells and the endothelial cells that line the blood vessel walls. IHC was performed on jejunal villi and blood vessels in the submucosa. In contrast to studies in rats and mice where Claudin-5 was shown to be expressed on intestinal epithelial cells, Claudin-5 was not expressed on the surface of jejunal or ileal villous epithelial cells (Figures 4(a) and 4(b)) in the piglet, but it was expressed on the blood vessel endothelial cells from this age group (Figures 4(c) and 4(d)). In a similar fashion, Claudin-5 was absent from the villi on the 6-week-old animals (Figures 4(e) and 4(f)) but it was present on their blood vessel endothelial cells (Figures 4(g) and 4(h)). (The red fluorescent cells in the lamina propria (Figures 4(c) and 4(d)) are autofluorescent cells and the red fluorescent cells within the blood vessel (Figures 4(g) and 4(h)) are red blood cells.) Our data shows that Claudin-5 surface localization on endothelial cells does not change with age and likely is not critical for regulating macromolecule transport into blood vessels as the piglet gut matures. It may be that another TJ protein besides Claudin-5 fulfills this role or it may be that paracellular leakage is not the method by which cells or macromolecules absorbed by jejunal or ileal epithelial cells enter into the neonatal blood stream. Vascular permeability assays or transendothelial leukocyte migration assays may provide insight into whether the blood vessels are transiently permeable in the neonatal piglet gut prior to 2 days of age.

We recognize that it would be of significant interest to characterize the surface expression of many other claudin family members as well as members of the zonodulin, occludin, and junction adhesion molecule families. We evaluated several commercial antibodies, which were indicated to be cross-reactive with pig but, despite using several antigen-retrieval methods, only Claudin-4 and Claudin-5 were successful for use in IHC in our hands (data not shown).



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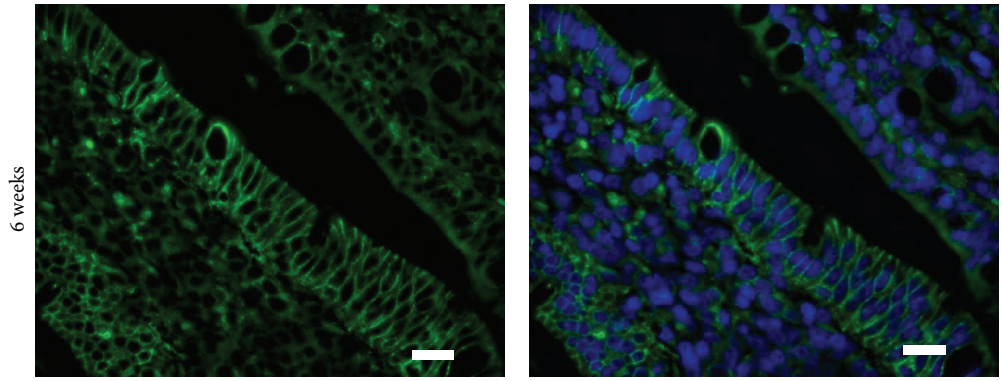

Figure 3: Claudin-4 surface localization does not change over time in piglet ileum. Claudin-4 is highly expressed on the surface of ileal intestinal enterocytes with the highest expression on the surfaces between adjacent cells at 24 hours of age ((a), (b)), 48 hours of age ((c), (d)), 3 days of age ((e), (f)), 5 days of age ((g), (h)), and 6 weeks of age ((i), (j)). These images are representatives of IHC performed on tissue from 3 animals per time point. Primary antibody: rabbit anti-Claudin-4. Secondary antibody: FITC-labeled goat anti-rabbit (green). Nuclear stain: DAPI (blue). Scale bar represents 40 μm.

3.4. Commensal Microbiota Does Not Influence Claudin-4 Surface Localization. The newborn intestine is sterile at birth, but it quickly becomes colonized with microbes derived from the maternal birth canal and the external environment. *E. coli* and other coliforms are the earliest colonizers of the pig digestive tract followed by *Clostridium* and *Lactobacillus* species which supplant *E. coli* as the dominant isoform within 48 hours [57, 58]. We previously reported that early colonizing nonpathogenic *E. coli* and *Lactobacillus fermentum* differentially affect villous structure [46], enterocyte turnover [59], and intestinal maturation specifically pertaining to digestive function [45]. Here, we wanted to determine whether Claudin-4 surface localization on gut epithelial cells was influenced by colonization of the piglet gut with commensal flora. We compared intestinal Claudin-4 surface expression on the villi from piglets raised for 14 days as GF animals, animals monocolonized by *EC* or *LF* or colonized with microbiota from sow feces (SF) spiked with EC and LF. The gut segment at 50% of the length of the small intestine (previously shown to be devoid of ileal Peyer's patches (IPP) and therefore likely represent the jejunum) was chosen for examination [46]. Because Claudin-4 was already expressed in the regions of the TJs in ileal villous epithelium at 24 hours of age (Figures 3(a) and 3(b)), this region was not investigated in the gnotobiotic piglets. Regardless of whether piglets were raised GF (Figures 5(a) and 5(b)) or colonized with EC (Figures 5(c) and 5(d)), LF (Figures 5(e), and 5(f)), or SF (Figures 5(g) and 5(h)), the jejunal villi and crypts (crypt data not shown) showed comparable Claudin-4 surface localization on the region of the cells in contact with adjacent enterocytes. These data suggest that either Claudin-4 localization was not influenced by microbiota or, if it did have an impact, it was rectified by 14 days of age.

We find it intriguing that commensal microbiota did not appear to impact Claudin-4 surface localization in jejunum of 14-day-old gnotobiotic piglets. Studies in mice showed that although the gut is functionally mature at 3 weeks of age, mice treated for the first weeks of life with antibiotics exhibited decreased and immature expression of Claudin-3 and immature barrier function compared with control mice [53]. These authors go on to show that mice enterally administered live or heat-killed probiotic bacteria *Lactobacillus rhamnosus GG* showed significantly improved barrier function with decreased intestinal permeability [53]. These results indicate that, at least in mice, intestinal barrier function, as regulated by TJ protein expression, can be influenced by gut microbiota. Others showed that *B. infantis* positively impacted TJ formation and barrier-preserving properties [42]. Probiotic bacteria may act by normalizing microbial populations or by directly improving host defense mechanisms, specifically by strengthening intestinal barrier function, which, in turn, may reduce systemic entry of gut luminal microbes or toxins. These data suggest that, in mice, Claudin-3 and Claudin-4 may play a pivotal role in probiotic-induced barrier maturation [42]. Likewise, a study in seven-week-old dairy calves showed that calves fed milk replacer plus calf starter had different expression patterns for the genes coding for Occludin and Claudin-4 (but not Claudin-1) in the jejunum and the ileum when compared to calves fed milk replacer alone [60]. The authors speculate that these differences may be due to diet-specific bacterial diversity [60]. Using IHC, our data indicates that, in 14-day-old piglets, Claudin-4 localization to TJ of jejunal epithelial cells was not impacted by floral colonization.

Colostrum is rich in maternal leucocytes and it is estimated that piglets absorb several hundred million maternal cells daily [61, 62]. The mechanism of maternal cell uptake is not yet clear. Fluorescently labeled colostral leucocytes ingested by 2–4-hour-old, colostrum-deprived piglets penetrated the duodenal and jejunal epithelium, whereupon they migrated through the lymphatics and the peripheral blood and seeded nonlymphatic tissues, including mesenteric lymph nodes, spleen, liver, and lungs [54]. These cells were not absorbed across the ileum suggesting that the process

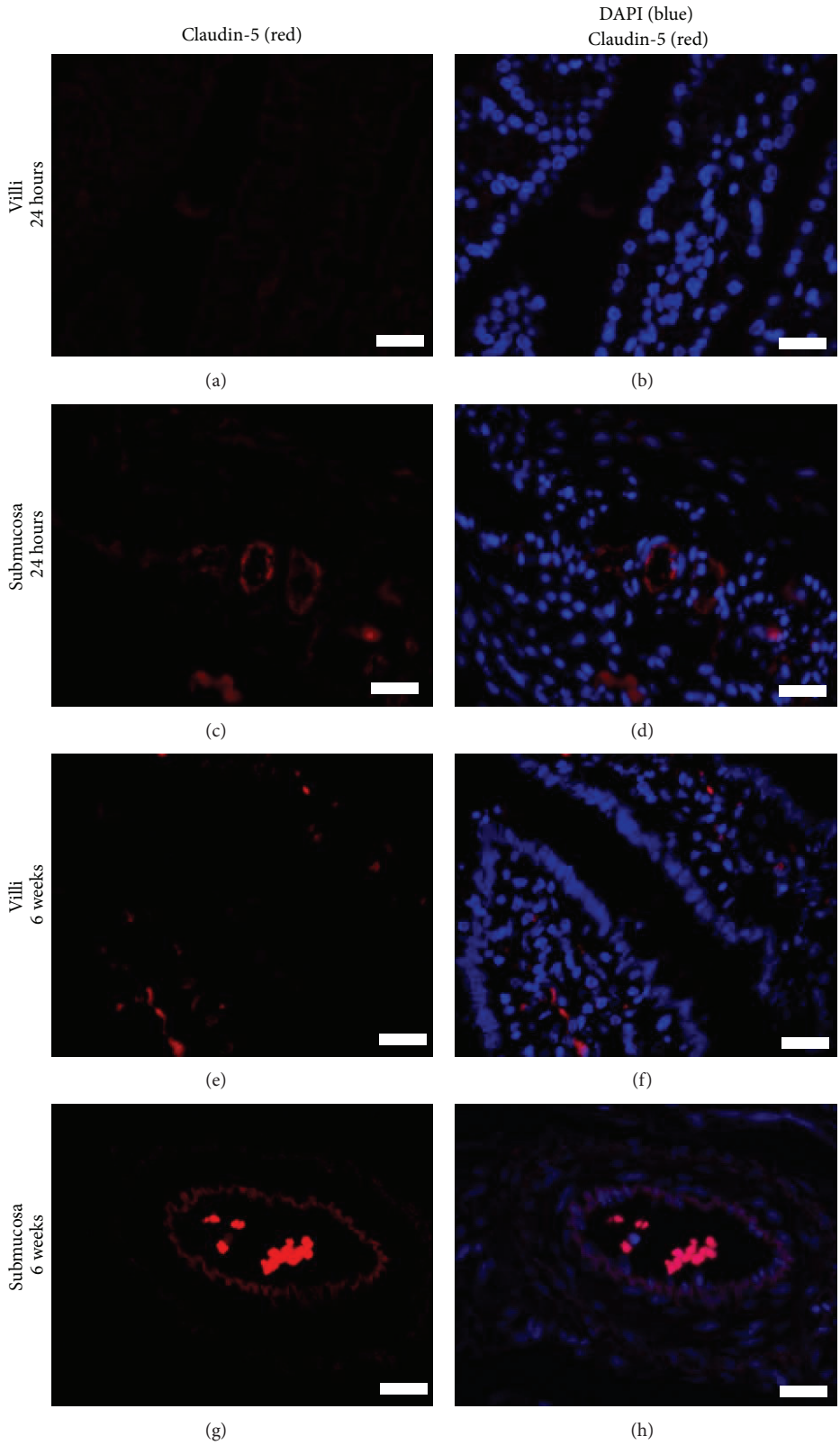

Figure 4: Claudin-5 surface localization on blood vessel endothelial cells does not change over time. Claudin-5 is absent from the surface of jejunal enterocytes in 24-hour-old ((a), (b)) and 6-week-old piglets ((e), (f)), respectively but it is present on the blood vessel walls in the submucosa for both age groups ((c), (d), (g), and (h)). Red cells within villi are from autofluorescent cells and red cells within the blood vessels are red blood cells. These images are representatives of IHC performed on tissue from 3 animals per time point. Primary antibody: rabbit anti-Claudin-5. Secondary antibody: PE-labeled goat anti-rabbit (red). Nuclear stain: DAPI (blue). Scale bar represents 40 μm.

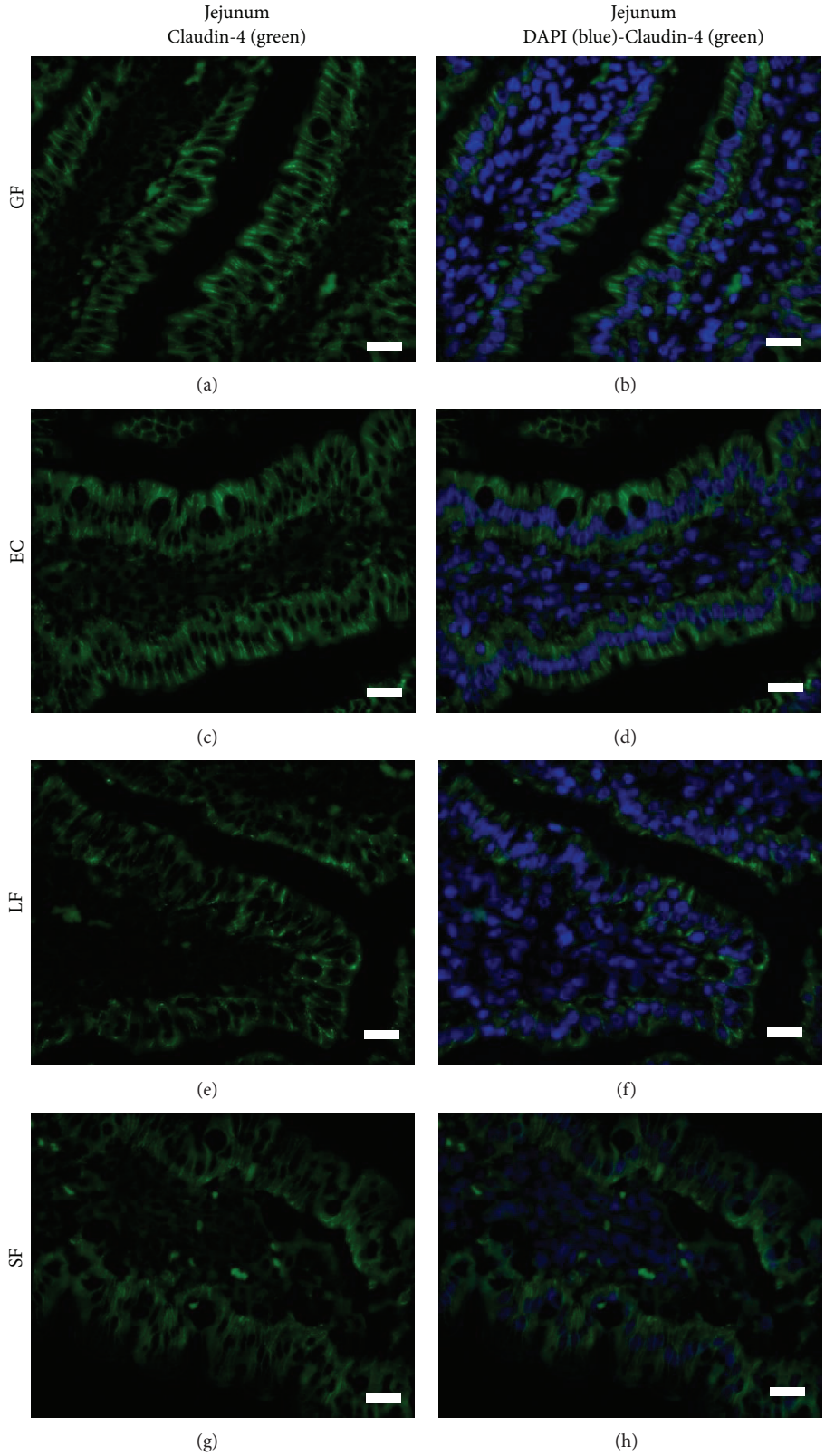

Figure 5: Claudin-4 surface localization in the piglet jejunum is not influenced by colonization by commensal microbiota.In the jejunum of 14-day-old germ-free piglets ((a), (b)), piglets colonized with EC ((c), (d)), LF ((e), (f)), or conventional microbiota (SF; (g), (h)), Claudin-4 is localized to the surface between adjacent enterocytes. These images are representatives of IHC performed on tissue from 4 animals per time point. Primary antibody: rabbit anti-Claudin-4. Secondary antibody: FITC-labeled goat anti-rabbit (green). Nuclear stain: DAPI (blue). Scale bar represents 40 μm.

is regionally selective [54]. Further, electron microscopy showed that radiolabeled, colostrum-derived lymphoid cells administered to piglets between 7 and 10 hours after birth were absorbed but if the cells were heat-treated, derived from the sow's blood, or obtained from another sow's colostrum, the cells did not traverse the gut wall, suggesting that the cells themselves may facilitate uptake [61]. Piglets born to vaccinated sows received sufficient *M. hyopneumoniae*-specific cell-mediated immunity to elicit significant delayed-type hypersensitivity responses three days after *M. hyopneumoniae* exposure, indicating that maternal cells taken up by the piglet are functional [63]. A study in calves showed that blood-derived PBMCs traversed the gut wall of 6-hour-old calves but only if the cells were incubated with acellular colostrum suggesting that factor(s) in colostrum promotes changes in the leucocytes which is necessary for uptake [64]. It is not yet clear how maternal cells traverse the gut wall but the above experiments suggest that colostrum-derived lymphoid cells play an active role in uptake and that the route may be through the paracellular route [61]. Future experiments will be performed to elucidate whether maternal cells and macromolecules indeed traverse the neonatal gut wall between adjacent enterocytes until Claudin-4 has relocated to the lateral surface of the cell. We will also use Ussing chambers to show that, upon Claudin-4 translocation to the lateral membrane, paracellular transport of labeled cells or macromolecules ceases. These experiments will provide indirect evidence that Claudin-4 is critically required for functional TJs.

4. Conclusions

Thus, our data indicates that, in the mixed cell populations that comprise jejunal and ileal tissue, FCGRT was minimally expressed in the ileum but showed higher expression in the jejunum from piglets less than 24 hours old. In contrast, PIGR gene expression showed a striking increase in both regions in the 6-week-old piglet gut relative to the tissues from the 24-hour-old piglets. CLDN4 and CLDN5 transcript abundance was conserved in jejunum and ileum in age-matched animals and striking differences in CLDN4 expression did not occur in either region of the gut with age. CLDN5 showed significantly higher expression in the jejunum and ileum from the 24-hour-old animals relative to the older animals. At the time period when the piglet gut is considered "leaky" (i.e., within the first two days of life) [4], jejunal enterocytes showed Claudin-4 protein localization at the apical aspect of jejunal enterocytes whereas ileal intestinal epithelial cells showed Claudin-4 localization at the surface associated with TJ formation. At 6 weeks of age, Claudin-4 was localized to lateral membranes in both the jejunum and ileum enterocytes. These data are intriguing as they provide evidence that Claudin-4 is not localized to the region of the jejunal TJs at the time when the jejunum is permeable. Microbiota did not impact Claudin-4 localization in 14-day-old piglets. More experiments are needed to directly establish whether Claudin-4 localization on jejunal villous epithelial cells directly impacts paracellular permeability in the neonatal piglet gut and thus impacts uptake and subsequent transport of maternal cells, antibodies, and other macromolecules into the blood.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Review Article

Transcriptional Regulators of Claudins in Epithelial Tight Junctions

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Human gastrointestinal tract is covered by a monolayer of specialized epithelial cells that constitute a protective barrier surface to external toxic and infectious agents along with metabolic and digestive functions. Intercellular junctions, among epithelial cells, such as desmosomes, adherens, gap, and tight junctions (TJs), not only provide mechanical integrity but also limit movement of molecules across the monolayer. TJ is a complex structure composed of approximately 35 different proteins that interact with each other at the apical side of two adjacent epithelial cells. Claudin family proteins are important members of TJ with so far 24 known isoforms in different species. Claudins are structural proteins of TJ that help to control the paracellular movement by forming fence and barrier across the epithelial monolayer. Altered function of claudins is implicated in different form of cancers, inflammatory bowel diseases (IBDs), and leaky diarrhea. Based on their significant role in the molecular architecture of TJ, diversity, and disease association, further understanding about claudin family proteins and their genetic/epigenetic regulators is indispensable.

1. Introduction

Epithelial monolayer (EM) is the largest body tissue lining many organs in the human body. In the intestine, EM provides protection to the internal body from toxic and infectious agents while at the same time it facilitates absorption of digested food and water from the gut. Epithelial monolayer integrity and paracellular transport are the important features that can be protected and maintained with the help of epithelial barrier function [1]. Epithelial cells are connected with each other by four types of junctions, that is, desmosomes, gap junctions, adherens junctions, and TJs [2–4]. Tight junctions are impermeable and control the movement of molecules and ions via a paracellular pathway. Until recently, tight junction functions were categorized as "fence" as they separate the apical and basolateral cell surface domain defining cell polarity or a "barrier" due to their control over solutes and liquid flow through the paracellular space between the epithelial cells [5–8]. However TJs are not restricted to the fence and barrier function but have been defined to participate in signal transduction processes, gene expression, cell proliferation, and differentiation [9–11]. Various unidentified external and internal regulators impair the normal function of TJs causing loss of water and solute in the passive manner that leads to leaky-flux watery diarrhea. The unwanted invasion of noxious luminal antigens prolongs the existence of mucosal inflammatory processes [12].

Tight junction (TJ) is a complex structure constituting of growing numbers of components, including integral membrane proteins (claudins, occludin, and junctional adhesion molecules "JAMs") and peripheral membrane proteins. The peripheral membrane proteins include (1) scaffold PDZ (postsynaptic density protein (PSD95), Drosophila discs large tumor suppressor (Dlg1), and Zonula occludens-1 protein (ZO-1)), multi-PDZ domain protein-1 (MUPP-1), and membrane-associated guanylate kinase (MAGI-1); (2) no-PDZ expressing proteins such as cingulin, symplekin,

atypical protein kinase C, Ras-related protein Rab-3B (Rab-3b), Ras-related protein Rab-13 (Rab-13), phosphatase and tensin homolog (PTEN), and 7H6 antigen; (3) cell polarity molecules ASIP/PAR-3, partitioning defective 6 homolog alpha (PAR-6), and PALS1-associated TJ protein (PATJ) [13, 14]. Besides these proteins, tricellulin protein has recently been identified at the epithelial cell junctions with involvement in the barrier function [15].

Claudin family so far includes 24 reported members in different types of mammalian cells; among them 21 are known components of TJ in EM in the kidneys, liver, brain, and intestine [25]. These are involved in various physiological processes such as regulation of paracellular permeability and conductance. Claudins are found in homo and heterotypic manner in single TJ [13, 26]. They can be divided into two main categories, "pore-sealing" and pore-forming claudins. Claudin-1, -3, -4, -5, -7, and -19 are known as pore-sealing claudins and an increased expression of these claudin proteins leads to increased tightness of EM and increased transepithelial electrical resistance (TEER) and decreases solute permeability across the monolayer [27–31]. On the other hand, claudin-2 and -15 are considered as the "pore-forming claudins," because of their ability to form paracellular anion/cation pores as well as water channels and therefore they decrease epithelial tightness and increase solute permeability [13, 32].

Epithelial barrier dysfunctions occur in inflammatory bowel diseases (IBDs) like Crohn's disease (CD) or ulcerative colitis (UC) that contribute to leaky-flux diarrhea, that is, loss of solutes and water in increased amount dependent upon the components of TJ proteins. Downregulation of pore-sealing claudins (e.g., 4, 5, and 8) while upregulation of pore-forming claudin-2 is observed in active Crohn's disease patients [33, 34]. Similarly downregulation of pore-sealing claudin-4 is also associated with UC disease [34]. Numerous studies have reported leaky diarrhea in patients undergoing immunosuppressive therapy after organ transplantation [35–38]. Recently our group has reported mycophenolic acid-(MPA-) mediated increased expression of myosin light chain kinase (MLCK), myosin light chain-2 (MLC-2), and MLC-2 phosphorylation and redistribution of ZO-1 and occludin in Caco-2 and in HEK-293 cells [39, 40] as a possible mechanism of diarrhea in patients undergoing immunosuppressive therapy. Transcription factors (TFs) play an important role in the gene regulation at the promoter level working either as an activator or as a repressor of a specific gene. The current review will focus major claudins family members (Table 1) and their regulators, which alter claudins gene activity at promoter level and therefore modulate TJs structure and function.

2. Claudin-1

Claudin-1 protein is a key constituent of TJs and its altered expression is reported in a variety of cancers, most prominently colorectal cancers [13, 17, 41, 42]. Promoter region (−1160 bps to −850 bps) of claudin-1 consists of putative binding sites for caudal-related homeobox (cdx-1, -2), GATA4, and T-cell factor/lymphoid enhancing factor-1 (Tcf/Lef-1) transcription factors. There is a direct correlation between claudin-1 and cdx-2 expression in human colon cancer patient [17]. Cdx-2 is a homeobox domain-containing nuclear transcription factor that plays an important role in intestinal development by regulating the proliferation and differentiation of intestinal cells [43–45], and it is expressed in all cells along the crypt villus axis. Cdx-2 transcriptional activity is controlled through mitogen-activated protein kinase/extra cellular signal regulated kinase pathway (MAPK/ERK pathway) which phosphorylates it at ser-60 position and resultantly reduces cdx-2 transcription activity in crypt and lowers villus cells. On the other side, cyclin-dependent kinase 2 (CDK2) phosphorylates cdx-2 at Ser-281 which coordinates cdx-2 polyubiquitination and degradation by the proteasome [43, 46–49].

Specificity protein-1 (Sp-1) is the first identified transcription factor of specificity protein/Krüppel-like factor (Sp/XKLF) family, consisting of 785 amino acids (aa) with molecular weight of 100 to 110 kDa. Sp-1's DNA binding domain is the most conserved among other domains of SP family members which consisted of Cy2His2 Zinc (Zn) fingers. Mutational analysis has revealed that Zn fingers 2 and 3 are essential for DNA binding activity [50]. Sp-1 binds to the GC-rich elements [51] that are common regulatory elements in the promoters of numerous genes. Sp-1 binds its individual binding sites as a multimer and is capable of synergic activation of promoters containing multiple binding sites [52] and regulates transcription by dynamically recruiting and forming complexes with many factors associated with transcription [53]. Normally Sp-1 has been described as a transcriptional activator but it can also act as a repressor [54]. Claudin-1 promoter region (−138 to −76 bp) contains Sp-1 binding site and a mutation in this region results in a significant loss of claudin-1 transcription [16].

3. Claudin-2

Claudin-2, also known as leaky protein, forms paracellular water channels in TJs and mediates paracellular transport of water molecules across the EM. EM permeability is enhanced by increased expression of claudin-2 in TJs. It is also involved in many signaling pathways, including vitamin D receptor, epidermal growth factor receptor (EGFR), and c-Jun N-terminal kinases (JNK) signaling pathways, and contributes to inflammatory bowel disease and colon cancer [33, 55–58]. *Salmonella* infection facilitates bacterial invasion across the EM by inducing claudin-2 expression and altering its localization in TJs which is reversible by specific inhibitors (EGFR (Gefitinib) and JNK (SP600125)), making claudin-2 as a potential therapeutic target to prevent bacterial invasion and inflammation [59].

Interleukin-6 (IL-6) increases TJ permeability of Caco-2 monolayer from the basal side by inducing caludin-2 expression. IL-6 activates the mitogen-activated protein kinases/extracellular signal-regulated kinases (MEK/ERK) pathway by inducing phosphorylation of ERK and phosphatidylinositol 3′-kinase (PI3K/Akt) by phosphorylating

Table 1: Regulators of claudins.

TJ proteins	Regulator	Promoter binding region	Expression/reference
Claudin-1	Sp-1	−138 to −76 bp	↑ [16]
	cdx-2	−1160 to −850 bp	↑ [17]
Claudin-2	cdx-2	−1067 to −1 bp	↑ [18]
Claudin-3	Sp-1	−112 to −74 bp	↑ [19]
Claudin-4	Sp-1	−105 to −49 bps	↑ [20]
Claudin-5	FoxO1	−2,906 to −2,871 bps	↓ [21]
Claudin-7	ELF-3	−150 bps	↑ [22]
Claudin-15	Hnf4α	−693 to −47 bps	↑ [23]
Claudin-19	Sp-1	−139 to −75 bps	↑ [24]

Note: arrow (↑) = upregulation, arrow (↓) = downregulation.

Akt, which in turn enhances cdx-2 expression. In the claudin-2 promoter region (−1067 to −1), four cdx-2 (cdx-A, -B, -C, and -D), STAT, and nuclear factor-kappa-light-chain-enhancer of activated B cells (NF-κB) putative binding sites are identified. IL-6 induced expression of claudin-2 can be reversed by using either specific inhibitors of MEK/ERK and PI3K/AKT pathways (U0126 (a MEK inhibitor) and LY294002 (a PI3K inhibitor)) or site directed mutagenesis in the putative cdx-2 binding sites in the promoter region of claudin-2 gene [18].

4. Claudin-3, Claudin-4, and Claudin-5

Both claudin-3 and claudin-4 are overexpressed in ovarian cancer. A Sp-1 binding site (−112 and −74 bps) in the promoter region of claudin-3 is crucial for its activation. Claudin-3 expression is significantly decreased at mRNA and protein levels, by knocking down the Sp-1 with siRNA, indicating an essential role of Sp-1 in claudin-3 activation [19]. Claudin-4 is mainly expressed in the EM of colon, renal tubules, mammary gland, and thyroid gland and is considerably raised in their cancers [60]. There are two known Sp-1 binding sites (between −105 and −49 bps) in the promoter region of claudin-4 [20].

Caludin-5 is mostly expressed in the TJs of EM of pancreatic acinar cells, alveolar lung cells, colon, and endothelial cells forming the blood-brain barrier and endoneurial blood-nerve barrier. In colonic regions, its expression is mainly involved in the paracellular sealing of TJs [33, 61–63]. Both downregulation and redistribution of claudin-5 can alter TJs structure leading to barrier dysfunction in active Crohn's disease [33]. Forkhead box (foxO) gene family members are potent transcriptional activators with four known members; foxO1 (also known as foxO1a), foxO3 (also known as foxO3a), foxO4, and foxO6 which bind to conserved consensus core recognition motif TTGTTTAC [64–66]. Four pairs of putative binding sites for foxO and tcf-β-catenin (Tcf-β-catenin act as a stabilizer) are identified in the three regions of claudin-5 promoter (region 1, position −2,906/−2,871; region 2, position −2,317/−2,287; region 3, position −1,103/−1,008). Both foxO1 and tcf-β-catenin interact with region 1 of the caludin-5 promoter to repress its transcription [21].

5. Claudin-7

Claudin-7 is expressed prominently in the biphasic type of synovial sarcoma of adults. E74-like factor 3 (ELF3) belongs to E26 transformation-specific sequence (ETS) family of transcription factors and binds to the Ets binding site in the promoter region (−150 bps) of claudin-7 [22]. Members of ETS family are mainly involved in cell differentiation, proliferation, and cell transformation [67]. Regulation of the target genes by ETS factors depends upon their activation by MAPK and their association with other cofactors [68, 69]. An essential role of ELF3 is reported in epithelial cell differentiation [70–72] and small interference RNA (siRNA) treatment downregulates the claudin-7 expression validating the central role of ELF3 in claudin-7 activation.

6. Claudin-15

Claudin-15 is a pore-forming protein expressed in the EM of intestine, liver, and kidney tissues. Downregulation of claudin-15 decreases permeability of EM layer and can initiate IBD. Four putative binding sites (BS1-4) of transcription factor hepatocyte nuclear factor 4 alpha (hnf4α) are present in the (−693 to −47 bps) region of claudin-15 promoter [23]. Hnf4α is considered as an important regulator of EM barrier integrity and is involved in the regulation of metabolism, cell junction, differentiation, and proliferation of liver and intestine epithelial cells [73]. Both animal model and IBD patients' biopsy studies have shown that an altered expression of hnf4α directly influences the expression and distribution of claudin-15 [23].

7. Claudin-19

The kidney is responsible for the filtration of excretory material from the blood. However, 25–40% of filtered Na^+ [74], 50–60% of filtered Mg^{2+} [75], and 30–35% of filtered Ca^{2+} [76] are reabsorbed into the body by thick ascending limb, the loop of Henle. Claudin-16 and -19 play a main role in the regulation of Mg^{2+} reabsorption and loss of either claudin-16 or -19 leads to excessive renal waste of Mg^{2+} [77]. Four putative transcription factor (not characterized, AP2,

NF-E, and Sp-1) binding sites are located between −139 and −75 in the promoter region of mouse claudin-19. However, only Sp-1 is described for having an important role in the expression of claudin-19 and a mutation in Sp-1 binding site significantly reduces the claudin-19 expression [24].

8. Conclusion

Tight junctions play an important role in the regulation of paracellular movement of molecules across the EM, impart mechanical strength, maintain the polarity of cells, and prevent the passage of unwanted molecules and pathogens through the space between the plasma membranes of adjacent cells. The efficiency of the junction in preventing ion passage increases exponentially with the number of strands of claudins family proteins which are having important role in the structure as well as controlling paracellular movement across the tight junctions. Altered expression of claudins family proteins in TJs plays a key role in numerous abnormalities like cancers, IBDs, and leaky diarrhea and a better understanding of their regulatory mechanism could help in designing innovative therapeutic strategies.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Oxidative Stress in Patients with Alzheimer's Disease: Effect of Extracts of Fermented Papaya Powder

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Brain tissue is particularly susceptible to oxidative stress (OS). Increased production of reactive oxygen species (ROS), reduced antioxidant systems, and decreased efficiency in repairing mechanisms have been linked to Alzheimer's disease (AD). Postmortem studies in AD patients' brains have shown oxidative damage markers (i.e., lipid peroxidation, protein oxidative damage, and glycoxidation). Fermented papaya (FPP, a product of *Carica papaya Linn* fermentation with yeast) is a nutraceutical supplement with favorable effects on immunological, hematological, inflammatory, and OS parameters in chronic/degenerative diseases. We studied 40 patients (age 78.2 ± 1.1 years), 28 AD patients, and 12 controls. Urinary 8-OHdG was measured to assess OS. Twenty AD patients were supplemented with FPP (Immunage, 4.5 grams/day) for 6 months, while controls did not receive any treatment. At baseline, 8-OHdG was significantly higher in patients with AD versus controls (13.7 ± 1.61 ng/mL versus 1.6 ± 0.12 ng/mL, $P < 0.01$). In AD patients FPP significantly decreased 8-OHdG (14.1 ± 1.7 ng/mL to 8.45 ± 1.1 ng/mL, $P < 0.01$), with no significant changes in controls. AD is associated with increased OS, and FPP may be helpful to counteract excessive ROS in AD patients.

1. Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disorder, and its incidence increases with age [1]. AD is characterized by the presence of several pathological hallmarks including neuronal loss, formation of senile plaques composed by extracellular deposits of amyloid beta (Aβ) caused by an abnormal processing of amyloid-beta precursor protein (APP), intracellular neurofibrillary tangles (NFT) composed of aggregated hyperphosphorylated tau proteins in brain, proliferation of astrocytes, and activation of microglial. These features are accompanied by mitochondrial dysfunction and alterations in neuronal synapses [1]. The molecular and pathophysiological mechanisms that underlie AD still have many dark sides. Even though AD is multifactorial, its etiology and the exact mechanism that triggers the pathological alterations are still not clear. Although most studies have suggested that the Aβ peptide (amyloid cascade hypothesis) may initiate and/or contribute to the pathogenesis of AD, the mechanisms through which it causes neuronal loss, and tau abnormalities still remain poorly understood. Reactive oxygen species (ROS) and reactive nitrogen species (RNS), including superoxide anion radical, hydrogen peroxide, hydroxyl radical, singlet oxygen, alkoxyl radicals, peroxyl radicals, and peroxynitrites, contribute to the pathogenesis of numerous human degenerative diseases [2] and have been implicated in the pathogenesis of neurodegenerative disorders including AD and Parkinson's disease, among others [3]. The production of reactive oxygen species (ROS) seems to be involved in triggering and maintaining the degeneration cycle of AD, causing the damage of mitochondrial DNA and of the electron transport chain, which leads to an increased production of ROS [4]. Brain tissue is particularly susceptible to oxidative damage. The metabolism of brain tissue requires high energy levels and it consumes approximately 20% of the total body oxygen despite the fact that it comprises less than

2% of total body weight. It is very rich in easily oxidizable polyunsaturated fatty acids and transition metal, such as iron and ascorbate, which are key players in oxidation and facilitate the formation of oxygen free radicals. The brain is also characterized by a low content of antioxidant systems [5].

The generation of ROS, which are toxic, is a part of normal metabolism in a biological system. Free radicals are extremely reactive species, which once formed can start a series of reactions that are harmful to the cell. It is important to emphasize that even under normal conditions there is a physiological cellular production of free radicals, which is normally counterbalanced by endogenous enzymatic cellular antioxidants systems. The balance between the production of reactive oxygen species and antioxidants is essential in a biological system to prevent adverse effects of oxidative stress. The damage caused by free radicals is caused by an imbalance between their production and their neutralization by cellular antioxidant systems in the human body [6, 7]. Both systems (production and neutralization) seem to be altered in AD and these changes have been suggested to play a major role in the process of age-related neurodegeneration and cognitive decline [8]. The free radicals thus generated are known to attack macromolecules such as deoxyribonucleic acid, proteins, lipids, and carbohydrates. This leads to either onset or acceleration of degenerative disorders. The main damage occurs for integration with cellular macromolecules essential to survival, such as DNA, proteins, and polyunsaturated fatty acids (which make up the cell membrane) [9]. Thus, ROS have been shown to trigger a variety of damage to cellular DNA and RNA, causing peroxidation of membranes and neuronal damage. In addition, the alterations of oxidative metabolism may render the brain more susceptible to further damage from Aβ, which in turn has a prooxidant action [10]. Accumulating evidence suggests that brain tissues in AD patients are exposed to oxidative stress during the development of the disease [11]. Oxidative stress and the following cellular damage caused by protein oxidation, lipid oxidation, DNA oxidation, and glycoxidation are closely associated with the development of cognitive decline in AD [9, 12].

Because free radicals and oxidative DNA damage may have a central role in age-related diseases such as AD, a protection from oxidative stress, and subsequent DNA damage may represent a basic approach for elongation of healthy age and treatment of such age-related diseases. In vitro antioxidant such as N-acetylcysteine or genetic disruption of the DNA damage response pathway by checkpoint kinase deletion can rescue many deficits and eventually elongates significantly lifespan [13]. These observations further indicate the important role of mitochondria, ROS, and DNA damage in aging and neurodegenerative diseases. In the past, randomized controlled intervention studies in AD, with antioxidants, such as selegiline or vitamin E, have produced modest but significant results [14]. Fermented papaya preparation (FPP), produced by fermentation of *Carica papaya* Linn by using yeast, is a food supplement that possesses beneficial and potent antioxidant properties that may be helpful against age-related and disease-related increase in oxidative stress [15]. FPP exhibits anti-inflammatory, antioxidant, and immunostimulatory action and induction of antioxidant enzymes [16].

In neurological conditions, oral administration of FPP in mice attenuated the reduction of short- and long-term memory induced by scopolamine [17]. Because of the above-described role of free radicals in the pathophysiology of chronic neurodegenerative diseases, it has been suggested a possible role for the antioxidant action of FPP in counteracting the oxidative stress associated with these conditions [15]. Therefore, we conducted the present study aiming to explore the effects of oral FPP on oxidative stress in AD patients.

2. Methods

We have measured oxidative stress in patients with initial or mild AD compared to age-matched control patients without AD. We have also tested the ability of FPP to reduce the excessive production of free radicals in patients with AD [12]. Oxidative stress was assessed by means of an enzyme immunoassay for the measurement of 8-hydroxy-2′-deoxyguanosine (8-OHdG) in the urine. Detection of 8-OHdG, a nucleic acid modification predominantly derived from hydroxide attack of guanidine, allows for assessment of more immediate oxidative damage [18]. We studied 40 patients (23 women and 17 men, mean age 78.2 ± 1.1 years) evaluated at the Alzheimer Evaluation Units of the University Hospital of Palermo. Twenty-eight patients were recruited after being diagnosed with early mild AD according to the criteria of the DSM-IV and NINCDS-ADRDA, while the other 12 were control patients of the same age.

The patients were not being treated with any other neurotrophic drug during the whole duration of the study. The 28 AD patients were divided into two groups; participants in group 1 ($n = 20$ patients) were treated for 6 months with a supplement of FPP (known commercially as Immunage, prepared by fermenting the *Carica papaya* Linn at the Osato Research Institute, Gifu, Japan) at a dose of 4.5 grams per day p.o. in a single dose. Patients of group 2 (8 AD patients) did not receive any treatment.

3. Results and Discussion

The clinical characteristics of the study participants are shown in Table 1. At baseline, 8-OHdG was significantly higher in patients with AD versus controls (13.7 ± 1.61 ng/mL versus 0.12 ± 1.6 ng/mL, $P < 0.01$, Figure 1). In group 1, supplementation with FPP significantly reduced 8-OHdG levels (from 14.1 ± 1.7 ng/mL to 8.45 ± 1.1 ng/mL, $P < 0.01$, Figure 2), while 8-OHdG did not change significantly in group 2 (not supplemented), showing a nonsignificant trend towards an increase (from 12.5 ± 1.9 ng/mL to 19.6 ± 4.1 ng/mL, P = NS). In the 20 patients treated with FPP, oxidative stress as measured by 8-OHdG was reduced in all but one patient (Figure 3). There were no significant changes in clinical MMSE evaluation and/or on any other laboratory parameters examined.

Numerous alterations of oxidative metabolism such as increased production of ROS metabolites and/or a reduction in the efficiency of antioxidant systems and repair capability of damaged molecules are present in AD and have been

Table 1: Clinical characteristics of study patients.

	AD (group 1) baseline before FPP supplementation	AD (group 2) not supplemented	Controls without AD	
Age (years)	78.1 ± 1.1	78.3 ± 1.0	77.9 ± 1.2	NS
8-OHdG (ng/mL)	14.1 ± 1.7	12.5 ± 1.9	1.6 ± 0.12	<0.001
SBP (mmHg)	132.9 ± 1.9	130.7 ± 2.1	131.0 ± 2.3	NS
DBP (mmHg)	78.6 ± 1.1	77.7 ± 1.2	77.9 ± 1.2	NS
CHOL (mg/dL)	207.9 ± 39	205.8 ± 38	195.7 ± 41	NS
TG (mg/dL)	127.5 ± 47	118 ± 57	112 ± 49	NS
HDL (mg/dL)	43.8 ± 12	47.9 ± 14	47.6 ± 13	NS
LDL (mg/dL)	136.8 ± 35	128.9 ± 40	127.7 ± 41	NS
BMI	24.9 ± 5.5	24.8 ± 6.4	24.1 ± 6.1	NS
MMSE	22.1 ± 1.5	21.9 ± 1.4	28.8 ± 2.1	$P < 0.01$

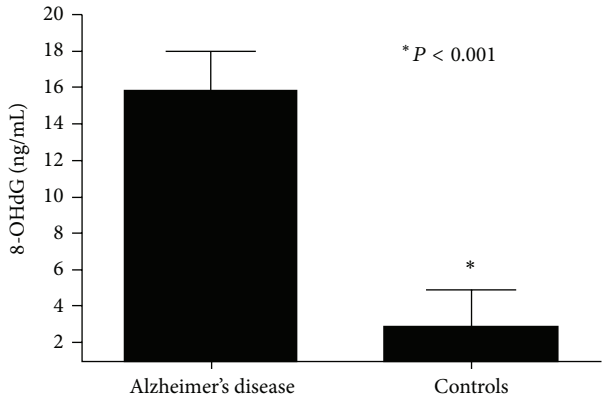

Figure 1: 8-Hydroxy-2′-deoxyguanosine (8-OHdG) level in patients with Alzheimer's disease and in controls.

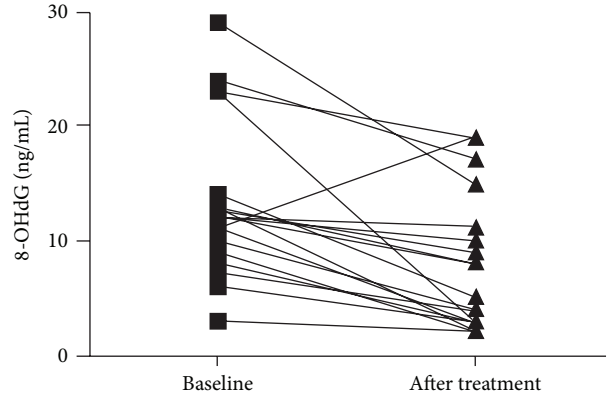

Figure 3: 8-Hydroxy-2′-deoxyguanosine (8-OHdG) level in each of the 20 patients with Alzheimer's disease (group 1) before and after fermented papaya powder (FPP) supplementation.

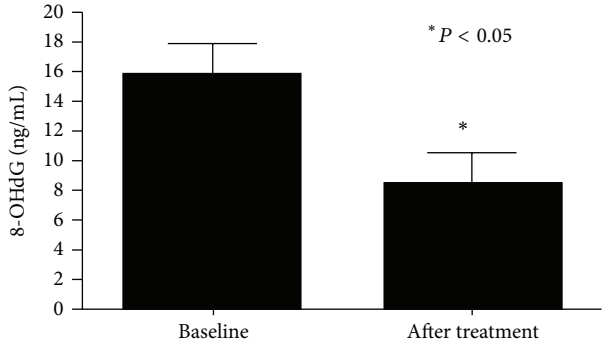

Figure 2: 8-Hydroxy-2′-deoxyguanosine (8-OHdG) level in patients with Alzheimer's disease (group 1) before and after fermented papaya powder (FPP) supplementation.

connected to its onset. Mitochondrial oxidative damage has been found to be excessive in the brains of aged people, especially AD patients and AD-like transgenic animal models. The damage caused by oxidative stress is one of the earliest pathophysiological events in the development of AD; it also seems to precede the formation of amyloid plaques and neurofibrillary tangles. Markers of DNA damage, particularly oxidative DNA damage, have been largely found in brain regions, peripheral tissues, and biological fluids of AD patients. Moreover, there is evidence that oxidative damage is one of the earliest detectable events within the progression from a normal brain to dementia [9, 19]. Almost one decade ago, a decrease in the DNA base excision repair activity was observed in postmortem brain regions of AD individuals, leading to the hypothesis that the brain in AD might be subjected to the double insult of increased DNA damage, as well as deficiencies of DNA repair pathways [20]. Autopsy studies on brain tissue from AD patients' brain tissue from AD patients have confirmed the presence of numerous signs of oxidative stress, such as Aβ-induced oxidative DNA damage and mitochondrial dysfunction, together with an increase in lipid peroxidation, proteins, and glycides oxidation [21], and a reduction of the antioxidant enzyme systems [22]. In vitro studies have shown that the neurotoxic properties of Aβ may be mediated by oxygen radicals. Amyloid deposits are associated with an overexpression of markers of oxidative stress, increased structural abnormalities of mitochondria, and mitochondrial DNA damage [23].

Age is the greatest risk factor for AD. Aging and chronic diseases are themselves associated with an increase in oxidative stress. The concentrations of oxidative-damaged proteins, lipids, and DNA have been reported to increase with age. This increase of oxidative stress during the aging process may contribute in part towards neurodegeneration in AD. The temporal association of the age related increased levels of ROS with the formation of the senile plaque provides further evidence that aging-induced alterations in brain oxidative status may be a major factor in triggering enhanced production and deposition of Aβ in AD [19].

We have previously shown that chronic diseases, such as diabetes mellitus type 2 or cardiovascular conditions, accelerate the age-dependent increase in oxidative stress [24]. A further derangement in oxidative stress balance may be caused by chronic inflammation of aging. Aging is characterized by a chronic, low-grade inflammation, and this phenomenon has been termed as inflammaging [25]. Aging and chronic disease create a cascade of events that can be best characterized as an asymptomatic inflammatory process. This cascade of events is mediated by cytokine interleukins 1 and 6 (IL-1alpha, and IL-6), nitric oxide (NO), and oxidative stress [26]. Inflammation has been suggested to be another responsible factor in AD and is presumed to be mediated through the cross talk among the amyloid, astrocytes, and microglia [27]. These reactions lead to altered neuronal function and the inflammatory injury. Thus, in patients with AD we have recently shown an increase of oxidative stress [12] and an alteration of the immunoinflammatory responses, with an increased cytokine production, that may have a potential causal role in contributing to an augmented oxidative stress [28]. Several studies have shown that Aβ may produce an increase in oxidative stress via several mechanisms, either increase in ROS production, decrease in the enzyme activities involved in the antioxidant defense system, or altering mitochondria function. Nerve cell insults caused by Aβ brain deposition may itself induce oxidative changes [29], and metals concentrated in amyloid deposits, such as copper, may as well contribute to the oxidative insults observed in AD-affected brains. Several studies suggest that Aβ increases oxidative stress by increasing lipid peroxidation measured by increased levels of thiobarbituric acid-reactive substances in brain [29]. In addition to lipids, it has been suggested that ROS-mediated reactions with proteins lead to oxidative damage of proteins and DNA in the brain tissue [30]. The accumulation of oxidative stress metabolites present in old age may itself cause an increase susceptibility of the brain to damage from neurotoxic peptides such as soluble or fibrillar Aβ. As the accumulation of Aβ can in turn cause a further production of ROS, it is still unclear whether the excess of oxidative stress is a primary or secondary event in AD. Although there are accumulating evidences suggesting that oxidative stress may be an early event in the onset of AD, this aspect seems to be of relative importance, as the production of ROS, even if secondary, is in turn detrimental to the brain tissue and can further contribute to neuronal damage [31]. This suggests that any effort to the removal and/or prevention of ROS formation may be useful in people with AD [29]. For example, when Aβ has started to aggregate and deposit in the brain, this protein elicits a neuroinflammatory response via the activation of microglia and astrocytes [32, 33]. Following the initial neuroinflammatory response, the neurotoxic by-products of inflammation cause additional oxidative damage to cells. Similarly, the hyperphosphorylated tau fibrils create cytoskeletal stresses and promote neuronal dysfunction [34].

Oxidative stress-induced cell damage and inflammation are implicated in a variety of age-related diseases other than neurodegenerative disorders (such as cancers, diabetes, arthritis, and cardiovascular dysfunctions) and aging. All these conditions could potentially benefit from functional nutraceutical/food antioxidant supplements. Antioxidant defenses may potentially protect the body from the detrimental effects of free radicals. Physiological antioxidants in food such as fruits and vegetables provide a reasonable amount of antioxidants. Although existing knowledge is not definitive, there is rational basis to suggest that antioxidant supplementation and food plant extracts may help protect against a number of neurological diseases in which oxidative stress is implicated and may have a role in the prevention and treatment of age-related disease.

It has been suggested that substances with antioxidant properties or that enhance the endogenous defense system against free radicals may have a role in preventing the onset or in slowing the progression of AD [35]. Dietary antioxidants contribute to increased levels of cellular antioxidant ability, thus decreasing the toxicity of ROS.

FPP has been shown to reduce apoptosis related to oxidative stress and activation of inflammatory cytokines [36, 37] and to contrast the DNA damage related to the production of free radicals induced by several prooxidant substances, including iron ions, copper, benzopyrene, methylguanidine, and aluminum, among others [15, 38–40].

In particular, it has been shown that FPP exerts several protective actions; it inhibits lipid peroxidation [41]; it enhances enzyme antioxidant activities such as glutathione S-transferase in hepatocytes [36] and showed remarkable hepatoprotective activity [42]. In vitro in a cellular model of AD, FPP has neuroprotective activity against β-amyloid-mediated copper neurotoxicity in β-amyloid precursor protein in a cell culture system [36]. FPP has shown protective properties against iron-mediated oxidative damage to DNA and proteins [43]. In the brain tissue, FPP has been shown to have a neuroprotective effect from oxidative damage, from lipid peroxide level, and superoxide dismutase activity in iron-induced epileptic foci of rats [41]. At the neuronal level, FPP has been shown to have a neuroprotective action, to improve the oxidative status in human neuronal cells and to protect from insults by oxidative stress linked, for example, to the cytotoxicity by aluminum in neuronal cells [44]. Neuroprotective potential evaluated in an AD cell model showed that the toxicity of the Aβ can be significantly modulated and/or reduced by FPP. In vitro FPP has been shown to protect cells by the oxidative damage related to the deposition of Aβ. Treatment with FPP increased the survival of neuronal cells, preventing apoptosis, and was able to decrease the production of hydroxyl radicals and superoxide anion in the cells, as well as decreasing nitric oxide

accumulation and intracellular calcium ion [36]. Noda et al., using an ESR technique, confirmed the potent antioxidant inhibitory effect of the FPP, by demonstrating its hydroxyl radical scavenging activity, its superoxide anion radical scavenging activity (SOD-like activity), and its inhibitory effect on hydroxyl radical generation from methylguanidine [40]. At the clinical level, studies in chronic and degenerative disease conditions (i.e., thalassemia, cirrhosis, and diabetes) showed that FPP favorably modulates immunological, hematological, inflammatory, vascular, and oxidative stress damage parameters [15].

Our results suggest that FPP has antioxidant actions in AD patients and that it may be prophylactic food against the age-related and neurological diseases associated with free radical overproduction. Our data confirm that AD is associated with an increased oxidative stress and that the FPP can be useful in helping to counteract the excessive production of free radicals present in patients with AD [12]. The previous in vitro studies are promising and proven preventive action on the damage from Aβ suggesting that it would also be useful to evaluate the action of FPP in the more advanced stages of the disease and in combination with neurotrophic drugs. It would also be interesting to identify the component of FPP neurotrophic action.

In conclusion, dietary factors can modulate physiological functions (including brain function) thereby increasing the economic productivity of a population as a function of health. A greater understanding of the molecular mechanisms of neuroprotection, oxidative stress, and immune function will facilitate definition of the prophylactic potentials of diet, nutritional/food supplements, medicinal plants, and herbal extracts. Although the role of oxidative stress in aging and neurodegenerative and other related diseases is largely accepted, the value of antioxidant strategies is still debatable. This becomes more important when, apart from foods or reasonable lifestyle changes, antioxidant supplements are considered. Well-defined long-term trials are still needed to assess the efficacy of antioxidant strategies or of antioxidant-rich nutritional intervention. Future studies of longer duration and with a larger number of subjects would be useful to assess the potential clinical actions of FPP and the possible relevance to the reduction of oxidative stress on the natural history of the disease.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Research Article

The Impact of ATRA on Shaping Human Myeloid Cell Responses to Epithelial Cell-Derived Stimuli and on T-Lymphocyte Polarization

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Vitamin A plays an essential role in the maintenance of gut homeostasis but its interplay with chemokines has not been explored so far. Using an *in vitro* model system we studied the effects of human colonic epithelial cells (Caco2, HT-29, and HCT116) derived inflammatory stimuli on monocyte-derived dendritic cells and macrophages. Unstimulated Caco2 and HT-29 cells secreted CCL19, CCL21, and CCL22 chemokines, which could attract dendritic cells and macrophages and induced CCR7 receptor up-regulation by retinoic-acid resulting in dendritic cell migration. The chemokines Mk, CXCL16, and CXCL7 were secreted by all the 3 cell lines tested, and upon stimulation by IL-1β or TNF-α this effect was inhibited by ATRA but had no impact on CXCL1, CXCL8, and CCL20 secretion in response to IL-1β. In the presence of ATRA the supernatants of these cells induced CD103 expression on monocyte-derived dendritic cells and when conditioned by ATRA and cocultured with $CD4^+$ T-lymphocytes they reduced the proportion of Th17 T-cells. However, in the macrophage-T-cell cocultures the number of these effector T-cells was increased. Thus cytokine-activated colonic epithelial cells trigger the secretion of distinct combinations of chemokines depending on the proinflammatory stimulus and are controlled by retinoic acid, which also governs dendritic cell and macrophage responses.

1. Introduction

The adult human intestine is referred to as "physiologically inflamed" due to the presence of enormous number of B- and T-lymphocytes, as well as macrophages (Mf), dendritic cells (DC), eosinophils, and mast cells. If all these immune cells were present in other tissues at such concentrations, they would be regarded as an abnormal chronic inflammatory cell infiltrate [1]. In such a scenario, the main players maintaining gut homeostasis should be those mechanisms that provide tolerogenic signals for specialized myeloid cells with antigen presenting function.

The vitamin A (VitA) metabolite retinoic acid (RA) is a key regulator of the cytokine TGF-β, which promotes Treg differentiation [2]. VitA also contributes to the formation of epithelial linings of mucosal surfaces [3], and its multifunctional metabolite RA [4] acts as a critical driver of lymphocyte trafficking to the intestinal mucosa [5]. All-trans retinoic acid (ATRA) induces the expression of the gut homing integrin $\alpha4\beta7$ on myeloid cells and the chemokine receptor CCR9 on T-lymphocytes, while the lack of the αv or β8 integrin chains in DC impairs Treg functions and Th17 responses *in vivo* [6]. ATRA also modulates Th17 effector T-lymphocyte differentiation in the gut [7]; however, the *in vivo* effects of ATRA in intestinal and extraintestinal compartments result in controversial outcomes presumably due to targeting multiple cell types with diverse functional activities [8]. VitA deficiency has an effect on epithelial cell integrity and the composition of the gut microbiota [9].

A single layer of colonic epithelial cells (CEC) forms the first line of defense against luminal pathogens. It communicates with other immune cells by direct contacts and by secreting an array of cytokines and chemokines. Chemokines represent low-molecular-weight proteins with pleiotropic effects on the recruitment and activation of leukocytes at inflammatory sites [10]. The dominant cell populations in the gut involve $CX3CR1^+$ Mf, which directly sense luminal content by their extended membrane protrusions across the epithelium [11], and migratory $CD103^+$ DC with tolerogenic potential. Apart from chemokines, colony-stimulating factor (CSF-2/GM-CSF) in the gut is a multifunctional cytokine that has an impact on DC and Mf numbers and can impair the ability of immune cells to produce regulatory factors such as RA and IL-10 and thus may lead to disrupted Treg homeostasis in the large intestine [12]. It also acts as an important regulator of human DC homeostasis by promoting *in vivo* expansion and differentiation from hematopoietic progenitors and monocytes [13]. Under steady state conditions, the low number of gut migratory DC is critically dependent on GM-CSF, but its level is dramatically increased during infection or inflammation and supports the development of DC precursors such as monocytes and inflammatory migratory DC thus modulating the composition of the DC pool [14].

Cytokines have been shown to be the causative factor and outcome of IBD pathogenesis. The major conclusive result has been shown by improvement in the IBD symptoms by blocking TNF-α. And a decrease in the IL-1 receptor antagonist in comparison to IL-1 was observed in IBD patients [15]. These data confirm that both TNF-α and IL-1β are able to trigger inflammatory conditions such as those observed in Crohn's disease (CD) or ulcerative colitis (UC) but the comparison of their effects at molecular and functional levels in context of the human intestinal microenvironment has not been elucidated so far. Despite similarities in the functional and regulatory mechanisms in human and mouse, major differences have been observed in their cytokine secretion [16] and mucus layer organization [17].

Based on these data and to overcome the discrepancies between the human and mouse systems, we designed experiments with human CEC in resting state and in an inflammatory milieu mimicked with TNF-α or IL-1β stimulation in the presence or absence of ATRA. This was performed by monitoring the levels of secreted chemokines measured at the protein level and by investigating their impact on the phenotype and functional attributes of myeloid cells generated by different growth/differentiation factors. Considering that DC have the potential to instruct T-cells for inflammatory or regulatory directions, our final goal was to identify the impact of stimulated CEC-induced and DC-mediated effects on $CD4^+$ effector T-lymphocyte responses. We could detect the secretion of CCL19, CCL21, and CCL22 chemokines by unstimulated CEC, which has not been shown before. We also observed that both IL-1β and TNF-α were able to trigger the secretion of Midkine (Mk), CXCL16, and CXCL7 by CEC, but their expression could efficiently be downregulated by ATRA. However, the secretion of CXCL1, CXCL8, or CCL20 by IL-1β-stimulated CEC was not influenced by ATRA. Our results also revealed that the *in vitro* induced inflammatory milieu created by proinflammatory chemokines was sufficient to increase the migratory potential of DC driven by GM-CSF but not by the other growth factors, and ATRA could further potentiate this effect. Furthermore, the molecular information collected by CEC and transmitted to DC could be translated to T-lymphocytes, which responded to CEC-initiated and DC-mediated stimulation by mounting Th17 responses. All these steps seemed to be under the control of ATRA as the response of CEC to both IL-1β and TNF-α was higher in the presence of ATRA.

2. Materials and Methods

2.1. Cell Culture of Caco2 Colon Epithelial Cells. The human colorectal adenocarcinoma cell line Caco2 is from ATCC-number HTB-37 and HT-29 is from ATCC-number HTB-38. The colorectal carcinoma cell line HCT116 was a generous gift from Dr. György Vereb, Department of Biophysics, University of Debrecen. Caco2 and HCT116 cells were cultured in RPMI-1640 medium supplemented with 1% antibiotic-antimycotic solution and 20% fetal bovine serum (GIBCO by Life Technologies, EU) in tissue culture flasks (Nunclon, Rochester, NY) at 37°C in 10% and 5% CO_2, respectively. HT-29 cells were cultured in RPMI-1640 medium supplemented with 1% antibiotic-antimycotic solution and 10% fetal bovine serum in 5% CO_2. Cell culture medium was replaced every 2-3 days and the cells were passaged when subconfluent.

2.2. Protein Array for Chemokine Analyses. CEC of 70–80% confluency were plated overnight in RPMI supplemented with 10% FCS-followed by stimulation with 10 ng/mL proinflammatory cytokines (IL-1β or TNF-α) in combination with or without 10 nmol ATRA or left untreated for 1 hour. The cells were washed and replaced with fresh medium for 5 hr, when the supernatants were collected for chemokine analysis performed by a commercially available protein array (Proteome Profiler Arrays—ARY017, R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Sample controls (transferrin R, gp130, and fibrinogen) included in the array allowed us the detection and quantitation of the secreted chemokines. Considering that ATRA dissolved in DMSO may have toxic effects on resting CEC, which could be further enhanced by activation with IL-1β or TNF-α, we performed preliminary titration experiments to optimize the cell culture conditions by using 24 h 7AAD-based viability assays performed by FACS analysis. These results indicated 98% viability of Caco2 and HT-29 cells in both the presence and absence of 10 nmol ATRA that was similar to those measured for untreated CEC.

2.3. In Vitro Cell Migration and the Chemotaxis Assay. Migration of three different groups of monocyte-derived cells, differentiated in GM-CSF+IL-4, GM-CSF, and M-CSF, was tested for cell migration to chemokines and cytokines secreted by Caco2, HT-29, and HCT116 cells. Monocytes (3×10^5) differentiated in the presence of the 3 different growth factors were placed on the upper chamber of a 5-micron Corning transwell plate and the CEC supernatants

were added to the lower chamber of the transwell. After 24 h the monocyte-derived cells that migrated to the lower chamber were collected. 10,000 polystyrene beads (15 micron) were added to each sample (Fluka Analytical, Germany) and the number of migrating cells was counted by FACS Calibur (BD Biosciences, Franklin Lakes, NJ, USA). The data were analyzed by the FlowJo software (Tree Star, Ashland, OR, USA).

2.4. Peripheral Blood Monocyte-Derived Cells. Leukocyte enriched buffy coats were obtained from healthy blood donors drawn at the Regional Blood Center of the Hungarian National Blood Transfusion Service (Debrecen, Hungary) in accordance with the written approval of the Director of the National Blood Transfusion Service and the Regional and Institutional Ethics Committee of the University of Debrecen, Medical and Health Science Center (Hungary). PBMCs were separated by a standard density gradient centrifugation with Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden). Monocytes were purified from PBMCs by positive selection using immunomagnetic cell separation with anti-CD14 microbeads, according to the manufacturer's instruction (Miltenyi Biotec, Bergisch Gladbach, Germany). After separation on a VarioMACS magnet, 96–99% of the cells were $CD14^+$ monocytes, as measured by flow cytometry. Monocytes were divided and cultured in 12-well tissue culture plates at a density of 2×10^6 cells/mL in 10% RPMI medium supplemented with four different growth factors: 80 ng/ml GM-CSF (Gentaur Molecular Products, Brussels, Belgium), 100 ng/mL IL-4 (PeproTech EC, London, UK), and M-CSF 50 ng/mL (MACS, Miltenyi Biotec, Germany).

2.5. Peripheral Blood Lymphocytes and $CD4^+$ T-Cells. Autologous naive T-cells were separated from human blood mononuclear cells using the naive $CD4^+$ T-cell isolation kit based on negative selection according to the manufacturer's instruction (Miltenyi Biotec).

2.6. Phenotypic Characterization of Myeloid Cells by Flow Cytometry. Detection of the cell surface expression of monocyte-derived myeloid cells was performed by flow cytometry using anti-CD1a-PE, anti-CD209-PE, anti-CD14-PE, anti-CD83-PE, anti-CD103-PE, anti-CX3CR1-PE, and anti-CCR7-PE (Beckman Coulter, Hialeah, FL, USA). The growth factor receptors were characterized by anti-GM-CSFRα-PE and anti-M-CSF R/CD115-PE (R&D Systems, USA) and isotype-matched control antibodies (BD PharMingen, San Diego, CA, USA). Fluorescence intensities were measured by FACS Calibur (BD Biosciences, Franklin Lakes, NJ, USA), and data were analyzed by the FlowJo software (Tree Star, Ashland, OR, USA). The human chemokines Mk, CXCL7, CCL20, and CXCL16 were ordered from PeproTech, UK; CXCL8 and CXCL1 are from Miltenyi Biotec.

2.7. IL-17 and IFNγ ELISPOT Assays. The monocyte-derived cells were cultured in GM-CSF+IL-4, GM-CSF, and M-CSF for 3 days along with the supernatant of unstimulated or cytokine activated Caco2 cells at 2×10^5 cells/well density. The cells were washed to remove all growth factors and supernatants and were cocultured with naïve autologous $CD4^+$ T cells (10^6cells/well) in 10% RPMI medium for 2 days at 37°C in a humidified atmosphere containing 5% CO_2. PHA and Con A activated T cells were used as positive controls. Negative controls involved $CD4^+$ T-cells and untreated monocyte-derived cells cocultured with $CD4^+$ T-cell. Detection of cytokine-secreting T cells was performed by the avidin-HRP system (NatuTec GmbH, Germany). Plates were analyzed by an ImmunoScan plate reader (CTL, Shaker Heights, OH, USA).

2.8. Statistical Analysis. Statistical analysis was performed by one-way analysis of variance (ANOVA) for multiple comparisons. Results are expressed as mean ± SD. Two group differences were analyzed by Student's t-test. P value (two-tailed) less than 0.05 was considered statistically significant.

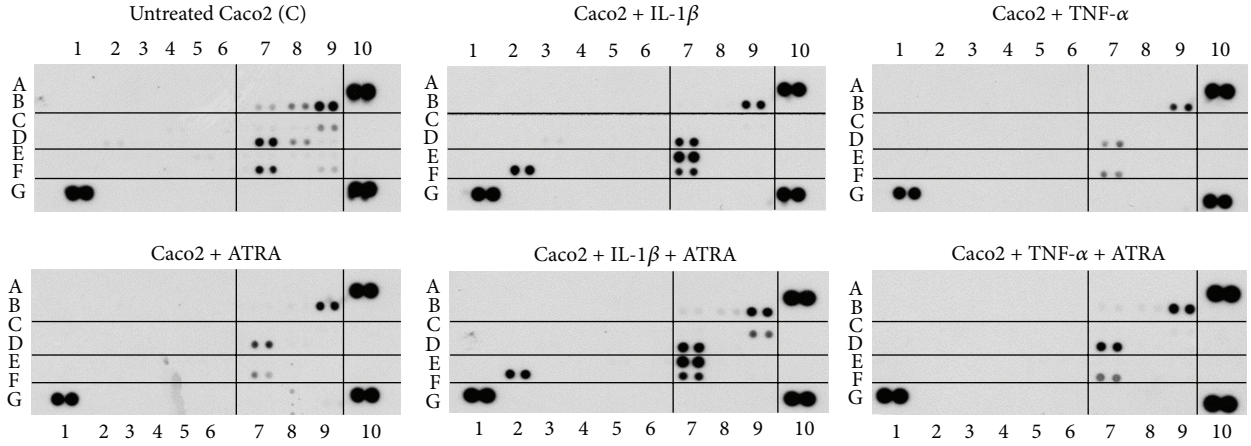
3. Results

3.1. Identification of Chemokines Secreted by Resting and Activated CEC. The single cell monolayer of CEC plays an essential role in the maintenance of gut homeostasis by supporting barrier function and defense against microbes preferentially through the secretion of chemokines [18]. It is also well established that the proinflammatory cytokines IL-1β and TNF-α act as potent activators of CEC [19]. In this study we applied a high throughput approach for identifying the chemokines secreted by CEC (Caco2, HT-29, and HCT116) in response to IL-1β and TNF-α by using a commercially available Human Chemokine Array to quantify the relative levels of chemokines released by resting and activated CEC at the protein level. The sample controls provided (transferrin R, gp130, and fibrinogen) were used for calculating mean pixel densities of the respective dot blots. The results showed that resting Caco2 cells secrete detectable levels of CCL19, CCL21, and CCL22 constitutively (dots 2D, 9F, and 8D in Figure 1(a), summarized in Figure 1(c)). Similar results were obtained for HT-29 cells, but HCT116 secreted only CCL19 at detectable levels. Comparison of the relative cytokine levels secreted by the Caco2, HT-29, and HCT116 cell lines are summarized in Table 1. Considering that these CCL chemokines are known to attract myeloid cells, they may maintain a population of myeloid cells in the vicinity of CEC to support cellular interactions. It has previously been shown that MDC/CCL22 attracts Th2 cytokine producing cells and its mRNA and protein expression is upregulated against enteroinvasive bacteria, but inhibition of the NF-κB pathway abolished CCL22 expression in response to proinflammatory stimuli [1]. The chemokines CXCL7, CXCL16, and Mk with different functional activities were also constitutively secreted by resting CEC (dot 9C, 7F, and 7D in Figure 1(a)) suggesting their role in the maintenance of epithelial cell homeostasis.

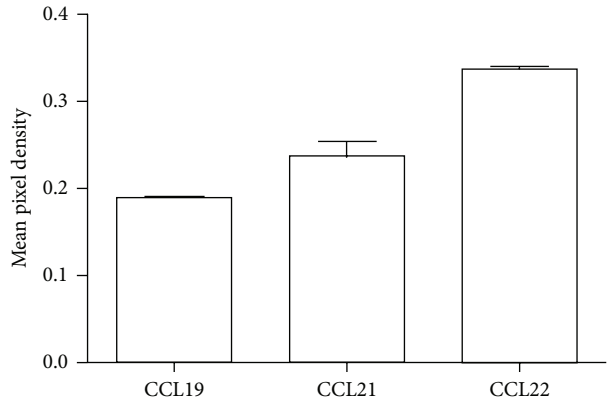
3.2. The Effect of ATRA on the Chemokine Secretion by CEC Prestimulated by IL-1β or TNF-α. In our model system, CEC were left untreated or stimulated by IL-1β or TNF-α in combination with or without ATRA for 6 hr and the cell culture supernatants were subjected to chemokine array analysis by calculating pixel densities using the relevant

	1	2	3	4	5	6	7	8	9	10
A										Reference spot
B							Transferrin R	gp130	Fibrinogen	
C			CXCL17	CCL17	CXCL12	CCL5	CXCL4	CCL18	CXCL7	
D		CCL19	CCL20	CCL15	CCL3	CXCL9	Mk	CCL22	CCL7	
E		CCL2	XCL1	CXCL11	CXCL10	IL-16	CXCL8	CCL1	CCL14	
F		CXCL1	CX3CL1	CCL26	CXCL5	TIG-2	CXCL16	CCL28	CCL21	
G	Reference spot									Reference spot

(a)



(b)



(c)

Figure 1: Chemokine secretion of human epithelial cells activated by IL-1β or TNF-α in the presence or absence of ATRA. Caco2 cells were activated by 10 ng/mL IL-1β or TNF-α in the presence or absence of 10 nmol ATRA. After 1 hr of activation, the supernatants were removed and the cells were washed and cultured in fresh medium. After 5 hr the supernatants of nontreated and activated Caco2 cells were collected and the relative levels of chemokines were determined by a Proteome Profiler Array used according to the manufacturer's instructions. The relative levels of the Caco2 cell-derived chemokines were determined by calculating mean pixel densities of the individual blots normalized to sample control fibrinogen. The mean ± SD of 4 independent measurements is shown. (a) Localization of the chemokine probes in the membranes related to the positive controls and the reference spots. (b) Representative dot blots showing the relative expression of chemokines produced by untreated Caco2 cells without or with ATRA (upper and lower panels 1) as compared to Caco2 cells stimulated by IL-1β (upper panel 2) and IL-1β in the presence of ATRA (lower panel 2). Upper and lower panels 3 correspond to Caco2 cells stimulated by TNF-α in the absence or presence of ATRA, respectively. (c) Relative expression levels of CCL chemokines produced by unstimulated Caco2 cells.

sample control (Figure 1). When Caco2 cells were activated by TNF-α or IL-1β, the secretion levels of the chemokines CXCL7, CXCL16, and Mk did not change significantly as compared to unstimulated cells (dots 9C, 7F, and 7D in Figure 1(a), summarized in Figures 2(a) and 2(b)). However, the expression of CXCL16 and Mk was downregulated in the presence of ATRA suggesting that the secretion of these chemokines may contribute to the maintenance of epithelial cell homeostasis. However, under inflammatory conditions they do not mediate positive signals for DC. Remarkably, the secretion of CXCL7 (dot 9C in Figure 1(a), summarized in Figures 2(a) and 2(b)) could be induced only when IL-1β or

Table 1: Relative expression of chemokines secreted by colon epithelial cell lines. Caco2, HT-29, and HCT116 cells were treated with IL-1β or TNF-α in the presence or absence of ATRA for 60 min. After removing the cell culture supernatant the cells were washed and replaced with fresh medium and the supernatants were collected after 5 hours and used for the Chemokine Array analysis according to the manufacturer's instruction to determine the variety of chemokines secreted by these cell lines. The values indicate the results of the densitometric analysis of dot blots and are normalized to the relevant sample control according to the manufacturer's instructions. For simplicity the value of 1 obtained after dividing with the sample control is referred to as 100.

		CCL19	CCL21	CCL22	Mk	CXCL16	CXCL7	CXCL1	CXCL8	CCL20
C	Caco2	30	30	30	90	70	40	—	—	—
	HT-29	40	30	40	80	90	60	—	—	—
	HCT-116	150	—	—	170	170	—	—	120	—
IL-1β	Caco2	—	—	—	100	80	—	120	140	30
	HT-29	—	—	—	80	90	—	100	90	30
	HCT-116	—	—	—	150	160	100	100	100	100
IL-1β + ATRA	Caco2	—	—	—	110	80	50	100	130	—
	HT-29	—	—	—	100	80	40	100	90	—
	HCT-116	—	—	—	160	140	90	110	110	—
TNF-α	Caco2	—	—	—	90	60	—	—	—	—
	HT-29	—	—	—	80	60	—	—	—	—
	HCT-116	—	—	—	150	150	—	10	10	—
TNF-α + ATRA	Caco2	—	—	—	90	60	—	—	—	—
	HT-29	—	—	—	90	80	20	—	—	—
	HCT-116	—	—	—	150	130	20	10	—	—
ATRA	Caco2	—	—	—	50	30	—	—	—	—
	HT-29	—	—	—	60	20	—	—	—	—
	HCT-116	40	20	30	80	80	—	—	—	—

TNF-α was combined with ATRA treatment demonstrating the dependence of its secretion on ATRA. DMSO, which is the standard solvent of ATRA, also decreased CXCL7 secretion (Figures 2(a) and 2(b)). Depending on CEC sensitivity to DMSO, certain effects of this solvent have previously been reported, as inhibition of prostaglandin E2 production upon treatment of Caco2 cells with IL-1β and attenuation of mRNA levels of IL6, IL-1α, and IL-1β [20]. These findings might be in line with inhibition of CXCL7 observed in our system. In the IEC-18 cell line IL-1β induced increased mRNA levels of MCP-1/CCL2, MIP-1α/CCL3, inducible NO synthase, and RANTES/CCL5, which are upregulated in a NF-κB dependent manner [21].

The secretion levels of CCL20 and CXCL1 were not affected by ATRA (dots 3D, 2F, and 7E in Figure 1(a), summarized in Figure 2(c)) and could be induced exclusively by IL-1β but not by TNF-α. Surprisingly, CXCL8 secretion was upregulated not only by IL-β with or without ATRA, but also by DMSO used as a vehicle for ATRA (Figure 2(c)). DMSO induced strain on the actin cytoskeleton and integrins expressed by Caco2 cells was suggested to be transferred to actin-associated molecules like α-actinin-1, acting as a scaffold protein and interacted directly with ERK1/2 leading to phosphorylation and increased secretion of CXCL8. The *in vivo* relevance of this effect is underscored by the physical deformation of CEC during peristalsis and villous motility [22]. Along with IL-8/CXCL8 and GROα/CXCL1, Caco2 cells also express MCP-1/CCL2 as a result of IL-1 stimulation, but our chemokine array-based method did not detect CCL2 [23]. These results suggest that the expression of individual chemokines depends on the means of activation and also on ATRA, which can modulate the outcome of chemokine secretion, whereas the group of chemokines not affected by ATRA indicates the complexity of chemokine-mediated regulation in the gut. Similar results were obtained for HT-29 and HCT116 CEC as summarized in Table 1. Even though HCT116 showed a similar overall pattern of chemokine secretion as the other CEC, trace amounts of NF-κB-dependent inflammatory chemokines (CCL2, CXCL2, and CXCL10), not observed in Caco2 and HT29 cells, were detected indicating CEC type-dependent regulation of chemokine secretion [10, 21, 24].

3.3. ATRA Regulates the Chemokine-Dependent Migration of Myeloid Cells Generated by Different Hematopoietic Growth/Differentiation Factors. Based on the results showing the inhibitory effect of ATRA on the secretion of some chemokines, we next sought to assess the chemokine-driven migratory potential of myeloid cells. We set up a transwell system and measured myeloid cell migration *in vitro* by using CCL19 and CCL21 chemokines as positive controls of cell recruitment. Myeloid cells differentiated from primary human monocytes by GM-CSF+IL-4 or GM-CSF to DC exhibited detectable but low migratory potential as compared to cells mobilized by high concentration (200 ng/mL) of CCL19 and CCL21 chemokines (Figures 3(a) and 3(b)), while monocytes cultured in M-CSF gave rise to macrophages with undetectable migratory activity (data not shown). The highest migratory potential could be attributed to cells differentiated in the presence of GM-CSF or GM-CSF+IL-4 and stimulated by the supernatant of Caco2 cells preactivated by IL-1β, but this process could be downregulated by ATRA. To analyze

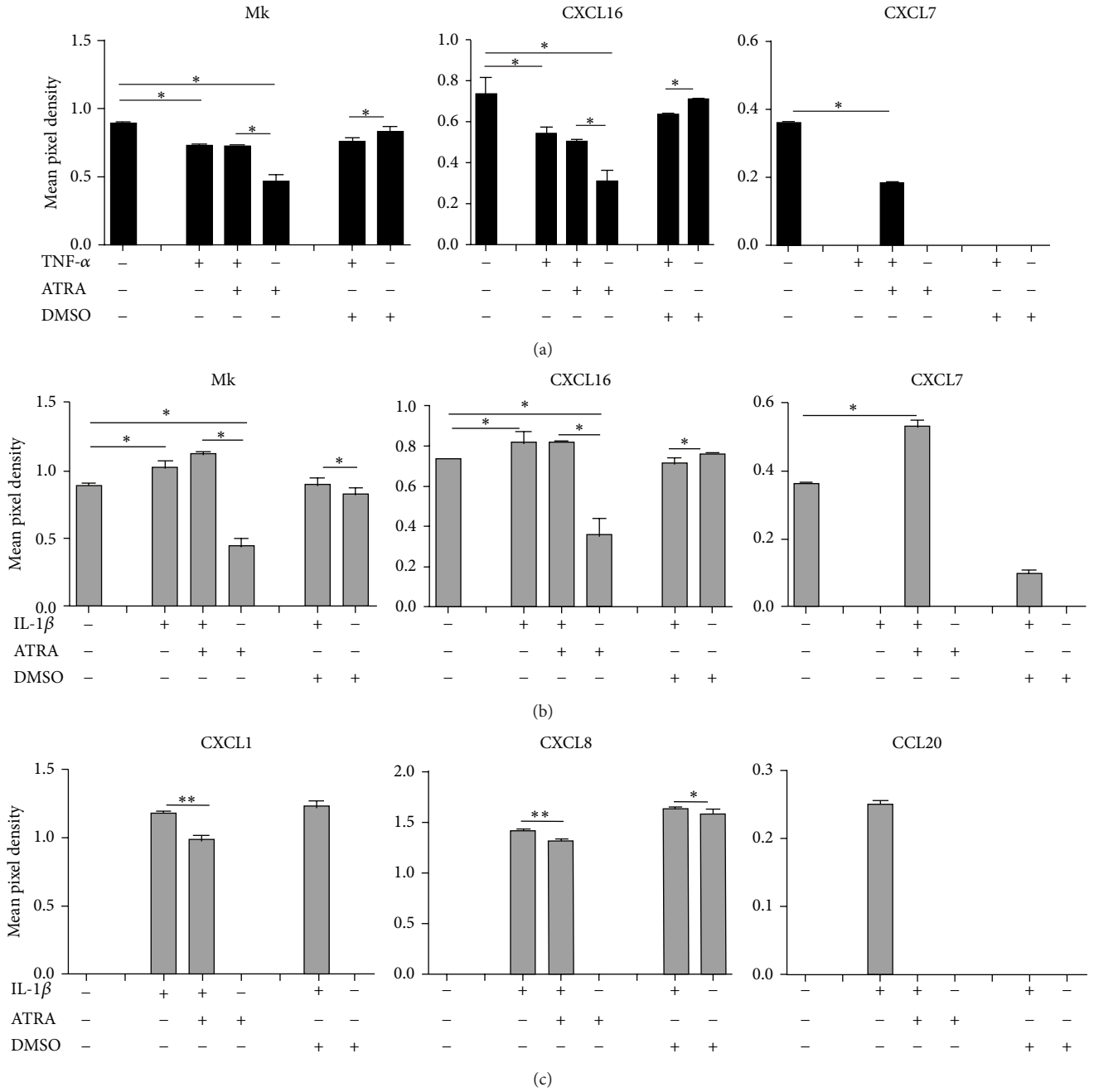


Figure 2: Effects of ATRA on the expression of chemokines in cytokine-stimulated Caco2 cells. Relative expression of chemokines in Caco2 cells, prestimulated by TNF-α or IL-1β in the presence or absence of ATRA, was determined as described in Figure 1 and was compared to unstimulated cells cultured with or without ATRA. The possible contribution of DMSO used as a solvent control of ATRA was tested in Caco2 cells cultured in the presence of TNF-α or IL-1β with or without DMSO. (a) Expression of chemokines secreted by Caco2 cells prestimulated by TNF-α, in the presence or absence of ATRA. (b) Expression of chemokines secreted by Caco2 cells prestimulated by IL-1β in the presence or absence of ATRA. (c) Chemokines induced exclusively by IL-1β stimulation in Caco2 cells. $^{*}P < 0.05$, $^{**}P < 0.01$. Bar diagrams indicate mean ± SD of 4 dot blots which was averaged after densitometry analysis and normalized with the "sample control" provided with the kit.

whether the supernatant of IL-1β-stimulated CEC has a direct effect on the migration of DC differentiated by GM-CSF+IL-4 or GM-CSF, the cells were subjected to direct cell migration assays toward the chemokines exclusively secreted by IL-1β used at pretitrated concentrations (CXCL1 (1 ng/mL), CXCL8 (100 ng/mL), and CCL20 (50 ng/mL)). We observed the high migratory capacity of DC differentiated by GM-CSF+IL-4 (Figure 3(c)) and Mf developed by GM-CSF (Figure 3(d)) toward CXCL1 and CXCL8 known to be involved in the chemotaxis and migration of polymorphonuclear leukocytes

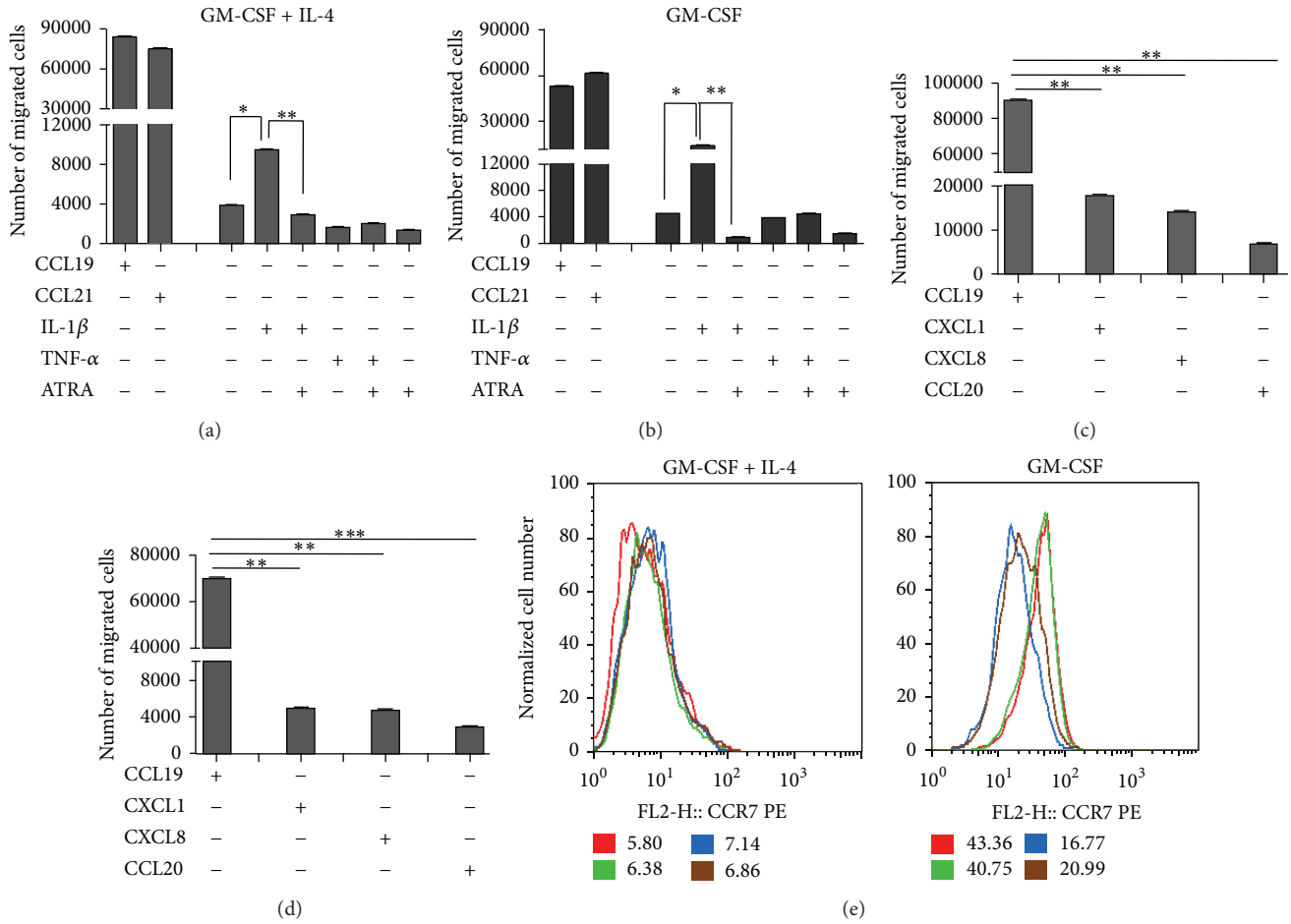


FIGURE 3: ATRA modifies the chemokine-dependent migration of *in vitro* differentiated myeloid cells. Caco2 cells were activated as described in Figure 1. The migratory potential of the monocyte-derived cells generated by GM-CSF+IL-4 or GM-CSF was tested in transwell chambers. 3×10^5 cells were placed on the upper chamber and the Caco2 cell supernatants on the lower chamber of the transwell plate. After 24 hr, the number of cells, which migrated to the lower chamber in response to the Caco2 cell supernatants, was counted by flow cytometry. The migratory potential of cells generated in the presence of GM-CSF+IL-4 (a) or GM-CSF (b) is shown as compared to the migratory potential of CCL19 and CCL21 chemokines, used as positive controls. To determine the cause of high migration in response to IL-1β treated CEC supernatant, the GM-CSF+IL-4 (c) and GM-CSF (d) differentiated monocytes were treated with CXCL1, CXCL8, and CCL20 chemokines which were secreted exclusively by CEC on IL-1β treatment. Bar diagrams indicate mean ± SD of 3 independent experiments $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Another fraction of monocyte-derived cells, differentiated by GM-CSF+IL-4 or GM-CSF, was also stimulated by the supernatant of IL-1β pretreated CEC in the presence or absence of ATRA. The cell surface expression of CCR7 in the differentiated monocyte derived cells was measured by flow cytometry (e). Red line depicts untreated monocyte-derived cells, green is for monocyte-derived cells treated by the supernatant of Caco2 cells previously activated by IL-1β, blue line is for monocyte-derived cells pretreated with the supernatant of Caco2 cells activated by IL-1β and ATRA, and brown line corresponds to cells treated by the supernatant of unstimulated Caco2 cells in combination with ATRA. Results of 3 independent experiments are shown as mean ± SD of MFI.

to inflammatory sites [25, 26] as compared to CCL20. Interestingly, the supernatant of Caco2 cells prestimulated with TNF-α had no such effect on cell migration (Figures 3(a) and 3(b)). When the migratory potential of myeloid cells was related to the cell surface expression of CCR7 we found that DC differentiated by GM-CSF in the presence of ATRA-conditioned CEC supernatant exhibited decreased CCR7 expression, while in cells differentiated in GM-CSF+IL-4 it remained unchanged (Figure 3(e)). Similar results were obtained by using the HT-29 and HCT116 cell lines (data not shown). These results suggest that in an inflammatory environment ATRA also modulates the migratory potential of myeloid cells in a cell type-dependent manner.

3.4. ATRA Supports the Development of Migratory CD103$^+$ Myeloid Cells. The dominant DC population of the gut is represented by CD103$^+$ migratory cells that express the enzymes required for the metabolism of VitA [4], while the CX3CR1$^+$ resident Mf population samples the microenvironment by protruding dendrites [11]. To assess how efficiently we could manipulate the effects of ATRA on CEC, we differentiated blood-derived monocytes with GM-CSF+IL-4 or M-CSF to generate DC and Mf, respectively, followed by the stimulation of cells with the supernatant of activated Caco2 cells. The cell surface expression of CD103$^+$ and CX3CR1$^+$ measured by FACS analysis revealed that the presence of Caco2 cell supernatants obtained from ATRA+IL-1β, ATRA+TNF-α, or

ATRA-pretreated CEC could increase the expression of the CD103 integrin in cells differentiated by GM-CSF+IL-4 or M-CSF to obtain DC and Mf, respectively (Figures 4(a) and 4(b)). These results also indicated that even in the presence of inflammatory stimuli (supernatant of IL-1β or TNF-α activated CEC) ATRA was able to promote the development of CD103$^+$ myeloid cells. In a similar experimental system, the frequency of CX3CR1$^+$ cells generated by GM-CSF+IL-4 and stimulated by cytokine-activated CEC supernatant was also increased in case the cell culture was conditioned by ATRA. In contrast to this finding, the expression of CX3CR1 remained unchanged in cells generated by GM-CSF (data not shown) and was decreased when the ATRA-conditioned CEC supernatant was added to Mf differentiated from monocytes with M-CSF and activated by IL-1β or TNF-α (Figures 4(c) and 4(d)). Similar results were obtained with HT-29 and HCT116 cells (data not shown). To confirm that the level of ATRA could be reconstituted in CEC after the washing procedure, we also added ATRA directly to DC and detected increased expression of CD103. To rule out the effect of other chemokines in the development of CD103$^+$cells, the chemokines secreted by activated CEC were added directly to monocytes at pretitrated concentrations (Mk 10 ng/mL, CXCL16 100 ng/mL, CXCL7 10 ng/mL, CXCL1 1 ng/mL, CXCL8 100 ng/mL, and CCL20 50 ng/mL) along with the differentiating growth factors GM-CSF+IL-4 or M-CSF and the expression of CD103 was measured by FACS. When the *in vitro* generated myeloid cells were treated directly with the different chemokines in the absence of ATRA, no phenotypic changes of DC and Mf could be detected (see Supplementary Figure 4 in Supplementary Material available online at http://dx.doi.org/10.1155/2015/579830), showing their direct dependence on ATRA.

3.5. Translation of the Molecular Information Collected by CEC-Stimulated Myeloid Cells to CD4$^+$ T-Lymphocytes. Considering the sensitivity of myeloid cells to proinflammatory signals provided by activated CEC and the modulatory effects of ATRA, we set out to test whether DC and Mf as antigen presenting cells could activate and polarize T-lymphocytes. To test this scenario, myeloid cells differentiated by GM-CSF+IL-4, GM-CSF, and M-CSF, respectively, were activated by supernatants of cytokine-activated Caco2 cells followed by coculturing them with autologous CD4$^+$ T-lymphocytes, and the number of IL-17 and IFNγ cytokine producing T-cells was detected by ELISPOT assays. We found that CD4$^+$ T cells cocultured with myeloid cells differentiated from monocytes to DC with GM-CSF+IL-4 (Figure 5(a)) or GM-CSF (Figure 5(b)) and "educated" by the supernatants of activated Caco2 cells in combination with ATRA resulted in significant suppression of IL-17 producing cell numbers. In contrast, monocyte-derived cells generated by M-CSF pretreated with Caco2 cell supernatant (Figure 5(c)) and subsequently co-cultured with CD4$^+$ T-cells, the number of IL-17 cytokine secreting cells was increased significantly indicating that DC and Mf exhibit different T-cell polarizing activities. Although slight differences could be observed in the magnitude of T-cell responses provoked by CEC supernatants activated by IL-1β or TNF-α, the T-lymphocyte responses were polarized to the Th17 direction in both cases independent of the proinflammatory cytokine used for CEC stimulation underpinning the role of DC-mediated inflammatory signals in driving CD4$^+$ T-lymphocyte responses. Under similar culture conditions IFNγ-secreting cells could not be detected in the CD4$^+$ T-cell population. Similar "education" of myeloid cells by ATRA-conditioning was observed also for HT-29 and HCT116 cell lines (data not shown). Thus, ATRA is able to exert different effects on monocyte-derived myeloid cells differentiated upon coculturing with CD4$^+$ T cells. This may indicate a broad range of RA-mediated effects involved in shaping the gut microenvironment.

4. Discussion

The cytokines secreted at increased levels in patients with IBD have been identified as TNF-α and IL-1β, but the complete spectrum of chemokines and chemokine receptors involved in these regulatory networks has not been analyzed in detail. We designed an *in vitro* experimental system to study the effects and the interplay of cytokines, chemokines, and RA in resting CEC and under inflammatory conditions for identifying the possible outcomes of myeloid cell-induced T-cell collaboration (Supplementary Figure 1). We observed that unstimulated CEC secrete CCL chemokines with the potential to attract DC and Mf thus ensuring continuous contact with CEC to support LP homeostasis [27, 28]. It has previously been observed that TNF-α and IL-1β do not induce the secretion of CCL22 in monocytes, macrophages, and B cells from human peripheral blood *in vitro*, but prolonged (12 hr) treatment could induce production of MDC/CCL22 protein by cultured human intestinal epithelial cells [1]. The detailed analysis of chemokine expression induced by the supernatants of CEC preactivated by IL-1β or TNF-α demonstrated that (1) the secretion of the CCL20, CXCL1, and CXCL8 chemokines could be induced only by IL-1β and was not affected by ATRA, (2) constitutive expression of the chemokines Mk, CXCL16, and CXCL7 was not modified by the supernatant of activated CEC, (3) ATRA downregulated the expression of Mk and CXCL16, and (4) the secretion of CXCL7 could be induced by both IL-1β and TNF-α in the presence of ATRA. Consistent with previous results, we also observed in our *in vitro* model that CXCL8 and CXCL1 are secreted upon activation of CEC by IL-1β but not by TNF-α showing that IL-1 family cytokines may exert dichotomous or opposing effects in maintaining gut homeostasis or inducing intestinal inflammation [29]. When Caco2 cells were treated with TNF-α in combination with *Clostridium difficile* toxin A, TNF-α itself did not influence the secretion of CXCL8 [10]. The chemokine CCL20 exhibited unique features as it could be induced exclusively by the supernatant of IL-1β stimulated CEC that could completely be inhibited by ATRA. This chemokine is highly specific for its receptor CCR6, that is, expressed by intestinal CEC and in human lymphoid tissues [30–32] and its expression is associated with IBD [33, 34]. The importance of the CCR6-CCL20 axis was also verified in CCR6 double negative mice showing decreased intestinal M-cell numbers and low IgA secretion upon rotavirus infection [35, 36]. Mk acts as a

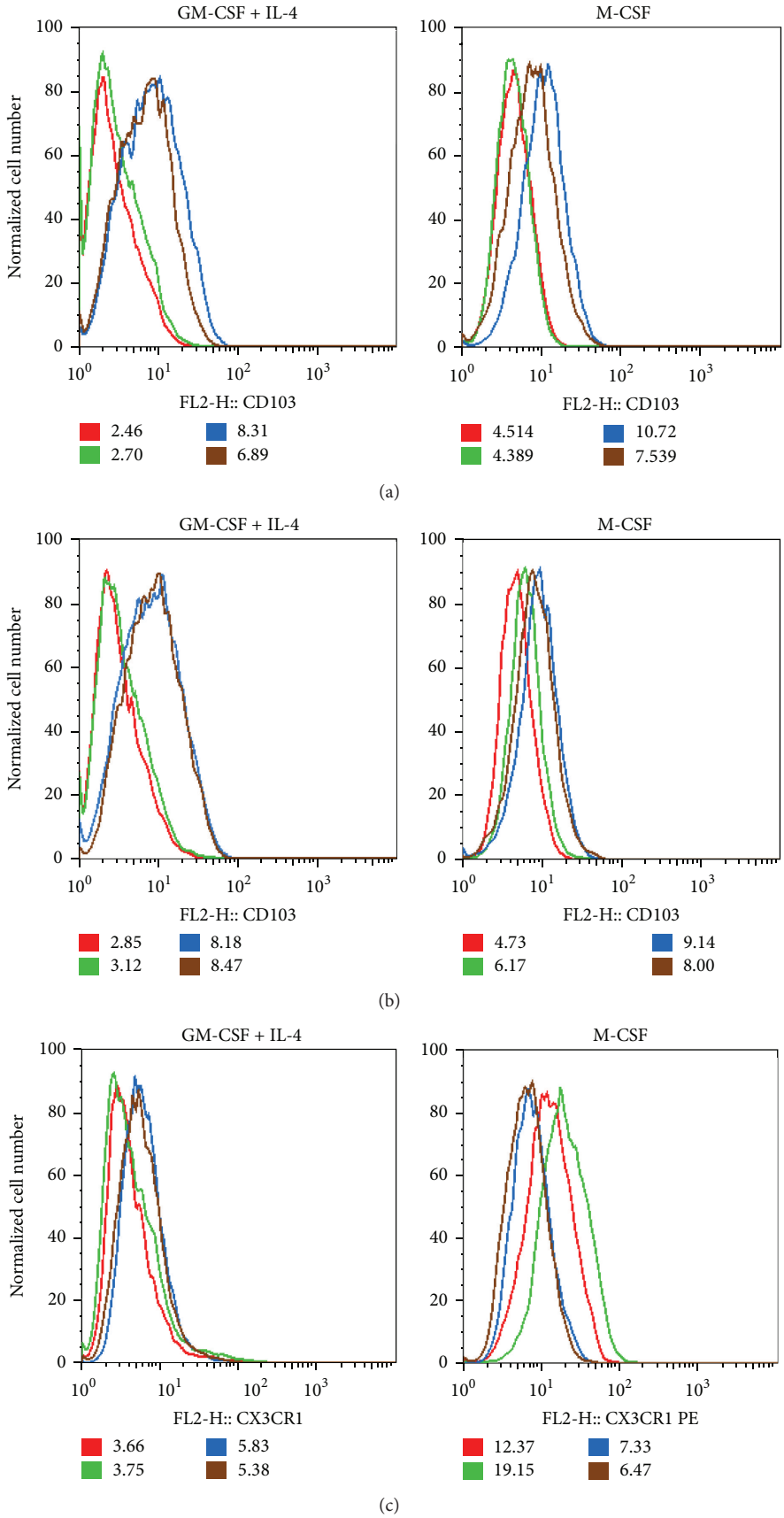
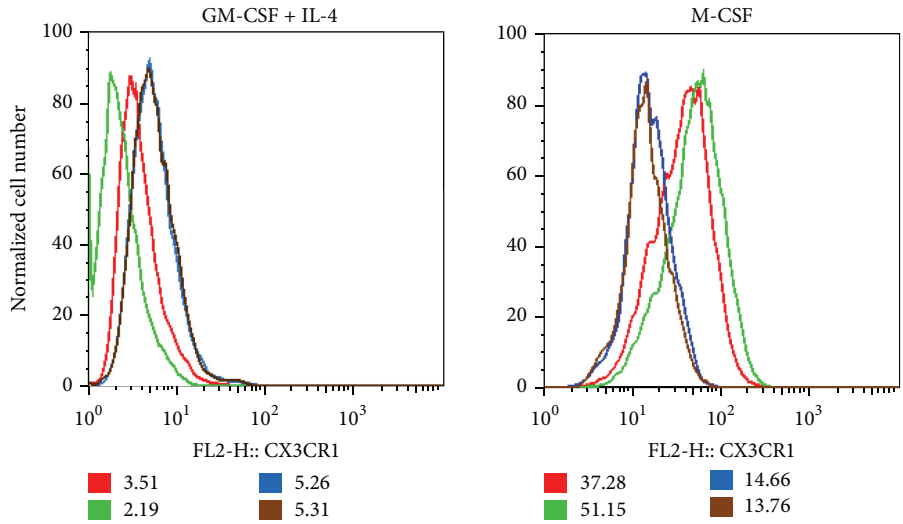


Figure 4: Continued.



(d)

Figure 4: ATRA promotes the expression of CD103$^+$ cells in *in vitro* differentiated myeloid cells. Monocytes were differentiated in the presence of GM-CSF+IL-4 or M-CSF, respectively. The differentiated cells were treated by the supernatants of Caco2 cells preactivated by IL-1β or TNF-α in the presence or absence of ATRA. The phenotype of the myeloid cells was characterized by measuring the cell surface expression of $\alpha 4\beta 7$/CD103 and CX3CR1 on day 3 of *in vitro* myeloid cell differentiation by flow cytometry. Histograms show the cell surface expression of CD103 in cells differentiated by GM-CSF+IL-4 (or M-CSF) and stimulated by the supernatants of Caco2 cells pretreated by IL-1β (a) or TNF-α (b) and the cell surface expression of CX3CR1 in cells differentiated by M-CSF (or GM-CSF+IL-4) and stimulated by the supernatants of Caco2 cells prestimulated by IL-1β (c) or TNF-α (d). MFI of a typical measurement out of 3–5 independent experiments is shown. Red line depicts untreated cells, green is for cells pretreated with the supernatant of Caco2 cells activated by IL-1β or TNF-α, blue is for myeloid cells pretreated with the supernatant of Caco2 cells activated by IL-1β or TNF-α in combination with ATRA, and brown corresponds to cells treated by the supernatant of unstimulated Caco2 cells and ATRA.

multifunctional cytokine and growth factor with bactericidal and fungicidal activity. Upregulation of Mk expression was also detected in the rat large intestine during DSS-induced colitis and was shown to activate CD4$^+$ T cells [37] leading to enhanced mucosal restitution during the repair process of colitis [38]. Ligation of the CXCR6 receptor by its CXCL16 ligand results in the activation of the MAP-kinase pathway observed in patients with Crohn's disease and was associated with clinical benefits and rapid ulcer healing [39]. When CEC were stimulated by IL-1β or TNF-α, the secretion levels of Mk, CXCL16, and CXCL7 remained constant while the physiological concentrations of ATRA could decrease the secretion of these chemokines significantly. CXCL7 was also shown to promote neutrophil adhesion and transmigration [40].

GM-CSF is a critical factor for DC development and expansion *in vivo;* it induces DC differentiation from monocytes and its absence can reduce the number of migratory DC [41, 42].

Based on this information and to further characterize the cell types involved, we generated myeloid cells with characteristic phenotypic properties by differentiating them in the presence of various growth factors (Table 2). We observed that the myeloid cells differentiated with ATRA exerted cell type-specific modulatory effects on their phenotype shown by the increased expression of CD14, GM-CSF, and M-CSF receptors, while decreasing CCR7 expression and inhibiting memory T-cell migration to secondary lymphoid organs

Table 2: Expression of CD1a, DC-SIGN, and CD14 cell surface molecules on monocyte-derived cells generated in the presence of the colony stimulating factors GM-CSF+IL-4, GM-CSF, or M-CSF. Peripheral blood-derived monocytes were differentiated in the presence of GM-CSF+IL-4, GM-CSF, or M-CSF in RPMI + 10% FCS for 3 days and the expression of the cell surface markers CD1a, DC-SIGN/CD209, and CD14 was monitored by flow cytometry using fluorescent labeled specific antibodies. The expression levels of CD1a, DC-SIGN, and CD14 are indicated as mean fluorescence intensity (MFI).

	CD1a	DC SIGN	CD14
GMCSF+IL-4	200.88	32.6	75.63
GMCSF	66.31	10.5	183.29
MCSF	6.01	4.3	369.47

[43] (Supplementary Figure 2). The role of ATRA in this process was confirmed by *in vitro* migration assays detecting moderate cell migration towards the supernatant of activated CEC containing a selected set of chemokines induced by activated CEC. ATRA inhibited the migration of myeloid cells differentiated in the presence of various growth factors even when they were stimulated by the supernatant of IL-1β-activated Caco2 cells. These results suggest that ATRA is a potent regulator of the tolerogenic microenvironment in the gut acting at least partially via modulating chemokine

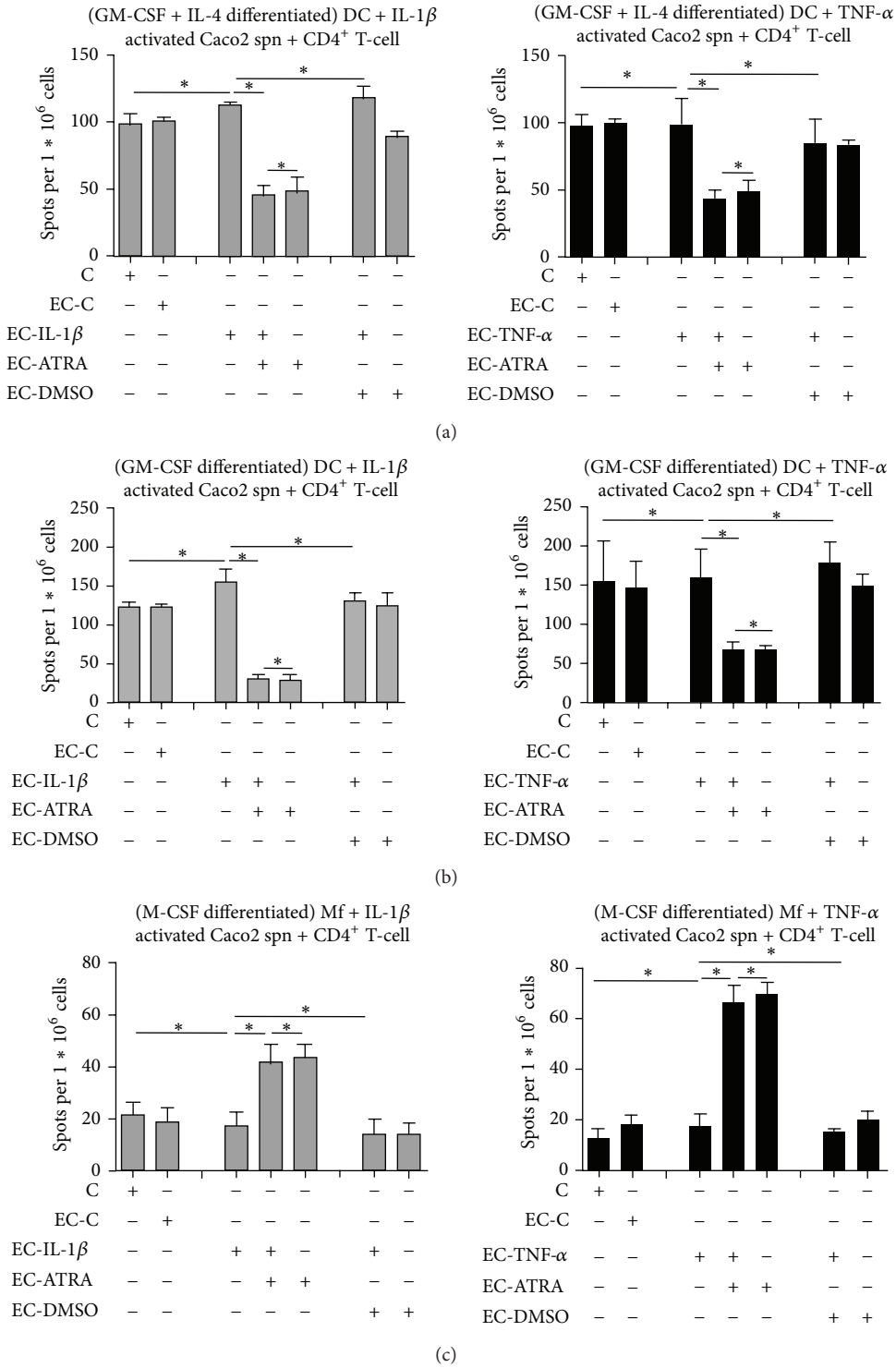


Figure 5: The effect of gut epithelial cell-mediated myeloid cell stimulation on the activation and polarization of T-lymphocytes. To assess the effects of stimulated myeloid cells on effector T-cell polarization, the supernatants of activated Caco2 cells were added to myeloid cells differentiated by GM-CSF+IL-4, GM-CSF, or M-CSF for 3 days and the washed cells were cocultured in fresh medium with autologous $CD4^+$ T-lymphocytes for another 2 days. The activation and polarization of $CD4^+$ T-cells c-incubated with myeloid cells generated by GM-CSF+IL-4 (a), GM-CSF (b), or M-CSF (c) was detected by the IL-17 ELISPOT assay. In the figure legends, C stands for unstimulated myeloid cells coincubated with $CD4^+$ T cells, EC-C for monocyte-derived cells "educated" by resting Caco2 cell supernatant followed by coincubation with $CD4^+$ T cells, and EC-IL-1β, EC-TNF-α, EC-ATRA, and EC-DMSO correspond to cultures containing monocyte-derived cells "educated" with the supernatants of Caco2 cells pretreated with IL-1β or TNF-α in the presence or absence of ATRA, or with DMSO used as solvent control, followed by coincubation with $CD4^+$ T cells. $^*P < 0.05$. Mean ± SD of the number of IL-17 secreting cells measured in 4 independent experiments is shown.

responses. Despite slight differences in the levels of CEC-derived chemokine secretion, the overall effects on modulating the myeloid cell phenotype, the migratory potential, and the DC- and Mf-mediated polarization of $CD4^+$ T-lymphocytes were comparable for the tested CEC that involved the Caco2, HT-29, and HCT116 cell lines suggesting a common regulatory mechanism.

Inhibited expression of the LPS-binding receptor CD14 (Supplementary Figure 2(a)) was shown to be associated with increased susceptibility to gastroenteritis and UC [44] and the expression of GM-CSF receptor was shown to be higher in healthy individuals than in UC or CD patients [45]. Microbial signals sensed by Mf in the colon are dependent on GM-CSF and result in increased IL-1β secretion. In the absence of microbes, decreased IL-1β secretion of mice led to low GM-CSF levels in the gut, while DC and Mf support the generation of Tregs by producing RA and IL-10 in the presence of TGF-β. The production of these regulators was drastically decreased in the absence of GM-CSF, as shown in $Csf2-/-$ mice [12]. In accordance with these findings we also observed the secretion of IL-1β when monocyte-derived cells generated in GM-CSF were treated with the supernatant of Caco2 cells conditioned by ATRA (Supplementary Figure 3). In this setting ATRA also decreased GM-CSF receptor expression (Supplementary Figure 2(b)) which could contribute to keep the overall concentration of IL-1β in the gut environment to controllable limits. ATRA is also responsible for the homeostatic regulation of $CD11b^+CD103^+$ DC [46] and under inflammatory conditions its production is increased to keep the local environment under check. When the chemokines were added directly to DC to test their effects on CD103 expression, DC could not acquire CD103 surface expression in the absence of ATRA (Supplementary Figure 4). Based on these results, we suggest that human monocyte-derived $CD103^+$ cells, induced by GM-CSF+IL-4 or GM-CSF together with appropriate activation signals, that is, the supernatant of activated CEC, are able to support the acquisition of the gut phenotype of human DC. To confirm this finding, we also performed experiments with blood-derived $CD1c^+$ DC and obtained a similar outcome (unpublished results) indicating that in the human system both cell types can acquire the capability to support $CD103^+$ myeloid cell development. Differentiation of the $CX3CR1^+$ population in the presence of GM-CSF+IL-4 was also promoted by ATRA but it was inhibited in Mf. These results are in line with previous results showing that $CX3CR1^+$ cells are inefficient in synthesizing ATRA and exhibit poor T-cell stimulatory capacity *in vitro* and *in vivo* when injected into intestinal lymphatics [47]. In contrast, $CD103^+$cells are able to migrate and can trigger adaptive immune responses by expressing gut homing receptors on T-cells [47].

The expression of M-CSFR (Supplementary Figure 2(b)) was shown to be enhanced by IL-1β stimulated CEC supernatant and the number of Th17 cells was increased when $CD4^+$ T cells were coincubated with Mf educated by the supernatant of CEC preactivated by IL-1β in the presence of physiological concentration of ATRA. The decreased number of Treg cell as compared to Th17 cells has been indicated in Crohn's patients and the number of Tregs was influenced by RA and IL-10 in the gut environment [10]. Bone marrow-derived DC could be "educated" by direct contact with murine epithelial MODE-K cells and the development of surface expression of $CD103^+$ was observed, which is involved directly in the development of Treg differentiation [48]. In our *in vitro* model with human CEC (Caco2, HT-29, and HCT116) and monocyte-derived DC, the development of Tregs was not observed. However, we were able to demonstrate the inhibition of Th17 cell numbers when autologous T-cells were cocultured with "ATRA educated" DC. We also observed the enhanced secretion of IL-10 by $CD4^+$T-cells after coculturing them with ATRA-conditioned DC and Mf (Supplementary Figure 5) indicating suboptimal conditions for Treg differentiation. It has also been shown that Th17 cells can exhibit both anti- and proinflammatory properties depending on the cytokine signals received [49]. These results altogether indicated that the detailed characterization of the gut milieu under different conditions is of utmost importance for understanding the complexity of regulation leading to the maintenance or loss of gut homeostasis.

5. Conclusion

The proinflammatory cytokines IL-1β and TNF-α, associated with inflammatory bowel diseases, exert different effects on gut epithelial cells monitored by the secretion of different combinations of chemokines in CEC. The metabolite RA, produced by both epithelial and myeloid cells, has the potential to downmodulate some, but not all chemokine responses to support a tolerogenic microenvironment in the gut environment. Myeloid cells differentiated by GM-CSF+IL-4 or GM-CSF (DC) and M-CSF (Mf) respond differently to activated epithelial cell-mediated stimuli and to RA. The signals provided by activated epithelial cells for DC and Mf can be translated to T-lymphocytes, but the number of Th17 producing cells is modulated differentially in DC and Mf indicating the complementary role of these myeloid cell types in regulating adaptive effector T-cell polarization.

Conflict of Interests

The authors declare no financial or commercial conflict of interests.

Acknowledgments

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Review Article

Gut Inflammation and Immunity: What Is the Role of the Human Gut Virome?

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The human virome comprises viruses that infect host cells, virus-derived elements in our chromosomes, and viruses that infect other organisms, including bacteriophages and plant viruses. The development of high-throughput sequencing techniques has shown that the human gut microbiome is a complex community in which the virome plays a crucial role into regulation of intestinal immunity and homeostasis. Nevertheless, the size of the human virome is still poorly understood. Indeed the enteric virome is in a continuous and dynamic equilibrium with other components of the gut microbiome and the gut immune system, an interaction that may influence the health and disease of the host. We review recent evidence on the viruses found in the gastrointestinal tract, discussing their interactions with the resident bacterial microbiota and the host immune system, in order to explore the potential impact of the virome on human health.

1. Introduction

The human virome is essentially a collection of all the viruses that are found in or on human beings. Continuously being updated, the human virome comprises eukaryotic and prokaryotic viruses, viruses that cause acute, persistent, or latent infection, and viruses that can integrate themselves into the human genome, for example, endogenous retroviruses [1, 2].

Both eukaryotic and prokaryotic viruses share lytic or latent life-cycles, which allow different virome/host interactions and promote virus survival and evolution [2]. As a result, human eukaryotic viruses can affect host physiology, mainly when chronically infecting particular sites, and virus-derived genetic elements can modify host gene and protein expression once integrated into host chromosomes [3–5]. Moreover, it has recently been shown that interactions between archaeal viruses and host cells in mammals are comparable with the well-documented relationships that exist between prokaryotic viruses and bacteria [6].

Nevertheless, the size of the human virome is not fully known. As discussed by Mokili et al. [7], our own cells are outnumbered about 10-fold by our bacteriome, and it has been postulated that the number of viruses in our body could be 10-fold higher still. Furthermore, the emerging evidence of new RNA viruses, unknown before the advent of innovative sequencing platforms, suggest that the eukaryotic virome may be far larger than previously thought [8].

The human gastrointestinal tract in particular plays host to one of the most complex microbial ecosystems and an intricate group of viruses. Progress in sequencing technology research is enabling us not only to detect the presence of such microorganisms, but also to evaluate how the intestinal microbiome affects human health. Such approaches have already shown how the gut microbiome, by interacting with the mucus layer, epithelial cells, and underlying lamina propria immune cells, can contribute to the health or disease of the host [9]. It is likely that similar studies into the complex interactions between the resident gut virome and immune and inflammatory processes could shed light on the pathogenesis of intestinal and extraintestinal diseases.

2. Human Gut Virome

Human faeces are known to contain at least 10^9 virus-like particles per gram [10]. Sequencing of gut viruses from faecal samples has shown that bacteriophages, which can harbour up to 10^{14} bacterial cells, are the most prevalent enteric viruses [11]. That being said, as discussed by Minot et al. [12], prokaryotic viruses are almost 10-fold more abundant in the gut than prokaryotes. This indicates that there is a dynamic community structure within the gastrointestinal tract, characterized by predator-prey interactions and thereby providing a source of horizontal gene transfer [13].

Although many gut bacteriophages have not yet been fully classified, the most abundant prokaryotic viruses in the intestine are currently thought to be the tailed, double-stranded DNA viruses of the order Caudovirales (Podoviridae, Siphoviridae, and Myoviridae), together with the tailless, cubic, or filamentous single-stranded DNA viruses (Microviridae) [14]. Prokaryotic viruses are known to influence human health by affecting bacterial community structure and function [12, 15, 16], but the intricate pathways by which this influence is exerted are yet to be fully clarified. Thus far, however, it has been discovered that (i) temperate phages are common; (ii) bacteriophages vary widely between individual hosts but not within a single subject; and (iii) the variety of bacteriophages present increases in adulthood, and the diet affects the composition of phage communities [12].

There are far fewer eukaryotic viruses than bacteriophages in the gut [15, 17, 18]. Nevertheless, sequencing of faecal samples from healthy children has revealed a complex community that includes viruses of the family *Picobirnaviridae, Adenoviridae*, *Anelloviridae*, *Astroviridae* and species such as *bocaviruses, enteroviruses*, *rotaviruses*, and *sapoviruses* [19]. Despite being fewer in number, these viruses also have significant effects on human health, both in healthy and immunocompromised subjects, causing acute gastroenteritis, acute enteritis, or colitis [19–22]. Picobirnaviruses, for instance, have been found in stool samples from individuals with diarrhoea of unknown aetiology [23–25], as well as in healthy subjects [19], leaving their pathogenic capability up for discussion. Among the RNA viruses found in the gut, a prevalence of plant viruses has been demonstrated, presumably introduced in the diet [12, 17].

Interesting studies have recently described the temporal dynamics of the human gut virome. It appears that the symbiotic relationships between host and virome develop at a young age, with specific variations occurring during the first two years of life, coinciding with environmental and dietary changes. As a result, individuals on the same diet showed similar gut virome composition [12, 17].

Given these findings, it is timely to evaluate the potential role of the gut virome in homeostasis, intestinal immunity, and inflammation.

3. Interaction and Recognition between the Virome and the Host Immune System

The enteric immune system exists in a continuous but dynamic equilibrium with all components of the gut microbiome, including the virome [26, 27]. It is likely that this interaction may influence the host's health and disease [2] by modulating the immune system itself [2, 28].

3.1. Viromal Effects on the Immune System. The virome is an important regulator of intestinal homeostasis and inflammation [29]. In this regard, as discussed by Foxman and Iwasaki [3], the virome is able to stimulate continuous low-level immune responses without causing any overt symptoms. This capacity has been documented for several systemic viruses, including Herpesviruses and Polyomaviruses, as well as Hepatitis B (HBV) and C (HCV) viruses in some individuals. Given what we know about virus-host interaction at the molecular level, it is feasible that variations within systemic and local gut virome, acting as commensal viruses, could even shape the immunophenotype of the host [2].

3.2. Interaction and Recognition between Phages and the Immune System. In addition to the role of the virome in regulating the bacterial microbiome (see below), there is evidence that bacteriophages may also directly interact with the human immune system. For example, as both Duerr et al. [30] and Hamzeh-Mivehroud et al. [31] showed, orally administered phages translocate *in vivo* to systemic tissue, wherein they trigger innate and adaptive immune responses. The humoral immune response induced by bacteriophages has also been documented in several different studies [32–35].

Nonetheless, little is known of the mechanism by which bacteriophages elicit innate antiviral immune responses. In asymptomatic individuals, the dynamic balance between the virome and the intestinal immune system is finely regulated by cytokines secreted by immune cells. These cells are able to recognize antigenic components or pathogen-associated molecular patterns (PAMPs), including those produced by viruses [2]. Toll-like receptors (TLRs) have also been postulated as innate antiviral immune sensors, with TLR3, TLR7, TLR8, and TLR9, as well as RIG-I—a cytoplasmatic double-stranded RNA helicase—and the cytoplasmatic DNA sensor cyclic-GMP-AMP (cGAMP) synthase reportedly involved in the recognition of viral structure. Activation of such receptors triggers signalling cascades that activate the transcription of nuclear factors such as NF-kB, IRF3, and IRF7, which in turn promote the expression of antiviral effectors such as type I interferon, proinflammatory cytokines such as Interleukin-6 and Interleukin-1 beta (β), and chemokines such as Interleukin-8 and CXCL-10 [36]. In an asymptomatic host, commensal bacteriophages activate one or more of these pathways, thereby inducing tonic stimulation of the antiviral immune response, and therefore a continuous cycle of cytokine production. These cytokines also exert their action on nonimmune cells and may continuously induce inflammatory processes therein, thereby conferring constant protection against pathogenic viral infections [28, 37].

Another mechanism by which bacteriophages interact with the immune system is through their association with the bacterial microbiome. Some bacteriophages use commensal bacteria as a vehicle for their own genome, and in specific conditions, immunodeficiency among others, induce the expression of phage particles, which can be detected by the immune system [38]. Other bacteriophages modulate bacterial antigenicity through the production of enzymes capable of modifying the O-antigen component of lipopolysaccharide (LPS) in microorganisms such as salmonella, *E. coli*, *Shigella,* and *Vibrio cholera* [39–42].

In addition, as discussed by Cuesta et al. [43], bacteriophage proteins enhance the potency of DNA vaccines.

However, intestinal bacteriophages are able to circumvent the adaptive immunity of their hosts, thanks to hypervariable regions found within their genomes. These regions are known to collocate into genes that encode for phage tail-fibre proteins and immunoglobulin super-family (IgSF) proteins, which could act as scaffolds for the presentation of diversified phage peptide sequences. Although the physiological relevance of these hypervariable regions still remains to be clarified, it is plausible that such a diversity-generating mechanism could enable phages to evade the antibodies targeting the phage particles [44, 45].

3.3. Interaction between Eukaryotic Viruses and the Immune System. To date, scant information is available about the relationship between eukaryotic intestinal viruses and the host immune system. However, the few studies performed so far suggest that the eukaryotic virome could have a significant impact on host defence mechanisms against viral and/or bacterial pathogenic infections.

It has also been suggested that other viruses that chronically reside in the healthy tissue of individuals, such as Herpesviruses, Poliomaviruses, Adenoviruses, Papillomaviruses, Hepatitis B and C viruses, and Human Immunodeficiency Virus (HIV), can cause acute or latent infections that protect the host from further viral and bacterial infections [46]. Indeed, an interesting mutual symbiosis experiment has shown that chronic infection with a gamma-herpes virus increases resistance to both *Listeria monocytogenes* and *Yersinia pestis* in mice [46]. It is also known to activate natural killer (NK) cells, resulting in increased resistance to tumour grafts [47].

However, other chronic viral infections can bring about a reduction in host immunity and increase susceptibility to infection. In particular, pathogenic immunodeficiency viruses, including Simian Immunodeficiency Virus (SIV)—which causes AIDS in rhesus monkeys—have been associated with damage to the intestinal barrier, resulting in an expansion of the gut virome [28]. Chronic immune suppression inevitably results in global immune deficiency of the host, allowing select enteric viruses to damage the intestinal epithelial cells, in turn promoting translocation of enteric viruses, commensal bacteria, and bacterial antigens across the epithelial surface, resulting in inflammation and systemic infection [48].

4. The Gut Virome in Health and Disease

The realization that viruses in asymptomatic hosts do not always cause the death of infected cells has prompted the emergence of a paradigm wherein the virome independently influences the host, aside from the classical immune response triggered to fight disease [5]. Indeed, since studies on the human bacterial microbiome demonstrated the presence of mucosal viruses in healthy individuals, the traditional concept of viral infection has been overturned. It has been established that viruses are prevalent in the gastrointestinal tract, despite the absence of symptoms, which suggests that even in health the gut mucosa is characterized by frequent infections that become part the virome and may in turn bring beneficial and/or damaging effects on the host. It is likely, therefore, that the gut virome is able to influence the host phenotype during health, as well as inflammation and disease, by interacting with both other members of the gut microbiome and host genetics factors. In particular, phages may modulate host-bacterial interactions by infecting bacteria, and it is equally feasible that the gut bacteriome may regulate the gut virome [28].

4.1. From Dysbiosis to Chronic Disease through Inflammatory Pathways. The intestinal phages may contribute to the transition from health to disease by helping to bring about dysbiosis—an imbalance between symbiotic bacteria and pathobionts [49].

Although little data regarding the role of phage in shaping intestinal bacterial dysbiosis is available to date, de Paepe et al. have postulated several mechanisms by which commensal bacteriophages could affect the ecosystem of the gut microbiota [49].

One such mechanism, termed "*Kill the winner*" suggests that phages shape the intestinal bacterial microbiota through density-dependent predation. In other words, phages kill only the dominant commensal bacteria (the "winning" microorganisms) in the intestinal ecosystem, thereby reducing their numbers. Indeed, just such a relationship has been demonstrated by Reyes et al. in adult germ-free mice colonized with 15 symbiotic bacteria and infected with a cocktail of faecal phages [50]. Phage predation is also suggested by the presence of clustered regularly interspaced short palindromic repeat (CRISPR) systems in human commensal bacteria. CRISPR spacers recognize and silence exogenous genetic elements such as phages, thereby conferring a type of acquired immunity [28].

According to another potential mechanism, described as the "*biological weapon*" model, commensal bacteria would use their phages to kill another bacterial competitor for the intestinal environment [51, 52]. In this scenario, the phage would provide immunity to its carrier bacteria against further infection [53]. Acting as "*biological weapons*", phages would cause massive lysis of competing microorganisms and a consequent shift in the composition of the population, leading to dysbiosis, and, in some cases, an inflammatory response [49]. Although this is an appealing hypothesis, further work is needed to confirm the existence of this mechanism.

Indeed, still other models have been put forward to explain the contribution of phages to intestinal dysbiosis. In one such model, so-called "*community shuffling*" [54], conditions of stress, such as antibiotic therapy, inflammation, and oxidative stress, have been theoretically implicated as triggers in prophage induction in several bacterial species like *E. coli* [55] and *Clostridium difficile* [56]. This theory is supported by the 30-fold increase in virus-like particles seen in biopsy specimens from patients with Crohn's disease with respect to healthy controls [57]. In the "*community shuffling*" model, this prophage induction would contribute to intestinal dysbiosis by altering the relationship between bacterial symbionts and pathobionts [54].

It is also feasible that temperate phages could affect the ecosystem without killing bacteria by carrying genes that modify bacterial phenotypes. This model, that is, the "*emergence of new bacterial strains*" has been demonstrated in *Escherichia coli* strain O104:H4, which can undergo lysogenic conversion, acquiring a Shiga-toxin encoding phage [58].

However it occurs, the intestinal dysbiosis promoted by the virome can be a triggering factor for inflammatory bowel disease (IBD) [59], Crohn's Disease (CD) [60], and colon cancer [61].

Inflammatory bowel diseases comprise a group of chronic inflammatory conditions that affect the gastrointestinal tract. This condition depends on individual genetic susceptibility, functional alterations in the intestinal epithelial barrier, dysbiosis, and immune factors.

As discussed by Lawlor and Moss [62] cytomegalovirus (CMV) is present in up to 70% of IBD patients, and that its reactivation could be associated with a type of colitis that displays some symptoms of IBD. Despite the implication of viral factors, it has been shown that antiviral treatment for CMV in IBD patients has no discernable impact on the outcome of the inflammatory disease [63]. It is therefore legitimate to ask whether CMV reactivation actively worsens the disease, or whether it is merely a "bystander" of inflammation [59].

Studies in mice deficient for the IBD susceptibility gene Atg16L1, which is involved in the autophagy pathway, have suggested a role for enteric viral infection in the pathogenesis of CD [16, 64–67]. Indeed, the Atg16L1 protein plays an important role in the biology of Paneth cells—specialized secretory cells located within the intestinal crypts—which release antimicrobial compounds and other substances that affect the gut microbiota [68]. Moreover, it found that intestinal noroviruses cause an abnormal phenotype of Paneth cell in mice with reduced expression of Atg16L1 (Atg16L1 hypomorphs), thereby highlighting an unexpected role of viruses in CD pathogenesis and showing how viral infection can have a profound influence on the expression of complex diseases [68].

5. Future Perspectives

In recent years, the development of high-throughput sequencing techniques has enabled partial characterization of the microbial composition of the healthy human gut, showing that viruses are important components of such communities (virome is described as follows).

Virome. Major Advances

(i) The human virome is the collection of all viruses found in or on humans, including eukaryotic and prokaryotic viruses that cause acute, persistent, or latent infection, and viruses integrated into the human genome, such as endogenous retroviruses [1, 2]. It also contains viruses that infect plants, presumably taken in with the host diet [15, 17, 18].

(ii) The virome has a profound impact on the composition and functional properties of the bacterial microbiota, which could in turn shape the development and function of the immune system [49].

(iii) The gastrointestinal virome, by interacting with the mucus layer, epithelial cells, and underlying lamina propria immune cells, can contribute to the health or disease of the host [60].

(iv) The enteric viruses are involved in the pathogenesis of dysbiosis and intestinal disorders, including inflammatory bowel disease (IBD), Crohn's disease (CD) [60], and colon cancer [61].

(v) The virome may contribute to phenotypic variation by regulating immunophenotype and the transcriptional state of the healthy host, reflecting their role in gene transfer and evolution [2].

(vi) Phages may serve as important reservoirs of genetic diversity in the microbiota by acting as vehicles for the horizontal transfer of virulence, antibiotic resistance and metabolic determinants among bacteria [49].

This raises major questions for research, which is currently being focused on clarifying the qualitative and quantitative composition of the human intestinal virome.

Although it is generally taken for granted that the intestinal virome is mainly composed of eukaryotic viruses and bacteriophages, there are suggestions that the counts used to make this assertion may be erroneous. Indeed, metagenomic sequencing analyses have often ignored RNA viruses, and the isolation procedures currently in use may prevent detection of some viruses in gut virome samples [2].

Another question to be answered is how the virome populations shape the composition, functional properties, and antigenicity of commensal bacteria, and what repercussions such interactions may have on host immunity and health.

In particular, better identification of PAMPs and viromal antigens that stimulate innate and adaptative systems should provide further insight into how our immune system protects against pathogenic viral and/or bacterial infections.

We also need to ascertain whether virus-host interactions covertly influence other disease phenotypes. In this regard it is vital to search for hitherto undetected effects that common viral infections might have on the pathogenesis of complex human diseases, the effects of noroviruses on Crohn's disease being a case in point [3]. Indeed, a better understanding of the mechanisms by which host-virus interactions contribute

to complex diseases may promote the development of new, more effective treatments.

The virome plays an important role in regulating the transcriptional state of a healthy host, which may respond differently to disease triggers, depending on the individual genetic constitution and virome composition of the latter. In this scenario, variations in the virome may contribute to phenotypic variation by regulating the immunophenotype, rather than by acting as pathogens.

Hence metagenetics—in essence the integrated study of the genetic impact on the host of the microbiome (and therefore virome) and vice versa *in vivo*—is set to become a major field of research.

Indeed, by helping us unravel the complex interactions between the virome and host genome, particularly as regards immunity, it is likely to shed considerable light on our genetics, health, and disease.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Central Role of Gimap5 in Maintaining Peripheral Tolerance and T Cell Homeostasis in the Gut

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Inflammatory bowel disease (IBD) including Crohn's disease and ulcerative colitis is often precipitated by an abnormal immune response to microbiota due to host genetic aberrancies. Recent studies highlight the importance of the host genome and microflora interactions in the pathogenesis of mucosal inflammation including IBD. Specifically, genome-wide (GWAS) and also next-generation sequencing (NGS)—including whole exome or genome sequencing—have uncovered a large number of susceptibility loci that predispose to autoimmune diseases and/or the two phenotypes of IBD. In addition, the generation of "IBD-prone" animal models using both reverse and forward genetic approaches has not only helped confirm the identification of susceptibility loci but also shed critical insight into the underlying molecular and cellular pathways that drive colitis development. In this review, we summarize recent findings derived from studies involving a novel early-onset model of colitis as it develops in GTPase of immunity-associated protein 5- (*Gimap5*-) deficient mice. In humans, *GIMAP5* has been associated with autoimmune diseases although its function is poorly defined. Here, we discuss how defects in Gimap5 function impair immunological tolerance and lymphocyte survival and ultimately drive the development of $CD4^+$ T cell-mediated early-onset colitis.

1. Introduction

The gastrointestinal tract is endowed with a complex immune network that has a major interface with the external environment and thus presents a site with a significant immunological challenge to maintain homeostasis. The maintenance of immune tolerance and gut homeostasis is achieved by an integrated regulation of innate and adaptive immunity but also involves the microbiome itself. The dysregulation of one of these biological components or a combination thereof often precipitates intestinal inflammation or IBD. In general, IBD encompasses two major chronic relapsing inflammatory conditions in the gastrointestinal tract: ulcerative colitis (UC) and Crohn's disease (CD). UC typically involves bloody diarrhea and inflammation involving the rectum that is often extended towards the proximal colon. Infiltration of inflammatory cells is chronic and restricted to the superficial layers of the colonic mucosa. On the other hand, CD is more pleomorphic and is characterized pathologically by discontinuous segments of transmural inflammation that can affect all parts of the GI tract, most commonly the ileocecal region. CD is often presented with development of fistulae and/or strictures while histological granulomata are a key feature. Importantly, the etiology or how dysregulation of the biological components required for gut homeostasis contributes to UC and CD remains poorly defined. An in-depth understanding in the development and/or causes of IBD will require a critical understanding of the interplay between several factors, including genetic susceptibility loci, the host immune system function, the development and composition of the intestinal microflora, and environmental factors such as diet, antibiotic treatment, appendectomy, and hygiene status [1–3].

Recent technical advances that allow for whole genome/exome sequencing [4, 5] and large scale genome wide association studies (GWAS) [6, 7] have led to a dramatic expansion of genetic studies and significantly advanced our understanding of the importance of susceptibility loci

associated with chronic (auto-)immune diseases including IBD [4–9]. Not only have NGS approaches been used to identify new and rare variants causing IBD using whole genome and/or whole exome sequencing, but also they have been used to facilitate transcriptome profiling in tissues from IBD patients (RNAseq analysis) and perform epigenomic characterization using CHIP-seq technology. In addition, next-generation sequencing allows for an in-depth analysis of the intestinal microbiome through 16S rRNA sequencing and thus promises to identify the role of microflora in IBD development. To date, more than 160 IBD genes and/or loci have been identified by GWAS [10, 11], most of them contributing modestly (relative risk of <2-fold) to disease susceptibility [12]. The identified loci predominantly represent polymorphisms in genes involved in the innate and/or adaptive immune function [13–15] but also involve genes required for autophagy [16, 17], epithelial barrier function [18], and/or activation of the endoplasmic reticulum stress response [19], indicating the diverse etiology of IBD [13, 20, 21]. The biological consequences and establishment of causality for associated variants still remain a challenging endeavor that relies on in-depth prior knowledge of gene function [22, 23]. As a consequence, for a large number of IBD loci, the functional alleles have not been confirmed and often the causal gene itself is unclear. Thus, the identification of causative genes and alleles remains a significant challenge. Nonetheless, traits that currently have been confirmed as susceptibility genes for IBD and are subject of intense research efforts include *NOD2* [20], *HLA class II* [24], *IL23R* [14], and genes involved in autophagy (e.g., *Leucine-rich repeat kinase 2* [*LRRK2*] [25], *ATG16L1* [16], and *immunity related guanosine triphosphate M* [*IRGM*]) [17]. For some, gene function is well defined [26, 27]; however, the functional implications of gene variants and how they predispose to colitis often remain elusive [8, 28, 29]. Whereas CD and UC behave as polygenic traits, rare cases of early-onset severe IBD presenting in infancy mostly behave as Mendelian disorders resulting from autosomal recessive mutations in single genes [30–34]. Mutations in *IL10RA*, *IL10RB* [35], or *X-linked inhibitor of apoptosis* (*XIAP*) [36] that cause severe forms of CD in infants born to consanguineous parents are prime examples [37]. Unfortunately, because of the disease severity often seen in early-onset IBD and the low frequency of patients carrying (unique) variants that may be life-threatening, identification of the genetic cause has often proved to be challenging. Current strategies involve resequencing of candidate genes and/or sequencing the whole genome/exome of individual patients by next-generation sequencing. While NGS has the potential to unveil all genome-/exome-wide variants, the understanding of the biological consequences of such variants again is challenging and requires a priori knowledge of gene function [22].

The use of (genetic) animal models has been helpful in providing biological insights into how genetic susceptibility loci affect gut homeostasis and, for instance, has revealed critical immunological pathways that are required for immunological tolerance in the gut [22, 38, 39]. Moreover, such models have revealed insight into the intricate balance between (altered) immune function and the role of microflora to IBD development [40]. To this extent, both forward and reverse genetic approaches have been valuable tools to improve our understanding of genes function, their regulation, and other complex interactions at the cellular and organismal level [22].

Our laboratory has applied an N-ethyl-N-nitrosourea (ENU) mutagenesis approach to identify genes with nonredundant function in lymphocyte development, priming, or effector function. As a result, we have identified a number of germ-line mutants that exhibit impaired peripheral tolerance, lymphocyte survival, and/or T cell activation [22, 41–43]. Among these, an ENU germline, designated *sphinx*, exhibited reduced peripheral T cell survival while developing spontaneous early-onset colitis development. The development of IBD-like intestinal inflammation in Gimap5-deficient mice exhibits hallmark features of IBD development in humans that include (1) a critical role for microbial flora; (2) colitis that is $CD4^+$ T cell driven; and (3) a concomitant loss of immunological tolerance, exemplified by a progressive decline in regulatory T cells (T_{reg}) numbers and function. Here, we discuss these critical aspects in the context of human IBD and consider the mechanistic pathways by which loss of Gimap5 leads to a loss of immunological tolerance ultimately causing the development of early-onset and severe colitis.

2. Gut Homeostasis, Immune Tolerance, and the Microbiome in IBD Development

The intestine represents a potential gateway for microbial pathogens but also contains commensal flora and dietary antigens that require strict immune tolerance. It is therefore no surprise that the gut constitutes the largest lymphoid organ in the body containing an extensive network of secondary lymphoid organs, with an enormous number of leukocytes, including several lymphocyte subpopulations that are uniquely observed in the gut [44, 45]. Upon activation, the intestinal immune system can mount a range of immune effector functions that have the potential to damage host tissue and reduce epithelial barrier function. Thus, a failure to maintain immunological tolerance against commensal flora often results in chronic intestinal inflammation [35].

The intestinal microbiota profoundly affects the immune system development under healthy conditions and thus represents an important environmental determinant of IBD development [46]. This is supported by evidence derived from human studies and studies using mouse models, as reviewed elsewhere [9, 47, 48]. For instance, (genetic) mouse models of intestinal inflammation generally do not develop disease when housed under germ-free conditions [49]. Moreover, T cell-mediated colitis is largely driven by bacterial antigens and fails to develop following nonspecific activation of host T cells. For example, transfer of OVA-specific $CD4^+$ T cells from RAG-$2^{-/-}$ OT-II transgenic mice into RAG-$2^{-/-}$ recipients developed colitis only when recipient mice were colonized with OVA-expressing *Escherichia coli*, not with control *Escherichia coli* [50, 51]. This finding has led to a particular focus in understanding the role of intestinal microbiota, that is, its composition, regulation, and interaction with

the host immune system, in the development of IBD. The gastrointestinal tract harbors more than 10^{14} microorganisms of ~1000 species [52, 53], mostly contained within the colon [54]. Over 90% of these consist of Bacteroidetes (gram negative) and Firmicutes (gram positive) bacteria. Specific *Bacteroides* species directly regulate antimicrobial peptide expression by intestinal epithelium through activation of Toll-like receptors (TLR) expressed on Paneth cells [55]. Moreover, the presence of specific bacterial species shapes adaptive immune functions within the intestines, including Enterobacteriaceae and Bacteroidaceae [56] for TCR$\alpha\beta$ intraepithelial lymphocytes; *Bacteroides fragilis* [57] and a mixture of Clostridia strains [58] for T regulatory cells; and *cytophaga-flavobacterium-bacteroidetes* and segmented filamentous bacterium for Th17 cells [59–61]. Thus, changes in the composition of commensal microbiota-(dysbiosis) may present a critical determinant of host immune responses and thereby contribute to the development of IBD [29]. Interestingly, studies involving 16S rRNA sequencing from gut biopsy or stool samples revealed a detectable difference between the intestinal microbiota in the two forms of IBD (CD and UC) compared to healthy controls [62]. However, whether the observed dysbiosis in microbiota is directly associated with the presence of IBD susceptibility loci or a consequence of intestinal inflammation *per se* is currently unclear and an area of intense inquiry. A prime example of bacterial species driving colitis is provided by studies involving *Helicobacter hepaticus*—a commensal bacterium with opportunistic pathogenic potential [57, 63]. Although colonization of wild-type C57BL/6J mice with *H. hepaticus* does not result in inflammation or disease, *H. hepaticus* induces colitis in IL10$^{-/-}$ [64] or SCID/Rag2$^{-/-}$ hosts that received naïve CD4^{+}CD45RB^{high} T cells [63]. This colitis model is driven by homeostatic proliferation of naïve T cells through bacterial antigens including the flagellar antigen of *H. hepaticus* [65]. Colitis induction in this model is only observed in the absence of T_{reg} cells allowing for robust CD4^{+} T cell effector responses. Overall, these observations suggest that perturbations in gut microbiota and host immune system underlie the development of intestinal inflammation and IBD—an etiology referred to as the two-hit hypothesis [66].

3. Monogenic Causes of IBD

Interestingly, a large number of IBD susceptibility loci identified by GWAS studies are shared with other complex (auto-)immune diseases such as type-1 diabetes, celiac disease, multiple sclerosis, and systemic lupus erythematosus [10]. This is primarily due to the fact that these loci represent genes involved in immune cell signaling, including T cell differentiation, immune tolerance, and/or innate immune responses [28, 67, 68]—immunological pathways that are critical determinants for (auto-)immune disease. Clear examples of such loci are loss-of-function mutations in either IL10RA or IL10RB [35]. These mutations are linked with severe, early-onset enterocolitis in children—a pathology that is also observed in mice lacking either *Il10* [69, 70] or *Il10rb* [31, 35]. Changes in *Il-10r* variants are functionally linked to alterations in hematopoietic cell function and colitis can generally be cured through hematopoietic stem cell transplantation [71].

Interleukin-10 (IL-10) is a pleiotropic cytokine with a multitude of anti-inflammatory and immunoregulatory functions, which is secreted by a variety of cell types and is critical for maintaining immune homeostasis of the gut [72, 73]. For instance, IL-10 modulates the function of APCs through inhibiting phagocytosis, downregulating the expression of MHCs and costimulatory molecules, and decreasing the production of proinflammatory cytokines and chemokines in IBD [74]. Moreover, IL-10 directly restricts the differentiation of Th cells [70, 74] and maintains the suppressive activity of T_{reg} cells [75]. Consistent with this, T cell-specific [76] or FoxP3^{+} T_{reg}-specific [77] deletion of *Il10* results in spontaneous colitis, highlighting the importance of T_{reg}-derived IL-10 in preventing intestinal inflammation. On the other hand, a recent study suggests that macrophages are a prime cell target for IL-10 activity in the gut in that loss of *Il-10ra* specifically on macrophages resulted in spontaneous colitis development [78]. Overall, these studies establish IL-10 as a central mediator in gut homeostasis affecting both innate and adaptive immune responses.

4. The Role of CD4^{+} T Cells in Colitis

The key challenge of the intestinal immune system is to properly respond to pathogens while maintaining immune tolerance towards commensal bacteria and food antigens [79]—a process that requires complex cellular and molecular regulatory mechanisms [45, 80]. Particularly, the presence of unique immunosuppressive CD4^{+} T cell populations has been described in the intestine that control immune homeostasis and prevent inflammation towards harmless foreign antigens [81]. Importantly, increased accumulation of CD4^{+} T cells in the intestine is a key feature of inflammatory bowel disease [9, 82] and presents an important therapeutic target. Intestinal CD4^{+} T cell populations can be broadly classified based on function into effector CD4^{+} T cells and regulatory CD4^{+} T cells.

Effector CD4^{+} T cells, also referred to as helper T (Th) cells, play a critical role in the execution of immune functions. These include the development of antigen-specific CD8^{+} T and B cell responses and inflammatory cytokine production causing the recruitment of effector cells such as neutrophils. Whereas early studies primarily focused on the functional distinction between Th1 (or IFN-γ^{+} producing CD4^{+} T cells) and Th2 cells (interleukin 4-producing T cells), more in-depth studies in mice suggested that Th2 cells were largely absent in healthy mouse colonies in the absence of intestinal parasites [83]. Importantly, a third subset, the Th17 subset of CD4^{+} T cells, has recently been described as the major T cell population within both healthy and inflamed intestinal mucosa [84]. The identification of this subset has almost entirely shifted the focus on this cell type as a driver of disease in both experimental models and human IBD. Th17 cells produce a large number of cytokines, including IL-17A and IL-17F—key cytokines involved in the

recruitment and activation of granulocytes and critical to the host response against extracellular bacteria. Importantly, microbiota-specific memory Th17 cells are far more potent in inducing colitis in recipient mice compared to Th1 cells [85]. Moreover, a correlation between IL17 levels and disease severity in human IBD patients has been observed [86] suggesting a key role for Th17 cells and cytokines in IBD. Although the classification of these T helper cells suggests a specific and unique cytokine production profile, the $CD4^+$ T cells isolated from lamina propria undergoing active colitis can express both IL-17 and IFNγ, indicating the unique plasticity of Th17 cells and their ability to convert into Th1 cells [87]. Given that Th17 cells are the main $CD4^+$ T cell population in the intestinal tract, this plasticity is thought to be of critical importance to adapt to changes in the local intestinal environment and mount a proper immune response while maintaining gut homeostasis.

The importance and dominance of regulatory T cells and their immunosuppressive function are demonstrated by the fact that the majority of individuals do not develop gut inflammation despite an enormous microbial and antigenic load within the intestine. Moreover, transfer of naïve $CD4^+CD45RB^{high}$ $CD4^+$ T cells in lymphopenic hosts such as $Rag1/2^{-/-}$ or SCID mice induces lymphopenia-induced T cell activation and colitis only in the absence regulatory T cells (reviewed in [45, 88]).

Thus, T_{regs} cells play a critical role in maintaining immune homeostasis and limiting autoimmune responses by modulating cells of both the innate and the adaptive immune systems. The main types of regulatory cells in the gut are the natural (thymic) and adaptive (induced) $CD4^+FoxP3^+$ T_{regs}, as well as Tr1 and Th3 cells [89]. The effector pathways by which T_{regs} induce tolerance are multiple and include secretion of inhibitory cytokines such as IL-10 and transforming growth factor-β (TGF-β), granzyme-mediated cytolysis of target cells, expression of cytotoxic T-lymphocyte antigen-(CTLA-) 4 resulting in T cell inhibition, and metabolic disruption [45, 90, 91]. Impaired immune regulation by T_{reg} cells will result in a loss of immunological tolerance in the gut and cause colitis. Such deficiencies may stem from inadequate numbers of T_{reg} cells—due to impaired development, proliferation, or survival—or defects in immunosuppressive function intrinsic to T_{reg} cells. Alternatively, pathogenic effector T cells may be resistant to suppression by T_{reg} cells. At the site of inflammation, effector T cells are reported to develop mechanisms of resistance to T_{reg} regulation [92, 93], although the underlying mechanisms remain poorly defined.

In humans, the critical role for T_{reg} cells in preventing gut inflammation is further supported by the finding that individuals with genetic abberations in IPEX causing functional impairment of the transcription factor FoxP3 develop severe bowel inflammation [94]. Moreover, patients with genetic mutations in FoxP3 who lack or have nonfunctional T_{regs} exhibit severe intestinal inflammation associated with lymphocytic infiltration of the intestinal mucosa [95, 96]. Similarly, mice lacking $FoxP3^+$ T_{regs} [92, 97] or lacking the ability to suppress via T_{reg}-derived cytokines such as IL-10 [45, 89], IL-35 [98], and TGFβ [99] develop severe colitis. Together, these studies highlight the importance of $CD4^+$ T cells, particularly T_{reg} cells, in maintaining gut homeostasis. In addition, they point to monogenic causes of IBD that specifically affect T_{reg} function ultimately leading to loss of immunological tolerance and gut inflammation.

5. GIMAP5: A Critical Determinant of T Cell Survival and Peripheral Tolerance

Recently, studies have identified the GTPase of immunity-associated protein 5 (GIMAP5) as a key factor in maintaining T cell homeostasis and immunological tolerance. GIMAP5 is part of the family of GIMAP proteins, which are predominantly expressed in lymphocytes and regulate lymphocyte survival during development, selection, and homeostasis [100–106]. Members of this family share a GTP-binding AIG1 (avrRPT2-induced gene-1) domain, derived from an AIG1 resistant gene first described in *Arabidopsis thaliana* that was induced upon infection with *Pseudomonas syringae type III*. The AIG domain is conserved across vertebrates and angiosperms and, in vertebrates, the family consists of seven (human and rat) and eight (mouse) members that are clustered within a tight single region on chromosomes 7, 4, and 6, respectively, ([107–110] and (Figure 1)). Mouse Gimap5 is a 308-amino acid protein that contains an AIG1 domain (residues 24–227) comprising five GTP-binding motifs (G1–G5), a P-loop NTPase domain (residues 1–168), two coiled-coil domains (residues 187–221 and 239–265), and a transmembrane domain (residues 284–304) ([105, 106] and (Figure 1)). Recent crystallographic studies revealed that the Gimap proteins manifest a nucleotide coordination and dimerization mode similar to dynamin GTPase—a component essential for the scission and fusion of cellular vesicular compartments such as endosomes [111, 112]. Members of the Gimap family appear to be expressed in different subcellular compartments, with Gimap5 localizing in multivesicular bodies (MVBs) and lysosomes in lymphocytes [113]. Their function in lymphocytes, however, remains poorly defined.

Genetic aberrancies of *GIMAP5* have been linked to impaired immunological tolerance, lymphocyte survival, homeostasis, and autoimmunity in a variety of species including humans, mice, and rats. In humans, polyadenylation polymorphisms in *GIMAP5* are associated with increased concentrations of IA2 autoantibodies in type 1 diabetes (T1D) patients [114] and an increased risk of systemic lupus erythematosus SLE [115, 116]. Moreover, in patients with T1D, expression of several GIMAP genes including GIMAP5 is reduced in T_{reg} cells compared to healthy individuals [117]. In a spontaneous rat model of type I diabetes (the BioBreeding diabetic prone (BB-DP) rats), abnormal thymocyte development and premature death of peripheral $CD4^+$ and $CD8^+$ T-cells [110, 118, 119] were linked to a frame shift mutation in GIMAP5, designated *lyp*, causing a truncated nonfunctional protein ($GIMAP5^{lyp/lyp}$) [100–106]. In the presence of the diabetogenic MHC locus IDDM1, this *lyp* mutation is essential for diabetes onset in BB-DP rats ultimately triggering lethal disease [105, 106, 110]. A similar loss of lymphocyte survival is observed in $Gimap5^{-/-}$ null mice [120]. However, the loss

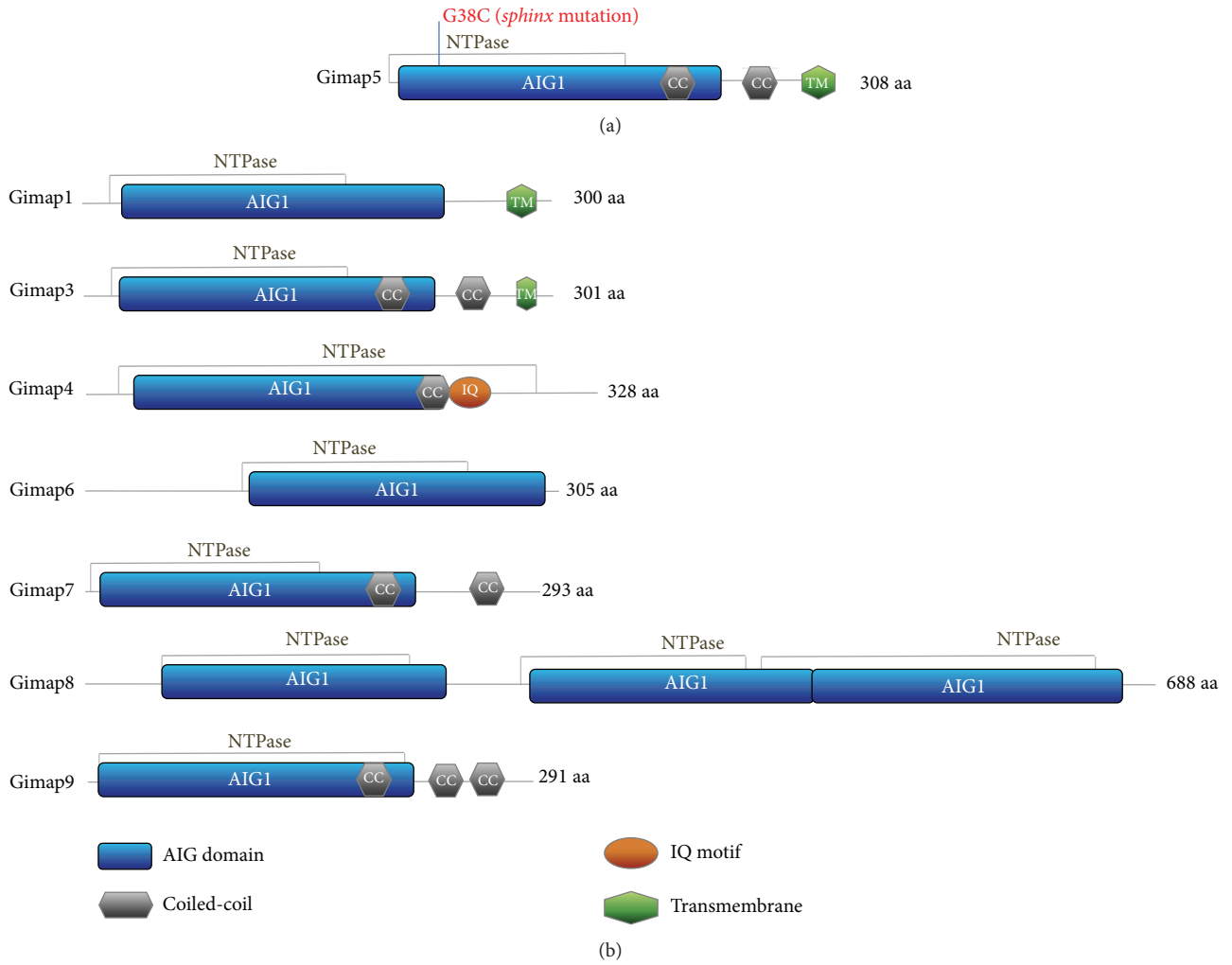

FIGURE 1: Predicted structural domains within mouse Gimap5. (a) Mouse Gimap5 is a 308-amino acid protein that contains an AIG1 domain (residues 24–227), a P-loop NTPase domain (residues 1–168), two coiled-coil domains (residues 187–221 and 239–265), and a transmembrane domain (residues 284–304). The G → C missense mutation in *sphinx* mice at residue 38 is indicated. (b) Schematic overview of the domain features present in the different Gimap family members.

of lymphocyte survival in *Gimap5*$^{-/-}$ mice is not limited to T cells, but also extends to reduced survival of NK, iNKT, and B cells with extensive extramedullary hematopoiesis observed in the liver [120].

These observations were confirmed by an N-ethyl-N-nitrosourea (ENU) induced *Gimap5*-germline mutant identified in our laboratory—designated *sphinx*. ENU is a widely used mutagen to create random germline point mutations in mice and has proven to be an effective approach to probe and identify critical genes for any phenotype of interest, for example, colitis or development/function of the immune system [22, 121]. Phenotypes causing ENU mutations primarily involve missense mutations (~61%) or nonsense mutations (10%) (source: http://mutagenetix.utsouthwestern.edu/), the type of genetic variants that can be found in humans. The *sphinx* mutation involved a G → T point mutation in *Gimap5* resulting in a G38C substitution in the predicted GTP-binding domain of Gimap5 [42]. The mutation destabilized the protein and caused a complete loss-of-function similar to the published Gimap5 KO [26]. Specifically, the *sphinx* mutant exhibited a similar reduced lymphocyte survival, including loss of NK cells, CD4$^+$ T, CD8$^+$ T, and B cells to the *Gimap5* knockout mice reported. The causative germline mutation involved a single G → T point mutation in Gimap5. This mutation resulted in a G38C substitution in the predicted GTP-binding domain of Gimap5, destabilizing the protein and causing a complete loss-of-function. Interestingly, from birth until weaning, *sphinx* (or *Gimap5*$^{sph/sph}$) mice appear outwardly healthy. However, after 7-8 weeks of age, mice lose weight and develop severe colitis, exemplified by goblet cell depletion, lamina propria leukocyte infiltration, epithelial cell hyperplasia, and crypt loss [42, 43]. The severe colitis likely contributed to the early mortality of *Gimap5*$^{sph/sph}$ mice, which generally occurred by 14 weeks of age. Interestingly, antibiotic treatment blocked intestinal inflammation in *Gimap5*$^{sph/sph}$ mice, suggesting a critical role

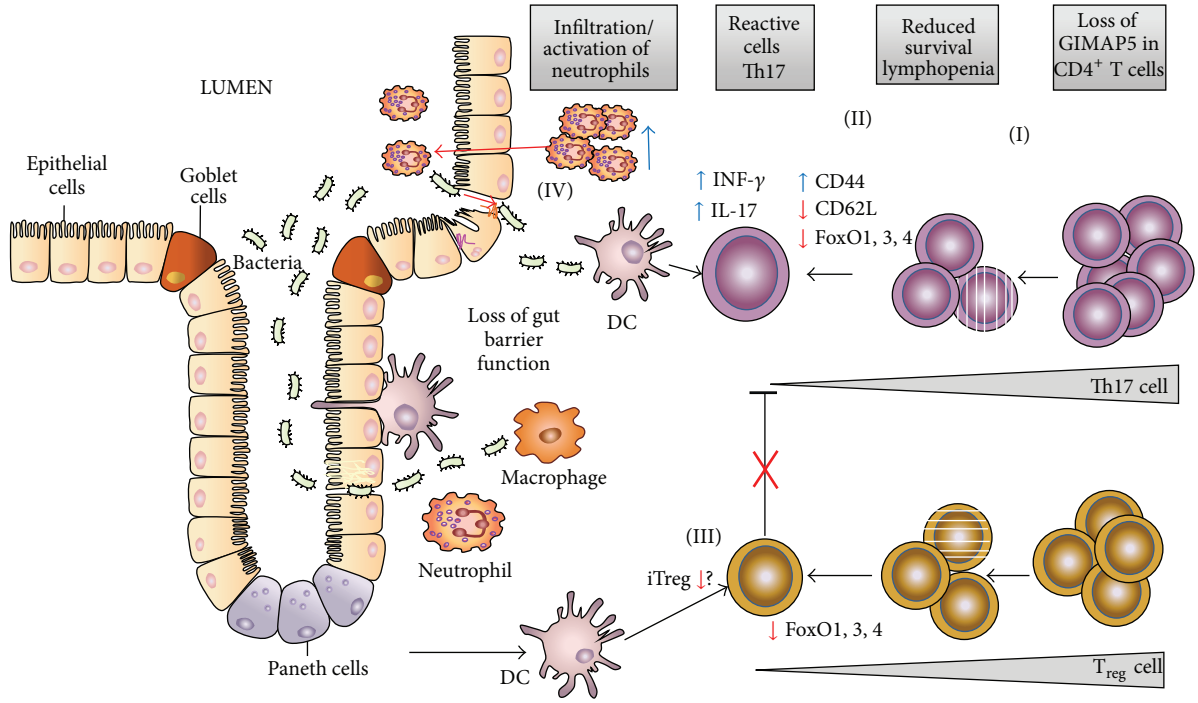

FIGURE 2: Schematic representation of the key events causing colitis in *Gimap5*-deficient mice. Loss of Gimap5 leads to reduced survival of lymphocytes (I) including $CD4^+$ T cells with remaining T cells exhibiting a characteristic LIP phenotype ($CD44^{high}$; $CD62L^{low}$) and polarization towards Th17 (II). Importantly, during the onset of $CD4^+$ T cell lymphopenia, a progressive loss of full-length FoxO1, FoxO3, and FoxO4 expression is observed that correlates with a loss of T_{reg} induction (iTreg) and function in the gut tissue (III). The lack of T_{reg} immunosuppressive activity (indicated by the red X) triggers activation of $CD4^+$ Th1/Th17 cells in the gut causing production of IL17 and IFNγ cytokines and subsequent infiltration of macrophages/neutrophils that further amplify intestinal inflammation and a loss of epithelial barrier function (IV) and may ultimately lead to neutrophil transepithelial migration (for an extensive review on neutrophils in IBD pathogenesis, see [133]).

for the microbiome also in this spontaneous model of colitis. Overall, inflammation of the gut in *Gimap5*$^{sph/sph}$ mice is early-onset and behaves as a monogenic trait, thus very similar to mutations in IL10RA, IL10RB, or XIAP [32, 33, 122, 123].

6. *Gimap5*$^{sph/sph}$ Mice: A Novel T Cell-Mediated Colitis Model

Gimap5$^{sph/sph}$ mice exhibit an absence of NK or $CD8^+$ T cell populations in peripheral lymphoid organs. Interestingly, relatively normal thymocyte development occurs, including $CD4^+$ T cell, $CD8^+$ T cell, and $Foxp3^+$ regulatory T cell lineages [42]. Nonetheless, *Gimap5*$^{sph/sph}$ mice exhibit a progressive reduction in circulating $CD4^+$ T cells and the $CD4^+$ T cells that remain after five weeks of age exhibit a lymphopenia-induced proliferation (LIP) phenotype ($CD44^{high}$ and $CD62L^{low}$), a T cell phenotype associated with autoimmunity [124]. Interestingly, despite their reduced survival, *Gimap5*$^{sph/sph}$ $CD4^+$ T cells produced exceeding amounts of IFNγ and IL-17A compared to wild-type $CD4^+$ T cells and exhibited spontaneous activation in the *Gimap5*$^{sph/sph}$ gut tissue pointing to a potentially critical role of $CD4^+$ T cells in this disease model. Indeed, antibody-mediated CD4-depletion *in vivo* prevented colitis in these mice corroborating the importance of $CD4^+$ T cells in the pathogenesis. The lymphopenia and expression of $CD44^{high}CD62L^{low}$ markers by $CD4^+$ T cells (Figure 2) are indicative of lymphopenia-induced proliferation and resemble the $CD4^+$ T cell phenotype first described in the adoptive transfer T cell model of colitis [125]. As mentioned, the development of $CD4^+$ T cell-induced colitis in *Gimap5*$^{sph/sph}$ can be prevented by antibiotic-treatment, again confirming the critical role of the microbiota in T cell activation [43]. Although the intestinal microbiota provide a potentially large source of foreign antigens that may drive the T cell response towards gut tissue, it is important to note that many autoimmune diseases are associated with immune-deficiencies which result in lymphopenia and subsequent "homeostatic" proliferation. The genetic and molecular basis of how these complex processes are controlled still remains incompletely defined. T_{reg} cells have been implicated as a critical factor in the development of disease following homeostatic proliferation and a similar critical role for T_{reg} cells was observed in the colitis development in *Gimap5*$^{sph/sph}$ mice. Specifically, *Gimap5*$^{sph/sph}$ mice fail to maintain a T_{reg} population with immunosuppressive function. Whereas relatively normal numbers of $Foxp3^+$ T_{reg} cells were found in spleen and LNs of 3-week-old mice, T_{reg} cell numbers were significantly reduced in 6-week old mice [43]. More

importantly, a progressive loss of T_{reg} function in MLN of *Gimap5*$^{sph/sph}$ mice was observed. Whereas T_{reg} cells from 4-week-old *Gimap5*$^{sph/sph}$ mice showed a slight but significant reduction in their ability to suppress wild-type $CD8^+$ T cell proliferation *in vitro*, T_{reg} cells from older (6-week-old) *Gimap5*$^{sph/sph}$ mice were incapable of suppressing wild-type $CD8^+$ T cell proliferation, suggesting a critical loss of T_{reg} function and survival to be responsible for colitis development in these mice (Figure 2). Indeed, transfer of wild-type $CD4^+CD25^+$ T_{reg} cells into *Gimap5*$^{sph/sph}$ early on prolonged survival, prevented increased $CD4^+$ T cell effector function in the MLN, and protected these mice from colitis [43]. Together, these data indicate that Gimap5 is a critical determinant of T_{reg} survival and function, thereby controlling gut homeostasis. The critical role of Gimap5 in T_{reg} survival/function is also evident in Type 1 diabetes in Bio-Breeding rats [105, 106] and may clarify why polyadenylation polymorphisms in *GIMAP5*, leading to rather subtle changes in gene expression, are associated with human autoimmune diseases such as T1D [114] and SLE [115].

7. Molecular Determinants of Peripheral Tolerance in the Absence of Gimap5

Given the loss of T_{reg} development/function, key questions currently center on understanding the molecular pathways by which Gimap5 controls T cell survival and peripheral tolerance. A number of studies have implicated Gimap5 to interact with Bcl2 members in mitochondria and implicated a critical role for Gimap5 in controlling proapoptotic pathways in T cells. Data in our laboratory, however, revealed no improved survival of lymphocytes (or prevention of colitis for that matter) when *Gimap5*$^{sph/sph}$ mice were crossed to *Bim*-deficient or *Bax/Bak*-deficient backgrounds (Aksoylar and Hoebe; unpublished data) suggesting that the reduced T cell survival is likely independent of the classical proapoptotic pathways. In terms of peripheral tolerance, a striking similarity is observed with the phenotypes reported in mice deficient in the family of Fork-head box group O (Foxo) transcription factors. The family of Foxo transcription factors contains 4 members of which three (Foxo1, Foxo3, and Foxo4) have overlapping patterns of expression and transcriptional activities and they play an essential role in the quiescence and survival of $CD4^+$ T cells [126, 127]. In addition, Foxo expression has been reported to be essential for T_{reg} cell development and function [128, 129]. The potential mechanisms by which Foxo transcription factors control T_{reg} development and function have been described in detail and include their role as coactivators downstream of the TGFβ signaling pathway by (1) interacting with SMAD proteins [130, 131] and by (2) directly regulating the induction of a number of T_{reg} cell associated genes, including Foxp3 itself but also CTLA-4 and CD25 [128, 129]. Importantly, $CD4^+$ T cells from *Gimap5*$^{sph/sph}$ mice revealed a complete absence of Foxo1, -3a, and -4 proteins. This effect was predominantly observed at the protein level with relatively normal RNA levels in $CD4^+$ T cells, suggesting that regulation of Foxo3 and Foxo4 protein expression occurs predominantly at the posttranslational level. Interestingly, the loss of Foxo expression was progressive and correlated with the loss of immunological tolerance in Gimap5-deficient mice. Importantly, the loss of Foxo expression in *Gimap5*$^{sph/sph}$ $CD4^+$ T cells was specifically observed in cells undergoing LIP, which may suggest degradation of Foxo expression due to constitutive homeostatic activation of T cells (Figure 2). Although T cell activation in general results in a brief transient loss of Foxo expression [132], loss of Foxo expression is not observed following transfer of wild type $CD4^+$ T cells into lymphopenic Rag2-deficient hosts (Aksoylar, Hoebe; unpublished results), suggesting that the loss of Foxo proteins in Gimap5-deficient $CD4^+$ T cells involves a unique degradation mechanism. Importantly, the loss of Foxo expression in *Gimap5*$^{sph/sph}$ $CD4^+$ T cells correlated with a loss of T_{reg} population and function and likely represents an important determinant of the colitis pathology observed in these mice.

8. Conclusion

A genetic alteration in Gimap5 has been strongly linked with reduced T cell survival and loss of immunological tolerance in both animal models and human studies. This results in predisposition to a variety of autoimmune related diseases including T1D, SLE, and colitis. Despite the profound impact of Gimap5 deficiency in terms of both lymphoid survival and peripheral tolerance, very little is understood about the molecular mechanisms underlying these robust phenotypes. Thus, a number of critical questions remain to be addressed that include (i) what is the molecular function of Gimap5 in T cells following activation?, (ii) what are the mechanistic pathways by which loss of Gimap5 causes reduced lymphocyte survival and peripheral tolerance *in vivo*?, and (iii) why do $CD4^+$ T cells in Gimap5-deficient mice exhibit loss of Foxo expression at the posttranslational level?

Finally, given the severe phenotypes related to the host immune system observed in both mouse and rat Gimap5-deficient models, a *GIMAP5* null phenotype in humans is expected to result in a severe immunodeficiency, although the phenotype has yet to be described. Such a severe immunodeficiency would be predicted to present in infancy as a monogenic trait and, with the current sequencing capacity and efforts, *de novo* mutations in *GIMAP5* should be considered prime causal candidates. Regardless, the detailed mechanistic insight into the loss of T cell survival and immunological tolerance in *Gimap5*$^{sph/sph}$ mice may ultimately help our understanding as to how polyadenylation polymorphisms in GIMAP5 predispose to T1D or SLE in humans. In addition, these studies point to a new candidate genetic susceptibility locus that should be taken into consideration for variants identified in early-onset colitis in pediatric patients.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Pathophysiological Role of Extracellular Purinergic Mediators in the Control of Intestinal Inflammation

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Purinergic mediators such as adenosine 5′-triphosphate (ATP) are released into the extracellular compartment from damaged tissues and activated immune cells. They are then recognized by multiple purinergic P2X and P2Y receptors. Release and recognition of extracellular ATP are associated with both the development and the resolution of inflammation and infection. Accumulating evidence has recently suggested the potential of purinergic receptors as novel targets for drugs for treating intestinal disorders, including intestinal inflammation and irritable bowel syndrome. In this review, we highlight recent findings regarding the pathophysiological role of purinergic mediators in the development of intestinal inflammation.

1. General Features and Metabolism of ATP in the Intestinal Compartment

Damage, trauma, and pathogenic infection cause inflammatory responses in tissues. Clinical pathologic responses involve the release of a series of inflammatory mediators, including cytokines (e.g., IL-1β, IL-6, and TNFα), lipid mediators (e.g., leukotrienes, platelet activating factor, and prostaglandins), and chemical mediators (e.g., histamine).

Accumulating evidence clearly demonstrates the importance of purinergic mediators, especially adenosine 5′-triphosphate (ATP), in the development of various inflammatory disorders [1]. In general, ATP is generated during glycolysis and the tricarboxylic acid cycle in the intracellular compartment and acts as an energy source. However, ATP is occasionally released into the extracellular compartment as so-called extracellular ATP (eATP). Biological roles of eATP were first reported in synaptic neurotransmission and neuromodulation [2]. eATP is released from nerves as a transmitter or cotransmitter and causes pain [2]. In the intestine, purinergic signaling is important for synaptic transmission in the enteric nervous system [2]. The excitatory postsynaptic potential of myenteric neurons is mediated by eATP together with nicotinic acetylcholine [3, 4]. Thus, stimulation by eATP is important for maintaining physiological intestinal motility.

In addition to nerve cells, dead, activated, or infected cells release eATP, recruiting and activating both innate and acquired immunity [5]. For instance, bacterial stimulation leads to eATP release from monocytes and enhances the production of cytokines in an autocrine manner [6]. Some gap junction hemichannels, such as pannexin and connexin hemichannels, are important for ATP release during cell activation [7]. In the steady state intestine some commensal bacteria also have the potential to release eATP [8]; thus, germ-free mice have lower luminal ATP levels than do specific pathogen-free mice. This commensal-derived eATP

stimulates $CD70^+$ $CD11c^{low}$ cells in the intestinal compartment and recruits Th17 cells into the colon [9].

Hydrolysis of the released eATP is catalyzed by cell surface-located enzymes, such as ectonucleoside triphosphate diphosphohydrolase family enzymes (e.g., e-NTPDase I (CD39), ectonucleotidase, and NT5E (CD73)). Consistent with the activity of eATP in the induction of intestinal Th17 cells, a deficiency of eATP-degrading enzymes elevates the concentration of luminal eATP and subsequently enhances the generation of Th17 cells in the gut [10]. By the sequential enzymatic activity of CD39 and CD73, eATP is hydrolyzed to adenosine in the extracellular compartment [11] (Figure 1). Finally, adenosine is metabolized by two pathways: one is intracellular uptake by equilibrative nucleoside transporters and the other is metabolism to AMP or inosine by adenosine kinase and adenosine deaminase, respectively [11].

Recognition of eATP is mediated by purinergic receptors, which comprise P2X ($P2X_{1-7}$) and P2Y receptors ($P2Y_{1,2,4,6,11-14}$). $P2X_{1-7}$ receptors are ATP-gated ion channels and are specific for ATP, whereas P2Y receptors are G protein-coupled receptors that are specific for ADP, UTP, and ATP [5]. Each eATP-specific purinergic receptor requires a different concentration of eATP for activation. For instance, activation of $P2X_7$ receptors requires a high concentration (mM level) of eATP, whereas other P2X receptors require lower concentrations (nM to μM) [5]. In addition, heterooligomeric assembly occurs within P2X receptor subunits (e.g., $P2X_{1-3}$, $P2X_{1-4}$, and $P2X_{2-4-5}$) and alters their functional properties, providing versatile signaling pathways mediated by eATP [12, 13].

Among several P2X and P2Y receptors, $P2X_7$ is involved mainly in the induction of inflammatory responses. $P2X_7$ uniquely has 200 amino acid residues in its C-terminus, which is longer than that of other P2X receptors [14]. C-terminal residues are important for receptor localization at the cell surface [14]. Stimulation of $P2X_7$ by prolonged high concentrations of eATP induces pore formation in the cell membrane and increases membrane permeability [14, 15]. These pores allow influx and efflux of particles with molecular masses of up to 800 Da [11]. These changes also mediate the production of reactive oxygen species and activate inflammasome, a key molecule in the production of inflammatory cytokines such as IL-1β and IL-18 [5] that is responsible for inducing inflammatory responses. In addition, eATP-$P2X_7$ pathways are involved in molecular shedding. Molecules responsible for adhesion (e.g., CD44 and CD62L) are shed from the cell surface by $P2X_7$ activation; stimulation by eATP is thus involved in cell migration [16, 17].

2. Role of eATP in Prevention and Development of Infectious Diseases

Some kinds of pathogens use intestinal tissues as invasion sites. Upon infection, pathogenic components from the microorganisms stimulate innate immune cells such as macrophages and neutrophils via innate receptors such as toll-like receptors (TLRs). This stimulation induces the release of eATP through pannexin-1 hemichannels and subsequently activates $P2Y_2$ and $P2X_7$ receptors in an autocrine or paracrine manner and enhances cytokine production [6, 18]. In microglial cells and macrophages, initial stimulation of lipopolysaccharide- (LPS-) TLR4 pathways with subsequent signaling by the $P2X_7$ pathway induces Ca^{2+} influx and IL-1β secretion [19]. In fact, eATP-$P2X_7$ pathways play important roles in eliminating intracellular pathogens. Activation of $P2X_7$ by selective agonists induces effective clearance of *Toxoplasma gondii* from infected macrophages and of chlamydia from epithelial cells [20, 21]. These signals are required for protective immunity against pathogens. In addition, a recent study found that eATP production was induced by administration of vaccine adjuvant, which is required for an effective response in vaccination against infectious agents and cancer [22].

Reciprocally, the pathogenicity of some pathogens is determined by their ability to induce eATP release. For instance, enteropathogenic *Escherichia coli* induces eATP release from host cells by killing them via type III secretion systems as well as cell-permeable cystic fibrosis transmembrane conductance regulator-mediated pathways [23]. Similarly, cholera toxin from *Vibrio cholerae* is capable of inducing eATP production [24]. Another study in colon epithelial cell lines found that adenosine, a metabolite of eATP, bound to A_{2B} receptors, resulting in short-circuit current responses causing diarrhea [23, 24].

Some kinds of pathogens have unique systems that inhibit eATP release from host cells and thus prevent the spread of infection to the host's immune system. For instance, infection of epithelial cells with *Shigella flexneri* induces eATP release via connexin hemichannels in the early phase of infection, and this release alerts the host to the pathogenic infection. However, prolonged infection with *Shigella* is accompanied by the production of Ptdlns5P, a lipid mediator, to close the connexin hemichannels [25]. Another example is that of *Streptococcus agalactiae*, a commensal bacterium that resides in the intestine or vaginal mucosa but occasionally shows pathogenicity, causing neonatal pneumonia. *Streptococcus agalactiae* releases ecto-5′-nucleoside diphosphate phosphohydrolase and degrades extracellular nucleotides, including eATP; it thus turns off the eATP-mediated alerting of the host defenses to danger [26, 27].

3. Pathological Aspects of eATP in the Mucosal Compartment

eATP-purinergic receptor-mediated pathways are now considered to be targets for the treatment of inflammatory disorders in the systemic compartment, including inflammatory pain and rheumatoid arthritis [28]. Accumulating evidence suggests that eATP-purinergic receptor-mediated pathways are also potential targets for the treatment of inflammatory diseases of mucosal tissues in, for example, the respiratory and gastrointestinal tracts [4, 5, 29]. In the asthma model, migration of eosinophils, dendritic cells, and Th2 cells into the inflamed lung is mediated by the $P2Y_2$ receptor; therefore, $P2Y_2$-deficient mice show reduced inflammatory

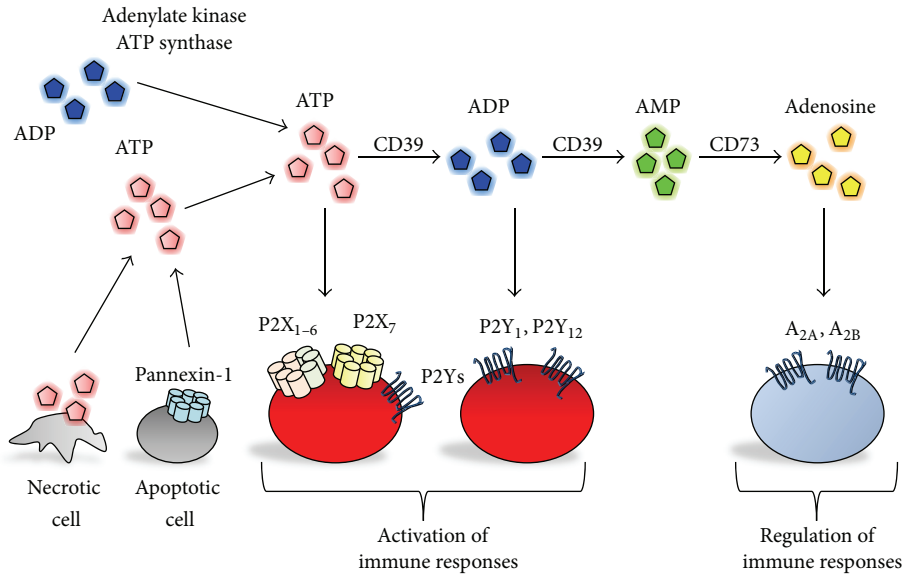

FIGURE 1: ATP is released from necrotic and apoptotic cells as extracellular ATP (eATP). Also, adenylate kinase and synthase mediate the generation of ATP in the extracellular compartment. Extracellular purines (e.g., ATP and ADP) stimulate their receptors and modulate various biological processes. Once eATP is released, the ATP is soon hydrolyzed to AMP and adenosine by the ectonucleotidases CD39 and CD73. Adenosine binds to adenosine receptors (e.g., A_1, A_{2A}, A_{2B}, and A_3).

responses [30]. Th2-type immune responses are also induced by dendritic cells expressing $P2X_7$. Indeed, depletion of eATP by apyrase treatment or $P2X_7$ deficiency reduces signs of inflammation in the upper respiratory tract [31]. It was recently found that the functional capacity of $P2X_7$ (i.e., its ability to promote pore formation) is associated with asthma risk or disease severity in humans [32]. Moreover, *in vivo* imaging analysis has revealed eATP release in the intestinal compartment and peritoneal cavity of mice with acute graft-versus-host disease (GVHD) [33]. Treatment with apyrase or with inhibitors of various purinergic receptors inhibits GVHD-associated intestinal inflammation. In this case, the eATP-P2X7 pathway activates dendritic cells and consequently induces Th1 immune responses (e.g., IFNγ production) and expansion of donor T cells, thus contributing to the onset of inflammation.

Several studies have revealed the pathologic roles of eATP and purinergic receptors (especially $P2X_7$) in the development of intestinal disorders, including irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD) [1, 34] (Table 1). IBS is a common gastrointestinal disorder characterized by discomfort, chronic abdominal pain, and altered bowel habit. Sometimes it occurs after intestinal infection. One meta-analysis has demonstrated that the risk of IBS increases 600% after gastrointestinal infection [35]. Consistently, it has been reported that transient intestinal infection with *Trichinella spiralis* in mice causes postinflammatory visceral hypersensitivity, which is associated with IL-1β production mediated by eATP-$P2X_7$ pathways [34] (Table 1). Because mast cells are considered to play a critical role in the development of IBS and express high levels of $P2X_7$, it is possible that the eATP-$P2X_7$ pathway in mast cells is involved in the development of IBS [36].

The eATP-purinergic receptor pathway, especially the eATP-$P2X_7$ pathway, is also involved in the development of IBD. Overexpression of $P2X_7$ receptors has been observed in the intestinal mucosa of patients with IBD—especially Crohn's disease [44]. Experimentally, $P2X_7$-deficient mice do not develop experimental colitis, and inhibition of $P2X_7$ by A-740003, Brilliant Blue G, or KN-62 ameliorates experimental colitis by reducing the recruitment of neutrophils, T cells, and macrophages, as well as collagen deposition [44] (Table 1). The eATP-P2X7 pathway is therefore now considered to be a novel therapeutic target in the treatment of IBD [43, 44] (Table 1).

Several mechanisms of eATP-mediated inflammation in the development of IBD have been proposed. First, eATP from damaged intestinal epithelial cells, which are frequently observed in IBD patients, and inflammatory cells (e.g., neutrophils and macrophages) stimulates dendritic cells to produce IL-6, IL-12, and IL-23 and TGFβ, thus inducing the production of inflammatory Th1 and Th17 cells [42, 43] (Figure 2) (Table 1). Enteric neuronal cell death is frequently observed in intestinal inflammation and causes colonic motor dysfunction. The eATP–P2X7 pathway is involved in enteric neuronal cell death through the pannexin-inflammasome cascade, and thus colonic motor dysfunction during colitis is prevented by targeting these pathways [41] (Table 1). We previously established mast cell-specific antibody libraries and showed that $P2X_7$ is expressed at high levels in mast cells in the colonic tissues [40]. eATP stimulates mast cells to induce the production of inflammatory chemokines (e.g., CCL2, CCL4, CCL7, CXCL1, and CXCL2), cytokines (IL-1β, IL-6, and TNFα), and mediators (histamines and leukotriene). Thus, blockade of $P2X_7$ by a specific antibody (1F11 monoclonal antibody) inhibits mast cell activation in the colonic

Table 1: Recent reports indicating the critical roles of eATP in the adverse conditions of intestines (inflammatory bowel diseases and irritable bowel syndrome).

Enteric diseases	Receptors	Functions	Reference
Inflammatory bowel disease	P2R/A2BR	Enhance co-transmigration of neutrophils and platelets across intestinal epithelial cells in IBD patients. Platelets release large amount of ATP in the lumen metabolite to adenosine via CD73 and ecto-NTPDases expressed in epithelial cells. Adenosine-A2BR pathway induces electrogenic Cl- secretion with water movement to lumen.	[37]
	P2XR	T cell receptor stimulation induces ATP synthesis and release from activated T cells through pannexin-1 hemichannels. Released ATP activates T cells and produce IL-2 and proliferation in autocrine manner. Blockage of P2X receptors (oxidative ATP) impairs the development of colitis in mice.	[38]
	P2R	ATP released from commensal bacteria acts on CD70+ CD11c+ cells reside in the intestinal lamuna propria and induces Th17 cells in mice; degradation of ATP (by apyrase treatments) ameliorates colitis in mice.	[9]
	P2Y2	Increase of P2Y2 expression in epithelial cells is observed during colitis. P2Y2 stimulation induces release of prostaglandin E2 release from the cells and promotion of intestinal microtubule stabilization and mucosal reepithelization. Those pathways take part in the wound healing during colonic inflammation. Treatment with P2Y2 agonist improves recovery from colitis in mice.	[39]
	P2X7	ATP induces activation of mast cells and enhances inflammatory responses, upregulation of P2X7 in mast cells of Crohn's disease patients, anti-P2X7 antibody treatment inhibits colitis in mice.	[40]
	P2X7	Induction of enteric neuronal cell death and alteration of intestinal motility.	[41]
	P2R	ATP induces IL-6 and CXCL1 productions from epithelial cells; ATP influences the response of epithelial cells to various TLR ligands and induces inflammatory T cells by affecting DC maturations.	[42]
	P2X7	Prophylactic systemic P2X7 blockade (A740003 and brilliant blue G) reduces inflammatory cytokines in rats.	[43]
	P2X7	Upregulation of P2X7 in epithelium, macrophage, and dendritic cells of Crohn's disease patients, P2X7-deficient mice did not develop colitis.	[44]
Irritable bowel syndrome	P2X7	Induction of IL-1β and the development of postinflammatory visceral hypersensitivity in the *Trichinella spiralis*-infected mouse	[34]

tissues and consequently prevents the development of intestinal inflammation [40] (Table 1). In this pathway, $P2X_7$ expression on mast cells is important for the development of colitis, because mast cell-deficient mice reconstituted with $P2X_7$-deficient mast cells show amelioration of inflammatory signs. Of clinical relevance, we have found that the number of $P2X_7^+$ mast cells is increased at sites of inflammation in Crohn's disease patients [40]. eATP is produced by injured epithelial cells and inflammatory cells, including neutrophils, via gap junction molecules such as connexin 43 [45]. It was reported that P2Y2 and P2X7 receptors are important for the migration of neutrophils and macrophages. In the inflammatory condition, neutrophils transmigrated between epithelial cells to the luminal part of the intestine. In this condition, platelets translocate along with neutrophils and released eATP at the mucosal surface (Figure 2) (Table 1). Additionally, mast cells express ectoadenylate kinase and ATP synthase to mediate the extracellular conversion of ADP to ATP, which in turn promotes mast cell activation in an autocrine and paracrine manner (Figure 2). We have recently found that, in contrast to the abundance of $P2X_7$ expression on mast cells in the colon, there are limited levels of $P2X_7$ expression on skin mast cells, which is regulated by skin fibroblasts [46]. Skin fibroblasts uniquely express Cyp26b1 to

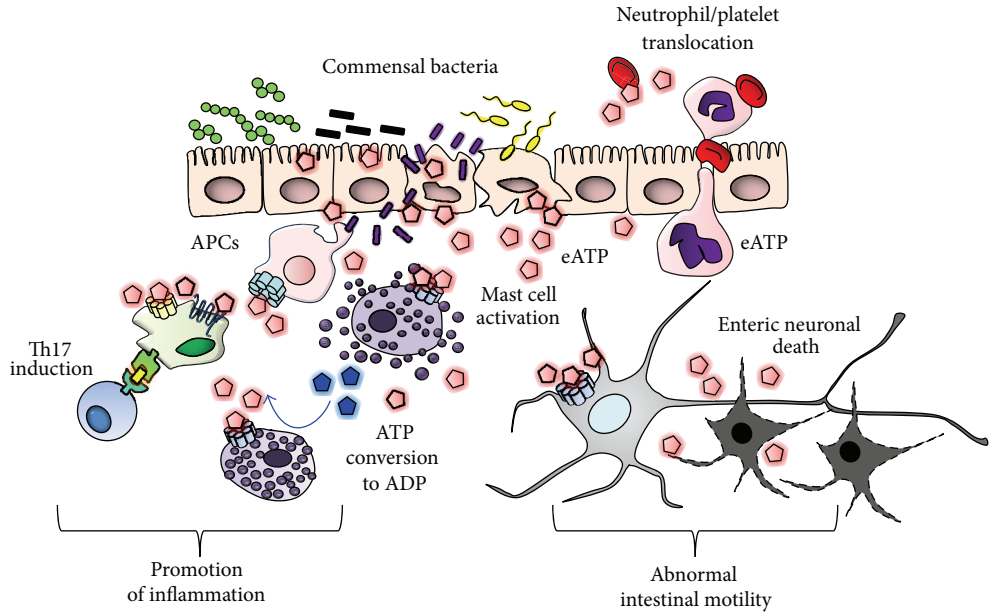

FIGURE 2: In the intestinal compartment, extracellular ATP (eATP) is released from damaged epithelial cells and commensal bacteria. Macrophages, platelets, mast cells, and neutrophils are potential source of eATP upon their activation. Neutrophils facilitate translocation of platelets across intestinal epithelium. eATP also induces Th17 cell generation, activation of mast cells, and neuronal cell death, promoting intestinal inflammation. APCs: antigen-presenting cells. eATP stimulates mast cells to induce the production of inflammatory chemokines (e.g., CCL2, CCL4, CCL7, CXCL1, and CXCL2), cytokines (IL-1β, IL-6, and TNFα), and mediators (histamines and leukotrienes).

degrade retinoic acid within tissues or microenvironments; Cyp26b1 is responsible for inhibiting $P2X_7$ expression [46]. Thus, unique tissue environments determine $P2X_7$ expression on mast cells, which is a critical factor in the development of local inflammation.

4. Resolution of eATP-Mediated Inflammation for Maintenance of Mucosal Homeostasis

Once eATP is released, it is soon hydrolyzed to ADP, AMP, and adenosine by the ectonucleotidases CD39 and CD73; this is essential for resolving inflammatory responses (Figure 1). Indeed, CD39-deficient mice, as well as humans who have CD39 polymorphism and thus low levels of CD39 expression, have increased susceptibility to IBD [47]. Similarly, CD73 deficiency or administration of CD73 inhibitor (e.g., α,β-methylene ADP) enhances susceptibility to intestinal inflammation in mice [48–50].

Adenosine, which is derived from the dephosphorylation of eATP via CD39 and CD73 or diffuses directly from the intracellular compartment via equilibrative nucleoside transporters, binds to adenosine receptors such as A_{2A} and A_3 receptors, which are involved in both the promotion and the resolution of inflammatory responses [51–53]. A_{2A} and A_3 receptor expression on T cells and myeloid cells is a prerequisite for the inhibition of intestinal inflammation [54]. In fact, A_{2A} and A_3 adenosine receptor-selective agonists (e.g., ATP-146e and IB-MECA, resp.) ameliorate intestinal inflammation by impairing the recruitment of inflammatory cells and the production of inflammatory cytokines [55, 56]. In addition, cyclosporine, salicylates, methotrexate, and sulfasalazine, which are used to treat IBD in humans, all decrease eATP levels and increase adenosine production, partly via the stimulation of CD73-dependent adenosine production [57]. Similarly, upregulation of CD39 expression induced on dendritic cells by IL-27 hampers Th1 and Th17 cell production and consequently prevents eATP-mediated inflammation [58]. All of this evidence indicates that inhibition of eATP signaling, together with the promotion of adenosine-mediated regulatory pathways by targeting receptors or ectoenzymes, would be a beneficial strategy for the treatment of intestinal inflammation.

5. Closing Remarks

The importance of purinergic signaling was recognized almost 70 years ago. Accumulating evidence has since revealed the underlying molecular and cellular mechanisms of purinergic signal-mediated maintenance and disruption of mucosal homeostasis. Currently, the clinical relevance of some of the drugs used to treat intestinal inflammation is explained by their regulation of eATP-adenosine balance. Additionally, drugs that target purinergic receptors have now undergone clinical trials [11]. Notably, ATP-adenosine balance, as well as receptor expression levels and the cells expressing these receptors, differs among tissues and environmental conditions. Further investigations using new technologies such as *in vivo* monitoring of eATP release [59, 60] will clarify the complex mechanisms of purinergic signal-mediated immune regulation. This in turn will provide further advances in the design of drugs for preventing and

treating inflammatory diseases and maintaining immunologic health.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Moderate Exercise Training Attenuates the Severity of Experimental Rodent Colitis: The Importance of Crosstalk between Adipose Tissue and Skeletal Muscles

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Although progress has been recently made in understanding of inflammatory bowel diseases (IBD), their etiology is unknown apart from several factors from adipose tissue and skeletal muscles such as cytokines, adipokines, and myokines were implicated in the pathogenesis of ulcerative colitis. We studied the effect high-fat diet (HFD; cholesterol up to 70%), low-fat diet (LFD; cholesterol up to 10%), and the normal diet (total fat up to 5%) in rats with TNBS colitis forced to treadmill running exercise (5 days/week) for 6 weeks. In nonexercising HFD rats, the area of colonic damage, colonic tissue weight, the plasma IL-1β, TNF-α, TWEAK, and leptin levels, and the expression of IL-1β-, TNF-α-, and Hif1α mRNAs were significantly increased and a significant fall in plasma adiponectin and irisin levels was observed as compared to LFD rats. In HFD animals, the exercise significantly accelerated the healing of colitis, raised the plasma levels of IL-6 and irisin, downregulated the expression of IL-1β, TNF-α, and Hif1α, and significantly decreased the plasma IL-1β, TNF α, TWEAK, and leptin levels. We conclude that HFD delays the healing of colitis in trained rats *via* decrease in CBF and plasma IL-1β, TNF-α, TWEAK, and leptin levels and the release of protective irisin.

1. Introduction

Inflammatory bowel diseases (IBD) such as ulcerative colitis (UC) and Crohn's disease (CD) are characterized by chronic relapsing inflammation of the gastrointestinal (GI) tract. Their etiology still remains unknown and it is believed that a combination of environmental agents and a dysfunctional mucosal immune system in genetically susceptible individuals could play an important role in their development [1, 2]. The composition of the gut microbiome could be an important environmental factor in IBD and a number of studies suggested a mucosal immune response to commensal bacteria in the pathogenesis of IBD and particularly in CD. The typical inflammatory response starts with an infiltration of neutrophils and macrophages known to release a variety of cytokines and chemokines that aggravate the immune response. It was established that CD is a Th1 cytokine-mediated disease characterized by increased production of interferon- (IFN-) γ, while UC bears a resemblance to a modified Th2 profile, with an augmented release of interleukin- (IL-) 5 but normal IFN-γ production [3]. Cytokines, such as TNF-α, IL-1β, and IL-6, that are more promiscuous in their

function, are associated with both forms of IBD to a lesser or greater degree [3, 4]. Each of these cytokines activates NF-κB and the mitogen-activated protein (MAP) kinases and induces various "downstream" proinflammatory effects responsible for the tissue and organ pathology in IBD [3]. Epidemiological studies have indicated that the incidence and prevalence of IBD rapidly increased particularly in Western countries and the rise observed in the rest of the world closely correlates with adopting a Western lifestyle including dietary habits, physical inactivity, and obesity [5]. Although the majority of CD patients are undernourished, there is now evidence that increasing body mass index (BMI) and overweight are emerging features of CD and may be associated with more severe course of disease activity [6, 7]. In Crohn's disease-relevant mouse model, the high-fat diet feeding actually accelerated the intestinal inflammation [8]. Characteristic feature for CD is hypertrophy of the mesenteric fat tissue located around the inflamed parts of the intestine [9]. Recent research suggests that this fat wrapping contributes actively to disease severity and may influence onset of complications [10–17]. This mesenteric fat is present from the onset of disease and is associated with overexpression of TNF-α, leptin, and other adipokines and correlates with the severity of intestinal inflammation and tissue injury, suggesting an important role for adipose tissue in the intestinal inflammatory process in CD [9]. Well-documented observations that physical activity is correlated inversely with systemic low-level inflammation lead to the suggestion that the anti-inflammatory activity induced by regular exercise may be responsible for some beneficial health effects in patients with chronic diseases including IBD [18]. Exercise may exert its anti-inflammatory effect *via* a reduction in visceral fat mass and/or by induction of an anti-inflammatory environment with each bout of exercise. Such effects may in part be mediated *via* muscle-derived peptides, so-called "myokines" [19].

While no single animal model fully captures the clinical and histopathological features of human IBD, the 2,4,6-trinitrobenzenesulfonic acid- (TNBS-) induced colitis is believed to be the most relevant to study CD-related immune responses [20]. The intrarectal administration of TNBS hapten reagent in ethanol solution causes disruption of the epithelial layer and exposes the *lamina propria* to bacterial and host haptenized protein. The experimental colitis induced by TNBS has many of the typical features of CD including the severe transmural inflammation, diarrhea, weight loss, and induction of IL-12-driven inflammation with a massive Th-1-mediated response [20]. In rodent with TNBS-induced colitis, a characteristic reduction of food intake and loss of weight together with a decrease in skeletal muscle mass associated with the severity of colitis have been described [21].

Our present study was designed to determine the effect of moderate forced treadmill on experimental colitis caused by intrarectal administration of TNBS in rats fed normal, low-fat, or high-fat diet. We hypothesized that diet-induced obesity augments the severity of experimental colitis and that the possible beneficial effects of physical exercise contribute to healing of colitis by modifying muscle-adipose tissue crosstalk. This particular issue has not been so far closely elucidated in relation to different diets and the possible alterations in plasma levels of myokines and adipokines and the expression of proinflammatory factors (cytokines and hypoxia inducible factor-1 alpha) considered as markers of inflammatory reactions associated with development and healing of colitis.

2. Material and Methods

2.1. Studies in Animal Model. Animal studies were carried out on eighty male Wistar rats 200–220 g with free access to water and food and adapted to laboratory conditions and 12/12 h day/night cycles. The study was approved by the local Ethical Committee at the Jagiellonian University Medical College in Cracow, Poland, and run in accordance with the Helsinki declaration.

One hundred twenty animals were randomized into the three experimental series (A–C) and fed for 8 weeks with either (A) high-fat diet (HFD; cholesterol up to 70%), (B) low-fat diet (LFD; cholesterol up to 10%), or (C) normal rodent diet (C1000 containing less than 5% of total fat), all in form of regular chow pellets and availability of tap water. All rodent diets were purchased from Altromin Company, Lage, Germany.

2.2. Forced Treadmill Exercise Training. Animals (series A–C) were randomly assigned to exercise groups and were subjected to running on a two-lane treadmill (Harvard Apparatus, MA, USA) at a speed of 20 m/min for 30 min each day, 5 days/week (total of 6 wks), prior to development of colitis. The treadmill used in our study has an endless conveyor-type belt, driven by a DC servomotor with optical encoder for precise speed control. The animals were separated from each other by opaque partitions. The motor drive electronics permits the user to select any speed from 0 up to 100 m per min. The front of the treadmill is made of dark acrylic so that the animals can run towards the darkened section of the channel.

After 6 weeks of exercise training, the colitis was induced in groups of trained and untrained rats fed different diets by intracolonic administration of 2,4,6-trinitrobenzenesulfonic acid (TNBS, Sigma, Slough, UK) at a dose of 10 mg/kg, dissolved in 50% solution of ethanol as reported in our previous studies [22, 23]. Briefly, the animals were anaesthetized with phenobarbital (60 mg/kg i.p.) and TNBS was administered into the colon in a volume of 0.25 mL per rat at a depth of 8 cm from the rectum with the use of a soft polyethylene catheter. Until the moment of awakening, the rats were positioned in the Trendelenburg position so as to avoid loss of the TNBS solution *via* rectum. Animals in the control group were given 0.9% saline in a volume of 0.25 mL per rat, corresponding to the rats that were administered TNBS. Following the induction of colitis, animals were housed individually, and daily food intake and body weight were monitored. With the induction of colitis, the exercise sessions have stopped. At day 14 from induction of colonic lesions with TNBS, the animals were weighed and anaesthetized to determine CBF using the H_2-gas clearance technique. The abdominal cavity was opened and, after separation of the colon, the CBF in

the areas of the mucosa not affected by inflammatory lesions was measured. CBF was expressed as a percentage of the CBF in the vehicle-control rats without TNBS administration as reported by our group elsewhere [22].

The area of colonic damage were evaluated planimetrically (Morphomat, Carl Zeiss, Berlin, Germany) by two independent researchers. Subsequently, fragments of the colon (2 mm × 10 mm) with colonic lesions were sampled, fixed with formaldehyde, embedded in paraffin, and routinely stained with haematoxylin and eosin (H&E) for histological assessment. The presence and intensity of histological changes were evaluated for the following criteria: presence, area, and depth of ulceration and presence and intensity of inflammatory infiltrations, ulcerations, and fibrosis. The microscopic changes in the colonic mucosa were graded with a compounded histological score including the extent of (1) crypt damage, (2) regeneration, (3) metaplasia/hyperplasia, (4) lamina propria vascular changes, (5) submucosal changes, and (6) presence of inflammatory infiltrates. The sections were graded with a range from 0 to 4 for each of the previous categories and data were analyzed as a normalized compounded score [22, 23].

2.3. Determination of Plasma Cytokines and MPO Activity. Immediately after the CBF measurements, a venous blood sample was drawn from the *vena cava* and placed into EDTA-containing vials and used for the determination of plasma IL-1β, TNF-α, TWEAK, IL-6, leptin, adiponectin, and irisin levels. Briefly, blood was collected and placed into sterile, plastic syringes and kept in ice till centrifugation. The blood samples were centrifuged with the speed of 1000 G for 10 minutes in 15°C temperature and the plasma was stored in −80°C. The plasma TNF-α, IL-1β, TWEAK, IL-6, leptin, and adiponectin were determined by a solid phase sandwich ELISA (BioSource International Inc., Camarillo, CA, USA) according to the manufacturer's instructions [24]. Plasma irisin protein concentrations were measured using a specific ELISA kit (Phoenix Pharmaceuticals Inc., USA). The fragments of colonic tissue weighing about 200 mg were collected and frozen in −70°C for the determination of MPO activity by ELISA as reported previously [25].

2.4. Expression of IL-1β, TNF-α, and Hif1α Transcripts in the Rat Colonic Mucosa Determined by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). The mRNA expression for IL-1β, TNF-α, and Hif1α was determined by RT-PCR in the unchanged colon mucosa of intact rats or those with TNBS colitis fed with different diets. Biopsy samples of colonic mucosa weighing about 200 mg were scraped off from oxyntic mucosa using a slide glass and immediately snap frozen in liquid nitrogen and stored at −80°C until analysis. The total RNA was extracted from the mucosal samples by a guanidium isothiocyanate/phenol chloroform method using a kit from Stratagene (Heidelberg, Germany) according to methods described by Chomczynski and Sacchi [26]. The concentration of RNA in RNase-free Tris-EDTA buffer was measured at absorption of 260 nm wavelengths by spectrophotometry. Five μg of total cellular RNA single-stranded cDNA was generated using StrataScript reverse transcriptase and oligo(dT) primers (Stratagene). The polymerase chain reaction mixture was amplified in a DNA thermal cycler (Perkin-Elmer-Cetus, Norwalk, CT). The nucleotide sequences of the primers used in PCR were as follows: β-actin (size of PCR product 764 bp), forward: 5′-TTG TAA CCA ACT GGG ACG ATA TGG-3′, reverse: 5′-GAT CTT GAT CTT CAT GGT GCT AGG-3′; IL-1β (size of PCR product 543 bp), forward: 5′-GCT ACC TAT GTC TTG CCC GT-3′, reverse: 5′-GAC CAT TGC TGT TTC CTA GG-3′; TNF-α (size of PCR product 295 bp), forward: 5′-TAC TGA ACT TCG GGG TGA TTG GTC C-3′, reverse: 5′-CAG CCT TGT CCC TTG AAG AGA ACC-3′; Hif1α (size of PCR product 510 bp), forward: 5′-TCT GGA CTC TCG CCT CTG-3′, reverse 5′-GCT GCC CTT CTG ACT CTG-3′.

PCR products were separated by electrophoresis in 2% agarose gel containing 0.5 μg/mL ethidium bromide and then visualized under UV light as described previously [23, 24]. The signal intensity of expression of mRNAs for IL-1β, TNF-α, and Hif1α was analyzed by densitometry (Gel-Pro Analyzer, Fotodyne Incorporated, Hartland, WI, USA) [24].

2.5. Statistical Analysis. Results are expressed as means ± SEM. The data was processed by the statistical analysis software SPSS version 16.0 (SPSS Inc., Chicago, IL). Statistical analysis was done using Student's t-test or analysis of variance and two-way ANOVA test with Tukey post hoc test where appropriate. Differences of $P < 0.05$ were considered significant.

3. Results

3.1. The Effects of Exercise on the Healing Process of Colonic Lesions and CBF in Rats Fed with Different Diet. As shown in Figure 1, the intrarectal administration of TNBS in rats induced severe mucosal injury characterized by necrosis of the epithelium and focal ulcerations of the mucosa in rats fed with normal diet. This colonic damage was significantly aggravated in rats with HFD ($P < 0.05$) and also significantly increased in those fed with LFD. The CBF was significantly decreased in HDF and LDF rats ($P < 0.05$) as compared with those fed with normal diet. In rats subjected to exercise prior to administration of TNBS, a significant reduction in the area of colonic lesions ($P < 0.05$) and a significant increase in CBF ($P < 0.05$) were observed as compared to those recorded in sedentary animals with TNBS colitis (Figure 1).

Figures 2(a), 2(b), 2(c), and 2(d) show the macroscopic appearance of the intact colonic mucosa (a) and that with TNBS colitis in rats fed with normal diet (b) or HFD without exercise (c) or HFD with exercise (d). The intact colonic mucosa showed normal macroscopic appearance but, in rats administered with TNBS, the severe colonic damage as manifested by the area of mucosal damage was observed and this damage was markedly exacerbated in rats fed with HFD (Figure 2(b) versus Figure 2(c)). In TNBS rats fed HFD and subjected to exercise, the area of colonic lesions was reduced comparing to that observed in TNBS rats fed HFD without exercise training (Figure 2(d) versus Figure 2(c)).

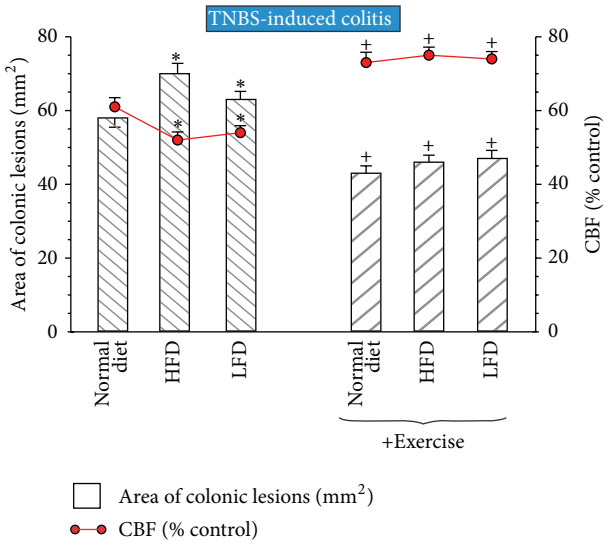


Figure 1: Effect of exercise on the area of colonic lesions and the alterations in CBF in animals fed normal diet, high-fat diet (HFD), and low-fat diet (LFD). Results are mean ± SEM of 8 animals per each group. An asterisk indicates a significant change ($P < 0.05$) as compared to respective values in rats fed normal diet. Cross indicates a significant change ($P < 0.05$) as compared to the respective values obtained in animals fed different diets but not subjected to exercise.

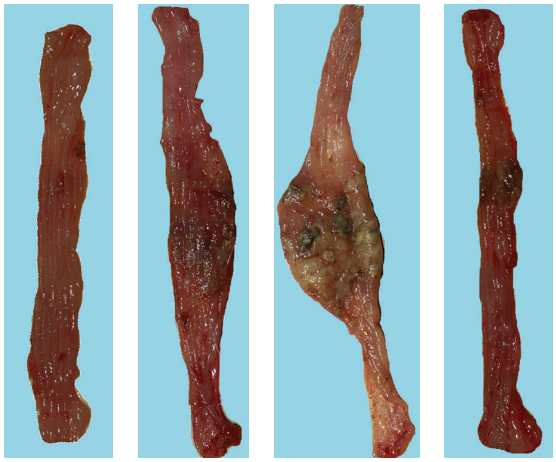
Figures 3(a), 3(b), 3(c), and 3(d) show that the representative colonic tissue pathomorphologic changes were evidently more severe in rats with TNBS colitis fed normal diet or those with TNBS colitis fed HFD compared with intact colonic mucosa. The histological appearance of mucosa of rat fed normal diet was illustrated by a desquamation of colonic epithelium and deep ulcerations reaching *muscularis mucosa* followed by neutrophil infiltration. In HFD rats, there was a total disorganization of mucosal structure, deep ulceration with necrosis, and heavy inflammation followed by intense infiltration with neutrophils, fibrosis, and lesser regeneration comparing with rats fed with normal diet. In contrast, the less pronounced histologic changes and neutrophil infiltration were observed in HFD rats subjected to exercise as manifested by the partial restoration of colonic mucosa architecture, a more pronounced regeneration, and the lack of deep mucosal ulcerations indicating more advanced healing of colonic mucosa compared with that in sedentary HFD rats without exercise (Figure 3(d) versus Figure 3(c)).

3.2. Effect of Exercise on the Weight of Colon and MPO Activity. As shown in Figure 4, the weight of colonic tissue was not significantly affected in rats without colitis fed different diets: either normal diet, HFD, or LFD. However, the weight of examined colonic tissue was significantly increased in rats with TNBS-induced colitis fed with normal diet when compared with that measured in rats without colitis (Figure 4). The weight of colonic tissue in TNBS exercising rats was significantly decreased in comparison with the untrained animals (Figure 4). Comparing with LFD or normal diet, TNBS rats subjected to HFD showed the significant increase in the weight of colonic tissue ($P < 0.05$) (Figure 4). MPO activity, as the marker of neutrophil infiltration of colonic mucosa which corresponded with the intensity of inflammation, was significantly increased in the colonic mucosa of TNBS colitis ($P < 0.02$) as compared with MPO activity in rats without colitis (Figure 4). In HFD rats, a significant elevation of MPO activity over the values measured in rats fed both of the normal diet and LFD ($P < 0.05$) was observed. As shown in Figure 4, in TNBS rats fed HDF and subjected to exercise, a significant fall of MPO activity ($P < 0.05$) in the colonic mucosa comparing with group of sedentary rats fed different diets was recorded.

3.3. Effect of Exercise on Plasma Proinflammatory Cytokines IL-1β, TWEAK, and TNF-α Levels. Plasma levels of proinflammatory cytokines IL-1β, TWEAK, and TNF-α were significantly elevated in animals with TNBS-induced colitis ($P < 0.02$) in comparison with intact rats (Figure 5). In rats fed with HFD, a significant increase in plasma IL-1β, TNF-α, and TWEAK levels ($P < 0.05$) was observed comparing to normal and LFD group. In exercising TNBS rats, a significant attenuation in plasma levels of proinflammatory cytokines IL-1β, TNF-α, and TWEAK was observed ($P < 0.05$) as compared to the values of these cytokines in sedentary rats fed with different diets. However, there were no significant differences between HFD, LFD, and normal diet groups in plasma levels of IL-1β, TNF-α, and TWEAK in TNBS-rats subjected to exercise (Figure 5).

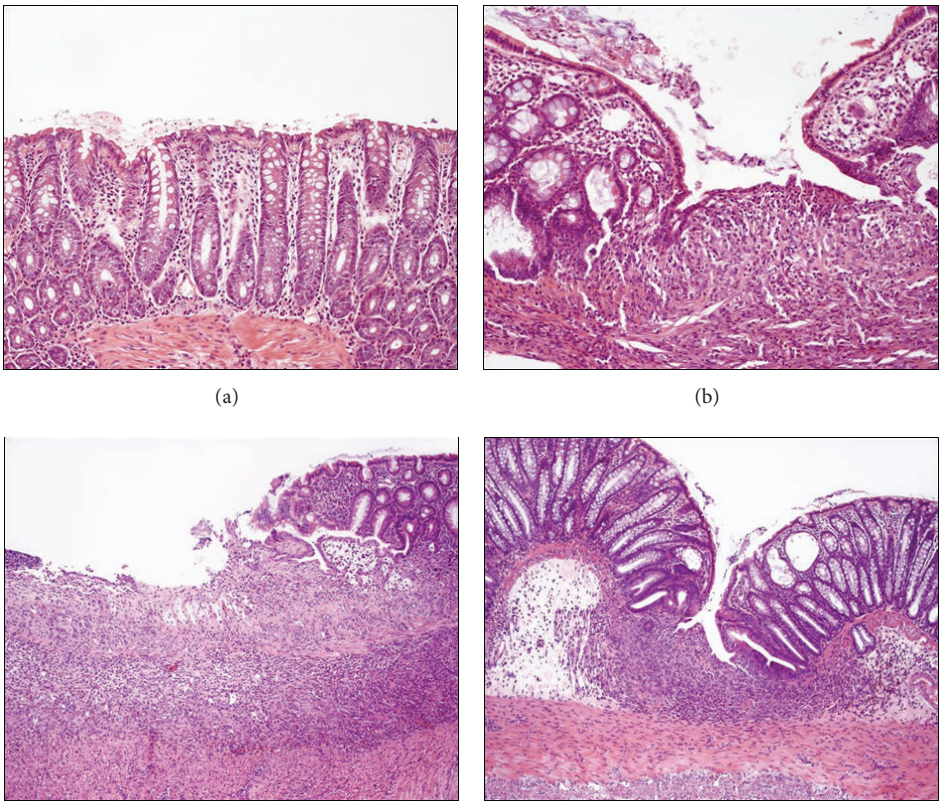
3.4. Effect of Exercise on Plasma Adipokine Leptin and Adiponectin Levels. Plasma level of leptin was significantly elevated and the plasma adiponectin level was significantly decreased in animals with TNBS-induced colitis fed normal diet ($P < 0.05$) comparing with those measured in intact animals (Figure 6). HFD significantly increased plasma leptin level and significantly decreased the plasma adiponectin levels ($P < 0.05$) as compared to values obtained in rats fed either normal diet or LDF (Figure 6). Exercise significantly decreased plasma levels of leptin and also significantly increased the plasma adiponectin levels in rats fed HFD ($P < 0.05$) as compared to the values of this adipokine recorded in untrained rats.

3.5. Effect of Exercise on Plasma Myokine Irisin and IL-6 Levels in Rats with TNBS Colitis Fed Different Diets with or without Exercise. As shown in Figure 7, the plasma levels of irisin and IL-6 were not significantly altered in TNBS rats fed normal diet as compared with intact rats. However, in TNBS colitis rats fed with HFD, the plasma levels of irisin and IL-6 were significantly decreased ($P < 0.05$) as compared with those fed LFD. Exercise significantly increased plasma levels of irisin and IL-6 ($P < 0.05$) in TNBS rats fed normal diet, HFD, and LFD comparing to those recorded in sedentary rats (Figure 7).



(a) (b) (c) (d)

Figure 2: The representative macroscopic appearance of intact colonic mucosa (a) and the colonic mucosa of rat with TNBS colitis fed normal diet (b) or HFD without exercise (c) and HFD with exercise (d) at day 14 after TNBS induction. Note, the normal appearance of colonic mucosa (a) and the severe colonic damage in TNBS rat fed normal diet (b). This damage was exacerbated in untrained rat fed HFD as reflected by necrotic damage occupying larger area of colonic mucosa (c). However, in trained rat fed HFD, the colonic damage was reduced and smaller area of the colonic damage was clearly confirmed by gross inspection (d).



(a) (b)

(c) (d)

Figure 3: The histological appearance of colonic mucosa of intact rat (a) and the colonic mucosa of rat with TNBS colitis fed normal diet (b) or fed HFD without exercise (c) and HFD with exercise (d) at day 14 after TNBS induction. Note the normal architecture of the colonic crypts in intact rat (a) and the severe damage as manifested by a partial loss of normal architecture and deep ulceration reaching *muscularis mucosa* (b). This histological damage was potentiated in untrained rat fed HFD showing complete loss of architecture accompanied by severe inflammation (c). In contrast, in trained rat fed HFD, both the area of colonic damage and the inflammatory reaction were reduced and more regeneration around the ulceration was evidently seen (d).

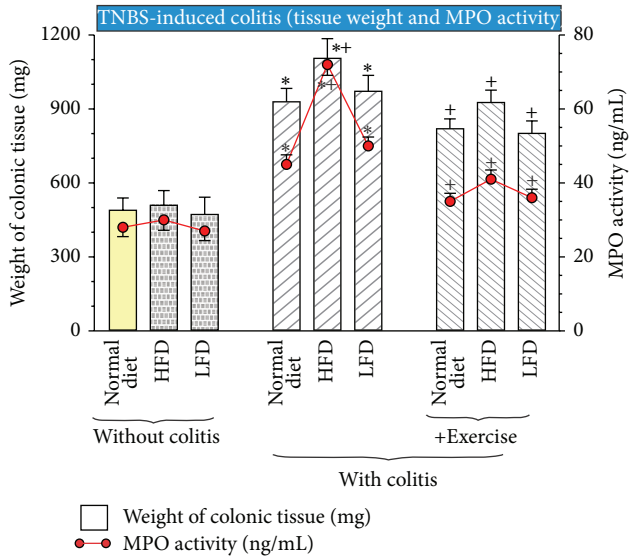


Figure 4: The effect of exercise on the weight of colonic tissue and the colonic MPO levels in rats with or without TNBS colitis fed normal diet, HFD, and LFD. Results are mean ± SEM of seven animals per each group. An asterisk indicates a significant difference ($P < 0.05$) as compared with the control groups of animals (without colitis). An asterisk and cross indicate a significant difference ($P < 0.05$) as compared to the values obtained in rats fed normal diet. Cross indicates significant difference ($P < 0.05$) as compared to the values obtained in TNBS rats fed different diets without exercise.

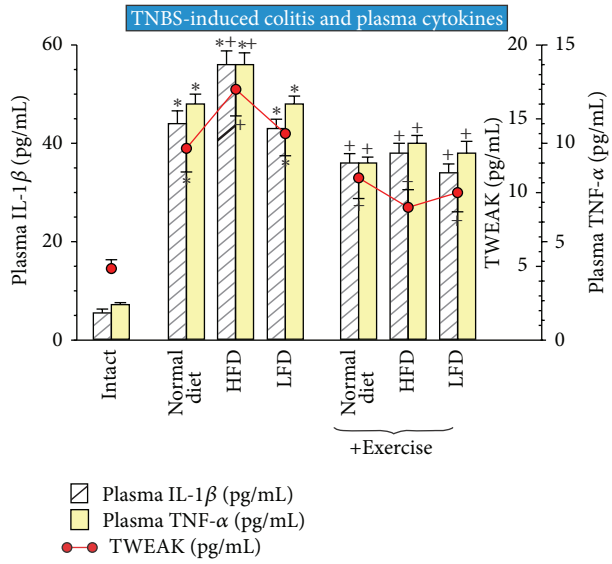


Figure 5: The effect of exercise on the plasma levels of proinflammatory cytokines IL-1β and TNF-α and TWEAK in TNBS rats fed normal diet, HFD, or LFD with or without exercise. Results are mean ± SEM of eight animals per each experimental group. Asterisk indicates a significant change ($P < 0.05$) as compared with respective values in intact rats. Asterisk and cross indicate a significant change ($P < 0.05$) as compared with respective values in animals fed with normal diet. Cross indicates a significant change ($P < 0.05$) as compared with those obtained in untrained animals.

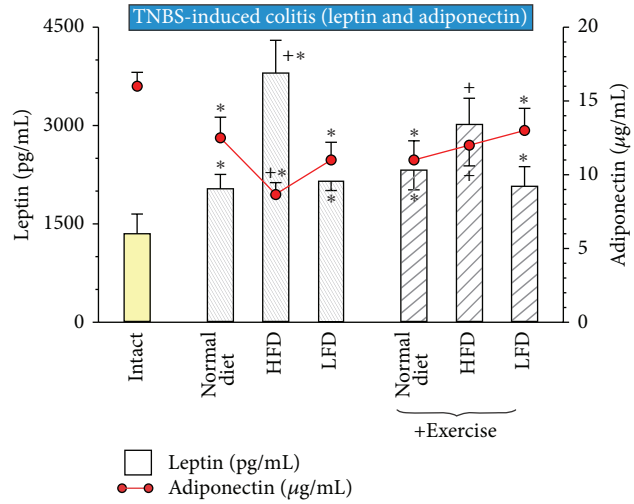


Figure 6: The effect of exercise on the plasma levels of leptin and adiponectin in trained and untrained rats fed normal diet, HFD, or LFD. Results are mean ± S.E.M. of seven animals per each experimental group. Asterisk indicates a significant change ($P < 0.05$) as compared with respective values in intact rats. Asterisk and cross indicate a significant change ($P < 0.05$) as compared with respective values in animals fed with normal diet or LFD. Cross indicates a significant change ($P < 0.05$) as compared with those obtained in untrained animals.

3.6. Effect of Exercise on the Mucosal Expression of β-Actin, IL-1β, TNF-α, and Hif1α in Rats with TNBS Colitis Fed HFD or LFD with or without Exercise. Figures 8(A)–8(D) show the effect of HFD alone or HFD and LFD with exercise on the mRNA expression of proinflammatory cytokines IL-1β and TNF-α and proinflammatory marker Hif1α in the colonic mucosa of rats with TNBS-induced colitis fed different diets. The weak signals for expression of IL-1β, TNF-α, and Hif1α mRNAs were recorded in intact mucosa (Figures 8(B), 8(C), and 8(D)). In untrained rats fed HFD, the signal intensity for these factors was significantly enhanced compared to that observed in the intact colonic mucosa (Figure 8(a)). This increase in signal intensity observed in rats fed HFD without exercise was significantly inhibited in trained rats fed HFD. The semiquantitative ratio of IL-1β-, TNF-α-, and Hif1α mRNAs over β-actin mRNA confirmed that the IL-1β, TNF-α, and Hif1α mRNAs were upregulated in TNBS rats fed HFD as compared with the expression of mRNA for these factors detected in intact colonic mucosa (Figure 8(b)). The signal intensity of mRNAs expression of IL-1β, TNF-α, and Hif1α in rats fed HFD or LFD was significantly inhibited in those subjected to exercise. The ratio of mRNAs for IL-1β, TNF-α, and Hif1α over β-actin mRNA confirmed that the expression of mRNAs for IL-1β, TNF-α, and Hif1α was significantly decreased in TNBS rats subjected to exercise (Figures 8(a) and 8(b)).

4. Discussion

The results of our present study indicate that diet-induced obesity delayed the healing of experimental colitis in rats and

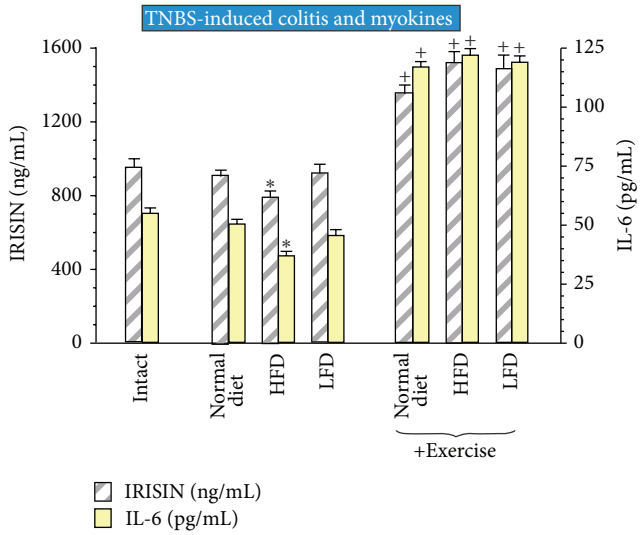


FIGURE 7: The effect of exercise on the plasma levels of irisin and IL-6 in trained and untrained rats fed normal diet, HFD, or LFD. Results are mean ± SEM of eight animals per each experimental group. Asterisk indicates a significant change ($P < 0.05$) as compared with respective values in intact rats. Cross indicates a significant change ($P < 0.05$) as compared with those obtained in animals not subjected to exercise.

that the forced moderate treadmill running 6 weeks prior to colitis induction significantly attenuated the severity of colonic damage induced by colonic application of TNBS in these rats. The mechanism of a beneficial effect of exercise observed in our study should be further elucidated but we found that this effect could be attributed to exercise-induced increase in the CBF and the release of myokines that can attenuate the gross, histological, and functional changes in the inflamed colon leading to an improvement in the mucosal healing of colitis. The development of experimental colitis in HFD rats was accompanied by the fall in CBF, the increased expression of proinflammatory cytokines IL-1β, TNF-α, and TWEAK, and decreased release of protective adipokines (e.g., adiponectin). All of these alterations possibly contributed to the exacerbation of colitis in these rats when compared to those that were fed a normal diet or low fat diet. We provide evidence that exercise applied prior to the development of colitis, especially in rats with diet-induced obesity as reflected by an increase in weight of colonic tissue and increased MPO activity, resulted in an enhancement in colonic microcirculation as a conglomeration of an increase in CBF, lower colonic tissue weight, an increase of MPO content, and the increased plasma levels of protective myokines, irisin, and IL-6. Moreover, exercise attenuated the expression of proinflammatory cytokines IL-1β and TNF-α and their plasma levels, as well as the expression of inflammatory state and cytokines of the colonic mucosa as well as Hif1α, in rats subjected to HFD, which suggested that moderate exercise selected in our study exerts a beneficial influence on the healing of colonic mucosa in this rodent model of colitis.

It is now generally accepted that obesity represents a low-grade chronic inflammatory state, characterized by abnormal profile of cytokine secretion, increased synthesis of acute-phase reactants, such as C-reactive protein (CRP), and the activation of proinflammatory signaling pathways [27]. Adipocytes are now recognized as new members of the immune system, producing several cytokines such as IL-6, TNF-α, and chemokines, in addition to adipokines (leptin, adiponectin, and resistin) [28, 29]. Abdominal adiposity and especially mWAT has been implicated in a wide range of gastrointestinal disorders from fatty liver and GI cancers to acute pancreatitis and Crohn's disease (CD) [30]. Furthermore, the macrophages infiltrate adipose tissue during obesity, thus contributing to production and release of additional inflammatory mediators [31], and the adipose tissue depots can be altered due to inflammatory pathologies such as CD [11, 14, 28]. Interestingly, CD has not necessarily been associated with obesity and is characterized by hypertrophy of mesenteric adipose tissue [14]. Several mechanisms could be responsible for a link of general or local obesity with CD. In genetically obese mice, the increased intestinal permeability has been described and considered one of the major pathogenic mechanisms linked with CD and UC [19].

In the normal intestinal mucosa, a continuous low-grade, nonpathogenic inflammation is observed, and this is triggered by a perpetual exposure of intestinal mucosa to a marked antigenic load from dietary and microbial antigens and several ligands of toll-like receptors (TLRs) [32]. Despite the *lamina propria* infiltration with activated immune cells, the IBD might be developed due to disruption or weakening of the mucosal barrier and recognition of the normal microbiota as pathogens [32]. Both CD and UC patients exhibited the activation of innate (macrophage, neutrophils) and acquired (T and B cell) immune responses and loss of tolerance to enteric commensal bacteria [33]. Fat wrapping and mesenteric adipose tissue hypertrophy are consistent features recognized on surgical specimens in patients with CD [9] and recent research suggests that the hypertrophied fat contributes actively to disease severity and may influence onset of complications [10–16]. It is likely that the mesenteric fat in CD is exposed to gut microbial antigens. Adipocytes express toll-like receptors and CD14, and both were shown to interact with these microbial antigens activating NF-κB pathways [34]. About 95% of the total viable bacteria cultured from mesenteric tissues are physiologically located in adipocytes, and only 5% are translocated in mesenteric lymph nodes, indicating that adipocytes might be a main reservoir of bacteria in the mesentery. Intriguingly, obesity is associated with reduced microbial diversity in a similar pattern to that seen in CD [35, 36]. All these observations fuelled speculation about the potential roles of mesenteric mWAT in the development of CD by reacting to the microbial environment and by initiating and/or promoting local inflammatory reactions by autocrine and/or paracrine modulation of adipocytes [10, 13].

In our study, the impaired healing of TNBS colitis accompanied by the reduction in the colonic blood flow was observed in rats fed HFD compared with rats fed a normal diet or LFD. These changes were accompanied by increase in plasma levels of proinflammatory mediators such as IL-1β,

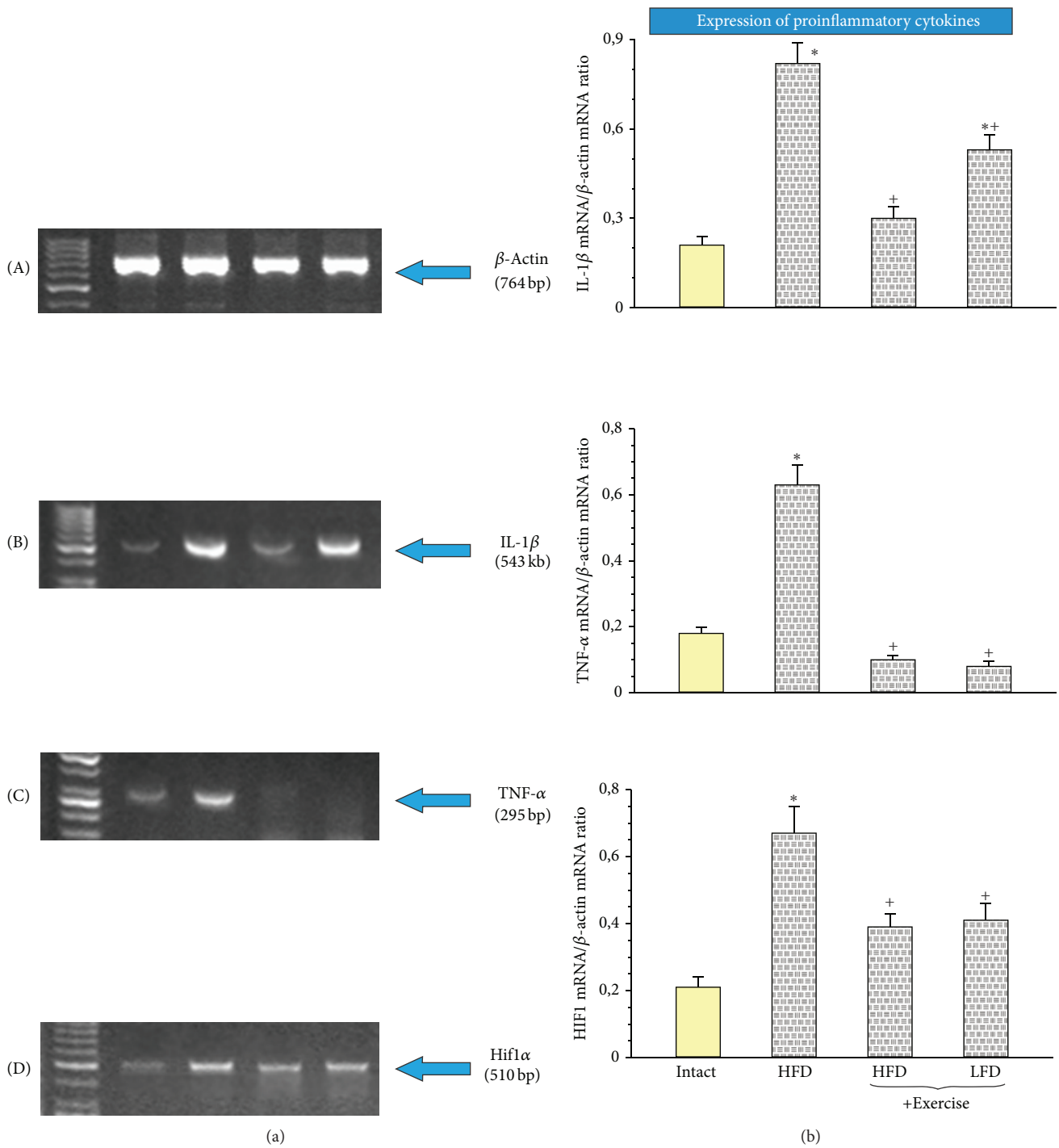


Figure 8: The RT-PCR analysis of mRNA expression for β-actin (A), IL-1β (B), TNF-α (C), and Hif1α (D) (a) in colonic mucosa of intact rats or untrained TNBS rats fed HFD or trained TNBS rats fed HDF or LDF and the semiquantitative ratio of IL-1β-, TNF-α-, and Hif1α mRNAs over β-actin mRNA (b). Results are mean ± SEM of 4 determinations. Asterisk indicates a significant change ($P < 0.05$) as compared with the values obtained in intact colonic mucosa. Cross indicates a significant change ($P < 0.05$) as compared with untrained rats fed with HFD.

TNF-α, TWEAK, and leptin and the reduction in plasma adiponectin levels. Particularly interesting to the study, is that the plasma TWEAK level was increased in rats with TNBS-induced colitis fed normal diet and this increase was potently elevated in rats fed HFD. This observation is consistent with the involvement of TWEAK cytokine in the inflammatory processes [37]. TWEAK actions are mediated by its binding to fibroblast growth factor-inducible 14 (Fn14), a highly inducible cell surface receptor that has been linked to several intracellular proinflammatory signaling pathways, including

the NF-κB pathway [17]. An increase in TWEAK and Fn14 gene expression in adipose tissue of severely obese patients was reported and inflammatory stimuli *in vitro* differentially increased the expression of TWEAK in macrophages and Fn14 in adipocytes [38]. Expression of Fn14 is upregulated in CD patients [37, 39] and the TWEAK/Fn14 pathway plays a pathological role in mouse models of CD by inducing inflammatory responses and regulating intestinal epithelial cell turnover [39–42].

Furthermore, we found that leptin, the product of adipose tissue, was increased in rats with colitis fed normal diet and this effect was markedly enhanced in rats fed HFD. This remains in agreement with previous observation that intestinal leptin, a cytokine produced by adipocytes, is increased in CD and can upregulate NFκB expression in colonic epithelial cells leading to development of inflammation [12, 43, 44]. Leptin is considered to be a proinflammatory cytokine and directly regulates production of several cytokines, particularly those produced by T cells [12]. An overexpression of leptin mRNA in mWAT was reported in IBD patients, indicating that leptin might participate in the inflammatory process by enhancing mesenteric expression of TNF-α [45]. Also, in rat model of experimental colitis, an elevated plasma leptin levels were observed which correlated with the degree of inflammation [46]. We also observed that LFD feeding increased the severity of experimental colitis although not to the same extent as observed in case of HFD.

It is of interest that plasma level of adiponectin was significantly decreased in HFD animals than in those fed normal diet or LFD. Adiponectin has a structure similar to TNF-α but antagonizes its effects by reducing secretion and attenuating the biological actions by competing for the receptor [47]. Conflicting data have been described for circulating levels of adiponectin in patients with IBD [48–50]. However, recent observations of lower levels of serum and mesenteric adiponectin in active CD patients but not those in remission support the notion of a defective regulation of anti-inflammatory pathways in CD pathogenesis [48]. The altered balance between proinflammatory and anti-inflammatory factors (increase in secretion of TNF-α, leptin, and release of chemoattractant protein-1 (MCP-1)) and decreased production of anti-inflammatory factors (e.g., adiponectin) could contribute to macrophage accumulation in adipocytes, as well as to an inflammatory transformation of the visceral adipose tissue, leading to the appearance of creeping fat. Moreover, the depletion of muscle mass and impaired muscle function are important features of IBD [51]. The IBD-related decreased muscle mass has been attributed to a variety of mechanisms including decreased nutrient intake, their malabsorption increased metabolic rate, and the inhibitory effects of inflammation on the growth hormone (GH)/insulin-like growth factor- (IGF-) I axis [52]. TWEAK has recently been shown to mediate the skeletal muscle atrophy in a variety of clinical settings [53]. Gruber et al. [8] observed in CD-relevant mouse model that HFD feeding, independently of obesity, accelerated disease onset of intestinal inflammation through mechanisms involving the increased intestinal permeability and altered luminal factors, leading to enhanced dendritic cell recruitment and promoted Th17 immune responses.

The potential benefits of exercise and physical activity in IBD patients recently raised major interest [54, 55]. Evidence suggests that the protective effect of exercise may to some extent be ascribed to its anti-inflammatory effects and/or specific effects on visceral fat mediated, in part, *via* muscle-derived peptides, so-called "myokines" [19, 56]. Presently identified myokines which exert endocrine, paracrine, or autocrine effects are LIF, IL-6, IL-7, BDNF, IGF-1, FGF-2, FSTL-1, and irisin [57]. The dysfunction of several organs and tissues of the body as well as an increased risk of chronic inflammatory diseases has been linked with lack of the endocrine and paracrine functions of the muscle that are not activated through contractions [57]. Myokines may balance and counteract the effects of adipokines such as leptin taking part in crosstalk between skeletal muscle and adipose tissue [57, 58].

Here we have shown for the first time that the plasma level of irisin was diminished in rats with TNBS-induced colitis fed HFD but not in those fed normal diet or LFD. Exercise training leads to marked increase in plasma irisin levels confirming that irisin identified as a putative myokine that is induced by exercising muscles could be involved in the mechanism of exercise-induced improvement of mucosal healing of colitis observed in our study. Interestingly, the circulating levels of irisin were also negatively associated with obesity and insulin resistance [59]. It was suggested that irisin could be therapeutic for human metabolic disease, obesity, and other disorders in which adipose tissue plays pathogenic role, and the exercise was found to exert beneficial influence [60]. We have also observed significant increases in circulating myokine IL-6 after exercise training. While typically regarded as a proinflammatory cytokine, IL-6 appears to mediate also metabolic effects associated with exercise. Until recently, it was accepted that the rise in plasma IL-6 level observed during exercise was a consequence of immune response to local damage. Today, it is known that muscle is unique in its ability to produce IL-6 during contraction in completely TGF-independent mode, which suggests a major role for this cytokine in a mechanism of regulation of metabolism rather than for this cytokine acting as an inflammatory mediator.

Our results are in variance with those observed in recent studies on the effects of exercise training in a mouse model of colitis [61]. The moderate forced treadmill running exacerbated dextran sodium sulfate- (DSS-) induced experimental colitis while the voluntary wheel running alleviated colitis symptoms and reduced inflammatory gene expression in these mice [61]. These contradictory effects of voluntary and forced exercise could be explained by effect of stress resulting from the extensive forced exercise in comparison with voluntary exercise. The differences between the results of our study and those by Cook et al. [61] could be explained by differences in experimental model of colitis and the bout of exercise used in both studies in different animal species. In the case of rats subjected to exercise, the applied intensity of forced exercise could be relatively smaller than for mice. Intensive exercise could lead to transient systemic inflammation and increased level of proinflammatory cytokines. It is well known that intensive exercise in humans could cause nausea, diarrhea, and gastrointestinal bleeding. For instance,

in marathon runners, the disorder called "runner's ischemic colitis" associated with bloody diarrhea, fatigue, and fever has been described [62]. Therefore, the effects of physical exercise on various immune parameters during the course of IBD may depend on type of exercise and its intensity and duration. Systematic, moderate exercise may be beneficial for IBD patients with respect to exercise-induced anti-inflammatory and anabolic mechanisms. In contrast, the acute intensive exercise may result in a release of proinflammatory cytokines predominantly in obese individuals leading to exacerbation of the inflammatory response and worsening of the pathology of CD [54].

5. Conclusions

Diet-induced obesity delays the healing of experimental colitis *via* decrease in CBF and the increase in MPO activity and the expression and release of proinflammatory mediators. Exercise diminished the severity of colonic damage mediated, at least in part, by an improvement of colonic microcirculation, the release of myokines such as protective irisin, and the restoration of adipokine adiponectin. We propose that the regular exercise could exert a beneficial effect in human IBD as documented by our translational research and accumulated evidence in experimental rodent colitis. This beneficial effect of exercise may be mediated by its long-term effects on abdominal adiposity and the anti-inflammatory environment that is created by each acute bout of exercise.

Conflict of Interests

The authors have no conflict of interests to declare.

Acknowledgments

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Research Article

An *In Vitro* Model to Evaluate the Impact of the Soluble Factors from the Colonic Mucosa of Collagenous Colitis Patients on T Cells: Enhanced Production of IL-17A and IL-10 from Peripheral $CD4^+$ T Cells

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Soluble factors from intestinal mucosal cells contribute to immune homeostasis in the gut. We have established an *in vitro* model to investigate the regulatory role of soluble factors from inflamed intestinal mucosa of collagenous colitis (CC) patients in the differentiation of T cells. Peripheral blood $CD4^+$ T cells from healthy donors were polyclonally activated in the presence of conditioned medium (CM) generated from denuded biopsies (DNB) or isolated lamina propria mononuclear cells (LPMCs) from mucosal biopsies from CC patients compared to noninflamed controls, to determine proliferation and secretion of cytokines involved in T-cell differentiation. Compared to controls, we observed significantly increased production of the proinflammatory cytokines IFN-γ, IL-17A, IL-6, and IL-1β and the anti-inflammatory cytokines IL-4 and IL-10 in the presence of CC-DNB-CM. The most pronounced effect of CC-LPMC-CM on peripheral $CD4^+$ T cells was a trend towards increased production of IL-17A and IL-10. A trend towards reduced inhibition of T-cell proliferation was noted in the presence of CC-DNB-CM. In conclusion, our *in vitro* model reveals implications of soluble factors from CC colonic mucosa on peripheral T cells, enhancing their production of both pro- and anti-inflammatory cytokines.

1. Introduction

The human gastrointestinal mucosa constitutes the largest mucosal surface area in the human body interfacing the external environment. A network of complementary regulatory interactions between different types of immune and nonimmune cells maintains mucosal homeostasis in the gut. These regulatory interactions occur in the midst of a complex mixture of proteins, known as extracellular matrix (ECM) or stroma [1, 2], which together with soluble mediators such as cytokines and growth factors from mesenchymal cells, immune cells, and epithelial cells regulate cell activation and differentiation [2]. Mesenchymal cells are actively involved in the inflammatory process in the gut and can perpetuate chronic gut inflammatory conditions like inflammatory bowel disease (IBD) [3–5]. Transforming growth factor-(TGF-) β is one of the potential soluble mediators in homeostatic mechanisms in the gut that downregulates effector T-cell responses in the mucosa by reducing proliferation and interferon-γ (IFN-γ) production [4, 6]. However, TGF-β together with IL-6 and IL-1β from the inflamed mucosa induces proinflammatory Th17 cells, suggesting an innate regulatory function of the gut mucosal microenvironment [4, 7, 8].

Research is ongoing to elucidate the role of soluble factors and ECM in immunopathological mechanisms in

IBD [4], but no such studies have so far been performed on microscopic colitis (MC) where the inflammation is subtler. MC comprises two entities, collagenous colitis (CC) and lymphocytic colitis (LC). Both conditions are characterized by chronic nonbloody, watery diarrhoea, often associated with abdominal pain and weight loss [9–11]. The colonic mucosa is macroscopically normal or almost normal and the diagnosis relies on microscopic assessment of mucosal biopsies. CC is presented with increased densities of lymphocytes and a thickened subepithelial collagen band ($\geq 10\,\mu$m) adjacent to the basal membrane. The pathophysiological data of CC are still limited, but it is postulated to be at least partially caused by disturbed immune responses to various luminal antigen(s), such as drugs, gluten, or infectious agents, in predisposed individuals [9]. Nonsteroidal anti-inflammatory drugs (NSAIDs), proton pump inhibitors, aspirin, and selective serotonin reuptake inhibitors have been associated with CC [12]. In the majority of patients, however, no precipitating factor is found. Dysregulated myofibroblast function has also been implicated for collagen deposition in CC patients [10, 13]. Recently, we reported on increased local activation of both $CD4^+$ and $CD8^+$ T cells in the lamina propria and epithelium of CC patients, demonstrated as increased expression of CD45RO and the proliferation marker Ki67, using flow cytometric analysis of freshly isolated lymphocytes from colonic biopsies [14]. In addition, mucosal transcript levels of IFN-γ, IL-12, IL-1β, IL-6, IL-17A, IL-21, IL-22, and IL-23 are enhanced in the inflamed mucosa of CC patients compared to normal mucosa, together with elevated protein levels of IL-6, IL-21, and TNF [15].

Although the above findings suggest that the mucosal microenvironment is involved in CC immunopathology, the interplay between these factors and the proinflammatory activity of local mucosal T cells in CC patients has not been elucidated. We therefore investigated the role of soluble factors from the intestinal mucosa of CC patients in the regulation of T cells using a novel *in vitro* model system with the aim of mimicking the *in vivo* exposure of newly recruited peripheral blood T cells to the soluble factors in the colonic milieu of inflamed CC and normal mucosa.

2. Material and Methods

2.1. Patients. CC diagnosis was confirmed by clinical symptoms: ≥3 loose stools/day and/or abdominal pain and a macroscopically normal colonic mucosa with characteristic histopathological findings: increased numbers of lymphocytes in the epithelium and lamina propria with deposition of a $\geq 10\,\mu$m thick subepithelial collagen layer [9]. Inclusion criteria were patients previously diagnosed with CC with clinically and histopathologically active disease. Patients with enteric infection, ischemic colitis, colonic cancer, or a previous history of Crohn's disease or ulcerative colitis were excluded. None of the patients were treated with immunosuppressive drugs or antibiotics.

We investigated colonic biopsies from 7 CC patients (female; $n = 6$) and 20 noninflamed controls (female; $n = 11$) without diarrhoeal symptoms, recruited among patients undergoing colonoscopy for examination of gastrointestinal bleeding or of abnormal radiological findings. All controls had a normal colonoscopy and histology.

Twelve biopsies from the hepatic flexure from each individual were obtained using standard biopsy forceps, placed in phosphate buffered saline (PBS), and processed within 1 hour. The colonoscopies were performed at the Division of Gastroenterology, Örebro University Hospital, Sweden, between November 2012 and November 2013.

Peripheral blood from healthy donors ($n = 6$) was collected in heparin tubes for $CD4^+$ lymphocyte isolation as described below.

The study was approved by the Regional Ethical Committee of Örebro-Uppsala County, Sweden (ID no. 2008/278; 081015). All patients in this study had provided written informed consent.

2.2. Preparation of Conditioned Medium from the Colonic Mucosa. We investigated the influence of two different preparations of the mucosa: one where the epithelium and intraepithelial cells were removed enzymatically, the denuded biopsies (DNB), and one where collagenase was used to digest the lamina propria after the removal of the epithelium. The DNB and the isolated mononuclear cells were cultured overnight, and the latter were termed lamina propria mononuclear cells (LPMCs). The DNB fraction contains collagen and mesenchymal cells, fibroblasts, and leukocytes. The cell populations are intact in the DNB fraction, whereas the lamina propria mononuclear cells (LPMCs) fraction is composed of a free leukocyte population as well as tissue, collagen, and cell debris.

Twelve biopsies were thoroughly washed with PBS and incubated with prewarmed Hank's balanced salt solution (HBSS) (Sigma Aldrich, St. Louis, MO, USA) containing 1 mM EDTA, 20 mM HEPES, and 5% heat inactivated fetal bovine serum (FBS) at 37°C, with constant stirring 4 times (15 min), to remove the epithelial layer. Six denuded biopsies were kept in serum-free RPMI-1640 containing 20 mM HEPES, 100 μg/mL streptomycin, 10 μg/mL gentamycin, and 100 U/mL penicillin (hereafter referred to as culture medium) on ice until further use, and the remaining six biopsies were further digested with collagenase type VIII and DNAse I type IV (Sigma Aldrich) for 1–1.5 hrs to digest the collagen. Isolated lamina propria mononuclear cells were washed twice in PBS and resuspended in culture medium. DNBs and LPMCs were cultured in culture medium overnight at 37°C under 5% CO_2-95% air, to generate conditioned medium (CM). CM from the DNB and LPMC fractions from CC patients and noninflamed controls, respectively, were pooled. Endotoxin levels were quantified using the Pierce LAL Chromogenic Endotoxin Quantitation Kit (Pierce, Rockford, IL, USA) according to the manufacturer's protocol. The maximum endotoxin level for DNB-CM and LPMC-CM was 4 EU/mL and 4.2 EU/mL, respectively. Total protein concentrations were determined using the Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA, USA). DNB-CM was used at the total protein concentrations of 250 and 62.5 μg/mL, whereas LPMC-CM was used at the total protein concentrations

125 and 62.5 μg/mL, the highest possible concentrations of the respective conditioned media. CM from the intestinal mucosa of noninflamed controls is referred to as Ctrl-DNB-CM and Ctrl-LPMC-CM, whereas CM derived from CC patients is referred to as CC-DNB-CM and CC-LPMC-CM.

2.3. T-Cell Proliferation and Cytokine Release Assays. $CD4^+$ peripheral blood lymphocytes were isolated from healthy donors using a Human $CD4^+$ T-cell Enrichment Cocktail kit (STEMCELL Technologies, Grenoble, France) according to the manufacturer's protocol. Cell viability was ~95%, as determined by Trypan blue exclusion. The purity of $CD4^+$ T cells was 90–95% as determined by flow cytometric analysis (Epics Altra, Beckman Coulter, Fullerton, CA, USA).

Purified $CD4^+$ PBLs were cultured in 96-well flat bottom assay plates (Sarstedt, Newton, NC, USA) precoated with 50 μL of 5 μg/mL anti-CD3 (UCHT1, BD Biosciences, San Diego, CA, USA) for 2 hrs at 37°C, followed by two washes with PBS.

1×10^5 $CD4^+$ T cells were added to the washed wells together with 1 μg/mL soluble anti-CD28 (BD Biosciences) and were incubated in culture medium with the addition of 2 mM L-glutamine and 5% AB serum, with or without DNB-CM or LPMC-CM from noninflamed controls or CC patients in a total volume of 200 μL. As controls, Ctrl/CC-DNB-CM and Ctrl/CC-LPMC-CM were cultured without $CD4^+$ T cells, and $CD4^+$ T cells alone were incubated without anti-CD3/anti-CD28. In addition, control wells containing culture medium only were included. The assay was performed in duplicate wells for cytokine analysis and triplicate wells for the proliferation assay. The cells were cultured at 37°C under 5% CO_2-95% air for three days; thereafter, supernatants were harvested and stored at −80°C until determination of cytokine content, and T-cell proliferation was measured using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, Promega, Madison, WI, USA). Forty microliters of AQueous One Solution Reagent was added into each well and incubated at 37°C for 4 hrs in a humidified, 5% CO_2 incubator followed by recording of the absorbance at 490 nm.

2.4. Cytokine Analysis. The pooled conditioned medium from inflamed CC mucosa and controls as well as supernatants from peripheral $CD4^+$ T cells were analyzed for IL-1β, IL-4, IL-6, IL-10, IL-17A, IFN-γ, and TNF using the xMAP technology developed by Luminex (Austin, TX, USA), using two Milliplex Map Kits (Cat. number HCYTOMAG-60K and Cat. number HCYP2MAG-62K), according to the manufacturer's instructions (Millipore, MA, USA). The assays were performed in duplicate and the levels of different cytokines were expressed as pg/mL, according to a standard curve with known amounts of each analyte (Millipore).

TGF-β levels were determined by ELISA, according to the manufacturer's instructions (BD Biosciences, San Diego, CA, USA).

Cytokine amounts were calculated as follows: cytokine amounts released by peripheral T cells incubated with CM minus cytokine amounts in CM alone. For each blood donor, the cytokine amounts released by $CD4^+$ T cells incubated with CM from CC patients were compared with the cytokine amounts released by $CD4^+$ T cells incubated with CM from controls.

2.5. Statistical Analysis. As the data obtained were not normally distributed, Wilcoxon's signed rank nonparametrical test was used for statistical comparison between groups. Differences were considered statistically significant when $P \leq 0.05$.

3. Results

3.1. An In Vitro Model to Study the Impact of the Mucosal Milieu on Peripheral T Cells: Increased Production of Both Pro- and Anti-Inflammatory Cytokines by Polyclonally Activated $CD4^+$ T Lymphocytes in the Presence of Soluble Factors from CC Mucosa. To mimic *in vitro* the exposure of peripheral T lymphocytes that have newly arrived into the colonic mucosa and to determine whether the local intestinal milieu affects T-cell activation and differentiation, we investigated T-cell cytokine production by α-CD3 plus α-CD28 stimulated $CD4^+$ peripheral blood T cells in the presence of soluble factors from the intestinal mucosa. There was significantly increased production of the proinflammatory cytokines IFN-γ, IL-17A, IL-6, and IL-1β in the presence of CM generated by culture of denuded biopsies (DNB-CM) from the colon of collagenous colitis patients, compared to DNB-CM from noninflamed controls (Figure 1). This was evident with the lower protein concentration of CM tested for IL-17A, IL-6, and IL-1β and with the higher concentration of CM tested for IFN-γ production.

We also noted a significantly increased production of the anti-inflammatory cytokines IL-4 and IL-10 in the presence of DNB-CM from CC patients compared to noninflamed controls, with the lower protein concentration of CM (Figure 1).

In contrast, no significant differences were noted for TGF-β production by peripheral $CD4^+$ T cells in the presence of DNB-CM from CC patients compared to noninflamed controls (data not shown).

In general, LPMC-CM had less impact on peripheral $CD4^+$ T-cell activation and differentiation. A trend towards increased production of IL-17A and IL-10 (both $P = 0.06$) was noted in the presence of LPMC-CM from CC patients compared to noninflamed controls in the lower protein concentration (Figure 2). The other cytokines investigated were not significantly altered (Figure 2).

3.2. Reduced Inhibition of $CD4^+$ Peripheral T-Cell Proliferation in the Presence of Conditioned Media from Colonic Mucosa from CC Patients. We next investigated the ability of soluble factors from the colonic mucosa to inhibit T-cell proliferation, as this has previously been demonstrated *in vitro* [4, 16, 17]. A tendency towards reduced proliferation

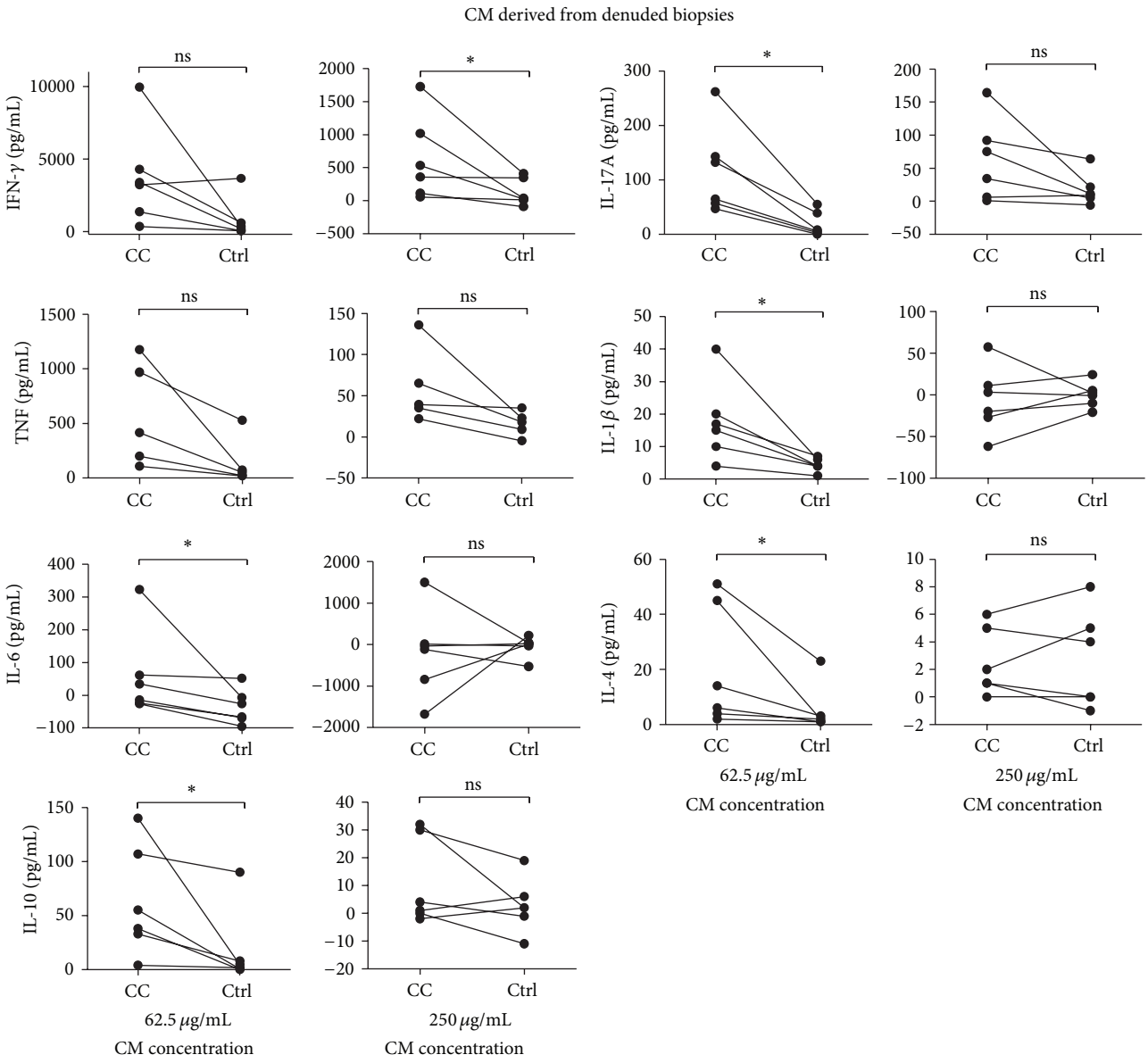

Figure 1: Luminex analysis for IL-1β, IL-4, IL-6, IL-10, IL-17A, IFN-γ, and TNF production by anti-CD3 plus anti-CD28 stimulated $CD4^+$ peripheral blood T cells ($n = 6$; for TNF analysis, $n = 5$) cultured for 3 days in the presence of two concentrations of CM derived from denuded biopsies (DNB) from inflamed CC mucosa or noninflamed controls. Cytokine amounts are expressed as the amounts released by peripheral T cells incubated with CM minus cytokine amounts in CM alone, in pg/mL. For each blood donor, cytokine amounts released by peripheral T cells incubated with CM from CC patients were compared with the amounts released by peripheral T cells incubated with CM from noninflamed controls. Each symbol represents data from one donor and the data points from CC and noninflamed controls for each donor are connected with a line. * = $P < 0.05$ versus noninflamed controls.

inhibition of peripheral $CD4^+$ T cells was noted in the presence of CM from culture of denuded biopsies (DNB-CM) ($P = 0.06$) from inflamed CC patients compared to noninflamed controls (Figure 3). This was evident with both total protein concentrations of DNB-CM tested for peripheral T-cell proliferation.

In contrast, no differences in proliferation inhibition were observed in the presence of LPMC-CM from noninflamed controls compared to CC patients (Figure 3).

3.3. Enhanced IL-1β and IL-6 Levels in Conditioned Medium from Inflamed CC Mucosa. As T-cell differentiation and function are regulated by different cytokines, we next analysed eight cytokines in pooled conditioned medium from DNB and LPMC fractions derived from inflamed CC mucosa compared to controls. We found more than twofold and eightfold increased levels of IL-6 and IL-1β, respectively, in DNB-CM from CC patients compared to controls, whereas no alterations were found in the levels of IFN-γ, IL-17A, TNF,

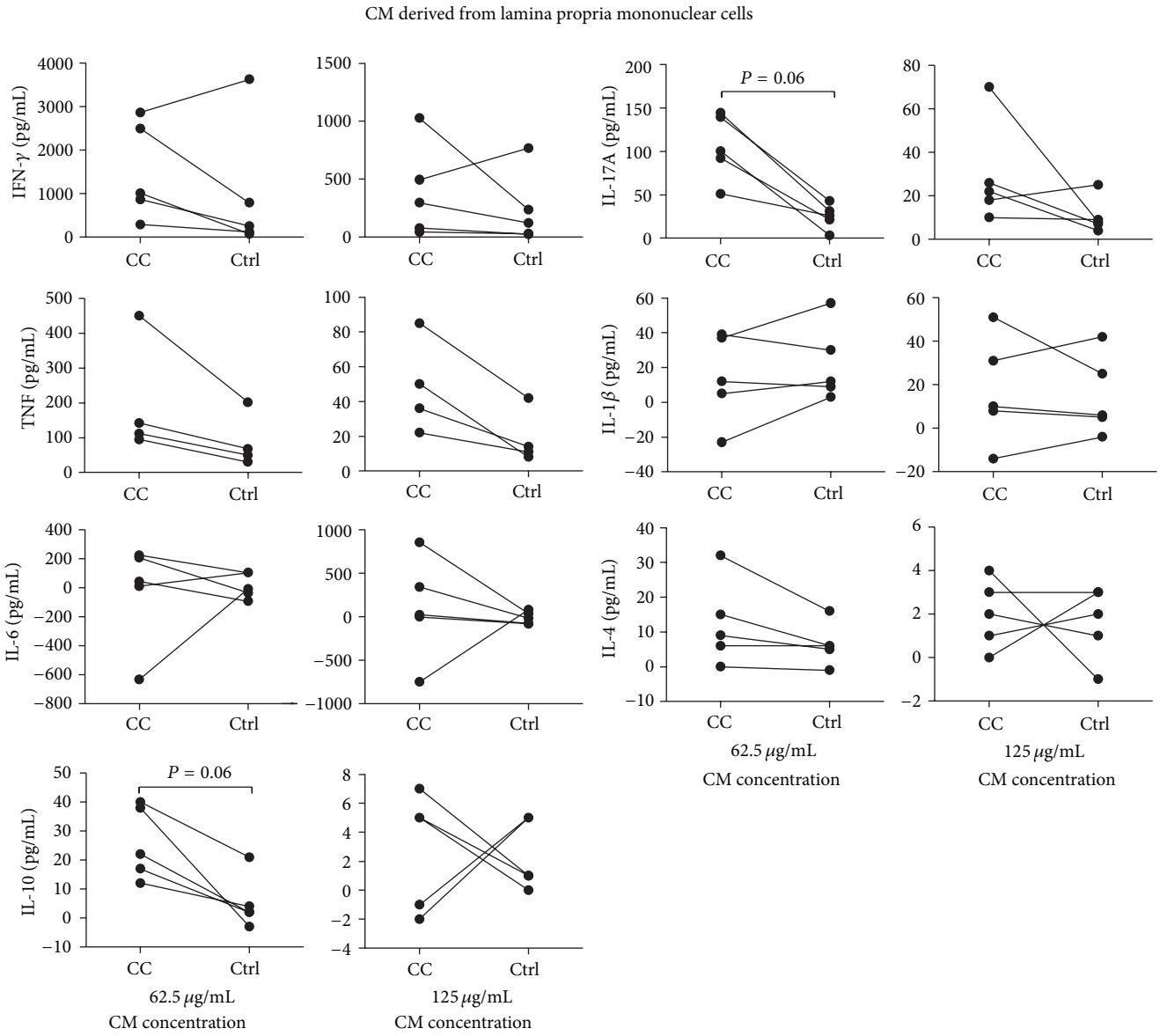

Figure 2: Luminex analysis for IL-1β, IL-4, IL-6, IL-10, IL-17A, IFN-γ, and TNF production by anti-CD3 plus anti-CD28 stimulated $CD4^+$ peripheral blood T cells ($n = 5$; for TNF analysis, $n = 4$) cultured for 3 days in the presence of two concentrations of CM derived from lamina propria mononuclear cells (LPMC) from inflamed CC mucosa or noninflamed controls. Cytokine amounts are expressed as cytokine amounts released by peripheral T cells incubated with CM minus cytokine amounts in CM alone, in pg/mL. For each blood donor, cytokine amounts released by peripheral T cells incubated with CM from CC patients were compared with the amounts released by peripheral T cells incubated with CM from noninflamed controls. Each symbol represents data from one donor and the data points from CC and noninflamed controls for each donor are connected with a line.

IL-4, and IL-10 (Table 1) or TGF-β (data not shown). Similar trends of increased levels of IL-6 and IL-1β were noted in LPMC-CM from CC patients compared to controls, though it was investigated in pooled CM from only two CC patients (data not shown).

4. Discussion

We here report on a novel *in vitro* model for analysis of the impact of the soluble factors from the colonic mucosa of CC patients on peripheral T lymphocyte activation and differentiation. We found that despite the subtle inflammation in the mucosa of collagenous colitis patients, not visible by the naked eye upon colonoscopy, soluble factors in the mucosa are sufficient to significantly enhance the production of IFN-γ, IL-17A, IL-6, IL-1β, IL-4, and IL-10 by peripheral $CD4^+$ T cells exposed to them *in vitro*. This is the first study to investigate the role of soluble factors from the intestinal mucosa of CC patients in the regulation of T cells, where this novel system reflects the impact of *in vivo* exposure of

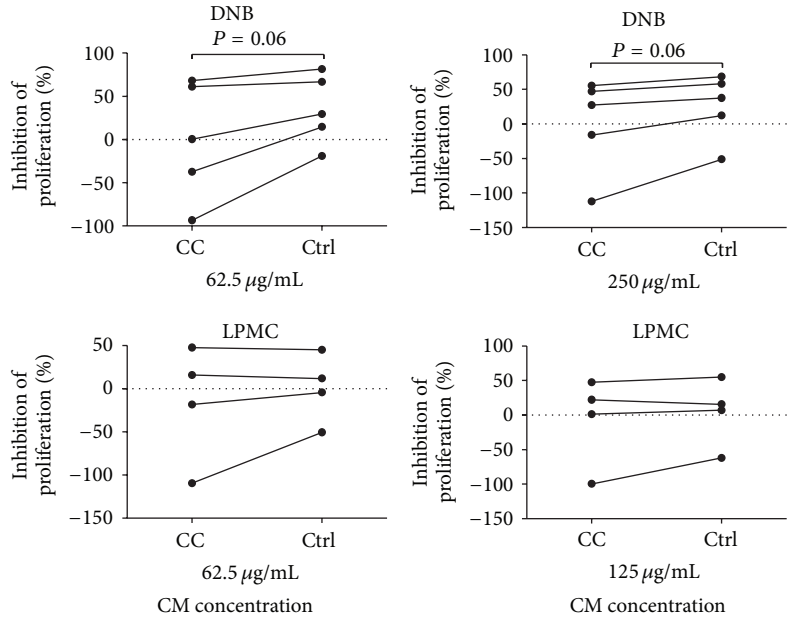

Figure 3: Proliferation of anti-CD3 plus anti-CD28 stimulated $CD4^+$ peripheral blood T cells ($n = 5$) cultured in the absence or presence of two concentrations of conditioned medium (CM) derived from denuded biopsies (DNB) or from lamina propria mononuclear cells (LPMC) from inflamed CC mucosa or noninflamed controls, as analysed on day 3 of culture. Data are presented as percentage inhibition of proliferation compared to cells cultured in the absence of CM. Each symbol represents data from one blood donor and the data points from CC and noninflamed controls for each donor are connected with a line. For LPMC fraction, $CD4^+$ peripheral blood T cells from four blood donors were tested for proliferation analysis.

Table 1: Protein levels (pg/mL) of cytokines analysed in pooled condition medium (CM) derived from denuded biopsies (DNB) from inflamed collagenous colitis (CC) mucosa or normal mucosa (control, $n = 20$; CC, $n = 7$).

	IFN-γ	IL-17A	TNF	IL-1β	IL-6	IL-4	IL-10
CC-DNB-CM	772	40	97	1831	9326	12	511
Ctrl-DNB-CM	509	36	70	228	4138	11	525

newly recruited peripheral blood T cells to soluble factors in the colonic milieu of inflamed mucosa from CC patients compared to normal mucosa.

CM from denuded biopsies from inflamed CC mucosa induced increased production of both pro- and anti-inflammatory cytokines by peripheral T cells. As microscopic colitis is subtler compared to ulcerative colitis and Crohn's disease, this may suggest that the colonic microenvironment in CC promotes production of anti-inflammatory cytokines to counterbalance inflammatory responses. Apparently this is not sufficient to keep the inflammation at bay. A study on the effects of stroma conditioned medium from Crohn's patients mucosa on cytokine production by T cells demonstrated increased IFN-γ and IL-17 production but provided no data on the effect of anti-inflammatory cytokine production [4].

No differences were noted in TGF-β production by peripheral $CD4^+$ T cells in the presence of CM from CC patients compared to controls. Whereas TGF-β in the normal mucosa likely suppresses T-cell function, the significantly increased amounts of IL-6 and IL-1β in the inflamed CC mucosa instead likely promote differentiation of proinflammatory Th17 cells [7, 18] producing large amounts of IL-17A. The colonic milieu from CC patients might also promote differentiation of peripheral $CD4^+$ T cells into IL-17/IFN-γ double producing Th17/Th1 cells that have been suggested to mediate gut inflammatory processes [19–21], corroborating our findings of enhanced levels of both IL-17A and IFN-γ.

We found a trend towards reduced inhibition of T-cell proliferation by soluble factors from denuded biopsies from the colonic mucosa of CC patients compared to controls. This is in accordance with the findings by Huff et al. on stroma conditioned medium derived from inflamed Crohn's mucosa [4]. Older studies have demonstrated that the mucosal microenvironment reduces the proliferative responses of lamina propria lymphocytes to antigen receptor stimulation [17, 22] but they are still active in their helper and cytolytic functions [22, 23]. The present study together with the study by Huff et al. [4] indicates that these effects are at least partly imprinted in the T lymphocytes by the local milieu, rather than an intrinsic characteristic.

In contrast to CM from denuded biopsies, lamina propria mononuclear cell- (LPMC-) CM from CC patients did not affect T-cell proliferation compared to LPMC-CM from controls. These different effects of the two types of CM are unclear and further experiments need to be performed to elucidate the differences in the composition of the CMs.

Despite our observed enhanced production of IL-10 by peripheral T cells in the presence of CM from CC patients, known to inhibit both T-cell proliferation and cytokine production [24], we found neither reduced proliferation of peripheral T cells nor production of proinflammatory cytokines. This indicates that other immunoregulatory molecules drive synthesis of these proinflammatory cytokines in collagenous colitis.

The production of cytokines did not increase with higher total protein concentrations in the CM. One explanation could be the presence of inhibiting and/or toxic factors in the CM limiting the T-cell responses. In addition, various molecules have different optimal concentrations for their function and high concentrations can limit their activity. To further elucidate this and explain the differences observed on $CD4^+$ T-cell differentiation we want to investigate a larger panel of cytokines and compare the protein profile between CM from CC patients and that from noninflamed controls and between DNB and LPMC fractions by proteomics.

In conclusion, we have set up an *in vitro* model for analysis of the impact of the soluble factors from the colonic mucosa of CC patients on peripheral T lymphocyte activation and cytokine production. Despite the subtle inflammation in CC, our data demonstrate significant alterations in cytokine production by peripheral $CD4^+$ T cells in the presence of mucosa-derived soluble factors from CC patients compared to controls. One of our future goals is to test this *in vitro* model on differentiation of $CD8^+$ T cells, as we have previously reported on their increased numbers in the colonic mucosa of CC patients. We also want to evaluate its use in evaluating the effect of drugs, including those in present use, on the colonic mucosal milieu and the lymphocytes there within, thereby facilitating the decision on optimum molecules as well as doses required for suppression of T-cell inflammatory responses.

Conflict of Interests

Nils Nyhlin has served as a speaker for MSD. Johan Bohr has served as a speaker for Dr. Falk Pharma, MSD, and AbbVie. Curt Tysk has served as a speaker for Dr. Falk Pharma, Tillotts Pharma, Ferring, MSD, and AstraZeneca.

Authors' Contribution

Ashok Kumar Kumawat participated in the creation of study design and carried out the studies, data analysis, and drafting of the paper. Olof Hultgren participated in data analysis. Nils Nyhlin, Anna Wickbom, Johan Bohr, and Curt Tysk participated in patient recruitment and in performing colonoscopies for biopsy collections. Elisabeth Hultgren-Hörnquist was in charge of the creation of study design, coordination, and data analysis. Ashok Kumar Kumawat, Elisabeth Hultgren-Hörnquist, Olof Hultgren, and Curt Tysk participated in finalization of the paper. All authors read and approved the final paper.

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Research Article

Tumor Necrosis Factor Induces Developmental Stage-Dependent Structural Changes in the Immature Small Intestine

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Background. Premature infants are commonly subject to intestinal inflammation. Since the human small intestine does not reach maturity until term gestation, premature infants have a unique challenge, as either acute or chronic inflammation may alter the normal development of the intestinal tract. Tumor necrosis factor (TNF) has been shown to acutely alter goblet cell numbers and villus length in adult mice. In this study we tested the effects of TNF on villus architecture and epithelial cells at different stages of development of the immature small intestine. *Methods*. To examine the effects of TNF-induced inflammation, we injected acute, brief, or chronic exposures of TNF in neonatal and juvenile mice. *Results*. TNF induced significant villus blunting through a TNF receptor-1 (TNFR1) mediated mechanism, leading to loss of villus area. This response to TNFR1 signaling was altered during intestinal development, despite constant TNFR1 protein expression. Acute TNF-mediated signaling also significantly decreased Paneth cells. *Conclusions*. Taken together, the morphologic changes caused by TNF provide insight as to the effects of inflammation on the developing intestinal tract. Additionally, they suggest a mechanism which, coupled with an immature immune system, may help to explain the unique susceptibility of the immature intestine to inflammatory diseases such as NEC.

1. Introduction

Intestinal inflammation is a common occurrence for premature infants. After a relatively protective intrauterine environment, the newborn is abruptly exposed to nonmaternal antigens and abnormal bacterial flora in the intensive care setting [1]. Even normal food sources for infants can induce inflammation in immature intestine. Exposure to formula has been shown to increase proinflammatory cytokines in the immature intestine [2], and breast milk from mothers exposed to a Western diet has been shown to contain increased amounts of long chain and saturated fatty acids that induce inflammation in the immature intestine [3]. While these data indicate that diets of premature infants put them at risk for chronic gut inflammation, the effects of chronic inflammation on the developing intestine are not fully understood. Notably, enteral feeding is one of the strongest risk factors for the development of necrotizing enterocolitis (NEC) [4], leading to questions regarding the role that feeding-induced chronic intestinal inflammation may play in the induction and pathophysiology of the disease.

NEC is predominantly a disease of premature infants, primarily affecting infants born at the shortest gestations [4, 5]. The incidence of NEC is the highest between the ages of 28 and 32 weeks relative gestation regardless of when an infant is born, suggesting that changes in gut development regulate susceptibility [6, 7]. A key component of NEC is exaggerated inflammation [8–10] mediated by potent inflammatory cytokines such as tumor necrosis factor (TNF) and platelet activating factor (PAF) [11]. In human studies, newborn infants who developed NEC expressed significantly higher serum TNF levels compared to controls [11], and in animal

models of NEC, animals treated with anti-TNF antibodies have lower incidence and severity of disease [12]. Our lab recently reported that significant developmental-stage dependent changes can be seen in intestinal goblet cell mucus secretion in the murine ileum eight hours following TNF exposure [13]. Furthermore, acute TNF exposure mediates the early stages of tissue damage seen in *Salmonella* infection of the intestinal tract [14] and can induce decreases in villus height in burn patients [15]. However, the specific role of TNF in the pathophysiology of NEC is not completely known and the mechanisms behind the initiation of inflammatory signaling represent a major gap in our understanding.

Considering that (1) susceptibility to NEC appears to be developmentally-dependent, (2) premature infants have frequent exposure to intestinal inflammation, and (3) TNF induced inflammation can have developmental dependent effects on the intestinal epithelium, we tested whether TNF induces other developmentally dependent changes in the small intestine. To better understand how different exposures of inflammation would affect the developing intestinal tract, we examined components of intestinal architecture following several different types of exposure to TNF. In the following studies, we show that both acute and chronic exposure to TNF have developmental-stage dependent effects on the developing small intestine.

2. Materials and Methods

2.1. Mice and TNF Injections. C57BL/6J, TNFR1$^{-/-}$, TNFR2$^{-/-}$, and TNFR1$^{-/-}$2$^{-/-}$ mice were purchased from The Jackson Laboratory. CD-1 mice were purchased from Charles River Laboratories. All genetically modified mice were maintained on a C57BL/6J background and C57BL/6J was used as wild type comparisons. For acute TNF experiments, C57BL/6J or CD-1 mice were injected intraperitoneally at the indicated ages with 0.5 μg/gbw TNF (PeproTech) and maintained for 8 hours separated from mothers and without feeds at 33°C in a humidified incubator (Petiatric.com). The distal 1/3 of the small intestine was harvested and isolated for ileal samples. Tissues were fixed by snap freezing or Carnoy solution; tissues prepared for immunohistochemistry were paraffin-embedded and sectioned at 5 μm. For chronic TNF experiments, CD-1 mice were given weekly intraperitoneal injections with 0.5 μg/gbw TNF. Injections began at age P7 and ended one week prior to euthanasia. During injections, mice were housed under normal conditions at the animal care facility at the University of Iowa. On the final day of experimentation, tissue was harvested as described above. All animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee at The University of Iowa.

2.2. Villus Morphometry and Cell Counts. Ileal sections were stained with H&E (Sigma-Aldrich). In an attempt to minimize sectioning variability, all sections were obtained from the center of the intestinal sample and only areas with full villi were included. In each sample used for measurement, at least 3 areas were counted to minimize sectioning variances. Measurements were obtained at each timepoint from at least five separate animals. A total of at least 20 consecutive villi from 3 separate areas per animal were analyzed in each experimental group. Villus length and surface area measurements were taken with a 10x objective (100x total magnification) from the tip of the villus to the entrance of the crypt opening. Villus length was measured by drawing a bisecting line through the center of the villi. Surface area was measured by drawing a line around the exterior of each structure. Villus epithelial cells were counted with a 60x objective (600x total magnification) from the entrance of the crypt opening to the beginning of the curve at the villus tip. Intestinal sections from at least five animals for a total of at least 70 villi per animal were analyzed for each experimental group. To detect Paneth cells, slides were stained with Alcian Blue/Periodic Acid Schiff stain (Sigma-Aldrich). Cells were quantified with a 60x objective (600x total magnification) by a single blinded investigator. Intestinal sections from at least five animals were analyzed for each experimental group and at least 100 crypts were counted per animal. All data were obtained using a Nikon NiU microscope using Nikon Elements software (Nikon).

2.3. Cell Lysates, PCR, and Western Blotting. Ileal samples were homogenized using a TissueLyser LT (Qiagen), then cleared, and boiled as previously described [16]. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked for 1 h in Tris-buffered saline with 0.05% Tween 20 (TBST) and 5% nonfat dry milk, incubated with anti-TNFR1 primary antibody (Santa Cruz Biotechnology) overnight at 4°C, and incubated with secondary antibody (Cell Signaling) for 45 minutes. Horseradish peroxidase was detected with the Western Lightning enhanced chemiluminescence kit (PerkinElmer Life Sciences). For mRNA quantification, ileal samples were homogenized as above and RNA was isolated using RNeasy Plus Mini Kit (Qiagen) according to manufacturer's directions. RNA concentration and quality were determined using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific). First-strand cDNA was synthesized using High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Life Technologies). Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) was performed using Taqman Fast Universal PCR Master Mix (2X) (Life Technologies) and Taqman Gene Expression Assays for TNFR1 primers (Life Technologies). qRT-PCR reactions were run in a C1000 Thermal Cycler (Eppendorf) and using the CFX96 Real-Time PCR Detection System (Bio-Rad). 10 ng of cDNA was used per reaction. The threshold cycle (CT) value for each well was obtained by using the instrument's software. Fold change in gene expression was determined by normalizing gene expression to β-actin in each sample. The 2ΔΔ-CT method was used to compare gene expression levels between samples.

2.4. Apoptosis Assays. Rates of apoptosis on histological specimens were determined by in situ oligo ligation DNA

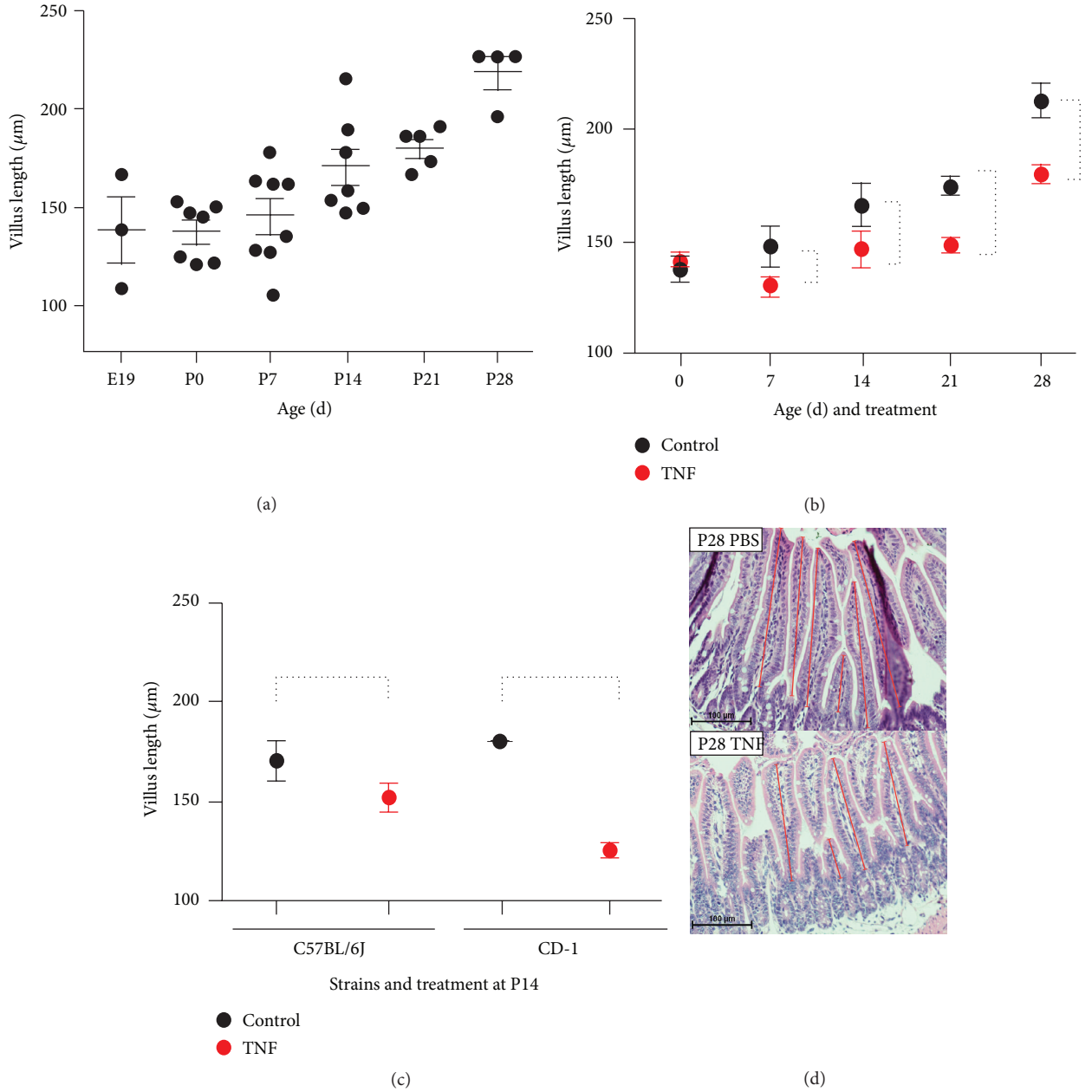

Figure 1: Acute TNF blunts ileal villus length at specific stages of development. (a) The ileal segment of the small intestine was harvested from mice at indicated ages and villus length was microscopically measured. Each dot represents one animal's average villus length which was generated by averaging at least 20 consecutive villi from 3 separate areas per animal. (b) Mice were treated with TNF at indicated ages and villus length was determined (red, TNF; black, control; $n = 5$ for each point; $P < 0.0002$, significance is denoted by dashed lines). (c) Small intestinal villus length was measured at P14 following TNF treatment and compared to controls in both C57BL6 and CD1 mice ($n = 5$; $P < 0.003$ for both strains). (d) Representative examples of histology sections at different developmental stages.

fragmentation assay (ISOL; Millipore), as we have previously reported [17].

2.5. Replicates and Statistical Analysis. All data are representative of at least three independent experiments. Statistical significance of differences was assessed with one-way analysis of variance (ANOVA). Post-test comparisons between groups were made using Holm-Sidak's multiple comparisons test. All statistics were performed using Prism software (Graph Pad). Minimum level of significance was set at <0.05 and error bars designate standard error of the mean.

3. Results

3.1. Ileal Villus Length Increases Significantly during Specific Stages of Development. We have previously shown that goblet cell numbers in the murine ileum increase in a developmental-dependent manner during the first four weeks of

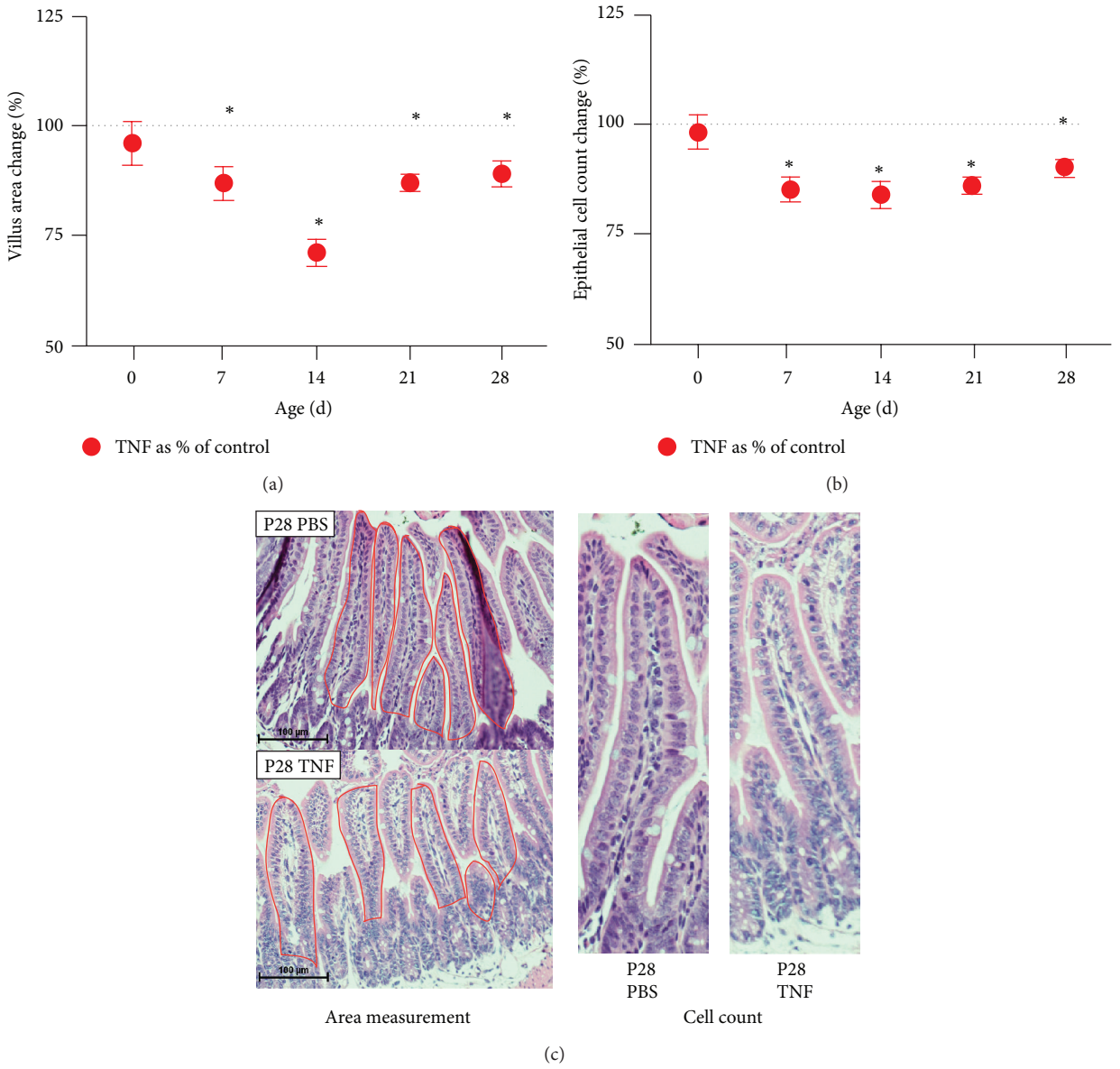

Figure 2: Acute TNF induces loss of ileal villus area and epithelial cell mass during development. Ileal segments of the small intestine were harvested from control and TNF-treated mice at indicated ages. (a) Villus areas were determined by tracing perimeters in Nikon Elements software; (b) epithelial cell numbers were microscopically quantified. Data are shown as the TNF-induced change versus controls (which are set to 100%). Significant differences from controls at each equivalent age are denoted by an asterisk ($n = 5$; $P < 0.0002$). (c) Representative tissue samples are shown.

life [13]. To determine if ileal height developed in a similar pattern, we measured the length of ileal villi at various stages of development throughout the first four weeks of life of the mouse. Ileal villus length increased steadily during development (Figure 1(a)). Birth did not affect villus length, as there was no difference between E19 and P0 mice.

3.2. Acute TNF Blunts Ileal Villus Length at Specific Stages of Development. We have previously shown that TNF has an age-dependent effect on intestinal mucus production and secretion [13]; however, the effects of TNF on the developing intestinal morphology in the immature intestine remain unknown. To test these effects, we examined the ileum eight hours after an intraperitoneal injection with TNF. This time point was based on our earlier studies of TNF exposure on goblet cell numbers in immature intestine [13, 16]. Villus lengths of treated mice were compared to age-matched controls. TNF induced significant villus blunting at P7, P14, P21, and P28 (Figure 1(b)). In contrast, P0 pups were protected from this effect, suggesting either the presence of a protective signaling pathway or decreased responsiveness to TNF at this stage of development. To determine if TNF-induced villus blunting was specific to the C57Bl/6J strain, we repeated this experiment in P14 CD-1 mice and compared these data to the results seen in C57Bl/6J mice from Figure 1(b).

TNF significantly blunted ileal villi in CD-1 mice as well (Figure 1(c)). Our data are thus consistent with a strain-independent effect, though it should be noted that showing true strain independence would require experiments with many more lines, which is beyond the scope of this study.

3.3. TNF-Induced Villus Blunting Is Mediated by Reduction in Villus Surface Area and Epithelial Cell Loss. To further characterize TNF-induced changes in intestinal morphology, we quantified the epithelial cells lining villus cross-sections. Similar to the effects seen on villus length, the number of villus epithelial cells was not affected by TNF at birth. However, in the subsequent 4 weeks, TNF induced a significant decrease in total epithelial cells compared to controls (Figure 2(b)). We next measured the average villus area of mice treated with TNF compared to controls. In control animals, villus area significantly decreased in the first week of life, followed by significant increases during the second, third, and fourth weeks of life. TNF treatment again had no significant effect at birth but induced significant reductions in villus surface area at later ages ($P < 0.0001$) (Figure 2(a)).

3.4. TNF Induces Increased Epithelial Apoptosis in Immature Intestine. To test if TNF-induced loss of villus height, surface area, and epithelial cell mass could be due to induction of apoptosis, histological samples from P14 mice treated with TNF were stained by in situ oligo ligation (ISOL) to detect DNA fragmentation and compared to controls. Mice exposed to acute TNF had significantly more apoptosis than age-matched controls ($P = 0.03$) (Figure 3).

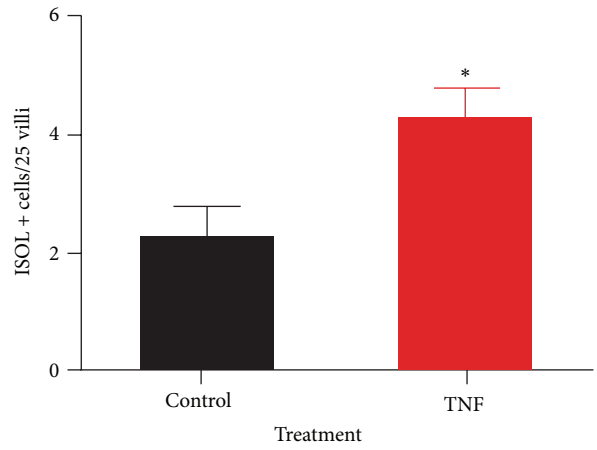

Figure 3: TNF induces increased villus apoptosis in immature intestine. P14 small intestinal samples were stained by in situ oligo ligation (ISOL) for DNA fragmentation and compared to controls. Positive events per 25 villi were counted microscopically. A significant increase in events was observed in the TNF-treated group ($n = 5$; $P = 0.03$).

3.5. TNF-Induced Villus Blunting Requires TNFR1. We have previously shown that TNF can affect both intestinal epithelial cells and ileal goblet cells through TNF receptor-1 (TNFR1) signaling [13, 16]. To determine if the TNF-dependent effects on villus length, area, and epithelial cell mass were also dependent on TNFR1, P14 TNFR1$^{-/-}$, TNFR2$^{-/-}$, and TNFR1$^{-/-}$2$^{-/-}$ mice were given intraperitoneal injections of TNF and their ileal villus measurements were compared to knockout controls. TNF induced villus blunting in wild type and TNFR2$^{-/-}$ mice, but not mice lacking TNFR1. This demonstrates a requirement for TNFR1, and no role for TNFR2. Similarly, TNF treatment induced loss of villus area only in wild type and TNFR2$^{-/-}$ mice, again demonstrating a requirement for the presence of TNFR1. However, only wild type mice showed a significant loss in epithelial cell number (Figure 4).

3.6. Ileal Expression of TNFR1 Is Constant during Development. Our findings indicate that the degree of TNF-induced villus blunting varies depending on the stage of development. To test whether these effects were due to differences in ileal maturity, rather than ontogenic differences in TNFR1 expression, ileal tissue was harvested from wild type mice at various ages and examined by Western blot analysis for expression of TNFR1. No significant differences in TNFR1 expression were detected during development (Figure 5). This finding is important, as it demonstrates a developmental stage-dependent change in downstream TNFR1 effectors that alter response without changing receptor expression.

3.7. Chronic TNF Exposure Induces Villus Blunting in a Dose Dependent Fashion. Since our data demonstrate a developmentally dependent effect of acute TNF on the architecture of the ileum, we investigated the effects of chronic exposure. To accomplish this, mice were treated with TNF once a week beginning on P7 until one week prior to euthanasia. In this fashion, we developed three groups of mice: control mice, mice exposed to a single dose of TNF one week prior to examination (brief exposure, or B-TNF), and mice exposed to weekly TNF treatments beginning on P7, and ending with the last dose occurring one week prior to euthanasia (chronic exposure, or C-TNF). Using this methodology, we examined the ilea of mice from each group for villus length. Similar to the acute exposure, mice exposed to chronic TNF showed a significant loss in villus length. Mice treated with brief exposure (B-TNF) had significantly shorter villi than controls, and mice treated with chronic exposure (C-TNF) had significantly shorter villi than those with brief exposure (Figure 6(a)). To determine if chronic exposure to TNF would impact the levels of TNFR1, we measured levels of TNFR1 mRNA in tissues of P21 mice treated with B-TNF and C-TNF compared to controls. We chose P21 as a time point because at that age the chronic mice had received more than one TNF dose, but the intestine was still immature (as opposed to the P28 intestine which is considered to be matured). There was no significant difference in TNFR1 levels in either B- or C-TNF groups compared to controls (Figure 6(d)).

3.8. Chronic TNF Has Age- and Dose-Dependent Effects on Villus Surface Areas and Epithelial Cell Counts. As chronic TNF (C-TNF) caused developmentally dependent villus blunting,

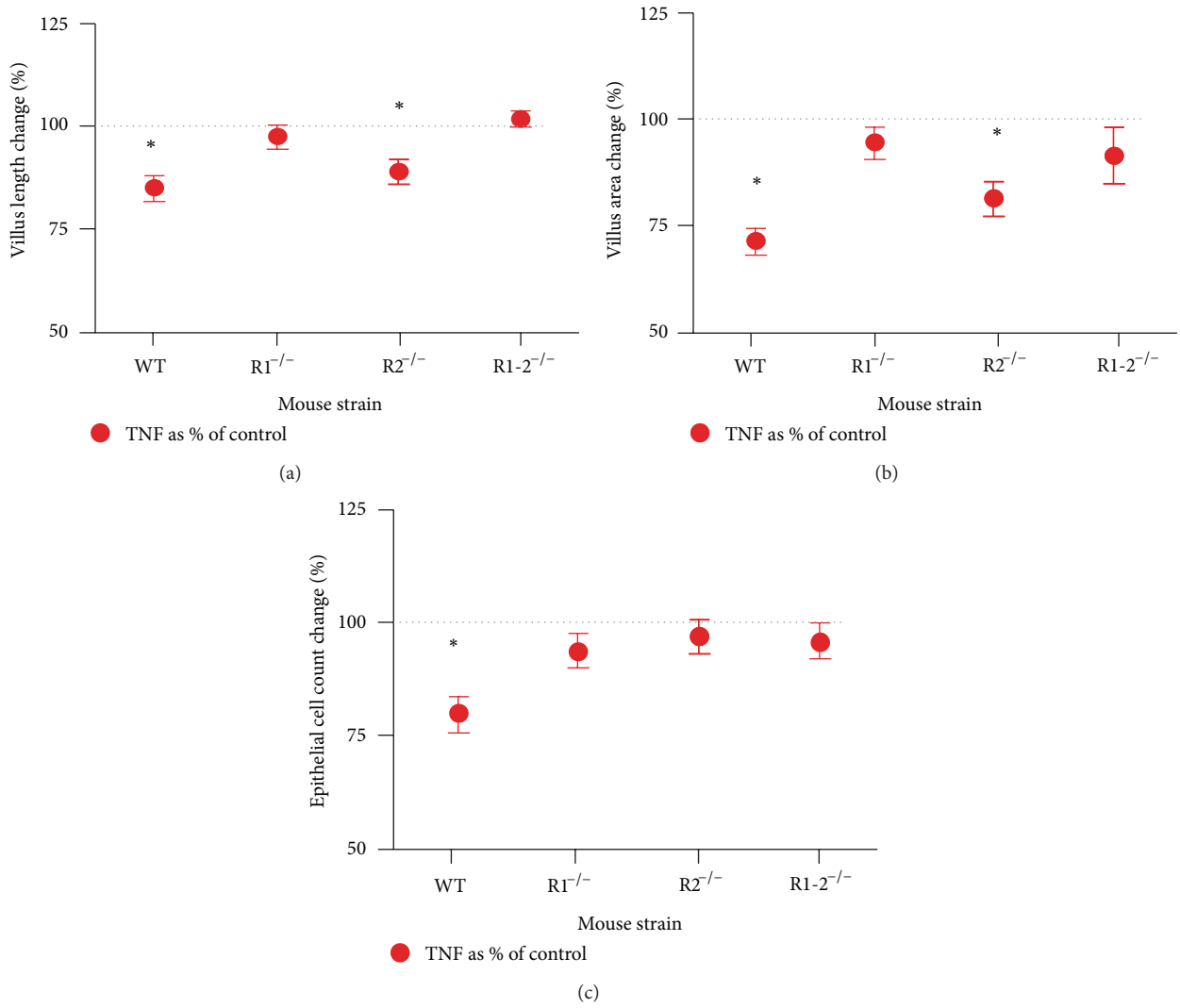

Figure 4: TNFR1 is required for TNF-induced ileal villus blunting and villus area loss. Small intestinal villus length, area, and epithelial cell counts were measured as above in mice lacking one or both TNFRs ($R1^{-/-}$, $R2^{-/-}$, or $R1\text{-}2^{-/-}$). Mice treated with TNF were measured and compared to controls of the same strain. Data are shown as the difference from controls, which are set to 100%. Significant differences ($P < 0.002$) from controls in TNF treatment are denoted by an asterisk.

we next investigated its effects on villus surface area and epithelial cell counts. While acute TNF treatment induced decreases in villus area and epithelial counts at P14 and 21 (Figure 2), brief (B-TNF) and chronic (C-TNF) treatments induced significant increases in both area and epithelial cell counts at these ages, suggesting a change in villus architecture from long thin villi to shorter, wider villi in the face of chronic inflammation (equal area with less length). Interestingly, this trend was not seen in P28 mice. Exposure to B-TNF induced a significant increase in villus area but a decrease in epithelial cell numbers, and exposure to C-TNF induced no change in villus area and a decrease in epithelial cell counts. These effects demonstrate a developmental stage-dependent effect of chronic exposure to TNF on the small intestinal architecture (Figures 6(b) and 6(c)) ($P < 0.0001$).

3.9. Acute but Not Chronic TNF Induces a TNFR1-Dependent Loss of Paneth Cell Populations. Our lab and others have described a key protective role for Paneth cells in the immature intestinal tract [8, 18–20]. TNF has been shown to be important in Paneth cell granule release in adult intestine [21, 22]; however, the effect of either acute or chronic TNF on Paneth cells from immature intestine is less well described. To examine this we treated mice with acute, brief, or chronic TNF exposures as above and quantified the number of granule containing Paneth cells in the ileum compared to controls. Mice were only examined from P14 to P28 as mature Paneth cells do not appear in mice until between P7 and P10 [23, 24]. Acute TNF induced significant loss of granulated Paneth cells in both P14 and P28 mice (Figure 7(a)) ($P = 0.002$). However, there was no difference in Paneth cell

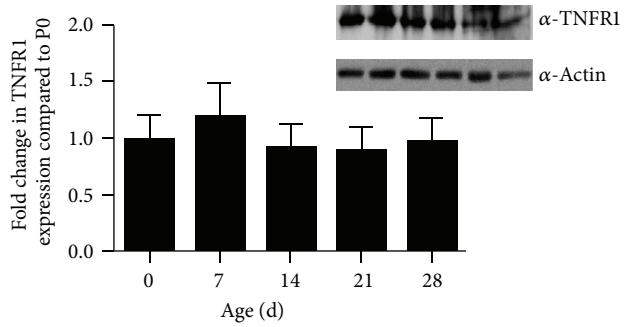

Figure 5: TNFR1 expression in small intestine is constant during development. TNFR1 protein expression levels were tested by western blot in small intestinal homogenates from P0 to P28 mice. A representative western blot is shown. The graph shows the average TNFR1 expression at noted ages ($n = 5$ animals/group; expression normalized to actin; all ages compared to P0). No significant difference was detected between any ages.

numbers in mice treated with brief or chronic treatments (Figure 7(b)). To determine if acute TNF-induced Paneth cell loss was TNFR dependent, we examined the effects of TNF on P14 mice lacking TNFR1, TNFR2, or both. TNF induced significant loss of granulated Paneth cells only in $TNFR2^{-/-}$ mice demonstrating a requirement for TNFR1 (Figure 7(c)) ($P = 0.035$).

4. Discussion

Premature infants are routinely subject to intestinal inflammation, both as a result of normal exposure to new environmental factors and through pathologic situations such as intestinal perforations and necrotizing enterocolitis. However, the ramifications of inflammation during development of the intestinal tract are not fully understood. In this study, we describe the normal growth patterns of intestinal villi. We next provide evidence that acute TNF exposure can blunt normal villus height during development. We also demonstrate that chronic TNF exposure can blunt height even more significantly. This blunting occurred through a TNFR1-mediated mechanism. Since TNFR1 expression remains constant through development of the small intestine, TNF-induced villus blunting may be dependent on downstream TNF signaling targets that are differentially expressed during development. Lastly, we show that acute TNFR1-mediated signaling also induces a significant decrease in the number of granule containing Paneth cells. Taken together, these morphologic changes caused by TNF exposure may help to explain why this cytokine plays a prominent role in the development of NEC in the immature intestine.

Our data demonstrate that both acute and chronic TNF exposures induce significant changes in the immature ileum. TNF mediates distinct physiological effects through two separate transmembrane receptors, TNFR1, and TNFR2 [25, 26]. Physiological levels of TNF result in a preferential ligation to TNFR2, which promotes cellular migration, proliferation, and wound healing [27], while higher concentrations of TNF lead to ligation of TNFR1 and subsequent activation of inflammatory responses [25]. Our lab has recently shown that TNF causes TNFR1-dependent, developmental stage-dependent changes in intestinal goblet cell secretion of mucus [13]. Similarly, we demonstrate in these experiments that TNF-induced villus blunting through a TNFR1-dependent, TNFR-2 independent mechanism. The developmental-stage dependent differential effects of TNFR1 are intriguing as protein levels of TNFR1 remain constant from birth through four weeks of life, when the ileum reaches maturation. It was also interesting to note that TNF had little effect on the most immature intestine (P0) despite having similar levels of TNFR1. Our initial hypothesis was that the most immature intestine would show the greatest effects of TNF exposure. However, TNF had no effect on villus length, villus area, or epithelial cell counts. This was similar to our data regarding TNF effects on goblet cells [13] and correlates clinically with a lack of development of NEC during extremely premature ages [28]. These data imply that either an unidentified downstream target of TNFR1 has developmental-stage dependent expression or that another developmental stage-dependent influence acts as a repressor of TNFR1 signaling. Either way, this alteration of TNFR1 signaling may be important, as intestinal inflammation is a common occurrence in premature infants.

The embryogenesis of the gastrointestinal tract is nearly identical in all mammals; however, the stage of development when birth occurs can vary dramatically between species [29]. While human infants have mature intestinal tracts at term birth, mice are born much earlier in the developmental process [29, 30] and do not reach a maturity level equivalent to term human infants until four weeks after birth [1]. Thus, the first four weeks of mouse life reasonably approximate the last half of human fetal development, the stage of development when premature infants are most susceptible to developing NEC. Thus it is important to note that villus length increased during the first four weeks of life as a function of age (Figure 1(a)).

In our experiments to study the effects of chronic inflammation on the intestine, we modeled two types of chronic TNF treatments. Brief dosing examined mice one week following a single TNF treatment, and chronic dosing examined mice one week following the last of multiple weekly TNF treatments. Our data show that both types of chronic exposure to TNF induced villus blunting at P14, P21, and P28. However, it was interesting to note that while chronic exposure to TNF induced loss of villus height at all ages, P14 and P21 mice had significantly increased villus area and epithelial cell counts with this exposure. This suggests a conformation change from thin long villi to shorter, wider villi in the face of chronic inflammation. This may be a compensation mechanism to place the villus epithelial layer closer in proximity to the lamina propria where the majority of adaptive immune cells reside. Interestingly, this trend was not seen in P28 mice, again demonstrating developmental stage-dependent effects of TNF on the small intestinal architecture.

We and others have recently reported a loss of Paneth cells in infants who developed NEC [18, 31], and we have recently developed a novel two-hit model of NEC in mice that utilizes Paneth cell loss to develop injury that is consistent

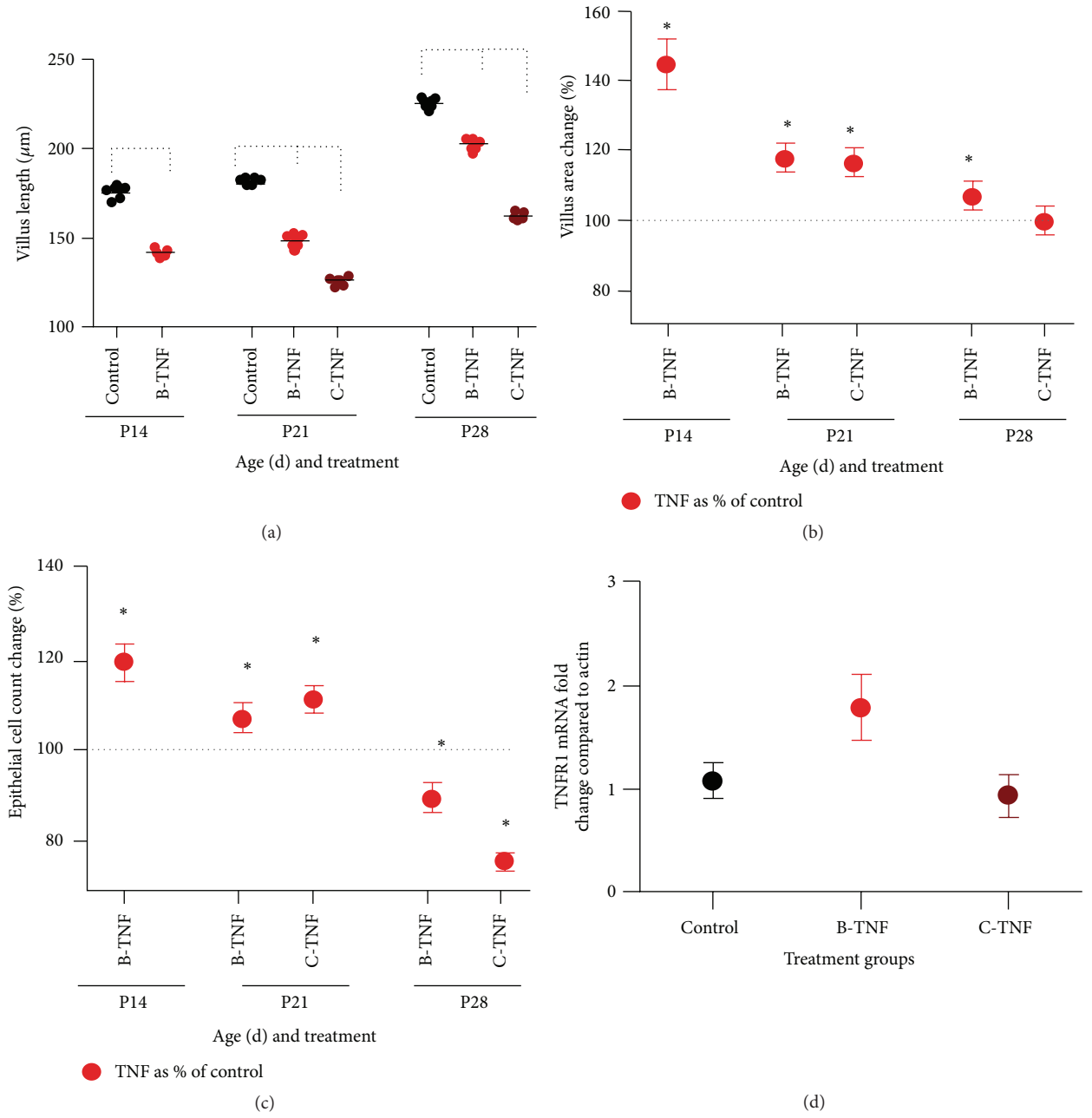

FIGURE 6: Chronic TNF treatment has exposure-dependent effects on small intestinal architecture. Mice were treated with TNF one week prior to euthanasia (brief exposure designated as B-TNF), or weekly starting on P7 until one week prior to euthanasia (chronic exposure designated as C-TNF). Small intestine was harvested and examined for (a) villus height, (b) villus area, and (c) epithelial cell counts as described above. For all groups, $n = 5$. Brackets in (a) indicate $P < 0.0001$. Asterisks in (b) and (c) indicate $P < 0.0001$ in TNF versus control. (d) To determine if chronic exposure to TNF impacts TNFR1 levels, mRNA levels of TNFR1 were quantified in tissues of P21 mice treated with B-TNF and C-TNF compared to controls. No significant differences in TNFR1 levels were observed.

with human disease [18]. In this model, significant injury only occurs following ablation of Paneth cells, suggesting that the injury to, or loss of, the Paneth cell is required to develop NEC. TNF has been shown to induce Paneth cell degranulation in adult mice [22], thus we desired to determine if TNF would have similar effects in the developing intestine. Our data show that acute TNF significantly reduces granule containing Paneth cells in both P14 and P28 mice, and that this reduction is due to a TNFR1-dependent mechanism. Interestingly, chronic exposure to TNF had no effect on the Paneth cell population, suggesting that while Paneth cells are responsive to TNF exposure, the ileum naturally compensates to preserve the Paneth cells in the face of chronic inflammation. These data correspond to additional

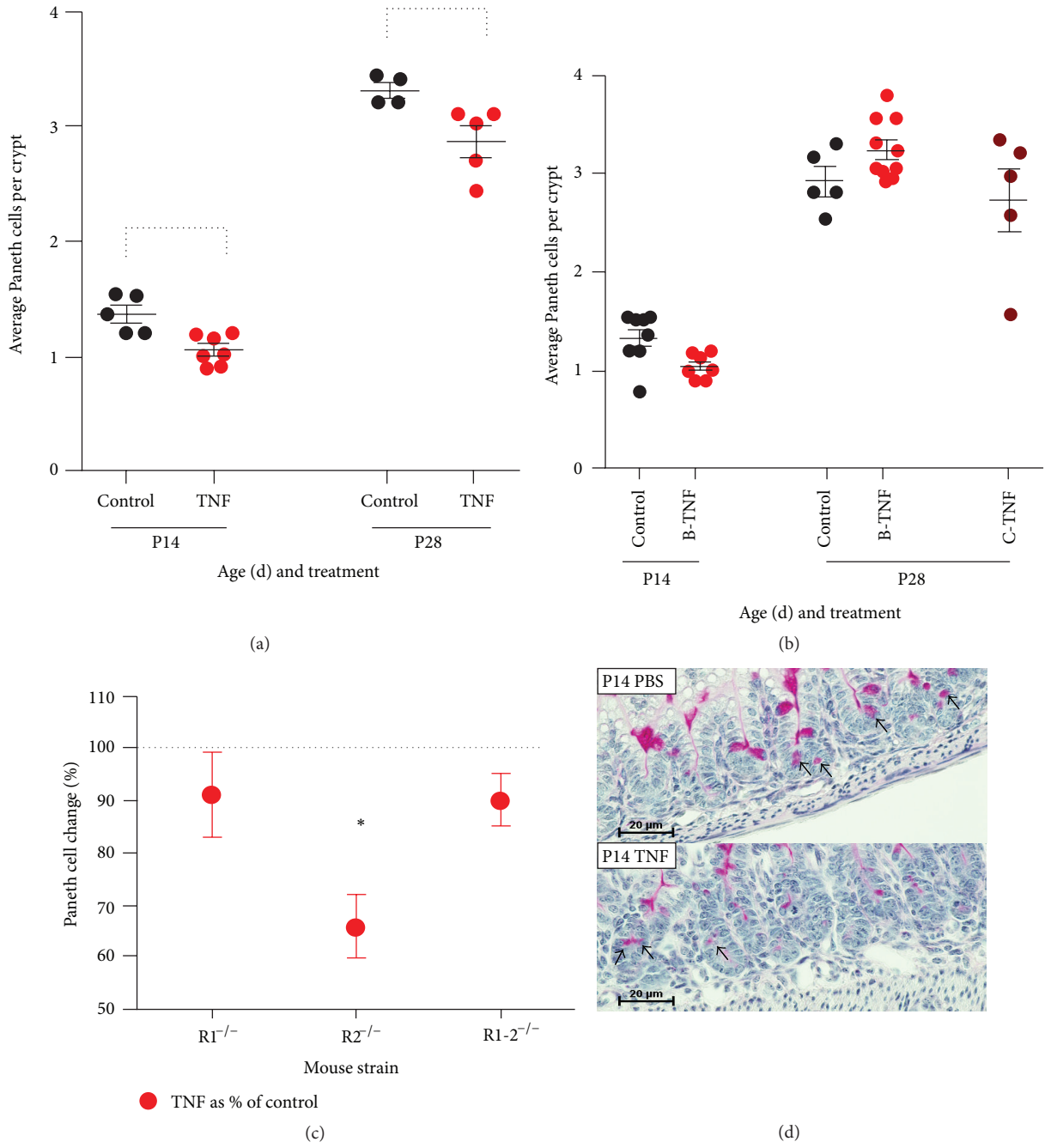

FIGURE 7: Acute TNF exposure induces loss of Paneth cells in a TNFR1-dependent manner. (a) Mice were treated with acute (injected 8 hours before collection) TNF exposure and granulated Paneth cells per crypt (100 crypts per animal) were counted histologically (n = 5 mice per condition; brackets, P = 0.002). (b) Mice were treated with brief (B-TNF; single injection with 1-week recovery) or chronic (C-TNF; weekly injections) exposure as above and granulated Paneth cells per crypt were counted; no significant changes were observed. (c) Representative tissue sections are shown. (d) To determine if acute TNF-induced Paneth cell loss was TNFR-dependent, mice lacking one or both TNFR were treated with acute exposure to TNF (n = 5, asterisk indicates P = 0.035 in $R2^{-/-}$ mice).

results from our laboratory that shows decreased expression of lysozyme, a key granular secretion of Paneth cells, in ileal enteroid cultures exposed to TNF [32].

In summary, we have shown that chronic exposure of the immature ileum to TNF induces not only functional changes but architectural changes as well. These findings may have important implications for elucidating the pathophysiology of NEC. Our recently proposed "bottom up" model of NEC hypothesizes that bacteria invade the intestinal tissue principally through the crypts, rather than through the villus tips as previously thought [8]. In this model, TNF-induced blunting caused by feeding-induced

or other chronic inflammation could subsequently decrease the distance between the luminal contents of the intestine and the lamina propria. This shortening of distance would greatly increase the ability of bacteria to reach the crypt and infiltrate the intestine. This, along with other effects of TNF such as depletion of the mucous layer and degranulation of Paneth cells, may allow for easier bacterial penetration into the intestinal lamina propria, leading to the inflammatory response and coagulation necrosis characteristic of NEC.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Research Article

The Possible Role of the Novel Cytokines IL-35 and IL-37 in Inflammatory Bowel Disease

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Interleukin- (IL-) 35 and IL-37 are newly discovered immune-suppressing cytokines. They have been described in inflammatory diseases such as collagen-induced arthritis and asthma. However, their expressions in inflammatory bowel disease (IBD) patients have not been yet explored. Our aim was to evaluate serum and inflamed mucosal levels in IBD patients. In 20 ulcerative colitis (UC) patients, 7 Crohn's disease (CD) patients, and 15 healthy subjects, cytokine levels in serum were determined using ELISA and mucosal expression studies were performed by immunohistochemistry, quantitative real-time PCR, and Western blot. The results showed that serums IL-35 and IL-37 levels were significantly decreased in UC and CD patients compared with healthy subjects. The cytokines levels correlated inversely with UC activity. IL-35 was expressed in infiltrating immune cells while IL-37 in intestinal epithelial cells as well as inflammatory cells. IBD patients had significantly higher *Ebi3*, *p35* (two subunits of IL-35), and *IL-37b* gene expressions; IL-35 and IL-37 protein expressions were higher in IBD patients compared with controls. The study showed that serums IL-35 and IL-37 might be potentially novel biomarkers for IBD. Intestinal IL-35 and IL-37 proteins are upregulated, suggesting that regulating the expression of the two cytokines may provide a new possible target for the treatment of IBD.

1. Introduction

Inflammatory bowel disease (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), is a kind of chronic inflammatory disorder of the gastrointestinal tract. Although the etiology is not completely understood, initiation and exacerbation of the inflammatory process seem to be due to a massive local mucosal immune response [1]. Analysis of immunoinflammatory pathways in the gut of patients with UC or CD has shown that tissue damage is driven by a complex and dynamic crosstalk between immune and nonimmune cells and that cytokines are key mediators of this interplay [2, 3].

Interleukin- (IL-) 35 and IL-37 are newly discovered immune-suppressing cytokines. IL-35 belongs to the IL-12 family, which contains IL-12, IL-23, and IL-27. It is composed of two subunits, Epstein-Barr virus-induced gene 3 (Ebi3) and p35 (IL-12a) [4]. IL-35 was shown to be secreted by Foxp3+CD4+CD25+ regulatory T cells (Tregs) in mice or a regulatory T cell population induced by IL-35 [5] and CD138+ plasma cells in experimental autoimmune encephalomyelitis (EAE) [6]. Using experimental database mining and statistical analysis methods, Li et al. reported that IL-35 is not constitutively expressed in human tissues but it is inducible in response to inflammatory stimuli [7]. IL-37, also known as IL-1F7, is a new member of the IL-1 family, which shares common characteristic symbolized by a similar β-barrel structure. IL-37b is the largest isoform of the five variants and is expressed in a variety of normal tissues and tumors in humans [8]. It was first found in bone marrow, and neutrophils were the main place for its synthesis. It is mainly expressed in blood cells, respiratory tract, gastrointestinal tract, and skin keratinocytes [9].

To investigate the possible role of IL-35 and IL-37 in the inflammatory process of IBD, we aim to evaluate serum and mucosal levels in IBD patients. To the best of our knowledge, this is the first study that explores expression through a quantitative real-time polymerase chain reaction (qRT-PCR), immunohistochemistry, and Western blot of IL-35 and IL-37 in inflamed colonic mucosa of IBD patients.

2. Materials and Methods

2.1. Subjects. A total of 27 patients with definitive diagnosis of IBD were recruited at Shandong University Qilu Hospital. 20 UC and 7 CD patients were included during the period from September 2013 to April 2014. Diagnosis was performed by the presence of history of abdomen pain, diarrhea, or blood in stool and macroscopic appearance by colonoscopy or double balloon endoscopy and biopsy compatible with IBD. The following were relevant medical records: gender, age at diagnosis, disease evolution, extension, extraintestinal manifestations, medical treatment, and clinical course of disease. UC activity was assessed by Mayo score activity index [10] and CD activity was assessed by Crohn's disease activity index (CDAI) [11]. Blood was drawn for the measurement of hemoglobin, hematocrit, and erythrocyte sedimentation rate (ESR). Additionally, 15 noninflamed controls (median age, 48 yr; 9 males/6 females) were recruited among healthy subjects undergoing a colonoscopy because of screening for colorectal cancer or polyp surveillance. These subjects were free from gastrointestinal symptoms and other inflammatory diseases. Only subjects with both macroscopically and microscopically normal colonoscopy were included. None of the healthy subjects in the study was taking any medications known to affect the gastrointestinal tract or the immune system.

2.2. Samples Collection. A fasting blood sample was taken from all patients and healthy subjects. It was centrifuged at 1500 ×g for 20 min at room temperature and the serum was collected and stored at −80°C until analysis. During endoscopy, biopsies from colon or ileocecum or small intestine were obtained from patients and healthy subjects. Two biopsies to be used for RNA and protein assessment were snap-frozen in liquid nitrogen and then transferred to −80°C for storage until processing. One biopsy was placed in formalin for pathology and immunohistochemical staining. The study was performed after receiving written informed consent from all study subjects, and the protocol was approved by the Regional Ethical Review Board at Qilu Hospital, Shandong University.

2.3. Enzyme-Linked Immunosorbent Assay. Serums IL-35 and IL-37 were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Bio-Swamp). All cytokines assays were performed in duplicate and in accordance with the manufacturers' protocols.

2.4. Immunohistochemistry. Formalin-fixed and paraffin-embedded 4 μm thick tissue slices were dewaxed and rehydrated before antigen retrieval. Microwave antigen retrieval method was then preformed with the slides immersed in EDTA antigen retrieval solution (ph 9.0) for 15 minutes. After that, 3% hydrogen peroxide (H_2O_2) was added on the slides to inhibit the endogenous peroxidase activity. Nonspecific binding was blocked by incubation with 10% normal goat serum in 37∘C, pH7.5, for 30 min. Subsequently, mouse anti-human IL-35 monoclonal antibody (Imgenex, USA) at a 1 : 200 dilution and mouse anti-human IL-37 monoclonal antibody (Abcam, USA) at a 1 : 250 dilution were applied, respectively, to the sections that were latter incubated at 4°C overnight. On the second day, biotinylated antibody and streptavidin-peroxidase reagent (Zhongshan Biotech, China) were successively applied for 30 min each at 37°C. Finally, 3′-diaminobenzidine tetrahydrochloride (DAB) was used for visualization and hematoxylin was added to counterstain. Samples were viewed with Olympus IX81 microscope and images were produced using DP Controller 1.2.1.108. All of the slides were independently analyzed by two pathologists.

2.5. RNA Isolation and Quantitative Real-Time PCR. RNA was extracted using Trizol Reagent (Takara, Japan), following the manufacturer's guidelines. First-strand cDNA was synthesized by Realtime PCR Master Mix Kit (TOYOBO, Japan) in a volume of 10 μL. For quantitative real-time PCR (qRT-PCR), the LightCycler 4.0 instrument (Roche Applied Science, Germany) and the SYBR Green Realtime PCR Master Mix Kit (TOYOBO, Japan) were used according to the protocol provided by the manufacturer. The following primers were used: *Ebi3*: forward 5′-GCA GCA GAC GCC AAC GT-3′, reverse 5′-CCA TGG AGA ACA GCT GGA CAT-3′; *p35*: forward 5′-CCT TCA CCA CTC CCA AAA C-3′, reverse 5′-TGT CTG GCC TTC TGG AGC AT-3′. *IL-37*: forward 5′-GCT CAG GTG GGC TCC TGG AA-3′, reverse 5′-GCT GAC CTC ACT GGG GCT CA-3′. Human glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as internal control from parallel samples because the reference gene was stably expressed, and its primers were forward: 5′-GGT GGT CTC CTC TGA CTT CAA CAG-3′ and reverse: 5′-GTT GTT GTA GCC AAA TTC GTT GT-3′. Melting curve analysis was used to confirm amplification specificity. The quantification data were analyzed with LightCycler analysis software version 4.0 (Roche Applied Science, Germany) and the relative target gene expression was normalized on the basis of *GAPDH*. Results were expressed as an x-fold difference relative to the calibrator.

2.6. Western Blot Analysis. Frozen tissue samples were lysed in RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium, deoxycholate, 0.1% SDS; Beyotime, China) and PMSF (Beyotime, China) followed by centrifugation (12,000 rpm, 4°C, 20 minutes), after which the supernatants were stored at −80°C until use. The protein concentrations of the lysates were determined using an enhanced BCA Protein Assay Kit (Beyotime, China). Extracted proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Samples transferred onto PVDF membranes were treated with a 1 : 200 dilution of mouse anti-human IL-35 monoclonal antibody (Imgenex, USA) and anti-human IL-37 monoclonal antibody (Abcam, USA) followed by a 1 : 5,000 dilution of sheep anti-mouse horseradish peroxidase-conjugated secondary antibodies (Zhongshan Biotech, China). As a control, rabbit anti-human GAPDH polyclonal antibody (Proteintech, USA) and peroxidase-conjugated anti-rabbit IgG (H+L) (Proteintech,

Table 1: Demographic and clinical characteristics of IBD patients.

Patients number	Sex	Age at diagnosis (years)	Disease duration (months)	Disease extent	Disease activity	Current therapy
					Mayo score	
UC1	F	46	4	E2	7	Mesalazine
UC2	M	29	70	E3	12	Mesalazine, corticosteroids
UC3	M	25	2	E3	9	Mesalazine, corticosteroids
UC4	F	51	10	E3	11	Mesalazine, corticosteroids
UC5	M	57	12	E1	5	Mesalazine,
UC6	M	34	6	E2	6	Mesalazine,
UC7	F	28	168	E2	3	Mesalazine,
UC8	M	34	3	E3	11	Mesalazine, corticosteroids
UC9	F	28	40	E2	11	Mesalazine, corticosteroids, and AZA
UC10	M	59	7	E3	11	Mesalazine, corticosteroids
UC11	M	24	1	E3	8	Mesalazine, corticosteroids
UC12	F	27	1	E1	4	Mesalazine, enema
UC13	M	27	1	E1	3	Mesalazine
UC14	M	58	2	E2	12	Mesalazine, corticosteroids
UC15	M	65	2	E2	5	Mesalazine
UC16	F	15	2	E2	11	Mesalazine, corticosteroids
UC17	M	22	12	E3	6	Mesalazine
UC18	M	37	1	E3	3	Mesalazine
UC19	F	63	1	E3	8	Mesalazine
UC20	F	28	5	E3	7	Mesalazine, enema
Average (SEM)		**37.85 (3.48)**	**17.50 (8.76)**		**7.65 (0.72)**	
					CDAI score	
CD1	M	60	2	L1	7	Mesalazine, corticosteroids, and AZA
CD2	M	39	180	L1	6	Mesalazine, AZA
CD3	M	28	5	L2	8	Mesalazine, corticosteroids
CD4	F	26	1	L3	9	Mesalazine, corticosteroids
CD5	M	49	60	L4	9	Mesalazine, corticosteroids
CD6	M	50	3	L3	6	Mesalazine
CD7	M	22	6	L1	7	Mesalazine, corticosteroids
Average (SEM)		**39.15 (5.44)**	**36.71 (25.18)**		**7.43 (0.48)**	

UC: ulcerative colitis; CD: Crohn's disease; E1: proctitis; E2: leftsided colitis; E3: pancolitis; L1: ileal; L2: colonic; L3: ileocolonic; L4: upper GI involvement; AZA: azathioprine.

USA) were used to normalize. Immunoreactivity was visualized with the Immobilon Western Chemiluminescent HRP Substrate (Millipore, USA) and quantified using Image J software (http://rsbweb.nih.gov/ij/index.html).

2.7. Statistical Analysis. Statistical evaluations were performed with GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA) and SPSS19.0 (SPSS Inc., Chicago, IL, USA). Data were expressed as mean ± standard error (SEM). The Mann-Whitney test was used to evaluate differences of cytokine expression in the serum. One-way analysis of variance, followed by post hoc *t* tests with Newman-Keuls test for multiple comparisons, was used to compare the 3 groups. Pearson correlation was used to calculate correlations between serum cytokines levels and Mayo score. One-way analysis of variance, followed by post hoc *t* tests with Tukey correction for multiple comparisons, was used to compare the 3 groups in differences of mucosal expression. In all tests, a P value < 0.05 was considered significant.

3. Results

3.1. Demographic and Clinical Characteristics of IBD Patients. They are described in Table 1. The Montreal classification was used to define the extent of UC: 50% had pancolitis (E3), 35% had left-sided colitis (E2), and 15% had proctitis (E1). According to Mayo score, six UC patients (30%) showed mild activity, seven (35%) moderate activity, and seven (35%) severe activity. Six UC patients (20%) had extraintestinal manifestations, including arthropathy (15%), primary sclerosing cholangitis (10%), and erythema nodosum (5%). All patients were under mesalazine treatment; 45% used oral or

Table 2: (a) Serum cytokines levels in each group. (b) Serum cytokines levels in UC groups.

(a)

	UC ($n = 20$)	CD ($n = 7$)	HC ($n = 15$)
Il-35 level (ng/mL)	$203.36 \pm 38.21^{**}$	$454.17 \pm 219.38^{**}$	1788.96 ± 209.43
Il-37 level (ng/mL)	$199.28 \pm 38.60^{**}$	$481.67 \pm 232.82^{**}$	2275.68 ± 261.24

(b)

	Mild UC (Mayo 3~5) ($n = 6$)	Moderate UC (Mayo 6~10) ($n = 7$)	Severe UC (Mayo 11~12) ($n = 7$)
Il-35 level (ng/mL)	$358.26 \pm 103.95^{*\#}$	157.29 ± 15.89	116.69 ± 14.48
Il-37 level (ng/mL)	$346.97 \pm 105.83^{*\#}$	154.21 ± 24.95	117.75 ± 17.14

$^{**}P < 0.001$ versus HC; $^{*}P < 0.05$ versus mild UC; $^{\#}P < 0.05$ versus severe UC.

systemic glucocorticosteroids; 5% were taking azathioprine. CD patients were few. Five were moderate activity and two were severe activity. Two patients had arthropathy.

3.2. Serums IL-35 and IL-37 Levels Are Decreased in IBD Patients. Serums IL-35 and IL-37 concentrations were significantly reduced in the active UC patients and active CD patients compared with healthy controls (HC) (Table 2(a)). There were also significant differences between mild UC and moderate UC and mild UC and severe UC ($P < 0.05$) (Table 2(b)). In contrast, UC and CD group and moderate and mild UC group seem to be statistically meaningless. We also assessed whether the serum cytokine levels were associated with the Mayo score in UC patients. The results showed that lower IL-35 and IL-37 levels were moderately negatively correlated Mayo score. ($R = -0.636$, $P < 0.05$; $R = -0.625$, $P < 0.05$, resp.) (Figures 1(a) and 1(b)).

3.3. IL-35 Expression in Colonic Mucosa from IBD Patients. In order to determine gene and protein expressions in UC and CD patients, mRNA relative expressions of *Ebi3* and *p35* and protein of IL-35 were quantified by qRT-PCR and Western blot analysis. IL-35 producing cells were determined by immunohistochemistry. As shown in the representative images of this analysis in Figures 2(a)–2(d), IL-35 was expressed in infiltrating immune cells but not in epithelial cells. Normal colon tissue from healthy subjects had no IL-35 expression at all (Figures 2(e) and 2(f)). IL-35 positive cells were localized mainly in inflammatory infiltrates, predominantly mononuclear cell (lymphocytes). The number of IL-35-expressing cells in inflamed colonic tissue of patients with UC (Figures 2(a) and 2(b)) was higher than that in CD patients' tissue (Figures 2(c) and 2(d)). IBD patients had significantly higher *Ebi3* and *p35* gene expression compared with healthy control group ($P < 0.0001$) (Figure 3). And the difference between UC and CD biopsies in *Ebi3* and *p35* mRNA expressions did not reach statistical significance ($P = 0.116$; $P = 0.779$). Western blot analysis showed a significant upregulation of IL-35 ($P < 0.05$) in inflamed mucosa of patients as compared to controls (Figure 4). IL-35 protein expression in UC biopsies was found higher than that in CD biopsies. No relationship between IL-35 mucosal expression and the activity was observed in UC and CD patients.

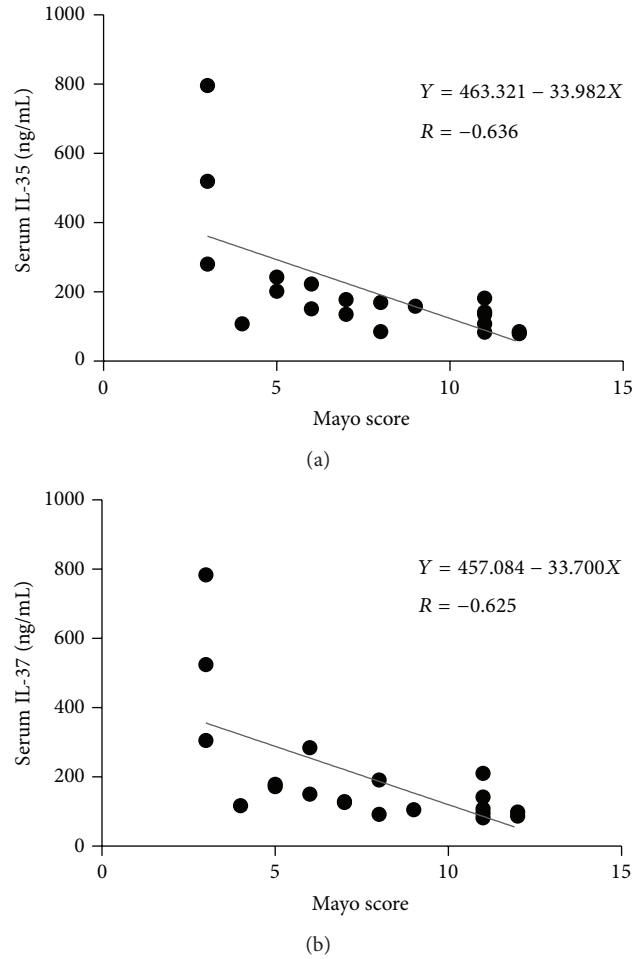

Figure 1: There was an intermediate inverse correlation between the Mayo score and serum IL-35 levels (a). IL-37 levels were moderately negatively correlated with Mayo score (b).

3.4. IL-37 Expression in Colonic Mucosa from IBD Patients. Immunohistochemistry showed that both immune and epithelial cells could express IL-37 (Figures 5(a)–5(d)), and normal tissue had IL-37 expression, though with relatively small amount (Figures 5(e) and 5(f)). Besides strong cytoplasmic staining, few single lamina propria mononuclear cells show nuclear expression of IL-37. Similarly, compared with

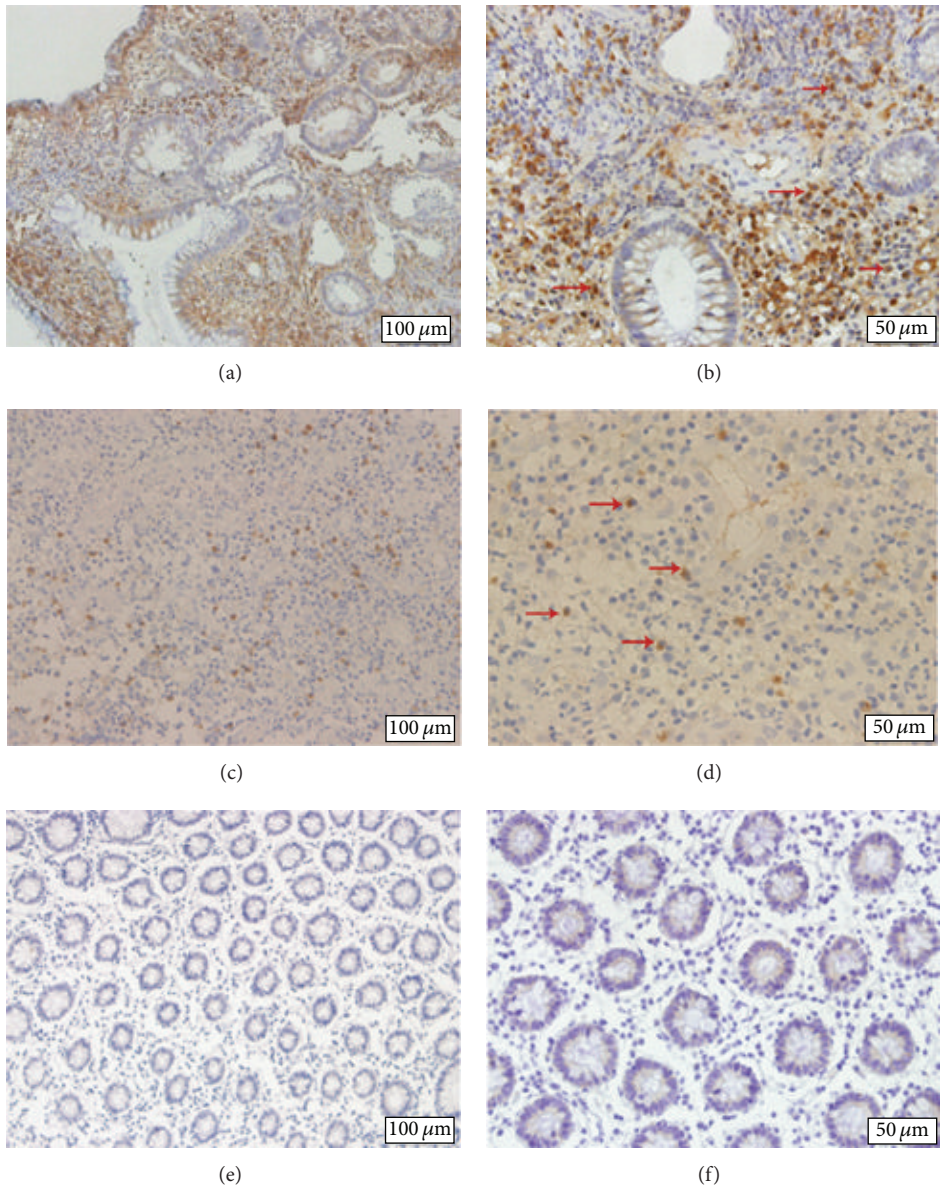

Figure 2: Photomicrographs of immunostaining for IL-35 in human colon from patients with UC (a and b), patients with CD (c and d), and healthy controls (e and f). No staining was found in the control group (e and f), and IL-35 was expressed in infiltrating immune cells (morphologically resembling lymphocytes) but not in epithelial cells. The staining was mostly cytoplasmic, and red arrows depicted immunoreactive positive cells. The number of IL-35-expressing cells in inflamed colonic tissue of patients with UC was higher than in CD patients' tissue. Original magnification: (a), (c), (e) ×200; (b), (d), (f) ×400.

control group, IBD group had higher *IL-37* mRNA expression ($P < 0.001$), whereas difference between UC and CD was still statistically significant ($P < 0.001$) (Figure 6). For IL-37, protein expression trend runs like IL-35. Western blot showed that IL-37 protein expression was found to be higher in UC patients and CD patients compared to healthy subjects and the expression levels of IL-37 protein were higher in the samples of UC patients than that of CD samples ($P < 0.001$) (Figure 7). The mean IL-37 expression tended to be higher in severe UC samples than in mild UC samples but was not statistically significant.

4. Discussion

In this study, we first demonstrated that serums IL-35 and IL-37 levels were significantly lower in active IBD patients than healthy controls and were moderately negatively correlated with Mayo score in UC patients. Takahashi et al. reported that the Treg cell frequency decreased in the active stage of UC and it correlated inversely with the disease activity [12]. Their results suggested that a deficiency of Treg cells was associated with the progression of ulcerative colitis. Treg cells are the main source of IL-35, which may result in the reduction

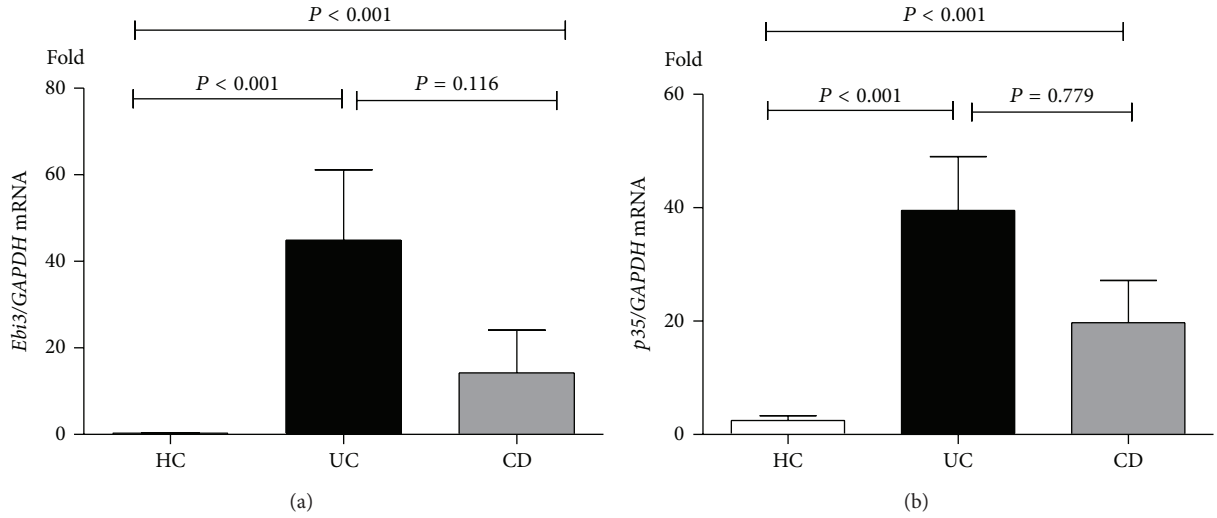

Figure 3: Detection of *Ebi3* and *p35* mRNA by qRT-PCR. *Ebi3* (a) and *p35* (b) were significantly overexpressed in endoscopic specimens in both UC patients and CD patients as compared to controls ($P < 0.001$). *Ebi3* and *p35* mRNA expression in UC and CD biopsies did not reach statistical significance.

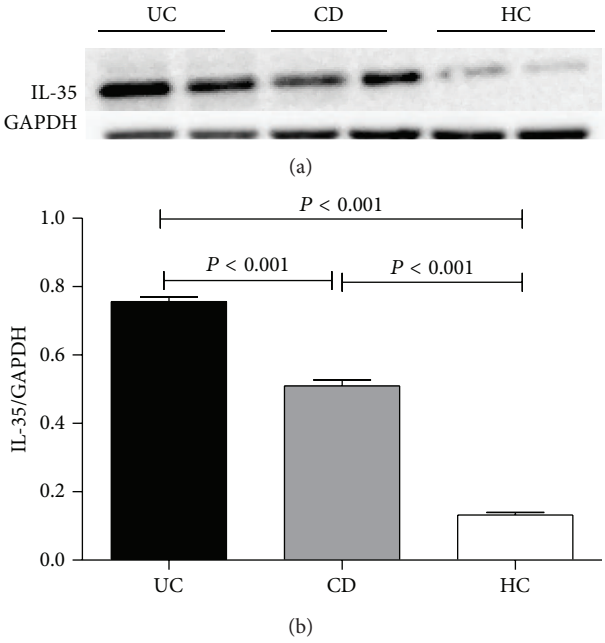

Figure 4: Detection of IL-35 in protein extracts of endoscopic specimens by Western blot. Representative Western blot of IL-35 and GAPDH (a) proteins levels in UC and CD patients and healthy controls. A significant upregulation of IL-35 was observed in inflamed mucosa of patients as compared to controls ($P < 0.001$) and IL-35 protein expression in UC biopsies was higher than that in CD biopsies (b).

of IL-35 in peripheral blood. Through immunocytochemical staining, IL-37 protein is present mainly in the cytoplasm of peripheral blood mononuclear cells (PBMC) and constitutively at low levels in normal people and can be upregulated by inflammatory stimuli and cytokines [13]. IL-37 is expected to have the function of translocation into nucleus [14] and can be redistributed between intracellular and extracellular. Perhaps IL-37 transferring to intracellular is the reason why the content is down in serum. The study shows that decreased serums IL-35 and IL-37 levels may represent insufficient anti-inflammatory activity in vivo and hold promise as novel biomarkers for monitoring disease activity in UC.

We have characterized the mucosal expressions of IL-35 and IL-37 in patients with IBD. The overexpression and enhanced production of IL-35 and IL-37 in colonic mucosa may play a role in the inflammatory process of IBD. Treg cells are highly infiltrated in the lamina propria (LP) of inflamed areas of UC colon compared to normal colon [15]. Treg cells induced the generation of induced regulatory T 35 cells (iTR 35 cells) in an IL-35- and IL-10-dependent manner in vitro and induced their generation in vivo under inflammatory conditions in intestines infected with *Trichuris muris*. So we think iTR 35 cells are increased to produce more IL-35 to inhibit effective T cell (Teff cell) proliferation and suppress Th17 development. Maybe the IL-35-producing B cells also participate the production of IL-35, for Shen et al. suggested that, during experimental autoimmune encephalomyelitis (EAE), CD138 (+) plasma cells were also the main source of B-cell-derived IL-35 and IL-10 [6]. In the gut, constitutive epithelial expression of anti-inflammatory immune mediators like IL-37 might be mandatory to maintain the homeostasis of the local immune response against commensal bacteria. The protein is induced by toll-like receptor (TLR) agonists in monocytes and is expressed in tissues from patients with inflammatory diseases [16, 17]. And the production of IL-37 by epithelial cells, neutrophils, and monocytes can form a positive feedback to promote more. We speculate that the increased IL-35 and IL-37, which are delivered by infiltrating immune cells, counteract mucosal inflammation in IBD. The UC group possessed the highest expression of IL-35 and IL-37 in colonic

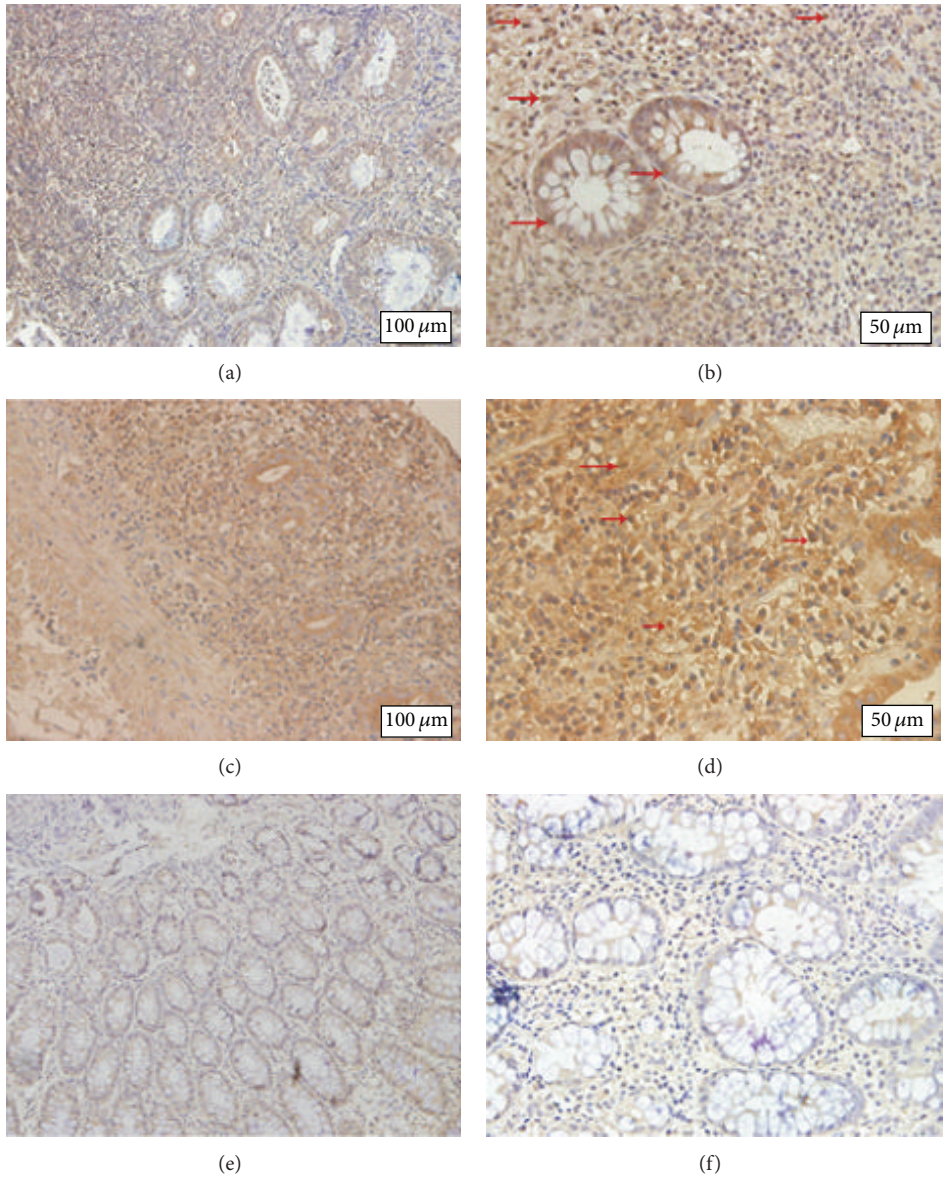

Figure 5: Photomicrographs of immunostaining for IL-37 in patients with UC (a and b), patients with CD (c and d), and control subjects (e and f). IL-37 was expressed by both immune and epithelial cells (a–d), and normal tissue had IL-37 expression, though with relatively small amount (e and f). Besides strong cytoplasmic staining, few single lamina propria mononuclear cells showed nuclear expression of IL-37.

tissue, followed by CD group, and HC group expressed the lowest. The lower anti-inflammatory cytokines in CD may explain why CD is a more chronic and continued disease. However, there was no relationship between IL-35 and IL-37 mucosal expressions and the activity in UC or CD patients. It is possible that, at the beginning of inflammatory disease, a large number of the immune-suppressing cytokines were stimulated to produce to limit inflammation in the affected colon. Despite their increased frequency and potent suppressor activity in vitro, they fail to reverse the disease process. Unlike *IL-37*, the mRNA levels of *Ebi3* and *p35* did not show differences between UC and CD. We should consider the fact that IL-27 and IL-35 shared the β-chain Ebi3 whereas IL-12 and IL-35 shared the p35 a-chain.

The mechanisms of IL-35 and IL-37 are not clear until now. IL-35 is involved in inflammatory diseases in the nervous system, alimentary system, bone and joint system, and respiratory system. Zandian et al. demonstrated that IL-35 had an inhibitory effect against demyelination by preventing the development of autoaggressive T cells [18]. Kochetkova et al. suggested that exogenous IL-35 could suppress the activity of CD4+T cells, and Th1 and Th17 cells and inhibit the inflammation of collagen-induced arthritis [19]. Meanwhile, it was indicated that IL-35 could help the respiratory system recover from inflammation [20]. Wirtz et al. recently confirmed that IL-35 could significantly suppress Th1 and Th17 cells' proliferation, reduce the development of experimental colitis, and protect the intestine from immune

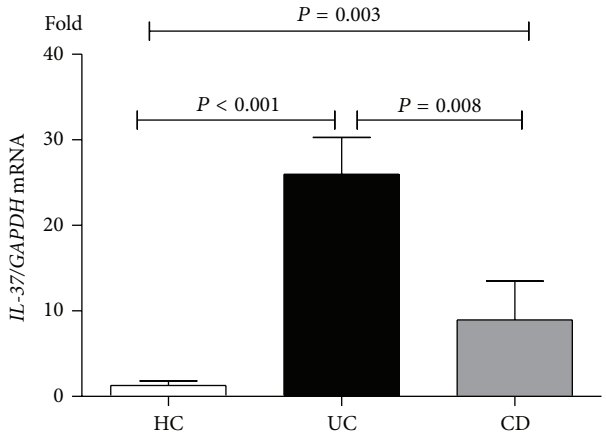

Figure 6: *IL-37* gene expression in colonic mucosa from IBD patients. A significant increase in *IL-37* mRNA was shown in inflamed mucosa of patients as compared to controls.

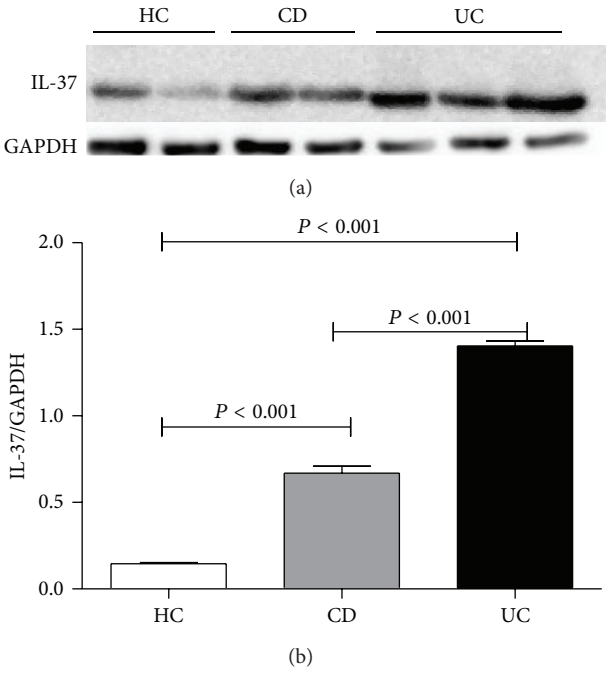

Figure 7: Detection of IL-37 in protein extracts of endoscopic specimens by Western blot. Representative Western blot of IL-37 and GAPDH (a) proteins levels in UC and CD patients and healthy controls. IL-37 protein expression was found to be higher in UC patients and CD patients compared to healthy subjects and the expression levels of IL-37 protein were higher in the samples of UC patients than that of CD samples (b).

responses in mice [21]. One subunit of IL-35, Ebi3, is widely expressed in EBV-transformed B-lymphocytes and tissues, such as tonsil and spleen [22]. Ebi3 could negatively regulate IL-17, IL-22, and Th17 transcription factor RORγt and exert protective immunity against inflammation [23]. The subunit of IL-12, p35, could lead to the progression of Herpes stromal keratitis (HSK) in mice, which is IL-12p40 independent [24]. The two subunits of IL-35 do have their own ability to regulate immunity and the process of inflammation. When they combine together to form the heterodimer, the p35 subunit may act as a ligand, and the other subunit EBI3 may mainly exert its immunological function [25]. So far, the signaling pathway of IL-35 is not clear yet. Meanwhile, the research confirmed that IL-35 signaled through a unique heterodimer of receptor chains IL12Rβ2 and gp130 or homodimers of each chain [26]. Signaling through the IL-35 receptor required the transcription factors STAT1 and STAT4, which formed a unique heterodimer that bounded to distinct sites in the promoters of the genes encoding the IL-12 subunits p35 and Ebi3. IL-35 can directly suppress Teff cell proliferation, convert naive T cells into IL-35-producing iTr35 cells, suppress Th17 development, and mediate IL-10 generation. Similarly, IL-37 is a cytokine for inflammation, autoimmunity, and other immunological disorders. The IL-37 protein is highly expressed in synovial cells of patients with rheumatoid arthritis but expressed at low levels in healthy human synovial cells [5, 27]. IL-37 expression was also significantly increased in the skin lesions of patients with psoriasis and in macrophages of Crohn's disease lesions [28]. IL-37 is synthesized as a proprotein which, after stimulation, is processed to its mature form [28]. Lipopolysaccharide (LPS), together with other inflammatory stimuli and cytokines, activates caspase-1 and is considered to be the major cleaving enzyme responsible for maturation of IL-1 family precursors [16]. With broad-spectrum function in antibacterial, antiviral, neutralizing endotoxins and antitumor and immune regulation, IL-37 can kill the microorganism in general. And its mechanism is mainly by changing the permeability of bacterial cells. It also has the ability to raise the production of several cytokines such as IL-8 to expand acquired immune function [29]. Studies of mouse models have reached the result that IL-37 downregulates inflammation [3]. TLR, tumour necrosis factor (TNF), and other cytokines can induce the production of inflammatory cytokines. Nold et al. reported that IL-37 attenuated the abovementioned process, thus exerting anti-inflammatory effects [27]. Besides, Liu et al. proved that IL-37 exerted a significant inhibition on TNF-α-induced IP-10 expression [30]. In the inflamed mucosa of IBD patients, T cell activation, as well as dendritic cells (DCs) activation, can be inhibited by epithelial cell-derived IL-37. The possible mechanism may be that IL-37 reduces the surface expressions of the costimulatory molecule CD86 (B7-2) and major histocompatibility complex (MHC) II on DCs. IL-37b mRNA expression induced by TNF-α was mediated by the activation of MAPK and PI3K and transcription factors NF-kB and AP-1. Conversely, these signalling molecules are major mediators of the induction of proinflammatory responses in the inflamed mucosa. Thus, it became clear that in the inflamed mucosa of IBD patients a negative feedback inhibitor of inflammatory responses (the induction of IL37b) and proinflammatory responses was induced via coupled signalling pathways [31].

5. Conclusion

IL-35 and IL-37 are brand new potential therapeutic cytokines for IBD. Our experiment group (namely, UC group

and CD group) includes 27 cases, and large-scale testing needs to be performed. Thus, further mechanism studies on the roles of IL-35 and IL-37 should be performed to make it available and useful in the future.

Abbreviations

IL-35: Interleukin 35
IL-37: Interleukin 37
CD: Crohn's disease
UC: Ulcerative colitis
IBD: Inflammatory bowel disease
PBMC: Peripheral blood mononuclear cells
qRT-PCR: Quantitative real-time polymerase-chain reaction
iTR35: Induced regulatory 35 cell
Teff: Effective T cell
TLR: Toll-like receptor
DCs: Dendritic cells.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Yanmei Li and Yanan Wang equally contributed to the paper.

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Research Article

Intestinal Mucosal Barrier Is Injured by BMP2/4 via Activation of NF-κB Signals after Ischemic Reperfusion

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Intestinal ischemic reperfusion (I/R) can cause dysfunction of the intestinal mucosal barrier; however, the mechanism of the intestinal mucosal barrier dysfunction caused by I/R remains unclear. In this study, using intestinal epithelial cells under anaerobic cultivation and an in vivo rat intestinal I/R model, we found that hypoxia and I/R increased the expression of BMP2/4 and upregulated BMP type Ia receptor and BMP type II receptor expression. We also found that exogenous BMP2/4 can activate the ERK and AKT signaling pathways in rat small intestine (IEC-6) cells, thereby activating NF-κB signaling, which leads to increased levels of inflammatory factors, such as TNF-α and IL-6. Furthermore, recombinant BMP2/4 decreased the expression of the tight junction protein occludin via the activation of the NF-κB pathway; these effects were abolished by treatment with the BMP-specific antagonist noggin or the NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC). All these factors can destroy the intestinal mucosal barrier, thereby leading to weaker barrier function. On the basis of these data, we conclude that BMP2/4 may act as the pathogenic basis for intestinal mucosal barrier dysfunction when the intestines suffer an I/R injury. Our results provide background for the development pharmacologic interventions in the management of I/R injury.

1. Introduction

Intestinal ischemic reperfusion (I/R) injury is a common pathophysiological process and can be caused by major vascular surgery, mesenteric artery occlusion, small bowel transplantation, cardiopulmonary bypass, hemodialysis, strangulated hernias, trauma, and shock [1, 2]. Acute intestinal ischemia is a life-threatening vascular emergency; however, the improvement in recent years has been minimal, and its in-hospital mortality rate remains high at approximately 60%–80% [3, 4]. Although many studies have focused on I/R, the mechanisms have not been fully elucidated. Many cytokines, such as TNF-α, IL-6, IL-1, and ICAM-1, have been reported to be involved in the process of intestinal I/R injury, and their expression can be regulated by nuclear factor-kappa B (NF-κB) [5–7]. These cytokines may increase capillary permeability and damage the intestinal microcirculation, which in turn may cause the intestinal mucosal membrane to lose its resistance to bacteria. This results in bacterial translocation and endotoxemia, ultimately leading to the systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction (MODF). It has been well demonstrated that the NF-κB pathway plays an important role in this cytokine-induced intestinal barrier dysfunction [8, 9]. The application of NF-κB inhibitors was found to significantly attenuate the expression of inflammatory cytokines and to alleviate intestinal I/R injury [10]. Therefore, understanding the NF-κB activation mechanism will provide new means for the clinical treatment of intestinal I/R injury and reduce the mortality of severe I/R-injured patients.

Bone morphogenic protein (BMP) is a key member of the transforming growth factor- (TGF-) β super family. More than 30 BMP proteins have been identified, and they can be further classified into several subgroups, including

the BMP2/4 group, the BMP5/6/7/8 group (OP-1 [osteogenic protein-1] group), the growth and differentiation factor-(GDF-) 5/6/7 group, and the BMP9/10 group [11]. The BMP2/4 and BMP5/6/7 groups are the best-characterized members of this family. After these receptors combine with their ligands, the BMP proteins function via either typical or atypical pathways. The canonical pathway activates the phosphorylation of Smad 1, 5, and 8, while the noncanonical pathway activates the NF-κB pathway [12]. Our previous studies have shown that the expression of BMP2/4 in intestinal epithelial cells increased in TPN mice, the ERK1/2 pathway was activated, and the proliferation of intestinal epithelial cells was weakened [13]. These effects may lead to weaker intestinal barrier function and thereby increase the possibility of endogenous infection. BMPs have recently been thought to influence inflammatory processes in adults due to their chemotactic activity on fibroblasts, myocytes, and inflammatory cells [14]. It has been shown that BMP2 can induce inflammatory reactions in endothelial cells, fibroblasts, preosteoblasts, and soft tissues. Sorescu et al. [15] found that BMP4 plays an inflammatory role in the early steps of atherogenesis by initiating an inflammatory cascade in an NF-κB-dependent manner through the stimulation of ICAM-1 surface expression in activated endothelial cells. The activation of NF-κB in human embryonic kidney (HEK) cells also depends on the BMP signaling pathway [12]. Therefore, we hypothesized that the BMP pathway would be upregulated and play an important role, through activation of the NF-κB signaling pathway, in the damage caused to the intestinal barrier function by I/R injury. Our data indicate that BMP2/4 expression is increased in both an early cell hypoxia model and a male Sprague-Dawley (SD) rat I/R model. BMP2/4 is able to directly increase the expression of the cytokines TNF-α and IL-6 and decrease the expression of the tight junction protein occludin by activating NF-κB signaling. This effect was attenuated in part by the BMP-specific antagonist noggin.

2. Materials and Methods

Anti-p-p65, anti-p65, anti-p-ERK1/2, anti-ERK1/2, anti-p-AKT, and anti-AKT were purchased from Cell Signaling Technology (Boston, MA, USA). Anti-GAPDH antibody was purchased from the ProteinTech Group (Chicago, IL, USA). Anti-BMP2, anti-BMP4, anti-BMPRIa, anti-BMPRII, anti-BMPRIb, and anti-occludin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Recombinant BMP2, BMP4, and noggin were purchased from Peprotech (NJ, USA). The inhibitor of NF-κB, PDTC, was purchased from Beyotime (Wuhan, China).

2.1. Cell Culture. IEC-6 intestinal epithelial cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and grown in Dulbecco's modified Eagle's medium (DMEM, Hyclone) supplemented with 10% fetal bovine serum (Gibco), 100 IU/mL penicillin, and 100 mg/mL streptomycin. The IEC-6 cells were cultured at 37°C in either normoxic (5% CO_2 and 20% O_2) or hypoxic (5% CO_2 and 1% O_2 in a hypoxia chamber) conditions (Thermo Fisher Scientific, Ohio, USA). For Western blot analysis, BMP2, BMP4, and noggin were added to the medium for 6 h or for a time gradient from 0 min to 120 min. For real-time PCR analysis, BMP2, BMP4, and noggin were added to the medium for 3 h. For immunofluorescence analysis, BMP2, BMP4, and noggin were added to the medium for 30 min.

2.2. Western Blot Analysis. The cells were washed twice with phosphate-buffered saline (PBS) before lysis in cold RIPA buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and 2 mM sodium pyrophosphate).

Samples were mixed with loading buffer and boiled for 5 min before electrophoresis. Proteins were loaded onto 8–10% SDS-PAGE gels at 100 V for 2 h. After electrophoresis, the proteins were electroblotted onto NC membranes at 200 mA for 2 h. Nonspecific binding was blocked by incubation in Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBS-T) and 5% skim milk. The transferred membranes were incubated overnight at 4°C with primary antibody as follows: anti-p-p65 (1 : 1000), anti-p65 (1 : 1000), anti-p-ERK (1 : 500), anti-ERK (1 : 500), anti-p-AKT (1 : 1000), anti-AKT (1 : 1000), anti-BMP2 (1 : 500), anti-BMP4 (1 : 500), anti-BMPRIa (1 : 200), anti-BMPRII (1 : 750), anti-BMPRIb (1 : 200), and anti-GAPDH (1 : 5000). After washing three times in TBS-T, the membranes were incubated with anti-rabbit IgG (Zhongshan Bio., China) conjugated to horseradish peroxidase at a dilution of 1 : 3000 in TBS-T containing 5% skim milk for 1 h at 37°C. After three additional washes with TBS-T, the signals were visualized using the Super Signal West Pico trial kit (Pierce, USA) and detected with Image Station 4000R (Kodak).

2.3. Animal Experiments. Male Sprague-Dawley (SD) rats weighing 200–250 g were obtained from our university's Laboratory Animals. After the rats were fasted for 12 hours, their abdomens were opened via a midline incision under sodium pentobarbital anesthesia. The rats were randomly assigned to three groups: (1) the I/R group, in which the superior mesenteric artery (SMA) was occluded for 30 minutes followed by defined times of reperfusion; (2) the noggin+ I/R group, in which an intraperitoneal injection of 4 μg/kg noggin was given 30 min before I/R, followed by ischemia, reperfusion, and sacrifice of the rats at defined times; and (3) the sham group, which included animals subjected to anesthesia and laparotomy without ischemia. The tissue was cut along the longitudinal axis, washed in physiological saline, and immediately frozen in liquid nitrogen and stored at −70°C for future use. All animal experiments were performed in compliance with our university's Guidelines for the Care and Use of Laboratory Animals.

2.4. Immunofluorescence Analysis. For the current study, 10 μm frozen sections were cut from the jejunum and fixed on slides. After fixation in 4% formaldehyde for 20 min, the sections were incubated in 3% H_2O_2 for 30 min to quench endogenous peroxidases. Nonspecific binding was blocked with 5% BSA in phosphate buffered saline for 30 min at room

temperature. Sections were incubated overnight at 4°C in 3% BSA in PBS with primary antibodies as follows: anti-BMP2, anti-BMP4, anti-BMPRIa, anti-BMPR2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) in a 1 : 50 dilution, and anti-P65 (Cell Signaling Biotechnology) in a 1 : 100 dilution. Sections were washed three times in BSA in PBS and incubated with Alexa 488-conjugated goat anti-rabbit antibody for 1 h at RT. After the nuclei were stained with DAPI, images were analyzed and collected with a Leica TCSSP confocal imaging system (Leica, Heidelberg, Germany).

The expression of NF-κB in IEC-6 cells was also assayed with an immunofluorescent technique. IEC-6 monolayers were washed three times with PBS before fixation in 4% paraformaldehyde for 20 min at room temperature. After another three washes, the IEC-6 cells were permeabilized with 0.2% Triton X-100 for 10 min and blocked in 5% BSA in PBS at room temperature for 30 min. Then, the IEC-6 cells were incubated overnight at 4°C in 5% BSA in PBS with anti-p65 at a 1 : 50 dilution. Monolayers were washed with PBS and incubated with FITC green-conjugated goat anti-rabbit secondary antibodies (1 : 100; Zhongshan) for 1 h in dark conditions. DAPI (Sigma-Aldrich, St. Louis, MO) was used to stain the nuclei, and the cells were imaged using a laser scanning fluorescence microscope (Leica, Heidelberg, Germany).

2.5. Real-Time PCR Analysis. Cells were washed two to four times with PBS prior to RNA isolation. Total cellular RNA was isolated with Trizol reagent and used for first strand cDNA synthesis with the Reverse Transcription System. Quantification of gene transcripts was performed with a 7500 Real-Time PCR System (Applied Biosystems, Foster City, California, USA) using Power SYBR Green (Applied Biosystems, Foster City, California, USA). PCR conditions were one cycle of 94°C for 2 min followed by 50 cycles of 94°C for 10 s, a specified annealing temperature for 15 s and 72°C for 15 s. Amplification was followed by melting curve analysis, which used the following program: one cycle at 65°C for 1 s, 94°C for 2 s, and 37°C for 5 s. The housekeeping gene β-actin was used as an endogenous reference gene to which the expression of the other genes was normalized using the comparative cycle of threshold value. The following PCR primers were used: TNF-a forward primer: 5′-GCG TGTTCATCCGTTCTCTA-3′, reverse primer 5′-CGTCTC GTGTGTTTCTGAGC-3′; IL-6 forward primer: 5′-AGT TGCCTTCTTGGGACTGA-3′, reverse primer: 5′-ACTGGT CTGTTGTGGGTGGT-3′; and β-actin forward primer: 5′-CCCATCTATGAGGGTTACGC-3′, reverse primer: 5′-TTTAATGTCACGCACGATTTC-3′.

2.6. Statistical Analyses. Statistical analyses were performed using SPSS 13.0 software. All experimental data are shown as means ± SD. Comparisons among 3 or more groups were made by analysis of variance (ANOVA), and 2 groups were compared by Student's t-test. A P value less than 0.05 was considered statistically significant in all cases. All reported significance levels represent 2-tailed P values. If not otherwise stated, all experiments were repeated for at least 3 individual experiments to ensure reproducibility.

3. Results

3.1. Hypoxia and I/R Induced the Expression of BMP2 and BMP4 in Intestinal Epithelial Cells. We analyzed the protein level of BMP2 and BMP4 with Western blotting. We found that the expression level of BMP2 and BMP4 was upregulated 2.5-fold (Figure 1(a)) and 3.1-fold (Figure 1(b)), respectively, in IEC-6 cells after 6 h of hypoxia. Meanwhile, we detected the expression of BMP2 and BMP4 in intestinal epithelial cells in an I/R rat model. IF analysis showed that these proteins were also significantly increased along the crypt/villus axis after 1 h of I/R, consistent with the significantly increased BMP2 and BMP4 levels in intestinal epithelial cells under hypoxia. Normally, BMP2 and BMP4 are expressed in both the epithelial and mesenchymal compartments, but BMP4 is highly expressed and enriched in the mesenchyme [13, 16]. In the present study, the BMP2 level significantly increased in the mid-to-distal villus region after 1 h of I/R, while the BMP4 level increased significantly in both the villi and mesenchyme in the I/R rat (Figure 1(c)).

3.2. BMP Receptor (BMPRIa and BMPRII) Expression Levels Were Upregulated with Hypoxia and I/R. The main BMP receptors include the type II BMP receptor (BMPRII) and the following type I receptors: the BMPRI group (BMPRIa and BMPRIb; also denoted as ALK-3 and ALK-6, resp.), the ALK-1 group (ALK-1 and ALK-2), and the TbR-I group (ALK-4/ActR-IB, ALK-5/TbR-I, and ALK-7). Typically, BMP2 and BMP4 bind to BMPRIa and BMPRIb, but BMPRIa has a high-affinity binding site for BMP2 [11]. To investigate whether the greater abundance of BMP2/4 led to an increase in intracellular BMP signaling, we evaluated the expression of BMPRII and BMPR-Ia in epithelial cells under hypoxia and I/R. At 6 h after hypoxia, BMPRIa and BMPRII expression levels were both significantly increased (Figures 2(a) and 2(b)). We also detected the expression of BMP receptors in the rat I/R model. The rats were euthanized after 1 h of I/R treatment. Sections of the small intestine were collected to detect changes in BMPRIa and BMPRII expression via immunofluorescence analysis. Immunofluorescence staining showed that the expression levels of the transmembrane receptors BMPRIa and BMPRII were significantly increased in the villi but had lower expression levels in the matrix (Figure 2(c)).

3.3. Exogenous BMP2 and BMP4 Activated the NF-κB Pathway. We used Western blotting to determine the effect of BMP2 and BMP4 on NF-κB transcriptional activity. BMP2 and BMP4 increased NF-κB transcriptional activity 3.5-fold and 3.4-fold, respectively, while BMP2/4 combined with noggin resulted in lower levels of NF-κB transcriptional activity (Figure 3(a)). NF-κB is normally sequestered in the cytoplasm. Once activated, NF-κB translates to the nucleus to trigger the transcription of genes involved in inflammatory cellular responses and other types of signals. We used IF

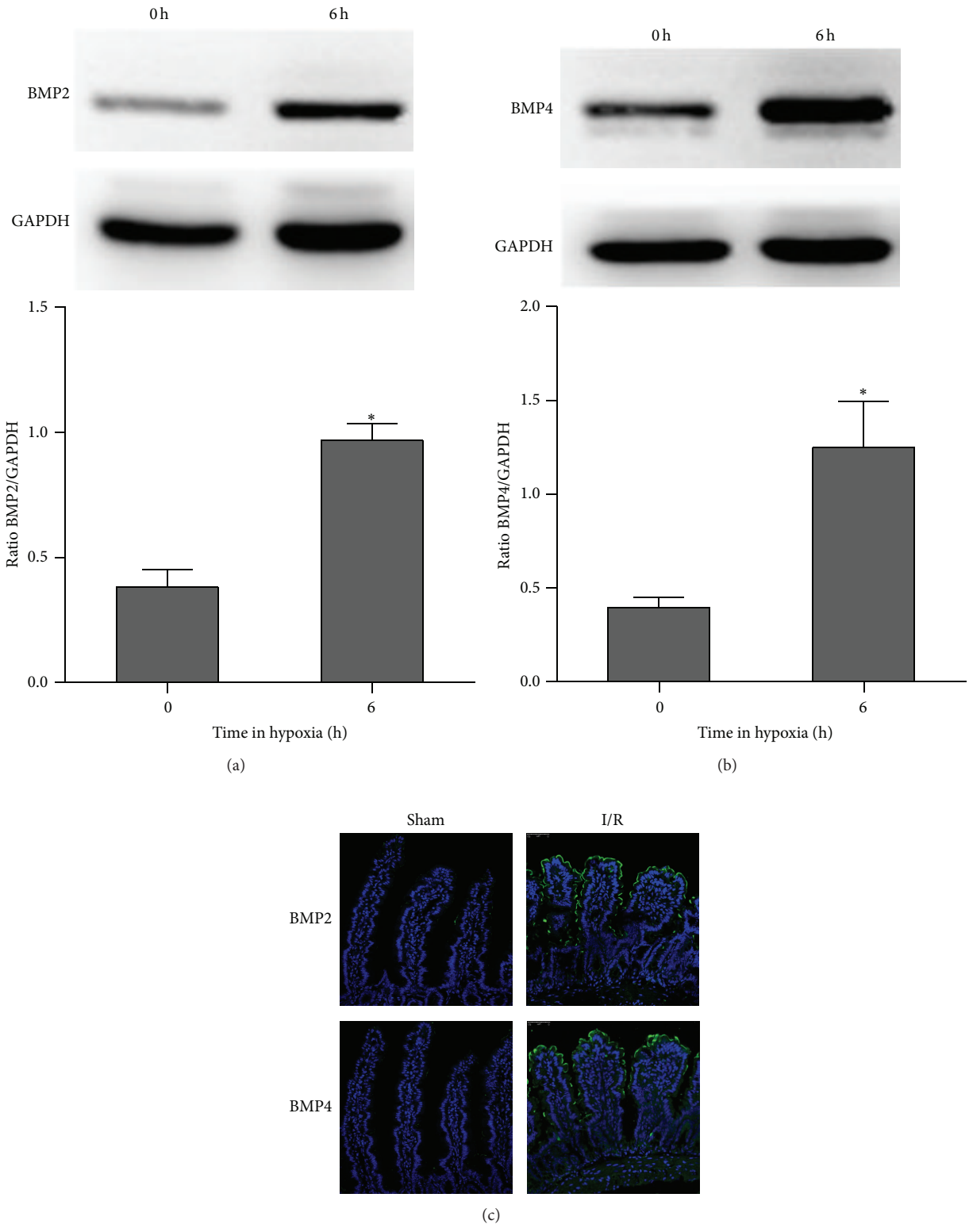


Figure 1: The expression of BMP2 and BMP4 in intestinal epithelial cells. (a) and (b) The IEC-6 cells were treated with hypoxia (1% O_2) for 6 h. Hypoxia caused a dramatic increase in BMP2 and BMP4 protein expression as detected by Western blotting. $^*P < 0.05$ versus control. Data are representative of 3 similar experiments. (c) The level of BMP2 protein expression significantly increased in the mid-to-distal villus region after 1 h of I/R, while the level of BMP4 protein expression also significantly increased in both the villi and mesenchyme.

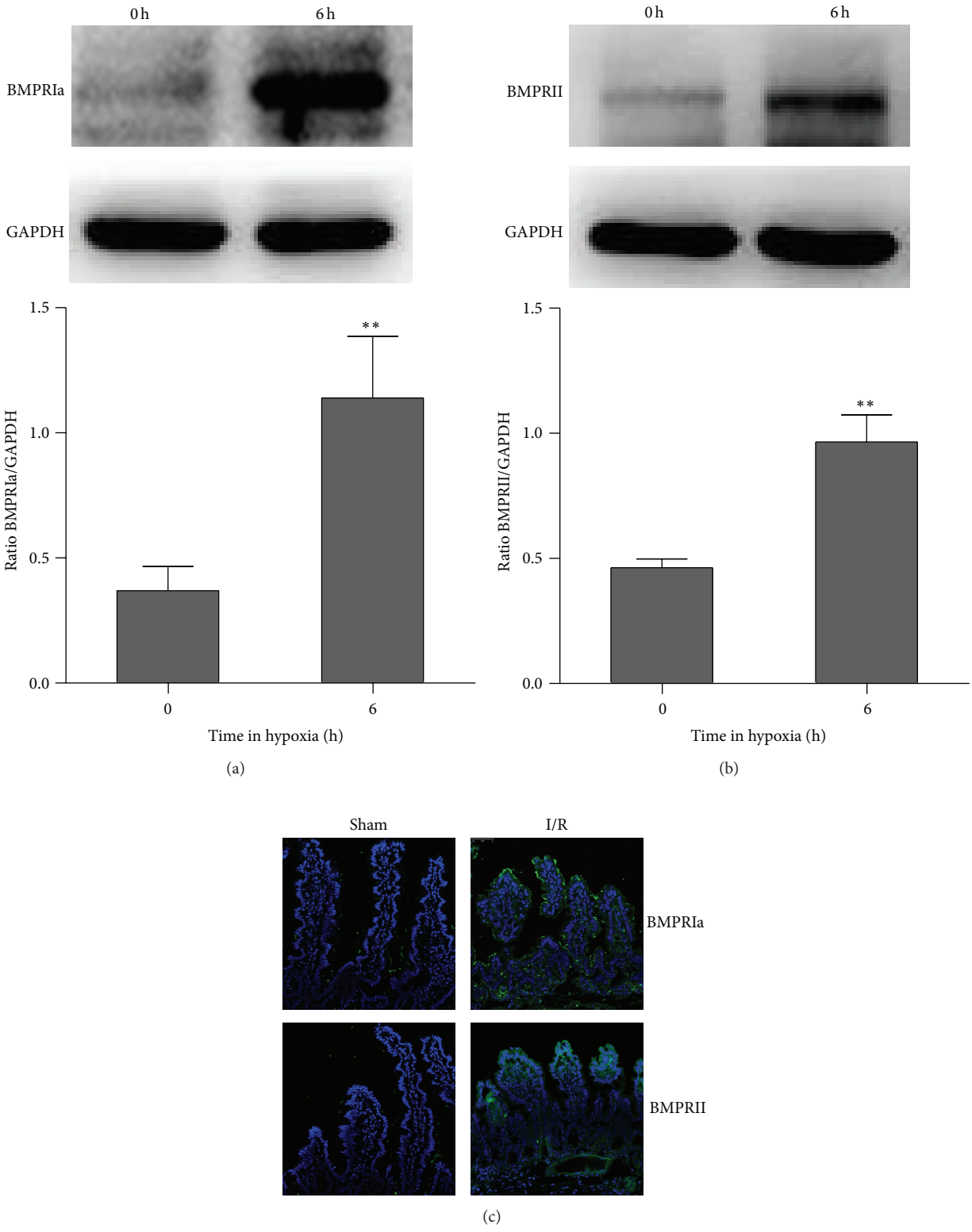
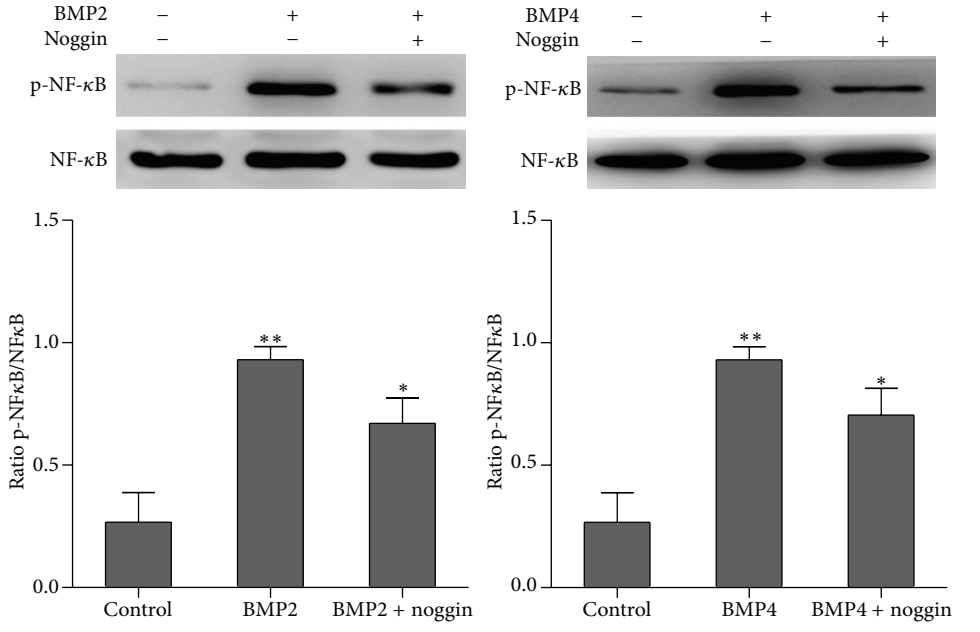
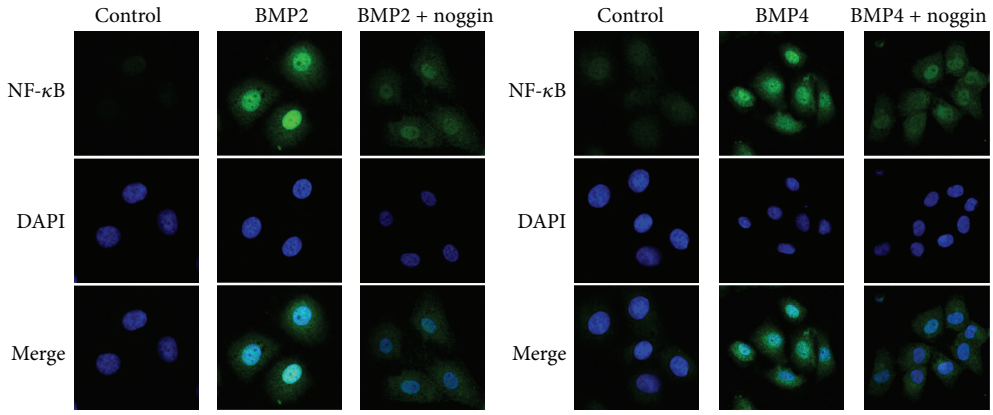


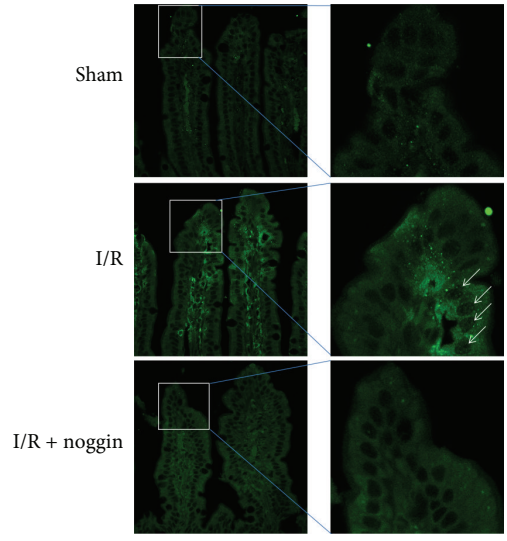
Figure 2: (a) and (b), (c) BMPRIa and BMPRII expression was detected by Western blotting and immunofluorescence staining. BMPRIa and BMPRII expression levels were both significantly increased after 6 h of hypoxia in IEC-6 cells. $^{**}P < 0.01$ versus control. BMPRIa and BMPRII expression in the intestinal mucosa also increased after I/R for 1 h compared to the control. Data are representative of 3 similar experiments.



(a)



(b)



(c)

Figure 3: Continued.

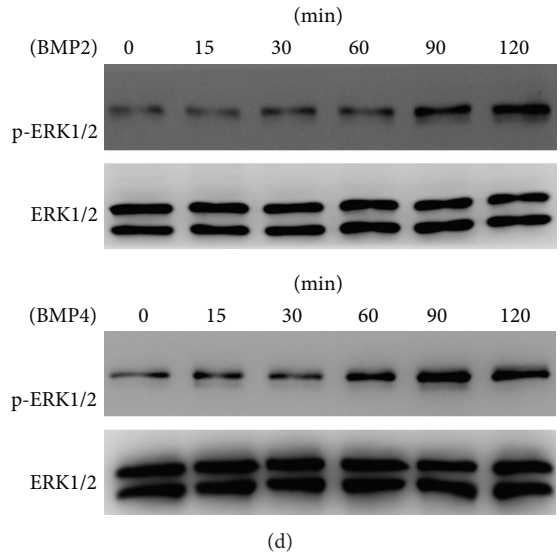


Figure 3: (a) Western blotting determined the expression of phosphorylated NF-κB in IEC-6 after treatment with BMP2 and BMP4 for 6 h. The phosphorylated NF-κB significantly increased compared with the control group, $^{**}P < 0.01$ versus control. Noggin partially decreased NF-κB transcriptional activity. $^{*}P < 0.05$, different from a single treatment with BMP2 or BMP4. (b) Immunofluorescence detected the translocation of NF-κB to the nucleus after treatment with BMP2 and BMP4 for 30 min, and noggin partially reversed the nuclear localization of NF-κB. (c) The fluorescence intensity of phosphorylated NF-κB was significantly increased in the I/R group compared to the sham group. In the I/R group, NF-κB exhibited significant nuclear localization in the distal villus, where abundant BMP2 and BMP4 are secreted after I/R (as shown in Figure 1(c)). The abundant BMP2 and BMP4 directly activated NF-κB resulting in its nuclear localization, and noggin decreased the nuclear localization of NF-κB in the I/R + noggin group. (d) Western blotting detected ERK expression upon BMP2 and BMP4 treatment at the defined time points. Phosphorylated ERK1/2 expression progressively increased in a time-dependent manner.

to detect the expression of NF-κB after stimulation by exogenous BMP2/4 for 30 min. The fluorescence intensity was greater compared to the control group, and blockade of Bmp2/4 signaling by noggin completely reversed the nuclear localization of NF-κB induced by BMP2 and BMP4 (Figure 3(b)). Meanwhile, the in vivo results showed that NF-κB signaling was obviously activated in intestinal epithelial cells after 1 h of I/R treatment. In contrast, after intraperitoneal injection of 4 μg/kg noggin 30 min before I/R, the expression of NF-κB was significantly inhibited (Figure 3(c)). Because mitogen-activated protein kinase (MAPK) is known as an upstream regulator of NF-κB [17], we evaluated the three common proteins of the MAPK pathway, ERK, P38, and JNK. Exogenous BMP2 and BMP4 activated ERK (Figure 3(d)) but not P38 or JNK (data not shown). These results are consistent with the results from S. O. Kim and M. R. Kim [17], who found that treatment with an ERK-specific blocking agent completely inhibited NF-κB activity. These results indicate that NF-κB may be activated by BMP2 and BMP4 via an increase in ERK phosphorylation.

3.4. The Expression of the Inflammatory Cytokines TNF-α and IL-6 Induced by BMP2 and BMP4 in Intestinal Epithelial Cells. NF-κB plays a central role in regulating the transcription of cytokines, adhesion molecules, and other mediators involved in acute respiratory distress syndrome (ARDS), sepsis, and multiple organ dysfunction syndrome (MODS) [6]. To test whether the activation of NF-κB induced by BMP2 and BMP4 resulted in an increase in inflammatory cytokines, we used RT-PCR to detect the expression of TNF-α mRNA and IL-6 mRNA in IEC-6 cells after treatment with BMP2 and BMP4 for 3 h. Treatment of IEC-6 cells with 100 ng/mL BMP2 caused the level of TNF-α mRNA to increase 6.3-fold compared to the control group (Figure 4(a)), while the effect of BMP4 in inducing the expression of TNF-α mRNA was weaker (Figure 4(b)). These effects were decreased by noggin. Tumor necrosis factor is one of the most powerful inducers and promoters of inflammation [3], and NF-κB both is activated by cytokines and induces the expression of inflammatory cytokines. This gives rise to the potential for NF-κB activation to spread from cell to cell within a tissue and beyond [18]. We also evaluated the expression of IL-6 mRNA. BMP2 and BMP4 both increased the expression of IL-6 mRNA, and these effects were decreased by noggin (Figures 4(c) and 4(d)).

3.5. BMP2 and BMP4 Disrupted Tight Junctions via the Activation of the NF-κB Pathway. The activation of NF-κB by IFN-γ enhances the permeability of T84 cells and decreases the levels of intercellular tight junction proteins, whereas the inhibition of NF-κB will block the increase in T84TJ permeability and alter occludin expression [8, 19]. We asked whether BMP2/4 would disrupt the intestinal mucosal barrier function via the activation of NF-κB. We used recombinant BMP2 and BMP4 to stimulate intestinal epithelial cells for 24 h, and the tight junction protein occludin was

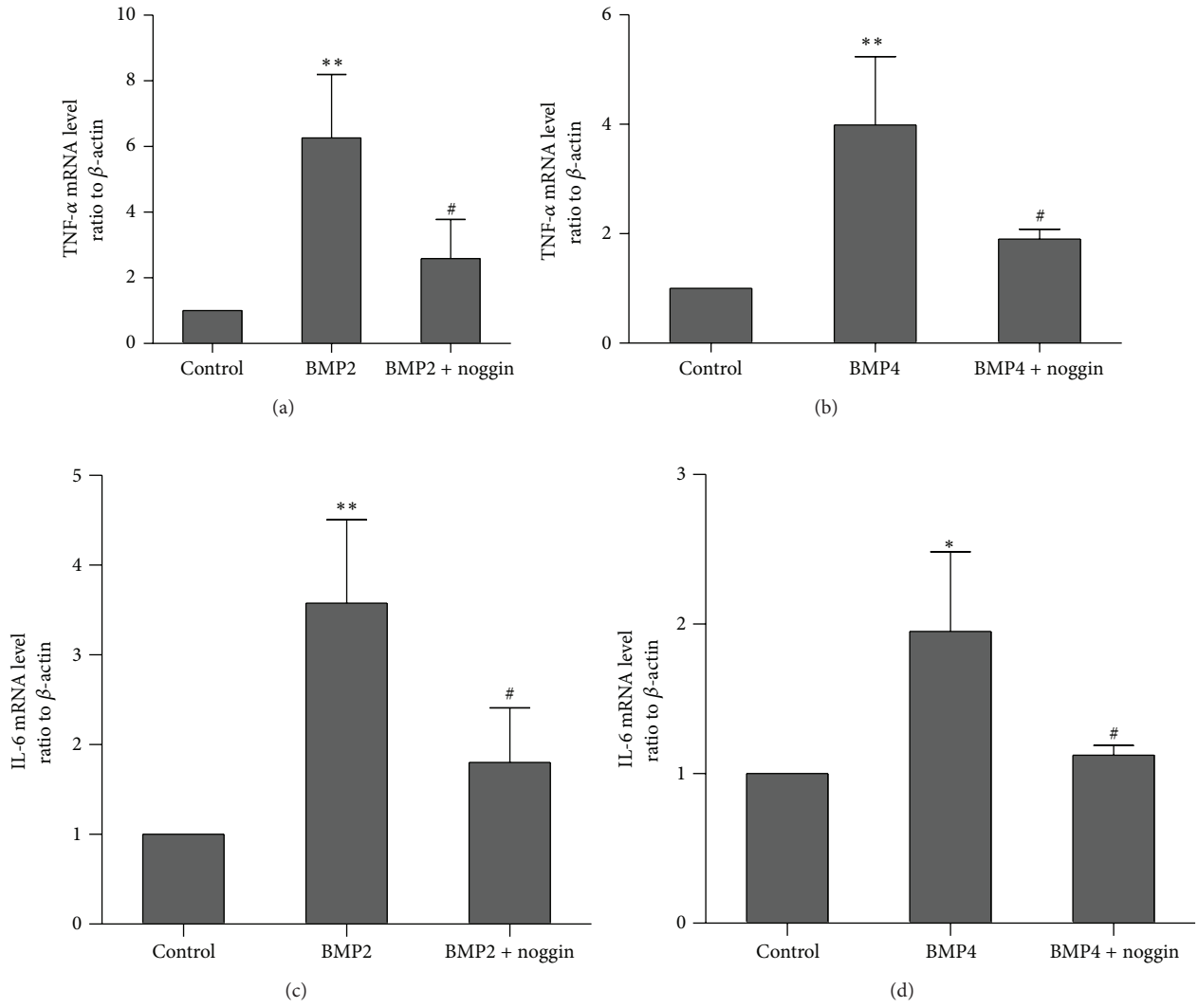


Figure 4: (a) BMP2 (100 ng/mL) significantly induced the expression of TNF-α mRNA. BMP2 combined with noggin partially reversed the increase in the TNF-α mRNA. (b) The effect of BMP4 in inducing the expression of TNF-α mRNA was weaker compared to BMP2, and the expression of TNF-α mRNA induced by BMP4 was also decreased by noggin. (c) and (d) BMP4 and BMP2 both increased the expression of IL-6 mRNA, and these effects were decreased by noggin. *Different from the control after BMP2 and BMP4 treatment, $^{**}P < 0.01$, $^{*}P < 0.05$. $^{\#}$Different from a single treatment with BMP2 or BMP4, $P < 0.05$. Data are representative of 3 similar experiments.

detected by Western blotting. The expression of occludin decreased after treatment with BMP2 and BMP4, while this effect was abolished by noggin or the NF-κB inhibitor PDTC (Figure 5(a)). These results may indicate that BMP2 and BMP4 disrupt the integrity of the intestinal mucosal barrier via the activation of NF-κB and that PDTC can reverse the decrease in TJ proteins. Boivin et al. have shown that PI3-K/Akt activation was required for the activation of NF-κB pathways in the modulation of the TJ barrier by treatment with IFN-γ [8]. We also detected the expression of AKT at the indicated times after stimulation of the IEC-6 cells with BMP2 and BMP4. The phosphorylation of AKT progressively increased in a time-dependent manner (Figure 5(b)).

4. Discussion

BMP belongs to the TGF-β super family. Previous research has focused on the roles of the BMP pathway in early intestinal development and in the proliferation and differentiation of intestinal epithelial cells [20–22]. However, the relationship between the BMP pathway and intestinal mucosal barrier dysfunction caused by I/R has rarely been examined. Shen et al. [23] found that, in rats after acetabular surgery, treatment with 4 mg/mL BMP2 protein significantly induced local inflammation, including an early and pronounced polymorphonuclear cell infiltration accompanied by the increased expression of TNF-α and IL-6. Intestinal I/R results in the release of abundant inflammatory factors, the generation of

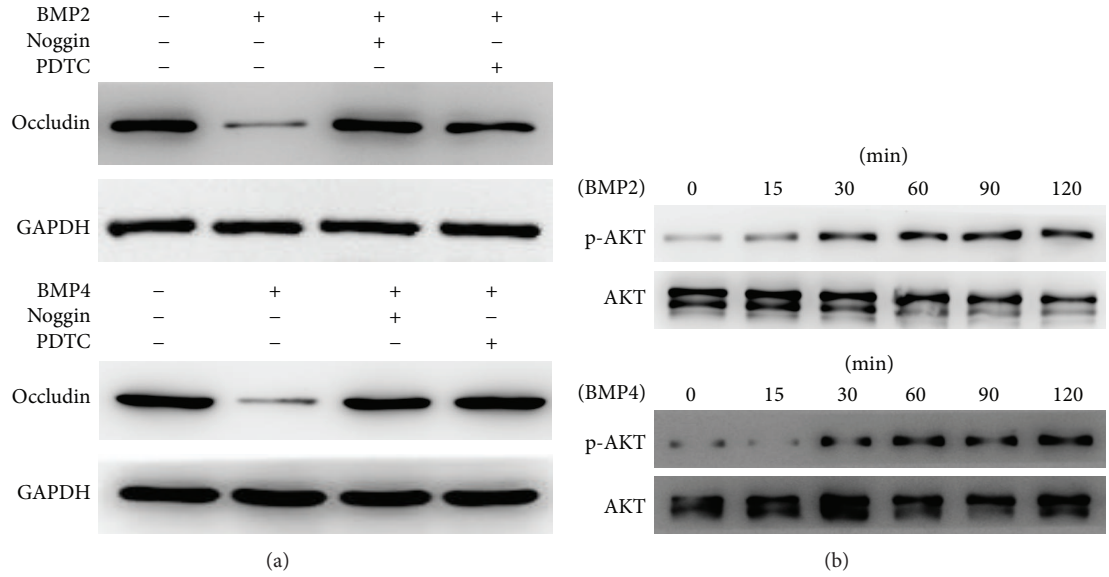


Figure 5: (a) Occludin protein expression was decreased in IEC-6 cells upon BMP2 or BMP4 treatment for 24 h, but both noggin and PDTC reversed the decrease in occludin expression in IEC-6 cells. (b) AKT phosphorylation progressively increased from 15 min to 120 min with BMP2 or BMP4 treatment.

oxygen free radicals (ROS), and the activation of NF-κB. These factors lead to enhanced permeability in the intestinal barrier and systemic inflammatory reactions [24, 25].

Because of the critical role of BMP in inflammatory reactions, we hypothesized that the BMP signaling pathway would contribute to the mechanisms involved in promoting I/R-associated intestinal mucosal barrier injury. Our present study shows that, with hypoxia and I/R, intestinal epithelial cells produce abundant BMP2 and BMP4 and that BMPRIa and BMPRII expressions are also enhanced. Western blotting and IF showed that recombinant BMP2 and BMP4 directly activate NF-κB in IEC-6 cells. BMP2/4 is able to directly increase the expression of the cytokines TNF-α and IL-6 and decrease the expression of the tight junction protein occludin by activating NF-κB signaling. This effect was attenuated in part by either the BMP-specific antagonist noggin or the NF-κB inhibitor PDTC. Our study may provide new evidence that the activation of NF-κB in intestinal I/R injury involves the BMP signaling pathway.

It is interesting to note that BMP signaling is complex; different BMP subgroups can mediate antagonistic effects, or the same ligand can produce different effects in similar tissues. For example, in an in vivo model, BMP4 inhibits liver proliferation, and the BMP4 antagonist noggin enhances regeneration [26], whereas another report demonstrates that BMP7 is an endocrine factor expressed in the kidney that enhances liver regeneration [27]. Our findings demonstrate that hypoxia and I/R increased intestinal epithelial cell BMP2/4 signaling, thereby activating NF-κB signaling. These changes led to increased expression of inflammatory factors, such as TNF-α and IL-6, and decreased expression of the tight junction protein occludin, which could result in disruption of the intestinal barrier. Our results are consistent with those of Masterson et al. [28] that, after 6 h in injured airways, BMP signaling is activated, E-cadherin expression is downregulated, and migration in normal adult airway epithelial cells is increased. Furthermore, inhibition of BMP activity protects epithelial barrier function in cases of lung injury [29].

However, our results contrast with another report that BMP7 has protective and anti-inflammatory functions in acute ischemic renal injury [30, 31]. In addition, BMP7 administration conferred intestinal mucosal protection and reduced systemic IL-6 expression levels in an inflammatory bowel disease model [32]. Additionally, BMP7 administration before intestinal I/R injury protects against intestinal mucosal injury and liver injury, preserves intestinal function, and prevents intestinal inflammation [33]. One possible explanation is that BMP7 preferentially acts through Alk2 (a stimulatory pathway), whereas BMP2/4 preferentially acts through Alk3 (an inhibitory pathway). Another possibility is that different doses of BMPs could activate different pathways. Alternative signal transduction mechanisms may also play a role: the canonical BMP pathway regulates gene expression via the SMAD-dependent pathway, while the noncanonical BMP pathway regulates NF-κB via the MEK/ERK pathway. Our studies have demonstrated that phosphorylated ERK1/2 and AKT expression levels progressively increased in intestinal epithelial cells upon treatment with BMP2/4. Thus, we believe that NF-κB may be activated by BMP2 and BMP4 via the MEK/ERK pathway in the hypoxia and intestinal I/R model.

Previous studies have shown that NF-κB is activated in the process of intestinal I/R injury and that the amount of activated NF-κB correlates with the degree of mucosal inflammation [34, 35]. The activation of NF-κB could regulate the expression of several inflammatory genes, such as cell factors, adhesion molecules, and enzymes. In turn, the released

inflammatory factors (e.g., TNF-α and IL-6) can activate NF-κB, thereby aggravating the deterioration of intestinal barrier function. Additionally, the upregulated expression of TNF-α and IL-6 induced in intestinal epithelial cells by BMP2 and BMP4 treatment confirms that BMP2 and BMP4 can mediate the intestinal mucosal injury caused by I/R via the activation of NF-κB. Moreover, BMP2 and BMP4 have been shown to activate ERK1/2, while the activation of the ERK pathway reduces the proliferation of intestinal epithelial cells [13]; thus, persistent BMP expression is unfavorable for the repair of intestinal mucosal injury. In addition to the amount of proinflammatory cytokines released by I/R, ischemia and hypoxia also disrupt the integrity of the intestinal mucosal barrier. We asked whether BMP2 and BMP4 could decrease the expression of tight junction proteins, and we evaluated changes in the expression of the tight junction protein occludin. Our results show that the expression of occludin decreased after treatment with BMP2 and BMP4, while these effects were abolished by treatment with noggin or PDTC. BMP2/4 can induce intestinal mucosal barrier dysfunction via the activation of NF-κB. These results are consistent with those of Boivin et al., who used NF-κB blockers to prevent the IFNγ-induced increase in epithelial permeability and the destruction of tight junction proteins [8]. Our previous study also found that IFN-γ induced intestinal barrier function injury via the NF-κB/HIF-1a pathway [19].

There are many types of extracellular BMP antagonists, such as noggin, chordin, cerberus, and follistatin [36, 37]. By binding BMP, these secretory factors act as competitive antagonists and are important negative regulatory factors in the BMP pathway. In mouse intestine, transient expression of the BMP antagonist noggin has been observed in both pericryptal mesenchymal cells and intestinal epithelial stem cells, which may contribute to maintaining intestinal stem cell self-renewal by activating Wnt signaling and inhibiting BMP signaling of the basal crypt epithelial cells [38]. Our data indicate that BMP2/4 expression increased in both an early intestinal epithelial cell hypoxia model and a rat I/R model. Furthermore, NF-κB was directly activated by recombinant BMP2/4 in IEC-6 cells by Western blotting and immunofluorescence (IF). Finally, BMP2/4 induced the expression of intracellular TNF-α mRNA and IL-6 mRNA in IEC-6 cells, and this effect was abolished by the BMP-specific antagonist noggin. The in vivo rat model also showed that, with an intraperitoneal injection of noggin before I/R, the activation of NF-κB was obviously inhibited. Thus, in future investigations, it will be important to examine the expression of a number of BMP antagonists (noggin, chordin, gremlin, and follistatin) in a rat I/R model.

Additionally, BMP expression regulation is complex. Hypoxia-inducible factor 1 (HIF-1) is one of the master regulators that orchestrate the cellular responses to hypoxia. Recent studies have found that BMP expression is modulated by HIF-1 expression, BMP2 expression was increased in primary chondrocytes under hypoxic conditions, and addition of the HIF-1 activator DFO significantly increased BMP2 expression [39]. Additionally, hypoxia stimulation increased BMP2 mRNA and protein expression levels in osteoblasts via an HIF-1-alpha-dependent mechanism involving the activation of the ILK/Akt and mTOR pathways [40]. Further work is required to better understand the mechanisms guiding the increased BMP expression in the hypoxia and intestinal I/R model.

To the best of our knowledge, the present study is the first to report that BMP2 and BMP4 can directly activate NF-κB, induce the expression of the inflammatory cytokines TNF-a and IL-6 in the intestinal epithelial cells, and decrease the expression of the tight junction protein occludin, which could result in disruption of the intestinal barrier. All of these effects may contribute to the mechanism by which BMP2 and BMP4 mediate intestinal mucosa injury in ischemic reperfusion.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Review Article

An Overview of the Role of Innate Lymphoid Cells in Gut Infections and Inflammation

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Innate lymphoid cells (ILCs) are a group of hematopoietic cells devoid of antigen receptors that have important functions in lymphoid organogenesis, in the defense against extracellular pathogens, and in the maintenance of the epithelial barrier. Three distinct groups of ILCs have been identified on the basis of phenotypic and functional criteria and termed ILCs1, ILCs2, and ILCs3. Specifically, ILCs1 express the transcription factor T-bet and secrete T helper type-1- (Th1-) related cytokines, ILCs2 are dependent on the transcription factor RORα and express Gata-3 and the chemokine receptor homologous molecule (CRTH2) and produce Th2-related cytokines, and ILCs3 express the transcription factor RORγt and synthesize interleukin- (IL-) 17, IL-22, and, under specific stimuli, interferon-γ. ILCs represent a relatively small population in the gut, but accumulating evidence suggests that these cells could play a decisive role in orchestrating both protective and detrimental immune responses. In this review, we will summarize the present knowledge on the distribution of ILCs in the intestinal mucosa, with particular focus on their role in the control of both infections and effector cytokine response in immune-mediated pathologies.

1. Introduction

Cells of the innate immune system (i.e., dendritic cells, macrophages, NK cells, and neutrophils) have the ability to recognize and rapidly respond to pathogens with production of various cytokines, which in turn regulate the antigen-driven differentiation of cells of the adaptive immune system (i.e., T and B lymphocytes). This process triggers an effective and specific response, which eliminates pathogens and resolves inflammation and tissue damage [1]. Effector cytokines are also involved in the orchestration of tissue-damaging immune response in states of chronic inflammation [2]. In recent years, the advent of sophisticated techniques of cellular biology has led to the identification of a new class of innate cells, termed innate lymphoid cells (ILCs), which have the ability to produce a vast array of cytokines mainly depending on their state of differentiation [3, 4]. ILCs play broad roles in lymphoid organogenesis, in the defense against extracellular pathogens, and in the maintenance of the epithelial barrier and are supposed to contribute to the amplification of immune-inflammatory responses in various organs [3, 4]. ILCs lack some cell lineage markers associated with T and B lymphocytes, dendritic cells, macrophages, and granulocytes but express CD90, CD25, and interleukin- (IL-) 7 receptor (R)α (CD127) (Table 1) [3, 4]. ILCs develop from hematopoietic precursors and their development is partially or wholly dependent on the common γ-chain, Notch, the transcription factor inhibitor of DNA binding-2 (Id2), IL-7, a cytokine involved in hematopoietic cell development and proliferation [3–6], and other transcription factors such as T-bet for ILC1, RORα and Gata-3 for ILC2, and TCF1 and Gata-3 for ILC3 (Figure 1). Mice deficient for Id2 show largely normal development of T and B cells but lack all ILC subsets, suggesting the existence of a common Id2-expressing progenitor to ILC subsets [3–6].

ILCs are currently classified into three distinct populations on the basis of the expression of specific transcription factors and/or cell surface markers and their ability to secrete some profiles of effector cytokines. Thus, they closely resemble the heterogeneity of $CD4^+$ T helper (Th) cell subsets. ILCs1 include classical NK cells and ILCs expressing T-bet, a Th-type-1-associated transcription factor, and producing

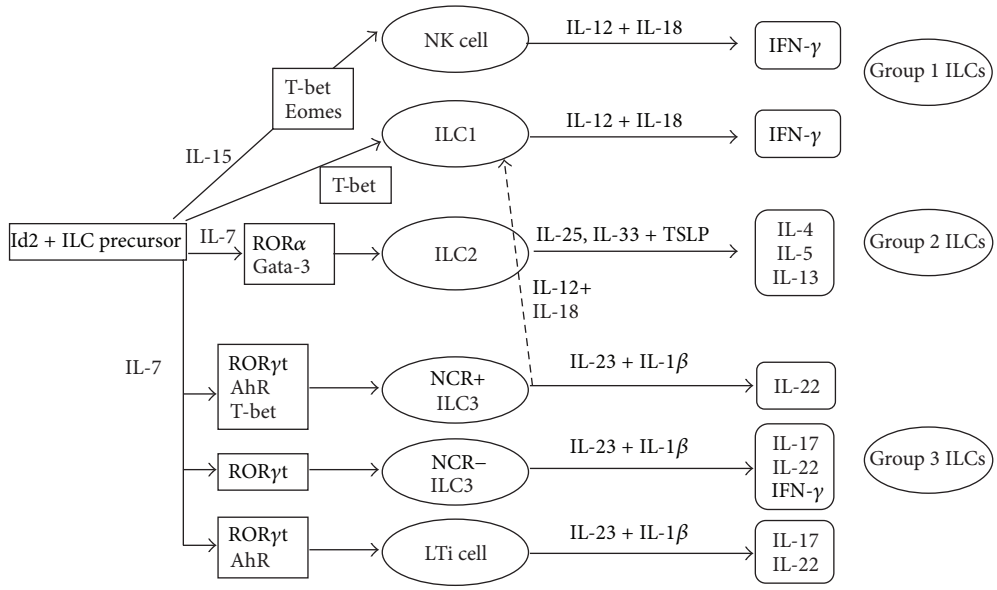

Figure 1: Schematic view of the differentiation of various innate lymphoid cells (ILCs), of the factors involved in such a differentiation, and of the cytokines produced by distinct subsets of ILCs. Id2, inhibitor of DNA binding-2; Gata-3, Gata-binding protein 3; RORα, retinoic acid receptor-related orphan receptor-α; Eomes, eomesodermin; NK, natural killer; LTi, lymphoid tissue-inducer; IL, interleukin; NCR, natural cytotoxicity triggering receptor; IFN-γ, interferon-γ; AhR, aryl hydrocarbon receptor; TSLP, thymic stromal lymphopoietin.

Table 1: Markers expressed by various subsets of innate lymphoid cells (ILCs).

ILC group	ILC lineage	Marker
1	ILC1	LIN−, CD25 low, CD56−, IL-7Rα+, CD161+/−, NKp44−, NKp46−, IL-1R+, ICOS+, IL-12Rβ2+, and CRTH2−
	NK cells	LIN−, CD56+, CD25+/−, IL-7Rα+/−, CD161+/−, NKp44+/−, NKp46+, IL-12Rβ2+, and CRTH2−
2	ILC2	LIN−, CD25 low, CD117+/−, IL-7Rα+, ICOS+, IL-1R+, ST2+, IL-17RB+, CRTH2+, and CD161+
3	ILC3	LIN−, CD25 low, CD117+, IL-7Rα+, CD161+, NKp44+, NKp46+, CRTH−, IL-1R+, IL-23+, and ICOS+
	LTi cells	LIN−, CD117+, IL-7Rα+, CD161+/−, IL-1R+, and IL-23+

LIN, lineage; NK, natural killer; LTi, lymphoid tissue-inducer; IL, interleukin; CRTH2, chemoattractant receptor homologous molecule; ICOS, inducible T cell costimulator; R, receptor; ST2, subunit of IL-33R.

interferon- (IFN-) γ, a potent stimulator of phagocyte activity against intracellular bacteria [3, 4, 7]. Conventional NK cells differ from ILCs1, because their development is dependent on the transcription factor eomesodermin (Eomes) and independent of Id2 [3, 4, 7, 8]. ILCs2 express the Th2-related transcription factor Gata-3 and, in humans, the chemokine receptor homologous molecule CRTH2 and produce IL-5, IL-9, IL-13, IL-4, and/or amphiregulin [3, 4] and play important roles in immunity to helminth infections [9–11] and in the pathogenesis of asthma and allergies [12–14]. ILCs3 express the transcription factor RORγt, synthesize IL-17A and IL-22, and, under specific stimuli, IFN-γ, and are involved in the recruitment of neutrophils, release of antimicrobial peptides, and epithelial cell proliferation (Figure 1) [3, 4]. Therefore, ILCs3 are required for the defense against bacterial infections [3, 4] and provide "help" to marginal zone B cells [15].

Here we review the available data on the role of ILCs in the control of both intestinal infections and effector cytokine response in immune-mediated pathologies of the gut.

2. ILCs1

At the present time, it is debated if typical ILCs1, which are predicted to be RORγt-independent, really exist in the gut, as IFN-γ-producing ILCs1-like cells described so far seem to originate from ILCs3 that upregulate T-bet and downregulate RORγt. NKp44-negative, c-kit-low ILCs expressing T-bet and IFN-γ and responding to IL-12, but not IL-23, with enhanced IFN-γ production are present in the intestinal lamina propria of patients with Crohn's disease (CD) but not in the fetal gut and in the noninflamed intestine of adults [16]. These cells are not seen in the intestinal lamina propria of alymphoid mice reconstituted with a human immune system under homeostatic conditions, but they appear following induction of colitis by dextran sodium sulphate (DSS) [16]. IFN-γ-producing, T-bet-positive, NKp44-negative, c-kit-low ILCs maintain, however, low levels of RORγt raising the possibility that they differentiate from RORγt-expressing ILCs3 in inflamed tissues. Fuchs and colleagues described an ILCs1-like subset, characterized by the expression of NKp44,

NKp46, CD56, CD103, granzyme, and perforin and located in the intestinal epithelial compartment (intraepithelial ILC1-like cells). These cells respond to IL-12 and IL-15, but not IL-18, with enhanced secretion of IFN-γ [17]. Despite sharing the NKp44 marker with human ILCs3, intraepithelial ILC1 subset clearly differs from ILCs3 in terms of phenotype, function, and transcription factors involved in development. In particular, studies in mice demonstrated the requirement of NFIL3 and Tbx21 (the gene encoding T-bet) for their development, while RORc (the gene encoding RORγt) and aryl hydrocarbon receptor (AhR) were dispensable [17]. However, these cells differ also in various aspects from ILCs1, as they did not express IL-7Rα and are independent of IL-15 for development and/or maintenance. Intraepithelial ILCs1 are increased in inflamed gut of CD patients and produce high amounts of IFN-γ in Rag1$^{-/-}$ mice treated with anti-CD40, a model of colitis characterized by wasting syndrome and severe intestinal inflammation [17]. Depletion of such cells ameliorates colon inflammation, supporting their role in orchestrating detrimental responses in the gut [17].

Recently, a new common progenitor to all IL-7R-expressing ILC lineages, expressing Id2, has been identified and named CHILP. CHILP gives rise to peculiar RORγt-independent, T-bet-dependent, Eomes^{-}, NKp46^{+}, NK1.1^{+}, IL-7Rα^{+} ILCs1. This cell type is present in the lamina propria of the small intestine and produces IFN-γ and TNF in response to IL-12 and to intestinal infection with the intracellular parasite *Toxoplasma gondii* [18]. Moreover, these cells express high levels of CXCR3, CXCR6, and CCR9, all of which are involved in lymphocyte migration to tissues. Studies in mice lacking specific genes revealed also that maintenance or differentiation of these cells requires T-bet, NFIL3, and Gata-3 as well as IL-15, but not IL-7 [18]. Another lymphoid precursor has been described in mouse fetal liver and adult bone marrow. It expresses high amounts of PLZF, a transcription factor previously associated with NK-T cell development, and has the potential to differentiate in ILC1, ILC2, and ILC3 [19].

3. Role of ILCs2 in the Control of Helminth Infections

Different types of Th2 cytokine-producing innate cells (e.g., natural helper cells, nuocytes, and type 2 innate helper cells), which express markers commonly found on ILC subsets (IL-7Rα, c-kit, CD25, and CD90), have been described [5, 9, 10]. These cells, now collectively referred to as ILCs2, act downstream to IL-25 and IL-33 and make a substantial contribution to antihelminth immunity through their ability to produce IL-13, a cytokine that drives many of the physiological responses required for worm expulsion, such as mucus production and smooth muscle contractility [3, 4, 9, 10]. In this context it was shown that adoptive transfer of nuocytes into *Nippostrongylus brasiliensis*-infected IL17βR/IL1R1 mice (which are severely impaired in their ability to expel worms) enables these animals to efficiently eliminate the parasite and IL-13 secretion from nuocytes is essential for worm expulsion [9]. The ability of ILCs2 to combat parasites is dependent on T cells, because nuocytes fail to undergo sustained expansion in helminth-infected Rag2-deficient mice [9]. Similarly, adoptive transfer of natural helper cells into γ-chain-Rag2-deficient mice infected with *Nippostrongylus brasiliensis* resulted in the restoration of goblet-cell hyperplasia in the recipient mice [5]. Along the same line are the results obtained with adoptive transfer of type 2 innate helper cells into *Nippostrongylus brasiliensis*-infected γ-chain-Rag2-deficient mice, in which worm expulsion requires IL-25 administration [20].

ILCs2 also produce amphiregulin, an epithelial growth factor family member that is crucial for tissue repair following virus-induced inflammation [21] as well as enhancing the immunosuppressive properties of regulatory T cells during colitis [22]. ILCs2 express elevated levels of Gata-3, which is crucial for their development and maintenance [23]. Development of ILCs2 is also dependent on RORα as mutant mice lacking this transcription factor do not have ILCs2 but display normal development of ILCs3 [24].

4. Regulation of ILCs3 Development and Activation

The factors/mechanisms that regulate differentiation/activation of ILCs3 are not fully understood, even though there is evidence that these cells can be activated by cytokines released by the intestinal epithelium and antigen presenting cells. While IL-7, stem cell factor, and TSLP are necessary for the development of ILCs3 and, together with IL-1, IL-2, and IL-15, regulate cell proliferation, IL-23 and IL-1β play an important role in inducing ILCs3 effector functions [25–30]. IL-23, a heterodimeric cytokine produced mainly by dendritic cells and macrophages, stimulates production of IL-22 by ILCs3 during intestinal infections [31, 32], but it is not essential for ILCs3 functions at steady state. Indeed, IL-23p19-deficient mice exhibit normal production of IL-22 by ILCs3 [33]. IL-23 induces also IL-17 production in CD56-negative ILCs3 isolated from the gut of patients with CD [34]. Another cytokine involved in the ILCs3 functions is IL-1β. IL-1β induces the accumulation and activation of ILCs3 during the course of *Helicobacter* (*H.*) *hepaticus* infection and synergizes with IL-23 or IL-7 in enhancing ILCs3-derived IL-22 production [35, 36]. Like CD4^{+}Th17 cells, ILCs3 display a certain degree of context-dependent plasticity, as they are capable of acquiring functional characteristics of ILCs1. A subset of intestinal ILCs3, which is negative for CCR6 and either positive or negative for NKp46, coexpresses T-bet and RORγt [37, 38]. T-bet in ILCs3 controls expression of various target genes such as those encoding IFN-γ, Fas ligand, IL-12Rβ1, and CXCR3 [37, 38]. Interestingly, IL-22 production is evident in both T-bet^{+} and T-bet^{-} ILCs3, albeit at different levels, and T-bet is highly expressed in NKp46^{+} IL-22-producing ILCs3 in the intestine. Accordingly, mice deficient in Tbx21 have a reduced number of NKp46^{+} IL-22-producing ILCs3 in the intestinal lamina propria and failed to produce IFN-γ [37].

Collectively the available data indicate that CCR6-negative ILCs3 progress from a T-bet-negative to a T-bet-positive state and then acquire NKp46 expression. This later

phenomenon can be followed by downregulation of RORγt [38, 39]. IL-7 is crucial not only for the development of ILCs but also for stabilizing in vivo the expression of RORγt in ILCs3, thus preventing their full conversion into IFN-γ-producing NKp46, T-bet-expressing ILCs [39].

The signals that induce T-bet expression in CCR6-negative ILCs3 are not completely understood. IL-12, the major Th1 inducing factor, seems to be uninvolved as mice deficient for IL-12 signaling have normal numbers of T-bet-expressing ILCs3 [38]. In contrast, IL-23-deficient mice have reduced numbers of T-bet-expressing ILCs3 [38], suggesting a role for IL-23 in inducing or maintaining T-bet expression in ILCs3. ILCs3 can also recognize and directly respond to environmental cues. For example, both mouse and human ILCs3 express AhR, a ligand inducible transcription factor that mediates a wide range of cellular events in response to halogenated aromatic hydrocarbons and nonhalogenated polycyclic aromatic hydrocarbons, small synthetic compounds, and metabolites of tryptophan and arachidonic acid. In its inactive state, AhR resides in the cytosol bound to several cochaperones. Following ligand binding, AhR dissociates from the chaperones and translocates to the nucleus, where it binds to its dimerization partner aryl hydrocarbon receptor nuclear translocator, and this complex activates the expression of a battery of genes with promoters containing a dioxin responsive element consensus sequence or a xenobiotic responsive element consensus sequence [40]. The number of postnatal ILCs3 as well as IL-22 but not IL-17 expression by ILCs3 in small intestine and colon is reduced in AhR-deficient mice [41]. The decrease in ILCs3 observed in AhR-deficient mice is not evident until the third week, suggesting that environmental stimuli may contribute to the differentiation, survival, and postnatal expansion of these cell subsets [41]. The pathways involved in the AhR-dependent ILCs3 development and function in the gut remain to be clarified, even though AhR ligands derived from food components could be involved. Indeed, it was shown that mice fed with phytochemical-free diets have a phenotype similar to AhR-deficient mice [42]. Another possibility is that AhR-dependent modulation of ILCs3 function is mediated by bacterial metabolites, as, under conditions of unrestricted tryptophan availability, *Lactobacilli* species produce indole-3-aldehyde, an AhR ligand, which enhances IL-22 expression in ILCs3 [43]. Interestingly, human ILCs3 express also RNA transcripts of Toll-like receptors (TLR) 1, 2, 5, 6, 7, and 9, though it seems that only TLR2 agonists induce cytokine production by human ILC3 in the presence of IL-2, IL-15, and IL-23 [44], supporting the hypothesis that bacteria can directly stimulate ILCs3 to synthesize effector cytokines.

Both human and mouse ILCs3 express NK cell activating receptors (e.g., NKG2D, DNAX accessory molecule-1, 2B4, CD94/NKG2C, NKp46, NKp44, and NKp30) that are known to mediate NK cell cytotoxicity and production of cytokines upon recognition of cognate cellular and viral ligands. In particular, NKp44 is detectable on ILCs3 and selectively marks the IL-22-producing subset in human tonsil and gut lamina propria [32, 36]. Engagement of NKp44 in ex vivo isolated ILCs3 selectively induces the expression of TNF and IL-2 while stimulation with IL-23, IL-1, and IL-7 preferentially induces IL-22 and GM-CSF expression [36]. Therefore, ILCs3, whose development is Notch dependent [45], can switch between IL-22 and TNF production, depending on the triggering stimulus.

5. The Role of ILCs3 in the Control of Intestinal Epithelial Barrier, Infections, and Inflammation

ILCs3 are involved in the development of intestinal lymphoid organs such as cryptopatches, which are located in the lamina propria between the gut crypts, and isolated lymphoid follicles, which represent important sites of T-cell-independent IgA production [3, 4]. For a detailed description of the regulatory functions of ILCs3 in the development of intestinal lymphoid organs, the reader is directed towards recent reviews [3, 4].

In healthy mammals, commensal bacteria are anatomically restricted either to the intestinal lumen, to the epithelial surface, or within the underlying gut-associated lymphoid tissues, a process that is essential to limit inflammation and maintain normal systemic immune homeostasis. ILCs3 have a critical role in this phenomenon, as their depletion in mice results in peripheral dissemination of commensal bacteria such as *Alcaligenes* species, residing within Peyer's patches and mesenteric lymph nodes of healthy humans and mice, and systemic inflammation [46]. RORγt-expressing ILCs3 express major histocompatibility complex class II (MHCII) and can process and present antigen to $CD4^+$ T cells. Among ILCs3, MHCII is highly expressed on cells that lack T-bet and NKp46, while minimal expression occurs in cells positive for those markers. Moreover, MHCII is seen on ILCs2 but not ILCs1 [46]. Interestingly, RORγt-expressing, MHCII-positive ILCs3 lack expression of classical costimulatory molecules, such as CD40, CD80, and CD86, and therefore antigen presentation by these cells limits, rather than promoting, $CD4^+$ T cell responses, through a mechanism that is independent of the ability of ILCs to produce IL-22 or IL-17A [33]. Mice lacking MHCII selectively on ILCs3 exhibit increased frequencies of proliferating $CD4^+$ T cells in the blood, significant increase in commensal bacteria-specific serum IgG, and development of colitis characterized by enhanced production of IFN-γ, IL-17A, and TNF by mucosal $CD4^+$ T cells [46]. All these phenomena can be prevented by administration of antibiotics, demonstrating a crucial role for commensal bacteria in the development of the disease [33, 46]. The ILC-mediated containment of commensal bacteria and regulation of mucosal homeostasis could also rely on the ability of ILCs3 to produce IL-22 in response to AhR-activating stimuli [41]. Indeed, binding of IL-22 to its heterodimeric receptor, comprising IL-10R2 and IL-22R1, on epithelial cells triggers the transcription factor STAT3, thereby promoting synthesis of antimicrobial peptides and proteins (i.e., β-defensins, RegIIIβ and RegIIIγ, calgranulins S100A8 and S100A9, and lipocalin-2) and elevated levels of mucus-associated molecules (i.e., Muc1, Muc3, Muc10, and Muc13), with the downstream effect of limiting the translocation of commensal bacteria across the epithelial barrier during inflammation [47]. Although microbiota can

modulate production of IL-22 by ILCs3 [33], the development of such cells seems to be independent of commensal bacteria, as the frequencies of IL-22-producing ILCs3 are similar in conventional versus germ-free mice [46].

ILCs3 also play an important role in the defense against pathogen infections, such as *Citrobacter rodentium*, a murine pathogen that models human enterohemorrhagic and enteropathogenic *Escherichia coli* infections [31, 32]. In particular, it was shown that mice lacking T and NK cells but retaining NKp46-expressing ILCs3 developed an IL-23-driven IL-22-mediated response and were resistant to infection [48]. ILCs3 also provide an early source of IL-22 during *Candida albicans* fungal infection [49]. IFN-γ produced by T-bet-dependent CCR6-ILCs3 has been shown to contribute to the response against *Salmonella typhimurium* infection in mouse [38].

Due to their ability to modulate epithelial cell functions as well as respond against commensal bacteria and pathogens, it is tempting to speculate that ILCs3 can participate in the complex regulation of IBD-related mucosal response, given that there is evidence that IL-22 is protective in murine models of IBD [27, 50]. On the other hand, as above specified, IL-17A and IFN-γ from NKp46-negative ILCs3 contribute to sustain inflammation in innate IBD models, such as anti-CD40 or *H. hepaticus*-induced colitis [25, 51]. These later findings are supported by the demonstration that IL-17A and IFN-γ-producing $CD127^+CD56^-$ILCs3 accumulate in inflamed gut of patients with CD [52]. However, another study documented a reduced frequency of $NKp44^+NKp46^-$ILCs type-3-like cells in CD [34]. Additional support to the hypothesis that ILCs3 can be proinflammatory in the gut comes from research into Tbx21/Rag2 double-knockout mice that develop spontaneous intestinal inflammation resembling UC [53]. In these mutants, ILCs3 produce high levels of IL-17A, a finding not observed in control mice, and depletion of all ILCs or neutralization of IL-17A improves colitis [54]. In addition, alymphoid mice on a Tbx21-deficient background do not develop colitis, indicating that lymphoid cells are required for inflammation [54].

A more complex scenario emerged however from studies in chronic CD45RB (high) $CD4^+$ T cell transfer and anti-CD40 antibody-induced acute innate colitis models in Rag1-deficient mice showing that IL-23R signaling in ILCs3 is protective in the former and pathogenic in the latter [51]. Furthermore, it was shown that IL-23R signaling promotes innate colitis via IL-22 as neutralization of IL-22 protects mice from colitis and the adding back of IL-22 to IL-23R-deficient animals restores the disease [51].

6. Conclusions

In recent years, it became evident that ILCs play a fundamental role in immune responses, not only as first barrier against pathogens but also for their ability to influence downstream adaptive immune steps. These advances have been facilitated by the better characterization of the factors involved in the differentiation and maintenance of such cells as well as identification of the ILC subsets involved in specific immune responses. Environmental cues can promote the activation of ILCs as well as shifting from a subset to another one. In this context, it has been demonstrated that commensal microbiota-driven induction of IL-7 is determinant in the maintenance of ILCs3 and activation of AhR, an intracellular receptor for various environmental molecules, regulates the function of these cells in the gut. The intestine also contains additional stimuli, such as TLR ligands and cytokines, that could contribute to ILC development and activation. Much work remains however to be performed in order to ascertain the exact contribution that each ILC subset plays in the maintenance of immune homeostasis in the gut and how this task is accomplished. Further experimentation will be also needed to investigate whether and which ILCs are involved in the pathogenesis of chronic inflammatory diseases of the gut, such as CD, UC, and celiac disease, since cytokines produced by these cells are known to regulate the tissue-damaging immune responses in these disorders. At the same time, it would be relevant to investigate whether ILCs play a role in the process of colitis-associated colon cancer by either providing an immunosuppressive environment that eventually promotes tumor growth or amplifying cytotoxic pathways that kill tumor cells. In this context, it is noteworthy that IL-22 produced by ILCs facilitates the growth of cancer cells through a STAT3-dependent mechanism in a bacteria-driven mouse model of colorectal cancer [55].

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Silvia Sedda, Irene Marafini, Michele M. Figliuzzi, Francesco Pallone, and Giovanni Monteleone solely contributed to this paper. All authors read and approved the final paper.

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Research Article

Glutamine Supplementation Attenuates Expressions of Adhesion Molecules and Chemokine Receptors on T Cells in a Murine Model of Acute Colitis

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Background. Migration of T cells into the colon plays a major role in the pathogenesis in inflammatory bowel disease. This study investigated the effects of glutamine (Gln) supplementation on chemokine receptors and adhesion molecules expressed by T cells in mice with dextran sulfate sodium- (DSS-) induced colitis. *Methods*. C57BL/6 mice were fed either a standard diet or a Gln diet replacing 25% of the total nitrogen. After being fed the diets for 5 days, half of the mice from both groups were given 1.5% DSS in drinking water to induce colitis. Mice were killed after 5 days of DSS exposure. *Results*. DSS colitis resulted in higher expression levels of P-selectin glycoprotein ligand- (PSGL-) 1, leukocyte function-associated antigen- (LFA-) 1, and C-C chemokine receptor type 9 (CCR9) by T helper (Th) and cytotoxic T (Tc) cells, and mRNA levels of endothelial adhesion molecules in colons were upregulated. Gln supplementation decreased expressions of PSGL-1, LFA-1, and CCR9 by Th cells. Colonic gene expressions of endothelial adhesion molecules were also lower in Gln-colitis mice. Histological finding showed that colon infiltrating Th cells were less in the DSS group with Gln administration. *Conclusions*. Gln supplementation may ameliorate the inflammation of colitis possibly via suppression of T cell migration.

1. Introduction

Inflammatory bowel disease (IBD), which includes Crohn's disease (CD) and ulcerative colitis (UC), is a relapsing and remitting disorder characterized by chronic inflammation of the gastrointestinal (GI) tract. The etiology of IBD remains unclear; however, both CD and UC are associated with enhanced leukocyte trafficking to the inflamed intestine [1, 2]. Previous studies indicated that effector T cells including CD4-positive T helper (Th) cells and CD8-positivecytotoxic T (Tc) cells play pivotal roles in the pathogenesis of mucosal lesions and chronic intestinal inflammation [3–5]. Blockage of lymphocyte migration to mucosal sites has therefore become a potential therapeutic strategy for IBD [6].

Lymphocytes migrate to specific tissues via a multistep process which is strictly regulated by adhesion molecules and chemokine receptors. Adhesion molecules present on the vascular endothelium and lymphocytes participate in the tethering, rolling, and adhesion of lymphocytes [7]. P-selectin and E-selectin appearing on activated endothelial cells interact with P-selectin glycoprotein ligand- (PSGL-) 1 expressed by lymphocytes, which promotes the initial tethering and subsequent rolling of lymphocytes over vessel walls. Integrins participate in rolling and firm adhesion of lymphocytes. Lymphocytes expressing $\alpha 4\beta 7$ integrins roll on mucosal addressin cell adhesion molecule- (MAdCAM-) 1, which is required for homing of lymphocytes to intestinal sites [8]. Lymphocyte arrest mediated by integrins, such as lymphocyte function-associated antigen- (LFA-) 1 (also known as CD11a/CD18 and $\alpha L\beta 2$ integrins) which interacts with their endothelial-cell ligands intercellular adhesion molecule- (ICAM-) 1, precedes extravasation into the underlying tissue [9].

Chemokines are small peptides which bind to chemokine receptors expressed on leukocytes and function as chemoattractants. They regulate lymphocyte homing to secondary lymphoid organs and transmigration into tissues by forming a chemokine concentration gradient which attracts lymphocytes to move towards an increasing concentration [10]. Dysregulation of chemokines and chemokine receptors was implicated in various autoimmune diseases, including IBD [11, 12]. Recent studies indicated that C-C chemokine receptor type 9 (CCR9) and $\alpha 4\beta 7$ integrins are required for the localization of lymphocytes to the GI mucosa [13]. Agents developed for disrupting actions of CCR9 and $\alpha 4\beta 7$ integrins showed promising results in IBD clinical trials [6].

Glutamine (Gln) is an immunomodulatory nutrient which is widely used in clinical practice [14]. Previous studies showed that Gln treatment has beneficial effects in different experimental models of colitis. Gln attenuates the expression of proinflammatory mediators [15] and improves outcomes which may be due to upregulation of heat shock proteins (HSPs) [16]. Recent work in our laboratory demonstrated that pretreatment with Gln suppresses cytokine expression of Th cells and ameliorates the severity of acute dextran sulfate sodium- (DSS-) induced colitis [17]. However, whether the beneficial effects of Gln are mediated by modulating lymphocyte trafficking in colitis is still unclear. Therefore, we investigated the influence of dietary Gln supplementation on T cell adhesion molecules and CCR9 expression in mice with DSS-induced acute colitis.

2. Materials and Methods

2.1. Study Protocols. Six-week-old male C57BL/6 mice were used in this study. Conventional mice were maintained in a temperature- and humidity-controlled room and were fed a standard chow diet ad libitum before the study. Care of laboratory animals was in full compliance with the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996), and protocols were approved by the Institutional Animal Care and Use Committee of Taipei Medical University.

2.2. Experimental Design. After 1 week of acclimation, 40 mice were randomly assigned to a control group or a Gln group in this study, with 20 mice in each group. Mice in the control group were fed a common semipurified diet, while the Gln group received a diet in which part of the casein was replaced with Gln. Gln provided 25% of the total amino acid nitrogen. This amount of Gln was proven to have an immunomodulatory effect in rodents [18–20]. Two diets were formulated to be isonitrogenous and isoenergetic (Table 1). After 5 d of being fed the diets, mice in the control and Gln groups were further divided into 2 respective subgroups. One subgroup was given distilled water, while the other subgroup received 1.5% (wt/vol) DSS (MW 40 kDa; MP Biomedicals, Solon, OH, USA) in the drinking water for 5 d to induce colitis. A flow diagram of the study design is shown in Figure 1. There were 4 groups in this study: control diet with distilled water (C group), Gln diet with distilled water (G group), control diet with DSS water (DC group), and Gln diet with DSS water (DG group). The respective experimental diets were given during the DSS exposure period. Body weights (BWs) were recorded daily, and all mice had free access to food and water throughout the study. At the end of the experiment, mice were anesthetized and sacrificed by cardiac puncture. Fresh blood samples were collected in heparinized tubes for measurements of the leukocyte population. Mesenteric lymph nodes (MLNs) were removed and processed for further analysis by flow cytometry. The colon was cut close to the ileocecal valve, and its length and weight were measured. Sections (1 cm) of the distal colon were cut. Colon tissues were fixed with buffered 4% paraformaldehyde for an immunohistochemical analysis.

Table 1: Composition of the semipurified diets.

Component	Control diet	Gln diet
	g/kg	
Soybean oil	100	100
Casein	200	150
Glutamine	0	41.7
Salt mixture[a]	35	35
Vitamin mixture[b]	10	10
Methyl cellulose	31	31
Choline bitartrate	2.5	2.5
Methionine	3	3
Corn starch	626.8	618.5

[a]The salt mixture contained the following (mg/g): calcium phosphate diabasic: 500; sodium chloride: 74; potassium sulfate: 52; potassium citrate monohydrate: 20; magnesium oxide: 24; manganese carbonate: 3.5; ferric citrate: 6; zinc carbonate: 1.6; curpric carbonate: 0.3; potassium iodate: 0.01; sodium selenite: 0.01; and chromium potassium sulfate: 0.55.

[b]The vitamin mixture contained the following (mg/g): thiamin hydrochloride: 0.6; riboflavin: 0.6; pyridoxine hydrochloride: 0.7; nicotinic acid: 3; calcium pantothenate: 1.6; D-biotin: 0.05; cyanocobalamin: 0.001; retinyl palmitate: 1.6; DL-α-tocopherol acetate: 20; cholecalciferol: 0.25; and menaquinone: 0.005.

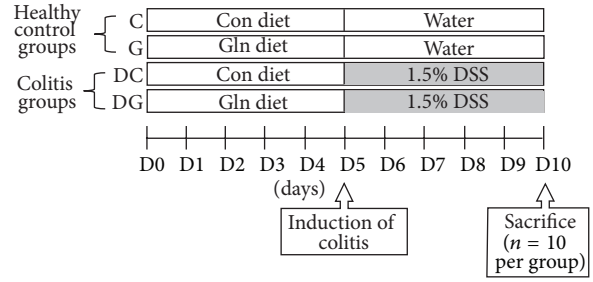

Figure 1: Flow diagram of the study design.

2.3. Blood Leukocyte Distribution. A five-color flow cytometric analysis was performed to determine the distribution of peripheral blood leukocytes. Antibodies against mouse leukocyte surface antigens were added to 100 μL aliquots of whole blood. The antibodies used to detect different subsets of leukocytes were as follows: PerCP-conjugated anti-CD45 (Biolegend, San Diego, CA, USA) for leukocytes, PE-conjugated anti-F4/80 (eBioscience, San Diego, CA, USA) for

monocytes/macrophages, FITC-conjugated anti-Ly6G (BD Biosciences, San Jose, CA, USA) for neutrophils, APC-conjugated anti-CD3ε (eBioscience) for T cells, and Pacific blue-conjugated anti-CD19 (Biolegend) for B cells. Antibodies were used at the concentration recommended by manufacturer. After a 30 min incubation at 4°C in the dark, red blood cells were lysed, and cells were suspended in staining buffer and then analyzed with a FACS Canto II flow cytometer (BD Biosciences). CD45-positive cells were gated, and results are presented as a percentage of specific CD-marker-expressing cells in blood leukocytes. Representative flow cytometry plots are shown in Figure 2(a).

2.4. Lymphocyte Populations in MLNs. Cell suspensions from MLNs were obtained by passing the tissues through a nylon cell strainer with a 40 μm pore size (BD Biosciences) in RPMI1640 medium (Biological Industries, Kibbutz Beit Haemek, Israel). After centrifugation at 300 ×g for 10 min, pelleted MLN cells were suspended in 1 mL of staining buffer. One hundred microliters of cell suspension was incubated with APC-conjugated anti-CD3ε (eBioscience) and Pacific blue-conjugated anti-CD19 (Biolegend) for 30 min at 4°C in the dark. Stained cells were washed and resuspended in staining buffer to measure the lymphocyte population by flow cytometry. Percentages of T and B lymphocytes were determined by CD3ε- and CD19-expressing cells in MLN cells. Representative flow cytometry plots are shown in Figure 2(b).

2.5. Expressions of Adhesion Molecules and Chemokine Receptors by T Cells. Whole blood and MLNs were used to analyze adhesion molecule- and chemokine receptor-expressing T cells. Whole blood and MLN cells obtained as described above were split into 2 vials with 100 μL in each aliquot, and these were incubated with Pacific blue-conjugated anti-CD4 (BD Biosciences) or Pacific blue-conjugated anti-CD8 antibodies (Biolegend). To investigate expressions of adhesion molecules and chemokine receptors, PE-conjugated anti-PSGL-1 antibodies (BD Biosciences), APC-conjugated anti-$\alpha 4\beta 7$ integrin, FITC-conjugated anti-CD11a, and PerCP-Cy5.5-conjugated anti-CCR9 (Biolegend) were added. Stained cells were analyzed by five-color flow cytometry. Lymphocytes were gated on the basis of their forward- and side-scatter profiles. Fluorescence data were recorded, and results are presented as percentages of adhesion molecule- and chemokine receptor-expressing $CD4^+$ and $CD8^+$ lymphocytes. Representative flow cytometry plots are shown in Figure 2(c).

2.6. RNA Extraction and Real-Time PCR. Total RNA was isolated from colon tissue using the Trizol reagent (Invitrogen, Carlsbad, CA). RNA (1 μg) was reverse-transcribed with a complementary (c)DNA synthesis kit (Fermentas, Glen Burnie, MD, USA) according to standard protocols. For real-time PCR, 5 μL of 1/10 diluted cDNA was amplified in a 25 μL PCR volume containing 12.5 μL of 2X SYBR green master mix reagent (Applied Biosystems, Foster City, CA, USA). The reaction was performed with ABI 7300 Real-Time PCR System (Applied Biosystems) according to the thermocycling protocol recommended by the PCR system. Primer sequences were as follows: mouse ICAM-1 (5′-AGCACCTCCCCACCTACTTT-3′ and 5′-AGCTTGCACGACCCTTCTAA-3′), mouse P-selectin (5′-TCCAGGAAGCTCTGACGTACTTG-3′ and 5′-GCAGCGTTAGTGAAGACTCCGTAT-3′), mouse E-selectin (5′-TGAACTGAAGGGATCAAGAAGACT-3′ and 5′-GCCGAGGGACATCATCACAT-3′), and mouse 18S rRNA (5′-CGCGGTTCTATTTTGTTGGT3′ and 5′-AGTCGGCATCGTTTATGGTC-3′). All samples were analyzed in triplicate, and fold change for each target gene was calculated by the equation $2^{-\Delta\Delta}$Ct (ΔCt indicates the difference in threshold cycles between the test gene and 18S rRNA, and ΔΔCt indicates the difference of ΔCt between the experimental and C groups).

2.7. Immunofluorescence Staining. Double-staining combinations CD3-CD4 and CD3-CD8 were performed on 5 μm paraffin-embedded colon sections. After antigen retrieval, sections were incubated with an antibody against CD3ε (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C and amplified with a rabbit anti-goat immunoglobulin G (IgG) secondary antibody conjugated with FITC (Santa Cruz Biotechnology). For colocalization, sections were then costained overnight at 4°C with secondary antibodies against CD4 (Abcam, Cambridge, UK) or CD8 (Novus Biologicals, Littleton, CO, USA) and amplified with the respective appropriate secondary antibodies: goat anti-mouse IgG or goat anti-rabbit IgG conjugated with rhodamine (Santa Cruz Biotechnology). Cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma, St. Louis, MO, USA) for 10 min at room temperature. Digital images at 400x magnification per section were acquired using appropriate filters of a Zeiss Axiophot fluorescence microscope (Carl Zeiss MicroImaging LLC, Thornwood, NY, USA) fitted with a Nikon D1X digital camera (Tokyo, Japan). Cells containing both FITC and rhodamine labels appeared yellow. These images were then overlaid with DAPI-staining images to determine the infiltration of T lymphocyte subpopulations in the colon mucosa.

2.8. Statistical Analysis. All data are expressed as the mean ± standard error of the mean (SEM). Differences among groups were analyzed by an analysis of variance (ANOVA) with Tukey's test. A two-way ANOVA with Bonferroni correction was used to analyze differences in BW changes. A P value of <0.05 was considered statistically significant.

3. Results

3.1. BW and Weight/Length Ratio of the Colon. Initial BWs ranged 21~25 g and did not differ among the 4 groups. There was no significant difference in BWs during the study between the C and G groups. At 4 d (d 9) and 5 d (d 10) after DSS administration, weight loss was observed in the DC group compared to the C group, whereas mice with Gln supplementation maintained their BWs during the DSS exposure period. At the end of the study, BWs were significantly higher in the DG group than the DC group (Figure 3(a)).

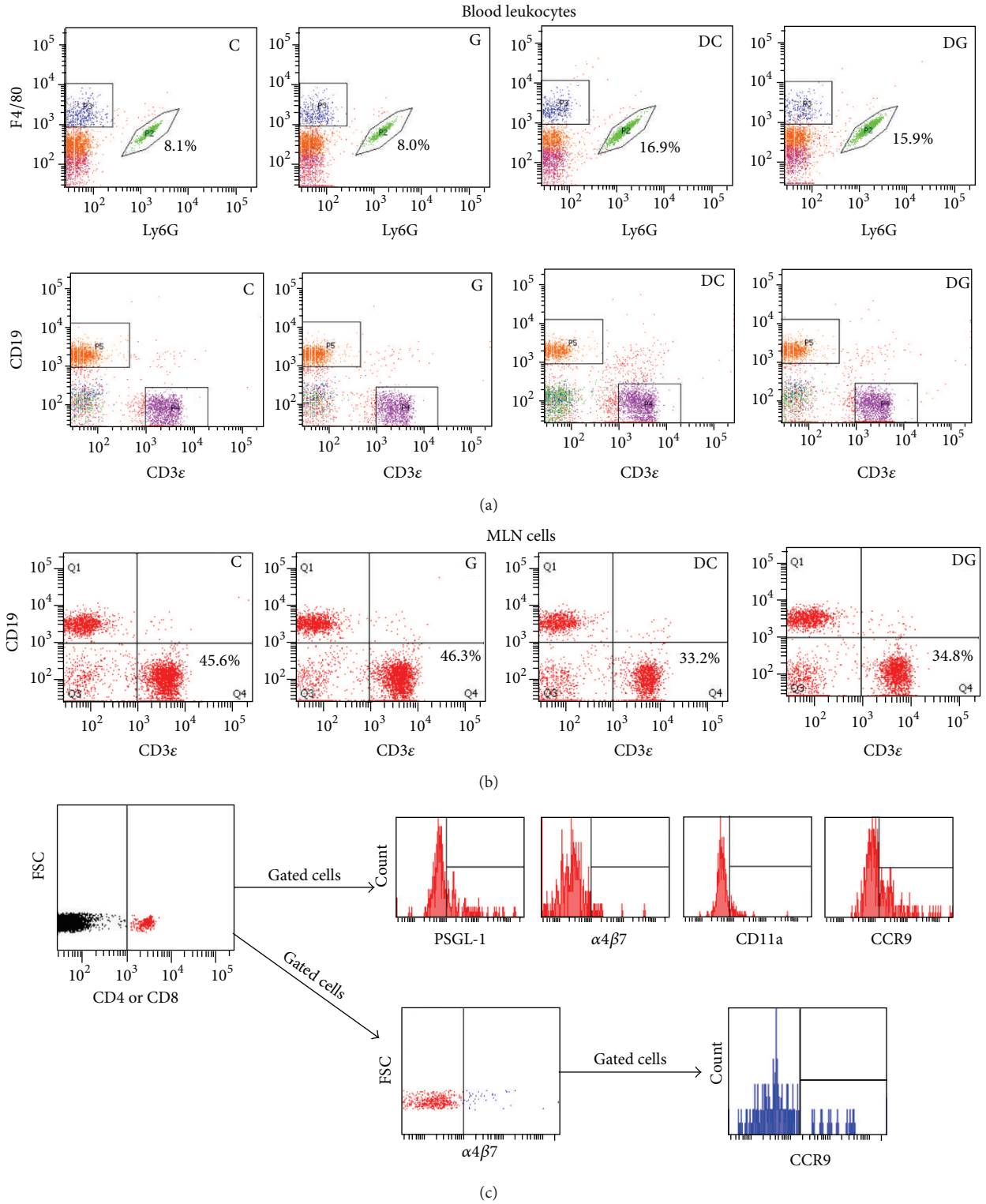

Figure 2: Representative flow cytometry plots. Blood leukocytes (a) were defined by gating on CD45-positive cells. The percentage of Ly6G-positive neutrophils from an individual representative mouse per group is listed. For analyzing the lymphocyte population in MLNs (b), MLN cells were first gated to exclude debris. Numbers indicate the percentage of CD3ε-positive lymphocytes in MLN cells. For analyzing the expression of adhesion molecules and chemokine receptors by T cells (c), lymphocytes were first identified based on low FSC and SSC characteristics. CD4- or CD8-positive lymphocytes were gated to analyze the percentages of adhesion molecule- and chemokine receptor-expressing $CD4^+$ and $CD8^+$ lymphocytes. Representative dot plots of leukocytes in blood are shown.

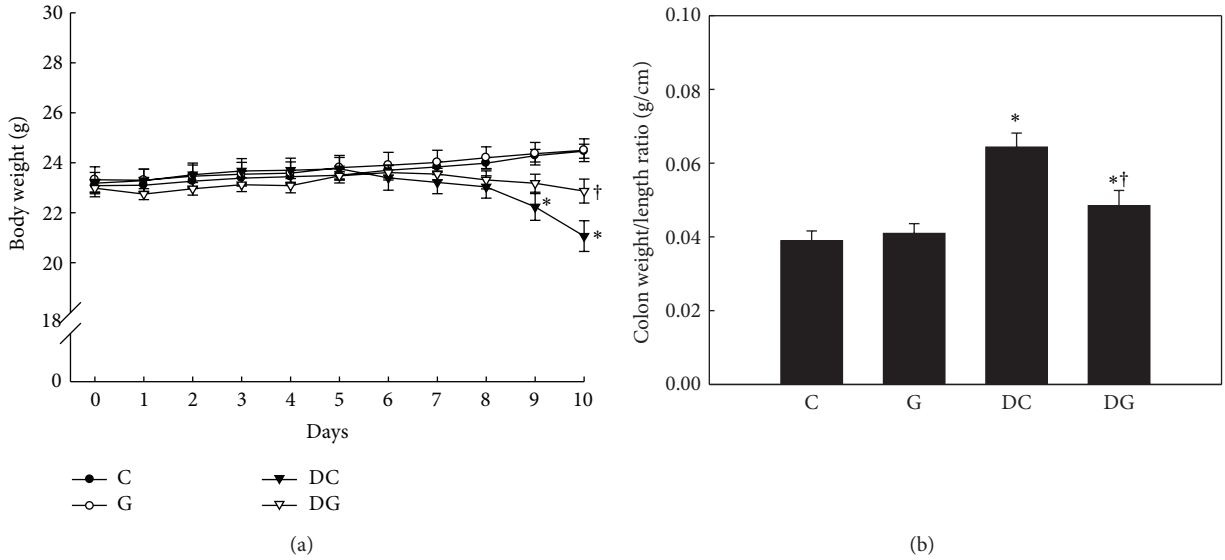

Figure 3: Body weight (a) and weight/length ratio of the colon (b). Data are presented as the mean ± SEM. C, normal mice fed the control diet; G, normal mice fed a glutamine-enriched diet; DC, DSS group fed the control diet; DG, DSS group fed a glutamine-enriched diet. *Significantly different from the C group ($P < 0.05$). †Significantly different from the DC group ($P < 0.05$).

The weight/length ratio of the colon, an indicator of colonic edema, was significantly higher in the colitis groups than the C group (Figure 3(b)). Treatment with Gln attenuated colonic edema associated with DSS-induced inflammation.

3.2. Leukocyte Populations in Blood and MLNs. There was no significant difference in blood or MLN leukocyte subpopulations between the C and G groups. Compared to the C group, the DSS colitis groups had a higher percentage of blood neutrophils and lower T-cell population in MLNs. DSS exposure did not alter the blood monocyte and lymphocyte distributions. Also, subsets of effector T cells in the blood and MLNs did not change. Gln supplementation had no influence on blood leukocyte or MLN lymphocyte populations in normal or colitic mice (Table 2).

3.3. Adhesion Molecule and CCR9 Expressions by Th Cells. Percentages of adhesion molecules and CCR9 expressed by blood and MLN Th cells did not differ between the C and G groups. DSS administration resulted in higher PSGL-1, CD11a (LFA-1 αL subunit), and CCR9 expressions by Th cells in both blood and MLNs, whereas no difference in $\alpha 4\beta 7$ integrin expression was detected among the control and DSS groups. Mice in the DG group had lower percentages of PSGL-1-, CD11a-, and CCR9-expressing Th cells in blood (Figures 4(a)–4(d)) and MLNs (Figures 5(a)–5(d)). The expression level of CCR9 on $\alpha 4\beta 7$-positive Th cells was also suppressed in the DG group (Figures 4(e) and 5(e)).

3.4. Adhesion Molecule and CCR9 Expressions by Tc Cells. No differences in adhesion molecules and CCR9 expressed by blood and MLN Tc cells were observed between the C and G groups. The DSS colitis groups had higher percentages of PSGL-1-, CD11a-, and CCR9-expressing blood and MLN Tc cells, whereas expression levels of $\alpha 4\beta 7$ integrins did not differ among the 4 groups. Compared to the DC group, the DG group had lower expression of CD11a by blood (Figures 6(a)–6(d)) and MLN Tc cells (Figures 7(a)–7(d)). There was no difference in expression levels of CCR9 on $\alpha 4\beta 7$-positive Tc cells between the 2 DSS colitis groups (Figures 6(e) and 7(e)).

Table 2: Leukocyte populations in blood and mesenteric lymph nodes (MLNs) (%).

	C	G	DC	DG
Blood				
Neutrophils	8.7 ± 0.5	8.0 ± 0.9	16.1 ± 2.7*	16.9 ± 2.1*
Monocytes	7.2 ± 0.9	6.7 ± 0.7	8.2 ± 1.3	7.7 ± 1.5
T cells	11.9 ± 0.4	11.7 ± 0.4	9.3 ± 1.2	9.3 ± 1.4
B cells	50.3 ± 0.9	53.4 ± 3.1	53.2 ± 2.7	49.0 ± 2.1
Th cells	6.7 ± 1.6	6.4 ± 0.4	7.3 ± 1.2	8.1 ± 0.8
Tc cells	3.6 ± 0.8	4.4 ± 0.3	4.5 ± 0.2	4.1 ± 0.3
MLNs				
T cells	45.2 ± 2.4	46.7 ± 2.2	32.1 ± 2.3*	34.9 ± 1.0*
B cells	30.3 ± 1.8	29.0 ± 3.3	31.3 ± 2.1	29.7 ± 1.4
Th cells	11.8 ± 0.3	12.3 ± 0.7	11.2 ± 0.9	12.1 ± 0.9
Tc cells	6.3 ± 0.4	6.7 ± 0.6	5.8 ± 0.3	6.5 ± 0.5

CD45-positive cells were considered to be leukocytes and gated to determine the population of leukocytes using a flow cytometer. Staining for Ly-6G, F4/80, CD3ε, and CD19 was used to respectively identify neutrophil, monocyte, T cell, and B cell populations. For the analysis of T cell subpopulations, lymphocytes were gated on the basis of their forward-and side-scatter profiles. Percentages of T helper (Th) and cytotoxic T (Tc) cells were respectively determined by CD4-and CD8-expressing cells in lymphocytes. Values are presented as the mean ± SEM. *Significantly differs from the C group ($P < 0.05$).

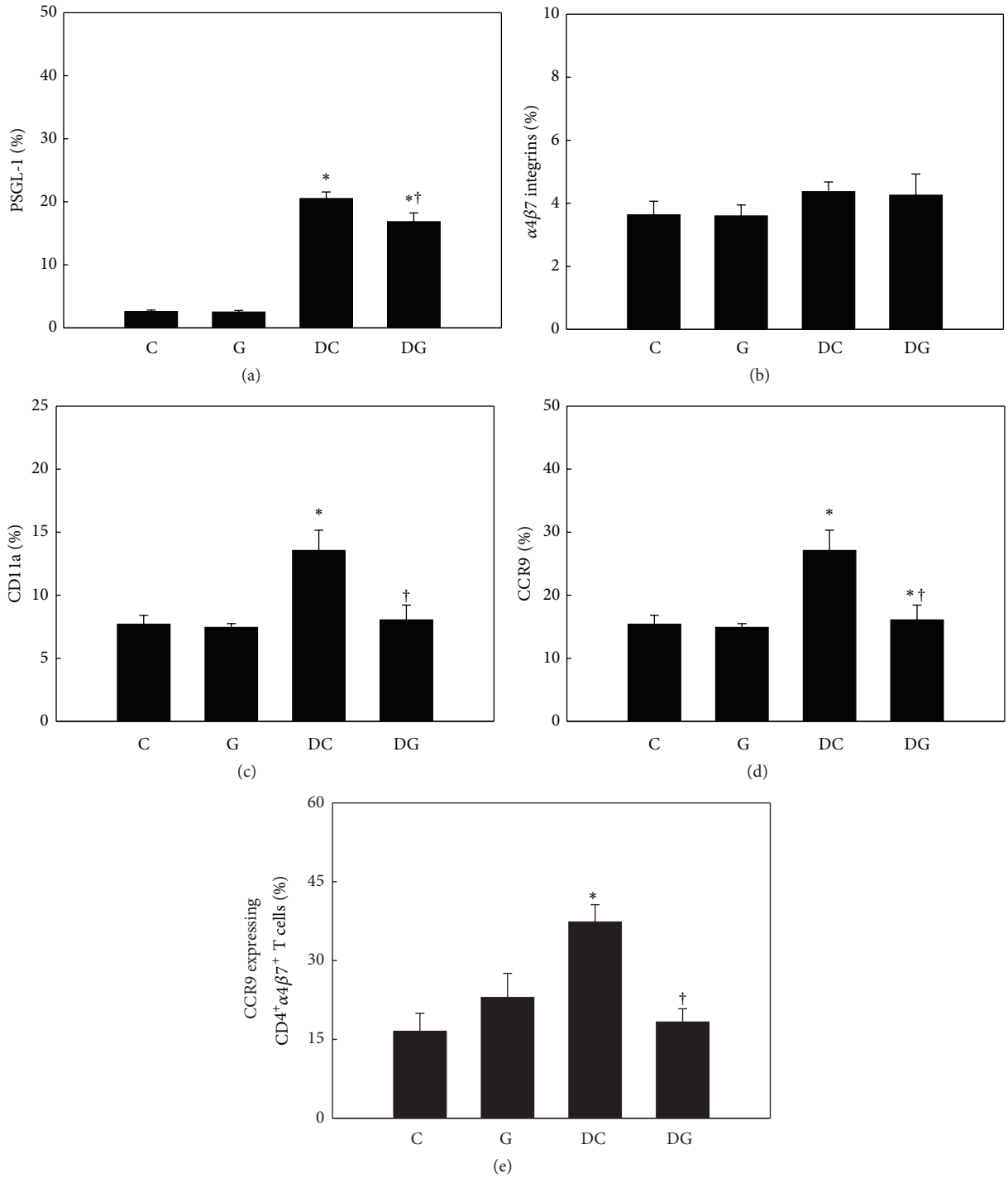

Figure 4: Percentage of adhesion molecule- and chemokine receptor-expressing T helper (Th) cells in blood. CD4-positive blood lymphocytes were gated to analyze expressions of PSGL-1, $\alpha 4\beta 7$ integrins, CD11a, and CCR9 by flow cytometry ((a)–(d)). (e) Expression of CCR9 by CD4 and $\alpha 4\beta 7$ integrin double-positive blood lymphocytes. Values are shown as the mean ± SEM. *Significantly different from the C group ($P < 0.05$). †Significantly different from the DC group ($P < 0.05$).

3.5. Gene Expression of Endothelial Adhesion Molecules in Colon Tissues. There was no difference in mRNA levels of ICAM-1, P-selectin, and E-selectin between the C and G groups in colon tissues. DSS-induced colitis greatly upregulated the adhesion molecule genes expressed by activated endothelial cells. Compared to the DC group, the expression levels of ICAM-1, P-selectin, and E-selectin mRNA were suppressed in the DG group (Figure 8).

3.6. T Lymphocyte Subsets in the Colon Mucosa. CD3 is the cell surface marker of T cells. CD3 and CD4 double-positive cells are considered Th cells, whereas Tc cells coexpress CD3

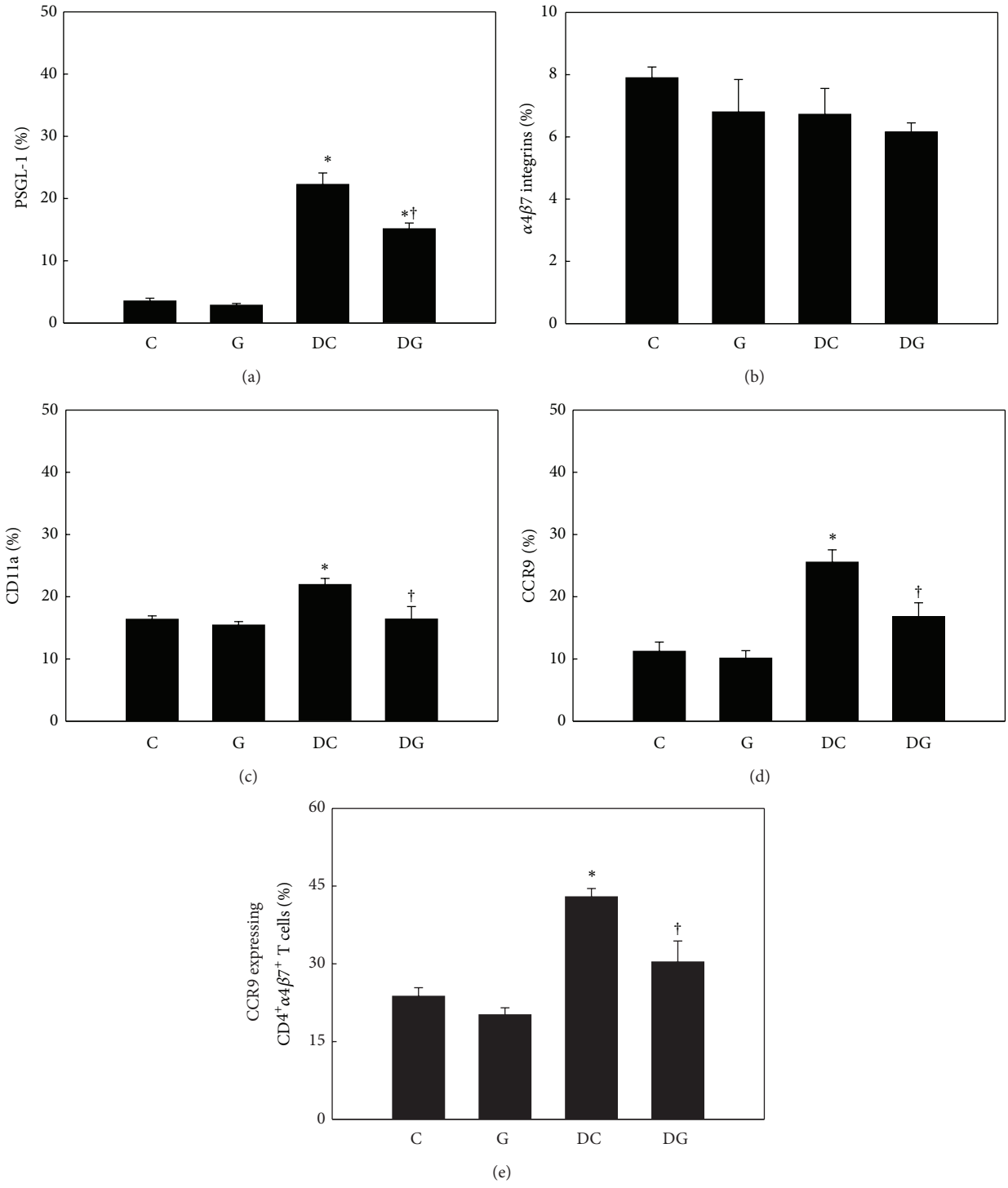

Figure 5: Percentage of adhesion molecule- and chemokine receptor-expressing T helper (Th) cells in mesenteric lymph nodes (MLNs). CD4-positive MLN lymphocytes were gated to analyze expressions of PSGL-1, $\alpha 4\beta 7$ integrins, CD11a, and CCR9 by flow cytometry ((a)–(d)). (e) Expression of CCR9 by CD4 and $\alpha 4\beta 7$ integrin double-positive MLN lymphocytes. Values are shown as the mean ± SEM. *Significantly different from the C group ($P < 0.05$). †Significantly different from the DC group ($P < 0.05$).

and CD8. As shown in Figure 9, the immunoreactive intensity of Th cells was higher in the DC group than the DG group. However, intensities of Tc cell populations did not differ between the DC and DG groups (Figure 10).

4. Discussion

DSS is a heparin-like polysaccharide which results in acute chemical toxicity that disrupts the intestinal epithelial cell

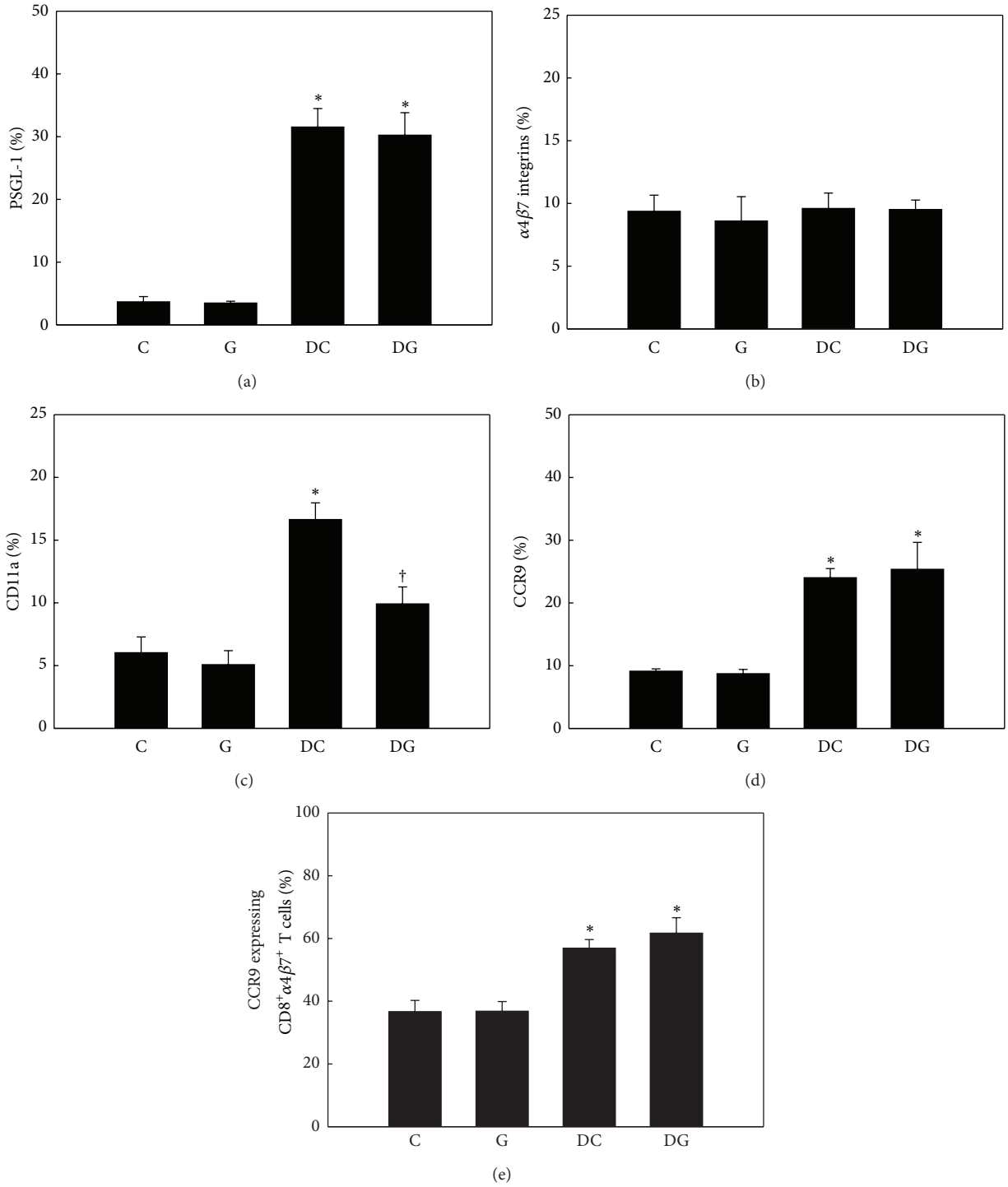

Figure 6: Percentage of adhesion molecule- and chemokine receptor-expressing cytotoxic T (Tc) cells in blood. CD8-positive blood lymphocytes were gated to analyze expressions of PSGL-1, $\alpha 4\beta 7$ integrins, CD11a, and CCR9 by flow cytometry ((a)–(d)). (e) Expression of CCR9 by CD8 and $\alpha 4\beta 7$ integrin double-positive blood lymphocytes. Values are shown as the mean ± SEM. *Significantly different from the C group ($P < 0.05$). †Significantly different from the DC group ($P < 0.05$).

barrier [21]. DSS-induced colitis is characterized by extensive crypt and epithelial cell damage with ulceration, tissue edema, and infiltration of immune cells predominantly in the distal colon that mimics the histological features of UC [22]. Recent studies indicated that DSS-induced morphological and biochemical damage also extends to the small intestines [23]. Susceptibilities to DSS-induced colitis differ in various inbred mouse strains [24]. A single cycle of DSS exposure to the C57BL/6 strain was found to develop acute colitis which later proceeds to chronic inflammation, and T cell

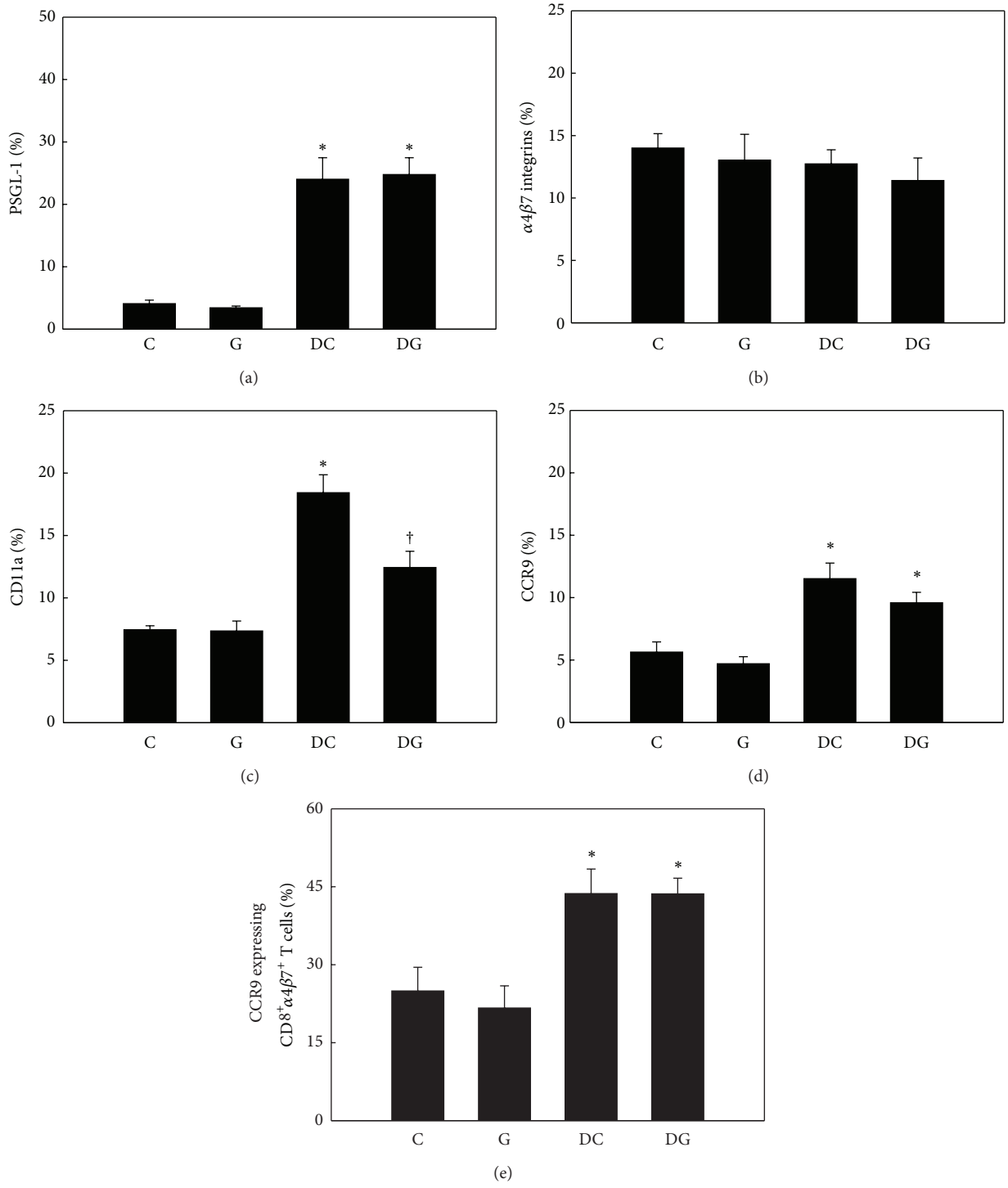

Figure 7: Percentage of adhesion molecule- and chemokine receptor-expressing cytotoxic T (Tc) cells in mesenteric lymph nodes (MLNs). CD8-positive MLN lymphocytes were gated to analyze expressions of PSGL-1, $\alpha 4\beta 7$ integrins, CD11a, and CCR9 by flow cytometry ((a)–(d)). (e) Expression of CCR9 by CD8 and $\alpha 4\beta 7$ integrin double-positive MLN lymphocytes. Values are shown as the mean ± SEM. *Significantly different from the C group ($P < 0.05$). †Significantly different from the DC group ($P < 0.05$).

migration to the colon plays an important role in the progression to chronicity [25]. Regarding the high sensitivity to DSS, C57BL/6 mice were used in this study to analyze the consequences of DSS exposure on adhesion molecules and chemokine receptors involved in T cell trafficking to the intestines.

IBD is associated with a massive influx of immune cells into the gut. Previous studies indicated that increased local

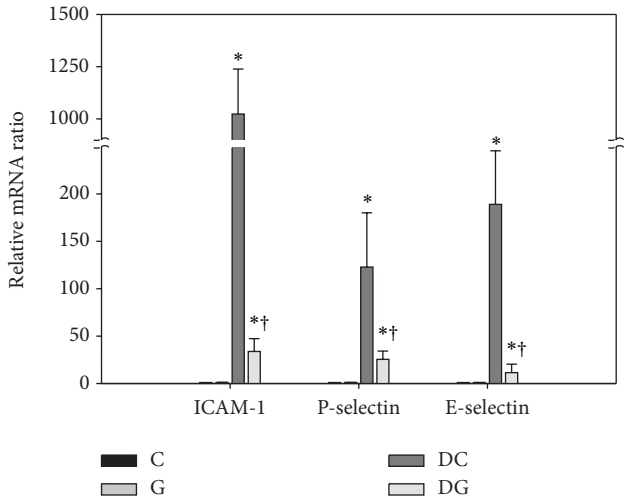

Figure 8: Gene expression of endothelial adhesion molecules in colon tissues. mRNA levels were analyzed by a real-time PCR. The C group was used as a calibrator, and the data were presented as the fold change in gene expression relative to the calibrator. Values are shown as the mean ± SEM. *Significantly different from the C group ($P < 0.05$). †Significantly different from the DC group ($P < 0.05$).

secretion of proinflammatory cytokines by the inflamed colon leads to upregulated expressions of vascular adhesion molecules, resulting in a sustained influx of inflammatory cells [26]. Although inflammation predominantly occurs in the GI tract, IBD patients are likely to develop extraintestinal manifestations [27] that may be attributed to aberrant activation and homing of T cells [28]. A recent study indicated that circulating $CD4^+$ and $CD8^+$ T cells are activated in both CD and UC patients, and these cells were correlated with leakage of microbial products from the impaired intestinal barrier [5]. Also, the severity of DSS-induced colitis was correlated with the immune response of MLNs [29]. A similar phenomenon was also observed in this study. Our results indicated that expressions of adhesion molecules and CCR9 on Th cells and Tc cells significantly increased in both blood and MLNs after DSS exposure. These findings suggest that both local and systemic T cells are activated. The role of Th cells in the pathogenesis of IBD has been widely studied. Dysregulation of Th cells can lead to immune cell infiltration into the intestinal mucosa and cause persistent inflammation [3, 6]. The pathogenic role of Tc cells in IBD has been less investigated. A previous study showed that antigen-specific Tc cells caused relapsing colitis in normal mice due to the cytolytic function against the intestinal epithelium [30]. Lee et al. [4] reported a gene expression profile of circulating $CD8^+$ T cells that predicted a more aggressive disease course for IBD patients. However, the modulatory mechanism of Tc cells in IBD is still under investigation.

Naïve T cells constantly recirculate between the blood and secondary lymphoid organs. Once activated in secondary lymphoid organs, they become effector T cells that express adhesion molecules and chemokine receptors which control their extravasation into nonlymphoid tissue sites [31]. T cell trafficking to the gut and gut-associated lymphoid tissues (GALTs) requires $\alpha 4\beta 7$ integrins. The ligand MAdCAM-1 is constitutively expressed by the mucosal endothelium in the small intestine and colon [32]. CCR9 is thought to participate in the specific localization of T cells to the small intestines because the sole ligand for CCR9, C-C chemokine ligand 25 (CCL25), is strongly expressed by the small intestinal epithelium [33]. However, Wurbel et al. [34] revealed that CCL25 expression increased during the recovery phase after acute DSS administration, suggesting a regulatory role of CCR9/CCL25 interactions during colonic inflammation. In this study, percentages of PSGL-1-, LFA-1-, and CCR9-expressing T cells were upregulated in acute DSS colitis, whereas expression levels of $\alpha 4\beta 7$ integrins in colitic mice did not differ from those of normal mice. It is possible that T cell trafficking into the gut during acute DSS exposure is less dependent on $\alpha 4\beta 7$ integrins. In support of our findings, Wang et al. [35] reported that localization of T cells to the intestines was relatively unaffected by $\alpha 4\beta 7$ blockade during acute DSS-induced colitis.

Gln is a critical fuel source for enterocytes and immune cells. Gln supplementation attenuates gut injury by a complex mechanism, which involves protecting the epithelial barrier function, reducing oxidative stress, and modulating inflammatory responses [36]. The local and systemic immunomodulatory effects of Gln have been discussed in various experimental colitis models via different administration routes. Studies using rodents with trinitrobenzene sulfonic acid-induced colitis indicated that Gln given by the rectal route inhibits nuclear factor (NF)-κB- and STAT-mediated inflammation in colon tissues [15] and further prevents colon fibrosis through downregulating gene pathways that contribute to the accumulation of matrix proteins [37]. We recently demonstrated that intraperitoneal pretreatment with alanyl-Gln, a Gln-containing dipeptide widely used in parenteral nutrition, suppresses cytokine expression in blood Th cells, reduces NF-κB-mediated inflammatory responses in the colon, and upregulates expressions of genes which promote recovery of the colonic mucosa [17, 38].

Because most exogenous Gln is absorbed in the proximal small intestine, it might not reach the inflamed colon at a sufficient concentration to modulate inflammatory responses [36]. However, the enteral route of Gln administration still showed protective effects against DSS-induced damage. Oral Gln supplementation reduced the feces water content, enhanced expression of HSPs in the colonic mucosa, and ameliorated colon injury caused by DSS exposure [16, 39, 40]. A previous study indicated that oral Gln attenuated leukocyte adhesion and emigration in a rodent model of indomethacin-induced ileitis [41]. In this study, we demonstrated that oral Gln administration suppressed adhesion molecules and CCR9 expressed by T cells and downregulated the mRNA levels of adhesion molecules expressed by endothelium in colon tissues. The histological findings also support the results that Gln administration suppressed the infiltration of Th cells into the colon mucosa. Gln consumption is an important component of T cell activation [42, 43]. Different susceptibilities of Th and Tc cells to Gln supplementation may be explained by a higher cell population of Th cells, which

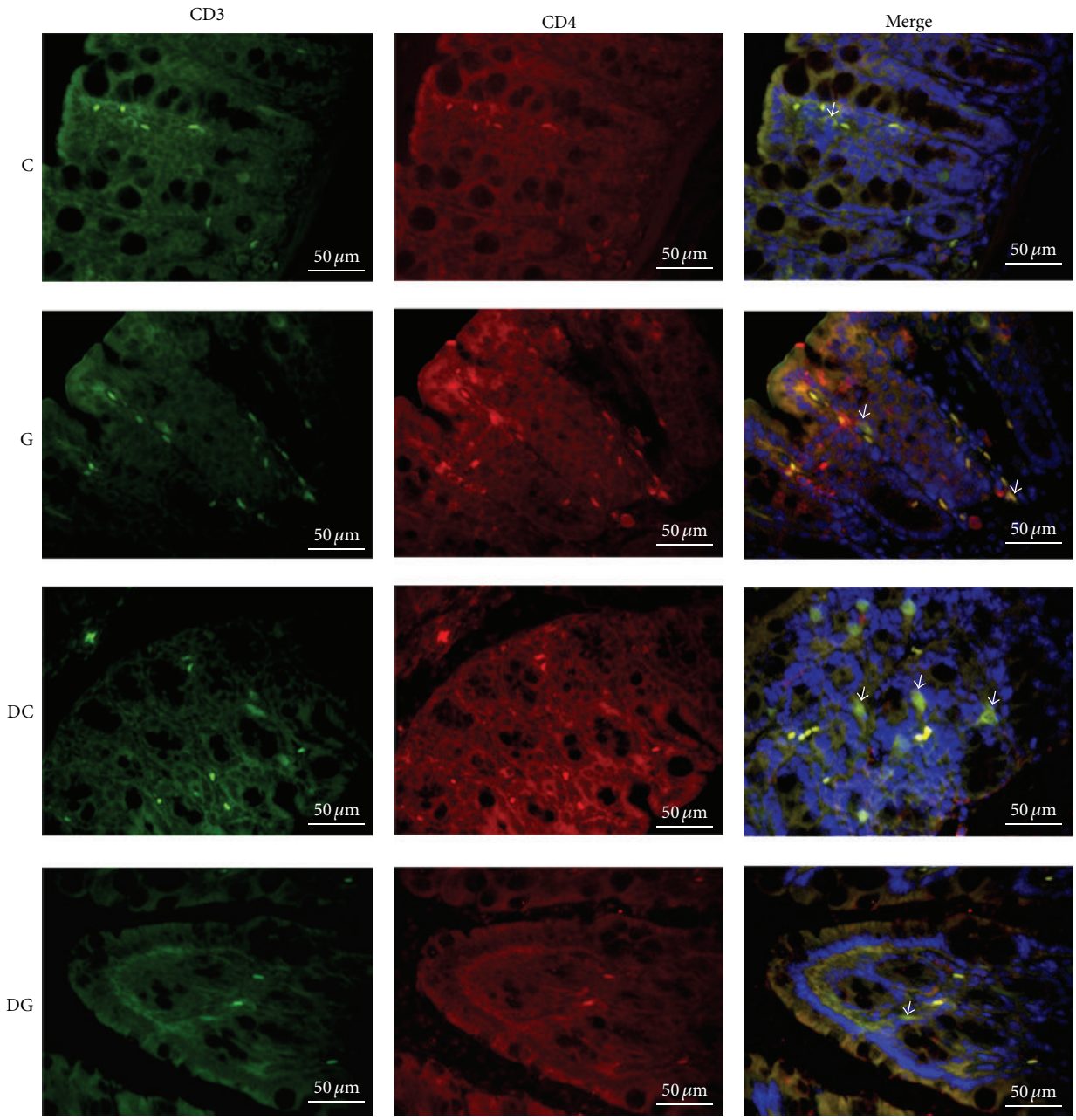

FIGURE 9: Representative examples of the immunofluorescence staining of T helper cells. In the left column, cell surface antigens were stained for CD3 (green, FITC), and staining of CD4 cells (red, rhodamine) is illustrated in the middle column. The nucleus is stained with DAPI (blue), and the last column presents a colocalized fluorescence image of $CD3^+CD4^+$ T cells.

competes as a Gln source with Tc cells. Further studies are needed to investigate the molecular mechanisms involved in gene expressions of adhesion molecules and CCR9 regulated by Gln.

In conclusion, this study showed for the first time that pretreatment with oral Gln reduced adhesion molecule- and CCR9-expressing T cells induced by DSS exposure. The inhibitory abilities against adhesion molecule and CCR9 expressions were more obvious in Th cells than Tc cells. Also, Gln supplementation reduced gene expressions of endothelial adhesion molecules in colons, prevented BW loss, and attenuated colon edema in colitic mice. Our results imply that dietary Gln prevented Th cell trafficking into colon tissues and provide a new mechanism of Gln supplementation that has beneficial effects on ameliorating the severity of acute DSS-induced colitis.

Conflict of Interests

The authors declare that they have no conflict of interests.

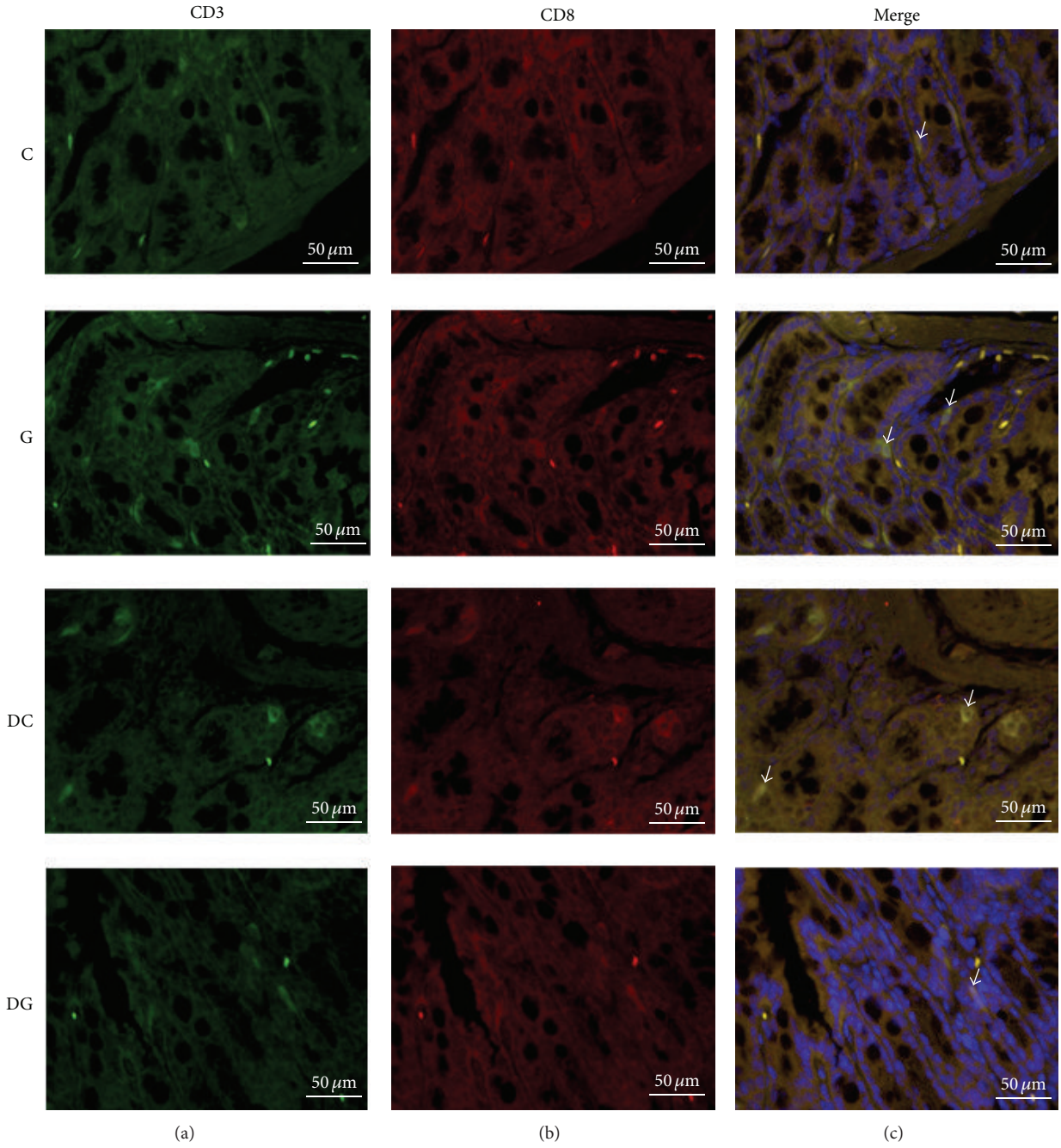

Figure 10: Representative immunofluorescence images of cytotoxic T cells. In the left column, cell surface antigens were stained for CD3 (green, FITC), and the staining of CD8 cells (red, rhodamine) is illustrated in the middle column. The nucleus is stained with DAPI (blue), and the last column presents a colocalized fluorescence image of $CD3^+CD8^+$ T cells.

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Clinical Study

Effect of Probiotic (VSL#3) and Omega-3 on Lipid Profile, Insulin Sensitivity, Inflammatory Markers, and Gut Colonization in Overweight Adults: A Randomized, Controlled Trial

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To evaluate the effects of probiotic (VSL#3) and omega-3 fatty acid on insulin sensitivity, blood lipids, and inflammation, we conducted a clinical trial in 60 overweight (BMI > 25), healthy adults, aged 40–60 years. After initial screening the subjects were randomized into four groups with 15 per group. The four groups received, respectively, placebo, omega-3 fatty acid, probiotic VSL#3, or both omega-3 and probiotic, for 6 weeks. Blood and fecal samples were collected at baseline and after 6 weeks. The probiotic (VSL#3) supplemented group had significant reduction in total cholesterol, triglyceride, LDL, and VLDL and had increased HDL ($P < 0.05$) value. VSL#3 improved insulin sensitivity ($P < 0.01$), decreased hsCRP, and favorably affected the composition of gut microbiota. Omega-3 had significant effect on insulin sensitivity and hsCRP but had no effect on gut microbiota. Addition of omega-3 fatty acid with VSL#3 had more pronounced effect on HDL, insulin sensitivity and hsCRP. Subjects with low HDL, insulin resistance, and high hsCRP had significantly lower total lactobacilli and bifidobacteria count and higher *E. coli* and bacteroides count.

1. Introduction

Inflammatory mediators have been recognized as factors that increase the risk of insulin resistance, diabetes, and cardiovascular diseases. Inflammation is also associated with metabolic syndrome, which in turn increases the risk of coronary heart disease [1–3]. Forty-four percent of the diabetes burden, 23% of the ischaemic heart disease burden, and between 7% and 41% of certain cancer burdens are attributable to overweight and obesity [4]. In India, 12.6% of women and 9.3% of men are obese [5].

Lipids and lipoproteins are well known risk factors for ischemic heart disease. Elevated levels of triglyceride, cholesterol, and LDL are documented as risk factors for atherogenesis. It is noteworthy that CRP plasma levels even slightly higher from the conventional upper limit of normal (1 mg/dL) have been associated with a 2-3-fold increase in risk of future myocardial infarction, stroke, and peripheral atherosclerosis among apparently healthy middle-aged men and women [6]. The association between CRP and cardiovascular disease is more than a mere epiphenomenon; in other words, this acute-phase reactant is not just a marker of increased inflammatory activity, but it is also directly involved in the pathogenesis of atherothrombosis through several mechanisms and is associated with several cardiovascular risk factors, such as age, smoking, hypertension, exercise, plasma lipids, homocysteine, and body mass index (BMI) [7, 8]. CRP has been suggested to be higher both in overweight (BMI 25–29.9 kg/m^2) and obese (BMI $\geq$ 30 kg/m^2) patients than in normal weight (BMI < 25 kg/m^2) subjects and has been attributed to be mediated by elevated cytokines, such as interleukin-6 (IL-6) and tumor necrosis

factor a (TNF-α), which are both expressed in adipose tissue [9–13].

Past studies have demonstrated that probiotics could improve lipid disorders by lowering blood cholesterol levels and increasing resistance of low density lipoprotein to oxidation [14, 15]. A combination of probiotic strains was also found to reduce the onset of insulin resistance and diabetes in animals [16]. VSL#3 is a commercially available mixture of probiotics containing a high concentration (450 billion colonies/sachet) of viable, lyophilized bifidobacteria, lactobacilli, and *Streptococcus thermophilus*. Li et al. [16] found that dietary supplementation with VSL#3 improved hepatic insulin resistance in diabetic ob/ob mice after 4 weeks of treatment. These findings provided the rationale for our present study, in which we tested the effectiveness of VSL#3 on inflammation, lipid profile, and insulin sensitivity in apparently healthy overweight human subjects. Omega-3 has also been found to improve obesity-induced metabolic syndrome through regulating chronic inflammation [17–19]. Therefore in the present study, we compared the effects of probiotics with omega-3 fatty acid, which is well known for its anti-inflammatory properties and cholesterol lowering effect [20].

Thus, this study investigated whether VSL#3 alone or with omega-3 as adjunct improved lipid profile, insulin sensitivity, and inflammatory responses which are indicators of risk for metabolic syndrome and, ultimately, heart disease, diabetes, and stroke, in a healthy overweight population.

2. Materials and Methods

The study protocol was approved by the Institutional Ethics Committee (IEC), Indian Council of Medical Research (ICMR). The trial was registered under Clinical Trials Registry India (CTRI/2012/08/002856) (ICMR). Before entering the study, the subjects gave their written informed consent. All clinical investigations were conducted according to the principles expressed in declaration of Helsinki.

Apparently healthy adult volunteers of both sexes, aged between 40 and 60 years and with BMI > 25, were recruited. Those with hypertension, diabetes mellitus, any metabolic disorder, acute gastrointestinal disorders, pregnancy or lactation, alcohol consumption, smoking, antibiotic therapy, or use of related medications before and during the 6-week study period were excluded.

The study was a randomized, placebo-controlled trial. The volunteers were randomly assigned to receive either placebo ($n = 15$), VSL#3 capsules ($n = 15$), omega-3 fatty acid capsules ($n = 15$), or omega-3 capsule + VSL#3 capsule ($n = 15$), for 6 weeks. They were asked not to consume any other probiotic-containing products (from a supplied list) during the study. Subjects were given an identification number and were assigned a treatment code by a scientist blind to the treatments corresponding with the codes. The probiotic and placebo groups could be blinded but it was not possible to blind subjects or field staff to the omega-3 supplementations, as the capsules looked different. Nevertheless, all the investigators, including the medical doctor collecting clinical data and those collecting anthropometric measurement, the laboratory technician (who carried out all the biochemical parameters and caecal colonization), and the statistician, were blind to the treatment. After completion of the biochemical and statistical analysis, the groups were decoded. Hence, there was minimum chance of bias entering the study results or interpretation.

The placebo and VSL#3 groups were given identical-looking coded wraps containing either placebo or probiotic and were instructed to take one capsule every day before any meal. The third group was advised to take one capsule of omega-3 fatty acid before breakfast, and the fourth group was advised to take one omega-3 capsule and one probiotic capsule before breakfast.

2.1. Probiotics (VSL#3), Omega-3, and Placebo. VSL#3 (manufactured in India by Sun Pharmaceutical Ind. Ltd.) is a freeze-dried pharmaceutical probiotic preparation containing 112.5×10^9 CFU/capsule of three strains of bifidobacteria (*Bifidobacterium longum, Bifidobacterium infantis, and Bifidobacterium breve*), four strains of lactobacilli (*Lactobacillus acidophilus*, *Lactobacillus paracasei,* Lactobacillus *delbrueckii* subsp. *bulgaricus,* and *Lactobacillus plantarum),* and one strain of *Streptococcus salivarius* subsp. *thermophilus.* Identical-looking placebo capsules containing 40 mg microcrystalline cellulose were used for blinding. The capsules were stored at 2 to 8°C prior to distribution and the subjects were instructed to refrigerate the capsules. Bacterial viability was confirmed at the Department of Microbiology and Immunology, National Institute of Nutrition (ICMR), Hyderabad, by plating serial dilutions of bacterial suspensions onto *Bifidobacterium* Agar, *Lactobacillus* MRS Agar (HiMedia, India), and *Streptococcus thermophilus* Agar (HiMedia Laboratories Pvt. Ltd., India) followed by incubation in an anaerobic jar with anaerobic gas pack (HiMedia, India) at 37°C for 48 hours. The omega-3 capsule contained 180 mg EPA and 120 mg DHA per capsule (Dr. Reddy's Laboratories Ltd. Hyderabad, India).

2.2. Biochemical Analyses. Blood samples were drawn at baseline and after 6 weeks of intervention (study end), after an overnight fast. Inflammatory markers such as IL-1 β, TNF-α, and IL-6 assays were carried out using ELISA. Measurements of all the serum parameters were done in duplicate and mean concentrations were calculated.

Serum glucose was estimated using Serum Glucose Biosystems kit (Barcelona, Spain). Total cholesterol, triglycerides, and high-density lipoprotein were measured using kits from Lab Care Diagnostics (India) Pvt. Ltd. The Friedewald equation [21] was applied to analyse all the lipid fractions. HOMA was used to evaluate insulin resistance before and after treatment [22]. Serum high-sensitivity C-reactive protein (hs-CRP) was estimated by dbc-hs Krishgen, Biosystems (India) CRP kit.

2.3. Stool Sampling and Colony Counting. Fecal samples were obtained from the subjects at the beginning of the trial and after 45 days, for stool culture. The specimens were

collected in sterile plastic containers and were immediately preserved at 4°C and were analysed on the same day or within 2 days. Fecal samples were homogenized using PBS and serially diluted. Plates were incubated in triplicate using selective media for enumeration of total aerobes (Nutrient agar, HiMedia India), total anaerobes (Schaedler agar, HiMedia India), coliforms (Violet Red Bile Agar, HiMedia India), *E. coli* (Eosin Methylene blue Agar, HiMedia India), bacteroides (Bacteroides Bile Esculin Agar Base, HiMedia India), bifidobacteria (*Bifidobacterium* Agar, HiMedia), lactic acid bacteria (*Lactobacillus* MRS Agar, HiMedia India), and *Streptococcus thermophilus* (*Streptococcus thermophilus* Agar, HiMedia India). Plates were incubated aerobically or anaerobically as appropriate and the colonies were counted after 48 hours.

2.4. Statistical Analysis. Assuming that the probiotics and omega-3 supplementation would reduce hsCRP concentration, a sample size of 15 in each arm was calculated to detect a 20% reduction in hsCRP with treatment, with a power of 80% and 5% significance using power and Sample Size Calculation software, version 3.0.14. Variation between groups at baseline was evaluated by one-way ANOVA. Changes from baseline to endline after treatment were evaluated by applying a one-way ANOVA. Repeated-measures analysis of variance (ANOVA) was used to determine if there were treatment and/or time differences. When there was a significant difference, one-way ANOVA was used followed by Newman-Keuls multiple-comparisons test to identify differences within the same treatment group over time. When there was significant change over time ANCOVA was used to analyse difference between groups after adjusting for significant differences at the baseline. Statistical software SAS 9.1 (SAS Institute, Inc.) was used throughout, and $P < 0.05$ was considered to indicate statistical significance.

3. Results

After screening for inclusion criteria, a total of 60 subjects were recruited. Mean age was 49 years (range 40–60) and mean BMI was 28.79 kg/m^2 (range 27–30). There were 30 females and 30 males. None of the subjects had diabetes or hypertension. Nevertheless, lipid abnormalities (triglycerides $\geq$ 150 mg/dL and HDL < 40 and < 50 mg/dL in men and women, resp.) and insulin resistance (<40 in males and <50 in females) were prevalent in 23.3% and 18.2%, respectively. Low HDL cholesterol (<40 in males and <50 in females) alone was prevalent in 88.3%. Mean ± SD total cholesterol, triglycerides, LDL, and HDL were 186.0 ± 42.83, 131.2 ± 66.60, 124.0 ± 42.75, and 34.6 ± 8.18 mg/dL, respectively. Mean ± SD fasting blood glucose and insulin concentrations were 87.9 ± 8.18 mg/dL and 18.2 ± 1.35 U/mL, respectively. HsCRP levels were elevated (>3 mg/L) in 83% mean ± SD 5.7 ± 2.21 mg/L. All the inflammatory markers (IL-1β, TNF-α, and IL-6) except hsCRP were maintained at low levels, but the baseline inflammatory markers including hsCRP were positively correlated ($P < 0.05$) with LDL, VLDL, triglyceride, and total cholesterol and negatively correlated with HDL. Moreover, hsCRP and IL-1β and TNF-α and IL-6 positively correlated with insulin resistance ($P < 0.05$).

The baseline characteristics and biochemical parameters of the subjects are given in Table 1. The baseline fasting blood glucose, insulin levels, and hsCRP concentration were comparable between groups. Insulin resistance as measured by HOMA was also comparable. As for the lipid profile, the baseline HDL level was significantly lower in the probiotic group and LDL was significantly higher in the probiotic + omega-3 group. Triglycerides and VLDL were comparable.

Total fecal aerobes and anaerobes (means ± SE) were $6.0 \times 10^7 \pm 0.076$ and $3.4 \times 10^9 \pm 0.169$, respectively. Total lactobacillus, bifidobacteria, streptococcus, coliforms, *E. coli,* and bacteroides were $6.3 \times 10^6 \pm 0.121$, $5.8 \times 10^8 \pm 0.167$, $5.1 \times 10^2 \pm 0.148$, $4.5 \times 10^6 \pm 0.196$, $4.1 \times 10^6 \pm 0.122$, and $7.9 \times 10^6 \pm 0.325$, respectively. Subjects with lipid abnormalities had lower total lactobacilli, bifidobacteria, and streptococcus and higher *E. coli* and bacteroides. A similar trend was observed when subjects were categorized as those with insulin resistance and those without insulin resistance based on HOMA (3.6). Also, subjects with more than 3 mg/L hsCRP had significantly lower lactobacilli, bifidobacteria, and streptococcus and higher *E. coli* when compared with those who had less than 3 mg/L.

3.1. Inflammatory Markers after Probiotic Supplementation. The baseline mean ± SE of hsCRP was comparable between the groups (Table 1). Mean hsCRP reduced significantly with probiotic ($P < 0.01$), and there was 24.5% and 34.6% reduction in hsCRP with probiotics ($P < 0.01$) and probiotic plus omega-3 ($P < 0.01$) groups, respectively (Table 1). Though there was a modest decrease in hsCRP with omega-3, there was no significant change when compared to the placebo group. When we assessed the relative change versus baseline, probiotics and probiotic + omega-3 were significantly different from the placebo (Table 1). There was modest reduction in proinflammatory cytokines, IL-1β, TNF-α, and IL-6 with probiotics and probiotic + omega-3.

3.2. Lipid Profile after Probiotic Supplementation. In the probiotic group HDL increased by 18.5% ($P < 0.01$); LDL ($P < 0.05$), triglycerides, and VLDL ($P < 0.01$) decreased by 7.04%, 5.8%, and 12.98%, respectively (Table 1). In the omega-3 group, total cholesterol, triglycerides, LDL, and VLDL decreased and HDL increased significantly by 6.7% ($P < 0.01$). HDL increased by 23.2% and LDL decreased by 10.7%; triglycerides decreased by 7.78% and VLDL by 7.78% ($P < 0.01$) in the probiotic with omega-3 group, compared with baseline levels (Table 1). The relative change versus baseline in probiotics was significantly different from the placebo group and probiotic + omega-3 (Table 1).

3.3. Insulin Resistance after Probiotic Supplementation. Fasting blood glucose (FBG) rose slightly in the placebo group but reduced significantly ($P < 0.05$) in the probiotic, omega-3, and probiotic + omega-3 combination groups. Similarly, the insulin levels reduced significantly ($P < 0.05$) in the

Table 1: Lipids, inflammatory markers, and insulin sensitivity after treatment.

	Placebo		Probiotic		Omega-3		Probiotic + omega-3	
	Pretreatment	Posttreatment	Pretreatment	Posttreatment	Pretreatment	Posttreatment	Pretreatment	Posttreatment
Fasting blood glucose (mg/dL)	89.30 ± 3.3	91.92 ± 3.18^{*a}	88 ± 1.01	79.38 ± 1.05^{*b}	87.60 ± 1.4	83.93 ± 1.23^{*c}	86.70 ± 2.15	74.46 ± 1.188^{*cd}
Cholesterol (mg/dL)	197.80 ± 13.92	197.91 ± 13.93^{a}	165.10 ± 5.76	156.06 ± 5.85^{*a}	165.50 ± 9.73	161.06 ± 9.74^{*a}	215.10 ± 7.86	204.46 ± 7.73^{*a}
Triglyceride (mg/dL)	128 ± 2.26	128.85 ± 27.7^{a}	140.70 ± 17.6	133.13 ± 17.39^{*ab}	105.90 ± 6.53	102.62 ± 6.44^{*a}	150.00 ± 8.49	138.74 ± 8.35^{*b}
LDL (mg/dL)	136.30 ± 14.19	136.67 ± 14.2^{a}	102.80 ± 4.91	94.50 ± 4.31^{b}	106.10 ± 10.1	99.87 ± 10.08^{*b}	150.30 ± 8.41	134.58 ± 7.93^{*a}
HDL (mg/dL)	35.90 ± 2.85	35.46 ± 2.85^{a}	29.60 ± 1.12	34.93 ± 1.08^{*a}	38.20 ± 1.65	40.66 ± 1.62^{*b}	34.80 ± 2.23	42.13 ± 1.89^{*b}
VLDL (mg/dL)	25.60 ± 5.45	25.77 ± 5.54^{a}	32.60 ± 4.38	26.62 ± 3.47^{a}	21.10 ± 1.3	20.51 ± 1.28^{*a}	30.00 ± 1.69	27.74 ± 1.67^{*a}
Atherogenic factor	196.80 ± 13.92	196.91 ± 13.93^{a}	164.10 ± 5.76	155.75 ± 5.8^{a}	164.50 ± 9.73	160.06 ± 9.74^{*a}	214.10 ± 7.86	203.46 ± 7.73^{*a}
Insulin level (μU/mL)	17.90 ± 0.52	18.26 ± 0.43^{a}	18.40 ± 0.27	17.59 ± 0.28^{*b}	18.50 ± 0.23	17.84 ± 0.22^{*a}	18.00 ± 0.3	16.75 ± 0.25^{*b}
Insulin resistance	3.90 ± 0.1	4.11 ± 0.12^{*a}	4.00 ± 0.03	3.44 ± 0.04^{*b}	4.00 ± 0.08	3.69 ± 0.07^{*c}	3.80 ± 0.08	3.07 ± 0.04^{*d}
CRP (mg/L)	5.30 ± 0.58	5.35 ± 0.58^{*a}	5.60 ± 0.52	4.36 ± 0.49^{*a}	5.60 ± 0.74	5.26 ± 0.72^{*a}	6.20 ± 0.41	4.22 ± 0.41^{*a}

Values are mean ± SEM.

*Superscript indicates a significant difference between baseline and after treatment.

* <0.05 significantly different from the pretreatment level.

^{a,b,c,d}Superscript indicates significant <0.05 difference in relative changes versus baseline compared between supplemented and placebo group.

probiotic, omega-3 and probiotic + omega-3 combination groups (Table 1).

When ≥2.5 was considered as the cut-off point for insulin resistance (IR) by HOMA, all the subjects before and after supplementation were insulin resistant. However, the mean insulin resistance, which was 4.0 ± 0.3 in the probiotic group, decreased to 3.4 ± 0.04 ($P < 0.05$) after treatment. Similarly, there was a modest improvement in insulin sensitivity in the probiotic + omega-3 combination group. When ≥3.6 was considered as the cut-off point for insulin resistance (IR), all 15 subjects in the probiotic group were resistant at baseline, which reduced to 86.% (13/15) after treatment with probiotic. In the omega-3 group 86.6% (13/15) subjects were IR which was reduced ($P < 0.05$) to 46.6% (7/15) after treatment. In the probiotic and omega-3 combination group 80% (12/15) subjects were IR (≥3.6), but none were IR (≥3.6) after treatment ($P < 0.01$). In the placebo group 73% (11/15) were IR before treatment, and 93% (14/15) were IR after treatment. When we assessed the relative change versus the baseline, the probiotic group and the probiotic + omega-3 groups were significantly different from the placebo and omega-3 group.

3.4. Atherogenic Index. Atherogenic index was significantly ($P < 0.01$) decreased in the probiotic, omega-3, and combination groups.

3.5. Stool Microbiota after Probiotics and Omega-3 Supplementation. There was a significant increase in the concentration of total aerobes, total anaerobes, lactobacillus, bifidobacteria, and streptococcus in the probiotic group and probiotic + omega-3 supplemented groups. In the probiotic + omega-3 group there was a significant effect on bacteroides, coliforms, and *E. coli* as well. In the omega-3 group there was no effect on gut microbiota (Table 2).

4. Discussion

This randomized, controlled clinical trial showed that the probiotic preparation VSL#3 affected insulin sensitivity, lipid profile, and atherogenic index favourably and reduced hsCRP, a marker of inflammation, in overweight/obese adults. Probiotic given in combination with omega-3 was more effective than probiotic alone. Omega 3 intake is usually low in Indian population. However, omega-3 supplementation showed only marginal effects on all the parameters. Nevertheless, when it was given along with probiotic, the beneficial effect of VSL#3 observed was enhanced.

Human studies evaluating the hypocholesterolemic potential of probiotics showed beneficial effect both with *Lactobacillus* and *Bifidobacterium* strain [23, 24]. VSL#3 used in our study contained both lactobacilli and bifidobacteria strains, and the hypocholesterolemic effect was similar to that reported elsewhere [23–27]. Similarly, a study on 48 volunteers for a period of ten weeks showed significant reduction in serum cholesterol concentration with daily consumption of 200 gms of yoghurt containing *Lactobacillus acidophilus* L1 [23]. Increase in serum HDL was also observed with prolonged consumption of 300 g/day of yoghurt supplemented with *Lactobacillus acidophilus* 145 and *B. longum* 913 over 21-week period [25]. Apart from reduction in LDL-cholesterol some studies observed reduction of fibrinogen and proatherogenic markers with *Lactobacillus plantarum* [26, 27]. In the current study we observed reduction in total cholesterol and hsCRP and increase in HDL. However, there are quite a few studies that have failed to register any effect on cholesterol with probiotics consumption [28–30]. Administration of *Lactobacillus rhamnosus* LC705 did not influence blood cholesterol levels in 38 men and another study with *Lactobacillus fermentum* or *Lactobacillus acidophilus* failed to demonstrate any change in serum lipid levels in volunteers [28–30]. These controversial observations may be attributed to factors such as strains of probiotics used in the study or dosage and duration of treatment. One mechanistic study from our lab showed that cholesterol reduction was possible only with probiotics containing bile salt hydrolase (BSH) gene. BSH gene-negative probiotics had no effect on lipid profile [31, 32]. Though the present study does not show the mechanism of cholesterol reduction by VSL#3, it may be speculated that VSL#3 bacteria may contain the BSH gene that may be responsible for cholesterol reduction. Other proposed mechanisms include fermentation of dietary fibre in the large intestine, which releases short-chain fatty acids (SCFA) especially acetic and propionic acids which are absorbed in the blood, pass into the liver, and enter the metabolic pathways [33, 34]. There are other studies suggesting that the probiotics might reduce serum cholesterol levels due to their ability to compete with cholesterol for intestinal absorption [15].

Plasma cholesterol and triacylglycerol concentrations have been shown to be lowered by fish oil, which is rich in omega-3, through inhibition of triacylglycerol and VLDL synthesis in the liver [35–37]. Consumption of fish oil in comparison with vegetable oils such as safflower or olive oil reduces apolipoprotein B production [33]. The cholesterol-regulating effect was more pronounced when VSL#3 was given along with omega-3 fatty acid in the current study though the mechanism of synergistic effect needs to be explored.

Probiotic yogurt containing *Lactobacillus acidophilus* La5 and *Bifidobacterium lactis* Bb12 improved fasting blood glucose in humans with type 2 diabetes [38]. Our study showed a similar effect on fasting glucose and insulin and improved insulin sensitivity in overweight nondiabetic subjects. Effect of VSL#3 on insulin resistance was shown by Li et al. in a mice model, which demonstrated decreased hepatic insulin resistance. Inhibition of proinflammatory cytokines that would induce insulin resistance has been proposed by Nestel et al. [37]. Effect of VSL#3 on insulin resistance could have been due to reduction in hsCRP and amelioration of inflammation as suggested elsewhere [39].

Insulin resistance and increased CRP concentrations have been shown to be significantly associated with several cardiovascular risk factors, such as age, smoking, hypertension, exercise, plasma lipids, homocysteine, and body mass index (BMI) [8]. Concerning the relationship between CRP

Table 2: Fecal bacterial count before and after treatment.

	Placebo		Probiotic		Omega-3		Pro + Omega-3	
	Pretreatment	Posttreatment	Pretreatment	Posttreatment	Pretreatment	Posttreatment	Pretreatment	Posttreatment
Total bacteria	7.74 ± 0.21	7.71 ± 0.18[a]	8.77 ± 0.09	9.13 ± 0.18[*b]	7.7 ± 0.19	7.78 ± 0.16[a]	8.83 ± 0.09	9.55 ± 0.12[**b]
Total anaerobes	9.32 ± 0.17	9.27 ± 0.12[a]	9.66 ± 0.06	10.71 ± 0.07[**b]	9.44 ± 0.18	9.54 ± 0.09[a]	9.61 ± 0.1	10.86 ± 0.05[**b]
Lactobacillus	6.87 ± 0.12	6.86 ± 0.14[a]	6.77 ± 0.1	7.95 ± 0.13[**b]	6.76 ± 0.26	6.8 ± 0.12[a]	6.76 ± 0.12	7.96 ± 0.12[**b]
Bifidobacteria	8.89 ± 0.17	8.88 ± 0.14[a]	8.71 ± 0.11	9.94 ± 0.01[**b]	8.71 ± 0.08	8.75 ± 0.17[a]	8.71 ± 0.08	9.9 ± 0.03[**b]
Streptococcus	2.81 ± 0.21	2.80 ± 0.31[a]	2.66 ± 0.1	8.96 ± 0.16[**b]	2.67 ± 0.19	2.89 ± 0.29[a]	2.67 ± 0.11	8.99 ± 0.13[**b]
Coliform	6.82 ± 0.19	6.83 ± 0.13[a]	6.59 ± 0.1	6.35 ± 0.2[a]	6.54 ± 0.13	6.44 ± 0.12[a]	6.99 ± 0.14	6.58 ± 0.11[**a]
E.coli	6.59 ± 0.14	6.93 ± 0.19[**a]	6.58 ± 0.09	6.47 ± 0.13[b]	6.55 ± 0.18	6.53 ± 0.09[a]	6.69 ± 0.19	6.39 ± 0.09[b]
Bacteroides	6.61 ± 0.19	6.98 ± 0.15[**a]	6.99 ± 0.18	7.57 ± 0.17[**b]	6.59 ± 0.1	6.45 ± 0.25[c]	6.96 ± 0.15	7.87 ± 0.11[**d]

[*] <0.05 significantly different from the pretreatment level.

[**] <0.01 significantly different from the pretreatment level.

[a,b,c,d]Superscript indicates significant <0.05 difference in relative changes versus baseline compared between supplemented and placebo group.

Bacterial count (log 10 CFU/g fecal dry weight ± standard error mean).

concentration and BMI level, the Third National Health and Nutrition Examination survey found that the prevalence of elevated CRP levels (i.e., CRP concentrations ≥0.22 mg/dl) is higher both in overweight (BMI 25–29.9 kg/m^2) and obese (BMI ≥ 30 kg/m^2) patients than in normal weight (BMI < 25 kg/m^2) subjects [40]. In line with this report we found elevated concentration (>3 mg/L) of hsCRP in 83% percent of our overweight subjects. It has been suggested that the association between BMI and CRP might be mediated by cytokines, such as IL-6 and TNF-α, which are both expressed in adipose tissue [9, 10] and are referred to as main regulators of CRP production in the liver [11, 12]. Indeed, in our study, high hsCRP and IL1β, IL-6, and TNF-α were positively correlated with lipid parameters (total cholesterol, triglyceride, LDL, and VLDL) and insulin resistance and negatively correlated with HDL, similar to that observed by Yudkin et al., 1999, in a study of 107 subjects [13].

In addition, the subclinical inflammation that plays a central role in most of the chronic noncommunicable diseases, such as diabetes type 2, has been shown to be linked with the composition of intestinal gut microbiota [41, 42]. Some studies have reported improvement in the mucosal barrier function and decreased intestinal endotoxin levels with bifidobacteria, leading to reduction in systemic inflammation and subsequent reduction in the incidence of diabetes [41]. In line with this, we found lower lactobacilli, bifidobacteria, and streptococcus and higher *E. coli* and bacteroides in subjects with insulin resistance (HOMA = 3.6), higher HDL and those with higher hsCRP (3 mg/L).

The value of using a single-strain probiotic over a combination of probiotic strains or species is a topic of ongoing debate. VSL#3 preparation is a cocktail of eight different probiotics, thus leaving the question of which specific probiotic(s) might be responsible for the beneficial effects described in this study. Nevertheless, the study provides important leads to conduct large clinical trials in subjects with diabetes.

To summarize, we found improved HDL, insulin sensitivity, and amelioration of inflammation (hsCRP). The study also showed increase in lactobacilli and bifidobacteria and reduction in gram negative bacteria with VSL#3 supplementation; nevertheless, improvement in insulin sensitivity and reduction in hsCRP with probiotic + omega-3 was greater than probiotic alone.

Conflict of Interests

The authors report no conflict of interests.

Authors' Contribution

Hemalatha Rajkumar conceived and designed the study, analysed and interpreted data, and finalized the paper. Manoj Kumar wrote the first draft of the paper. Dr. Shiva Prakash Myakala and Naseha Mahmood conceived the project and carried out experiments. Dr. Manoj Kumar and Hanumanth Reddy Challa supervised all the experiments. Dr. Sudarshan Reddy Varikuti carried out experiments. All authors commented on the paper.

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