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# The role of physical and biological gut barriers in modulating crosstalk between the microbiota and the immune system

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Marika Falcone, Federica Facciotti and Julien Diana

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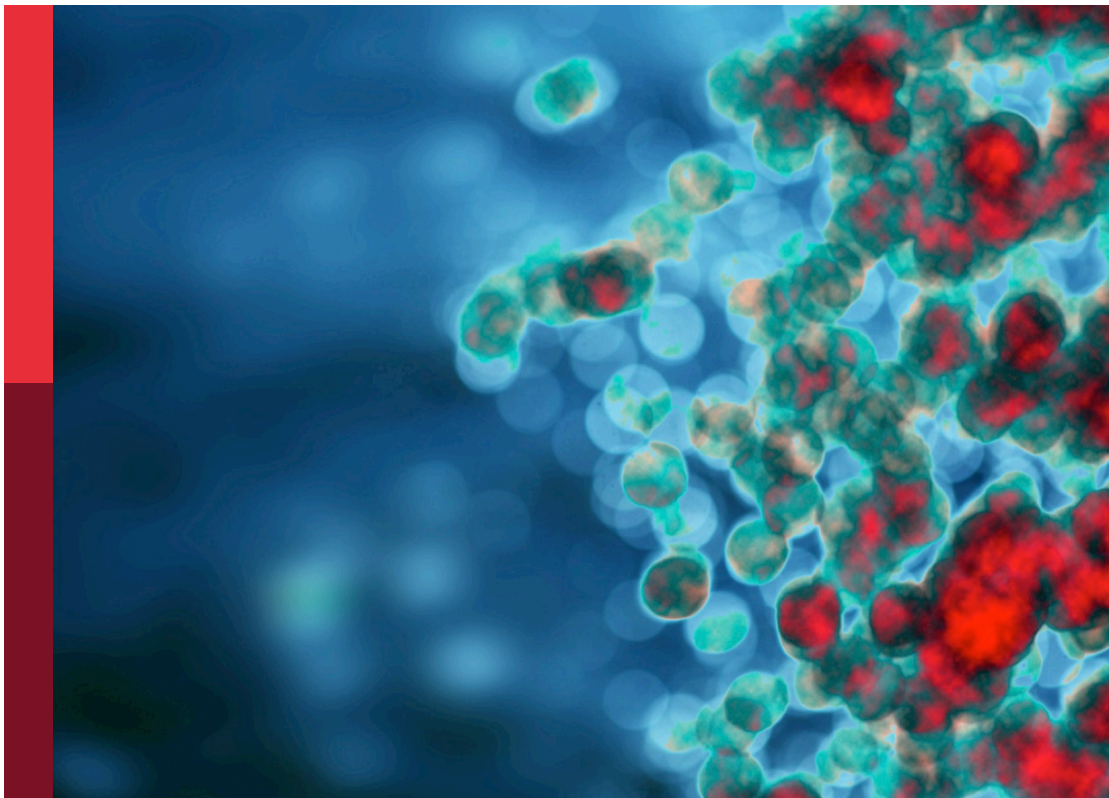

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# The role of physical and biological gut barriers in modulating crosstalk between the microbiota and the immune system

**Topic editors**

Marika Falcone — San Raffaele Hospital (IRCCS), Italy

Federica Facciotti — European Institute of Oncology (IEO), Italy

Julien Diana — Institut National de la Santé et de la Recherche Médicale (INSERM), France

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Pietro Invernizzi,
University of Milano-Bicocca, Italy

*CORRESPONDENCE
Marika Falcone
falcone.marika@hsr.it

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# Editorial: The role of physical and biological gut barriers in modulating crosstalk between the microbiota and the immune system

Vittoria Palmieri and Marika Falcone*

Autoimmune Pathogenesis Unit, IRCCS San Raffaele Scientific Institute, Milan, Italy

KEYWORDS

**gut barrier disruption, microbiota, mucosal immunity, autoimmune disease, allergy**

Editorial on the Research Topic
The role of physical and biological gut barriers in modulating crosstalk between the microbiota and the immune system

The intestine has several means to maintain immune homeostasis and avoid inflammation despite the massive antigenic stimulation from food components and commensal bacteria that are present in the gut mucosa. These include physical barriers such as the mucus layer, the intestinal epithelial barrier (IEB) and the gut vascular barrier (GVB). The integrated response of these combined defense systems is fundamental to containing microbes and their products within the intestine and preventing their systemic spread and ability to activate immune and autoimmune responses in the gut and in extra-intestinal tissues. The main function of the mucus layer is to limit contact between the gut mucosa and harmful molecules present in the intestinal lumen but it is also fundamental in regulating the interaction between the commensal microbiota and the immune system. For example, the mucus layer contains mucins that play key immune regulatory functions (1, 2), such as MUC2, which induces tolerogenic dendritic cells (3). Furthermore, the mucus layer hosts commensal species that are crucial for modulating immunity such as short-chain fatty acid-producing bacteria (4, 5). The IEB is represented by a single layer of epithelial cells held together by a complex junctional system consisting of tight junctions, adherens junctions and desmosomes. Originally, the IEB was believed to be an impermeable barrier that blocks the paracellular passage of macromolecules, but it is now clear that the IEB and the tight junctions are dynamic structures that regulate the continuous antigen trafficking between the intestinal lumen and the gut mucosa (6). Molecules, including bacterial and food antigens, that cross the IEB reach the lymphatic circulation and stimulate immune cells either through pattern recognition receptors on innate immune cell subsets such as DCs or through TCR-mediated antigenic stimulation with molecular mimicry mechanism (7). Finally, the GVB has been recently identified as another important intestinal barrier structure that determines which microbial components can migrate from the gut into the systemic circulation to reach the liver, spleen and peripheral organs and possibly stimulate innate and adaptive immune responses (8).

In this Research Topic several reports have demonstrated the key role of the gut barrier in preventing abnormal immune activation in the gut and in extra-intestinal tissues in sepsis, allergies, and autoimmune diseases like multiple sclerosis (MS) and type 1 diabetes (T1D).

The importance of the gut microbiota in modulating the pathogenesis of autoimmune T1D has been amply demonstrated both in humans and in preclinical models (9, 10). Here, two papers have highlighted the innovative aspects of this interaction. Miranda-Ribera et al. reviewed the lines of evidence supporting a causal link between increased gut permeability and T1D pathogenesis, supporting the notion that uncontrolled antigen trafficking leads to inflammation and skewed effector Th17 cell responses that favor T1D. On the other hand, Pearson et al. demonstrated that enteric viruses of the norovirus family protect against T1D by reducing inflammation and favoring the differentiation of Treg cells in pancreatic lymph nodes. In their review, Jayasimhan and Mariño proposed the innovative concept that intestinal inflammation and breakage of the GB promote T1D pathogenesis by exposing enteric glial cell antigens, which are potential autoantigens in T1D. GB integrity is also important for other extra-intestinal autoimmune diseases. For example, Buscarinu et al. collected in their review numerous reports indicating that intestinal permeability is altered in MS patients, resulting in bacterial translocation and passage of microbiota-derived endotoxins and metabolites into the systemic circulation, which have a pro-inflammatory effect on the central nervous system. GB integrity is also essential in regulating allergic reactions as demonstrated by the work of Ruohtula et al. who showed that the presence of a microbial community dominated by butyrate producers early in life lowers the risk of developing allergies by promoting the maturation and proper function of the GB.

Importantly, several articles included in this Research Topic provided important evidence on key factors that regulate GB function such as the commensal microbiota composition, psychological stress, diet and other environmental factors (i.e., cigarette smoking). Two reviews summarized the importance of the gut microbiota in regulating GB function. For example, Wang et al. highlighted the importance of dysbiosis in shaping intestinal epithelial cells and mucosal immunity, thereby reducing their ability to contain microbial pathogens and prevent gut-derived sepsis. Antonini et al. discussed how dysbiosis may lead to extra-intestinal autoimmune diseases by altering GB integrity and favoring the passage of bacterial components that could reach peripheral organs to activate autoimmune responses in the central nervous system. Not only bacterial dysbiosis but also alteration of the fungal component can lead to GB damage, inflammation and T1D as shown by Honkannen et al. The review by Taleb et al. reported evidence that dietary components such as tryptophan by regulating the activity of the enzyme indoleamine 2,3 dioxygenase 1 (IDO1) impacts gut immunity and also the GB defense mechanism via the induction of antimicrobial peptides, mucins and tight junction protein expression. In light of the recent evidence on the importance of the brain-gut axis and the ability of the CNS to regulate GB function, Ilchmann-Diounou et al. reviewed published work on how psychological stress promotes intestinal inflammation and breaks the integrity of the intestinal epithelial and mucosal barriers. Finally, Berkowitz et al. demonstrated that the administration of cigarette smoke condensate compromises the gut barrier architecture by inducing Paneth cell damage and reducing the GB defense system. The review by Klepsch et al. dissected the possible mechanism by which these diverse external factors regulate GB integrity. Specifically, the Authors discussed the complex biology and immunoregulatory function of the nuclear receptor (NR) family, a class of receptors that are highly expressed in the gastrointestinal tract and function as sensors of microbial metabolites as well as nutrients and food components. Several NRs have been found capable of regulating gut immune homeostasis by sustaining the integrity of the physical and biological gut barriers.

Restoration of GB integrity could be an innovative therapeutic approach to treat different immune-mediated diseases such as autoimmune diseases and allergies. In this Research Topic an innovative therapeutic approach in this direction was suggested by Xie et al. The Authors showed that administration of porcine β-defensin 129, an anti-microbial peptide normally present in the mucus layer, reinforces GB integrity and reduces intestinal inflammation.

## Author contributions

MF: Writing – review & editing, Writing – original draft. VP: Writing – review & editing, Writing – original draft.

## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Publisher's note

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ORIGINAL RESEARCH
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# A Novel Non-invasive Method to Detect RELM Beta Transcript in Gut Barrier Related Changes During a Gastrointestinal Nematode Infection

*Norus Ahmed[1], Emanuel Heitlinger[2,3], Nicole Affinass[1], Anja A. Kühl[4], Natasa Xenophontos[1], Victor Hugo Jarquin[2,3], Jenny Jost[2], Svenja Steinfelder[1,5] and Susanne Hartmann[1]**

[1] Department of Veterinary Medicine, Institute of Immunology, Freie Universität Berlin, Berlin, Germany, [2] Research Group Ecology and Evolution of Molecular Parasite Host Interactions, Leibniz Institute for Zoo and Wildlife Research, Berlin, Germany, [3] Institute for Biology, Molecular Parasitology, Humboldt Universität, Berlin, Germany, [4] Charité–Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, iPATH.Berlin, Berlin, Germany, [5] Department of Neuroscience, Max Delbrück Center for Molecular Medicine, Berlin, Germany

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**Edited by:**
Julien Diana,
Institut National de la Santé et de la Recherche Médicale (INSERM), France

**Reviewed by:**
David Voehringer,
Friedrich-Alexander-Universität Erlangen-Nürnberg, Germany
Bertrand Kaeffer,
Institut National Recherche Agronomique, France

***Correspondence:**
Susanne Hartmann
susanne.hartmann@fu-berlin.de

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Currently, methods for monitoring changes of gut barrier integrity and the associated immune response via non-invasive means are limited. Therefore, we aimed to develop a novel non-invasive technique to investigate immunological host responses representing gut barrier changes in response to infection. We identified the mucous layer on feces from mice to be mainly composed of exfoliated intestinal epithelial cells. Expression of RELM-β, a gene prominently expressed in intestinal nematode infections, was used as an indicator of intestinal cellular barrier changes to infection. RELM-β was detected as early as 6 days post-infection (dpi) in exfoliated epithelial cells. Interestingly, RELM-β expression also mirrored the quality of the immune response, with higher amounts being detectable in a secondary infection and in high dose nematode infection in laboratory mice. This technique was also applicable to captured worm-infected wild house mice. We have therefore developed a novel non-invasive method reflecting gut barrier changes associated with alterations in cellular responses to a gastrointestinal nematode infection.

**Keywords: intestinal nematode, gut barrier, RELM-β, non-invasive method, feces**

## INTRODUCTION

Soil transmitted, intestinal nematodes affect around 24% of the world's population (1) and are prevalent in wild animals. The majority of parasitic helminths live in the gut and are in close contact with the host's epithelial cell (2), representing an important barrier during infection (3). The gut barrier is composed of specific enterocytes interspersed by goblets cells that secrete the cysteine rich cytokine RELM-β and antimicrobial peptides, together forming the epithelial barrier during infection (4). This barrier defends against pathogen invasion but also leads to the activation of the mucosal immune system in the underlying lamina propria. In helminth infections, the activation of the mucosal immune system leads to the activation of a T helper 2 (Th2) immune response. During a mouse gastrointestinal (GI) infection with *Heligmosomoides polygyrus,* the Th2 immune response is characterized by the expression of the transcription factor GATA-3, the cysteine-rich cytokine

resistin-like molecule-beta (RELM-β), the cytokines interleukin (IL)-4, IL-5, IL-9, IL-13 and the antibodies IgE and IgG1 (5–7). This Th2 response leads to reduced worm fecundity and parasite expulsion (8, 9). In this scenario, RELM-β has been shown to play multiple roles in different aspects of host defenses. It aids in spatial separation of the colonic epithelium and the microbiota by acting as a bactericidal protein (10, 11). Additionally, RELM-β plays a role in immune regulation and host defenses against intestinal nematode infections. In a *H. polygyrus* infection it prevents worm feeding on host tissues and contributes to the weep and sweep response (12, 13). Notably, during *H. polygyrus* infection significant changes in the composition of the gut microbiota have been documented (13, 14).

Currently, different diseases are detectable using non-invasive techniques that utilize samples from urine, saliva, and stool. Urine samples can be used to detect selected viral, bacterial and parasitic diseases (15, 16), including typhoid fever (17) and eggs of the blood fluke *Schistosoma haematobium* (18). Saliva has been a useful source in the early detection of foot and mouth disease in wild boar (19). Moreover, bacterial infections, such as *Helicobacter pylori* (20) and the parasite *Plasmodium falciparum* are detected non-invasively using saliva from infected patients (16). Stool is regularly used to monitor numerous wildlife populations, including detection of virus infections in gorillas (21) or wild apes (22), bacterial shedding in the European badger as well as helminth eggs in Asian elephants (23, 24) *or African buffalo*s (25). In addition, stool is also used in humans to detect cytomegalovirus DNA instead of using mucosal biopsies (26).

In gastrointestinal helminth infections various helminth eggs are detectable using stool, however this method does not detect the early stages of infection. Neither does it investigate host parameters associated with disease, such as gut barrier related changes. Therefore, a non-invasive sampling technique that provides further information about cellular changes during infection and enables the monitoring of disease progression is urgently required for both laboratory and field settings. A non-invasive assessment reflecting cellular and immunological gut parameters would provide a better understanding of pathogen burdens, detection of communities prone to diseases and the identification of immunologically naïve populations (15, 27). Previously, it has been described that the gastrointestinal epithelium is frequently renewed and cells are shed daily into the fecal stream. A large number of these exfoliated cells have been shown to be intact and viable (28).

Here, we describe a novel non-invasive method that uses exfoliated intestinal cells to monitor cellular and immunological changes of the gut barrier in response to infection. We establish and demonstrate this method in laboratory mice infected with the small intestinal nematode *H. polygyrus*. By comparing acute vs. chronic infection, dose-dependent responses and reinfection after abrogation of infection, we illustrate the potential of detecting cellular responses after gut barrier changes using RNA extracted from exfoliated intestinal cells. In addition, we applied this method to wild mouse stool samples. Thus, this study uses the gene expression from exfoliated cells present on stool to detect changes in gut barrier function due to infectious diseases by non-invasive means.

# MATERIALS AND METHODS

## Animals

Female BALB/c mice were used (8 weeks old; purchased from Janvier, Saint Berthevin, France). The experiments performed followed the National Animal Protection Guidelines and were approved by the German Animal Ethics Committee for the protection of animals (G0253/14 and G0113/15).

## Infection

*H. polygyrus* was maintained by serial passage in C57BL/6 mice, described previously (30). Mice aged 8 weeks were infected by oral gavage with either 20 or 200 infective L3 larvae diluted in drinking water. On either 14 or 35 days post infection (dpi) mice were sacrificed by isofluorane inhalation. Mice were treated during the acute phase of infection (day 14 and day 15) with 2 mg/animal pyrantel pamoate for adult worm expulsion (Sigma, St. Louis, MO, USA) in 200 μL water.

## Fecal Collection and Storage

4-10 freshly excreted fecal pellets were collected from mice. Fecal pellets were collected in cryotubes, placed into liquid nitrogen and stored at −80°C until processing.

## Wild Mice Sampling

House mice (*Mus musculus*) were captured in autumn 2017 using live traps (approval number 35−2014–2347). Traps were set overnight in farms and private properties in the state of Brandenburg in Germany. All animals were transferred to individual cages and remained there until fecal samples were collected on the following day. Around 4–5 pellets of fresh fecal samples were kept in liquid nitrogen during transportation and maintained at −80°C until processing. Mice were euthanized, digestive tracts were dissected and helminths were identified and counted under a binocular microscope.

## Oocyst Flotation and Counting

For detection and quantification of *Eimeria,* parts of fecal samples were stored in potassium dichromate solution 2.5% (w/v). Oocyst were flotated using a saturated salt solution (specific gravity = 1.18−1.20), collected by centrifugation (3,234 × *g*/room temperature/ 10 minutes) and washed with distilled water (1,800 × *g*/room temperature/10 min). To estimate the intensity of infection, flotated oocysts were counted using a Neubauer chamber under a Leica® DM750 M light microscope at 10X magnification. Results were expressed in oocyst per gram (OPG) of feces.

## *Eimeria* qPCR

To quantify *Eimeria* in intestinal tissue, DNA from cecum and ileum was isolated using the innuPREP DNA Mini Kit (Analytik Jena AG, Jena, Germany) after disruption of the tissue with liquid nitrogen in a mortar. A short fragment of the mitochondrial cytochrome C-oxidase subunit I (mt COI) of *Eimeria* was amplified in a qPCR using the primers TGT CTATTCACTTGGGCTATTGT (Eim_COI_qX-F) and GGA TCACCGTTAAATGAGGCA (Eim_COI_qX-R). Every reaction

contained 1X iTaqTM Universal SYBR® Green Supermix (Bio-Rad Laboratories GmbH, München, Germany), 400 nM of each primer and 50 ng of DNA template in final volume of 20 μL. Cycling amplification were carried out in a Mastercycler® RealPlex 2 (Eppendorf, Hamburg, Germany) with the following program: 95°C initial denaturation (2 min) followed by 40 cycles of 95°C denaturation (15 s), 55°C annealing (15 s) and 68°C extension (20 s). Melting curve analysis was performed to detect primer dimer formation and non-specific amplification. The CDC42 gene of the mouse nuclear genome was amplified using primers Ms_gDNA_CDC42_F CTCTCCTCCCCTCTGTCTTG and Ms_gDNA_CDC42_R TCCTTTTGGGTTGAGTTTCC as an internal reference. Relative quantification of *Eimeria* DNA as achieved as the ΔCt between mouse and *Eimeria* (CtMouse–CtEimeria). The threshold for detection was estimated at ΔCt = −5 and results above this value were considered as an estimate of parasite tissue load. If both ΔCtIleum and ΔCtCecum indicated infection the higher value was used as estimate for tissue load.

Fresh fecal samples were kept in liquid nitrogen during transportation and maintained at −80°C until processing. Mice were euthanized, digestive tracts were dissected and helminths were identified and counted under a binocular microscope.

## Intestinal Exfoliated Epithelial Cell Extraction

### Flotation Method

Falcon tubes (15 mL) were filled with 3 mL PBS and put on ice. Fecal pellets previously stored at −80°C were used and one pellet was placed individually into each falcon tube. Tubes were placed on a rocker for 45 minutes at 4°C. Tubes were then turned upright on ice for a further 20–30 min to allow cells to loosen up. Another set of falcon tubes were prefilled with 3 mL PBS and stored on ice. Individual falcon tubes containing a single fecal pellet were slowly inverted until the layer of epithelial cells started to detach from the fecal pellet (**Supplementary Video 1**). The inverting force on the falcon tubes was slowly increased until the layer floated off. Epithelial cells could also be removed using a pipette while rotating the fecal pellet to completely peel off the layer. These cells were then collected using a pipette and placed into the freshly prepared falcon tubes to remove any fecal debris. This material was subsequently used for RNA extraction (Analytik Jena, Jena, Germany).

### Alternative Field Method

Fecal pellets were placed in 2 mL tubes filled with 1 mL RNA later (Sigma-Aldrich, St. Louis, MO, USA) and stored at −20°C.For the removal of exfoliated epithelial cells samples were placed in 3 mL PBS filled falcon tubes and left upright for 60–90 min before inverted until cells float off. A rocker was not required for this method.

## Histopathology

Mucus preparations taken from feces were fixed in formalin and embedded in paraffin. Paraffin sections of 1–2 μm thickness were cut, dewaxed and stained histochemically with hematoxylin and eosin (H&E) for overview and with periodic acid-Schiff (PAS) for mucus. Sections were cover slipped with corbit balsam (Hecht, Germany). For immunohistochemical detection of epithelial cells, paraffin sections were dewaxed and subjected to a heat-induced epitope retrieval step prior to incubation with anti-EpCAM (clone E6V8Y, Cell Signaling). For detection, EnVision+ System- HRP Labeled Polymer Anti-Rabbit (Agilent Technologies) was employed. Nuclei were counterstained with hematoxylin (Merck). Negative controls were performed by omitting the primary antibody. Images were acquired using the AxioImager Z1 microscope (Carl Zeiss MicroImaging, Inc.). All evaluations were performed in a blinded manner.

## Realtime PCR

RNA was isolated from exfoliated cells, intestinal tissue sections and whole fecal pellets that were previously stored at −80°C via homogenization in RNA lysis buffer. The homogenized exfoliated cells, fecal pellets and tissue samples were centrifuged and supernatants were treated with a innuPREP RNA kit (Analytik Jena, Jena, Germany) following manufacturer's instructions. 2 μg of RNA was reverse transcribed to cDNA using a High Capacity RNA to cDNA kit (Applied Biosystems, Foster City, CA). The relative expression of β-actin, resistin-like molecule-beta (RELM-β), IL-25, IL-33, and TSLP was determined by Real Time PCR using 10 ng of cDNA and the FastStart Universal SYBR Green Master Mix (Roche, Basel, Switzerland). Relative gene expression of two-three technical replicates is shown as mean. Primer pairs used for gene amplification were as follows: β-actin (*Actb*) forward: GGCTGTATTCCCCTCCATCG, reverse: CCAGTTGGTAACAATGCCATGT, Relm-β (*Retnlb*) forward: GGCTGTGGATCGTGGGATAT, reverse: GAGGCCCAGTCC ATGACTGA, IL-33 (*Il33*) forward: AGGAAGAGATCCTTG CTTGGCAGT, reverse: ACCATCAGCTTCTTCCCATCCACA, IL-25 (*Il25*) forward: ACAGGGACTTGAATCGGGTC, reverse: TGGTAAAGTGGGACGGAGTTG, TSLP (*Tslp*) forward: TGG TAAAGTGGGACGGAGTTG and reverse: TGTGCCATTTCC TGAGTACCGTCA. GAPDH (*Gapdh*) forward: TGGATTTGG ACGCATTGGTC, reverse: TTTGCACTGGTACGTGTTGAT; HPRT (*Hprt*) forward: TCCTCCTCAGACCGCTTTT, reverse: TTTCCAAATCCTCGGCATAATGA; GUS-β (*Gusb*) forward: GCTCGGGGCAAATTCCTTTC, reverse: CTGAGGTAGCAC AATGCCCA. The mRNA expression was normalized to the β-actin using the Δ-ct value and calculated by Roche Light Cycler 480 software.

## Cycling Conditions

Samples initially undergo denaturation at 95°C for 10 min. The samples are then amplified for 40–50 cycles at a denaturing temperature of 95°C for 15 s. The annealing temperature used was 60°C for 30 s and an elongation temperature of 72°C for 20 s.

## Cytokine Detection by ELISA

RELM-β protein was analyzed using Mouse Resistin-like beta(RETNLB) ELISA kit (CUSABIO, China) according to the manufacturer's instructions. A total of four fecal pellets were pooled from four individual mice and homogenized in 1.2 ml PBS. Samples were then centrifuged at 16,000 g for 5 min. Supernatant was collected and used undiluted for RELM-β detection.

## Fecal Egg Counts

Fecal pellets were collected and placed in glass tubes. Fecal pellets were homogenized and mixed with 1 mL tap water. Next, 6 mL of a saturated salt solution (NaCl) was added to the homogenized sample. The McMaster chamber was then filled with sample and eggs were counted.

## Flow Cytometry

For surface and intracellular staining, the following monoclonal antibodies from BioLegend/eBioscience, San Diego, CA, USA were used:

| Antibody/clone | Flurochrome | Vendor | Host species | Reactivity |
|---|---|---|---|---|
| CD4 (RM4-5) | PerCP | BioLegend (Biozol) | Rat | Mouse |
| IL-13 (eBio13A) | Alexa 488 | eBioscience | Rat | Mouse |
| Foxp3 (FJK-16s) | Alexa 488 | eBioscience | Rat | Bovine, Dog, Cat, Mouse, Pig, Rat |
| GATA-3 (TWAJ) | eFluor 660 | eBioscience | Rat | Human, Mouse, Pig, Rhesus monkey |
| Dead Cell Exclusion Marker | efluor 780 | eBioscience | | |

## Statistics

Experiments are displayed as either mean ± SD or mean ± SEM as indicated. Statistical analysis was completed using GraphPad Prism software (La Jolla, CA, USA). Significance was determined as indicated using the Kruskal-Wallis with Dunn's multiple comparison test or the Mann Whitney *U* Test. Also, multiple t tests were corrected for multiple comparisons using the Holm-Sidak method. Significance was measured as $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, and $^{***}P \leq 0.001$. Data from wild mice was analyzed in R (29) using linear regression. RELM-β expression as a response variable was modeled using the function "lm" with either a) presence of helminths or b) helminth species richness as predictors. For both predictors either i) *Eimeria* oocyst per gram feces, ii) intensity of *Eimeria* tissue stages (as ΔCt Mouse-Eimeria) or iii) presence of *Eimeria,* as determined by both methods, was included as additional predictor variable, allowing for interaction effects. The resulting six models were compared using the Akaike information criterion (AIC) and considered to possess equal explanatory power if $\Delta AIC < 2$.

# RESULTS

## Identification of Epithelial Cells and Mucus in a Layer of Exfoliated Intestinal Cells From Stool

A layer of exfoliated cells from stool (**Figure 1A**) was analyzed using different histological techniques. A thick mucus layer is apparent in an H&E stain (**Figure 1B**) and in a PAS-staining (**Figure 1C**) with cells either attached to the mucus or in between mucinous strands. These cells displayed a broad cytoplasm suggesting that they are of epithelial origin and were probably scaled off during defecation. Immunohistochemical detection of EpCAM expression confirmed that the majority of these cells are epithelial cells (**Figures 1D,E**). Thus, epithelial cells can be retrieved from the surface of stool (**Figure 1F**).

## Quantification of RNA Isolated From Exfoliated Intestinal Cells

Currently there is a lack of information on cellular and immunological changes of the gut barrier after infection using non-invasive techniques. Here, we established a method allowing the quantification of RNA from exfoliated intestinal cells. Exfoliated cells showed a lower Cycle threshold (Ct) value for the amplification of RELM-ß compared to homogenized whole fecal pellets. The Ct value is defined as the required number of cycles for the signal to exceed the fluorescence threshold in quantitative PCR (**Supplementary Table 1**). Furthermore, exfoliated cells showed very low Ct value variation between technical triplicates, whereas homogenized fecal pellets sometimes displayed a difference of 10 in Ct values within triplicates. We attributed the lower quality of the homogenized samples to higher degradation of mRNA based on the differences in Ct values within triplicates. Therefore, we regard the extraction of exfoliated cells to be superior to whole fecal pellet homogenization. To identify the ideal housekeeping gene, the different housekeeping genes β-actin, GAPDH, HPRT and GUS-β were analyzed. β-actin, GAPDH, GUS-β and HPRT were tested using 10 ng cDNA and 100 ng cDNA (**Supplementary Table 2**). While expression levels of all four housekeeping genes correlated with each other, β-actin produced a lower Ct value at 10 ng compared to the other three housekeeping genes at 100 ng. Thus, β-actin was the housekeeping gene of choice throughout. β-actin was also detectable using fecal samples stored at −20°C in RNA later for 30 days (data not shown).

## RELM-β Expression in Exfoliated Cells During Acute and Chronic Intestinal Helminth Infection

Depending on the infection, the immune responses vary in terms of quantity and quality. Here, we aimed to quantify mRNA expression of a selection of immune genes typical for a Th2 type response against helminth infections. We aimed to obtain infection-specific data using exfoliated intestinal cells isolated from stool. To establish this technique, a murine infection model with the small intestinal nematode *H. polygyrus* was used. During the early acute phase of *H. polygyrus* infection the larvae enter the intestinal tissue and develop into the fourth larval (L4) stage, then re-enter the lumen of the gut. In response to the tissue invasive phase and resulting tissue damage the epithelium-derived cytokines IL-25, IL-33, and TSLP are released (31). During an acute *H. polygyrus* infection (14 dpi), our data shows that mRNA expression of these tissue-derived cytokines from exfoliated cells were insufficiently detected and showed high variability between triplicates (**Supplementary Figures 1A-C**). We thus investigated whether exfoliated cells could be used to detect the Th2 cysteine-rich cytokine RELM-β. RELM-β is

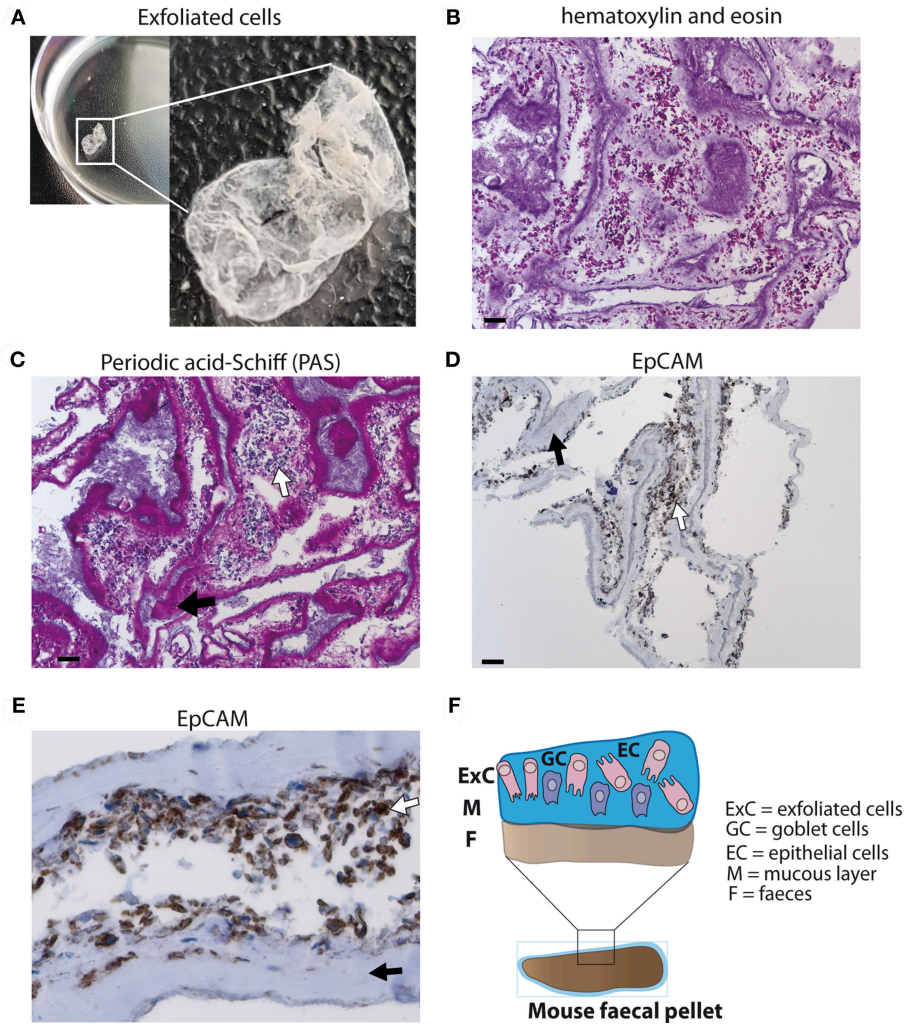


**FIGURE 1 |** Analysis of exfoliated intestinal epithelial cells isolated from murine stool. **(A)** A layer of exfoliated cells removed from the surface of mouse stool in PBS. **(B)** Hematoxylin and eosin (H&E) stain of exfoliated cells extracted from the surface of stool at 100x magnification (scale bar 100 μm). **(C)** Periodic acid-Schiff (PAS) staining for mucus detection in exfoliated cells at 100x magnification (scale bar 100 μm). **(D)** EpCAM staining identifying epithelial cells using exfoliated cells (brown staining) at 100x magnification (scale bar 100 μm) **(E)** EpCAM staining of epithelial cells at 400x magnification. **(F)** Schematic diagram of stool surface with exfoliated cells. **(C–E)** White arrows display cells and black arrows highlight mucus.

produced by intestinal goblet cells that significantly increase during intestinal helminth infection (32). Interestingly, RELM-β expression was significantly detected as early as day 6 post *H. polygyrus* infection (**Figure 2A**). This increase of expression was also significant at day 8 and 10, steadily decreasing thereafter, making it detectable throughout the course of an acute infection. In contrast, protein detection of RELM-β in feces by ELISA detected an increase in RELM-β at day 8–10 (**Supplementary Figure 1G**). In the case of chronic helminth infection, RELM-β expression continued to decrease from day 21 to levels similar to day 0 (**Figure 2B**). As a method to confirm infection, helminth eggs were counted in parallel during the acute and chronic phase (**Figures 2C,D**). As expected, egg counts and RELM-β mRNA expression peaked at different time points but overall mirrored a similar course of infection. Of note, the RELM-β detection in exfoliated cells directly correlated with the local gut Th2 immune response investigated via flow cytometry during the course of infection. An increase in the Th2 transcription factor GATA-$3^+$ in $CD4^+$ T cells in mesenteric lymph nodes (mLN) peaked at day 8 post *H. polygyrus* infection (**Figure 2E**). The same was observed for the Th2 cytokine IL-13 (**Figure 2F**). When investigating the systemic immune response, we observed a delayed peak in the expression of GATA-$3^+$ in $CD4^+$ T cells at day 14 in the spleen, decreasing onwards to day 35 (**Figure 2G**). The same was observed for the IL-13 expression in the spleen indicating a delayed systemic response to infection (**Figure 2H**). Thus, the local immune response in the infection draining mLN mirrored RELM-β detection in exfoliated cells.

To verify the observed mRNA levels in exfoliated cells, we examined the cytokine expression in host tissue along the gut. In parallel, intestinal tissues from the duodenum, jejunum, ileum, proximal colon, intermediate colon and distal colon

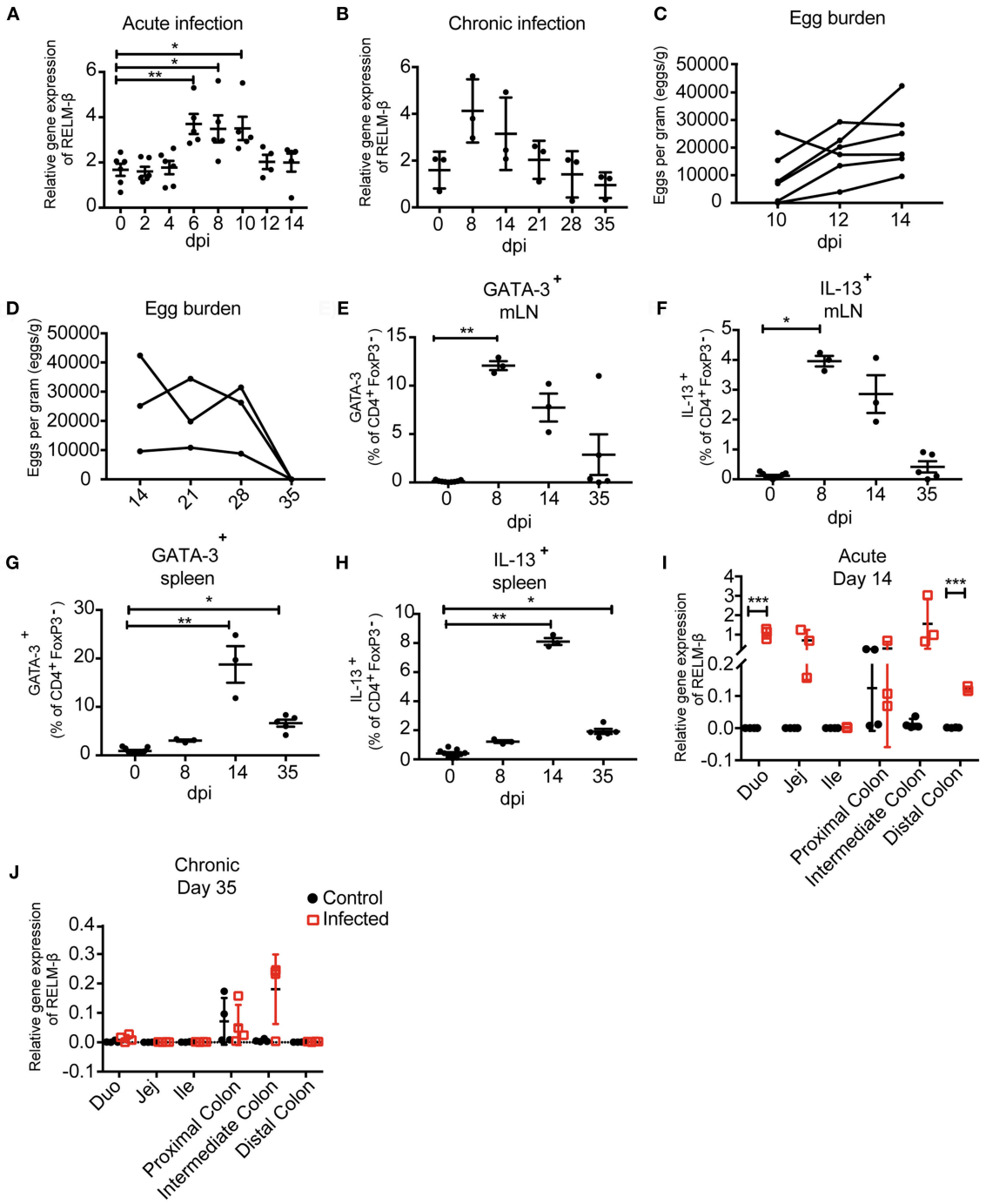


**FIGURE 2 |** Immunological and parasitological analysis of murine stool after infection with *Heligmosomoides polygyrus*. BALB/c mice were infected orally with 200 infectious L3 stage larvae of *H. polygyrus*. Parameters were measured at different time points and in different regions of the intestine. **(A)** Relative gene expression of RELM-β in exfoliated intestinal cells during acute infection (day 0–14 dpi). **(B)** RELM-β gene expression in exfoliated cells until chronicity of infection (day 0–35 dpi), shown as mean ± SD. **(C)** Fecal egg counts during acute *H. polygyrus* infection (day 14 dpi). **(D)** Fecal egg counts during chronic *H. polygyrus* infection (day 14–35 dpi). Frequency of CD4+ T cells expressing GATA-3 **(E)** and IL-13 **(F)** in mesenteric lymph nodes (mLN). **(G)** Frequencies of $CD4^+$ T cell expressing GATA-3 and IL13 **(H)** in spleen. **(I,J)** RELM-β expression in intestinal tissue (duodenum, jejunum, ileum, proximal colon, intermediate colon, and distal colon) at 14 and 35 dpi, respectively. All relative gene expression analysis is compared to β-actin using 10 ng cDNA. Data from **(A)** is pooled from two independent experiments with $n = 4$–10 fecal pellets from 4–6 animals. **(B)** is representative of two independent experiments, $n = 3$. **(C)** is pooled from two independent experiments, $n = 6$. **(D–H)** is representative of two independent experiments, $n = 3$. **(I,J)** is representative of two independent experiments, $n = 3$–4. **(A)** shown as mean ± SEM, $^*P \leq 0.05$, $^{**}P \leq 0.01$, and $^{***}P \leq 0.001$. **(B–H)** shown as mean ± SD; **(A–H)** Statistical analysis was performed using the Kruskal-Wallis with Dunn's multiple comparison test, **(I,J)** multiple *t* tests and corrected for multiple comparisons using the Holm-Sidak method, $^*P \leq 0.05$, $^{**}P \leq 0.01$, and $^{***}P \leq 0.001$. dpi: days post infection.

were analyzed to decipher the expression of the tissue derived cytokines IL-25, IL-33, TSLP (**Supplementary Figures 1D-F**) and the goblet cell produced RELM-β. Interestingly, at 14 days post-infection a significant RELM-β signal was detectable in the duodenum and distal colon during acute *H. polygyrus* infection (**Figure 2I**). However, RELM-β mRNA was not observed in intestinal tissue at day 35 post infection (**Figure 2J**) correlating with the RELM-β detection in exfoliated cells. This data indicates a reduced Th2 immune response against the worm infection, probably due to advanced clearance of the parasite.

In summary, RELM-β mRNA as an indicator for gut barrier changes was detectable as early as day 6 post nematode infection in exfoliated intestinal cells. The non-invasively detected RELM-β expression mirrored the immune responses at the gut barrier. Thus, exfoliated epithelial cells from stool can be used to detect infection.

## Pathogen Dose-Dependent Expression of the Cysteine-Rich Cytokine RELM-β in Exfoliated Epithelial Cells

Next, we aimed to investigate if the detection of the cysteine-rich cytokine RELM-β correlated to the intensity of infection. Changes in the gene expression of RELM-β in exfoliated gut cells were studied using different infection dosages. Mice were infected with either a low dose infection with 20 L3 of *H. polygyrus* or a high dose infection with 200 L3 and adult worm burden was assessed at day 14 (**Figure 3A**). The high dose infection was reflected by a higher RELM-β expression compared to a low dose infection. The expression of RELM-β during the 200 L3 infection was significantly higher compared to a low dose infection at day 8 p.i. (**Figure 3B**). This effect was most prominent at day 8 but a trend was detected from day 4 p.i. onwards. Thus, RELM-β expression in exfoliated cells does mimic the intensity of an intestinal infection.

In nature, animals frequently get re-infected with helminths. Therefore, we asked if our new non-invasive method is suitable to detect the expression of RELM-β as a marker for gut barrier changes after drug clearance of *H. polygyrus* and reinfection. Mice were re-infected 28 days after drug clearance with 200 L3 infective larvae. RELM-β mRNA was significantly detectable in exfoliated gut cells at day 4 and 6 in mice re-infected with *H. polygyrus* (**Figure 3C**). Interestingly, a significantly positive linear relationship was observed between the expression of RELM-β in exfoliated cells and adult worm counts (**Figure 3D**).

## Analysis of Intestinal Exfoliated Cell Gene Expression in Wild Mice Samples

In order to analyze the potential of the non-invasive cytokine detection method with wildlife animals, we tested stool samples collected from wild *Mus musculus* mice captured in Brandenburg, Germany. RELM-β gene expression was analyzed in exfoliated intestinal cells isolated from fecal pellets from wild mice with no worms detectable in the gut. The data was then compared to data obtained from wild mice with natural infections of eight species of helminths (**Supplementary Table 3**). We observed a significant increase of RELM-β expression in samples from wild mice in the presence of worms and with species richness of helminths in comparison to mice without helminths. In addition, we studied the effect of a coinfection with the protozoan gut parasite *Eimeria* spp. on the expression of RELM-β in helminth-infected mice vs. mice without helminths. We scrutinized these results using six statistical models in total (**Supplementary Table 4**). All models found a significant increase of RELM-β expression in the presence of the gastrointestinal helminths (**Figure 4A**). This was independent of *Eimeria* spp. load in the tissues and *Eimeria* spp. oocyst abundance in the feces (**Figures 4A,B**) or the presence of *Eimeria* as a second predictor. The statistical models predicted a significant increase in RELM-β expression with helminth richness (**Figure 4C**) when controlling for *Eimeria* spp. tissue load or oocyst abundance (**Figures 4C,D**). Furthermore, a trend for higher RELM-β expression and species richness was determined when including *Eimeria* presence in the model. All the models had similar quality as assessed by the Akaike information criterion (AIC). Models predicting RELM-β expression with the abundance of individual worm species did not show significant differences (data not shown).

In conclusion, our study with samples from wild animals shows that RELM-β is induced during an active infection with intestinal helminths and this expression was robust in presence of a coinfection with the gut protozoan parasite *Eimeria spp*.

# DISCUSSION

Here we investigated whether exfoliated intestinal cells released with feces mirror immunological host responses corresponding to gut barrier changes during a gastrointestinal nematode infection with *H. polygyrus*. Investigation into infection related genes revealed the goblet cell-secreted RELM-β as a reliable marker reporting gut barrier changes due to acute worm infection. RELM-β was significantly detectable during the early stages of an acute *H. polygyrus* infection and decreased steadily thereafter. Intriguingly the mRNA levels of RELM-β in exfoliated cells reflected the local GATA-3 and IL-13 expression in mLN during infection. Our data suggests that detection of RELM-β in exfoliated gut cells not only detects gut barrier related changes but also reflects the local Th2 immune response.

In the intestine, epithelial cells represent an essential barrier that undergoes continuous homeostatic turnover replacing old cells and lost or damaged epithelial cells during infection (33, 34). Additionally, epithelial cells are part of the mucosal immune response and aid in eliciting the prominent Th2 immune response during gastrointestinal helminth infection. Exfoliated cells are shed daily in feces and have been previously described to be of colonic origin (35). Interestingly during *H. polygyrus* infection goblet cells (GCs) play an important role in the secretion of RELM-β. The secretion of this cysteine-rich cytokine in intestinal tissue is essential for normal spontaneous worm expulsion (12). RELM-β acts on *H. polygyrus* by impairing feeding thus reducing protein and ATP content, leading to pathogen expulsion (31). Goblet cells also secrete mucin forming a mucosal surface barrier limiting interactions with luminal

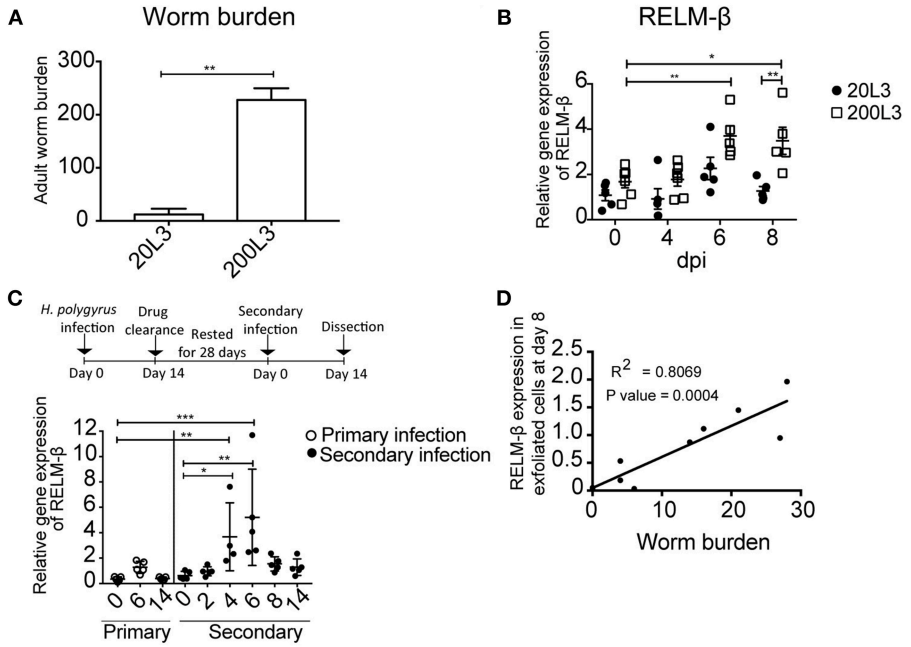


**FIGURE 3 |** RELM-β expression in exfoliated cells reflects the intensity and type of infection. BALB/c mice were infected orally with either a low dose (20 L3) or a high dose (200 L3) *H. polygyrus* larvae. **(A)** Worm burden assessed at 14 dpi. **(B)** RELM-β relative gene expression during early *H. polygyrus* infection until day 8 dpi after low dose (20 L3) and high dose (200 L3) infection. **(C)** BALB/c mice were infected orally with 200 L3 *H. polygyrus,* treated with pyrantel pamoate for worm clearance and 28 days later reinfected with 200 L3. RELM-β gene expression shown during primary and secondary infection. **(D)** Linear regression comparing RELM-β gene expression at day 8 in exfoliated cells with low worm burden. **(A)** is representative from two independent experiments with $n = 3$–5 animals and shows the mean ± SD. **(B)** is pooled from two independent experiments with $n = 4$–6 animals, data shown as mean ± SEM. All relative gene expression analysis is compared to β-actin using 10ng cDNA. **(C)** is representative of two independent experiments, $n = 4$–5. **(D)** is pooled from two independent experiments, $n = 10$. Statistical analysis using **(A)** Mann Whitney U Test, **(B)** multiple *t* tests and corrected for multiple comparisons using the Holm-Sidak method, **(C)** was performed using the Kruskal-Wallis with Dunn's multiple comparison test. All relative gene expression analysis is compared to β-actin. Significance was measured as $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, and $^{***}P \leq 0.001$.

microbes (36). Additionally, during homeostasis GCs are highly abundant in the colon compared to the upper intestinal tract (13).

Importantly, our method is applicable in the field where cooling and proper storage of biological material is limited. We tested fecal pellets from wild mice freshly placed in RNA later™ and then stored at −20°C for 30 days. Interestingly, β-actin expression was detectable with a Ct value of 25, similar to flash frozen and −80°C stored fecal pellets (data not shown). Thus, this non-invasive method allows stool to be stored for longer periods of time before analysis, whereas egg counts need to be processed as soon as possible for a reliable representation of infection (37). This suggests that both egg counts and exfoliated cells should be used together to complement the information not only on the infection status but in addition the cellular host responses at the gut barrier.

Nematodes dwelling in the small intestine, such as *H. polygyrus* have been shown to affect colonic permeability (38). This cellular response in the colon to an infection dwelling in the small intestine enables the detection of cellular responses using exfoliated cells likely originating from the colon. In addition, gastrointestinal nematodes have been described to alter the gut bacterial environment (13, 39). Increase in abundance of gram-negative bacteria during *H. polygyrus* infection (13, 14), accompanied by colonic barrier permeability (38) correlate with the increased early detection of RELM-β during infection in our study. Here, it is interesting to note that RELM-β is highly expressed (34) and plays multiple roles, such as recruiting $CD4^+$ T cells in *Citrobacter rodentium* infection (10). Additionally, RELM-β has been described as a bactericidal protein that kills gram-negative bacteria, limiting the association of bacteria with colonic tissues (11). Thus, the ability to detect RELM-β in exfoliated cells can possibly be attributed to the highly abundant goblet cells in the large intestine secreting RELM-β, to deal with gut barrier changes and the increase in bacteria during nematode infection. Thus, the previously described altered colonic permeability (38) and the increased gram-negative bacteria (13, 14) explain the high expression of RELM-β and the detectability in exfoliated cells compared to the tissue cytokines.

Comparison of the tissue cytokines in different tissue compartments at 14 dpi confirmed no significant changes in IL-25, IL-33 and TSLP in the distal colon (**Supplementary Figures 1A-C**). Additionally, expression of these tissue cytokines could not be detected using exfoliated cells from stool. The observed systemic tissue RELM-β expression and high expression in exfoliated cells displays a reliable marker that mirrors the local *H. polygyrus* Th2 immune responses non-invasively. Additionally, this method can be utilized for other

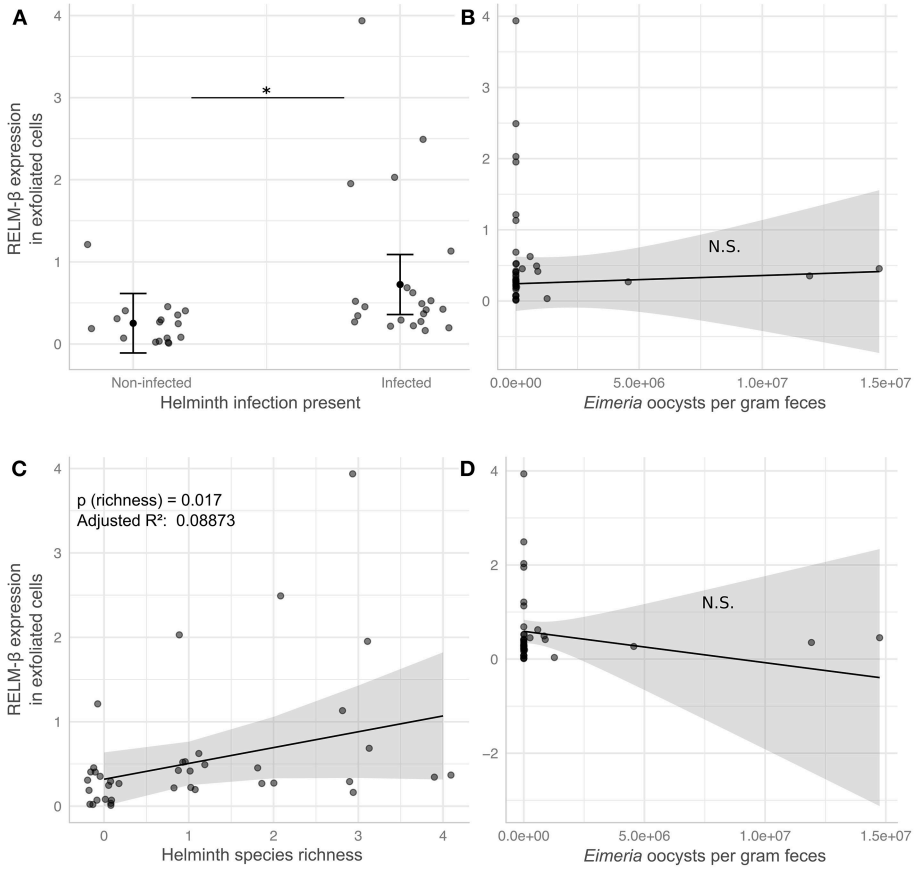


**FIGURE 4 |** RELM-β expression in exfoliated cells differs in response to helminth infection in wild house mice. Helminth and *Eimeria* infections were assessed in wild house mice. Linear models predict RELM-β expression to be significantly elevated by either presence **(A)** or species richness **(C)** of helminths, while the number of *Eimeria* oocysts shed per gram feces in the same mice has no significant effect in the respective models for presence **(B)** and richness **(D)**. Gray points depict values for individual mice and are jittered relative to the x-axis to avoid overplotting in **(A)**, solid black points and lines indicate marginal means estimated in statistical models (see **Supplementary Table 4**; Model 3 and Model 6), error bars and shaded areas represent 95% confidence intervals for these estimates. $^*P \leq 0.05$.

colonic infections and might display RELM-β as a marker of gut barrier changes.

In order to identify if exfoliated cells can be used as a measure of infection load, we tested a low vs. high dose infection and identified significant differences in RELM-β expression. This method detected a difference in infection dose highlighting its potential as a marker of infection load and also reported the quality of the immune response in a secondary infection. A linear regression comparison of exfoliated cells compared to worm burden revealed a significantly positive relationship. Interestingly, RELM-β expression in exfoliated cells mirrored the kinetics of the local immune response in our study. Consequently, detection of RELM-β in exfoliated cells might be useful not only as an additional measure to egg counts but also as a means to monitor cellular gut barrier changes correlating to infection loads. However, previous studies have shown that RELM-β is detectable on the protein level in feces via western blot (10) and ELISA (**Supplementary Figure 1G**). On the other hand, our method described here investigates transcript levels using exfoliated cells. This is especially interesting as the ELISA is dependent on the availability of species-specific antibodies against the gene of interest. Thus, the lack of antibodies for certain genes restricts the potential use of protein analysis. Antibodies are also restricted to specific animal species making it difficult to detect certain proteins. Therefore, this method allows for a cost effective alternative to protein studies by ELISA.

We believe that our novel method of using exfoliated cells to detect changes at the gut barrier might pave the way for further investigation into infection-specific markers under laboratory settings but also in infections of wild life animals.

In wildlife, animals are exposed to a variety of pathogens and monitoring of the health status is urgently needed for wildlife conservation, zoonotic diseases and disease control. However, the only methods applicable to wildlife studies are non-invasive methods. Over time there have been many advances in different molecular biology techniques allowing for in depth research into different diseases. However, not much is known regarding cellular changes during infection in natural animal populations. We demonstrate here that our method using exfoliated cells to investigate infection related gut barrier changes works in both

laboratory and wild animals. We found RELM-β to be a marker for helminth infection in wild house mice without being able to pinpoint a single worm species as most relevant driver of the RELM-β expression. A significant effect of worm species richness on RELM-β expression could indicate that the marker might be suitable to detect general stress on the gut barrier in wild systems. This prospect warrants further investigation.

*Eimeria* spp. is the most prevalent protozoan parasite in the studied house mice (Jarquin et al. unpublished) and could have a potential effect on RELM-β expression, such as inducing an opposing immune response. We were able to test the influence of *Eimeria* spp. on RELM-β expression, including co-infections with worms, but did not find an effect despite investigating a sufficient sample size. We thus conclude that there is no indication of protozoan infections affecting RELM-β expression at the gut barrier in helminth-infected wild mice hosts.

In conclusion, we describe a novel method to measure cellular parameters using exfoliated cells from stool to detect infection-related gut barrier changes in samples from wildlife and experimental laboratory conditions. This study paves the way for further investigation into infection-specific markers under laboratory settings but also in infections of wild animals. Further investigation into pathogen-specific infection markers is required. Thus far, our technique represents a potential addition to lab and field non-invasive techniques reflecting gut barrier changes. RELM-β detection in exfoliated cells can potentially provide a suitable marker for colonic inflammation in a variety of different diseases and colitis infections.

## DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the Supplementary Files.

## AUTHOR CONTRIBUTIONS

NoA and NX performed all the exfoliated cell extractions and gene expression experiments. SH and SS conceptualized and designed the research. NiA performed all the flow cytometry experiments and analysis. EH provided input into all wild mouse experimental data and analysis. AK performed all the histopathology experiments. VJ was involved in the capturing and sample collection from wild mice and JJ was involved in the identification of worms in wild mouse samples. NoA and SH wrote the manuscript. All authors approved the final version of the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00445/full#supplementary-material

**Supplementary Figure 1 |** BALB/c mice were infected orally with 200 infectious L3 stage larvae of *H. polygyrus*. Parameters were measured at different time points and in different regions of the intestine. **(A)** Relative gene expression of IL25, **(B)** IL33 **(C)** TSLP in exfoliated intestinal cells during acute infection (day 0–14 dpi). Relative gene expression of **(D)** IL25, **(E)** IL33, **(F)** TSLP in intestinal tissue (duodenum, jejunum, ileum, proximal colon, intermediate colon, and distal colon) at 14 dpi. **(G)** RELM-β protein detection via ELISA using undiluted fecal supernatant.

**Supplementary Table 1 |** Real Time PCR cycle threshold (Ct) values of β-actin in exfoliated cells and homogenized stool using a 10 and 100 ng cDNA concentrations.

**Supplementary Table 2 |** Exfoliated cells were used to detect the Ct values of the different housekeeping genes β-actin, GAPDH, HPRT and GUS-β using 10 and 100 ng cDNA concentrations.

**Supplementary Table 3 |** Represents the location, sex, and infection status of wild *Mus musculus* mice captured in Brandenburg, Germany.

**Supplementary Table 4 |** Represents the different statistical models used for wild *Mus musculus* mice captured in Brandenburg, Germany.

**Supplementary Video 1 |** Shows exfoliated cells being removed from whole fecal pellets.

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REVIEW
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# Nuclear Receptors Regulate Intestinal Inflammation in the Context of IBD

*Victoria Klepsch[1], Alexander R. Moschen[2], Herbert Tilg[2], Gottfried Baier[1] and Natascha Hermann-Kleiter[1]**

[1] Translational Cell Genetics, Department of Pharmacology and Genetics, Medical University of Innsbruck, Innsbruck, Austria, [2] Department of Internal Medicine I, Gastroenterology, Endocrinology and Metabolism, Medical University of Innsbruck, Innsbruck, Austria

Gastrointestinal (GI) homeostasis is strongly dependent on nuclear receptor (NR) functions. They play a variety of roles ranging from nutrient uptake, sensing of microbial metabolites, regulation of epithelial intestinal cell integrity to shaping of the intestinal immune cell repertoire. Several NRs are associated with GI pathologies; therefore, systematic analysis of NR biology, the underlying molecular mechanisms, and regulation of target genes can be expected to help greatly in uncovering the course of GI diseases. Recently, an increasing number of NRs has been validated as potential drug targets for therapeutic intervention in patients with inflammatory bowel disease (IBD). Besides the classical glucocorticoids, especially PPARγ, VDR, or PXR-selective ligands are currently being tested with promising results in clinical IBD trials. Also, several pre-clinical animal studies are being performed with NRs. This review focuses on the complex biology of NRs and their context-dependent anti- or pro-inflammatory activities in the regulation of gastrointestinal barrier with special attention to NRs already pharmacologically targeted in clinic and pre-clinical IBD treatment regimens.

**Keywords: nuclear receptor, intestinal barrier homeostasis, immune system, microbiota, inflammatory bowel disease**

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***Correspondence:**
*Natascha Hermann-Kleiter*
*natascha.kleiter@i-med.ac.at*

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## INTRODUCTION

This review is based on the most recent advances in our understanding of the complex biology of nuclear receptors (NRs) within both the healthy and inflamed intestinal tract (**Tables 1–3**) and the emerging number of ligands successfully used in preclinical and clinical trials (**Table 4**) to target inflammation and treat inflammatory bowel disease (IBD) without focusing on intestinal infections (1–5). In general, NRs enable fine-tuning of cellular processes to environmental changes such as external milieu signals and the cell-intrinsic metabolic state and are therefore ideally suited as targets for therapeutic interventions (6–9). Within the gastrointestinal (GI) system, NRs are highly expressed and well-known sensors of nutrients, hormones, and specific host-bacterial metabolites (1–5). Gut physiology is regulated by several nuclear receptors such as ERβ (NR3B2), GR (NR3C1), FXR (NR1H), PPARγ (NR1C3), PXR (NR1I2), RARα (NR1B1), VDR (NR1I1), HNF4α (NR2A1), or NR2F6 (Ear2) which have been demonstrated to play fundamental roles in epithelial intestinal cell integrity and especially in shaping intestinal immune cell composition and function (**Figure 1**) (9–14).

In humans, the NR family consists of 48 members and is, therefore, the most significant group of transcriptional regulators. It includes the receptors for steroid and thyroid hormones together with receptors for lipophilic vitamins and cholesterol metabolites (6, 7). The physiological ligands for approximately half of NRs are known, whereas the rest are classified as orphan receptors (8, 15–17). Members of the NR family are highly conserved; the modular domain structure consists of an activation domain (AF), the central DNA-binding-domain (DBD), the hinge region, the ligand-binding domain (LBD), and the activation function 2 (AF2) (15). NRs differ in their modes of action. In the classical steroid receptor signaling, for instance, the ligand (steroid) enters the cell to activate the receptor located in the cytoplasm. Due to the resulting conformational change, the receptor translocates to the nucleus and binds to its cognate nuclear receptor response element on the DNA within the target gene promoters thereby altering the transcription levels (18). However, other nuclear receptors, such as thyroid hormone receptors or the peroxisome proliferator-activated receptors (PPARs), are localized in the nucleus regardless of whether or not they are bound to a ligand and constitutively interact with DNA response elements (1). Of note, additional non-genomic functions of nuclear receptors in the cytosol have been firmly established such as the activation of cAMP, $Ca^{2+}$ or the MAPK signaling cascade (17–19). The specificity of transcriptional activation by a given NR is achieved by the tissue-selective expression of co-repressors or co-activators as well as post-transcriptional modifications of both.

Expression analysis of biopsies from IBD patients as well as animal studies with NR ligands suggests a significant correlation between NR biology and IBD pathology (**Tables 1**–**4**) (**Figure 1**). Interestingly, the presence of NRs or their ligand agonists seems to be mainly protective during IBD (**Tables 1**–**3**). The NR superfamily is one of the primary classes of therapeutic drug targets for human disease (1, 2). How NRs regulate gut homeostasis in the complex interplay between intestinal epithelial cells, the immune system, and the microbiota is an active area of research.

Ligands targeting NRs in IBD, either being tested in clinical trials or already in use to treat IBD patients, are dexamethasone and methylprednisolone (targeting GR), rosiglitazone, pioglitazone, bezafibrate, and curcumin (targeting PPARγ), and 1,25-di-hydroxyvitamin, calcitriol, and cholecalciferol (targeting VDR) (3, 4) (**Table 4**).

Current strategies to treat IBD include anti-inflammatory drugs, immunosuppressives, biological agents, antibiotics, and changes in dietary habits in combination with pain medication (20). These treatment options help relieve symptoms and reduce the risks of recurrence and complications, but in most cases, only a subgroup of patients responds to the available therapies. Surgery is the last therapeutic possibility when there is loss of response and adverse side effects. In the context of an increasing number of IBD patients, new approaches to treatment are needed, and molecular targets such as NRs represent a promising avenue to pursue in a search for more effective drugs. This review focuses on the complex relationship between nutrition, inflammation and nuclear receptor biology within the GI and the emerging number of NR ligands used in IBD therapy.

## Nuclear Receptors Regulate Intestinal Homeostasis

Nutrient uptake and elimination of toxic dietary components or xenobiotics within gut epithelium are dependent on the dietary lipid-activated NRs such as CAR (NR1I3), FXR, PXR, and VDR. Also, glucose, fatty acid, triglycerides, and lipoprotein metabolism in intestinal epithelial cells (IECs) are regulated by the PPAR family (α, β, δ), whereas cholesterol transport and absorption and bile acid metabolism are dependent on LXR and LRH (NR5A2) (21).

Furthermore, NRs such as ERβ, RARα, HNF4α, and NR2F6 regulate essential aspects of intestinal barrier functions such as mucus secretion, goblet and paneth cell numbers, autophagy and expression of tight junction proteins (**Figures 1**, **2**) (11–14, 22).

Microbiota, and their metabolites such as butyrate, propionate, or indole, influence NR biology directly, functioning as ligands to target FXR, PPARγ, or PXR (**Figure 1**) (5). Depletion of butyrate-producing microbes by antibiotic treatment reduces epithelial signaling through PPARγ showing that microbiota-activated PPARγ signaling prevents the dysbiotic expansion of potential pathogens (23) (**Figures 1**, **2**). However, FXR activation itself alters the intestinal microbiota and could provide opportunities for microbiome biomarker discovery or new approaches to engineering the human microbiome (24–26) (**Figure 2**). For detailed aspects of nuclear receptor and microbiota biology, we refer to a recent review by Duszka and Wahli (27).

Within the intestinal epithelium, NRs such as VDR, HNF4α, LXR, PPARγ, LRH1, and NR2F6 play protective roles in intestinal epithelial integrity (**Figures 1**, **2**); decreased mRNAs have also been validated in intestinal samples from IBD patients (10, 12) (**Table 1**). In mice, deletion of the VDR increases mucosal injury that leads to high mortality in DSS-induced experimental colitis (10). In parallel, the activation of the farnesoid X receptor (FXR) prevents chemically-induced intestinal inflammation, improves colitis symptoms, inhibits epithelial permeability, and reduces goblet cell loss (13) (**Figure 2**). Intestinal steroidogenesis controls PPARγ expression in the colon, and this axis is impaired in ulcerative colitis (11).

The microbiota-NR axis influences not only metabolism of the intestinal epithelium, but also the components of the circadian clock; in particular, RORα (NR1F1) and RevErbα (NR1D1) influence corticosterone synthesis in IEC whereas PPAR and LXR families can alter the hepatic circadian clock (28, 29) (**Figure 2**).

NRs contribute especially to gut homeostasis by shaping intestinal immune cells; on one side, they are constantly challenged in the face of stimulatory signals from nutrients and gut microbiota, and on the other, they shape the composition of the microbiota themselves (**Figures 1**, **2**) (5, 13, 19, 28, 29). Already the development of gut-associated lymphoid tissue is dependent on the expression of NRs like RORγt (NR1F3), which is required for the generation of lymphoid tissue inducer (LTi) cells and subsequent formation of Peyer's patches. As the amount

**TABLE 1 |** Steroidal nuclear receptors.

| Nuclear receptor | Spec | Model | Study outcome | References |
|---|---|---|---|---|
| ER α/β NR3A1/2 (protective) | hu | Biopsies | *ERB* mRNA is decreased in IBD patients | (1, 2) |
| | | Blood | Altered ERα expression (increased) and ERβ (decreased) in T lymphocytes from IBD patients | (3) |
| | mo | Spontaneous | Altered epithelial barrier in KO mice, decreased *Erb* mRNA levels | (2, 4) |
| | | AOM/DSS | $ER\beta^{-/-}$ mice are more susceptible to clinical AOM/DSS colitis-associated colorectal cancer | (5) |
| | | | Estrogens promote colon cancer development by impairing the mucosal responses | (6) |
| | | Chemical | *ERb* expression in female mice protected against DSS colitis (male mice are not protected) | (7) |
| GR NR3C1 (controversial) | hu | Biopsies | IBD patients without steroid treatment showed increased *GR* expression | (8) |
| | | | Enhanced *GR* mRNA levels in leukocytes of UC patients | (9) |
| | | | *hGRb* mRNA expression in PBMCs is a novel predictor of glucocorticoid response in UC patients | (10) |
| | | | *hGRb* mRNA expression was significantly enhanced in active stage of UC | (11) |
| | | | UC patients are positive for *GRa* and *GRb* expression; *GRb* expression negatively correlates with GC response in UC patients | (12) |
| | | | IBD may be associated with GR polymorphisms | (13) |
| | | | GR isoform expression does not predict steroid treatment response in IBD patients | (14) |
| | | | GR levels increase in UC patients responding to GCS therapy | (15) |
| | | | $GR\beta^{+}$ cells are increased in GC-resistant group than control and GC-sensitive group | (16) |
| | | | No significant associations between GR gene polymorphisms and GR resistance in IBD treatment | (17) |
| | | | GR expression was downregulated in IBD patients | (18) |
| | | | No difference in GR expression in patients vs. healthy controls could be detected | (19) |
| | mo | Chemical | GR in myeloid cells essential to achieve resolution of DSS-induced colitis | (20) |

*Hu, human; mo, mouse; mb, microbiota; UC, ulcerative colitis; CD, Crohn's disease; GWAS, genome-wide association study; IBD, inflammatory bowel disease; AOM, azoxymethane; IEC, intestinal epithelial cells; DSS, dextran sulfate sodium; TNBS, 2,4,6-trinitrobenzene sulphonic acid; mRNA, messenger RN; KO, knock-out.*

of RORγ protein is reduced in the absence of the vitamin A metabolite retinoic acid (RA), this suggests that RAR directly controls the fetal development of intestinal secondary lymphoid organs (SLOs) as well as the fitness of the immune system in adulthood [recently reviewed (19)].

Macrophages expressing LXR, NR4A1 (NUR77), PPARγ, or RARα are essential for gut immune homeostasis (30–34) (**Figure 3**). Especially the reciprocal differentiation potential of naïve $CD4^{+}$ T cells into either pro-inflammatory Th17 or tolerance-inducing regulatory T cells is dependent on several NRs such as RORγ, RORα, LXR, NR4A2 (NURR1), PPARγ, RAR, or VDR (35) (**Figure 3**). Whereas, RAR-related orphan receptors (RORγ and RORα) are key transcriptional activators, RAR, RXR, NUR77, PPARγ, LXR, GR, VDR, and ER contribute to anti-inflammatory effects (31, 32, 36–40) (**Figure 3**).

The NR network in CD4 Th17 cells is highly complex. PPARγ suppresses Th17 differentiation by directly interfering with the silencing mediator of retinoid acid and thyroid hormone receptor (SMRT) clearance from the *Rorc* promoter. PPARγ activation suppresses not only the expression of *Rorc* but also subsequently that of *Il17a*, *Il17f*, *Tnfa*, *Il22, Il21, Il23r, CCR6*, and *CCL20* (18). LXR reduces the expression of *Rorγt*, *Il17a, Il17f, Il22, Il23r,* and *Ahr* but does not affect that of *Il21* and *Rora*. RAR suppresses Th17 differentiation through retinoic acid-mediated inhibition of *Il23r*, *Il6r,* and *Irf4* expression and Smad2/3 phosphorylation via the TGFβ receptor pathway (43). NR2F6 directly binds to RORE sites within the *Il17a* locus and subsequently interferes with the transactivation of the *Il17a* promoter by RORc (41). It is still unclear how specificity is achieved despite highly homologous hormone response (HRE) DNA-binding core consensus sequences.

NRs, such as VDR or PPAR also regulate intestinal CD8 T cell responses (42–44). In parallel to the CD4 Th17 compartment, NR of the retinoic acid and retinoic acid-related family are key regulators of innate lymphoid cells (ILCs).

Innate lymphoid cells are tissue-resident immune cells that play essential roles in maintaining and protecting the gastrointestinal barrier against invading pathogens. In particular, ILCs themselves mediate immune responses but are controlled by dietary components and microbial metabolites. ILCs also directly regulate host metabolism and glucose tolerance (45). NR of the retinoic acid and retinoic acid-related family are key regulators of group ILC2 and 3 (46–48) (**Figure 3**). In a healthy state, ILC3 are responsible for mucosal homeostasis through the secretion of moderate amounts of IL-22, IL-17, and GM-CSF (49). Especially, regulation of gastrointestinal homeostasis and dysregulation by ILC3s result in overexpression of the IL-17, IFNγ, and IL-22 pro-inflammatory cytokines which can be seen in mice and IBD patients (50–57).

Thus, whereas on one side, microbiota and their metabolites shape the homeostasis of the gut immune system, innate and adaptive lymphocytes sequentially shape gut microbiota and lipid metabolism on the other. Thus, NRs occupy a center stage of gut immune homeostasis (27).

**TABLE 2 |** Non-steroidal nuclear receptors.

| Nuclear receptor | Spec | Model | Study outcome | References |
|---|---|---|---|---|
| CAR<br>NR1I3<br>(protective) | hu | Biopsies | *CAR* expression is reduced in CD and UC patients | (21) |
| | mo | Chemical | Colonic CAR expression is reduced in DSS-treated mice; $CAR^{-/-}$ mice exhibit reduced healing following DSS exposure | (21) |
| FXR<br>NR1H4/5<br>(protective) | hu | Biopsies | Reduced ileal *FXR* expression in CD patients | (22) |
| | | | Genetic variation of *FXR* is associated with IBD | (11) |
| | mo | Spontaneous | FXR protects the small intestine against bacterial overgrowth and the disruption of the epithelial barrier | (13) |
| | | Chemical | $FXR^{-/-}$ mice are more susceptible to DSS and TNBS, enhanced levels of pro-inflammatory cytokines | (23) |
| | | | FXR protects against colitis symptoms (DSS and TNBS) | (24) |
| LRH-1<br>NR5A2<br>(protective) | hu | Biopsies | Reduced mRNA and protein expression of LRH-1 in CD and UC patients | (25) |
| | mo | Chemical | $LRH\text{-}1^{+/-}$ mice are more susceptible to DSS and TNBS colitis and show enhanced inflammatory responses | (25) |
| | | AOM/APCmin | $LRH\text{-}1^{+/-}$ mice show reduced intestinal tumorigenesis | (26) |
| LXR<br>NR1H3/2<br>(protective) | hu | GWAS | *LXR* polymorphisms contribute to enhanced risk of developing IBD | (27) |
| | | Biopsies | Colonic *LXRα* and *LXRβ* expression is significantly reduced in IBD patients | (28) |
| | | Cell culture | Loss of *LXR* expression and function is believed to reduce fatty acid synthase expression in UC patients | (29) |
| | mo | Chemical | $LXR^{-/-}$ mice are more susceptible to colitis (DSS and TNBS) | (28) |
| NUR77<br>NR4A1<br>(protective) | hu | GWAS | *NR4A1* gene locus is associated with an increased risk for UC and CD; reduced *NR4A1* expression in colons from patients | (30) |
| | mo | Chemical | NR4A1 expression is reduced in DSS colitis, $Nur77^{-/-}$ mice are more susceptible to DSS-induced colitis | (30, 31) |
| NUR1<br>NR4A2<br>(protective) | mo | Chemical | Loss of NR4A2 in CD4 T cells only leads to an increased susceptibility to DSS-induced colitis | (32) |
| PPARα<br>NR1C1<br>(protective) | mo | Chemical | $PPAR\alpha^{-/-}$ mice are more susceptible to TNBS colitis | (33, 34) |
| | | | PPARα controls aspects of colonic inflammation (DSS) | (35) |
| PPARδ<br>NR1C2<br>(protective) | mo | Chemical | $PPAR\beta/\delta^{-/-}$ mice are more susceptible to DSS-induced colitis | (36) |
| PPARγ<br>NR1C3<br>(protective) | hu | Biopsies | $PPAR\gamma$ expression is reduced in UC patients | (37–40) |
| | | | $PPAR\gamma$ expression is decreased in intestinal samples from IBD patients | (41) |
| | | | SNPs in PPAR$\gamma$ are associated with CD | (42) |
| | | GWAS | $PPAR\gamma$ polymorphism is associated with susceptibility to IBD | (27, 43) |
| | mo | Chemical | PPAR$\gamma$ protein levels are decreased during DSS colitis | (44) |
| | | | Intestinal epithelial cell-specific $PPAR\gamma^{-/-}$ mice are more susceptible to induction of DSS colitis | (45) |
| PPARγ<br>NR1C3<br>(protective) | mo | Chemical | IEC-specific deletion of PPARγ enhances colonic inflammation (DSS) | (46) |
| | | | Induction of DSS colitis in $CD4^{cre}$ $PPAR\gamma^{fl/fl}$ mice enhances disease severity and histopathology | (46) |
| | | | Macrophage-specific PPARγ deletion in mice significantly exacerbated DSS colitis | (47) |
| | | | $PPAR\gamma^{+/-}$ mice are more susceptible to induction of TNBS colitis | (48) |
| | | | $PPAR\gamma^{+/-}$ mice are more susceptible to induction of TNBS colitis | (49) |
| | | Transfer | Treg-intrinsic PPAR$\gamma$ activation prevents colitis progression | (50) |
| | | Ischemia | $PPAR\gamma^{-/-}$ mice are more susceptible to tissue injury | (51) |
| | | Spontaneous | *Ppar*$\gamma$ as a susceptibility gene in SAMP1/YitFc mouse Crohn's disease | (42) |
| | | | PPAR$\gamma$ induces colon epithelial expression of β-defensins and therefore functions as an antimicrobial factor | (52) |
| PXR<br>NR1I2<br>(protective) | hu | Biopsies | *PXR* mRNA expression is significantly reduced in colons of UC patients (unaffected in CD) | (53) |

*(Continued)*

**TABLE 2** | Continued

| Nuclear receptor | Spec | Model | Study outcome | References |
|---|---|---|---|---|
| | | | Decreased *PXR* expression in intestinal samples from IBD patients | (41) |
| | | | PXR is associated with IBD | (54–57) |
| | | GWAS | Several PXR haplotypes contribute to CD susceptibility | (27, 58) |
| | mo | Chemical | PXR activation ameliorates DSS-induced colonic injury | (47, 59) |
| | | | Gut injury was more severe in $PXR^{-/-}$ mice challenged by experimental necrotizing enterocolitis | (60) |
| Rev-Erb α/β; NR1D1/2 (protective) | hu | Biopsies | NR1D2 expression is downregulated in UC patients | (61, 62) |
| RORα; NR1F1 (promotion) | hu | Biopsies | *RORα* expression is upregulated in colonic mucosa of CD patients | (62) |
| RORγt; NR1F3 (promotion) | mo | Transfer | Adoptive transfer of *RORγt*-deficient T cells into $Rag1^{-/-}$ mice failed to induce colitis | (63) |
| RXR; NR2B1,2,3 (protective) | mo | Chemical | $RXR\alpha^{+/-}$ mice are more sensitive to TNBS and DSS-induced colitis | (49, 64) |
| VDR NR1I1 (protective) | hu | Biopsies | IBD susceptibility and VDR polymorphism are genetically associated | (52, 65–72) |
| | | | Colonic epithelial *VDR* expression was reduced in CD or UC patients | (73, 74) |
| | | | Vitamin D deficiency associates with an increased risk of IBD in epidemiological studies | (75–81) |
| | mo | Transfer | $VDR^{-/-}$ T cells induced enhanced colitis symptoms in $Rag1^{-/-}$ mice | (74, 82) |
| | | Spontaneous | VDR/IL-10 d.k.o. mice developed accelerated IBD resulting in 100% mortality by 8 wks. of age | (82–84) |
| | | Infection | *Salmonella* infection induced colonic epithelial VDR expression, and VDR attenuates responses to infection | (85, 86) |
| | | Chemical | VitD deficiency predisposes mice to DSS colitis | (87) |
| | | | Intestine-specific $VDR^{-/-}$ mice developed enhanced DSS colitis (mucosal damage, increased pro-inflammatory cytokines | (86, 88) |
| | | | hVDR-expressing mice are highly resistant to DSS and TNBS-induced colitis | (74) |
| | | | $VDR^{-/-}$ mice are extremely sensitive to DSS colitis | (82, 89) |

## Diseases of the Gastrointestinal Tract

The two major forms of chronic inflammatory disorders within the gastrointestinal tract are Crohn's disease (CD) and ulcerative colitis (UC), characterized by clinical symptoms like severe diarrhea, pain, fatigue and weight loss (58). UC primarily affects the colon and rectum, whereas CD targets the small and large intestine, the mouth, esophagus, stomach, and the anus (59, 60). The etiology of the disease is multifactorial including genetic predisposition, the composition of gut microbiota, and environmental factors such as nutrition and antibiotic usage which subsequently can also alter immune responses (61). Intestinal barrier integrity is one of the most critical factors for a healthy GI tract, as an invasion of solutes, microorganisms and luminal antigens cause immune cell infiltration and inflammatory responses (62). Treatment options such as the corticosteroid prednisone or the anti-tumor necrosis factor-α antibody Infliximab suppress the immune system and relieve symptoms of patients.

Several members of the NR family have a protective role during disease progression, and their loss in different IBD animal models (DSS, TNBS, T cell transfer, or infection) leads to exacerbated colitis symptoms (**Tables 1**–**3**) (**Figure 1**). In human genome-wide association studies (GWAS), several nuclear receptor polymorphisms have been associated with IBD, and NR expression is mostly down-regulated in biopsies from UC and CD patient in comparison to healthy subjects (12, 63–73) (**Tables 1**–**3**). As IBD onset typically occurs in the second and third decade of life with a high number of patients progressing to relapse and chronic disease, an urgent need to develop new therapies with either low adverse side effects during long-term management or even curative potential is needed in the future.

## Nuclear Receptors as Therapeutic Targets in the Clinic

The characterization of NRs that either promote or suppress intestinal inflammation has led to efficacious therapeutics for IBD. One classic anti-inflammatory drug, namely 5-ASAs, augments PPARγ expression and promotes its translocation from the cytoplasm to the nucleus resulting in activation of peroxisome-proliferator hormone response element-driven genes to suppress colitis activity (74, 75). Regarding combinatorial therapy, the treatment with 5-ASAs and rosiglitazone (PPARγ agonist) had a better therapeutic effect in UC than 5-ASA alone (76–78) (**Table 4**).

Regulating the glucocorticoid receptor (GR), glucocorticoids play an important role in inducing remission in IBD (79). Unfortunately, response rates are low and vary between 20 and 30% of patients showing resistance with the therapy also inducing common side effects (80) (**Table 4**).

**TABLE 3 |** Orphan nuclear receptors.

| Nuclear receptor | Spec | Model | Study outcome | References |
|---|---|---|---|---|
| HNF4α NR2A1 (protective) | hu | Biopsies | *HNF4A* expression is decreased in intestinal samples from IBD patients | (41, 90, 91) |
| | | GWAS | *HNF4A* locus is associated with an increased risk for UC | (92–95) |
| | mo | Chemical | IEC-specific *Hnf4α*$^{-/-}$ mice are more susceptible to DSS-induced colitis | (41) |
| | | Spontaneous | Development of spontaneous colitis in aged mice | (90) |
| | mb | Meta-analysis | Interactions between HNF4α and microbiota gene expression patterns are associated with human IBD | (96) |
| NR2F6 EAR2 (protective) | hu | Biopsies | High *NR2F6* expression in healthy IECs, downregulated *NR2F6* expression in intestinal mucosa of IBD patients | (61, 97–100) |
| | mo | Chemical | *Nr2f6*$^{-/-}$ mice are more susceptibility to DSS induced colitis due to loss of barrier integrity and reduced Muc2 gene regulation | (101) |
| | | Spontaneous | Spontaneous colitis phenotype in aged mice | (101) |

*Hu, human; mo, mouse; mb, microbiota; UC, ulcerative colitis; CD, Crohn's disease; GWAS, genome-wide association study; IBD, inflammatory bowel disease; AOM, azoxymethane; IEC, intestinal epithelial cells; DSS, dextran sulfate sodium; TNBS, 2,4,6-trinitrobenzene sulphonic acid; mRNA, messenger RNA; d.k.o., double knock-out.*

There is strong evidence in support of vitamin D that targets the VDR having protective effects in IBD-related inflammatory responses (81). Treatment with vitamin D3 in mild to moderate CD patients significantly improved disease activity and quality of life after 24 weeks of treatment (**Table 4**) (82). More studies showed a positive effect of vitamin D supplementation in UC and CD patients (82–86). However, since the clinical efficacy and mechanism of action of vitamin D therapy are unclear, additional studies are necessary to fully explore its possible immunomodulatory and anti-inflammatory effects, also in relation to decreasing epithelial permeability and maintaining barrier integrity (87) (**Tables 2B**, **4**).

Rifaximin, an intestine-specific human PXR agonist, appears to have more antimicrobial efficacy in the therapy of CD than traditional medications like metronidazole or ciprofloxacin; it decreases intestinal permeability and targets (NFκB), regulating anti-inflammatory effects in IBD patients (88–90) (**Table 4**).

Besides clinical data on nuclear receptor therapy targets, so far, several NRs have only been tested pre-clinically in animal models.

## Steroid Hormone Receptors

### Estrogen Receptor (ER; NR3A)

The estrogen receptors, members of the steroid hormone receptor family, play an essential role in the maintenance of colonic homeostasis. Understanding the biological effects of ERα and ERβ within the gut and the immune system are important for unraveling the gender-dependent differences in intestinal inflammatory diseases. Interestingly, estrogen levels impact the composition of gut microbiota itself (91); however, the composition of the microbiota influences the bioavailability of estrogen (92) (**Figure 2**). Males are at greater risk than females for developing ulcerative colitis (UC) and experiencing worse clinical progression, whereas females are more likely to develop CD (93–96). Especially ERβ is expressed abundantly in the colonic epithelium, where it regulates maintenance of colonic architecture, tight-junction formation, and barrier function (97, 98). *ERα* and *ERβ* gene expression levels are comparable between male and female UC colon samples, suggesting that sex-based differences in ER-mediated effects are most likely not caused by differences in gene expression (**Table 1**) (99).

Nevertheless, *ERβ* expression has been found markedly decreased in colonic mucosa of CD/UC patients with active disease; specifically, *ERβ* expression in female mice protected against DSS colitis, whereas it failed to protect male mice (100). Recently, Wendy A. Goodman et al. (93) reported that fundamental differences in ERα/ERβ signaling ratios impact colitis in males and females. Analysis of gene expression from inflamed colonic tissues identified alteration of typical estrogen-responsive genes such as *Socs3*, *Ctsd*, and *Fos* as being up-regulated in colon tissues of DSS-treated *ERα*-knockout male mice compared with *ERα*-knockout females. In line with these data, similar gene expression profiles of *SOCS3*, *CTSD*, and *FOS* were found in colonic biopsy specimens from male and female patients suffering from UC (93, 99) (**Table 1**).

Experimental colitis studies in mice and rats have shown that ER has pre-clinical therapeutic implications (93, 101–103). Supraphysiological doses of 17β-estradiol have anti-inflammatory (in the DNB mouse colitis model) as well as pro-inflammatory (in the DSS mouse colitis model) effects demonstrating complex immunomodulation in female mice during intestinal inflammation (102). Additionally, studies in male rats and in the HLA-B27 transgenic rat IBD model demonstrated reduced colonic damage score with estradiol treatment during acute colonic injury (101, 103).

### Glucocorticoid Receptor (GR; NR3C1)

Glucocorticoids targeting the GR contribute to diverse biological processes including glucose metabolism, stress, or immune responses. Endogenous GCs are predominantly produced by the adrenal glands, but within the IEC the NR LRH-1 regulates extra-adrenal glucocorticoid synthesis in the intestine (104).

The GR is expressed in almost every cell in the body and is a multi-tasking transcription factor, changing its role and function from anti-inflammatory effects via direct gene

**TABLE 4 |** Human IBD therapy—clinical trials.

| NR | Compound | Mechanism | Cell type | References |
|---|---|---|---|---|
| GR | Glucocorticoids | Anti-infl | lmC | (1–4) |
| | Prednisolone | Anti-infl | lmC | UC:(5–7) |
| | | | | CD: (8, 9) |
| | Budesonide | Anti-infl | lmC | UC: (10–14) |
| | Prednisone | Anti-infl | lmC | CD: (15–17) |
| | | | | UC: (18) |
| | Prednisolone | Anti-infl | lmC | (6, 19–21) |
| | Methylprednisolone | Anti-infl | lmC | CD: (11, 22) |
| | Beclomethasone | Anti-infl | lmC | CD: (13) |
| | | | | UC: (23) |
| | Cortisone | Anti-infl | lmC | UC: (24) |
| | Fluticasone | Anti-infl | lmC | UC: (25–27) |
| PPARγ | 5-ASA (Sulfasalazine, Mesalazine, Mesalamine) | Anti-infl | lmC | CD & UC: (28) |
| | Rosiglitazone | Anti-infl | lmC | UC: (29–31) |
| | | Perm | IEC | UC: (31) |
| | 5-ASA + Rosiglitazone | Anti-infl | lmC | UC: (32) |
| PXR | Rifaximin | Anti-mic | mb | (33–37) |
| RORγt | Secukinumab | Anti-inflam | lmC | (38) |
| VDR | Vitamin D | Anti-infl | lmC | (39) |
| | | Perm | IEC | (40–44) |
| | | Anti-infl | lmC | (45–51) |
| | | Pro-bact | mb | (52, 53) |

*Anti-infl, anti-inflammatory; pro-infl, pro-inflammatory; anti-mic, anti-microbial; pro-bact, pro-bacterial; perm, epithelial permeability; mb, microbiota; lmC, immune cells; IEC, intra epithelial cells; UC, ulcerative colitis; CD, Crohn's disease.*

suppression or activation to potential pro-inflammatory actions as well [reviewed in (105)]. Nevertheless, rapid non-genomic mechanisms of GC signaling have also been reported [reviewed in (105)].

A mechanism for glucocorticoid-mediated inhibition of immune responses is the interference with activities and modulation of key pro-inflammatory transcription factors, including NF-κB, activator protein 1 (AP-1), members of the signal transducer and activator of transcription (STAT), CCAT/enhancer-binding protein (C/EBP), and nuclear factor of activated T cells (NFAT) families (106, 107). Through GR-mediated transrepression, expression of pro-inflammatory cytokines and chemokines like IL-1α, IL-1β, and IL-8 are down-regulated (**Figure 3**). Additionally, the GR can directly activate suppressive inflammatory mediators like TGF-β and IL-10, inhibit T and B lymphocyte proliferation, and promote a tolerant macrophage profile (M2), altogether increasing its anti-inflammatory function (105, 108) (**Figure 3**). The signaling pathways of GR and PPARα, another nuclear receptor, can cooperate and increase the inhibition of cytokine gene expression to alleviate inflammation (109).

Within the immune system, glucocorticoids are circadian mediators (110) and regulate diurnal oscillations in T cell distribution by inducing IL-7R and CXCR4 (111) and regulate T cell responses in gastrointestinal Peyer's patches. Dexamethasone suppresses IL-23–mediated IL-22 production in human and mouse ILC3s (112).

GCs have a long history in IBD therapy and are well-known immune suppressants. Nevertheless, the expression of the GR itself does not appear to predict steroid treatment responses in IBD patients although conflicting data exist (**Table 1**). GCs are especially able to protect mice and men against TNF-induced inflammatory symptoms, and GR dimers control intestinal STAT1 and TNF-induced inflammation in mice (113, 114).

The role of the GR in IBD has been reviewed recently (115). Human trials demonstrated that standard systemic corticosteroids (cortisone, prednisone, methylprednisolone, fluticasone) are effective in inducing remission in UC by suppressing immune responses, and might be of benefit in CD (3) (**Table 4**). Therefore, glucocorticoids are still the mainstay for induction of clinical remission in cases of acute relapse of both CD and UC, and second-generation corticosteroids such as budesonide or beclomethasone have been developed. Whereas, budesonide induces remission in active ileal CD, it shows less efficacy in and does not prevent CD relapse (3, 116) (**Table 4**). Many preclinical studies were performed using different IBD animal models to investigate the complex cellular and molecular basis of glucocorticoid action at the interface between the endocrine, the immune, and the intestinal system. As a future perspective, screening assays for GR agonists are ongoing in order to develop new effective medications against acute inflammation (117).

The role of other steroid hormone receptors such as the androgen receptor (AR) and the progesterone receptor (PR) during colitis progression have been investigated only poorly. Pre-diagnostic circulating testosterone is associated with a lower risk of CD but not UC in women (95). Progesterone therapy decreases oxidative damage, characterized by decreased MDA, MPO, TNFα and caspase-3 activity, in the colonic mucosa (118).

## Non-steroidal Nuclear Receptors

Despite a wide range of pre-clinical IBD trials with compounds specifically targeting NR family members, the following NRs have not yet reached UC or CD patients in clinical trials.

### Constitutive Androstane Receptor (CAR; NR1I3)

The xenobiotic NR CAR can be regulated by xenobiotics and endobiotics but also by steroid hormones (119). One of its diverse metabolic functions (119) includes the clearance of xeno- and endobiotics such as toxic bilirubin (**Table 2A**) (120). Its expression in the intestine and the liver is dependent on the presence of microbiota (119, 121, 122).

CAR is expressed in the healthy intestinal epithelium, but the expression is reduced within intestinal mucosal biopsies from patients with UC and CD, or tissue from DSS mice (67, 123) (**Figures 1**, **2**). In the pre-clinical DSS mouse model, especially wound healing of intestinal epithelial cells is reduced in *Car*-deficient mice whereas activation of

CAR using a selective CAR agonist 3,3',5,5'-tetrachloro-1,4-bis(pyridyloxy)benzene (TCPOBOP) enhances mucosal healing (67) in mice (**Figure 2**). In a rat DSS colitis model, CAR agonists reduced the mRNA expression of several pro-inflammatory cytokines in a CAR-dependent manner; CAR inhibited apoptosis by inducing Gadd45b within an *in vitro* cell analysis (124). Therefore, CAR activation may also prove effective in patients with IBD.

### Farnesoid X Receptor (FXR)

FXR functions as an enterohepatic regulator of bile acid homeostasis and regulates especially lipid (125) and glucose metabolism (126), as well as inflammation (13). *Fxr*-deficient mice are more susceptible to IBD models such as TNBS or DSS due to enhanced expression of pro-inflammatory cytokines in innate immune cells (127) (**Table 2A**) (**Figures 1**, **3**). Also monocytes and dendritic cells (DCs) are modulated by FXR and there is a decrease in epithelial expression of pro-inflammatory molecules both *in vivo* and in stimulated epithelial cultures after induction of FXR signaling, suggesting that the immunomodulation observed might be partly mediated through epithelial effects (13, 128) (**Table 2A**; **Figure 2**). Along with the FXR ligand, INT-747 represses the expression of various pro-inflammatory cytokines, chemokines and their receptors (13, 127). Colon inflammation in CD patients and rodent models of colitis is associated with reduced expression of *FXR* mRNA (**Table 2A**) (127). FXR also regulates gut barrier function due to its antibacterial growth effect (129) and its control of proliferating $Lgr5^{+}$ intestinal stem cells (130) (**Figure 2**). Bile acids are well known natural ligands of FXR and regulate the protective activity of FXR in shielding the intestine from bacteria-induced damage and thereby maintaining a competent gut barrier and preventing the development of IBD (**Table 2A**) (13, 131).

Several pharmacological modulators of FXR activity have been tested in human clinical trials, but its role in IBD has so far only been investigated in pre-clinical mouse models (13). Fexaramine is an intestinal-specific FXR modulator which is potentially safer than systemic FXR agonists as it preferentially activates FXR target genes in the intestine (132), but its functional role has not yet been investigated in IBD models.

### Liver Receptor Homolog-1 (LRH-1; NR5A2)

LRH-1 is mostly known for its regulatory role in cholesterol and bile acid homeostasis but has recently emerged as a key regulator of intestinal function. Unlike most of the other NRs, LRH-1 acts constitutively to drive the transcription of its target genes (133). Nevertheless, this atypical NR contains a well-ordered hormone-binding pocket, which binds signaling phospholipids including phosphoinositides (134, 135).

LRH-1 is expressed in intestinal crypts, where intestinal stem cells (ISCs) reside, and where it contributes to epithelial renewal by potentiating WNT/β-catenin signaling (136–138). GWAS meta-analyses of IBD patients found a significant association between LRH-1 and IBD (**Table 2A**). Subsequent analysis on IBD patients revealed a significant decrease in expression of *LRH-1* and its transcriptional targets such as *CYP11A1* and *CYP11B1* in the affected tissues (69, 139). Both *Lrh-1* haploinsufficiency and somatic deficiency of *Lrh-1* in the intestinal epithelium rendered mice more susceptible to experimentally induced DSS or TNBS colitis (**Table 1**) (69). One pathway how LRH-1 limits inflammation involves the regulation of extra-adrenal glucocorticoid production in the gut (69, 133). Apart from the immune-regulatory action on local immune cells, glucocorticoids may also induce intestinal tight junction proteins and improve epithelial barrier function (**Table 1**; **Figures 1**, **2**) (140). It is plausible that after hapten-induced mucosal inflammation, the cell cycle regulatory function of LRH-1 comes into play to promote mucosal renewal and regeneration (69). A recent study also underpins the human relevance, using humanized mouse intestinal organoids, a humanized *in vivo* IBD model, and human intestinal organoids (**Table 2A**). Thereby, Bayrer et al., uncovered an essential role for LRH-1 in intestinal epithelial homeostasis and cell survival, which mitigates inflammatory injury (135). As preliminary therapeutic results, the use of DLPC (dilauroyl phosphatidylcholine) as an extrinsic agonist ligand for LRH-1 has been reported to result in decreased colitis symptoms (69).

### Liver X Receptors (LXR; NR1H)

LXRs control lipid and glucose homeostasis and respond to physiological concentrations of sterols. Whereas, LXRα is mainly expressed in the liver, intestine, kidney, and immune cells, LXRβ is more ubiquitously expressed (141–143). Within the immune system, both LXRs are important anti-inflammatory transcription factors and physiological regulators of innate and adaptive immune responses, apoptosis, and phagocytosis (144).

Several LXR agonists are effective in pre-clinical models of diseases such as atherosclerosis or diabetes and are used as an anti-inflammatory agent (143, 145). *Lxr*-deficient mice are more susceptible to DSS colitis, show slower recovery and decreased survival (**Table 2A**). Expression of both *LXRA* and *LXRB* is significantly suppressed in the inflamed colon from both CD and UC patients compared with a non-inflamed colon (70), and LXR polymorphism has been linked to enhanced IBD risk (**Table 2A**) (146). While LXRα induces anti-inflammatory effects in innate immune cell populations (**Figure 3**), LXRβ has anti-inflammatory effects in colon epithelial cells (**Figure 2**). Addition of an LXR agonist GW3965 results in faster recovery and increased survival in pre-clinical mouse colitis models, making LXRs an exciting target to suppress inflammatory responses in IBD (70).

### NR4A Family: (Nur77; NR4A1, Nurr1; NR4A2 and Nor1; NR4A3)

The orphan NR4A subfamily includes three members, which are expressed in a wide variety of tissues, especially innate and adaptive immune cells (147).

Although NR4A family members belong to the nuclear receptors superfamily, their activity is not considered to be regulated by physiological ligands, because their ligand-binding pockets are hidden by bulky amino acids, and their ligand-binding domains are constitutively active (148). Nevertheless, structurally diverse synthetic ligands for NR4A2 and NR4A3 have recently been identified (147). Genetic variants of the

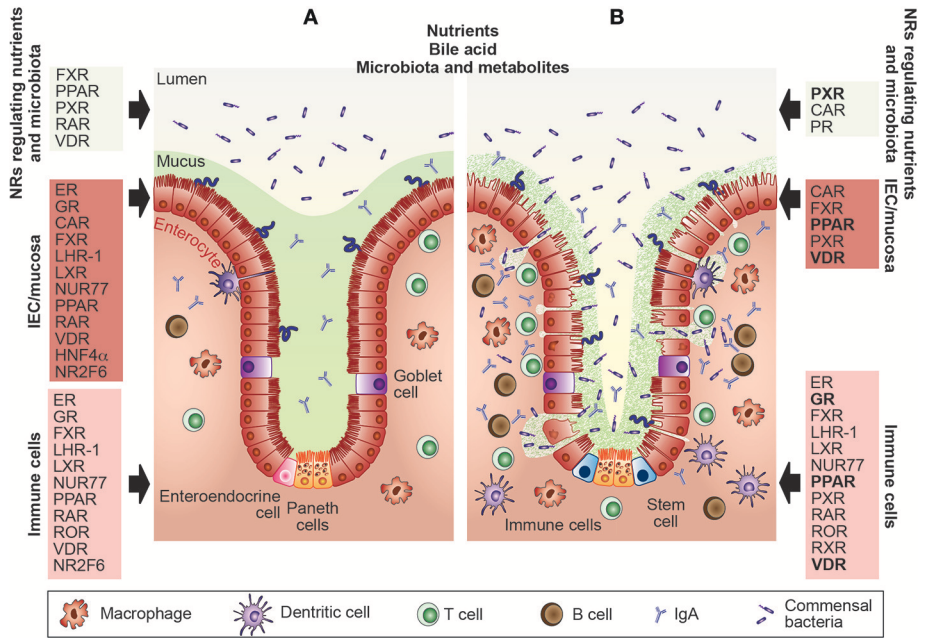


**FIGURE 1 |** Nuclear receptors are essential for the maintenance of gut homeostasis and have already been targeted in IBD patients. **(A)** Within the healthy gastrointestinal system, nuclear receptors (NRs) such as FXR, PPAR, PXR, RAR, or VDR are well-known sensors of nutrients, toxic dietary products, specific host-bacterial metabolites, and bile acid. Intestinal barrier function and epithelial intestinal cell integrity are dependent on the appropriate function of the ER, GR, CAR, FXR, LHR-1, LXR, NUR77, PPAR, RAR, VDR, HNF4α, and NR2F6 which regulate mucus secretion, expression of tight junction proteins autophagy, circadian clock as well as goblet and paneth cell numbers. Also, NRs such as the ER, GR, FXR, LHR-1, LXR, NUR77, PPAR, RAR, ROR, VDR HNF4α, and NR2F6 contribute to gut homeostasis by shaping intestinal immune cell development, and the composition and effector functions of macrophages, dendritic cells, T and B cells. **(B)** The primary protective role of the NRs in the pathophysiology of inflammatory bowel diseases has been validated in pre-clinical animal models and clinical trials. NRs targeted by therapeutic drugs in IBD patients are GR, PPAR, PXR, and the VDR (highlighted in bold), NRs tested in preclinical mouse models are CAR, ER, FXR, LHR-1, LXR, NUR77, PPAR, PXR, RAR, ROR, and RXR; thus, novel concepts integrating NR, and gastrointestinal physiology have been integrated into the development of effective therapies. CAR, constitutive androstane receptor; ER, estrogen receptor; FXR, farnesoid X receptor; GR, glucocorticoid receptor; HNF4α, hepatocyte nuclear factor-4-alpha; IBD, inflammatory bowel disease; IECs, intestinal epithelial cells; LRH, liver-related homolog; LXR, liver X receptor; NR2F6, nuclear receptor subfamily 2 group F member 6; NR4A1/2, nuclear receptor subfamily 4 group A member 1/2 (NUR77, NUR1); PPAR, peroxisome proliferator-activated receptor; PXR, pregnane X receptor; RAR, retinoic acid receptor; RevErb, nuclear receptor subfamily 1, group D, member 1; ROR, RAR-related orphan receptor; RXR, retinoid X receptor; VDR, vitamin D receptor.

*NUR77* gene locus are associated with increased risk for both UC and CD, and *NUR77* expression is significantly reduced in colon tissues from patients with UC or CD and mice treated with DSS (**Table 2A**) (31, 149). *Nur77*-deficiency increases the susceptibility of mice to DSS and TNBS colitis and prevents intestinal recovery (31, 149) (**Table 2A**) (**Figure 1**). Mechanistically, NUR77 negatively regulate the TLR–IL-1R signaling axis (149). An independent study demonstrated that loss of *Nur77* in mice leads to enhanced colon inflammation with larger numbers of infiltrating neutrophils, T-cells, and macrophages during DSS colitis. *Nur77* overexpression dampens the pro-inflammatory state of both RAW macrophages and epithelial Caco-2 cells (**Table 2A**) (31).

The family member NR4A2 also regulates immune cell function and subsequently colitis, as deletion of NR4A2 in T cells attenuates induction of Tregs and causes aberrant induction of Th1 $CD4^+$ T cells and subsequent exacerbation of colitis (**Figure 3**) (150). Treatment with cytosporone B (Csn-B), an agonist for Nur77, significantly attenuated excessive inflammatory response in mouse DSS colitis. Therefore, Nur77 has been suggested as a potential target for the prevention and treatment of IBD (149).

## Peroxisome Proliferator-Activated Receptors (PPARγ, NR1C3)

PPARs are involved in the control of energy metabolism, inflammation and immune responses activated by natural ligands such as fatty acids, eicosanoids, and phospholipids (151, 152). PPARγ is highly expressed in both IECs and immune cells. Impaired epithelial expression has been documented in preclinical animal models of IBD and UC patients (**Tables 2A,B**) (**Figures 1**, **2**).

Expression and activity of PPARγ are directly induced by microbial metabolites such as butyrate or propionate (153, 154).

Direct targeting of the activity of PPARγ to enhance anti-inflammatory effects via its agonistic ligand rosiglitazone is efficacious in the treatment of mild to moderately active UC (78, 155, 156) (**Table 4**). In combination with the anti-inflammatory drug 5-ASA (5-Aminosalicylate and its generics),

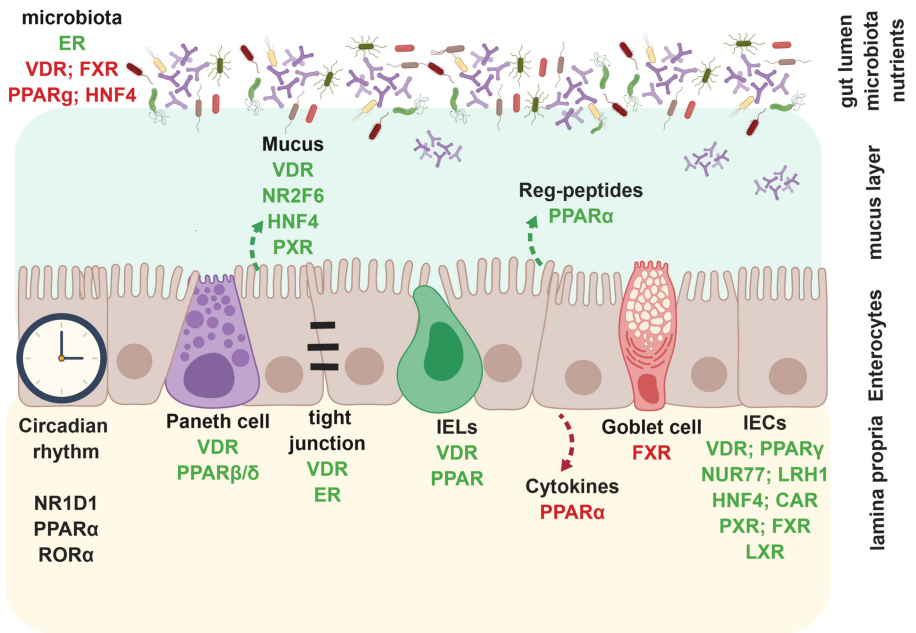


**FIGURE 2 |** Nuclear receptors regulate intestinal epithelial barrier. During homeostasis, nuclear receptors such as VDR, FXR, PPARγ, HNF4α inhibit bacterial outgrowth whereas ER enhances microbiota richness. VDR, NR2F6, HNF4α, and PXR promote mucus secretion and epithelial barrier integrity, whereas VDR and ER directly enhance tight junctions. PPARα specifically promotes the production of anti-microbial Reg-peptides, beneficial for an intact barrier. Circadian rhythm in enterocytes is dependent on NR1D1, PPARα, and RORα. VDR and PPARβ/δ positively regulate paneth cell development. VDR and PPARα also promote $CD8\alpha\alpha^{+}$ IELs. PPARα enhances expression of pro-inflammatory in enterocytes. FXR inhibits goblet cell development. VDR, PPARγ, NUR77, LRH-1, HNF4α, PXR, FXR, and LXR promote enterocyte development, whereas CAR is involved in wound healing of IECs. Created with BioRender.

rosiglitazone achieves better therapeutic effects without causing side effects in UC patients (76). Therefore, rosiglitazone is the most widely used therapeutic agent in conjunction with PPARγ activation that directly leads to trans-repressing of several pro-inflammatory target genes such as NF-κB and signal transducers and activators of transcription (STATs) (77, 157–161) (**Figure 3**). Also, microbiota-activated PPARγ signaling prevents dysbiotic expansion of potential pathogens by reducing the bioavailability of respiratory electron acceptors in the lumen of the colon (23) (**Figure 2**). Accordingly, several preclinical studies in IBD animal models have been and are being performed to investigate the molecular mode of action of new compounds that target PPARγ to enhance its anti-inflammatory effects within the immune and the epithelial compartment (**Tables 2A,B**) (162).

In addition to PPARγ, the two other family members, PPARα (NR1C1) and PPARδ (NR1C2) are used for therapeutic IBD intervention. Each PPAR isotype has a specific expression pattern within the gut, but all of them mediate the effects of the microbiota (163). Agonistic ligands targeting PPARα such as dexamethasone (164), fenofibrate (165), palmitoylethanolamide (166), or bezafibrate (167) have anti-inflammatory effects in pre-clinical animal studies. The first-generation PPARα agonists, the fibrates, have however been hampered by drug-drug interaction issues, statin drop-in, and ill-designed cardiovascular intervention trials reviewed in Bougarne et al. (168). Ambiguous results were obtained targeting PPARδ with GW0742 or dietary punicic acid showing either anti-, pro-inflammatory or no effects in experimental mouse IBD models (**Tables 2A,B**) (165, 169, 170).

## Pregnane X Receptor (PXR; NR1I2)

PXR protects the body from harmful foreign toxicants and endogenous toxic substances as it induces genes involved in drug transport and metabolism (171). Pregnane X receptor is primarily expressed in the liver and the intestine; the distribution and function of human PXR in the gastrointestinal system contribute to its emerging role as a modulator of inflammation and the intestinal mucosal barrier (**Table 2B**) (171, 172) (**Figures 1**, **2**). In contrast to most other NRs, PXR has a wide spectrum of ligands such as drugs, endogenous ligands or products of the gut microflora (173–175). Despite high homology between the LBD and DBD domain of human and mouse PXR, species-specific responses to ligand activation (such as rifampicin, or pregnenolone-16α-carbonitrile) are surprisingly different making results of pre-clinical mouse studies less extrapolatable for human trials (171).

PXR agonists reduce the mRNA expression of several pro-inflammatory cytokines in a PXR-dependent manner such as TNF-α and IL-1β in a rat DSS colitis model (124). PXR has been implicated in the pathogenesis of IBD, and its activator rifaximin (works only in humans, not in mice) has demonstrated efficacy in CD and UC (**Table 2B**). Antibiotic therapy with rifaximin, which was approved in 2004 for the treatment of traveler's diarrhea

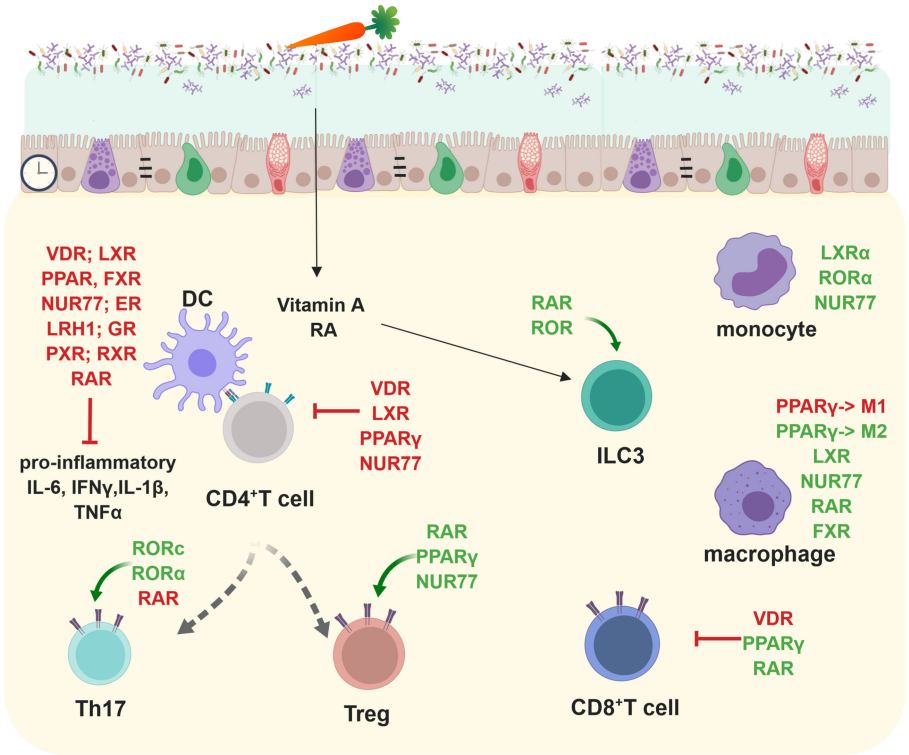


**FIGURE 3 |** Nuclear receptors shape adaptive and innate immune responses in the lamina propria and subsequently gut homeostasis. Most NRs (VDR, LXR, PPAR, FXR, NUR77, ER, LRH-1, GR, PXR, RXR, and RAR) target pro-inflammatory cytokine production (IL-6, IFNγ, IL-1β, TNFα). RORc and RORα are important in promoting Th17 development whereas RAR, PPARγ, and NUR77 positively influence regulatory T cells. VDR, LXR, PPARγ, and NUR77 inhibit $CD4^+$ T cell responses. RAR and ROR promote ILC3 differentiation. LXRα, RORα, and NUR77 positively influence monocytes. PPARγ inhibits M1 macrophages but promotes M2 macrophages which are important anti-inflammatory effector cytokine producers. LXR, NUR77, RAR, and FXR foster in parallel the anti-inflammatory responses of macrophages. VDR enhances inflammatory whereas PPARγ, and RAR suppresses pro-inflammatory $CD8^+$ T cell responses. Created with BioRender.

(176), was associated with the induction and maintenance of remission (90, 177). In CD patients, bile acid malabsorption is associated with deactivation of PXR (178). Despite the difference between human and mouse PXR species-specific responses, a lot of effort was made to test different ligands of PXR in mouse models of colitis utilizing its anti-microbial effects and its anti-inflammatory potential on immune cells as well as epithelial cells, helping the latter to maintain an intact epithelial barrier in the gut (**Figure 3**).

## Retinoic Acid Receptor (RAR; NR1B)

RARs usually form heterodimers and function as ligand-dependent transcription factors but also play extra-nuclear and non-genomic roles. The vitamin A metabolite all-trans-retinoic acid (atRA) acts as a ligand for RAR and is involved in the regulation of both the intestinal barrier function as well as immune homeostasis (19, 179, 180).

In general, the NRs of the RAR family play important pleiotropic roles in the regulation of innate immune cells such as dendritic cells, macrophages, and ILCs, and are especially important in the regulation of T cell homing to the gut as well as IgA class switching in B cells (**Figure 3**) (19). The pleiotropic roles of retinoic acid and RARs as modulators of the immune system, for example, induction of Th1, Th2, and Th17 responses together with the release of pro-inflammatory cytokines like IL-12 and Il-23 by DCs, have been reviewed in much detail recently and will therefore not be discussed here (19, 30).

The vitamin A metabolite retinoic acid (RA) can also enhance ILC3 responses in mice through multiple mechanisms, including direct binding to the *Rorc* or *Il22 loci*, promoting maturation of LTi-like ILC3s, and regulating ILC3 proliferation (47, 51, 181). In addition to promoting maintenance of the intestinal epithelium, during fetal development vitamin A and the metabolite RA control the size of secondary lymphoid tissues via LTi cells in mice, which can influence the efficiency of protection from viral infections later in life (47, 51, 182).

In mouse or rat colitis models Vitamin A inhibits the development of DSS colitis and colon cancer (183, 184) (**Table 2B**). Several pre-clinical studies in mice show anti-inflammatory effects of RAR ligands like ATRA (185–187), Neomangiferin (188), 13cis-retinoic acid (189), or RA (44, 190). Importantly, atRA supplementation reduced the tumor burden in a mouse model of colorectal cancer via enhancing protective $CD8^+$ T cell responses highlighting the relevance of NRs as a potential therapeutic option to treat colon cancer patients (191) (**Figure 3**).

### RAR-Related Orphan Receptor Gamma (RORγ; NR1F3)

RORγ has a broad pattern of expression but is observed at very high levels within the thymus. There RORγ regulates thymocyte and lymphoid development but is also involved in the regulation of metabolism and the circadian rhythm (192). The recently de-orphanized RORγ is known to bind to sterols, with certain oxysterols having a very high affinity for this receptor. Synthetic inverse agonists of RORγ are effective in treating and preventing autoimmunity in mouse models and are beneficial in glucose and lipid metabolism (1). A cross-talk between RORγt$^+$ ILCs and intestinal macrophages induces mucosal IL-22 production in Crohn's disease (193). In CD4$^+$ Th17, the splice isoform RORγt controls the secretion of the cytokines IL-17a and IL-17f. Especially IL-17f has recently been identified as highly pathogenic in gut inflammation. Therefore, RORγt-expressing Th17 cells induce murine chronic intestinal inflammation (**Table 2B**) (194, 195). In parallel, delivery of IL-15 to CD4$^+$ T cells in the colon downmodulates Foxp3 expression and enhances RORγt expression rapidly triggering IBD characterized by enhanced production of pro-inflammatory cytokines (such as interferon-γ, IL-6) and accumulation of Th1/Th17 cells (196) (**Figure 3**). Pharmacologic inhibition of RORγt via GSK805 provides therapeutic benefit in mouse models of intestinal inflammation and reduces the frequency of Th17 cells isolated from primary intestinal samples of individuals with inflammatory bowel disease (IBD) (197). In the course of IBD, RORα-dependent ILC3 functions are pivotal in mediating gut fibrosis, and they can offer an avenue for therapeutic intervention in Crohn's-like diseases (198).

### Retinoid X Receptor (RXR; NR2B)

RXRs have been implicated in a diversity of cellular processes. These pleiotropic effects originate from the ability of RXRs to dimerize with diverse NRs, which exert transcriptional control on specific aspects of cell biology, and the ability to stimulate transcriptional activation by RXR partner receptors (199). RXRs form heterodimers, either spontaneously or in a ligand-dependent manner, with NRs well known to play crucial roles in the regulation of intestinal homeostases such as VDR, PPAR, FXR, LXR, or CAR (199) (**Figure 2**). Especially the RXR/PPARγ heterodimers, which are permissive to activation by both PPARγ and RXR ligands, have been investigated in colitis models (200). *Rxra*$^{+/-}$ mice are highly sensitive to TNBS colitis and AOM/DSS colitis induction (**Table 2B**) (201). In the colon, the RXR ligand LG101305 is equally effective as PPARγ ligands in reducing intestinal inflammation during TNBS colitis. Also, rexinoids have a marked synergistic effect with PPARγ agonists on inflammation suggesting that co-administration of low doses of PPARγ and RXR agonists might be worth exploring in human IBD (200) (**Table 4**). Conventional full RXR agonists are known to show considerable adverse effects, but the partial RXR agonist, CBt-PMN, efficiently ameliorated the symptoms of colitis. This effect is attributed to the down-regulation of pro-inflammatory cytokines such as *Tnf* and *Il6* in colon-infiltrating monocytes probably by the activation of PPARδ/RXR and Nur77/RXR heterodimers by CBt-PMN (202).

### Vitamin D Receptor (VDR, NR1I1)

In the intestine, VDR signaling regulates microbial homeostasis, barrier integrity as well as immune cell distribution and function (**Table 2B**) (203). The immune system, in particular, is influenced by vitamin D3 via enhancement of chemotactic and phagocytic responses of macrophages and production of antimicrobial proteins, such as cathelicidin; it inhibits the surface expression of the MHC-II-complex antigen and costimulatory molecules and downregulates the production of many pro-inflammatory cytokines, such as IL-1, IL-6, IL-8, and TNF-α (204). Association studies showing a higher incidence of CD follow a "North-South gradient" and support animal and clinical data demonstrating an important role for vitamin D as a risk factor and potential therapeutic target in CD (205) (**Table 2B**).

IL-22 production is dependent on vitamin D and in the absence of vitamin D, mice develop a more severe enteric infection that takes longer to resolve (**Figure 2**) (206).

In contrast Chen et al., report that VDR KO mice have more interleukin-22 (IL-22)-producing ILCs and more antibacterial peptides than WT mice. The increased ILCs in the VDR KO mice was a cell-autonomous effect of VDR deficiency on ILC frequencies (207).

A double-blind, randomized placebo-controlled study of the effect of vitamin D supplementation over 3 months showed significantly increased 25(OH)D levels in patients in remission accompanied by maintenance of the intestinal permeability (86) (**Table 4**). Several therapeutic studies in experimental IBD animal models and patients have subsequently shown vitamin D to have therapeutic efficacy on epithelial permeability as well as anti-inflammatory properties, and the effects of different analogs have been summarized in a recent review (203) (**Figure 3**; **Table 2B**).

### Others

For two other non-steroidal nuclear receptors Rev-erb α/β (NR1D1/2) and RORα (NR1F1), data only from human patient biopsy is available. Whereas, *NR1D2* expression in UC patients is downregulated (72), *NR1F1* expression is upregulated in CD patients' colonic mucosa (208) (**Table 2B**).

## Orphan Nuclear Receptors

### Hepatocyte Nuclear Factor 4 Alpha (HNF4α; NR2A1)

The orphan NR HNF4α is considered to be an important actor in intestinal epithelial cell homeostasis and mucosal barrier integrity as this NR regulates proper intestinal epithelial cell differentiation (209–211), lipid metabolism (212), goblet cell maturation, epithelial junctions and *Muc* gene expression (**Table 3**) (12, 211, 213, 214) (**Figures 1**, **2**). Its role in liver and intestinal inflammatory networks has recently been reviewed in detail elsewhere (215). In humans, *HNF4A* expression is strongly reduced in intestinal biopsies of UC and CD patients (12, 64) and a GWAS has identified *HNF4A* locus as a susceptibility gene for UC (216). Besides, a single-nucleotide polymorphism within the *HNF4A* locus has also been associated with UC and pediatric CD (12, 216–219). Two HNF4α isoforms P1 and P2 are expressed in different compartments in the colonic epithelium, interact with distinct sets of proteins, and regulate the expression of unique sets of target genes, and thus play distinct roles during pathological

conditions such as colitis (**Table 3**) (220). Pre-clinical mouse and human association studies suggest a highly important role for this NR, but as an orphan NR family member, currently, no agonistic compounds targeting HNF4α are available for treatment options.

### Nuclear Receptor Subfamily 2 Group F Member 6 (NR2F6; EAR-2, COUP-TFIII)

We were the first to unravel the role of the COUP-TF family member NR2F6 in the pathogenesis of IBD (14). In immune cells, NR2F6 inhibits $CD4^+$ Th17 T cell responses and autoimmunity (38, 221) arnd suppresses $CD4^+$ and $CD8^+$ T cell-driven anti-tumor immunity (40, 222, 223). In the gut, NR2F6 directly protects the colonic intestinal epithelium and thus enhances gut barrier homeostasis. *Nr2f6*-deficient mice are highly susceptible to DSS-induced colitis; mechanistically, NR2F6 directly binds to a consensus sequence at −2 kb of murine and human MUC2 promoter and transactivates Muc2 expression. Loss of NR2F6, therefore, increases intestinal permeability and results in spontaneous late-onset colitis in *Nr2f6*-deficient mice (14) (**Figures 1**, **2**). Beside this pre-clinical dataset in mice, several studies from the literature document reduced *NR2F6* gene expression in patients with IBD (**Table 3**) (65, 72, 224, 225).

## CONCLUSION

NRs and NR ligands control important gastrointestinal functions ranging from nutrient uptake, the composition of the microbiota and intestinal immune cells. Mechanistic studies have identified several NRs involved in the pathophysiology of IBD; therefore, novel concepts integrating NR and gastrointestinal physiology have been integrated into the successful development of effective drug therapies into the clinic.

Despite this expanding use of NR targeting as a therapeutic approach, there are many unknown issues about some classes of NRs, especially orphan NRs. In addition to the translation of the existing knowledge on NR biology, advances in current knowledge especially assessing dynamic NR regulation throughout disease progression should lead to the development of new drug targets for treating IBD. In this context, it should be mentioned that currently NRs are investigated not only with a focus on gastrointestinal diseases but also from a broader perspective. NRs such as the GR (dexamethasone), RXR (bexarotene and alitretinoin), PPARα (fibrates), and PPARγ (thiazolidinediones) have already been successfully targeted by approved drugs for treating autoimmunity, cancer, hyperlipidemia, or type 2 diabetes, respectively. Understanding the molecular mechanism of NRs in other human diseases will hopefully provide important insights into how to optimize NR-targeting therapies in IBD.

## AUTHOR CONTRIBUTIONS

VK and NH-K wrote the manuscript. ARM, HT, and GB contributed to the colitis and nuclear receptor biology aspects and helped with writing the manuscript.

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REVIEW
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# Microbiota-Immune Interaction in the Pathogenesis of Gut-Derived Infection

*Chenyang Wang, Qiurong Li* and Jianan Ren**

*Research Institute of General Surgery, Jinling Hospital, Medical School, Nanjing University, Nanjing, China*

Gut-derived infection is among the most common complications in patients who underwent severe trauma, serious burn, major surgery, hemorrhagic shock or severe acute pancreatitis (SAP). It could cause sepsis and multiple organ dysfunction syndrome (MODS), which are regarded as a leading cause of mortality in these cases. Gut-derived infection is commonly caused by pathological translocation of intestinal bacteria or endotoxins, resulting from the dysfunction of the gut barrier. In the last decades, the studies regarding to the pathogenesis of gut-derived infection mainly focused on the breakdown of intestinal epithelial tight junction and increased permeability. Limited information is available on the roles of intestinal microbial barrier in the development of gut-derived infection. Recently, advances of next-generation DNA sequencing techniques and its utilization has revolutionized the gut microecology, leading to novel views into the composition of the intestinal microbiota and its connections with multiple diseases. Here, we reviewed the recent progress in the research field of intestinal barrier disruption and gut-derived infection, mainly through the perspectives of the dysbiosis of intestinal microbiota and its interaction with intestinal mucosal immune cells. This review presents novel insights into how the gut microbiota collaborates with mucosal immune cells to involve the development of pathological bacterial translocation. The data might have important implication to better understand the mechanism underlying pathological bacterial translocation, contributing us to develop new strategies for prevention and treatment of gut-derived sepsis.

**Keywords: gut-derived infection, gut microbiota, mucosal immunity, intestinal epithelium, bacterial translocation, intestinal barrier**

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***Edited by:***
*Julien Diana,*
*Institut National de la Santé et de la Recherche Médicale (INSERM), France*

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*Rita Carsetti,*
*Bambino Gesù Children Hospital (IRCCS), Italy*
*Gianluca Ianiro,*
*Università Cattolica del Sacro Cuore, Italy*

****Correspondence:***
*Qiurong Li*
*liqiurongjue@126.com*
*Jianan Ren*
*Jiananr@gmail.com*

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## INTRODUCTION

Bacterial infections are common complications in critically ill patients, likely leading to sepsis, multiple organ dysfunction, and even death (1). Infection and septic complications contributed to the majority of deaths in these cases, and are regarded as the leading cause for mortality in critical illness (2, 3). Elucidation of the mechanisms underlying the pathogenesis of infection and septic complications in critical illness is therefore of upmost importance, facilitating to develop potentially effective strategies for prevention and treatment.

In critical illness, the gut may serve as the motor of multiple organ dysfunction syndromes (MODS), probably derived from intestinal bacterial translocation and subsequent acute septic responses (4, 5). Early in this decade, studies regarding bacterial translocation mainly focused

on the structure and function of intestinal epithelial barrier (6). Disruption of the epithelial barrier and increased gut permeability have been frequently observed in critically ill patients, which was thought playing a central role in the development of bacterial translocation and systemic infections in these cases (7, 8). The gut microbiota has long been recognized as a key component of the intestinal barriers (9). It has been known that small intestinal bacterial overgrowth could predispose to bacterial translocation (10, 11), however, there have been few efforts to characterize the composition and dynamic changes of the gut microbiota in the process, due to technological limitations. Over the past 15 years, the introduction of next-generation DNA sequencing techniques has revolutionized this area of science, allowing us to define the microbial compositions and their potential functions in the intestine (12). Recently, the gut microbiotas in critically ill patients have been determined through high-throughput sequencing analyses, characterized by overgrowth of pathogenic organisms and the loss of commensal bacteria (13–16). The gut microbiota dysbiosis could contribute to bacterial translocation by increasing gut permeability and inducing the mucosal immune dysfunction (17). The findings demonstrate that the microbiota is probably an active participant in the development of gut-derived infection, sepsis, and multiple-organ dysfunction in critical illness (18, 19). Thereby, improved knowledge of the gut microbiota composition and function would facilitate more comprehensive understanding of the mechanisms behind the pathogenesis of gut-derived infection in critical illness and the design of new treatment options.

The gut microbiota serves as a critical player in preventing and sometimes in driving enteric infections (20). Trillions of commensal microorganisms residing in the gastrointestinal (GI) tract can compete for adhesion sites with pathogens, and comprise the first line of defense against bacterial translocation (21). Alterations in the intestinal microbiota induced by antibiotics treatment can lead to the translocation of enteric bacteria across the epithelium in mice (22), providing further evidence for the importance of the microbiota in host resistance against pathogens. In addition to this, the gut microbiota has a key role in maintaining the gut homeostasis by establishing and maintaining beneficial interaction with mucosal immune cells and intestinal epithelial cells (23). In critical illness, this interaction could become pathological due to alterations of the gut microbiota, leading to the loss of intestinal homeostasis, bacterial translocation, gut-derived sepsis, and deleterious clinical sequelaes (24). Thereby, it is needed to unravel the changes of the gut microbiota and the underlying mechanisms of microbiota–host interaction in critical illness, contributing to offer new strategies to reconstruct intestinal homeostasis and avoid some of the untoward outcomes.

Based on the current research data, gut microbiota perturbations, host immune deficiencies, and increased intestinal permeability are the three key factors responsible for promoting bacterial translocation and gut-derived infection. Given the crucial role of the microbiota in shaping intestinal barrier integrity, it is interesting to consider whether microbiota dysbiosis and altered microbiota–host interaction is causally linked to gut-derived infection and consequent septic complications. In this review, we presented the changing features of the intestinal microbiota structure and composition in critical illness and the potential roles of these changes in the pathogenesis of gut-derived infection. We also discussed how the gut microbiota drives bacterial translocation through alterations in microbial community architecture, modulation of innate and adaptive immunity, and disruption of the mucosal barrier in critical illness. The data presenting here have highlighted the alterations of the microbiota–immune interaction in critical illness and offer novel paradigms to understand the pathophysiology of gut-derived sepsis. We also reviewed the research advances on other components (fungi, parasites, and viruses) of the gut microbiota and their potential relationships with bacteria and host immunity in human health and diseases. Lastly, we discussed the therapeutic potential to modify the intestinal microbiota with fecal microbiota transplantation (FMT).

## BACTERIAL TRANSLOCATION AND GUT-DERIVED INFECTION

Bacterial translocation is defined as the process in which the intestinal bacteria and/or their products spread through the gut barrier into the extra-intestinal sites, including the mesenteric lymph nodes (MLNs), systemic circulation, and distant organs (25, 26). The phenomenon of bacterial translocation was initially described in 1949, when live enteric bacteria were observed in the peritoneal washings from dogs with hemorrhagic shock (27). Until 1990s, however, the translocation of enteric organisms into the mesenteric lymph node (MLN) was identified in surgical patients undergoing laparotomy (28–30), which offered direct evidence supporting this concept. Bacterial translocation was also associated with a striking increase in the post-operative sepsis, leading to the generation of the gut origin hypothesis of sepsis. Subsequently, a large amount of clinical studies further confirmed the presence of bacterial translocation in patients with critical illness and its involvement in the development of sepsis (31–34). Based on the findings, it began to be accepted that bacterial translocation is a major source of systemic infections and might play an important early step in the pathogenesis of sepsis in critically ill patients (35, 36).

In the last several decades, detection of bacterial translocation in patients is mainly dependent upon culture of peripheral blood (37). Owing to low sensitivity of this method, cultures of blood specimens are often negative, even in the patients with sepsis (38). As a result, specific interventions against infections are probably delayed in some cases, causing lethal complications. It is quite possible that enteric bacteria may translocate into systemic circulation, but escape from detection by culture-based methods. In recent years, the development of 16S rDNA-based molecular techniques has improved the ability to detect the microorganisms, allowing us to define the composition of translocating bacteria into the blood (39, 40). Using denaturing gradient gel electrophoresis, multiple organisms (5–8 bacterial species) were frequently observed in the blood specimens of severe acute pancreatitis (SAP) patients (41). Recent studies with

next-generation sequencing techniques showed that a diverse microbiota is present in the blood of septic patients and is mainly composed of gut-associated microorganisms (42–44), indicating the possibility for translocation of intestinal microbiota. Dickson et al. demonstrated that the lung microbiome is enriched with gut-associated bacteria both in a murine model of sepsis and in patients with acute respiratory distress syndrome (ARDS) (45). Furthermore, the lower GI tract, rather than the upper respiratory tract, was identified as the likely source community of post-sepsis lung microbiota, providing evidence for gut–lung translocation of intestinal microbiota (45, 46). Based on culture-dependent methods, previous studies have demonstrated that the bacterial translocation is usually characterized by migration of one or several organisms from the gut (29, 30). Discovery and identification of the blood and lung microbiota has prompted us to rethink the notion of bacterial translocation, which might be replaced by translocation of gut microbiota. Although many observations have strongly supported the hypothesis of the gut microbiota translocation, future studies with next-generation sequencing techniques are needed to characterize the microbial landscape in the MLN and distant organs in patients and experimental models. The findings would provide direct evidence for the translocation of intestinal microbiota and give us new perspectives to understand the pathogenesis of gut-derived sepsis. Interestingly, recent studies have revealed that in healthy individuals the blood and lung also harbor a diverse bacterial microbiota (44, 45, 47, 48), suggesting that translocation of intestinal microbiota may present under healthy condition. The observations are consistent with previous opinion that intestinal bacterial translocation probably occurs as a normal physiological event in healthy subjects (49). However, the pathological translocation of enteric bacteria in critically ill patients may increase owing to breakdown of intestinal barrier integrity (50), likely causing the alterations in the blood and lung microbiotas and the pathogenesis of systemic infections and sepsis (44, 45).

## DYSBIOSIS OF INTESTINAL MICROBIOTA AND GUT-DERIVED INFECTION

In the past few decades, our understanding into the structure and function of the gut microbiota has been largely enriched with advances of culture-independent techniques. The gut microbiota is involved in maintaining host homeostasis, with an important role in nutrition and energy metabolism (51), immune modulation (52), and host defense (53). Recently, numerous studies have highlighted the composition and role of the gut microbiota under a range of intestinal and extraintestinal diseases (54–62). The involvement and implication of the gut microbiota in the development of bacterial translocation and gut-derived infection have also been broadly recognized. The harmful roles that the intestinal microbiota plays in critical illness are multifactorial and may be separated into three aspects: disruption of microbial barrier, loss of colonization resistance and metabolic disorder (63–65).

### Disruption of Microbial Barrier and Gut-Derived Infection

The gut microbiota represents the first barrier of protection against pathogen invasion, and disruption of this barrier is probably required for gut-derived infection in critical illness. Recent data showed that the intestinal microbiotas in critically ill patients in intensive care unit (ICU) are significantly altered, as characterized by overgrowth of opportunistic Proteobacteria and decreases in commensals Firmicutes and Bacteroidetes (13–16). Of special note, the presence of specific pathogens at ICU admission was associated with subsequent infection with the same organism for *Escherichia coli*, *Pseudomonas* spp., *Klebsiella* spp., *Clostridium difficile*, and vancomycin-resistant *Enterococcus* (66). Furthermore, *Enterococcus* status at ICU admission was associated with risk for death or all-cause infection, indicating that the gut microbiota alterations have potential impact on mortality or the risk of healthcare-associated infections in critically ill patients (67). The patients with SAP also had significant alterations in the gut microbiota, including reduced microbiota diversity, increased *Enterococcus* and *Enterobacteriaceae*, and decreased *Bifidobacterium* (68). Additionally, the changes of the gut microbiota have been frequently seen in patients who underwent severe trauma (69), serious burn (70, 71), and major surgery (72, 73). The dysbiosis of the microbiota has been linked to occurrence of severely adverse events in critical illness, including sepsis, MODS, and even death (74, 75). Of special note, altered microbiota composition could cause increased penetrability and a deteriorated colonic mucus layer, contributing to lethal colitis and susceptibility to infection by enteric pathogens, such as *C. difficile* (76) and *Citrobacter rodentium* (77). Apparently, this is becoming clearer that the gut microbiota seems to provide disease-promoting influences in critically ill patients. A plethora of data from basic research with animal models also supports the prominent role of the gut microbiota dysbiosis in contributing to adverse outcomes in critical illness (78, 79). For instance, intestinal ischemia/reperfusion (I/R) injury could trigger a dysbiosis of gut microbiota and mucosal barrier damage, leading to enteric bacterial translocation and development of septic complications (80, 81). Altogether, the microbiota dysbiosis in critical illness is among the key factors that cause dysfunction of the intestinal barrier, contributing to pathological bacterial translocation and gut-derived infection. Yet, the extent to which this dysbiosis is causative to the subsequent acute septic response and multiple organ failures observed in critical illness remains to be determined.

### Decreased Colonization Resistance Against Intestinal Pathogens

The intestinal microbiota plays a critical role in resistance against colonization by exogenous bacterial pathogens, termed colonization resistance (82). This phenomenon has been described over 50 year ago, and it has long been thought as microorganism-mediated direct inhibition (83). Being present in such huge numbers, the microorganisms in intestinal tract can compete for limited nutrition and adherence sites

to the epithelia, preventing overgrowth, and invasion of potentially pathogenic microbes. Long-term antibiotic treatment could cause loss of commensal enteric bacteria, and thus decreases this direct inhibition. As a result, antibiotic-resistant bacterial species, such as vancomycin-resistant *Enterococcus faecium* (63), Gram-negative *Enterobacteriaceae* (84), and *C. difficile* (85), could proliferate and dominate mucosal surfaces, preceding severely enteric infection and bloodstream invasion. In addition to its direct roles in nutrition and niche competition, the gut microbiota can also combat invading pathogens indirectly by enhancing host immune defenses (immune-mediated colonization resistance) in the gut. The commensal bacteria are capable of augmenting mucosal immune responses for eradication of invading pathogens by various mechanisms (86–88). Overall, both direct and indirect mechanisms could cooperate to provide resistance against colonization and invasion by potential pathogens, preventing the occurrence of bacterial translocation and gut-derived infection. Although the mechanisms underlying colonization resistance remain incompletely defined, there is little doubt that reestablishing colonization resistance after antibiotic treatment could be a potentially effective strategy for prevention and therapy of antibiotic-resistant bacterial infection. Recent studies have proved that the commensal microbiotas can be successfully manipulated to cure *C. difficile* infection in patients (89), which has been regarded as a consequence of reestablishing microbiota-mediated colonization resistance.

### Potential Role of Microbial Metabolic Disorders

The gut microbiota has a huge metabolic activity and can convert host-derived and dietary components (lipids, carbohydrates, proteins, etc.) into various metabolites that are either beneficial or harmful for the host (90). Some of the metabolic products, including lactic acid, short chain fatty acids, bile salts, and bacteriocins are often considered as antimicrobial factors playing a critical role in protection against pathogenic infection (91, 92). On the contrary, a few metabolites deriving from microbial digestion of proteins, such as phenolic and sulfur-containing compounds, are potentially toxic to intestinal epithelial cells (93). The phenol expose could cause an increase of paracellular permeability in a dose-dependent manner, due to destruction of the intercellular tight junctions (94, 95). Likely, the microbiota alterations in critically ill patients might induce metabolic disorders and excessive production of such toxic metabolites, resulting in disruption of intestinal epithelial barrier and bacterial translocation (96, 97).

In total, increasing evidence has demonstrated that the microbiota dysbiosis is closely associated with the development of gut-derived sepsis and subsequent mortality in critically ill patients (19, 98). As such, the gut microbiota has also been successfully used as a therapeutic target in the management of sepsis and MODS (99–101). With emerging evidence from clinical trials and basic researches, the causality of the relationship between the microbiota dysbiosis and gut-derived sepsis would be demonstrated. It will raise hope for simple and effective adjunctive therapies based on our expanding knowledge of the gut microbiota that might benefit critically ill patients.

## MICROBIOTA-IMMUNE INTERACTION AND GUT-DERIVED INFECTION

The intestinal immune system is considered as the last but the most important defense line against invasion of enteric microorganisms. There is a dynamic and complex interaction between the gut microbiota and the mucosal immune system (102). Under normal conditions, the microbiota could maintain a delicate balance with the mucosal immune system, which is extremely important for host health (54). The critical illness and associated medical interventions can cause a rapid and extreme change in the gut microbiota composition and activation of mucosal immune response (103). Consequently, this interaction between the gut microbiota and mucosal immune system is strikingly altered and becomes pathological in nature, providing the possibility for bacterial translocation, gut-derived infection and deleterious clinical sequalae.

### Communication Between Gut Microbiota and Innate Immunity

In order to confront the microbial challenges, the intestine has developed a complex immune defense network containing the greatest number and diversity of immune cells in the body. As an important component of the intestinal immune network, the innate immune system plays a pivotal role in maintaining the balance between tolerance to commensal microorganisms and immunity to opportunistic pathogens (104). The innate immune cells in the intestine are usually non-responsive to the great number of commensal microorganisms. Yet, they can sense enteric microbial signals to restrict overgrowth of the pathobionts and assure a beneficial microbiota composition. At the same time, the innate immune cells also can rapidly respond to invading pathogens and prevent migration from the intestinal lumen to systemic circulation and distant organs. Once passing the mucous and epithelial barriers, invading bacteria would be recognized, phagocytosed, and eliminated by mucosal innate immune cells (e.g., macrophages, dendritic cells) under healthy state (105). Since the critically ill patients are usually accompanied by systemic immune deficiencies or immunosuppression, the innate immune cells in intestinal mucosa are likely dysfunctional and fail to eradicate invading pathogens, and thus lead to systemic translocation of intestinal bacteria (106–108). Translocating bacteria and their products can activate immune response through recognition of specific pathogen-associated molecular patterns (PAMPs) by host innate immune cells (e.g., neutrophils and macrophages), triggering a systemic inflammatory response (109). Under such pathological conditions, activated neutrophils are excessively recruited into the intestine, which further promotes a dysregulation of innate immune function and cause mucosal injury (110). Alterations of the enteric microenvironment, coupled with medical treatment, lead to an overgrowth in opportunistic pathogenic bacteria and a decrease of commensal bacteria in critical illness (13–16).

The dysbiotic microbiota, in turn, could aggravate the mucosal immune dysfunction and promote an increase in enteric bacterial translocation, ultimately resulting in gut-derived infection, sepsis, and MODS (111, 112). Unsurprisingly, the interaction between gut microbiota and mucosal innate immunity is severely perturbed during the process. The innate immune dysregulation, microbiota dysbiosis, and bacterial translocation seem to shape a positive-feedback loop, together leading to uncontrollable inflammatory response and septic complications in critical illness. In a mouse model, morphine treatment induced a shift of gut microbiota toward a proinflammatory phenotype, which may be a result of the innate immune changes and commensal bacterial translocation (113–115). Yet, fecal microbiota transplant successfully reversed morphine-induced microbial dysbiosis and restored gut immune homeostasis (113). The findings provide evidence supporting the existence of the feedback loop and its potential importance in the pathogenesis of gut-derived infection.

Several antimicrobial molecules generating from goblet cells, Paneth cells, and enterocytes, also have been identified as critical components of the innate immunity (116). These substances, including mucins, defensins, lysozyme, secretory phospholipase A2, and cathelicidins, have strong microbicidal activity and are able to directly kill microbes in the intestine, facilitating maintenance of gut homeostasis (117, 118). The generation and release of such antimicrobial molecules is also regulated by the gut microbes and their products (119, 120). Owing to lack of gut microbial stimulations, the intestinal mucous layer in germ-free mice is remarkably attenuated, despite the numbers of goblet cells are normal (121). Introduction of bacterial products, such as lipopolysaccharide (LPS) or peptidoglycan, can stimulate the release of mucin by goblet cells, leading to a rapid reconstitution of the inner mucous layer (122). The metabolites of the gut microbiota, i.e., butyrate, also can promote release of mucin for maintenance of the mucous barrier (123). The antimicrobials from Paneth cells, including defensins, lysozyme, and secretory phospholipase A2, are also expressed under the control of gut microorganisms (124). In return, the antimicrobial functions of these substances are required for stabilization of the gut microbiota (125) and integrity of the epithelial barrier (116, 126). In mice deficient for principal intestinal mucin (Muc2), there is an increased translocation of commensal and pathogenic bacteria (127), which is closely related to bacterial overgrowth in the intestine. In cynomolgus monkeys, administration of Campath-1H, a humanized monoclonal antibody against CD52, led to a significant decrease in the expression of defensin 5 and lysozyme in Paneth cells, altering the composition of the gut microbiota toward a pathogenic state (128). Likewise, it has been reported that decreased expression of α-defensins due to loss of Paneth cells can induce an expansion of pathogenic bacteria and a reduction in gut microbial diversity, leading to bacterial translocation (129). In addition, a lack of the antimicrobial cathelecidin can cause more severe disruption of intestinal mucosa in the colitis mouse models induced by dextran sodium sulfate (130). Evidently, diminished release of antimicrobial molecules is involved in increased bacterial translocation and is, at least in part, responsible for the pathogenesis of gut-derived infection.

In addition to bacterial translocation, one of the most interesting aspects regarding gut microbiota and host innate immunity involves *C. difficile* infection (CDI) and *C. difficile*-associated diarrhea (CDAD) (131). Many studies have indicated that the composition and diversity of the fecal microbiota in patients with CDI are pronouncedly altered, and the dysbiosis is associated with the infection and its resistance to antibiotic therapy (132, 133). A variety of factors, including antibiotics, NSAIDs, acid suppressing agents, and ages, can cause the microbiota dysbiosis. The loss of the protective microbial barrier allows for the formation of an ecological niche that favors the growth of *C. difficile*, and then leads to CDI and CDAD. Several mechanisms, such as alterations of fermentative metabolism (especially SCFAs), alterations of bile acid metabolism, and imbalance of antimicrobial substances production, have been proposed to explain the involvement of the microbiota in the process of the infection (131). Unsurprising, the innate immune system also participates in the pathogenesis of CDI, which is mainly mediated via toxin-dependent mechanism (134). Following colonization and growth of *C. difficile* in the intestinal tract, the innate immune cells (135, 136), including intestinal mast cells, macrophages, monocytes, and dendritic cells, are activated by *C. difficile* toxins, through the surface and intracellular innate immune sensors, for instance, the inflammasome and the TLR4, TLR5, and NOD1 signaling pathways (137). Multiple proinflammatory cytokines (IL-12, IL-18, IFN-γ, IL-1β, TNF-α) and chemokines (MIP-1a, MIP-2, IL-8, leptin) are produced in the process, which may be responsible for host inflammatory damages and the histopathological features associated with CDI, such as fluid accumulation, edema, increased mucosal permeability, mast cell degranulation, epithelial cell death, and intense local neutrophilic infiltration (138). Collectively, the microbiota dysbiosis and impaired innate immune response could play crucial roles in triggering *C. difficile* colonization and growth, and in the development of CDAD.

## Crosstalk Between Gut Microbiota and Mucosal Adaptive Immunity

Despite characterized by tolerance to enteric microorganisms, the intestinal immune system has the daunting task of protecting us from pathogenic insults. Apart from the innate immunity, a highly sophisticated adaptive immune system also has been evolved in the gut (139), which are of upmost importance for prevention of bacterial translocation and gut-derived infection. When the enteric microorganisms cross the epithelium, the adaptive immune cells in the intestine are activated by antigen-presenting cells (macrophages, dendritic cells) to eradicate pathogens and establish long-lasting protective immunity (140). In the intestine, there is a huge and diverse population of T lymphocytes, forming a large part of the adaptive immune response. Many studies have suggested that loss of mucosal T cells has significant adverse effects on the maintenance of intestinal barrier integrity and defense of enteric infection, leading to increased morbidity (141, 142). In burn-injured

rats, translocation of intestinal bacteria to MLN and systemic circulation is markedly increased following depletion of T cells (143). Gut I/R can induce a significant reduction in T-cell numbers and variations in lymphocyte phenotypes in intestinal mucosa, leading to enteric bacterial translocation and development of septic complications (144, 145). Depletion of intestinal mucosal lymphocytes induced by Campath-1H could cause dysbiosis of gut microbiota (128, 146, 147) and disruption of intestinal epithelial barriers (148, 149). Similar to the observations, severe impairment of gut barrier integrity was also seen in intestinal transplanted patients receiving Campath-1H administration (150, 151), which might be a major reason for high incidence of infectious complications after small bowel transplantation. In both septic patients and animal sepsis models, the lymphocytes within the intestinal epithelium undergo significant apoptosis, leading to pathologic bacterial translocation and gut-derived sepsis (152–165).

The adaptive immune system in the gut mucosa is mainly composed of intraepithelial lymphocytes (IELs) and lamina propria lymphocytes (LPLs) (156). They are essential to the adaptive immune response in intestinal mucosa, and have been shown to play a critical role in defending against the invasion of pathogens and infections. When the adaptive immune system is disrupted, the translocation of intestine-derived bacteria occurs and could trigger systemic inflammatory response and the onset of sepsis. γδ T cells are a unique subset of T cells with a distinct T-cell receptor (TCR), and serve as a key controller for the adaptive immune response to a broad range of pathogens (157). Intraepithelial γδ T lymphocytes can prevent mucosal dissemination of bacteria through the secretion of cytokines and antimicrobial molecules following mucosal injury (158). In the absence of intraepithelial γδ T cells, the host control of invasive bacteria is compromised and invasive bacteria populations are expanded (159). Additionally, the reduction of γδ T cells in the gut mucosa could induce transition of non-invasive intestinal bacterial types toward more invasive, causing bacterial translocation into the systemic circulation and pathological infections. In septic patients, γδ T cells in peripheral blood are significantly reduced, and this decrease is closely associated with the high mortality rate caused by infectious complications (160, 161).

The gut microbiota is actively involved in shaping and maintaining normal adaptive immune system in intestinal mucosa (139). The phenotypic differentiations of specific lymphocyte lineages in the mucosal immune system are reliant on the distinct component of the microbiota. In germ-free mice, the gut adaptive immune system is underdeveloped, and introduction of the commensal bacteria can induce enrichment and differentiations of mucosal lymphocytes (162–164). Development of the adaptive immune cell diversifications represents an establishment of a complete "firewall" in the gut, which could prevent against the translocation of indigenous bacteria and pathogen infection (165). The gut microbiota also plays an important role in modulating the production of secretory IgA, mainly targeting against the enteric commensals and their antigens (166, 167). In the absence of IgA, the gut commensal bacteria could more easily enter the lamina propria and submucosal tissue by leaky barrier, leading to enteric bacterial translocation (168–170). The individuals with secretory IgA deficiency have a tendency to develop gut-derived infections and functional disorders of the intestinal tract (171, 172). The interaction between gut microbiota and mucosal immunity is extremely complex. Consequently, the precise mechanism by which the alteration in commensal bacteria-specific adaptive immunity crosstalk involves the invasion and translocation of enteric bacteria remains incompletely clear and needs to be further elucidated.

## OTHER ORGANISMS BEYOND BACTERIA IN THE INTESTINAL TRACT

In addition to the bacteria, the human intestinal microbiota also contains fungi, viruses, parasites, and other organisms. Despite representing a smaller fraction of the gut microbiota, they also play a crucial role in maintaining host health and in driving the development of the intestinal diseases.

### Gut Fungal Microbiota

In GI tract, the fungi comprise a dynamic and ecologically diverse microbial community, termed the gut mycome. The fungal microbiota has been regarded as a critical player for the development of fungal infections and intestinal diseases, through interacting with enteric bacteria and host immune system (173). In ICU patients, the fungal overgrowth in the gut is frequently presented, which is usually considered as a result of commensal enteric bacteria loss after antibiotic or immunosuppressive therapy (174). Subsequently, the fungal pathogens, such as *Candida* and *Aspergillus*, could translocate impaired intestinal barrier into the bloodstream, leading to the fungemia. In a non-human primate model with lymphocyte depletion, the gut fungal microbiota is also perturbed, together with a dysbiosis of the bacterial flora (147). The findings indicate that a complex crosstalk may exist between the fungal and bacterial microbiota in the gut. It has been shown that *Candida albicans* has an ability to modify the bacterial microbiota (175), however, the detailed mechanisms underlying this interaction are still not well-known. There is also a complex interaction between the fungal microbiota and host immune system, which is mainly mediated via an innate immune receptor Dectin-1 (176). After recognizing β-1,3-glucans (a component of the fungal cell walls), Dectin-1 could activate intracellular signals through CARD9, resulting in release of inflammatory cytokines and induction of Th17-mediated immune responses (176, 177). Deficiencies in either Dectin-1 or CARD9 can lead to enhanced susceptibility to pathogenic fungal infections in humans and mice (178, 179), and are closely associated with ulcerative colitis in humans (180, 181). With improved understanding into host-fungus relationships, several fungal species with beneficial effects have been utilized in many acute and chronic diseases. For example, *Saccharomyces boulardii* has showed significant efficacy in preventing antibiotic associated diarrhea (182) and relapse of *C. difficile* infection (183). Despite these advances, in-depth studies on gut mycome

composition and their relationships with gut bacteria, host immunity and related diseases are still warranted.

## Intestinal Parasites

The intestinal parasites, mainly including Blastocystis and Amoebozoa, represent a unique microeukaryotic population, also termed gut eukaryome. Over the past few decades, the advances of DNA-based molecular techniques have enabled us to better estimate the presence of the intestinal parasites and its roles playing in human health and gastrointestinal diseases (184). Recent studies with real-time PCR showed that single-celled parasites, such as Blastocystis and Dientamoeba, are far more common than previously anticipated, even in developed countries (185, 186). Intriguingly, these parasites are most common in individuals with a healthy gut, while less prevalent in patients with irritable bowel syndrome (IBS) (187), and even less common in patients with inflammatory bowel disease (IBD) (188). The observations suggest that the parasites may be beneficial to human health rather than culprits of diseases (189). However, the parasites infection is possibly present in some individuals, which may be associated with specific ecological conditions in the gut, such as the microbiota dysbiosis. Gilchrist et al. showed that a high parasite burden was coupled with increased abundance of *Prevotella copri* in Bangladeshi children with *Entamoeba histolytica* infection (190). In a mouse model with n amoebic colitis, the microbiota dysbiosis induced by antibiotic treatment can increase the severity of amoebic colitis and delay the clearance of *E. histolytica* (191). *Giardia* infection was also related to the dysbiosis of gut microbiota, as characterized by an increase of facultatively and strictly aerobic bacteria (192). In contrast to this, some animal experiments showed that probiotics can prevent or modulate parasite infection, supporting the association of the gut microbiota with the parasites (193). Taking all these studies into account, it appears that the presence of intestinal parasites, are closely linked to certain microbial communities. However, the causative link between the presence of a given parasite and the microbiota dysbiosis is still incompletely clear. The gut microbiota may not only be driving the susceptibility to, but also the outcome of, parasite infection (194). Future investigations should be designed to strengthen our knowledge regarding associations between parasites and gut microbiota, and also explore whether the parasites can be transplanted to a diseased recipient as a potential therapy for functional and/or organic bowel diseases as well as metabolic disorders.

## Gut Virome

The human gut virome is composed of two main players: microbial viruses (bacteriophages) and eukaryotic viruses (195). It is estimated that the human GI tract contains $\sim 10^{15}$ bacteriophages, which represent the most abundant member of the gut virome (196). The vast majority of bacteriophages in the gut are a DNA phage named crAssphage (cross-assembly phage), mainly belonging to the family Podoviridae (197). Similar to the bacterial microbiome, the gut viral communities are established at birth and evolve over time to become "adult-like" virome (198, 199). The structure and composition of the virome are also influenced by age, host genetics and environmental factors, such as diet, antibiotic use, and location (198–202). The viruses also have cross-kingdom interaction with the bacteria and other constituents of the intestinal microbiota, which are usually beneficial to host health and sometimes could increase the risk of disease (203). Owing to their ability to kill host bacteria, the phages can play a role in maintenance of the intestinal homeostasis through affecting the structure and function of enteric bacterial community (204). Under certain conditions, however, changes of the phage populations could induce intestinal dysbiosis and contribute directly to the development of intestinal diseases, such as IBD (205). To explain the mechanisms underlying phage-driven intestinal dysbiosis, several hypothetical models (206), including "Kill the Winner" model, "Biological Weapon" model, and "Community Shuffling" model, have been put forward to elucidate the complex interaction between the phages and bacteria during the process. In addition to these, the phages can also transfer genes (i.e., bacteriophage transcription factors) into bacteria to change their phenotypes and further control their biological functions, which is termed as the "Emerging New Bacterial Strain" model. Meanwhile, enteric bacteria also develop defense mechanisms against the bacteriophages, through the restriction modification system (207), hiding membrane receptors (208), increasing production of competitive inhibitors (209), self-destruction (210), and CRISPR-Cas systems (211). The detailed mechanisms that maintain the balance between bacteriophages and bacterial populations and result in the intestinal dysbiosis and diseased states have been documented in the review article by Mukhopadhya et al. (212). Development and implementation of metagenomic techniques have allowed us to study the "entire virome" composition and its interaction with other elements of the gut microbiome. With discovery and identification of new viral genomic sequences in the coming years, our understanding on the gut virome as a cohesive ecological unit that can affect the intestinal homeostasis and lead to diseases will continue to improve.

# MANIPULATION OF GUT MICROBIOTA FOR TREATMENT OF GUT-DERIVED SEPSIS

Considering the gut microbiota dysbiosis as one of the most important factors that can lead to pathologically bacterial translocation and systemic infection, it may be feasible to develop novel therapeutic strategies against gut-derived sepsis by modulating the microbiota. More than 90% of the commensal organisms would be lost during the early stage of the critical illness insults, thereby, it may be impossible that a single or several probiotic species would be able to completely replenish the diversity of the gut microbiota (213). Transfer of healthy donor feces containing thousands of microbial species, termed FMT, would facilitate replenishment of diminished commensal bacteria and guide the patient's microbiota toward a healthy state (214). In the last several years, FMT has been successfully utilized in the treatment of recurrent CDI (215, 216). Yet, FMT is scarcely

used in the treatment of septic patients, due to that in such cases antibiotic therapy is frequent and its continuation would adversely influence remodeling of the microbiota after FMT. Recently, it has been reported on the use of FMT in septic patients with MODS and non-*C. difficile* diarrhea, refractory to standard medical management (99–101). At 2–3 weeks of post-FMT, the patients had resolution in their diarrhea and significant decreases in the blood levels of the inflammatory mediators, such as TNF-α, interleukin (IL)-1β, IL-6, and C-reactive protein. Following FMT, the stool microbiotas in the patients showed marked alterations toward that of the donors, with growing Firmicutes and reducing Proteobacteria. Even though this is a serial of case reports, the improved clinical outcomes in these patients following FMT are still exciting. This success raises the possibility for the use of the unconventional therapeutic procedure in the clinical management of gut-derived sepsis and MODS which is commonly complicated in critically ill patients. Although the efficacy of FMT observed in such cases reports remains to be further validated, manipulation of the microbiota with FMT for therapeutic benefits represents a new avenue in the future care of critically ill patients (16, 75, 217–219). Nonetheless, such early experiences with FMT curing ICU patients have strengthened enthusiasm for broader its use in critical illness.

## CONCLUDING REMARKS

The interplay between gut microbiota and host immune is exquisitely complex. Exploration of the relationship between the gut microbiota alterations and host immunological disorders has significant potential to enhance our understanding and future treatment of relevant diseases. Abundant evidence has demonstrated that disturbance of the microbiota-immune relationship is a key event in the development of pathological bacterial translocation (220, 221). However, studies of the microbiota-immune interaction in critical illness remain in their infancy, and the underlying mechanisms are still incompletely clear. Beyond just describing effects of the microbiota dysbiosis on mucosal immune cell phenotypes, future investigations need to move toward unraveling the molecular mechanisms of the interaction in the pathogenesis of gut-derived infection. Systems biology studies based gut metagenomics and immunogenomics under the conditions of critical illness have fundamental importance for identifying the critical signal pathways and molecules that promote translocation of enteric microorganisms. Elucidation of the cross-regulation of gene expression between commensal bacteria and cells of the mucosal immune system will provide us mechanistic understanding on the complex interaction in critical illness. The knowledge would enable the field to enter a stage in which interventional strategies could be designed to improve the immune defense against invading microorganisms while protecting from pathological bacterial translocation to systemic circulation. With deeper understanding of this interaction, the precision manipulations that can restrict bacterial translocation may be possible and offer new strategies to avoid some of the untoward outcomes related to gut-derived infection in critically ill patients.

## AUTHOR CONTRIBUTIONS

CW wrote the original draft and revised the manuscript. QL reviewed and edited the manuscript. JR critically revised the manuscript. All authors read and approved the final version of the manuscript for submission.

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REVIEW
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# How the Interplay Between the Commensal Microbiota, Gut Barrier Integrity, and Mucosal Immunity Regulates Brain Autoimmunity

*Martina Antonini*[1], *Marta Lo Conte*[1], *Chiara Sorini*[2] *and Marika Falcone*[1]*

[1] *Experimental Diabetes Unit, Division of Immunology, Transplantation and Infectious Diseases, IRCCS San Raffaele Scientific Institute, Milan, Italy,* [2] *Immunology and Allergy Unit, Department of Medicine, Solna, Karolinska Institute and University Hospital, Stockholm, Sweden*

The intestinal barrier provides the host with a strong defense line against the external environment playing also a pivotal role in the crosstalk between the gut microbiota and the immune system. Notably, increasing lines of evidence concerning autoimmune disorders such as Multiple Sclerosis (MS) report an imbalance in both intestinal microbiota composition and mucosal immunity activation, along with an alteration of gut barrier permeability, suggesting this complex network plays a crucial role in modulating the course of autoimmune responses occurring in tissues outside the gut such as the central nervous system (CNS). Here, we review current knowledge on how gut inflammation and breakage of gut barrier integrity modulates the interplay between the commensal gut microbiota and the immune system and its role in shaping brain immunity.

**Keywords: autoimminity, microbiota, T cells, gut barrier, central nervous system**

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*Lund University, Sweden*

***Correspondence:**
*Marika Falcone*
*falcone.marika@hsr.it*

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## INTRODUCTION

The human gastrointestinal tract is equipped with physical and biological barriers whose function is not only to isolate the internal host's milieu from the external environment but also to regulate the immune system, absorption of nutrients, and to limit the access of microorganisms, both commensal, and pathogens. Hence, the intestinal mucosa operates in a dynamic manner in order to maintain intestinal integrity and immune homeostasis. Alterations of gut barrier integrity have been associated not only to inflammatory bowel diseases (IBD) but also to autoimmune disorders occurring outside the gut, such as Multiple Sclerosis (MS) both in experimental models and humans. These alterations are associated with dysbiosis, i.e., modification of microbiota composition, along with a persistent activation of the immune system within the gut mucosa (1). The mechanisms through which gut dysbiosis, breakage of gut barriers, and brain autoimmunity are linked are still unknown, but it has been proposed that a condition of dysbiosis can promote inflammation and morphological and functional changes of intestinal mucosa thus favoring uncontrolled passage of macromolecules, microorganisms or their derivates from the intestine to the systemic circulation where they activate myelin-reactive T cells. Here we will review the current knowledge on how the commensal microbiota and the gut barriers modulate the immune system within the gut mucosa and systemically and how this could influence the pathogenesis of MS (2, 3).

## THE CROSSTALK BETWEEN THE COMMENSAL MICROBIOTA AND THE IMMUNE SYSTEM

More and more revalued and increasingly considered by the recent scientific bibliography is the concept of intestinal ecosystem, by which functions and interactions among mucosal barrier, local immune system, and intestinal microflora are defined. Inside the gut microbial community, the esteemed number of species changes, but it is generally accepted that the human microbial compartment includes at least $10^{14}$ microbial cells, overall 10 times more than the total human somatic cells (4). The advances of molecular biology techniques, including ribosomal RNA 16S sequencing and metagenomics, have been instrumental in identifying the large biodiversity of the mammalian intestinal microbiota. In fact, microbiota organization is extremely diverse and influenced by many environmental and time factors. In the mammalian microbiota are five predominant *phyla* present (Bacteroidetes, Firmicutes, Actinobacteria, Proteobacteria e Verrucomicrobia). Firmicutes include several genera such as *Clostridium*, *Lactobacillus* and *Ruminococcus*, as well as *Eubacterium*, *Fecalibacterium,* and *Roseburia*; Bacteroidetes include *Bacteroides*, *Prevotella* and *Xylanibacter* genera; Proteobacteria involve *Escherichia* and *Desulfovibrio*, while *Akkermansia* genus belong to Verrucomicrobia phylum (5). Although every mammalian intestine harbors a unique microbiota profile, there is a balance in its composition conferring benefits toward the host, whose alteration negatively affects the overall health status (6). Indeed, studies of germ-free (GF) or antibiotic-treated mice demonstrated that functions of commensal bacteria are important in several mechanisms, including, but not restricted to, the maturation of the immune system, and other physiological processes like resistance to pathogenic infections, nutrient absorption and maintenance of intestinal barrier functions (7, 8). All these functions are closely dependent on the heterogeneous and dynamic features of the surrounding environment and they are also fundamental in maintaining the homeostasis in the intestinal environment.

Over the past few years, broad evidence of a pivotal role of the microbiota in shaping the immune responses has been collected. It has long been acknowledged that, when compared to wild-type, germ free mice exhibit imbalanced immune development characterized by immature gut-associated lymphoid tissues (GALT), decreased numbers of intestinal lymphocytes and decreased levels of both antimicrobial peptides and immunoglobulin A, two components that are essential to contrast enteric infections (9). On the other hand, the intestinal immune system is crucial to discriminate between invasive or opportunistic organisms and harmless ones, i.e., the commensal microbiota, adopting an immunological tolerance toward the latter. Hence, intestinal microbiota regulates the development and function of the immune system which in turn, shape the microbial community and regulates immune responses against the bacterial species on mucosal surfaces.

Specific resident bacteria play an important role in shaping the immunological features of different immune cell subsets. Hence, knowledge of mechanisms through which the gut microbiota regulate T cells differentiation is highly relevant in the field of immunology and autoimmune disease.

For example, it has been observed that GF mice show reduced number of Th1 and Th17 cells, as well as a reduction of IL-17 and IL-22 cytokines and overall, a decrease of Lamina Propria (LP) associated -$CD4^+$ lymphocytes (10–12). Th17 cells, in particular, normally play a key role in host defense from pathogens, and several studies also describe their involvement in the pathogenesis of autoimmune diseases (13). A decrease in this cell subset has been correlated to the absence or reduction of segmented filamentous bacteria (SFB) in the human microbiota, suggesting their potent capacity in triggering Th17 cells differentiation and thereby, IL-17 and IL-22 induction (14, 15). Resident intestinal bacteria are also essential for the induction and the development of $Foxp3^+$ cells in the gut. In the absence of commensals, the amount of $Foxp3^+$ $Helios^-$ $T_{reg}$ (intestinal $T_{reg}$) is significantly reduced at colonic lamina propria level (16). However, by re-balancing the intestinal microbiota with certain bacterial species, intestinal $T_{reg}$ subset can be restored (16–18). Indeed, colonization of GF mice with *Clostridium* strains triggered the transforming growth factor β (TGF-β) expression by intestinal epithelial cells, and thereby, promoting the differentiation of $Foxp3^+$ T regulatory lymphocytes in the colonic lamina propria (18). Furthermore, in a mouse model of experimental colitis, through a polysaccharide-A (PSA)- mediated mechanism, a molecule univocally expressed on its surface, the commensal *Bacteroides fragilis* has been shown to be able to stimulate the development of T regulatory cells and to increase their suppressive capacity by inducing IL-10 anti-inflammatory cytokine (19). With similar mechanisms, the gut bacteria also have an important role in $CD8^+$ T cell-mediated immune responses in the intestine. In fact, a reduced number of intestinal $CD8^+$ T cells have been observed in GF mice, suggesting that signals from commensals are necessary in order to maintain the pool as well as the function of intestinal $CD8^+$ T lymphocytes (20). Furthermore, gut microbiota also affects the development of intestinal-resident B lymphocytes residing at lamina propria level, given that GF mice have a reduced number of B cell in the intestinal LP (21). Such cell population is also responsible for the IgA production, which in turn, are strong regulators of microbiota composition. Hence, this has importance not only in order to reach a broad diversification for IgA at mucosal sites, but also to establish immune tolerance toward commensal microorganisms (22). Collectively, these studies demonstrate that our intestinal microbial community has a broad and long-lasting effect on the development and induction of T and B cell responses in the gastrointestinal tract.

Recent studies showed that is not the presence of a single or a consortium of bacterial strains that modify gut mucosal immunity but rather the functional metabolic profile that different bacterial species induce within the intestinal milieu. In fact, the gut microorganisms produce a broad range of metabolites through the anaerobic fermentation of

exogenous and undigested dietary components as well as endogenous ones that are synthetized by both gut bacteria and the host. The interaction between microbial metabolic products and the host immune cells within the gut mucosa is fundamental to regulate differentiation of T cells with regulatory ($T_{reg}$ cells) or effector ($T_{eff}$ cells) properties. Among these metabolites there are, for instance, short-chain fatty acids (SCFAs), mainly acetate, propionate, and butyrate. Resulting all from dietary fiber fermentation, acetate and propionate are mainly produced by Bacteroidetes, while butyrate is produced by Firmicutes (23). SCFAs have been studied for a long time for their capacity to increase the number and function of regulatory T lymphocytes in the gut (24). In addition, it has been reported that these metabolites are involved in promoting gut barrier function and inducing anti-inflammatory effects through the inhibition of the transcription factor NF-κB, as well as in influencing gene transcription by counteracting histone-deacetylase (HDAC) activity (25–27). Another example of microbial metabolites that affect the intestinal immune system activity is provided by tryptophan derivatives metabolites that bind the aryl hydrocarbon receptor (AhR). Initially recognized for its involvement in xenobiotics metabolism, AhR was further investigated for its role in regulating mucosal immune responses (28). In such overview, tryptophan catabolism and production of indole-3-aldehyde by the commensal *Lactobacillus reuteri* has been shown to be able to inhibit the overgrowth of *Candida albicans* in mice gut by binding AhR and triggering the IL-22 pathway, thus protecting the mucosa from infection (29). In addition to tryptophan, intestinal bacteria are involved in the production of arginine derivatives such as polyamines, diamine, spermidine, and spermine, capable of exerting immune-modulatory effects, for instance, by enhancing the homeostasis of both intestinal mucosa and resident immune cells (30, 31). Hence, microbiota regulate immune responses in the gastrointestinal tract both directly and indirectly, by coordinating immune cell subsets to different conditions and regulating inflammatory responses. For these reasons, to elucidate molecular and metabolic pathways, regulatory metabolites as well as microorganisms involved in their production, is crucial to understand the pathogenesis of immune-mediated disorders.

## THE ROLE OF PHYSICAL AND BIOLOGICAL GUT BARRIERS IN REGULATING THE CROSSTALK BETWEEN THE COMMENSAL MICROBIOTA AND THE IMMUNE SYSTEM

The complex and dynamic interaction between the commensal microorganisms and the gut immune system is regulated at the gut barriers level through several mechanisms. Intestinal microorganisms and/or microbial metabolites may affect both immune system and epithelial barrier and, at the same time, intestinal epithelial cells (IECs) and the mucus layer are able to strongly influence immune responses and to shape the microbial composition. This complex network is fundamental to maintain immune homeostasis at the intestinal level.

Although gut commensals play a key role in orchestrating intestinal immune responses, an abnormal stimulation of mucosal immunity as well as a microbial systemic spreading, may lead to harmful intestinal inflammation and excessive immune activation. To avoid such an occurrence, intestinal microbes are compartmentalized in the lumen and remain isolated from intestinal immune system by physical and functional barriers working in a coordinated manner, consisting of the mucus layer, the intestinal epithelial barrier (IEB) and the gut vascular barrier (GVB) (8). In the colon, the mucus layer covering the epithelial one is organized in two zones: an inner dense layer, usually sterile and inhabited by immune system cells, and an outer layer colonized by commensal bacteria (32). The intestinal mucus is composed mainly by mucins which represent complex agglomerates of glycoproteins characterized by specific O-linked glycans produced by globet cells (33). The main function of the mucus layer is to limit the interaction between the gut mucosa and harmful molecules or pathogens present in the intestinal lumen and thus, to regulate the passage of food and microbial products into the gut tissue and systemic circulation. However, it is now clear that the mucus layer is also fundamental to both shaping the composition of gut microbial community and regulate the interaction between commensals and the immune system. The ability to adhere to host epithelial cells and mucus has long been considered a key property underlying colonization by both pathogenic and beneficial bacteria (34–36). Hence, under certain conditions, a host may favor the colonization and competitiveness of beneficial microbial strains, as well as can promote the secretion of specific factors able to reduce adhesion and thus, limiting a strain's colonization (34). For instance, the colonic mucus layer allows microbial adhesion and growth by exposing mucins glycans which serve as both a nutritional source and attachment sites for bacterial adhesins (37, 38). Since mucin glycosylation is distinctive for each species, this phenomenon likely favors the microbial colonization in a selective manner (39, 40). In this regard, several studies showed that altered glycosylation profiles of mucins are correlated with increased inflammation in both mouse models and humans, underlying the role of mucin glycosylation in maintenance of intestinal homeostasis (41–43). Furthermore, mucin proteins provide a nutrient harvest for intestinal microbiota. Many intestinal bacteria are known to be mucin-degrading microorganisms, involving, for instance, *Akkermansia muciniphila* (44), *Bacteroides thetaiotaomicron* (45), and *Bacteroides fragilis* (46). Released saccharides can then be used by the same bacteria as carbohydrate sources or by other resident commensals which are biologically unable to degrade polysaccharides constituting mucins (33, 47). Importantly, the mucin glycans can control microbial translocation by regulating bacterial mucin degradation.

The dense layer of transmembrane mucins that covers the entire intestinal mucosa (the mucin barrier) does not simply act as a diffusion barrier but exerts important dynamic functions that are able also to improve the intestinal immune properties, thereby regulating the interaction between commensals and the

gut immune system. For instance, MUC2 is important for the cooperation between goblet cells and $CD103^+$ dendritic cells that leads to $T_{reg}$ cell differentiation and immune tolerance (48). In addition, a recent study showed that glycans associated with MUC2 induced the assembly of galectin-3-Dectin-1-FcγRIIB receptor complex and the activation of β-catenin which in turn, by inhibiting the nuclear factor-κB gene transcription, suppressed inflammatory but not tolerogenic intestinal DCs (49). Besides MUC2, MUC1 also have a strong anti-inflammatory function acting as a decoy molecule on the apical cell surface of enterocytes to limit bacterial adherence, translocation and inflammation.

Another important intestinal barrier is represented by the IEB. This anatomical structure is mainly composed of a tight junction (TJ), a adherent junction (AJ) and desmosomes. This organization is fundamental in order to maintain its integrity as well as to regulate paracellular trafficking of solutes and fluids. While AJs are necessary for assembly of TJs, the latter are made up of several kind of proteins which include transmembrane proteins (claudin, occludin), peripheral membrane proteins (zonula occludens) and regulatory molecules (2). Overall, these proteins allow the stabilization of TJs and contribute to the regulation of molecular signaling across IECs and TJs, thereby regulating gut permeability (2, 50, 51). The passage of components from the lumen through the mucosal layer is achieved by two different mechanisms, which involve paracellular and transcellular routes. The first pathway allows the passage of water, solutes and ions, and is mainly due to TJs. The regulation of paracellular permeability involve cytoskeletal contraction, endocytosis of TJ proteins, transcriptional regulation of TJ genes and epithelial cell apoptosis (52). In contrast, macromolecules such as proteins, bacterial products or microorganisms use the transcellular route which is mainly ascribed to M-cells overlying isolated lymphoid follicles or Peyer's patches (PP) (52–56).

An impairment of intestinal epithelial barrier has been recognized as a crucial mechanism for the onset of several inflammatory and immune-mediate disorders. Interestingly, increased permeability of the intestinal barrier has been ranked among those factors that may be responsible for bacterial translocation (57). Indeed, as result of gut barrier imbalances, some microorganisms, bacterial products, or toxins may translocate across the epithelium in an uncontrolled manner, leading to the onset of inflammatory disorders which may arise both in the gut and in distal organs, as described by the recent literature (50, 58–62). Current evidences have identified two main routes through which bacterial translocation may occur: transcellular or paracellular routes. While the first pathway is under the control of membrane pumps and channel, the second is due to an impairment of TJs (63). Although only a few evidences have been reported, transcellular migration has been observed in rats, where *E.coli* and *P.mirabilis* have been detected within intact enterocytes (64). Since appear to be related to an impairment of TJs, paracellular translocation has been instead associated to direct damage to enterocytes and their support structures, along with significant changes in intestinal TJs gene expression, and downregulation of both zonulin 1 and occludin (65, 66). Hence, functions of intestinal mucosa depend, at least in part, on many structural and dynamic factors, including IECs, the integrity of cellular plasma membranes, TJs and their protein components which contribute to maintain a homeostatic intestinal environment that impacts the overall health of the host.

Interestingly, the crosstalk between IECs and the mucosal immune system plays a key role to ensure the correct functioning of the immunological network operating at the intestinal level. Indeed, by detecting antigens or secreting anti-microbial molecules in response to commensal bacteria, IECs provide immune signals to these cells, thereby controlling the mucosal immune response (67).For instance, IECs sensing of gut microbial components through toll-like receptors (TRLs) protects from epithelial damage following administration of dextran sulfate sodium (DSS) (68). At the same time, intestinal immune cells exert a control on IEC-derived cytokines, such as thymic stromal lymphopoietin (TSLP), by secreting IL-12 and thus, ensuring the host ability to mount appropriate immune response toward pathogens (69). Commensal microorganisms also contribute to maintain the IEB integrity, for instance, by regulating tight junction expression and IECs proliferation (70, 71). Importantly, IECs form a real network with components of the mucosal immune system such as intraepithelial lymphocytes and immune cells residing in the intestinal lamina propria, contributing also in shaping the regulatory features of the latter in order to ensure the homeostasis. In this context, by using a culture of human monocyte-derived DCs with epithelial cells, a recent study highlights the capacity of IECs to induce anti-inflammatory DCs (72). Investigating about this phenomenon, researchers showed how this process was mediated by a combination of TSLP and other factors expressed by epithelial cells. TSLP induced-DCs did not release interleukin 12 as well as they did not drive Th1 responses toward bacteria, demonstrating that in steady state conditions the interplay between DCs and IECs is instrumental in order to maintain an anti-inflammatory environment (72).

In the intestinal barriers, gut bacteria induce also the production of secretory IgA and anti-microbial peptides (AMPs), such as RegIIIγ and alpha-defensins, which have been shown to have a crucial role in maintaining spatial compartmentalization of commensal bacteria outside the host epithelium as well as in exerting modulatory functions on both chemotaxis and TRLs signaling (73, 74). IgA also contribute to the compartmentalization of commensals by binding them and preventing their interaction with epithelial cells and their subsequent internalization. Interestingly, it has been observed that in a steady state, non- invasive bacteria residing in mice gut are coated with IgA and thus, favoring microbes to reach PP in order to induce a broader and more diversified amount of species-specific IgA in mice (22). Thus, immunoglobulin A also play a pivotal role in maintaining intestinal homeostasis.

At mucosal surfaces level, IECs-associated inflammasomes also have been demonstrated as one of those mechanisms of regulation, necessary to provide tissue homeostasis and host defense to infection (75). Several studies have described that inflammasomes play a key role in shaping the intestinal

bacteria composition, such as that inflammasome-deficient mice show an aberrant microbiota, with an overgrowth of the bacterial *phyla* Bacteroidetes, particularly *Prevotellaceae* and *TM7*, and a decrease in the representation of *Lactobacillaceae* (76, 77). The subsequent dysbiosis enhances inflammatory responses in the intestine, thereby predisposing the host to develop inflammatory bowel disease (76). Moreover, mice lacking inflammasomes exhibit an imbalance in intestinal barrier functions that affect tissue homeostasis at distal organs (77, 78). Interestingly, studies in both humans and mice have identified the expression of NLRP6 inflammasome to be involved in colonic homeostasis, where it acts at the host-microbiome interface (76, 79). Specifically, it has been observed that *Nlrp6*$^{-/-}$ mice present alterations in globet cell mucus secretion, suggesting its putative role in the maintenance of mucus barrier (80). In addition, by triggering the production of epithelial IL-18 in response to commensals, it also promotes the release of AMPs (79). Therefore, dysfunctions in this regulatory system may result in imbalances of AMPs production, thereby leading to dysbiosis and intestinal auto-immune phenomena. This has been clearly demonstrated in NLRP6 deficient mice, in which colonic epithelial cells showed reduced levels of IL-18 and altered microbiota composition (76). Interestingly, a recent study demonstrated that microbiota-associated metabolites such as taurine, histamine and spermine can modulate NLRP6 inflammasome signaling, IL-18 secretion and release of AMPs, thereby promoting intestinal dysbiosis (81). Hence, by playing a pivotal role in both intestinal mucus secretion and induction of AMPs, NLRP6 inflammasome is indirectly involve in controlling bacterial adherence and colonization.

Recently, another important gut barrier has been identified, which also play a key role in modulating the interaction between the gut bacteria and the host: the GVB. The GVB is characterized by the presence of tight junctions and adherent junctions, in addition to several cell type such as endothelial cells but also pericytes and fibroblast which are involved in the maintenance of the endothelial wall integrity (82). GVB integrity is fundamental to prevent the entry of bacteria components into the gut mucosa and systemic circulation, where they can potentially trigger activation of immune cells including self-reactive T cells. The latter hypothesis is supported by the observation that damage of the GVB is present in patients affected by an extra-intestinal autoimmune disease ankylosing spondylitis (83).

In summary, it is now clear that the intestinal barriers play a central role in the biological network linking the intestinal microbiota and mucosal immune system, not only because it is physically and biologically positioned between them, but also because it participates actively in maintaining immune homeostasis in the gut. Hence, an imbalance of intestinal barrier or inflammation phenomena compromising its integrity, which might lead to the translocation of commensal or pathogenic bacteria, resulting in inflammation and activation of effector immune cells in the gut mucosa and, possibly, systemically and at sites distal from the intestine (**Figure 1**).

## HOW DOES THE GUT ENVIRONMENT MODULATE BRAIN AUTOIMMUNITY?

The intestinal environment can regulate the pathogenesis of extra-intestinal autoimmune diseases such as MS through different mechanisms (84). A pro-inflammatory gut microbiota profile and alterations of the immune-regulatory function of the gut barriers could drive immune cells residing in the intestinal mucosa toward an effector phenotype. An increased differentiation of T effector cells and reduction of tolerogenic mechanisms, i.e., differentiation of $T_{reg}$ cells (FoxP3$^{+}$ and IL-10-secreting) may affect the functional phenotype and enhance aggressiveness T cells that circulate in the gut mucosa possibly including autoimmune myelin-reactive T cells (84). Alternatively, a damage of the different physical and biological gut barriers could favor the uncontrolled translocation of bacterial components, e.g., bacterial antigens, metabolites, toxins, from the gut mucosa into the systemic circulation where they can activate myelin-reactive T cells either in the peripheral lymphoid organs (lymph nodes) or in the brain tissue (83).

## THE ROLE OF THE MICROBIOTA

The influence of the gut microbiota in immune-mediated diseases extends beyond the intestine. Although the underlying mechanisms are yet to be characterized, several recent reports demonstrated that the gut microbiota is implied in the pathogenesis of different extra-intestinal autoimmune diseases such as Type 1 Diabetes (T1D), Rheumatoid Arthritis (RA) and MS. In support of a role of dysbiosis in the autoimmune pathogenesis of MS, there are epidemiological observations showing that many environmental factors involved in MS pathogenesis such as diet composition, hygienic conditions and the use of antibiotics, act by modifying the gut microbiota (85). In animal models of relapsing-remitting (RR) MS, it was shown that the TCR transgenic SJL/J mouse model that spontaneously develop experimental autoimmune encephalomyelitis (EAE) within 3–8 months of age, is protected from brain autoimmunity in the absence of microbiota when hosted under specific pathogen-free (SPF) conditions (84).

Importantly, alterations in the composition of the gut microbiota have also been found in individuals with RRMS compared to healthy controls. For instance, recent lines of evidence revealed overall decreased levels in both Bacteroidetes and Firmicutes *phyla* as well as in *Prevotella* strains in patients with RRMS compared to controls (86, 87). Interestingly, the alterations of gut microbiota composition, i.e., decreased relative abundance of *Prevotella,* are selectively found in RRMS patients with an active disease, meaning that those modifications could be linked with activation of brain autoimmunity and onset of MS relapse (1). Also, it is interesting to note that RRMS patients treated with Interferon-β and glatiramer acetate, show an increase in *Prevotella* genus, notoriously associated with high-fiber ingestion and beneficial role via butyrate synthesis (88). By evaluating individuals with MS,

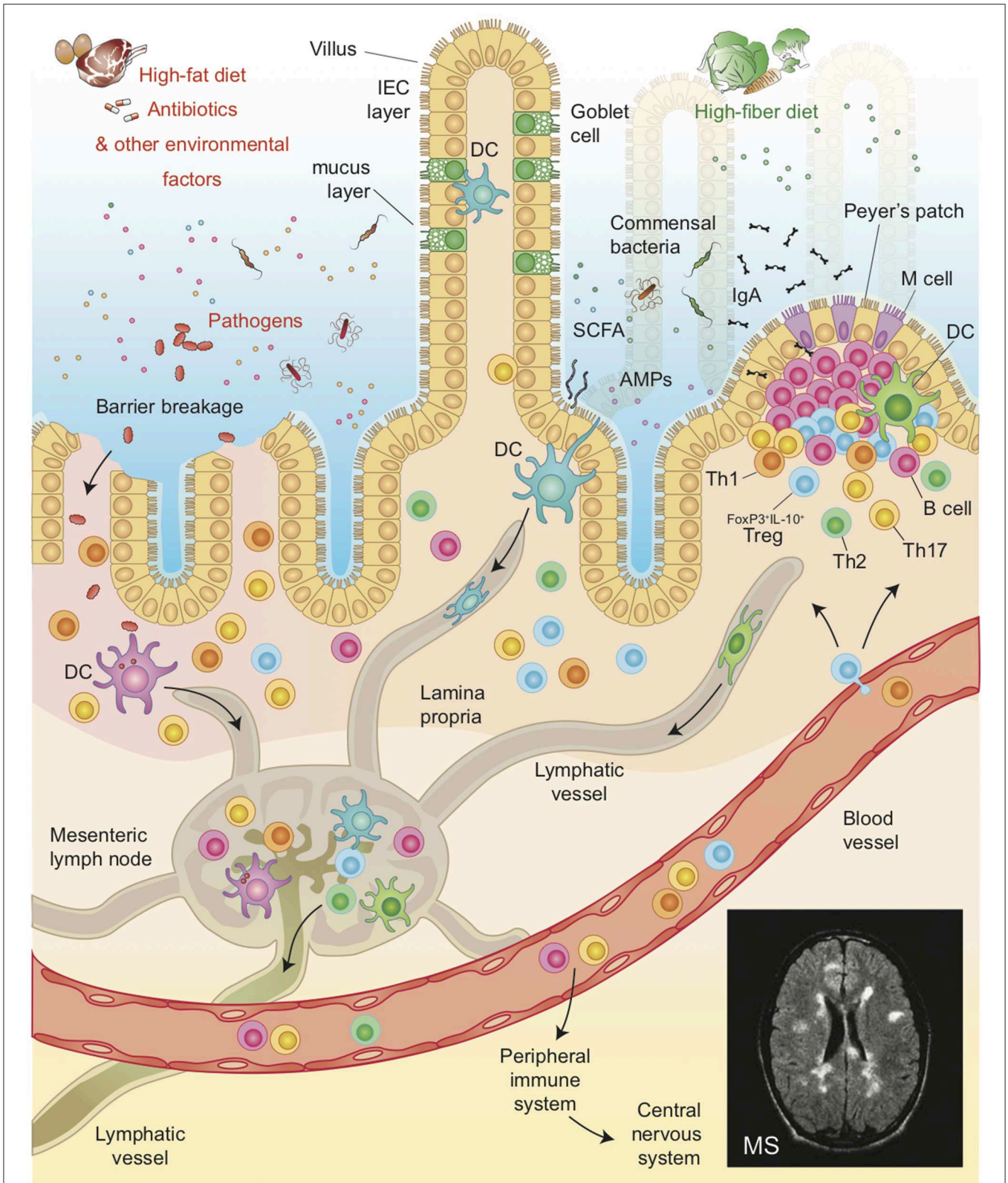


**FIGURE 1 |** Illustration of the host intestinal barrier, including the complex interaction among IEB, and mucus layer and its role in modulating the cross-talk between commensal bacteria and the GALT. Environmental factors such as diet, pathogens or antibiotics affect the pathogenesis of MS by altering gut microbiota composition, intestinal permeability and by favoring the translocation of bacteria or microbial products, thereby shaping auto-immune responses both in the gut and in peripheral organs such as the Central Nervous System in Multiple Sclerosis.

others observed a reduction in Firmicutes and *Butyricimonas*, which are butyrate-producing microorganisms (89). Likewise, another study detected an altered microbiota with decreased of bacteria with tolerogenic properties such as Clostridia XIVa and IV groups and Bacteroidetes members (87). In another study they have been found significant differences within the RR-MS patient cohort and particularly a trend toward reduced species richness in patients compared to controls (90, 91).

The mechanisms through which dysbiosis promotes MS pathogenesis are still unknown. The current hypotheses to link the gut dysbiosis with extra-intestinal autoimmune diseases such as MS hold that self-reactive T cells could be activated by mechanisms of molecular mimicry, bystander activation, and/or amplification of autoimmunity by the microbiota-induced pro-inflammatory milieu (58, 59, 92–96). For example, in animal models it has been clearly demonstrated that the gut microbiota modulate the Th17/$T_{reg}$ balance during the development of EAE. Those experiments allowed the characterization of some commensal bacterial species able to modulate brain autoimmunity. For instance, the colonization of GF mice with SFB, selectively promotes the differentiation of Th17 lymphocytes in both the lamina propria and CNS thus worsening disease activity in EAE (84). Conversely, *Bacteroidetes fragilis* was able to prevent intestinal inflammation and brain autoimmunity through a polysaccharide A- mediated mechanisms, which induced an increase of both $Foxp3^+$ T regulatory cell and IL-10-secreting Type 1 regulatory T cells (Tr1), thus limiting Th17 cell expansion (97, 98). Similarly, oral administration of *Lactobacillus* spp. and *Bifidobacterium bifidum* strongly ameliorated the EAE clinical score by increasing $T_{reg}$ cell frequency in mice (99). Furthermore, the treatment with human gut-derived commensal *Prevotella* suppressed EAE, by decreasing pro-inflammatory Th1 and Th17 cells and enhancing $T_{reg}$ cells frequency (100). A recent study also confirmed that in humans the gut microbiota could modulate MS pathogenesis by promoting Th17 cell differentiation in the gut. Specifically, a reduced abundance of *Prevotella* strains and increased in *Streptococcus oralis* in patients with active RRMS correlated with increased frequency of intestinal Th17 cells (1). Collectively, these research findings demonstrate that altered profiles in the microbiota composition may be directly responsible for brain autoimmunity. However, as previously mentioned, the metabolic profile induced by microbiota rather than the single bacterial strain is fundamental in shaping gut immunity and plays a role in regulating extra-intestinal autoimmune disorders and EAE. In line with this idea, mice lacking the Aryl-hydrocarbon Receptor (AhR), an environmental sensor that detects dietary, microbial and metabolic cues such as tryptophan derivatives, show an increased susceptibility to EAE and are more prone to develop infection with *Citrobacter rodentium* and *Listeria monocytogenes*. The latter finding suggests that the metabolic environment and, specifically, tryptophan derivatives metabolites, play an important role in modulating brain autoimmunity (29, 101).

## THE ROLE OF THE GUT BARRIER INTEGRITY

As previously mentioned, components of the gut microbial strains could directly trigger activation of autoimmune T cells and/or their acquisition of an effector Th17 cell phenotype. However, an intestinal dysbiosis with decreased microbial function and diversity could promote extra-intestinal autoimmune diseases also by inducing inflammation and alterations of the gut barrier integrity (102, 103). In support to the latter hypothesis, studies in humans and animal models indicate that, along with intestinal inflammation and dysbiosis, an increased intestinal permeability may play a pathogenic role in brain autoimmunity (59, 95). An intestinal barrier dysfunction has been observed at the onset of EAE, in which increased permeability and altered expression of TJs have been associated to a disruption of the mucosal balance between Th1/Th17 and $T_{reg}$ cell subsets in intestinal lamina propria, Peyer's patches and mesenteric lymph nodes (MLN) (104). A recent study furthermore showed that the degree of intestinal permeability impairment is closely related to EAE severity (105).

Also, in humans altered gut barrier functions and composition have been observed in MS patients. The intestinal barrier permeability in patients can be inferred by using a test that directly measures the ability of lactulose and mannitol to permeate the intestinal mucosa, then revealing the degree of gut permeability and mucosal alterations of the small intestine (95). A recent study, by applying this test, detected a significant increase in intestinal permeability in RRSM patients compared to healthy controls. In fact, the concentration of mannitol recovered in urine samples was strongly decreased in MS patients than in controls, suggesting the presence of abnormalities in the mechanism of intestinal absorption (95). A previous report, by using the lactulose/mannitol test, reported an increased intestinal permeability in individuals affected by RRMS and showed that the bowel inflammatory scenario correlated with systemic immune alterations (expression of the CD45RO isoform of the leukocyte common antigen on peripheral blood $CD20^+$ B cells) (106). Further confirmation of diminished intestinal barrier function in MS has been provided by the detection of elevated serum zonulin levels in both RRMS and Secondary Progressive Multiple Sclerosis patients (SPMS) (107). Interestingly, in this study RRMS patients in remission phase showed serum zonulin levels comparable to controls, suggesting that an impairment of gut barrier functions may be ranked not only among those factors involved in the onset of auto-immune response, but also among those implicated in the relapse phases (107). There is another finding showing that alterations of the gut barrier integrity may lead to MS pathogenesis, namely that elevated systemic lipopolysaccharide (LPS) levels, a biomarker of increased bacterial translocation, are detectable in the plasma samples of MS individuals (68). Interesting, in those patients the bacterial translocation was associated with the presence of increased *in vitro* Th17-like responses and higher neurological disabilities (68).

## CONCLUDING REMARKS

Collectively, several lines of evidence indicate that functional and morphological alterations of the intestinal barrier are associated with dysbiosis and are present in humans and animal models of RRMS (95, 108). Persistent dysbiosis, responsible for the overgrowth of harmful bacterial species, and chronic exposures to molecules originating from microbial translocation, may shape the autoimmune T cell repertoire in MS patients toward an effector Th17 cell type interfering with $T_{reg}$ function (109). However, although some preliminary indications exist suggesting that gut barriers are altered in MS and EAE, the putative role of the different physical and biological gut barrier in the pathogenesis of brain autoimmunity requires yet to be fully investigated.

## AUTHOR CONTRIBUTIONS

MA reviewed the literature and wrote the manuscript. ML reviewed the literature and the final manuscript. CS reviewed the manuscript and prepared the figure. MF wrote the manuscript.

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# The Contribution of Gut Barrier Changes to Multiple Sclerosis Pathophysiology

*Maria Chiara Buscarinu[1], Arianna Fornasiero[1], Silvia Romano[1], Michela Ferraldeschi[2], Rosella Mechelli[3], Roberta Reniè[1], Emanuele Morena[1], Carmela Romano[1], Giulia Pellicciari[1], Anna Chiara Landi[1], Marco Salvetti[1,4]* and Giovanni Ristori[1]**

[1] Department of Neurosciences, Mental Health and Sensory Organs, Faculty of Medicine and Psychology, Centre for Experimental Neurological Therapies, Sapienza University, Rome, Italy, [2] Department of Neurology and Psychiatry, Sapienza University, Rome, Italy, [3] Department of Human Science and Promotion of Quality of Life, San Raffaele Roma Open University, Rome, Italy, [4] IRCCS Istituto Neurologico Mediterraneo (INM) Neuromed, Pozzilli, Italy

The gut barrier consists of several components, including the mucus layer, made of mucins and anti-bacterial molecule, the epithelial cells, connected by tight junction proteins, and a mixed population of cells involved in the interplay with microbes, such as M cells, elongations of "antigen presenting cells" dwelling the lamina propria, intraepithelial lymphocytes and Paneth cells secreting anti-bacterial peptides. Recently, the influence of intestinal permeability (IP) changes on organs far from gut has been investigated, and IP changes in multiple sclerosis (MS) have been described. A related topic is the microbiota dysfunction that underpins the development of neuroinflammation in animal models and human diseases, including MS. It becomes now of interest to better understand the mechanisms through which IP changes contribute to pathophysiology of neuroinflammation. The following aspects seem of relevance: studies on other biomarkers of IP alterations; the relationship with known risk factors for MS development, such as vitamin D deficiency; the link between blood brain barrier and gut barrier breakdown; the effects of IP increase on microbial translocation and microglial activation; the parallel patterns of IP and neuroimmune changes in MS and neuropsychiatric disorders, that afflict a sizable proportion of patients with MS. We will also discuss the therapeutic implications of IP changes, considering the impact of MS-modifying therapies on gut barrier, as well as potential approaches to enhance or protect IP homeostasis.

**Keywords: gut barrier, intestinal permeability, microbiota, multiple sclerosis, neuro-inflammatory diseases**

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*Charles Kelly,*
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*Harvard University, United States*

***Correspondence:**
*Giovanni Ristori*
*giovanni.ristori@uniroma1.it*
*Marco Salvetti*
*marco.salvetti@uniroma1.it*

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## INTRODUCTION

The requirement for different functions is reflected by the structural complexity of the intestinal surface. Its role as a barrier relies on three components. The layer of mucus contributes to separate the microbiota from the upper part of the epithelium. The epithelial cells, with tight junction (TJ), regulate the paracellular permeability. A third component with immunological functions includes M cells and elongations of "antigen presenting cells" dwelling the lamina propria, that scan the luminal antigens, intraepithelial lymphocytes, and Paneth cells secreting anti-bacterial peptides. The passage of substances through this physical barrier is possible thanks to trans-cellular or para-cellular transport mechanisms. The first is closely related to the presence of selective

transporters, the second is under the control of the proteins that make up the TJ, especially occludin and claudins. The TJ can be assembled or disassembled according to the different signals coming from the intra- and extra-cellular environment. Dietary factors, microbiota composition, cytokines, enzymes and growth factors can all contribute to modulate TJ (1).

The enteric nervous system, consisting of the ganglia of enteric neurons and glial cells able to release important mediators in repair, cell proliferation, epithelial differentiation and TJ changes (2), regulates the intestinal permeability (IP) and represents a communication pathway between the intestinal microenvironment and the CNS. A recent review emphasized the bottom-up connections, which occur through neuroendocrine tissue, such as enterochromaffin cells, and neuroimmune mechanisms, that often involve the vagus nerve. Even the microbiota plays a pivotal role in the communication between intestine and brain through the production of certain substances, such as short chain fatty acids (SCFA) or tryptophan catabolites, that contribute to the homeostasis of IP (3).

The IP changes (IPC) and the dysbiosis appear as virtually co-occurring events, that trigger a vicious circle leading to pathogenic cascades in gut and far-from-gut tissues. In fact, recent evidences coming from children with beta cell autoimmunity, at risk for type 1 diabetes, showed that both increased intestinal permeability and differences in microbiota composition are contemporarily associated with the pre-pathological condition, being thus early events in the development of autoimmunity (4). However, investigations on IPC are relatively rare in neuroinflammation, especially in the human disease, while studies on dysbiosis are already very numerous, and microbiota alterations were deeply investigated in both experimental autoimmune encephalomyelitis (EAE) and MS. Over the last decade several studies on animal models showed that an immune response to gut microbiota is able to promote cerebral autoimmunity driven by the expansion of pro-inflammatory T cells and autoantibodies, at expenses of regulatory T cells (5–8).

Recent studies carried out in MS patients supported the importance of microbiota alterations in disease pathophysiology. Some authors tried to define metagenomic signatures of microbiomes associated to MS: a group reported higher Firmicutes/Bacteroidetes ratio, increase *Streptococcus* and decreased *Prevotella* strains in patients with active disease (9); others found increased *Akkermansia muciniphila* and *Acinetobacter calcoaceticus,* and reduced *Parabacteroides distasonis* in patients compared to controls (10). The effects of human-derived microbiota on EAE is an interesting approach to evaluate the impact of dysbiosis or commensal bacteria on neuroinflammation. The MS-derived microbiota was capable of inducing or worsening experimental models of disease (10, 11), while *Prevotella histicola*, a human gut-derived commensal bacteria, could suppress EAE (12).

Studies on IPC in MS and EAE are not very numerous. Approximately 20 years ago, studying co-morbidity between Crohn's disease and MS, a first finding of increased IP was reported in a minority of cases with MS (13). The recent momentum of the gut-brain axis role in the pathogenesis of neuroinflammation prompted studies on IP changes in EAE. A work showed that an increased IP preceded EAE development and worsened during disease with disruption of TJ. These changes were associated with unbalance of mucosal immunity (prevalence of pro-inflammatory Th1-Th17 subsets over T regulatory cells). The same work also showed that similar alterations of intestinal barrier occurred in the passive model of EAE after transfer of encephalitogenic T cells (14). Starting from the evidence of the plausible pathogenic role of mucosal-associated invariant T cells in MS (15), we decided to investigate further the role of gut in the neuroinflammation, and designed a pilot study on IPC in patients with relapsing-remitting MS and healthy controls, including MS-discordant twin pairs. We reported that an alteration of IP is a relatively frequent event in MS. Data on twins suggested a genetic influence on the determinants of gut barrier disruption. IPC included a deficit of the active mechanism of absorption from intestinal lumen in patients compared to controls (16).

The topic of IPC in neuroinflammation is currently under active scrutiny, and several lines of investigations are focusing on the plausible relationships between gut barrier disruption and pathophysiologic components of MS (**Figure 1**), as well as on translational implications based on IPC (**Table 1**).

## Intestinal Permeability Changes and MS Pathophysiology

An important issue to be faced for IPC evaluation regards the methodological improvement of IP metrics. Recent approaches, using multi-sugar tests, allow to study different segments of gastro-intestinal tract and may prove to be more informative to understand the level especially involved in MS pathogenesis (17). Among the peripheral markers of IP the role of zonulin has been recently recognized (18): this modulator of the tight junctions proved to be involved in autoimmune disorders typically associated with an IP dysfunction, such as celiac disease and type 1 diabetes (19). Notably, a recent work showed that zonulin can rapidly increase both IP permeability and blood brain barrier (BBB) *in vitro* by modifying TJs, having a synergic action with the pro-inflammatory cytokines typically involved in MS pathogenesis, and explaining, at least in part, how the gut-brain axis may mediate the pathogenesis of neuro-inflammatory diseases (20). Another peripheral biomarker recently used to quantify IPC in MS is the Intestinal Fatty Acid Binding Protein (IFABP). IFABP is a cytosolic protein exclusively expressed by enterocytes and rapidly released into blood circulation upon cell stress (21). Some data indicated that serum IFABP was higher in people with MS than in healthy controls (22), while others reported no differences between patients and controls (13); the discrepancy is possibly due to diverse analytical methods, suggesting that the approach still needs optimization. A consensus on the best method(s) to evaluate IPC is certainly desirable in this phase: it may help to replicate results in different conditions, and to understand whether specific or shared gut barrier changes characterize each immune-mediated disorder.

Recent works have tried to link IPC with risk factors known to be associated to MS, such as the epidemiological evidence of increased MS prevalence in countries at high latitudes, where the sunlight is limited and the populations tend to have vitamin D deficiency (23). A recent review suggests that vitamin

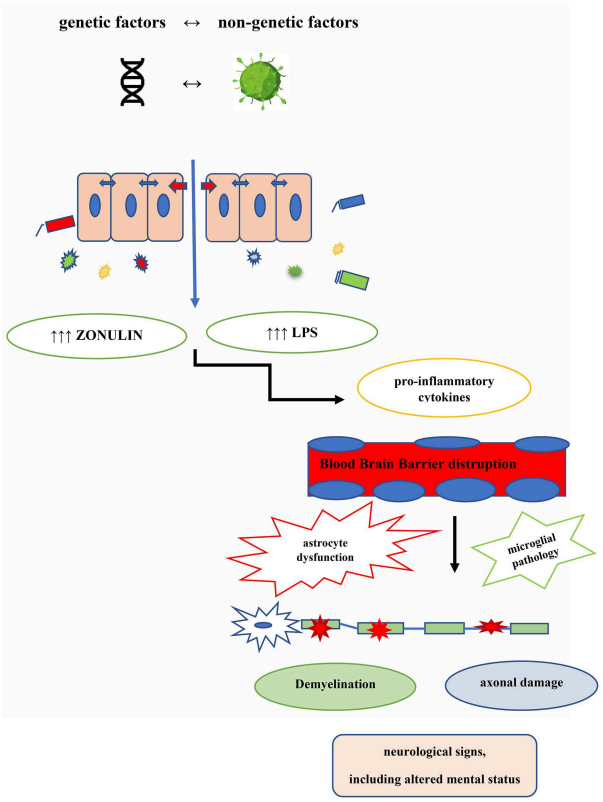


**FIGURE 1 |** MS pathogenic loop centered on the gut barrier disruption. Genetic and non-genetic aetiologic factors contribute to the co-occurring intestinal permeability changes (IPC) and dysbiosis, that in turn bring about the MS pathogenic cascade.

**TABLE 1 |** Gut barrier stabilizers or enhancers investigated in chronic inflammation.

| Intervention | Disorders with gut barrier disruption |
|---|---|
| Larazotide | Celiac disease |
| Divertin | Experimental inflammatory bowel disease |
| Food-grade bacteria engineered to produce elafin | Inflammatory bowel disease and gluten-related disorders |
| Vitamin D | Inflammatory bowel disease and other immune-mediated disorders |
| Escherichia coli strain Nissle 1917 | Experimental autoimmune encephalomyelitis |
| Approaches targeting the Mincle-Syk axis in gut dendritic cells | Commensals deprivation |
| Obeticolic acid | Alcoholic hepatitis, non-alcoholic steatohepatitis, and primary biliary cirrhosis |
| Microbiota transplantation | Clostridium difficile infection |

D deficiency reduces intestinal calcium absorption and leads to gut stasis and subsequent IPC. This would allow gut microbiota to transfer more endotoxins into the blood and to stimulate the production of inflammatory cytokines within the CNS (24). Another interesting link seems to be that between gut barrier and BBB breakdown: in a work above reported (20), the authors demonstrated that, at least *in vitro*, increase of zonulin, as well as of interleukin 17 and interferon gamma, provoked similar effects of IPC and BBB leakage, suggesting plausible vicious circles between intestinal dysfunction and neuro-inflammation. These data are in accord with those of Fasano and coworkers who measured serum levels of zonulin in relapsing–remitting MS, and found increased concentrations in phase of disease activity, while patients in remission showed serum levels comparable to those of controls (18).

A crucial point regarding the contribution of IPC to MS pathophysiology is the low-grade microbial translocation to systemic circulation and eventually to brain (25, 26). Along this line, gastrointestinal disorders with intestinal barrier breakdown, such as celiac diseases and inflammatory bowel diseases, show evidence of CNS demyelination or overt co-morbidity with MS in a proportion of patients (27). However, several works showed that, also in the absence of gastrointestinal diseases, a low-grade translocation of bacteria or bacterial products from the intestines into the circulation is present in MS, and correlates with changes of gut microbiota (26). In this context a failure of the protective function that commensal bacteria exert on the gut barrier can be hypothesized. Homeostatic microbiota may regulate IP through multiple mechanisms: production of short chain fatty acids (SCFA) that increase tight junctions; toll like receptors activation, that promote epithelial cell proliferation, IgA synthesis, and antimicrobial peptides production; metabolizing actions on dietary tryptophan and production of metabolites that play a role as anti-inflammatory mediators also far from gut (28). Conversely, a low-grade endotoxemia, possibly due to IPC, was demonstrated in MS patients (29, 30). These works showed increase levels of lipopolysaccharide (LPS) and LPS-binding protein in plasma of MS patients, that correlated with the concentrations of pro-inflammatory cytokine and with the expanded disability status scale. In fact, LPS is known to exert pro-inflammatory actions on microglia and astrocytes, and to participate in the disruption of BBB, all effects that can perpetuate the pathogenic loop of MS. Along this line, a recent work showed that the circulating bacterial peptidoglycan comes from host microbiota and acts as a natural immune potentiator that tunes the host immune response. The same work also showed that the neutralization of the circulating peptidoglycan suppressed the development of the experimental model of MS (31).

A recent interesting observation concerning the gut-brain axis is the potential neuroactive impact of the human microbiota on mental status, in particular the quality of life and the depressive status. Clear metagenomic profiles and specific metabolites production (SCFA and neuromodulators' precursors) by gut prokaryotes have been reported to correlate with indicators of mental health, by surveying a large microbiome population cohort, with validation in independent data sets (32). These data bear relevance to the long known relationship between MS and affective disorders. Patients with MS have an estimated prevalence of depression that is 2–3 times higher than that of the general population (33). Mechanisms underlying this condition may be multiple:

besides the reactive component due to the stressors of "living with MS," lesion burden and brain atrophy are often correlated to standard scales for measuring the mood status. However, a new evidence (34) on a wide array of biological abnormalities shared by MS and major depressive disorder (peripheral inflammation, neuroinflammation, chronic oxidative and nitrosative stress, mitochondrial dysfunction, neuroendocrine abnormalities and microglial pathology) make it plausible that IPC, with gut dysbiosis and bacterial translocation into the systemic circulation, could represent a significant (albeit not the sole) determinant of mood status disruption in MS. This perspective may have therapeutic implications, suggesting new treatments to deal with depression in MS, that are closer to etiopathogenic rather than symptomatic approaches.

## Translational Implications

Among the translational implication of IPC, two points should be emphasized: the possible effects of disease-modifying therapies (DMT) on gut barrier and the potential therapeutic approaches aimed at antagonizing the IPC through stabilizers or enhancers of intestinal integrity. A recent review reported that DMTs, currently used in clinical practice for MS, would be able to act at different levels on IPC, modulating the gut barrier, the gut microbiota and the interaction between the two. However, these actions, though plausible, seem indirect, and whether they actually play a meaningful role in the clinical response remains to be established. Among these drugs, dimethyl-fumarate and fingolimod seem to have an antimicrobial action and a positive direct effect on TJ (26). On the other hand, teriflunomide and dimethyl-fumarate provoke gastro-intestinal side effects in some people with MS, raising the question whether these unwanted consequences may be associated, at least in part, to IP disruption. Actually, the effects of current DMT on IP was not yet explicitly evaluated; a study on dimethyl-fumarate on IP and microbiota was recently carried out in our Center (manuscript in preparation).

Besides being of pathophysiological interest, the brain-gut axis abnormalities (microbiota unbalance, IPC and alterations in bile acid metabolism) could also open new avenues for therapeutic targets. The probiotics use and the successful modification of the microbiome could be one strategy to modulate and improve intestinal barrier function. However, probiotics do not modify the host microbiome in a satisfying and lasting manner (few clinical trials in MS showed modest beneficial trends in clinical variables and some biomarker changes in peripheral immune function). Fecal microbiota transplantation would constitute the optimal strategy and isolated cases were described with beneficial effects on disease course. Also supplementation of bile acids might have several beneficial effects, modulating the intestinal barrier function, shaping the gut microbiota toward homeostatic profiles, and also regulating inflammatory signaling in the central nervous system (35). At least some of these effects may be mediated by the nuclear hormone receptor, farnesoid X receptor (FXR), that has bile acids among its ligands. Obeticholic acid (6α-ethyl-chenodeoxycholic acid), a synthetic FXR agonist, that is an orally available drug currently in clinical trials for the treatment of inflammatory diseases (alcoholic hepatitis, nonalcoholic steatohepatitis, and primary biliary cirrhosis), was shown to be capable of ameliorating EAE (36).

The topic of IP enhancers or stabilizers recently received increasing interest, being the object of several works: this approach seems to target an early pathogenic event (the IPC), underlying many conditions of chronic inflammation in gut and far-from-gut organs. Many drugs come from studies conducted in chronic gastro-enteric inflammation: an interesting example is larazotide (a 8-mer peptide with activity as TJ regulator), that was tested in a model of celiac disease and was shown to inhibit gliadin-induced macrophage accumulation in the intestine and to preserve the TJ structure (37). Larazotide was then tested in several trials in celiac disease with encouraging results (38–40). Another compound of interest is divertin, a small molecule that diverts myosin light chain kinase from its effects on gut barrier dysfunction and disease progression in experimental inflammatory bowel disease (41). Other approaches exploit the effects of probiotic or engineered bacteria to revert IPC and restore the gut barrier homeostasis. Elafin, an endogenously produced inhibitor of elastase that is deficient in inflammatory bowel diseases and gluten disorders, was effective in stabilizing IP and restoring gut homeostasis in a pilot study with food-grade bacteria engineered to produce the molecule (42, 43). In a study on EAE, where the authors confirm the pathogenic role of a profound defect in the IP function, treatment with oral daily probiotic *Escherichia coli* strain Nissle 1917 (ECN), but not with another strain, repaired intestinal permeability dysfunction and induced a general modulation of immune effectors, with a beneficial effect on the disease progression (44). Vitamin D, that is currently under active scrutiny in several chronic inflammatory conditions, including MS, has a specific action as a gut barrier stabilizer; a recent controlled trial in patients with inflammatory bowel disease reported improvement of IPC at different segments of gastro-intestinal tract (45). Finally, a recent paper clarifies the molecular model through which commensals act as enhancers of gut barrier integrity, disclosing possible therapeutic targets to counteract systemic inflammation. Sensing of commensal species by the C-type lectin receptor Mincle, coupled to a dendritic cell Syk-kinase, activates an homeostatic cascade that regulates the function of group 3 innate lymphoid cells, fosters the IgA production, and impedes the systemic translocation of gut microbiota (46).

All these findings provide several hints to try therapeutic approaches in MS: repurposing compounds that have been studied especially for IPC in chronic gastro-intestinal inflammation, and/or reworking the increasingly growing data coming from microbiota studies in EAE and MS will plausibly yield fruitful lines of attack against neuroinflammation.

## AUTHOR CONTRIBUTIONS

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

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# Tryptophan Dietary Impacts Gut Barrier and Metabolic Diseases

*Soraya Taleb**

*Institut National de la Santé et de la Recherche Médicale (INSERM), Unit 970, Paris Cardiovascular Research Center, and Université Paris-Descartes, Paris, France*

The intestine has a major role in the digestion and absorption of nutrients, and gut barrier is the first defense line against harmful pathogens. Alteration of the intestinal barrier is associated with enhanced intestinal permeability and development of numerous pathological diseases including gastrointestinal and cardiometabolic diseases. Among the metabolites that play an important role within intestinal health, L Tryptophan (Trp) is one of the nine essential amino acids supplied by diet, whose metabolism appears as a key modulator of gut microbiota, with major impacts on physiological, and pathological pathways. Recently, emerging evidence showed that the Trp catabolism through one major enzyme indoleamine 2,3-dioxygenase 1 (IDO1) expressed by the host affects Trp metabolism by gut microbiota to generate indole metabolites, thereby altering gut function and health in mice and humans. In this mini review, I summarize the most recent advances concerning the role of Trp metabolism in host–microbiota cross-talk in health, and metabolic diseases. This novel aspect of IDO1 function in intestine will better explain its complex roles in a broad range of disease states where the gut function affects local as well as systemic health, and will open new therapeutic strategies.

**Keywords: tryptophan, indoleamine 2, 3-dioxygenase, gut microbiota, metabolic syndrome, cardiovascular disease and cardiometabolic diseases**

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***Edited by:***
*Julien Diana,*
*Institut National de la Santé et de la Recherche Médicale (INSERM), France*

***Reviewed by:***
*Nadia Corazza,*
*University of Bern, Switzerland*
*Paolo Puccetti,*
*University of Perugia, Italy*

***Correspondence:**
*Soraya Taleb*
*soraya.taleb@inserm.fr*

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## INTRODUCTION

The prevalence of Western diet-induced metabolic syndrome (MetS) is booming, affecting more than 2 billion people worldwide and accounting for at least 3 million deaths per year (1). This becomes worrying since MetS is the major contributor of the persistent increase in cardiovascular diseases (CVD), including myocardial infarction (MI), which is the main complication of atherosclerosis. Many patients with obesity suffer from adverse metabolic complications and associated atherosclerosis, whereas others remain "metabolically healthy obese" (MHO), although they still have a higher CVD risk than normal weight and metabolically healthy subjects (2). The inconsistency regarding individual susceptibility to cardiometabolic diseases is still an issue that is currently not sufficiently addressed. This susceptibility to cardiometabolic diseases is mainly associated with environmental factors such as diet. One link between environment and disease is gut microbiota and the disruption in host–microbiota cross-talk could be involved in disease pathogenesis. The intestinal epithelium is a single-cell layer that constitutes a physical barrier against the external entities due to the expression of epithelial tight and adherence junctions. It acts as a selectively permeable barrier permitting the absorption of nutrients such as amino acids, carbohydrates, lipids, electrolytes, and water, while avoiding pathogen invasion. The dysfunction of this barrier as observed in inflammatory diseases leads to enhanced permeability and translocation

of microbial entities such as lipopolysaccharide (LPS) to systemic circulation, which may cause observed inflammation responsible for obesity complications (3). A recent study pinpoints toward the importance of hyperglycemia as an initial trigger responsible for the disruption of tight and adherence junctions leading to the observed increase in intestinal permeability related to MetS (4). However, other actors should be involved as an increase in intestinal permeability is also observed in other diseases without glycemia disruption such as intestinal bowel diseases (5). In this context, it is not clear whether the permeability changes are a primary event in the disease development or a secondary result elicited by intestinal inflammation.

The association between altered gut microbiota or dysbiosis, inflammation, and cardiometabolic diseases is becoming increasingly clear but remains poorly understood (6, 7). In the CVD context, the interplay between dietary composition and gut microbiota-derived metabolites has been highlighted by the discovery of the role of Trimethylamine *N*-oxide (TMAO) in promoting atherosclerosis (8). Besides, L tryptophan (Trp) intake has recently emerged as a potential link between altered gut microbiota, impairment of intestinal immunity and disease development (9).

In this mini review, I summarize current evidence supporting the involvement of Trp catabolism by both the host, and gut microbiota in the context of MetS. Furthermore, I describe the potential mechanisms of action of Trp metabolites in modulating the local intestinal homeostasis, which may impact systemic metabolic parameters.

## TRYPTOPHAN CATABOLISM IN CARDIOMETABOLIC DISEASES

Trp is one of nine essential amino acids brought by the diet, which the metabolism appears now as a key modulator of gut microbiota impacting major physiological, and pathological pathways (10, 11). In mammalian cells, Trp is primarily degraded through the kynurenine pathway (KP), a cascade of enzymatic steps leading to the generation of several biologically active compounds. Subsequent to Trp absorption via enterocyte transporters in the large intestine, Trp transits into the hepatic portal system where it is utilized by the liver for the KP through tryptophan 2,3-dioxygenase (TDO). Unused Trp is then secreted into the bloodstream and is available for use by peripheral tissues. The Trp degradation step in peripheral tissues is mainly due to indoleamine 2,3-dioxygenase (IDO)1, which contributes to the major Trp catabolism in extrahepatic tissues as compared with that resulting from IDO2 isoform. Specifically, Trp is degraded into *N*-formylkynurenine, leading to the generation of several active metabolites, including kynurenine (Kyn), 3-hydroxykynurenine (3-OHKyn), kynurenic acid (Kna), 3-hydroxyanthranilic acid (3HAA), and quinolinic acid. A small fraction of Trp is converted to serotonin and melatonin via the serotonin pathway, mainly in the gastrointestinal tract (**Figure 1**). During inflammation, IDO1 is up-regulated mostly in macrophages, and dendritic cells by proinflammatory stimuli, notably interferon (IFN)-γ (12). IDO1 exerts its biological effects mainly through the generation of downstream metabolites that suppress effector T-cell function, and favor the differentiation of regulatory T cells (Tregs) (13). However, IDO1 does not appear instrumental in these functions, as IDO1 knockout mice do not develop an autoimmune phenotype. This may be due to a compensatory or counter-regulatory mechanism (14). In addition, the biological effects of IDO1 may go beyond its role in the regulation of the immune response. Indeed, IDO1 activity was shown to contribute to arterial vessel relaxation and to the control of blood pressure in the context of septic shock (15). IDO1 activity was also shown to play a critical role in aneurysm development through favoring vascular smooth muscle cell (VSMC) apoptosis (16), and the

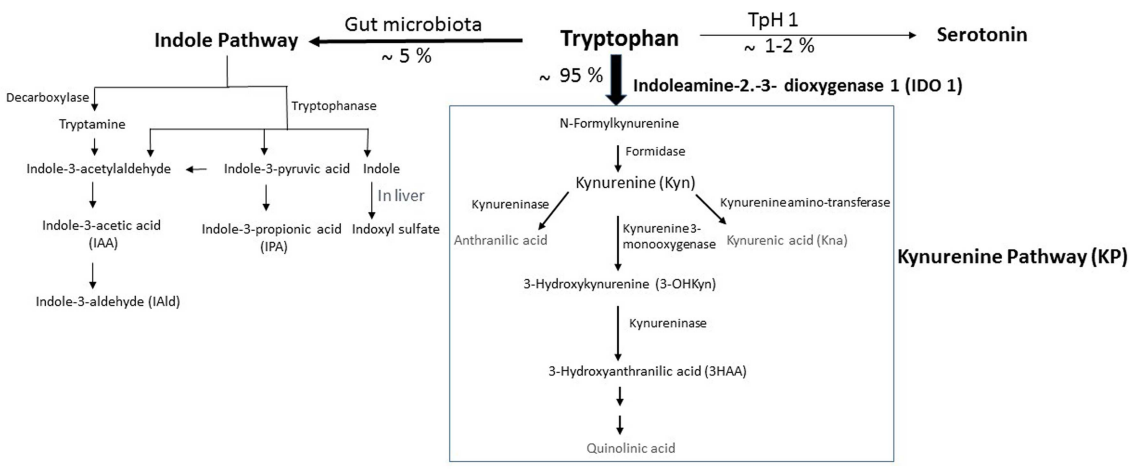


**FIGURE 1 |** Simplified illustration of the kynurenine pathway in the gastrointestinal tract. Tryptophan (Trp) is predominantly converted into kynurenine (Kyn) pathway (KP) by the indoleamine 2,3-dioxygenase 1 (IDO). A small amount of Trp is converted by gut microbiota through the action of the enzyme tryptophanase, into indole, and its derivatives and into tryptamine. Indole metabolites could be converted in the liver into indoxyl sulfate. Another fraction of Trp is converted through Trp hydroxylase 1 (TpH 1) into serotonin.

increase of metalloproteinase (MMP)-2 expression in VSMC (17). On the other hand, metabolites generated from kynurenine may regulate diverse cellular functions, including viability (18), adhesive and migratory properties (19), as well as inflammatory potential (20). Trp metabolism has also been involved in various diseases ranging from chronic granulomatous disease (21) and gastrointestinal diseases (22) to neurodegenerative diseases (23). In obesity, IDO activity is up-regulated in adipose tissue, in circulating blood, and likely in the digestive tract of obese compared to non-obese subjects (24–28). The use of mouse models has recently involved its enzymatic activity in the pathogenesis of MetS (28). On the other side, administration of Kna, a metabolite downstream of Kyn, to WT mice has been shown to activate G protein-coupled receptor (GPR) 35, and increase energy expenditure (29), suggesting a protective effect. However, this may not represent the role of the endogenous Kna with physiological concentrations, as that experiment was performed in WT mice. It has to be noted that the mechanisms of IDO1 actions could be different in an autoimmune context such as type I diabetes, where the observed low level of IDO1 may weaken the immunomodulatory microenvironment, and make the pancreatic β-cells more susceptible to inflammatory deleterious response (30).

In the context of CVD, we previously showed that, in contrast with previous studies (31–34), IDO1 does not protect but rather promotes the development of atherosclerosis (35). We showed that IDO1 activity sustains an immune-stimulatory potential through inhibition of a major immune-regulatory and athero-protective cytokine, interleukin (IL)-10 (36). Consistently, it was shown that the induction of IDO1 by dietary microbial oxazoles reduced IL-10 production in intestinal epithelial cells (IECs). This mechanism was dependent on KP such as Kna, which inhibited IL-10 production through the activation of the aryl hydrocarbon receptor (AHR) (20). However, on the other hand, KP was previously shown to induce the generation of protective Tregs through AHR activation (37). AHR receptor is activated not only by small molecules coming from the host such as KP but also by environmental sources such as the diet, the gut microbiota, and pollutants (38). Evidence showed that AHR activation could have either pro- or anti-inflammatory effects depending on ligand- and/or cell-specific effects to locally modulate the inflammatory response (38). Moreover, it is important to know whether physiologically relevant concentrations of those ligands can activate AHR *in vivo*.

In human atherosclerotic lesions, we found increased levels of the IDO1-generated metabolite, Kna, which were associated with an unstable plaque phenotype. Moreover, Kna blood levels predicted death, and recurrent MI in patients admitted for acute MI (35). These results are in agreement with previous clinical studies showing that circulating Kyn and Kyn-derived metabolites are associated with cardiovascular risk factors (39, 40) and with worse outcome in patients with coronary artery disease (41–43). Collectively, these data indicate a potential involvement of IDO1 in MetS and CVD, and an emerging concept suggests that Trp catabolism through host expressing IDO1 in gastrointestinal tract could contribute significantly to cardiometabolic diseases.

## TRYPTOPHAN METABOLISM IN GASTROINTESTINAL TRACT

In the gastrointestinal tract, Trp metabolism has major effects on the host and notably on immunity and metabolism, gut microbiota, intestinal barrier, and transit (11). In mice, dietary lack of tryptophan leads to impaired intestinal immunity and promotes microbiota dysbiosis (44). Moreover, patients with intestinal bowel disease exhibit increased IDO activity, supporting the importance of Trp metabolism in maintaining intestinal homeostasis (22). In turn, gut microbiota derivatives could affect KP. Short-chain fatty acids (SCFAs), such as acetate and butyrate, are the end products of fermentation of dietary fibers by the anaerobic intestinal microbiota, and have been shown to exert multiple beneficial effects (7). Recently, it has been shown that butyrate negatively regulates IDO expression by IECs (45), suggesting a potential role of gut microbiota-derived metabolites in the regulation of Trp metabolism. Consistently, the absence of gut microbiota in germ-free mice was shown to reduce host IDO activity as assessed by decreased plasma Kyn/Trp ratio (46).

In homeostatic conditions, Trp metabolism in the intestine follows three major pathways: (i) the KP in both immune cells (mainly macrophages) and IECs via IDO1, which is the major pathway (represents 95% of ingested Trp). (ii) The direct transformation of Trp (4–6%) by the gut microbiota into tryptamine and indole metabolites via the action of the enzyme tryptophanase, which is expressed in many Gram–negative and Gram–positive bacteria. Among indole metabolites, indole pyruvic acid can give rise to indole propionic acid (IPA) and to indole acetaldehyde that can be converted to indole acetic acid (IAA), and then to indole aldehyde (IAld) (**Figure 1**). Some of indole metabolites such as IAA, indole-3-acetylaldehyde, IAld, and tryptamine have been shown to maintain intestinal barrier integrity and immune cell homeostasis through activation of the AHR (10, 22). Other studies demonstrate that intestinal barrier function can be improved by IPA through the activation of the pregnane X receptor (PXR). (iii) A small portion of Trp (1–2%) can give rise to the serotonin production pathway in enterochromaffin cells via Trp hydroxylase 1 (TpH1) (**Figure 1**).

The conversion of Trp to Kyn through IDO1 represents thus the major pathway of Trp degradation in the intestine, indicating the importance of the enzyme beyond Kyn in the pathway of Trp metabolism (**Figure 2**). Notably, we recently showed that high-fat diet supplementation was associated with increased IDO1 activity and inversely to a decrease in indole derivatives such as IAA, whereas IDO1 deletion leads to higher intestinal IAA production (28). In accord with mouse data, we observed a shift of Trp metabolism toward more Kyn and less IAA in feces of obese, and non-treated type II diabetic compared to non-obese subjects (28), suggesting an increase in gut IDO activity and a decrease in indole pathway in the context of MetS (**Figure 2**). The same was observed in the context of intestinal inflammatory diseases (22), suggesting the involvement of common mechanisms in diseases with disruption of intestinal barrier. Consistently, gut microbiota of individuals with MetS and with intestinal inflammatory disease showed decreased AHR activation (22, 47), suggesting that

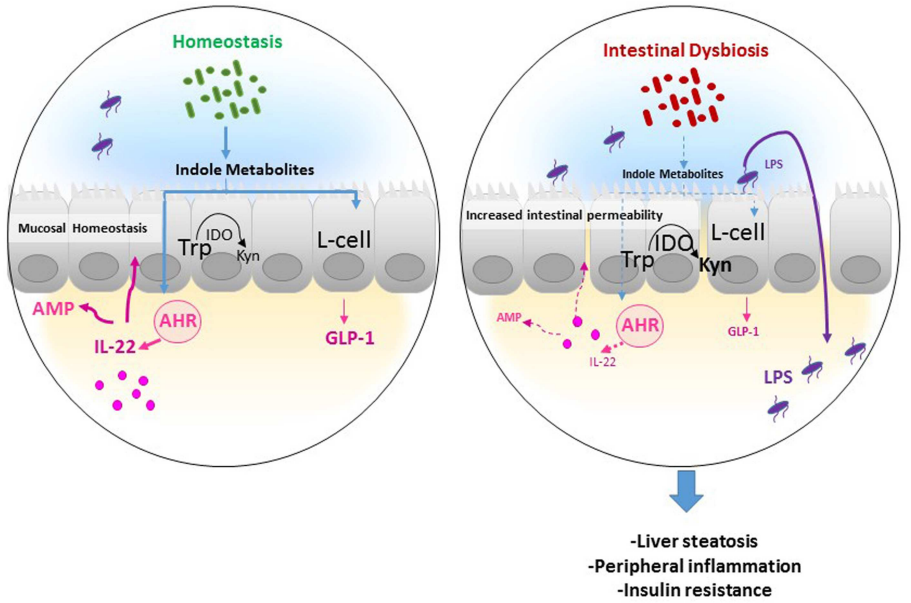


**FIGURE 2 |** Potential mechanism of actions of Trp metabolites in the gastrointestinal tract. In homeostatic condition, Tryptophan (Trp) is used by the host indoleamine 2,3-dioxygenase (IDO) to be converted to Kynurenine (Kyn), and by gut microbiota to produce indole metabolites. Several indole derivatives have been described as activators of the AHR that promotes IL-22 production, which stimulates mucosal defense via the induction of antimicrobial proteins (AMP). In addition, indoles may affect mucosal homeostasis by stimulating mucin production and promoting epithelial barrier function by enhancing tight junction proteins. Also, indoles induce the release of the incretin, glucagon-like peptide 1 (GLP-1), in enteroendocrine L cells, which is known to stimulate insulin secretion, suppress appetite, and inhibit gastrointestinal motility, and secretion. In an inflammatory disease condition such as obesity, the increase in IDO activity leads to decreased indole production from Trp and thus low GLP-1 and IL-22 production leading to increased intestinal permeability and lipopolysaccharide (LPS) translocation in the systemic circulation, resulting in peripheral inflammation, liver steatosis, and insulin resistance.

dysregulation of indole metabolites and AHR activation could be involved in inflammatory diseases.

Several bacterial Trp metabolites, such as indole metabolites, have been proven to be AHR ligands and to exhibit protective effects (48). Specifically, indole metabolites including indole, IPA, IAA, and tryptamine were shown to promote IL-22 production through AhR activation (10). IL-22 cytokine has been found to control epithelial cell proliferation and antimicrobial peptide (AMP) production, limiting the ability of commensal bacteria to cause inflammation, which has been shown in the context of MetS (**Figure 2**) (49), and more recently in atherosclerosis (50). Moreover, other studies demonstrate that intestinal barrier function can be regulated by indole derivatives, particularly IPA through activating the PXR, which inhibits inflammation locally and up-regulates tight junction expression (51). On the other hand, mouse models of obesity showed that peripheral serotonin produced by the gut favors MetS through a negative regulation of brown adipose tissue thermogenesis (52). However, this may be questionable in humans since the change of energy expenditure depending on brown adipose tissue in adult human is still under debate.

Indole absorption through the colonic mucosa is followed by its liver metabolism to indoxyl sulfate, the prototype of protein–bound uremic toxins. This "gut–liver axis," driven by the local gut microbiota, could then exert peripheral effects. For example, in patients with chronic kidney disease, accumulation of indoxyl sulfate due to insufficient renal removal has been involved in CVD in these patients (53). However, this represents a supraphysiological concentration of indoxyl sulfate, which may not be representative to what happens in CVD patients without renal diseases. On the other hand, certain indole metabolites seem to exert anti-inflammatory effects (54). Future studies are needed to determine the role of indole metabolites, particularly indoxyl sulfate in cardiometabolic diseases without renal failure.

Gut Trp catabolism may have peripheral effects and could impact the development of cardiometabolic diseases. We recently showed that IDO1 deletion or inhibition in the context of MetS improved insulin sensitivity, decreased endotoxemia, and chronic inflammation, and positively regulated lipid metabolism in liver, and adipose tissues. We found that these beneficial effects were due to rewiring of Trp metabolism toward a microbiota-dependent production of IL-22 and were abrogated after treatment with a neutralizing anti-IL-22 antibody. Moreover, microbiota transfer of feces from obese mice treated with IDO1 inhibitor (L-1 methyltryptophan, L-1MT) compared to non-treated mice increased IAA as well as IL-22 production and improved metabolic parameters in the recipient mice fed with high-fat diet (28). In addition, we and others have recently shown that indole metabolites and particularly IAA protect against MetS complications (28, 47), highlighting the importance of IDO1 activity on intestinal homeostasis and peripheral metabolism. The observed protective role of indoles may be

related to their local effects on intestine through promoting IL-22 production and/or the stimulation of enteroendocrine L cells to produce glucagon-like peptide-1 (GLP-1), an incretin stimulating the secretion of insulin by pancreatic β cells (47, 55). Moreover, indole was shown to alleviate liver inflammation in mice through preventing LPS-induced detrimental effects (56). In this context, the supplementation with Lactobacillus strain bacteria stain producing high levels of indole metabolites leads to improvement of metabolic parameters, through maintaining intestinal barrier function and promoting GLP-1 production (49).

## CONCLUSION AND PERSPECTIVES

Understanding the relationships between the diet and the complex cross-talk between the host and gut microbiota appears now as instrumental for the development of new therapeutic approaches to modulate metabolic dysbiosis and treating disease. Data demonstrate the importance of indole metabolites in the re-establishment of intestinal epithelial barrier integrity in the context of intestinal inflammatory diseases and MetS. However, because different bacteria may possess diverse catalytic enzymes, it is complicated to predict which indole metabolites are produced and which ones may activate AHR. Thus, the physiologic implications of AHR activation by tryptamine and the different indole metabolites in the gastrointestinal tract remain to be established. Also, future studies are needed to determine their involvements in co-associated diseases such as CVD. Although several bacteria capable of producing Trp catabolites such as lactobacilli have been identified, others may exist and should be identified through, for example, the use of bacterial metabolome approaches. One major achievement would be to identify the best way to modulate Trp catabolism to improve dysbiosis and metabolic health. For example, further studies are warranted to investigate whether enhancing indole derivative formation by bacteria or directly administering those metabolites is beneficial in inflammatory diseases characterized by gut barrier disruption. Also, future studies are required to determine therapeutic efficacy of whether these bacteria and/or indole metabolites should be used in a combined or separate manner.

The use of IDO inhibitor failed to show efficiency in cancer; one possibility is to reuse this inhibitor in patients with metabolic diseases. In this context, the development of other IDO inhibitors, for example, oral non-absorbable drugs, to inhibit locally IDO in gastrointestinal tract thereby enhancing effectiveness while reducing potential side effects, would be valuable. However, before the development of such potential treatments, a more comprehensive and mechanistic understanding of factors that influence the different Trp catabolism pathways is critical to elucidating the physiologic functions of IDO and the consequences of its disturbance.

## AUTHOR CONTRIBUTIONS

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

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# Exploiting the Zonulin Mouse Model to Establish the Role of Primary Impaired Gut Barrier Function on Microbiota Composition and Immune Profiles

*Alba Miranda-Ribera[1,2], Maria Ennamorati[1], Gloria Serena[1,2], Murat Cetinbas[3,4], Jinggang Lan[1], Ruslan I. Sadreyev[3,5], Nitya Jain[1,2], Alessio Fasano[1,2†] and Maria Fiorentino[1,2*†]*

[1] Department of Pediatric Gastroenterology and Nutrition, Mucosal Immunology and Biology Research Center, Massachusetts General Hospital, Boston, MA, United States, [2] Department of Pediatrics, Harvard Medical School, Harvard University, Boston, MA, United States, [3] Department of Molecular Biology, Massachusetts General Hospital, Boston, MA, United States, [4] Department of Genetics, Harvard Medical School, Boston, MA, United States, [5] Department of Pathology, Massachusetts General Hospital and Harvard Medical School, Boston, MA, United States

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***Correspondence:**
*Maria Fiorentino*
*mrfiorentino@mgh.harvard.edu*

*†These authors share senior authorship*

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The balanced interplay between epithelial barrier, immune system, and microbiota maintains gut homeostasis, while disruption of this interplay may lead to inflammation. Paracellular permeability is governed by intercellular tight-junctions (TJs). Zonulin is, to date, the only known physiological regulator of intestinal TJs. We used a zonulin transgenic mouse (Ztm) model characterized by increased small intestinal permeability to elucidate the role of a primary impaired gut barrier on microbiome composition and/or immune profile. Ztm exhibit an altered gene expression profile of TJs in the gut compared to wild-type mice (WT): Claudin-15, Claudin-5, Jam-3, and Myosin-1C are decreased in the male duodenum whereas Claudin-15, Claudin-7, and ZO-2 are reduced in the female colon. These results are compatible with loss of gut barrier function and are paralleled by an altered microbiota composition with reduced abundance of the genus *Akkermansia*, known to have positive effects on gut barrier integrity and strengthening, and an increased abundance of the *Rikenella* genus, associated to low-grade inflammatory conditions. Immune profile analysis shows a subtly skewed distribution of immune cell subsets toward a pro-inflammatory phenotype with more IL-17 producing adaptive and innate-like T cells in Ztm. Interestingly, microbiota "normalization" involving the transfer of WT microbiota into Ztm, did not rescue the altered immune profile. Our data suggest that a primary impaired gut barrier causing an uncontrolled trafficking of microbial products leads to a latent pro-inflammatory status, with a skewed microbiota composition and immune profile that, in the presence of an environmental trigger, as we have previously described (1), might promote the onset of overt inflammation and an increased risk of chronic disease.

**Keywords: gut permeability, microbial products trafficking, tight-junctions, zonulin transgenic mouse, microbiota, dysbiosis, immunity, chronic inflammatory diseases**

## INTRODUCTION

The interplay among host genetics, immune response, intestinal microbiota composition and function, and exposure to environmental factors is critical in the breakdown of the normally coordinated, homeostatic mucosal immune tolerance leading to the development of chronic inflammatory disorders. In this scenario, the role of increased gut permeability has been recently appreciated and seems to be a critical factor in the chain of events leading to break of tolerance and onset of inflammation, whose sequence is still largely unknown. Increased intestinal permeability has been reported more than 30 years ago in celiac and Crohn's disease patients and first-degree relatives suggesting that barrier dysfunction may be necessary but not sufficient to disease development (2–5). Animal studies suggest that epithelial barrier dysfunction can trigger intestinal disease (6–9) as the development of spontaneous colitis in the IL-10 KO mouse is prevented by blocking increased small intestinal permeability with the zonulin inhibitor AT1001 (8). A recent *in-vitro* study using a human "gut-on-a-chip" model of inflammation that allows manipulations of the gut barrier and inflammatory factors, has shown that barrier dysfunction plays a critical role in the initiation of inflammation (10). Increased intestinal permeability has been proposed in the pathogenesis of several diseases not only affecting the gastrointestinal system but also metabolic disorders such as obesity and diabetes and/or diseases of the brain and the nervous system such as Parkinson's disease, multiple sclerosis, and the autism spectrum (11–16).

Intestinal hyperpermeability may precede the clinical manifestations of disease (2, 6, 17) but since inflammation can induce permeability defects (18–20), the controversy regarding the role of barrier defects as the cause or the consequence of disease has not been resolved. Growing evidence suggest mutual influence of impaired gut barrier, microbiome composition/function, and immune profile (21–23), with subsequent break of tolerance leading to chronic inflammatory diseases in genetically susceptible individuals. The currently accepted view is that besides genetic predisposition, exposure to environmental stimuli, loss of gut barrier function, inappropriate immune response, and changes in gut microbiota composition and function seem to be all at play in the pathogenesis of CID (24, 25). Also, intriguing is the evidence that these five factors can influence each other in an intricate "interactome" that remains largely undefined. Identifying the key elements that initiate break of intestinal mucosal tolerance is hence of paramount importance to guide the development of therapeutic approaches to specifically target the initiator of the entire cascade that leads to inflammation. Despite research efforts, a primary role of increased intestinal permeability in the onset of inflammation has not been established.

Pre-haptoglobin 2 (pHP2) is the archetype of the zonulin family (26) that reversibly regulates intestinal permeability by modulating intercellular TJs (27, 28) and its release is triggered by specific microbiota (29) and exposure to gliadin (30, 31). Zonulin is integrally involved in the pathogenesis of several CID (30–32) and it is augmented in chronic inflammatory conditions associated with TJ dysfunction, both at mucosal level (for example celiac disease) (28) and systemically (for example type-1 diabetes) (33). Zonulin plays a role as specific modulator of innate immune response involving antigen trafficking through both epithelial and endothelial compartments as well as M2 polarized macrophages expansion (22, 34).

Mice do not express pHP2, which is present only in humans (26). The Zonulin transgenic mouse (Ztm), genetically engineered to express murine pHP2 under its natural promoter (35), is a model of zonulin-induced increased intestinal permeability and represents a unique and extremely valuable tool to understand how loss of control of antigen trafficking may influence the gut microbiota composition and the immune system development.

In this study, we have leveraged on the Ztm as an animal model of increased gut permeability and inflammation susceptibility (1) to get insights on the role of the gut barrier in orchestrating early mucosal events that eventually lead to the onset of inflammation and development of disease.

## MATERIALS AND METHODS

### Animals

A colony of C57Bl/6 (WT) was maintained in our facility at Massachusetts General Hospital (MGH). Breeding pairs of Ztm were created as previously described (35), and generously donated by Andrew Levy. Both colonies were maintained in our facilities under standard conditions (12 h light: 12 h dark cycle, standard humidity, and temperature), and housed in separate cages within the same facility during the study. Animals were fed standard pellet food and water *ad libitum*. Mice 4–8 weeks of age were used in this study. All animal studies in this paper were approved by the Institutional Animal Care and Use Committee at the MGH (2013N000013).

### Total RNA Extraction and Quantitative PCR (Real-Time PCR)

WT and Ztm mice were anesthetized using Forane (isoflurane, USP) from Baxter, and euthanized by cervical dislocation. Tissues (duodenum, jejunum and colon) were immediately collected and kept at −80°C until processed. Total RNA was extracted from frozen tissues using TRIzol™ reagent (Thermo Fisher) and the Direct-Zol RNA mini prep Kit (ZymoReserach) following the manufacturer's protocol. To ensure full removal of genomic DNA, samples were treated with DNA free™ Kit (Thermo Fisher,). Total mRNA was quantified and A260/280 and A260/230 measured using a Nanodrop 2000 (Thermo Fisher). cDNA was made with random hexamer primers plus Maxima $H^-$ First Strand cDNA Synthesis Kit (Thermo Fisher) according to manufacturer's instructions. Quantitative real-time PCR was performed using PerfeCTa SYBR® Green SuperMix (Quanta) and run in a Bio Rad CFX connect thermocycler. All primers were designed in house (**Table 1**) using NIH's Primer Blast and obtained from Integrated DNA Technologies. We analyzed for gene expression the two most proximal sections of the small intestine (duodenum and jejunum). Only the sections of the

**TABLE 1 |** List of all primers and FASTA accession numbers of the genes analyzed by real time qPCR analysis.

| Gene | Accession number | 5′ OLIGO | 3′ OLIGO | Gene function |
|---|---|---|---|---|
| Claudin 1 | NM_016674.4 | GGCTTCTCTGGGATGGATCG | CTTTGCGAAACGCAGGACAT | Barrier-forming |
| Claudin 2 | NM_016675.4 | CCGTGTTCTGCCAGGATTCTC | AGGAACCAGCGGCGAGTAG | Pore-forming |
| Claudin 3 | NM_009902.4 | CCTAGGAACTGTCCAAGCCG | CCCGTTTCATGGTTTGCCTG | Barrier-forming |
| Claudin 4 | NM_009903.2 | CGTAGCAACGACAAGCCCTA | TGTCCCCAGCAAGCAGTTAG | Barrier-forming |
| Claudin 5 | NM_013805.4 | GTTAAGGCACGGGTAGCACT | TACTTCTGTGACACCGGCAC | Barrier-forming |
| Claudin 7 | NM_016887.6 | GCATACTTTCTGGGGGCCA | TGAAGCGACACTCTCACAGC | Barrier-forming claudin |
| Claudin 8 | NM_018778.3 | AAGGTCTACGACTCCCTGCT | TTCACGTTCTCATCGTCCCC | Barrier-forming claudin |
| Claudin 10 | NM_001160096.1 | CCCAGAATGGGCTACACATA | CCTTCTCCGCCTTGATACTT | Pore-forming claudin |
| Claudin 12 | NM_001193661.1 | GAGCCGATGTGCTCCTGTT | GGAGGGCTTGAGCTGTATGG | Barrier-forming |
| Claudin 15 | NM_021719.4 | AGGCACACCTTATCTGGCAC | TGCCCCCTGAACAATCACAA | Pore-forming claudin |
| INFγ | NM_008337.4 | CAGCAACAGCAAGGCGAAA | CTGGACCTGTGGGTTGTTGAC | Pro-inflammatory cytokine |
| IL6 | NM_031168.2 | GTCCTTCCTACCCCAATTTCCA | CGCACTAGGTTTGCCGAGTA | Pro-inflammatory cytokine |
| IL8 | NM_008176.3 | ACTCAAGAATGGTCGCGAGG | GTGCCATCAGAGCAGTCTGT | Pro-inflammatory cytokine |
| IL10 | NM_010548.2 | TGGGTTGCCAAGCCTTATCG | TTCAGCTTCTCACCCAGGGA | Anti-inflammatory cytokine |
| IL17 | NM_010552.3 | TTTAACTCCCTTGGCGCAAAA | CTTTCCCTCCGCATTGACAC | Pro-inflammatory cytokine |
| JAM3 | NM_023277.4 | GCTGTGAGGTCGTTGCTCTA | AGTGGCACATCATTGCGGTA | Barrier-forming |
| Myo1C | NM_008659.3 | CCGATCACCCGAAGAACCAA | CGCCGGAGGTTCTCAATGAA | Scaffolding |
| Occldn | XM_011244634.2 | CTGACTATGCGGAAAGAGTTGAC | CCAGAGGTGTTGACTTATAGAAAGAC | Barrier-forming |
| TNFα | NM_013693.3 | GATCGGTCCCCAAAGGGATG | TTTGCTACGACGTGGGCTAC | Pro-inflammatory cytokine |
| TRIC | XM_006517605.1 | TGTGTGAAGCTGCCATCAGT | TTTGCCACGTAGTCAGGCAT | Barrier-forming |
| Zonulin | Sturgeon et al. (1) | GAATGTGAGGCAGATGACAG | GTGTTCACCCATTGCTTCTC | TJ modulator |
| ZO1 | NM_009386.2 | AAGAAAAAGAATGCACAGAGTTGTT | GAAATCGTGCTGATGTGCCA | Scaffolding |
| ZO2 | NM_001198985.1 | AGCTTGTAGTTCTGAGCCGC | CCGACACGGCAATTCCAAAT | Scaffolding |
| ZO3 | NM_001282096.1 | GGCTGATTGTTTCCAGGCCC | CCAGAGACAGCTATGCCGAA | Scaffolding |
| 18S | X03205 | AGAAACGGCTACCACATCCA | CCCTCCAATGGATCCTCGTT | Ribosomal RNA |

*All primers were designed in house using NIH's Primer Blast and acquired from Integrated DNA Technologies (USA).*

intestine where we found significant gene expression changes are shown on graphs.

## Microbiota Analysis

### Sample Collection and DNA Extraction

Stools were collected from a total of 71 mice divided into 3 groups: WT ($n = 38$), Ztm ($n = 28$), and $Ztm_{bWT}$ ($n = 5$) (Ztm receiving bedding from WT). To minimize cage effects (36), mice within each genotype were cohoused for 2 weeks before sample collection. Fecal samples were collected from each animal, flash frozen, and kept at −80°C until further processing. Genomic DNA, for each sample, was extracted using the DNeasy powersoil extraction kit (Qiagen) following Qiagen's instructions. DNA was quantified using Nanodrop. In order to carry out the phylogenetic profiling, the hypervariable V4 region of the 16S rRNA gene was amplified by PCR using 5X prime master mix (Prime), reverse 806 primers were barcoded and a unique forward 515 primer (Integrated DNA Technologies) was used. To confirm correct amplification of the V4 regions, a regular gel electrophoresis was run. PCR products were purified using the QIAquick PCR purification kit (Qiagen) and their concentration was measured by Quant-iT Picogreen dsDNA kit following manufacturer's instructions. Sequencing of the samples was done at the MGH NextGen Sequencing Core facility (Boston, US), on the Illumina system using the MiSeq v2 500 cycles reagent kit as per manufacturer's instructions. To allow maximum coverage of the amplicon the system sequenced a total of 250 paired-end cycles. The following primers were used for the sequencing (37):

read 1 (TATGGTAATT GT GTGYCAGCMGCCGCGGTAA)
read 2 (AGTCAGCCAGCCGGACTACNVGGGTWTCTAAT)
index (AATGATACGGCGACCACCGAGATCTACACGCT).

## Bedding Transfer

Timed mating of C57BL/6 (WT) and Ztm mice were set up and monitored for live births. On the day of birth, half the soiled bedding from a WT cage was mixed with autoclaved sterile bedding in a new cage. Ztm breeders with their newborn pups were transferred to this new cage with WT bedding ($Ztm_{bWT}$ mice). This process was repeated twice a week for 4 weeks until the pups were weaned. Colonic fecal content was collected 28 days after birth and analyzed for microbial composition.

## Preparation of Lymphocytes and Flow Cytometry

Spleens were mechanically disrupted, and single cell suspensions were depleted of RBCs with ACK lysis buffer (Invitrogen). Lymphocytes from intestines were prepared as described (38). Small and large intestines from 28 to 35 days old mice were collected in ice-cold HBSS, cleaned and chopped into 1 mm pieces. Samples were pooled from 2 mice and intestinal epithelial

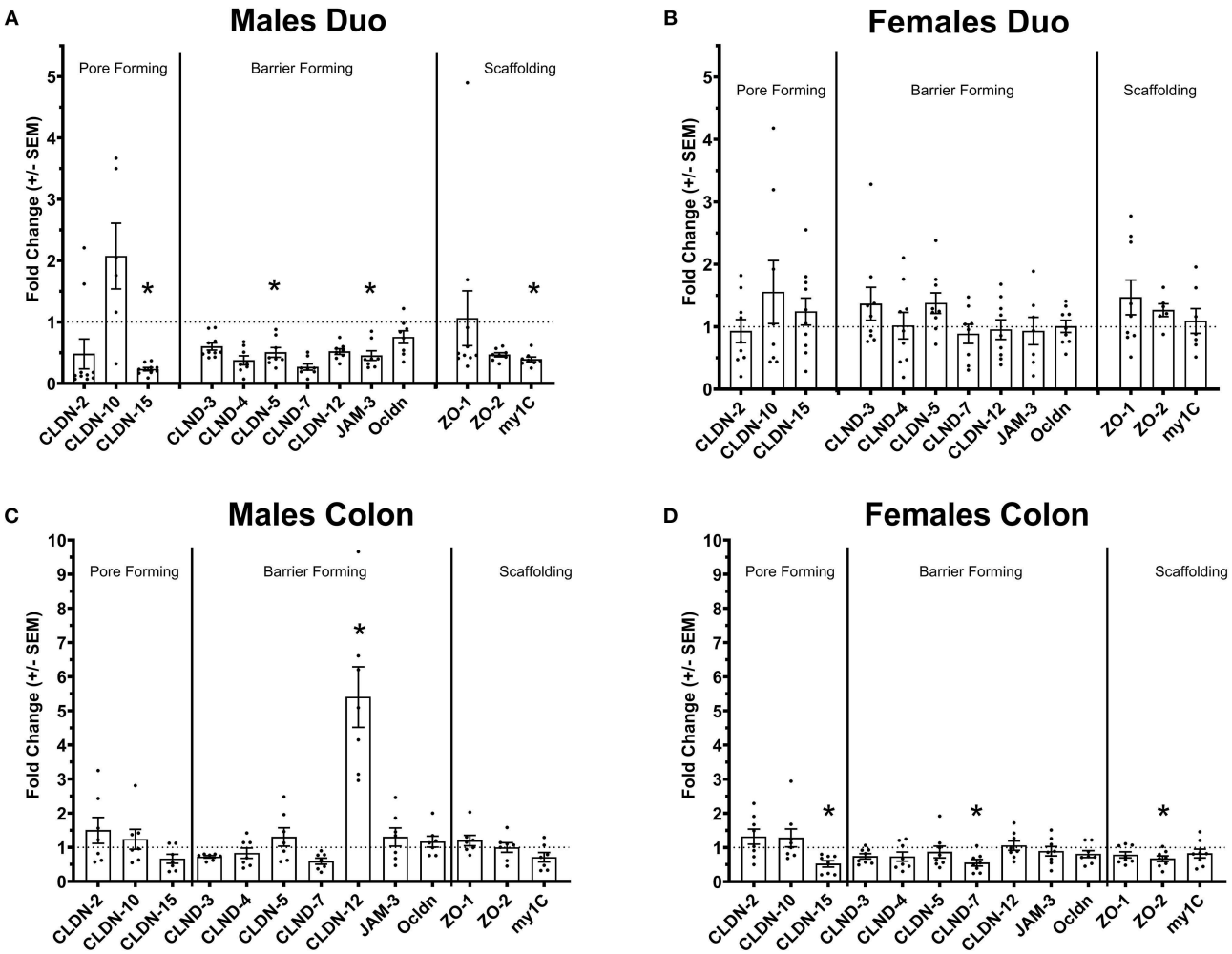



**FIGURE 1 |** Tight-junctions gene expression profile in the intestine of Ztm. **(A)** Down regulated tight-junction genes in the duodenum Ztm males ($n = 6$–10). **(B)** Unaltered tight-junction profile in the duodenum of Ztm females ($n = 7$–12). **(C)** Increased *CLDN-12* in the colon of Ztm males ($n = 7$). **(D)** Downregulation of tight junction genes in the colon of Ztm females ($n = 8$). mRNA expression was analyzed by qPCR, normalized to WT, and expressed as fold regulation. Statistical analysis was performed by the non-parametric Mann-Whitney test comparing each gene to its WT counterpart. $^*p < 0.05$.

cells were removed by shaking in HBSS containing 5 mM EDTA, 1 mM DTT, 5% FCS, and 15 mM HEPES for 30 min at 37°C. Tissues were digested in enzyme solution prepared in EDTA-free HBSS buffer containing 0.15 mg/mL Liberase TL (Roche) and 0.1 mg/mL DNase1 (Roche) for 45 min at 37°C. The LPLs liberated into the supernatant were filtered in a 40 μm strainer and stained for flow cytometry.

Cells were first stained for surface markers and then fixed in eBioscience Fix/Perm buffer. Cells were subsequently permeabilized in eBioscience permeabilization buffer and stained with antibodies against transcription factors. All data were acquired on custom-built BD FACS Aria or Fortessa X20 and data analyzed using FlowJo software (Treestar, CA). mCD1d-PBS57 tetramers were from NIH tetramer core. All batches of reagent were titrated for optimal staining of C57BL/6 splenocytes, with unloaded CD1d tetramers serving as negative controls. Changes in antibody staining panels (e.g., antibody clones, reagent batches) across experiments were minimized. Strong discrepancies in staining from different experiments were noted and the data in question were excluded from final analyses.

## Microbiota Data Analysis

Sequencing data were processed and analyzed with QIIME2 software package v. 2018.2.0 (39). The sequencing reads with low quality score (average Q < 25) were truncated to 240 bp followed by filtering using *deblur* algorithm with default settings (40). The remaining high quality reads were aligned to the reference library using *mafft* (41). Next, the aligned reads were masked to remove highly variable positions, and a phylogenetic tree was generated from the masked alignment using the FastTree method (42). Alpha and beta diversity metrics and Principal Component Analysis plots based on Jaccard distance were generated using default QIIME2 plugins (39). Taxonomy assignment was performed using *feature-classifier* method and

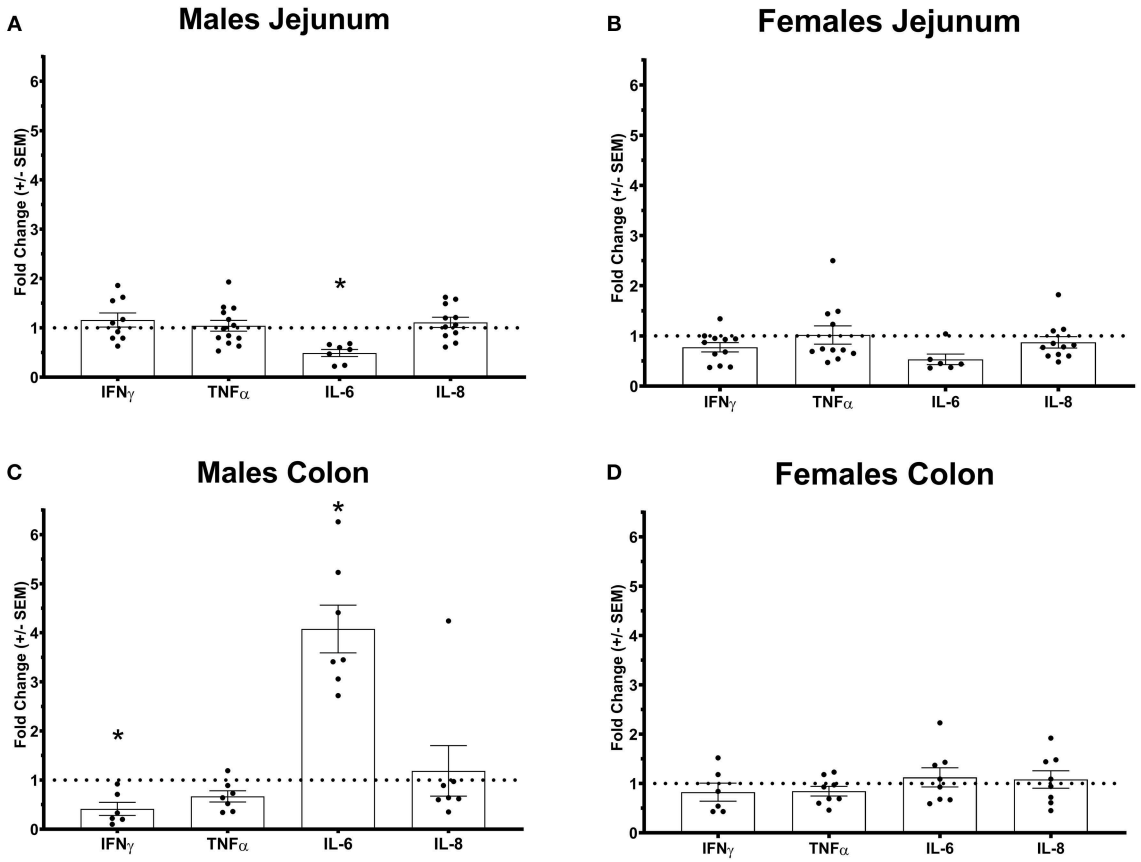



**FIGURE 2 |** Pro-inflammatory cytokines gene expression profile in Ztm intestine: **A)** Down-regulated IL-6 in the jejunum of Ztm males ($n = 13$). **B)** Pro-inflammatory cytokines profile in and g the jejunum of Ztm females ($n = 10$). **C)** Down-regulated IFN up-regulated IL-6 in the colon Ztm males ($n = 7$). **D)** Unaltered Pro-inflammatory cytokines profile in the colon of Ztm females ($n = 8$). mRNA expression was analyzed by qPCR, normalized to WT, and expressed as fold regulation. Statistical analysis was performed by the non-parametric Mann-Whitney test comparing each gene to its WT counterpart. *$p < 0.05$.

naïve Bayes classifier trained on the Greengenes 13_8 99% operational taxonomic units (OTUs). Differential abundance analysis of OTUs was performed using ANCOM (43). The Kruskal-Wallis test was used to assess the statistical significance of abundance differences, with the multiple testing corrections using Benjamini-Hochberg false discovery rate (FDR). The FDR cutoff was set at 0.05.

## Statistical Analysis

Statistical analysis was performed in Graph Pad Prism 8, all data are expressed as means ± standard error of the mean (SEM) using the non-parametric Mann-Whitney test for comparisons between 2 groups for the qPCR analysis and unpaired parametric *t*-test for comparisons between 2 groups for the flow cytometry data. $P < 0.05$ was considered statistically significant in both tests. **Figures 1**, **2** represent the expression levels of the genes analyzed. Multiple genes are shown in each graph for convenience. Please note that each gene has been compared to its WT counterpart by the method described above and represented as fold change. In **Table 2** is reported the fold change of all genes analyzed. Zonulin gene expression in the Ztm intestine has been expressed as dCt (**Table 2**) since no zonulin is present in the WT.

# RESULTS

## Ztm Mice Have an Altered TJ Gene Expression Profile in the Intestine

We have previously shown that Ztm mice exhibit a significantly increased small intestinal permeability both *in-vivo* and *in-vitro* at baseline associated to a higher susceptibility to DSS colitis, particularly in male mice, compared to WT (1). To elucidate the molecular basis of this altered permeability, we performed real time PCR of TJ and pro-inflammatory genes in different sections of the intestine (i.e., small intestine and colon) of WT and Ztm. Our results show an altered regulation of several TJs genes in Ztm compared to WT (**Figure 1**). In the small intestine of male Ztm we observed a significantly reduced expression of *Claudin* (*CLDN*)*-15*, *CLDN-5*, *JAM-3,* and *Myo1C* ($p < 0.05$; **Figure 1A**). We did not detect alterations in the expression of any of the analyzed genes in the small intestine of female Ztm (**Figure 1B**), in line with our previous finding

**TABLE 2 | (A)** List of all genes analyzed by real time PCR in the duodenum, jejunum, and colon of adult mice ($n$ = 6–12).

| A) | Duodenum | | | | Jejunum | | | | Colon | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Gene | Males | | Females | | Males | | Females | | Males | | Females | |
| | FC | *p* | FC | *p* | FC | *p* | FC | *p* | FC | *p* | FC | *p* |
| CLDN 1 | 0.02 | ns | 1.43 | ns | – | – | – | – | 2.19 | ns | 1.07 | ns |
| CLDN 2 | 0.48 | ns | 0.93 | ns | 0.99 | ns | 1.22 | ns | 1.50 | ns | 1.32 | ns |
| CLDN 3 | 0.60 | ns | 1.37 | ns | 1.56 | ns | 1.17 | ns | 0.72 | ns | 0.74 | ns |
| CLDN 4 | 0.37 | ns | 1.02 | ns | 0.90 | ns | 1.13 | ns | 0.83 | ns | 0.73 | ns |
| CLDN 5 | 0.51 | **<0.05** | 1.38 | ns | 1.22 | ns | 1.17 | ns | 1.30 | ns | 0.87 | ns |
| CLDN 7 | 0.27 | ns | 0.89 | ns | 0.92 | ns | 1.13 | ns | 0.59 | ns | 0.55 | **<0.05** |
| CLDN 8 | – | – | – | – | – | – | – | – | 1.83 | ns | 1.45 | ns |
| CLDN 10 | 2.08 | ns | 1.55 | ns | 1.20 | ns | 0.94 | ns | 1.24 | ns | 1.28 | ns |
| CLDN 12 | 0.52 | ns | 0.95 | ns | 1.00 | ns | 1.14 | ns | 5.40 | **<0.01** | 1.06 | ns |
| CLDN 15 | 0.23 | **<0.05** | 1.86 | ns | 1.65 | ns | 0.89 | ns | 0.66 | ns | 0.52 | **<0.05** |
| INFγ | bdl | – | bdl | – | 1.89 | ns | 1.37 | ns | 0.41 | **<0.05** | 0.82 | ns |
| IL6 | bdl | – | 1.27 | ns | 0.46 | **<0.05** | 0.54 | ns | 6.66 | **<0.001** | 1.13 | ns |
| IL8 | 0.41 | ns | 2.50 | ns | 1.11 | ns | 0.87 | ns | 1.19 | ns | 1.08 | ns |
| IL10 | – | – | – | – | – | – | – | – | 1.07 | ns | 1.04 | ns |
| IL17 | – | – | – | – | – | – | – | – | 0.55 | ns | bdl | - |
| JAM3 | 0.45 | **<0.05** | 0.93 | ns | 0.97 | ns | 0.99 | ns | 1.30 | ns | 0.89 | ns |
| Myo1C | 0.39 | **<0.001** | 1.09 | ns | – | – | – | – | 0.71 | ns | 0.82 | ns |
| Occldn | 0.75 | ns | 1.01 | ns | – | – | – | – | 1.17 | ns | 0.63 | ns |
| TNFα | 0.65 | ns | 2.69 | ns | 1.04 | ns | 1.02 | ns | 0.67 | ns | 0.85 | ns |
| TRIC | 0.53 | ns | 1.67 | ns | – | – | – | – | 1.76 | ns | 1.00 | ns |
| ZO1 | 1.06 | ns | 1.47 | ns | 1.10 | ns | 0.76 | ns | 1.20 | ns | 0.78 | ns |
| ZO2 | 0.47 | ns | 1.26 | ns | 0.85 | ns | 1.82 | ns | 0.99 | ns | 0.67 | **<0.05** |
| ZO3 | – | – | – | – | 1.09 | ns | 0.99 | ns | 1.45 | ns | 1.16 | ns |

| B) | Duodenum | | | | Jejunum | | | | Colon | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Gene | Males | | Females | | Males | | Females | | Males | | Females | |
| | dCt | st.dev | dCt | st.dev | dCt | st.dev | dCt | st.dev | dCt | st.dev | dCt | st.dev |
| zonulin | 17.2 | 1.25 | 16.3 | 0.70 | 14.2 | 2.37 | 15.5 | 1.13 | 14.5 | 1.02 | 15.8 | 1.75 |

*Expressed as Fold Change relative to same gene expression in the WT (FC, bdl = below detection level), statistics calculated with the non-parametric Mann-Whitney test (ns = non-significant, significant p-values provided in bold) comparing gene expression from the Ztm to its WT counterpart.* ***B)*** *Zonulin expression in the Ztm mice (males and females) duodenum, jejunum, and colon. Data are expressed as dCt (Ct normalized to its own 18S expression); standard deviation (st.dev) is also reported.*

of higher susceptibility to DSS-induced increased morbidity and mortality in male mice (1). Conversely, while in the colon of male Ztm only *CLDN-12* gene expression appeared significantly ($p < 0.05$) increased (**Figure 1C**), in the female colon *CLDN-15*, *CLDN-7,* and *ZO-2* were significantly ($p < 0.05$) reduced (**Figure 1D**). When looking at the expression of pro-inflammatory genes in the small intestine and colon, we detected a significant downregulation of IL-6 expression in the jejunum ($p < 0.05$) and its upregulation in the colon ($p < 0.05$) in Ztm males compared to WT males (**Figures 2A,C**). IFNγ was also significantly downregulated in the Ztm male colon ($p < 0.05$). Females exhibited a slightly decreased IL-6 expression in the jejunum (**Figure 2B**), while no changes were observed in the colon (**Figure 2D**) in any of the inflammatory cytokines analyzed. Please see **Table 2** for the fold change of all genes analyzed.

## Ztm Shows Gut Dysbiosis Skewed Toward Inflammation

To investigate whether constitutively increased gut permeability affects the host gut microbial composition, we analyzed the microbiome of 4–8 weeks old Ztm and WT mice. The gut microbiota composition in 4–8 week mice is established (44) but still susceptible to fluctuations by external factors (i.e., diet). Hence, this timeframe represents a window of opportunity to influence changes in the immune system and microbiome composition, and as such to investigate the impact of gut permeability. Principle component analysis (PCA) showed that Ztm microbiota clustered distinct from WT gut microbiota (**Figure 3A**). Analysis at phylum level showed that microbiota in Ztm was characterized by a marked reduced abundance of Verrucomicrobia (0.2%) compared to WT (5.2%), and the almost complete absence of Cyanobacteria

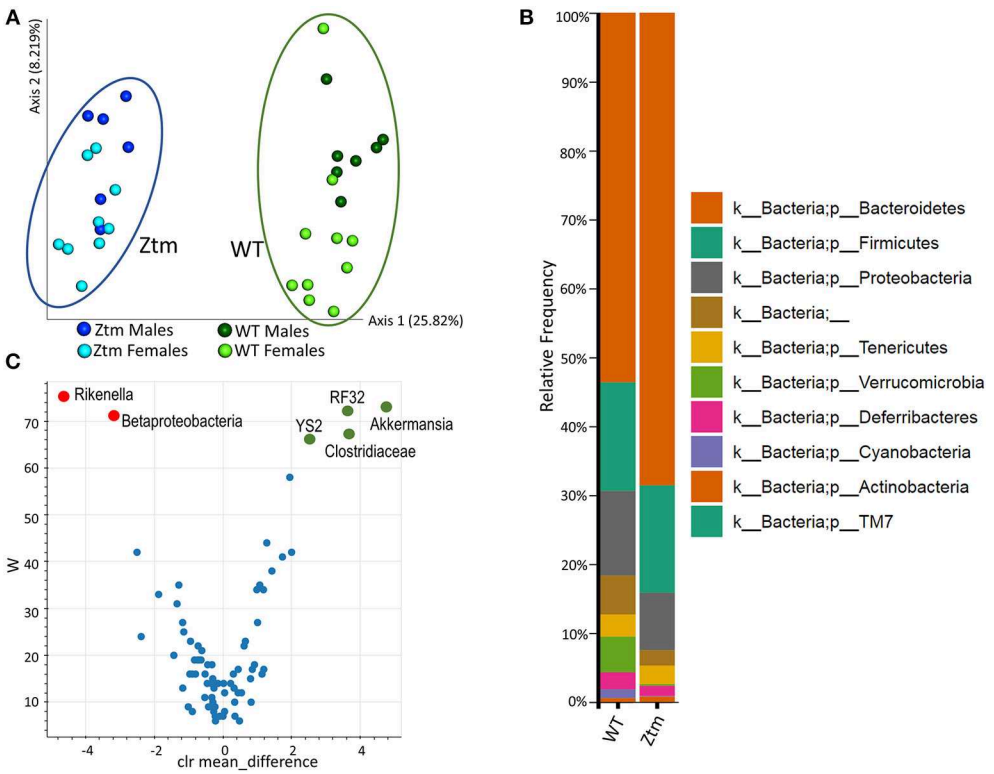



**FIGURE 3 |** Baseline dysbiosis in Ztm gut microbiota. Stools from 15 WT mice (WT males $n = 6$, WT females $n = 9$) and 17 Ztm (Ztm males $n = 7$, Ztm females $n = 10$) were analyzed. **(A)** Principal Component Analysis (PCA) of Jaccard distances for Ztm ($n = 17$) and WT ($n = 15$) mice show distinct clustering between Ztm and WT. No significant differences between males and females within each group were detected. **(B)** Microbiota composition at phylum level. WT ($n = 15$), Ztm ($n = 17$). Males and females within each group were analyzed together. Absence of Verrucomicrobia and Cyanobacteria in the Ztm is evident. **(C)** Volcano plot showing the statistical differences between Ztm and WT at genus level. In green overrepresented genus in WT mice, in red overrepresented genus in Ztm. Differential abundance analysis of OTUs was performed using ANCOM. Statistical significance was tested by the Kruskal-Wallis of abundance differences, with the multiple testing corrections using Benjamini-Hochberg false discovery rate (FDR) set at 0.05.

(Ztm = 0.1% WT = 1.3%) (**Figure 3B**). At the class level, Clostridiacea were also significantly reduced in Ztm compared to WT. A deeper analysis confirmed a significantly reduced abundance of the orders RF32 (Alphaproteobacteria) and YS2 (Cyanobacteria) and the genus *Akkermansia* in Ztm compared to WT mice. Conversely, Betaproteobacteria and the genus *Rikenella* were significantly more abundant in the gut of Ztm compared to WT (**Figure 3C**), pointing to a pro-inflammatory microbiota composition in Ztm.

To understand whether changes in the Ztm microbiota affected the development of the immune system (described below), we also analyzed the microbiota in Ztm mice that had received at birth bedding from a cage housing WT mouse ($Ztm_{bWT}$, Ztm receiving bedding from WT) with the goal of normalizing microbiota between WT and Ztm mice. The Shannon Diversity Index (indication of the microbiota's complexity within the group) showed a significant difference between WT and Ztm ($q = 0.007$) but not between WT and the bedding-transferred $Ztm_{bWT}$ (**Figure 4A**). Consistently with the Shannon Diversity Index, the PCA plot showed that Ztm microbial composition segregated from the WT cluster, while $Ztm_{bWT}$ microbiota clustered with the WT microbiota (**Figure 4B**), indicating the successful engraftment of WT microbiota in the $Ztm_{bWT}$. Verrucomicrobia and Cyanobacteria, that were severely reduced in Ztm, were engrafted in $Ztm_{bWT}$ where we observed the successful colonization of *Akkermansia* (**Figure 4C**) and, normalization of *Rikenella* abundance (data not shown).

## Immune Phenotype of Ztm Mice

Changes in intestinal barrier function may cause bacterial products including endotoxins and other molecules to leak through the gut barrier and disseminate in both the lamina propria and systemically, potentially instigating changes in the immune phenotype. To determine if increased intestinal permeability and/or an altered microbiota influence lymphocyte distribution in the gut and in secondary lymphoid organs, we compared WT mice with Ztm and $Ztm_{bWT}$, as described in the above paragraph (**Figures 4B,C**).

Overall, Ztm and $Ztm_{bWT}$ mice had similar distribution of major immune cell subsets compared to WT mice (**Figures 5**, **6**). Flow cytometry analysis of lymphocytes in the colon and small intestine revealed no significant differences in the frequency of $IL7R^+$ conventional $\gamma\delta$-$TCR^+$ cells, $CD4^+CD25^+$ cells, conventional $CD4^+CD25^{neg}$ and $CD8^+$ T cells, and $CD19^+$ B cells (**Figures 5A,C**). However, we noted an overall statistically

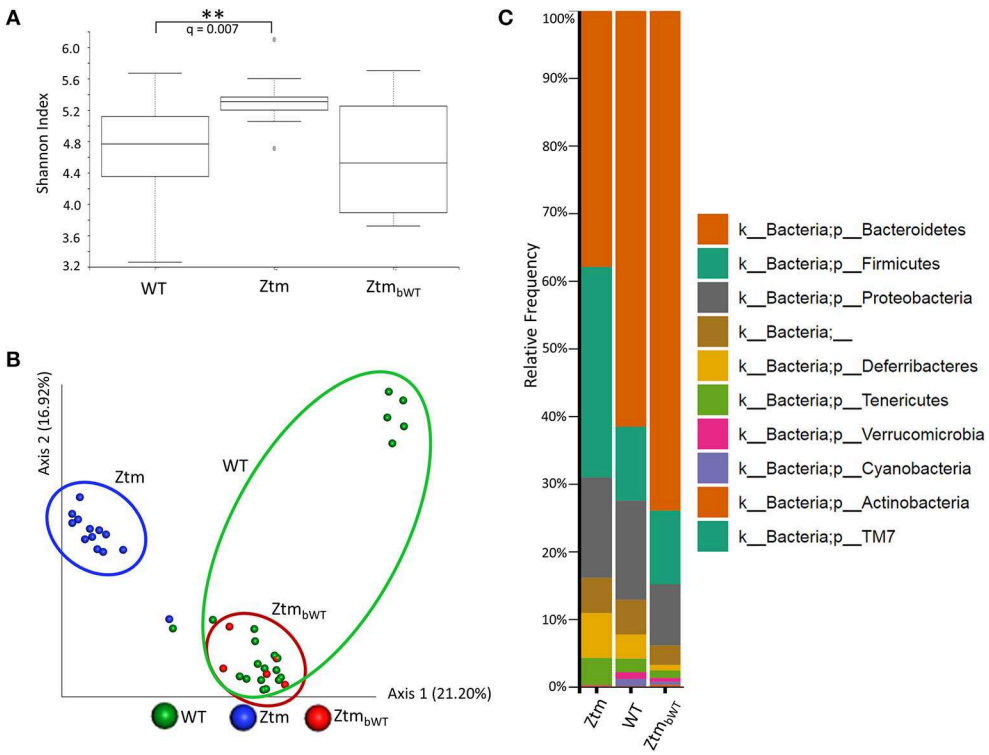
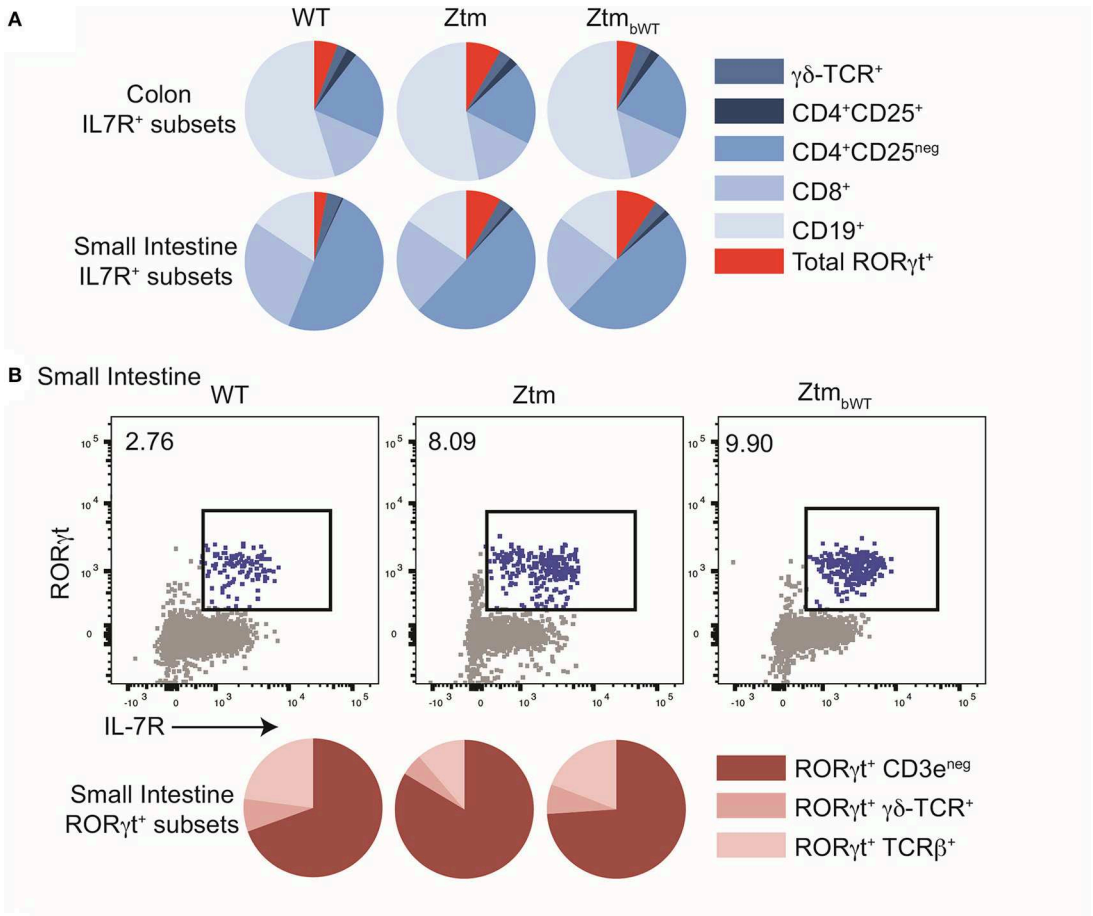



**FIGURE 4 |** Microbiota analysis of Ztm mice with and without bedding transfer. Stools recovered from the colon of both male and female pups were analyzed as described in the material and methods. Stools were collected at weaning. Ztm$_{bWT}$ represent pups that were subjected to bedding transfer from WT. Bedding transfer was carried out twice a week since birth. **(A)** Shannon Index, expression of alpha diversity (variation/complexity of the microbiome within the group). WT $n = 21$, Ztm $n = 13$, Ztm$_{bWT}$ $n = 5$. WT and Ztm have a significantly different ($q = 0.007$) microbiota diversity, whereas no statistical difference is observed between WT and Ztm$_{bWT}$. **(B)** Microbiota composition at phylum level. Successful engraftment of Verrucomicrobia and Cyanobacteria from WT to Ztm$_{bWT}$ following bedding transfer. **(C)** Principal component Analysis (PCA) of Jaccard distances. Ztm$_{bWT}$ (red) cluster with WT (green) and separately from Ztm (in blue). Differential abundance analysis of OTUs was performed using ANCOM. Statistical significance was tested by the Kruskal-Wallis of abundance differences, with the multiple testing corrections using Benjamini-Hochberg false discovery rate (FDR) set at 0.05. $^{**}q < 0.01$.

significant increase in the frequency of IL7R$^{+}$RORγt$^{+}$ cells in the small intestine but not in the colon of both Ztm and Ztm$_{bWT}$ mice (**Figure 5A**, red slice; **Figures 5B,C**). This increase was most likely due to elevated IL7R$^{+}$CD3$^{neg}$RORγt$^{+}$ cells that may be innate lymphoid cells (ILCs) (**Figure 5B**, bottom row; **Figure 5C**). Splenic cellularity was also comparable across all groups (**Figure 6A**) as was the distribution of CD4$^{+}$ and CD8$^{+}$ T cells (**Figure 6B**). There was no significant difference in the frequency of CD4$^{+}$CD25$^{+}$ cells in the spleen of Ztm and Ztm$_{bWT}$ mice compared to WT mice (**Figure 6C**). However, the frequency and numbers of CD4$^{+}$CD25$^{+}$ cells expressing RORγt$^{+}$ was significantly increased in Ztm$_{bWT}$ mice (**Figure 6C**). We also noted an increased frequency and numbers of CD4$^{+}$RORγt$^{+}$ Th17 cells in both Ztm and Ztm$_{bWT}$ mice (**Figure 6C**). Further analyses revealed an interesting distribution of transcription factor RORγt-expressing innate-like T cells. While the frequency and numbers of mCD1d-PBS57 tetramer$^{+}$ invariant NKT (iNKT) cells was decreased, the frequency of RORγt expressing subset of iNKT cells (NKT17 cells) increased in both Ztm and Ztm$_{bWT}$ compared to WT mice (**Figure 6D**). Similarly, there was a modest increase in the frequency of γδ T cells (γδ TCR$^{+}$ innate-like T cells) that expressed RORγt (**Figure 6E**). NKT17 cells and γδ-17 T cells are pro-inflammatory innate-like T cell subsets that produce IL-17 and have been implicated in the pathogenesis of various autoimmune diseases including type 1 diabetes and celiac disease (45, 46). Finally, we determined the frequency and phenotype of conventional CD11c$^{hi}$ dendritic cells (CDC) and CD11c$^{lo}$SiglecH$^{+}$ plasmacytoid dendritic cells (PDC) and noted a small but significant increase in the frequency of splenic PDCs but not CDCs in both Ztm and Ztm$_{bWT}$ compared to WT mice (**Figure 6F**). These data suggest that altered gut permeability subtly increased the baseline frequency of IL-17 producing T cells in mucosal tissue and in secondary lymphoid organs of Ztm mice in the absence of overt inflammation and disease. The fact that the engraftment of WT microbiota did not affect the immune phenotype in Ztm$_{bWT}$ (no significant differences were detected between Ztm and Ztm$_{bWT}$), suggests that the increased trafficking of microbial products through an impaired gut barrier rather than the function of an imbalanced microbiota primarily imprints the development of the immune system in the Ztm.




C

| | Mean± SD | | | P value | | |
|---|---|---|---|---|---|---|
| **COLON** | **WT (n=6)** | **Ztm (n=4)** | **Ztm$_{bWT}$ (n=6)** | **WT vs Ztm** | **WT vs Ztm$_{bWT}$** | **Ztm vs Ztm$_{bWT}$** |
| Total RORγt$^+$ | 4.82±1.75 | 7.135±0.54 | 4.263±0.63 | 0.1815 | 0.6317 | *0.0138* |
| γδ-TCR$^+$ | 2.14±0.27 | 2.435±1.39 | 3.10±0.98 | 0.7302 | 0.1792 | 0.5650 |
| CD4$^+$CD25$^+$ | 2.07±0.60 | 1.79±0.18 | 1.86±0.56 | 0.5779 | 0.6727 | 0.8808 |
| CD4$^+$CD25$^{neg}$ | 18.53±1.36 | 16.95±6.57 | 18.73±6.20 | 0.6908 | 0.9592 | 0.7779 |
| CD8$^+$ | 11.93±1.80 | 12.6±0.00 | 13.12±5.91 | 0.6306 | 0.7550 | 0.9207 |
| CD19$^+$ | 47.9±2.52 | 45.95±4.31 | 46.96±9.87 | 0.5559 | 0.8816 | 0.9033 |
| **SI** | **WT (n=4)** | **Ztm (n=4)** | **Ztm$_{bWT}$ (n=4)** | **WT vs Ztm** | **WT vs Ztm$_{bWT}$** | **Ztm vs Ztm$_{bWT}$** |
| Total RORγt$^+$ | 2.725±0.05 | 7.335±1.06 | 9.095±1.13 | *0.0258* | *0.0156* | 0.2518 |
| γδ-TCR$^+$ | 3.165±1.93 | 2.625±1.27 | 2.895±0.40 | 0.7729 | 0.4438 | 0.4974 |
| CD4$^+$CD25$^+$ | 0.32±0.12 | 0.695±0.16 | 1.255±0.79 | 0.0853 | 0.2438 | 0.4294 |
| CD4$^+$CD25$^{neg}$ | 44±15.41 | 44.75±10.11 | 46±5.37 | 0.9594 | 0.8784 | 0.8915 |
| CD8$^+$ | 25.3±6.64 | 20.05±1.90 | 21.8±3.25 | 0.3953 | 0.5724 | 0.5791 |
| CD19$^+$ | 14.05±4.59 | 13.8±6.64 | 14.05±3.18 | 0.9691 | 1 | 0.9661 |
| **SI** | **WT (n=4)** | **Ztm (n=4)** | **Ztm$_{bWT}$ (n=4)** | **WT vs Ztm** | **WT vs Ztm$_{bWT}$** | **Ztm vs Ztm$_{bWT}$** |
| RORγt$^+$ CD3e$^{neg}$ | 69.45±9.82 | 83.6±2.26 | 76.15±5.58 | 0.1857 | 0.4902 | 0.2226 |
| RORγt$^+$ γδ-TCR$^+$ | 7.635±0.70 | 5.155±1.76 | 7.165±3.33 | 0.2054 | 0.8632 | 0.5293 |
| RORγt$^+$ TCRβ$^+$ | 22.9±10.60 | 11.25±4.03 | 19.7±6.92 | 0.2836 | 0.7551 | 0.2745 |

**FIGURE 5 |** Distribution of immune cell subsets in colon and SI of WT and Ztm/Ztm$_{bWT}$ mice. Lamina propria and epithelial compartment lymphocytes from the colon (WT $n = 6$; Ztm $n = 4$; Ztm$_{bWT}$ $n = 6$) and small intestine (SI) (WT $n = 4$; Ztm $n = 4$; Ztm$_{bWT}$ $n = 4$) of 35 days old mice were analyzed by flow cytometry. **(A)** Pie chart representation of distribution of indicated IL7R$^+$ subsets in colon (Top row) and small intestine (SI) (Bottom Row). **(B)** (Top row) Representative flow cytometry dot plots show expression of IL-7R (x-axis) and RORγt (y-axis) in lymphocytes from the SI. (Bottom Row) Pie chart representation of distribution of indicated IL7R$^+$RORγt$^+$ subsets in SI. **(C)** Summary of distribution of immune cell subsets in colon and SI. Mean, SD, and *p*-values are indicated. Unpaired parametric *t*-test was used for comparisons between the 2 groups with significance set at $p < 0.05$.

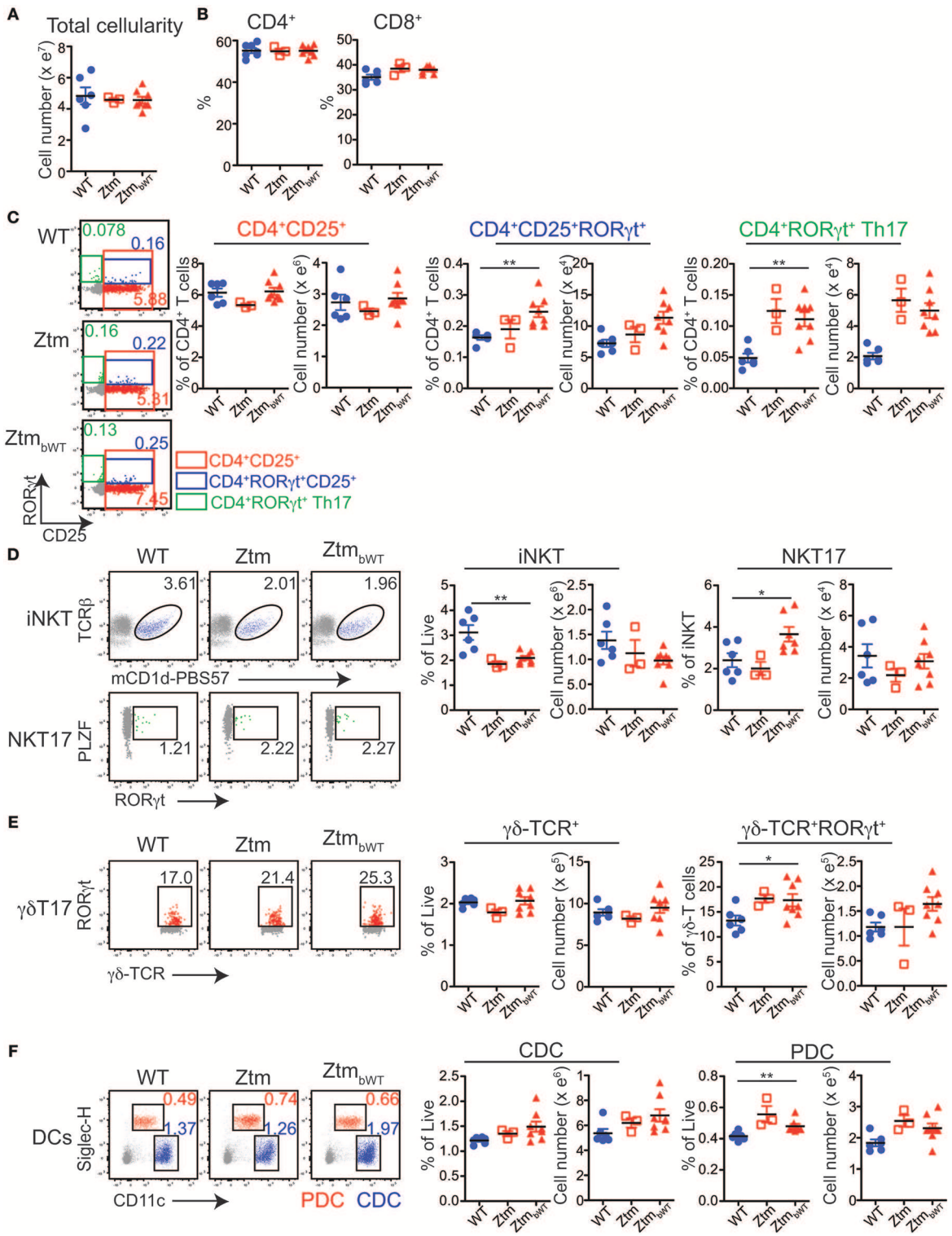



**FIGURE 6 |** Distribution of immune cell subsets in spleen of WT and Ztm/ZtmbWT mice: single cell preparations from the spleen of 28–35 days old WT ($n = 6$), Ztm ($n = 3$), and ZtmbWT ($n = 8$) mice analyzed by flow cytometry. **(A)** Total splenic cellularity. **(B)** Vertical scatter plots show frequency of IL7R+TCRβ+ CD4+ and CD8+ T cells. **(C)** (Left) Representative flow cytometry dot plots show expression of CD25 (x-axis) and RORγt (y-axis) on CD4+ T cells. (Right) Vertical scatter plots show frequency and cell numbers of CD4+CD25+ cells, CD4+CD25+RORγt+ cells, and CD4+RORγt+ Th17 cells. **(D)** (Left) Representative flow cytometry dot plots show expression of (Top row) mCD1d-PBS57 tetramer (x-axis) and TCRβ (y-axis) on IL7R+ cells and (Bottom row) RORγt (x-axis) and PLZF (y-axis) on mCD1d-PBS57 tetramer+ TCRβ+ iNKT cells. (Right) Vertical scatter plots show frequency and cell numbers of iNKT cells and RORγt+ NKT17 cells.

*(Continued)*

**FIGURE 6 | (E)** (Left) Representative flow cytometry dot plots show expression of δ-TCR (x-axis) and RORγt (y-axis) on IL7R+ cells. (Right) Vertical scatter plots show frequency and cell numbers of γδ T cells and RORγt+ γδ-T17 cells. **(F)** (Left) Representative flow cytometry dot plots show expression of CD11c (x-axis) and Siglec-H (y-axis) on live cells (Red cells: CD11cloSiglec-H+ plasmacytoid dendritic cells or PDCs; Blue cells: CD11chiSiglec-Hneg conventional dendritic cells or CDCs). (Right) Vertical scatter plots show frequency and cell numbers of CDCs and PDCs. Data in **(A–F)** are representative of 3 independent experiments. Error bars are SEMs. Unpaired parametric *t*-test was used for comparisons between 2 groups with significance set at $^{*}p < 0.05$ and $^{**}p < 0.01$.

## DISCUSSION

We have previously shown that Ztm express zonulin, exhibit constitutive increased gut permeability *in-vivo* and are more susceptible to DSS induced colitis (1). The increased morbidity and mortality affected predominantly Ztm males and is supported by our molecular data showing an altered TJ gene expression profile in the duodenum of male Ztm, with the significant decreased expression levels of several genes suggesting an impairment of small intestinal barrier function. Although, its function of gut barrier- or pore-forming claudin still has not been well-characterized, *CLDN-12* significant increase in the colon of Ztm males could represent a compensatory mechanism to re-establish barrier function and/or electrolytes absorption (47). Of interest was the significantly reduced expression of *Myosin-1C* gene, because of its proven involvement in the zonulin pathway and its disassociation from ZO-1, leading to TJ disassembly and increased intestinal permeability (48). It's important to point out that although changes in gene expression not necessarily correspond to changes in protein expression, the fact that multiple TJ genes are affected, strongly suggests an impaired gut barrier. Also, intriguing are our findings of *IL6* gene expression upregulation in the colon of Ztm males (and not females), suggesting that the increased permeability and trafficking of microbial products in the duodenum generates a pre-inflammatory imprinting in the immune system predisposing Ztm to develop colitis when exposed to an external inflammatory stimulus (1). Increased colonic expression of *IL-6* might also be the consequence of a constitutive increased passage of microbiota species into the colonic lamina propria.

Gut permeability defects in the Ztm seem to have a critical effect on both the gut microbiota composition and the immune system. The analysis of stool microbiota showed dysbiosis in the Ztm gut. Striking was the marked reduced presence of *Akkermansia* (sp. *muciniphila*) vs. a significantly more abundant *Rikenella* genus in the Ztm gut. *A. muciniphila* contribute to enterocyte monolayer integrity and the overall strengthening of the gut epithelial barrier (49–51) and low levels have been associated to many human diseases (52–54) and have been reported as intestinal microbiota feature of both genetic and diet-induced obese and diabetic mice (55, 56).

While *A. muciniphila* are indicators of a gut healthy status, on the other end high abundance of *Rikenella* has been found in obesity and diabetes (57, 58) and hence associated with a status of low chronic non-infective inflammation. Overall, the low abundance of *A. muciniphila* and the enriched *Rikenella* presence in the gut of Ztm, suggest that the Ztm gut microbiota is skewed vs. a more maladaptive and pathogenic profile.

It is well-known that commensal bacteria are involved in the development of the immune system and the establishment of oral tolerance (59–61). The balance between pro-inflammatory IL-17$^{+}$ helper Th17 cells and the anti-inflammatory Foxp3$^{+}$ regulatory T cells (Treg) in the mucosa (62, 63) plays a key role in the development of chronic inflammatory diseases (64, 65). Microbial products have been shown to regulate the balance between Treg cells and IL-17 producing RORγt+ Th17 cells (59, 66). It has been shown that specific components of the commensal microbiota induce Th17 cells in the lamina propria of the small intestine and that antibiotic treatment prevented such differentiation (67).

In the Ztm, despite an altered baseline trafficking of microbial products, major immune cell subset distribution appears unaffected. However, the modest increase in pro-inflammatory IL-17 producing innate and innate-like cells in the intestine as well as in gut distal secondary lymphoid sites indicate that the threshold of immune reactivity in these mice might be altered, rendering them more susceptible to lose tolerance to non-self-antigens with subsequent onset of inflammation. In fact, when challenged with DSS, Ztm develop a more severe colitis and exhibit a significantly increased morbidity and mortality that is associated to elevated zonulin gene expression (1).

Immune profile studies following bedding transfer with the successful colonization of WT microbiota into Ztm mice, show no changes indicating that the Ztm immunophenotype is largely unaffected by the microbial composition and suggest that increased intestinal trafficking of microbial products primarily drives changes in the host immune system. In other words, the reduced barrier function in Ztm supersedes an imbalanced microbiota for the enhanced differentiation of RORγt$^{+}$ and IL-17 producing subsets.

Our data indicate that zonulin-dependent increased gut permeability and microbial products trafficking orchestrate the development of the immune system and microbiota composition toward a pro-inflammatory status in the Ztm, with increased IL-17 producing cells and the lack of gut protective microbial species (i.e., *Akkermansia*) vs. an over-representation of pro-inflammatory strains (i.e., *Rikenella*). This low-grade pro-inflammatory-skewed status renders Ztm critically susceptible to develop overt inflammation leading to disease in the presence of exogenous stimuli (i.e., DSS) (1). Taken together, these data suggest that increased trafficking of microbial products in the small intestine might cause break of tolerance and onset of inflammation either locally or at distance. Indeed, the DSS experiments in Ztm (1) support our hypothesis of a key role of small intestinal microbial products trafficking in triggering inflammation at a distal site (colon). The observation that the onset of colitis with loss of the stem cell niche and its regenerative capacity can be rescued by blocking the zonulin pathway suggests a pivotal role of the mutually-influenced increased small intestinal trafficking of microbial products, a pro-inflammatory

immune system, and gut dysbiosis shown in this paper, rather than merely a local effect of DSS once it reaches the colon. This is also in line with data generated in the IL-10 KO mouse, another model of colitis in which increased small intestinal permeability was shown to have a pathogenetic role in leading to colitis (8).

Although, further investigations are needed, these observations support our hypothesis and a new paradigm in which the presence in Ztm of an increased gut permeability, an altered immune system and dysbiosis of the gut microbiota are not sufficient to initiate the chain of events leading to overt inflammation. In the absence of an exogenous stimulus, Ztm remain healthy. It is the action of an external trigger to drive Ztm over the threshold where they develop disease. The same chain of events can be hypothesized in CID, in which in genetically predisposed individuals, exogenous factors that trigger epithelial barrier dysfunction and activation of the immune system could selectively promote the growth of microbial species in the gut that in turn worsen changes in the intestinal tissue resulting in breaking mucosal tolerance and onset of chronic inflammation, locally, and/or systemically and clinical symptoms.

In conclusion, this study provides important insights on the understanding of the role of gut permeability in the initiation of inflammatory diseases, offering the basis for innovative and yet unexplored therapeutic approaches for the management of these debilitating chronic diseases aimed at re-establishing the intestinal barrier function by downregulating the zonulin pathway.

## DATA AVAILABILITY STATEMENT

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

## ETHICS STATEMENT

This study was carried out in accordance with the recommendations and guidelines of the Institutional Animal Care and Use Committee at the MGH. The protocol was approved by the Institutional Animal Care and Use Committee at the MGH (2013N000013).

## AUTHOR CONTRIBUTIONS

MF, AM-R, NJ, RS, and AF conceived and designed the experiments. AM-R, ME, GS, JL, and MC carried out the experiments. MF, AM-R, NJ, RS, MC, and AF contributed to the interpretation of the results. AM-R, MF, NJ, and AF wrote the manuscript. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

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# Mucosal Exposure to Cigarette Components Induces Intestinal Inflammation and Alters Antimicrobial Response in Mice

*Loni Berkowitz[1,2], Catalina Pardo-Roa[2], Geraldyne A. Salazar[2], Francisco Salazar-Echegarai[2], José P. Miranda[3], Gigliola Ramírez[1], José L. Chávez[1,2], Alexis M. Kalergis[2,4], Susan M. Bueno[2]* and Manuel Álvarez-Lobos[1]**

[1] Departamento de Gastroenterología, Escuela de Medicina, Pontificia Universidad Católica de Chile, Santiago, Chile, [2] Departamento de Genética Molecular y Microbiología, Facultad de Ciencias Biológicas, Millennium Institute on Immunology and Immunotherapy, Pontificia Universidad Católica de Chile, Santiago, Chile, [3] Departamento de Nutrición, Diabetes y Metabolismo, Escuela de Medicina, Pontificia Universidad Católica de Chile, Santiago, Chile, [4] Departamento de Endocrinología, Facultad de Medicina, Pontificia Universidad Católica de Chile, Santiago, Chile

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**Edited by:**
Julien Diana,
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Technical University of Munich, Germany

***Correspondence:**
Susan M. Bueno
sbueno@bio.puc.cl
Manuel Álvarez-Lobos
manalvarezl@gmail.com

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The main environmental risk factor associated with the development of Crohn's disease (CD) is cigarette smoking. Although the mechanism is still unknown, some studies have shown that cigarette exposure affects the intestinal barrier of the small bowel. Among the factors that may be involved in this process are Paneth cells. These specialized epithelial cells are located into the small intestine, and they are able to secrete antimicrobial peptides, having an essential role in the control of the growth of microorganisms. Alterations in its function are associated with inflammatory processes, such as CD. To study how cigarette components impact ileum homeostasis and Paneth cells integrity, we used intragastric administration of cigarette smoke condensate (CSC) in mice. Our results showed that inflammation was triggered after mucosal exposure of CSC, which induced particular alterations in Paneth cells granules, antimicrobial peptide production, and a reduction of bactericidal capacity. In fact, exposure to CSC generated an imbalance in the fecal bacterial population and increased the susceptibility of mice to develop ileal damage in response to bacterial infection. Moreover, our results obtained in mice unable to produce interleukin 10 (IL-$10^{-/-}$ mice) suggest that CSC treatment can induce a symptomatic enterocolitis with a pathological inflammation in genetically susceptible individuals.

**Keywords: cigarette smoking, Paneth cells, Crohn's disease, inflammatory bowel disease, antimicrobial peptides, microbiota**

## INTRODUCTION

Cigarette smoking has been associated as the major risk factor for several inflammatory gastrointestinal disorders, among them Crohn's disease (1). The impact of the toxic components of cigarette in the respiratory and vascular systems has been widely studied. However, how smoking affects the function of the gastrointestinal tract have not yet been clarified. Mucosal damage, alterations of mucosal immune response, and changes in gut irrigation are some of the proposed mechanisms that might explain the role of cigarette smoking in this disorder (2).

Accordingly, cigarette smoking is the main environmental risk factor associated with the development and progression of Crohn's disease (CD) (3). Although the pathogenesis of this disease is not completely understood, the evidence suggests that it would be the result of a complex interaction between genetic alterations, innate immune response, imbalance of intestinal microbiota, and environmental factors (4). Specifically, it has been postulated that after a certain stimulus, genetically-susceptible individuals develop an inadequate mucosal immune response against their intestinal microbiota. This response can lead to a pathological inflammation of the digestive tract, mainly involving the terminal ileum and the colon (5).

Interestingly, several studies show an association between cigarette smoking and the development of inflammation, specifically in the ileum, suggesting that the impact may be site-specific (6, 7). The particulate phase of cigarette smoke is composed mainly by low molecular weight components, such as nicotine, nitrosamines, polycyclic aromatic compounds, and heavy metals. The particulate phase can indeed be absorbed by epithelial cells of the body surfaces, such as the oronasal mucous membranes, skin, alveoli, and particularly through the gastrointestinal tract (1). As a matter of fact, high levels of nicotine, possibly the most studied particulate compound of cigarette, has been found in gastric fluids. These amount of nicotine could be 10 times higher than the levels found in arterial blood, and 80 times higher than the concentration observed in venous blood (8). Thus, the intestinal effects of cigarette smoking may be directly related to the amounts of particulate compounds swallowed that reach the intestine and could cause tissue damage.

Among the factors that may be involved in intestinal inflammation caused by cigarette smoking are Paneth cells (PCs). PCs are specialized epithelial cells of the small intestine that contain multiple secretory granules, filled with antimicrobial peptides and trophic factors, which are essential for the control microbial growth and maintaining intestinal homeostasis (9). Alterations in their function are associated with an imbalance of the normal microbiota, gastrointestinal infections, and inflammatory processes (10). Moreover, Paneth cells have been postulated as a site of origin for intestinal inflammation in ileal Crohn's disease, the location most commonly affected by the disease (11). However, the effect of cigarette smoking on Paneth cells has not been elucidated yet.

The aim of this study was to evaluate the impact of cigarette smoke particulate matter on the intestinal homeostasis and its bactericidal response, particularly on Paneth cells, using cigarette smoke condensate (CSC) in mice.

# MATERIALS AND METHODS

## Ethics Statement

All the experiments that used mice were conducted in agreement with the international ethical standards and following the local animal protection guidelines. Experimental protocol No. 170329009 were reviewed and approved by the Scientific Ethical Committee for Research Safety and the Scientific Ethical Committee for Animal and Environment Care of the Pontificia Universidad Católica de Chile.

## Mice Strains

Seven-to eight-week-old C57BL/6 wild-type male mice (WT) and C57BL/6 IL-$10^{-/-}$ mice (IL-$10^{-/-}$) were originally purchased from Jackson Laboratories (Bar Harbor, ME, USA) and maintained in the pathogen-free animal facility at the Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile. General condition and physiological score were evaluated and recorded every 2 days using a dedicated supervision protocol (**Supplementary Table 1**).

## Mouse Model of CSC Exposure

Mice received 200 μg CSC, 400 μg CSC or PBS by intraperitoneal or intragastric administration, 3 times a week for 2 weeks. CSC (Murty Pharmaceuticals, Lexington, KY, USA) is a commercial formula that contains total particulate matter prepared from a standard research cigarette (3R4F: University of Kentucky, KY, USA) and dissolved in DMSO. In each case (CSC treatment or vehicle), the total volume was 200 uL with a final concentration of 5% DMSO. According to the literature, these doses correspond to 8 and 16 cigarettes in a subject of average weight, based on the concentration of nicotine and the metabolic rate of rodents (12). For intragastric administration, mice were anesthetized using isoflurane (anesthesia was initiated with 5%, and maintained with 2% isoflurane). The intragastric administration (intragastric gavage) was done using a flexible cannula attached to a syringe and used to deliver the treatment directly into the stomach. One day after the last administration, mice were euthanized for organ recovery.

## Cotinine Levels

Blood samples were collected from the submandibular region 45 min and 24 h after the last administration of CSC. Total blood was maintained during 30 min at 37°C and then it was centrifugate at 2,000 rpm for 10 min to collect serum. Serum was stored at −80°C until use. Cotinine (nicotine metabolite) serum levels were determined by ELISA (Abnova, catalog no. KA2264), according to the manufacturer's recommendations.

## Tissue Treatment for Histological Procedures

Distal Ileum and colon were dissected and perfused with 2 mL sterile phosphate-buffered saline (PBS, pH 7.4), and then fixed in 10% formalin solution. Tissues were embedded in paraffin (Tissue Processor Leica ASP300), and transversal sections of 5 mm were adhered to positively charged glass slides, deparaffinized, and used for Hemathoxylin-eosin (H&E), alcian blue-PAS (AB-PAS), and immunofluorescent (IF) staining.

## Histopathology and Morphometrics Analysis

For hispathology review, sections were stained with H&E or AB-PAS (pH 2.5) by routine methods. The histological score was performed from 0 (non-inflamed) to 12 (highly inflamed), on transversal sections of terminal ileum, proximal colon and distal colon, according to Schultz et al. (13). Also, morphology and morphometrics were assessed in ileum sections stained with AB-PAS. At least 15 crypts and villi were assessed per section

by a single blinded observer, analyzing 3–5 section per mouse containing full villi. Villus-crypt unit (VCU) length and the number of goblet cells per VCU were measured or counted at 10× magnification. The number of Paneth cells per crypt and granules organization was examined at 100× magnification under immersion oil. Then, Paneth cells were classified according to the organization of their granules as normal (D0), disorganized (D1), depleted (D2), and diffuse (D3) (11). Also, the number of intermediate cells (between Goblet and Paneth cells) was assessed and classified according to their location as: adjacent to PCs (P1), in the middle zone of proliferation (P2), or in upper positions over differentiated Goblet Cells (P3).

## Immunofluorescent Staining

After deparaffinization, antigen demasking was performed by boiling the tissue samples in 0.01 M Sodium Citrate Buffer (pH 6.0)—Tween 0.05% for 45 min. After cooling down the samples to RT, slides were washed 2 times with distilled $H_2O$ for 5 min each, and then 3 times in TBS-Tween 0.1% for 5 min each. Sections were blocked with 50 mL of TBS-Tween 0.1%—FBS 5%—BSA 10%, for 60 min at RT in a humidified chamber. Then, the slides were incubated overnight at 4°C with primary antibody against Lysozyme (goat anti- Lysozyme C 1:200, Santa Cruz catalog no. sc-27958) or primary antibody against RegIIIγ (rabbit anti- RegIIIγ 1:200, Abcam catalog no. ab198216). Fluorochrome-conjugated secondary antibodies, Donkey anti-goat IgG (H+L) Cross-Adsorbed Alexa Fluor 488 (Invitrogen, catalog no. A11055, diluted 1:200) and Donkey anti-rabbit IgG (H+L) Alexa Fluor 594 (Abcam, catalog no. ab150080, diluted 1:400), were used to incubated the slides for 1 h at RT in a humidified chamber. Sections were mounted using Vectashield Antifade Mounting Medium with DAPI (Vector Laboratories, catalog no. H-1200), and visualized using an epifluorescence microscope (Nikon Eclipse E200).

## Transmission Electron Microscope

Sections of the distal ileum (1 mm) were fixed for 16 h by immersion in 2.5% glutaraldehyde in cacodylate buffer 0.1 M pH 7, and then, washed tree times with cacodylate buffer 0.1 M pH 7. The sections were post-fixed with 1% osmium tetroxide ($OsO_4$) for 90 min, and washed 3 times with bidistilled water. Then, sections were treated with 1% aqueous uranyl for 1 h, and sequentially dehydrated through graded acetones. Sections were left overnight in epon/acetone 1/1 and then in pure resin for 4 h. Finally, sections were included in fresh resin and polymerized in an oven at 60°C for 24 h. Ultrafine sections (80 nm) were obtained using an ultramicrotome Leica Ultracut R, which were incubated with 4% uranyl acetate in methanol for 1 min and lead citrate for 5 min. The grids were examined using a Phillips Tecnai 12 electron microscope operated at 80 kV at a magnification of 1700–2250X. Transmission electron microscopy was performed at the Advanced Microscopy Facility UC.

## Real Time PCR

The mRNA levels of *cryptdin-1*, *cryptdin-4*, *reg3γ*, and *lysozyme* were measured by quantitative real-time PCR analysis. Briefly, total RNA was extracted from distal ileum (whole tissue, 4 cm) using TRIZOL reagent (Invitrogen, catalog no. 15596026) according to the manufacturer's instructions, and then treated with DNase I Amplification Grade (Invitrogen, catalog no. 18068015) to eliminate DNA. A total of 1 μg RNA was reverse transcribed to cDNA using iScript RT Supermix (Biorad, catalog no. 1708890), according to the manufacturer's instructions. The resulting cDNA was amplified by real-time PCR in a StepOnePlus thermocycler (Applied Biosystems, CA) using the SsoAdvanced Universal SYBR Green Supermix (Biorad, catalog no. 1725270). The primers are listed in **Supplementary Table 2**. The amplification conditions were as follows: 30 s at 95°C and 40 cycles of 15 s at 98°C and 1 min of annealing and extension at 56°C. The relative quantification values were calculated using a comparative threshold cycle ($2^{-\Delta\Delta ct}$) program on StepOne software, using *gapdh* as housekeeping gene.

## Bacterial Strains and Culture Conditions

*S.* Typhimurium ATCC14028 strain (STM) was originally obtained from American Type Culture Collection and *S.* Typhimurium 14028s ΔphoPQ::Kan (STMΔ*phoPQ)* was generated from ATCC14028 and both strains were kindly provided by Dr. Carlos Santiviago (Universidad de Chile, Santiago, Chile). Strains of *S.* Typhimurium aliquots were stored at −80°C in Luria-Bertani (LB) broth (tryptone 1%, yeast extract 0.5%, and NaCl 0.5%) supplemented with 20% glycerol. To perform infection assays, aliquots were grown with agitation at 37°C in LB broth until OD600 equal to 0.6 was reached. Then, bacterial doses were resuspended in sterile phosphate-buffered saline (PBS).

## Infection Protocol and Bacterial Load Quantification

After 48 h of the last CSC administration, a group of mice was anesthetized with isoflurane and infected with $1 \times 10^5$ CFU of STM or $1 \times 10^6$ CFU STM Δ*phoPQ* by intragastric gavage in 200 μl of PBS. Stool samples were collected at the first and second day post-infection, to perform bacterial counts. To evaluate bacterial load in organs, mice were euthanized 5 days post infection with STM or 2.5 days post infection with STM Δ*phoPQ*. Spleen, liver, blood and MLN were recovered from infected mice and the homogenized tissues were serially diluted in sterile PBS and seeded on LB or McConkey agar plates, in triplicates. Plates with LB solid medium supplemented or not kanamycin were used, depending on the strain. All colony forming units (CFUs) were quantified 24 h later and normalized based on organ weight. Bacterial growth in the plates was expressed as colony-forming units per organ, per mL of blood o per mg of feces.

## 16S Ribosomal RNA Gene Sequence Analysis of Fecal Microbiota

Fecal samples were collected in sterile plastic containers with 10% v/v DNA/RNA Shield™ (Zymo Research, catalog. no R1100-250) and stored at −80°C until further processing. Genomic DNA was extracted from 250 ul of stored stool using the ZymoBIOMICS™ DNA Mini Kit (Zymo Research, catalog. no D4300) according to the manufacturer's protocol. Fecal samples were collected

from each group before and after treatment, under the same conditions. Only those differences that were not present at the initial time were reported.

Library preparation for the V3–V4 region was performed under the Illumina 16S Metagenomic Sequencing Library Preparation Protocol (Part # 15044223 Rev. B), using primers **319f** (5′-ACTCCTACGGGAGGCAGCAG-3′) and **806r** (5′-GGACTACHVGGGTWTCTAAT-3′) and sequenced on a MiSeq Illumina System (Illumina, San Diego, CA, USA), using the MiSeq Reagent kit V3 (Illumina, catalog no. MS-102-3003), with a 300 bp paired-end reads protocol. Raw sequence reads were generated and de-multiplexed by Theragen Etex Co, Ltd (Suwon, Korea). SRA files were deposited to NCBI database under Accession Number PRJNA525412.

Library adapters were trimmed using CutAdatp v1.11 (14) and paired-end reads merged using FLASH v1.2.11 (15). Reads with quality Q >20 and length >300 pb were kept using SICKLE v1.2.11 and chimeric sequences were discarded with ChimeraSlayer (16). The number of OTUs was determined by clustering the pre-processed sequences from each sample with a 97% identity cut-off using QIIME v1.8.0 (17). Taxonomic abundance was counted with RDP Classifier v2.11 (18).

Alpha diversity for each sample was determined using the Shannon index (19) and transformed to "effective number of species" (20). Beta diversity was measured according to Bray-Curtis distance (21). Principal component analysis (PCA) was then performed based on the measured beta diversities using Clustvis (22). For comparisons of relative abundances, the non-parametric Mann-Whitney *U*-test was used.

## Clinical Signs of Colitis

IL-10$^{-/-}$ mice treated with CSC or vehicle were evaluated every 2 days and classified based on the onset of diarrhea, mucus in stools, perianal edema, or prolapse according the clinical score of colitis signs (described in **Supplementary Table 1**).

## Statistics Analysis

Statistical analyses were performed to analyze the data obtained on this work. Unpaired Student's *t*-test was employed to assess whether the means of two normally distributed groups differed significantly, and Mann Whitney *U*-test as its non-parametric counterpart. One-way ANOVA with Tukey's post-test was performed to compare multiple means. Two-way ANOVA with Bonferroni's multiple comparisons post-test was also used in some experiments. All statistical analyses were performed using Prism v6 software (GraphPad Software, San Diego, CA, USA).

# RESULTS

## Intragastric CSC Exposure Causes Intestinal Inflammation

To evaluate the impact of cigarette components on the gastrointestinal mucosa, WT C57BL/6 mice received 200 or 400 μg of CSC by intragastric administration (i.g. CSC). To evaluate whether the intestinal impact depends on a systemic effect, another group of mice received equivalent doses of CSC by intraperitoneal administration (i.p. CSC). In both cases, we observed that there was absorption of the components of the cigarette, demonstrated by high levels of cotinine detected in serum of mice that received CSC (**Supplementary Figure 1A**). Indeed, the levels reached after 45 min of 400 μg of CSC administration were similar to those detected on an average smoker (23).

Histopathological analyzes showed that i.p. CSC treatment did not cause significant damage in the intestinal mucosa, as compared to the vehicle administration (**Supplementary Figures 1B,C**). However, administration of i.g. CSC caused a significant inflammation in the ileum (**Supplementary Figure 1B**). Moreover, the higher dose of i.g. CSC also generated histological alterations in the colon (**Supplementary Figure 1C**). This result was confirmed with a larger group of mice treated with 400 μg i.g. CSC, where the histopathology analyzes of the distal ileum, proximal colon and distal colon showed that i.g. CSC administration caused mild inflammatory infiltrate and distortion of the epithelial architecture, in all the areas evaluated (**Figures 1A,B**). Interestingly, in both ileum and colon sections, the i.g. CSC administration caused multifocal lesions, similar to those observed in Crohn's patients. According to the results obtained in these assays, we chose the treatment of 400 ug of i.g. CSC for subsequent analyzes of mucosal factors that may be involved in the inflammation observed in the gastrointestinal tract.

## Exposure to CSC Causes Morphometric Changes in the Ileal Epithelium

To evaluate in more detail the histological effects of CSC at ileal level, ileum sections of WT C57BL/6 mice were stained with alcian-blue PAS (AB-PAS) and morphometrics analysis were performed. We observed that the intestinal tissue of mice treated with CSC showed a reduction in villus-crypt length (**Supplementary Figure 2A**) consistent with the atrophic villi detected in the histopathological analysis of some animals with ileal inflammation. However, no differences were detected in the ileal perimeter (**Supplementary Figure 2B**), number of goblet cells per villus-crypt unit (**Figures 2A,B**), or in the number of Paneth cells per crypt (**Figure 2C**). Also, PCs were classified according to the organization of their granules as normal (D0), disorganized (D1), depleted (D2), and diffuse (D3), but no differences were observed between both groups (**Supplementary Figures 2C,D**).

Using AB-PAS, TEM, and immunofluorescence staining for lysozyme, it was possible to observe intermediate cells, which share characteristics between Goblet cells and Paneth cells (**Figure 2D** and **Supplementary Figure 2E**). These cells have been considered progenitor forms of secretory cells, originated from stem cells. Considering that they should be located in the proliferation zone of the crypt, we decided to classify them according to their location, in: adjacent to PCs (P1), in the middle zone of proliferation (P2), or in upper positions over differentiated Goblet Cells (P3). We observed that mice treated with CSC had a significantly higher number of intermediate cells outside the bottom of the crypt (**Figure 2D**), suggesting an increased requirement of

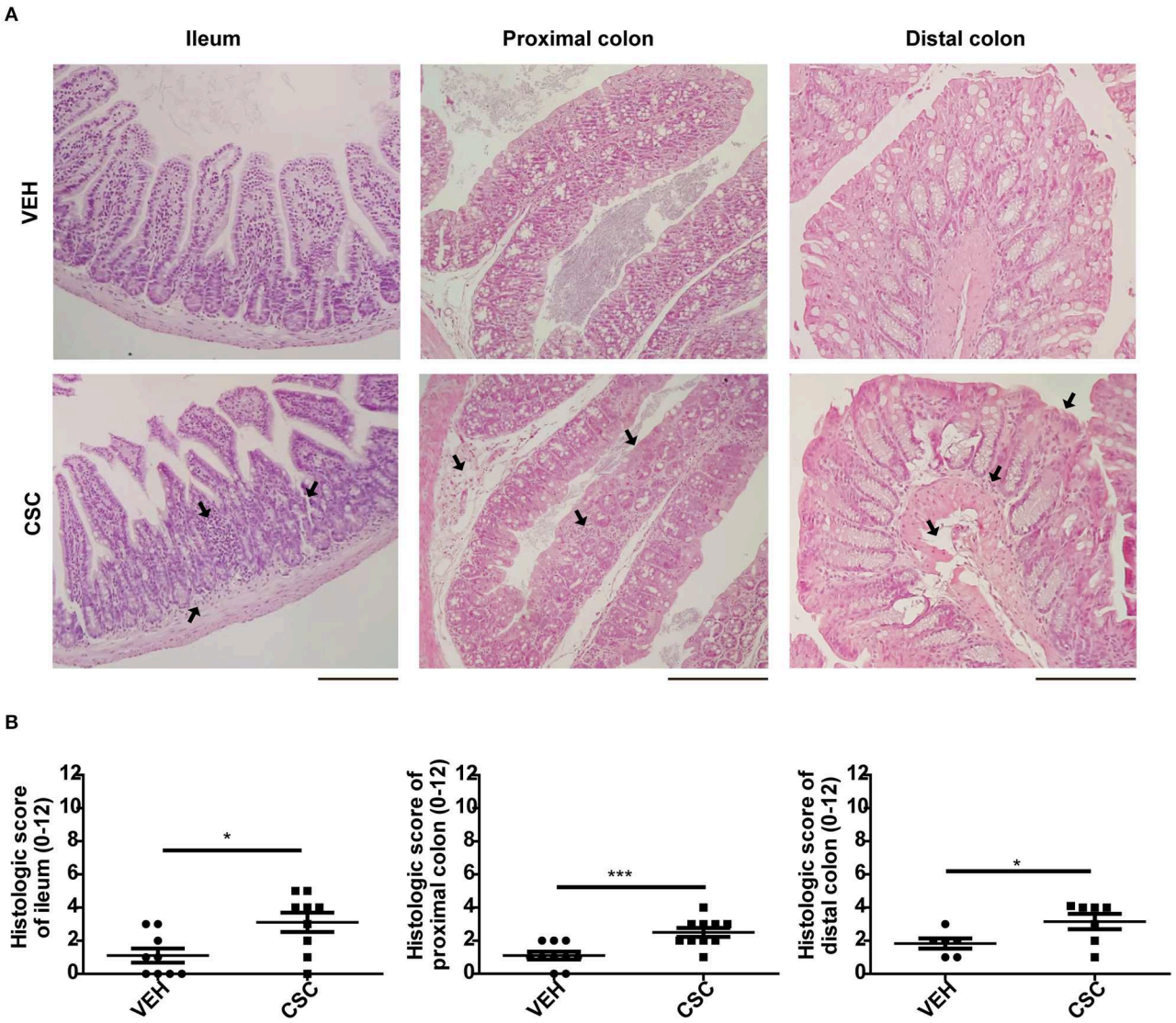


**FIGURE 1 |** Intragastric CSC exposure causes intestinal damage. **(A)** Representative images of ileum, proximal colon, and distal colon sections stained with hematoxilin-eosin, of mice treated with 400 μg i.g. CSC or vehicle. **(B)** Histopathological analysis of ileum, proximal colon and distal colon of mice treated with 400 μg i.g. CSC or vehicle ($n = 9$, *t*-student, $^{*}p < 0.05$, $^{***}p < 0.001$). Scales bars are 200 μm. The same magnification was used for each section.

epithelium regeneration or some alterations in the maturation of Paneth cells. Moreover, using TEM, it was possible to observe that mice treated with CSC presented a greater quantity of abnormal granules, with a semi-dense halo surrounding the electrodense core (**Figures 2E,F**). However, we did not observe differences in the number of granules per cell (**Figure 2G**) or in their size (**Figure 2H**). To assess whether the CSC generates death and regeneration of the epithelium of the crypts, we analyzed mitotic bodies and evidence of cell death by TEM. While we did not find signs of cell proliferation (mitotic bodies), we observed clear signs of cell damage and death, such as RE distention, degenerating granules, necrotic nucleus (**Supplementary Figure 3**).

## CSC Reduces the Expression of Antimicrobial Peptides

Considering the differences observed in the ultrastructure of PCs granules, we wanted to evaluate whether CSC treatment also generates functional changes in these cells. To address this aim, we analyzed the impact of CSC in the transcription of some of the gene encoding main antimicrobial peptides present in murine PCs. We observed that CSC resulted in reduced production of the mRNA for *cryptdin-1*, *cryptdin-4*, and *regIIIγ*, without affecting *lysozyme* mRNA production (**Figure 3A**).

In order to assess whether these changes also occurred at protein level, the presence of RegIIIγ and Lysozyme was evaluated by immunofluorescence in ileum sections (**Figure 3B**).

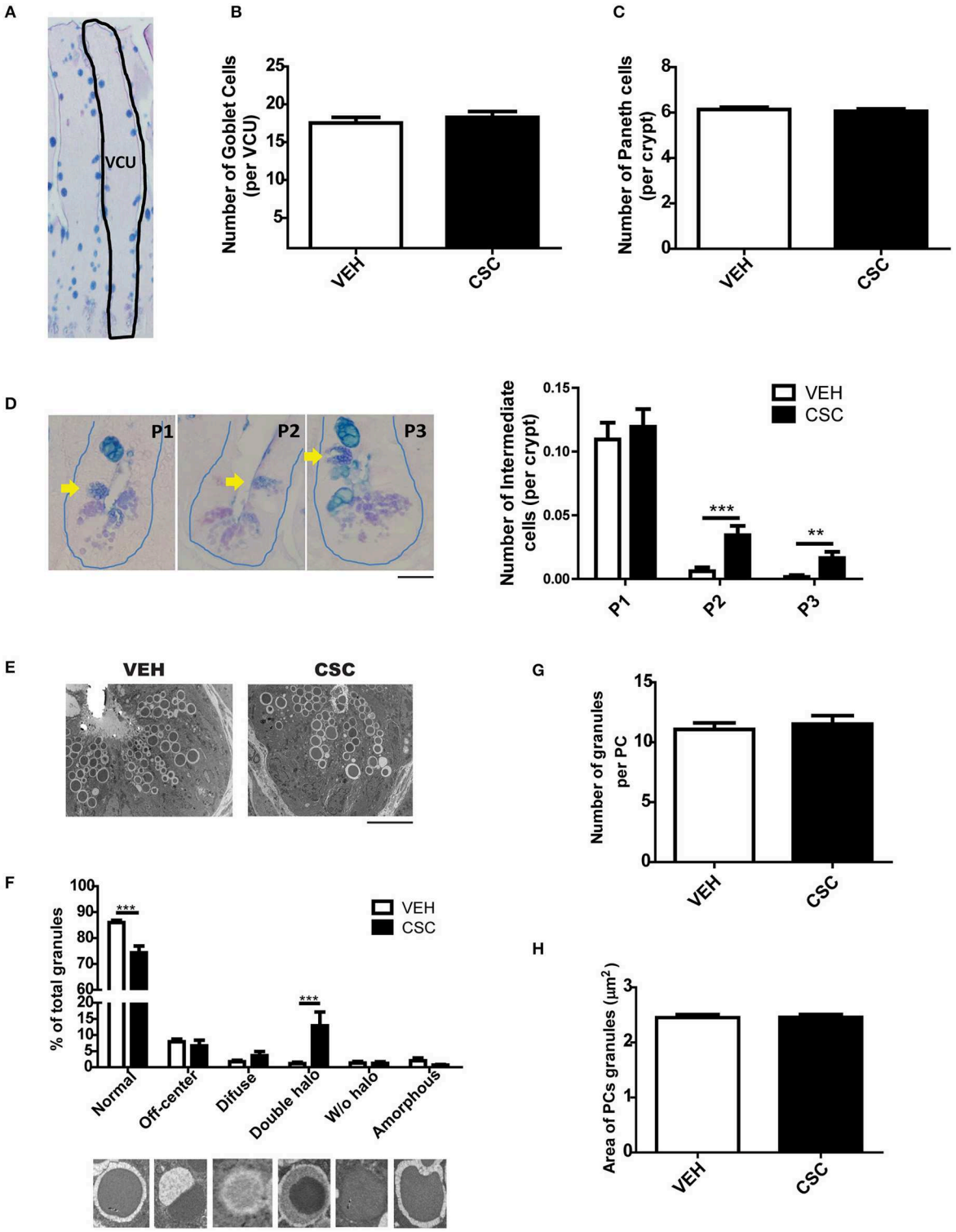


**FIGURE 2 |** Exposure to CSC causes cellular anomalies in the crypts of Lieberkühn. **(A)** Representative image of ileum epithelium stained with AB-PAS. The dashed line denotes a villus-crypt unit (VCU). **(B)** Number of Goblet cells per VCU of mice treated with 400 μg i.g. CSC or vehicle ($n = 6$ mice). **(C)** Number of Paneth cells per crypt of mice treated with 400 μg i.g. CSC or vehicle ($n = 6$ mice). **(D)** Quantification of Intermediate Cells per crypt of both groups, according to their location, in: adjacent to Paneth cells (P1), in the middle zone of proliferation (P2), or in upper positions over differentiated Goblet Cells (P3) (2-way ANOVA, *post-hoc* Bonferroni $^{**}p < 0.01$, $^{***}p < 0.001$, $n = 660$ crypts). Scale bar is 25 μm. **(E)** Representative electron micrograph of Liberkühn crypts of mice treated with 400 μg i.g. CSC or vehicle. Scale bar is 10 μm. **(F)** Classification of granules, according to their TEM morphology in: normal, off-center, diffuse, double halo, without halo and amorphous ($n = 3$ mice, 2-way ANOVA, *post-hoc* Bonferroni $^{***}p < 0.001$). **(G)** Number of granules per Paneth cell (VEH $n = 79$ PCs, CSC $n = 86$ PCs). **(H)** External area of Paneth cells granules (VEH $n = 79$ PCs, CSC $n = 86$ PCs).

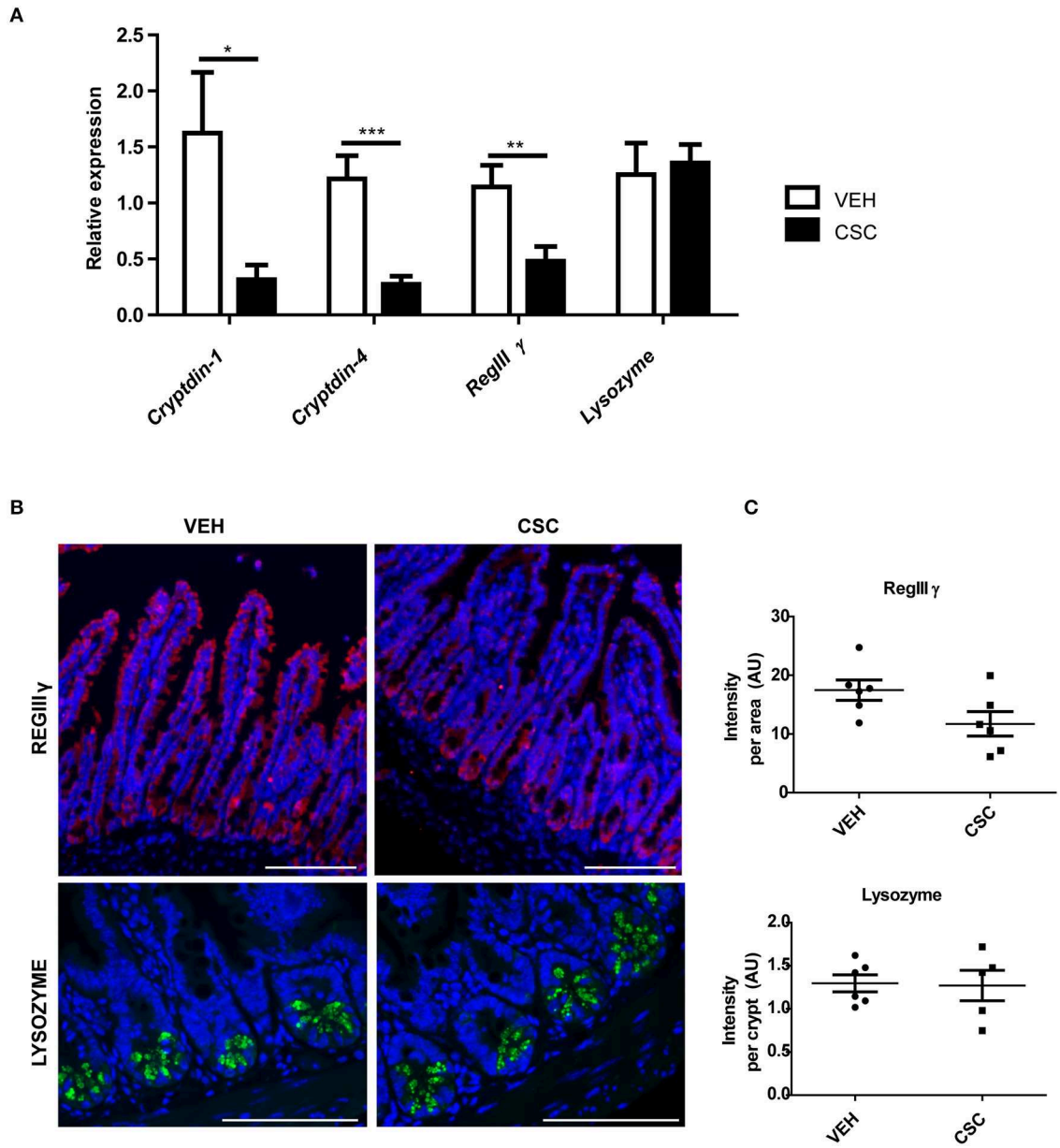


**FIGURE 3 |** CSC reduces the expression of some antimicrobial peptides. **(A)** Relative expression of *cryptdin-1*, *cryptdin-4*, *regIIIγ*, and *lysozyme* in ileum sections of mice treated with 400 μg i.g. CSC or vehicle ($n = 9$, *t*-student, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). **(B)** Representative images of RegIIIγ immunodetection in ileal epithelium (upper panel) and Lysozyme in Lieberkühn crypts (lower panel). RegIIIγ in red, Lysozyme in green, and DAPI in blue. Scales bar are 100 μm. **(C)** Red fluorescence intensity normalized by the epithelial area (upper panel, $n = 6$, *t*-student $p = 0.06$) and Green fluorescence intensity normalized by the number of crypts (lower panel, $n = 6$, *t*-student $p = 0.76$).

We observed that RegIIIγ was present throughout the entire epithelium (**Figure 3B**). However, the sections showed a reduced immunodetection of RegIIIγ in the epithelium of the mice treated with CSC, as compared to those treated with vehicle. In fact, the main difference was observed in the villi, and not in the crypts (**Figure 3B**, yellow arrows). However, when we quantified the fluorescence intensity per tissue, these differences were not statistically significant (**Figure 3C**). On the other hand, consistently with the results obtained by qRT-PCR, lysozyme immunodetection showed no difference between CSC-treated and vehicle-treated mice. In both cases, the presence of these proteins was limited to the granules of the Paneth cells (**Figure 3B**), which showed similar fluorescence intensity per crypt (**Figure 3C**). These results demonstrate that intragastric exposure of CSC alters the ileal production of proteins required to control microbial proliferation in the intestine of mice.

## Exposure to CSC Increases the Susceptibility of Mice to Develop Ileal Inflammation Against Bacterial Infection

To evaluate whether the impact of CSC on bactericidal peptides could increase the risk of gastrointestinal infections, WT mice pre-treated with CSC or vehicle were orally infected with *S.* Typhimurium 14028 (STM) or *S.* Typhimurium 14028s ΔphoPQ::Kan (STM Δ*phoPQ)*. STM has the ability of colonize the intestine and generate a systemic infection (13, 24, 25). Consistently, bacterial loads in organs (**Figure 4A**) and intestinal inflammation (**Figures 4C–E**) was detected in all mice after infection with STM, but no differences in these parameters were detected between mice pre-treated with CSC and vehicle. Only an apparent increase in the fecal bacterial load of mice pre-treated with CSC was observed in comparison to the vehicle group (**Figure 4A**). Next, we evaluated the intestinal inflammation and bacterial dissemination in groups of mice infected with STM Δ*phoPQ*, which is an attenuated bacteria with a low ability to generate a systemic infection under normal conditions (26). The lack of a functional PhoPQ system makes this bacterium sensitive to antimicrobial peptides (27, 28). Therefore, its capacity to colonize capacity the intestinal epithelium will depend on the bactericidal capacity of the tissue. We observed that treatment with CSC did not increase STM Δ*phoPQ* translocation to germinal centers or bacterial load in organs (**Figure 4B**). Again, only an apparent increase was observed in fecal bacterial load of the mice pre-treated with CSC (**Figure 4B**). Interestingly, infection with STM Δ*phoPQ* generated inflammation in the ileum of mice pre-treated with CSC (**Figures 4C–E**), but not in the intestine of mice treated with vehicle. These results suggest that the ileal damage caused by the CSC makes these mice susceptible to develop inflammation after bacterial infections.

## Exposure to CSC Generates Changes in Fecal Bacterial Population

To evaluate the impact of CSC treatment on the gut microbiota of mice, stool samples were collected before and after the treatment with CSC or vehicle. To rule out any change in the microbiota due to genetic-environmental differences not attributable to CSC treatment, we only reported those differences found between CSC-treated and vehicle-treated mice at the end point.

According to the results obtained, treatment with CSC did not generate significant changes in the microbial diversity of each sample (alpha diversity), evaluated as the Shannon index (**Figure 5A**). The analysis of relative abundance of the different Phylum, showed that the treatment with CSC did not generate significant changes in this taxonomic category (**Figure 5B**). However, it is possible to observe that the treatment with CSC significantly modified the relative abundance of some families in comparison to the vehicle (**Figures 5C,D**). Specifically, treatment with CSC generated an increase in Erysipelotrichaceae and a decrease in Rikenellaceae families (**Figure 5E**).

By comparing the relative abundance of the different genera, it was possible to observe a significant increase in the abundance of *Allobaculum* (**Figure 5F**). Its abundance after the treatment with CSC may explain the increase of Erysipelotrichaceae, the family to which *Allobaculum* belongs. Interestingly, a greater relative abundance of this genus has been previously associated with high levels of Cadmium (29), the most abundant heavy metal in the CSC. In the case of the Rikenellaceae family, no specific genus was altered. We also observed a significant reduction in the abundance of *Eisenbergiella* (**Figure 5F**), a genus belonging to the family *Lachnospiraceae*, known for its important capacity to produce butyrate (30). These results suggest that the direct exposure of the intestine to cigarette components generates an imbalance in the microbial population, which may contribute to the alteration of the intestinal homeostasis.

## CSC Induces Symptomatic Colitis in Susceptible Mice

Considering the intestinal inflammation caused by the exposure to CSC, it is feasible that this treatment could also stimulate the development of symptomatic colitis in susceptible individuals. To test this hypothesis we used IL-10-deficient ($IL10^{-/-}$) mice, which spontaneously develop colitis depending on microbial exposure. IL-$10^{-/-}$ were exposed to CSC in specific pathogen-free conditions, which induce IL-$10^{-/-}$ mice to spontaneously develop local colitis and mild inflammation from the 8th week of age, as described in previous studies (31).

According to the clinical score of colitis signs recorded for each group, we observed that CSC treatment stimulated the appearance of signs of colitis in an accelerated and increased manner, as compared to the vehicle group (**Figure 6A**). The main signs observed were loose stools and perianal edema. In addition, these mice had a significantly higher histopathological score in the ileum and colon (**Figures 6B,C**). As in WT mice, the CSC administration caused multifocal lesions, similar to those observed in Crohn's patients. Furthermore, a group exposed to CSC was stimulated at the same time with conventional microbiota. However, it reached a severe inflammation within a few days after the treatment started (data not shown). This result suggests that both factors could act additively.

# DISCUSSION

The results presented in this work show that intragastric exposure to the particulate matter of cigarette smoke generates intestinal inflammation and alteration of the antimicrobial response in mice, supporting the notion that cigarette smoke is a possible causal factor for Crohn's Disease. Interestingly, the damage generated by the treatment with CSC was substantially greater in response to intragastric administration than intraperitoneal administration. These results suggest that the effect depends on a local impact rather than a systemic one.

Cigarette smoking has been mainly associated with the development of ileal inflammation in humans (6). However, in our study we observed inflammation in both ileum and colon due to CSC treatment. These differences suggest that the impact of the cigarette is not necessarily site-specific. In fact, it could depend on the differential exposure of the cigarette components to the different segments of the intestine. In the case of humans, an important part of the particulate matter

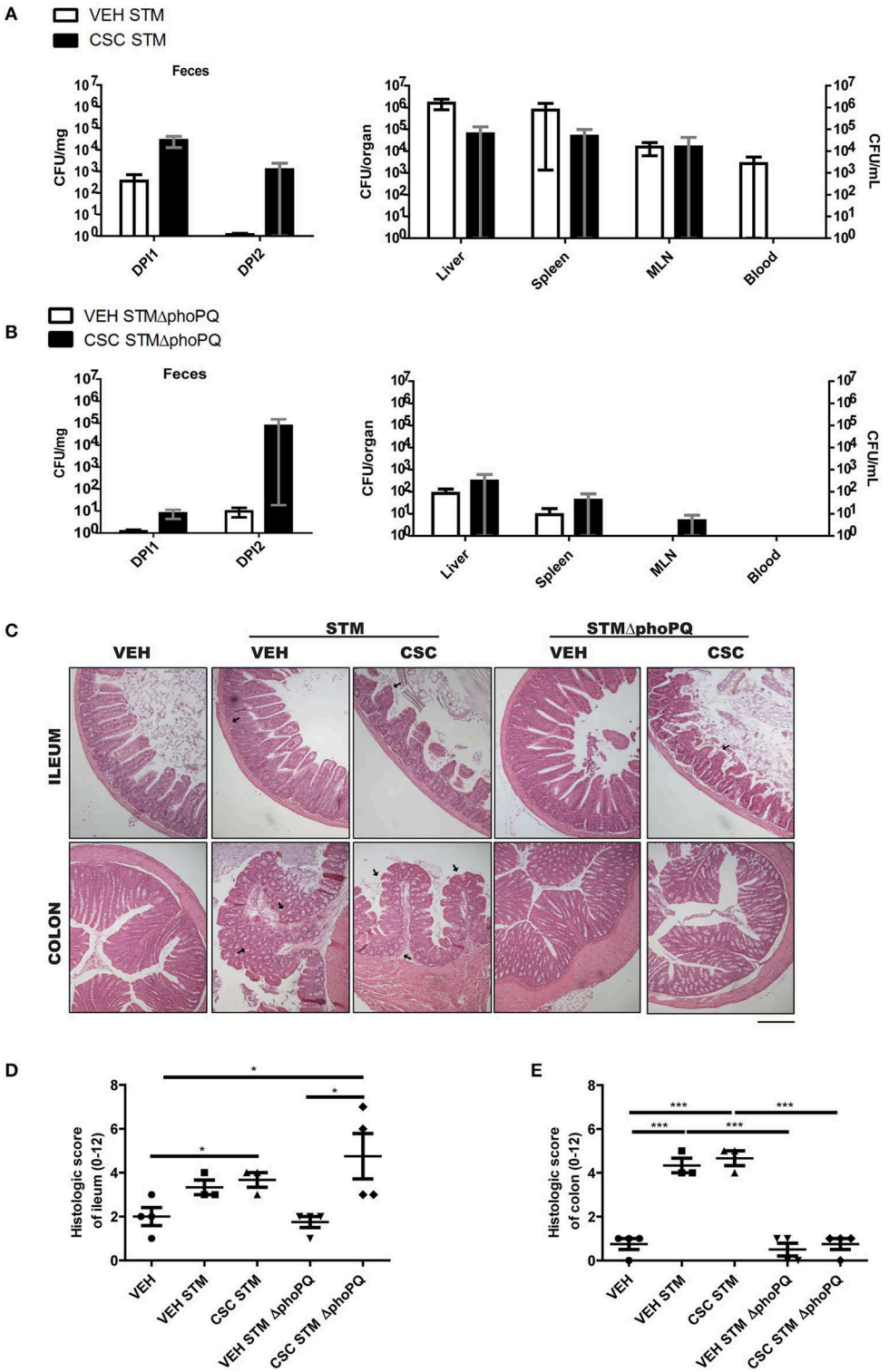


**FIGURE 4 |** Exposure to CSC increases the susceptibility to respond in a pathological way against bacterial infection ($n = 3$ or 4). **(A)** Bacterial burden of mice infected with *S*. Typhimurium wild-type, pre-treated with CSC or vehicle. Left panel: Bacterial burden in heces evaluated at day 1 (DPI1) and 2 (DPI2) post-infection. Right panel: Bacterial burden in liver, spleen, mesenteric lymph nodes (MLN) and blood, evaluated at day 5 post-infection. **(B)** Bacterial burden of mice infected with *S*. Typhimurium Δ*phoPQ,* pre-treated with CSC or vehicle. Left panel: Bacterial burden in heces evaluated at day 1 (DPI1) and 2 (DPI2) post-infection. Right panel: Bacterial burden in liver, spleen, mesenteric lymph nodes (MLN) and blood, evaluated at day 2.5 post-infection. No significant differences were found (Mann-Whitney). **(C)** Representative images of ileum and colon sections stained with hematoxilin-eosin, of mice treated with CSC or vehicle and infected with *S*. Typhimurium wild-type or *S*. Typhimurium Δ*phoPQ*. Scale bar is 200 μm. **(D)** Histopathological analysis of ileum and **(E)** colon sections (ANOVA, Tukey *post-hoc* $^{*}p < 0.05$, $^{***}p < 0.001$).

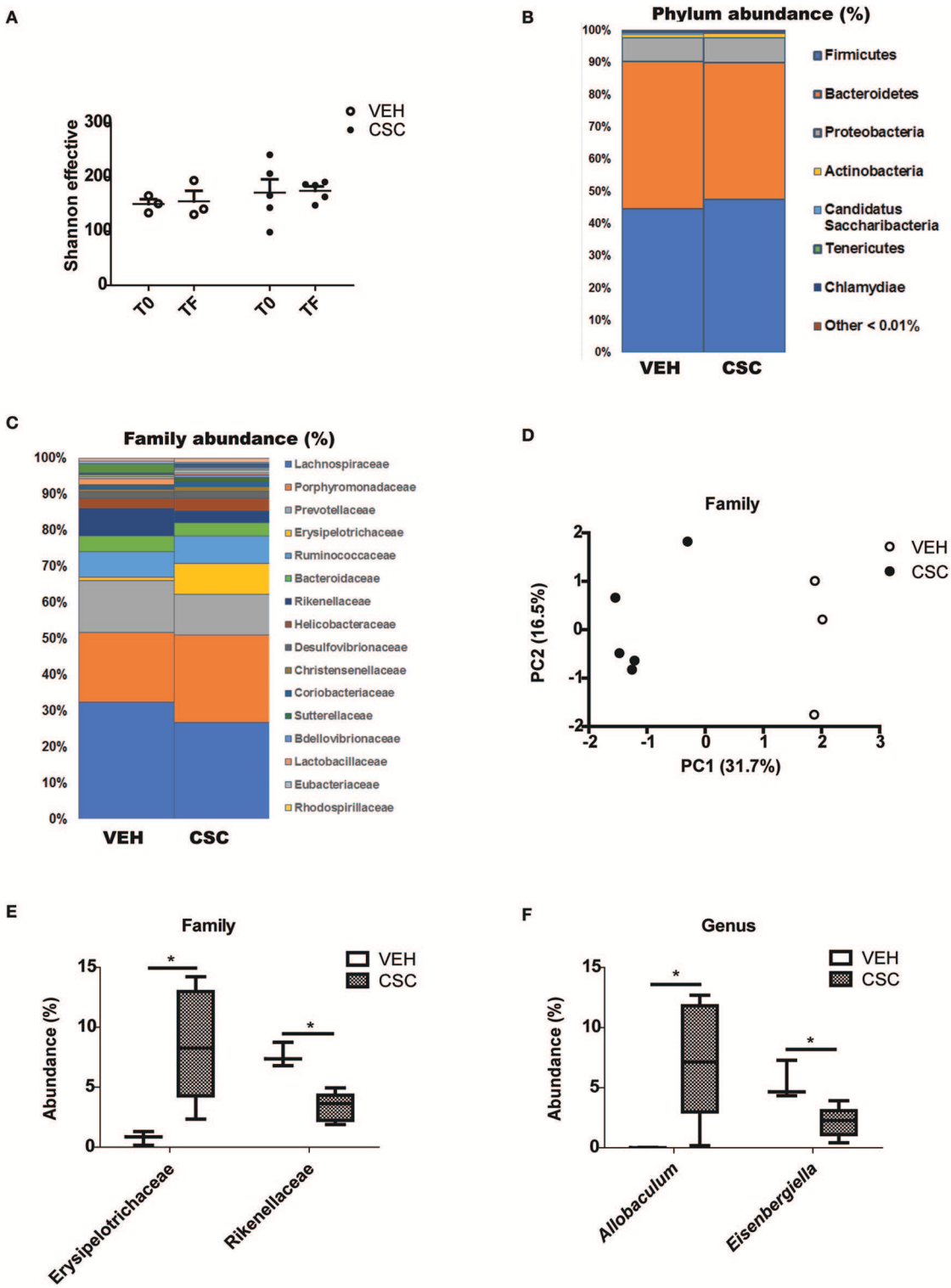


**FIGURE 5 |** CSC generates an imbalance in the fecal microbial population. Sequence analysis of the 16S gene of rRNA from mice treated with vehicle ($n = 3$) or CSC ($n = 5$). **(A)** Effective number of Shannon species, evaluated before (T0) or after (TF) each treatment. **(B)** Relative percentage of dominant phylum detected in each group. **(C)** Relative percentage of dominant families detected in each group. **(D)** Principal Components Analysis (PC1 and PC2) of the β-diversity of phylum evaluated in fecal microbiota of mice treated with vehicle or CSC. **(E)** Relative percentage of families Erysipelotrichaceae and Rikenellaceae in mice treated with vehicle or CSC (Mann-Whitney, $^*p < 0.05$). **(F)** Relative percentage of genus *Allobaculum* and *Eisenbergiella* in mice treated with vehicle or CSC, at the final time (Mann-Whitney, $^* p < 0.05$).

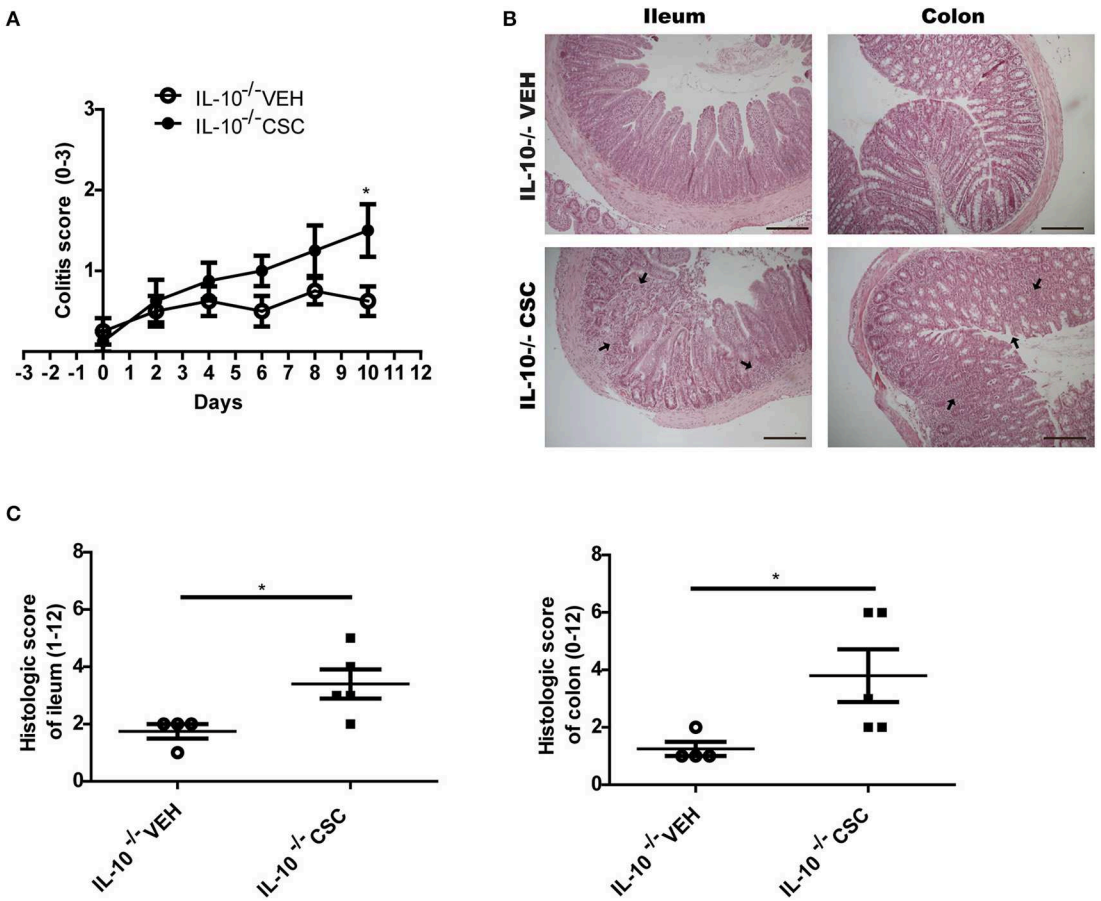


**FIGURE 6 |** CSC induces symptomatic colitis in susceptible mice. **(A)** Colitis score of IL-10$^{-/-}$ mice treated with vehicle or CSC ($n = 8$, 2-way ANOVA, post-Bonferroni *$p < 0.05$). **(B)** Representative images of ileum and colon sections stained with hematoxilin-eosin, of IL-10$^{-/-}$ mice treated with CSC or vehicle. Scale bars are 200 μm. **(C)** Histologic score of ileum and colon of IL-10$^{-/-}$ mice treated with CSC or vehicle (*t*-student, *$p < 0.05$, $n = 4$ o 5).

is swallowed by smokers (8). These components will reach the ileum slowly and chronically, being able to be absorbed mainly in this segment. In the case of the murine model described here, the intragastric administration of a bolus of CSC can stimulate gastric emptying and digestion, increasing the circulation of the treatment to more distal segments of the intestine. This could also explain the differences between our results and other studies in mice, where they only observe ileal inflammation after a long-term exposure to cigarette smoke in a whole-body chamber (7, 32).

After demonstrating the intestinal damage caused by the luminal arrival of CSC, we evaluated its effect on the bactericidal capacity of the intestinal mucosa. It has been reported that patients with ileal CD present a reduced expression of bactericidal peptides (33), and in addition, they have altered their intestinal microbiota (5). Specifically, we wanted to evaluate the impact of CSC on the integrity of Paneth cells, because they have been postulated as a possible site of origin of CD (11).

The results obtained in mice treated with CSC show that the recurrent arrival of components of cigarette smoke can damage the epithelium and alter the normal functionality of its cellular components, including Paneth cells. In fact, it is likely that cell damage caused by CSC does not concentrate on a single cell type, but rather encompasses several factors that participate in intestinal homeostasis. Particularly, in this study we observed that CSC effectively alters the bactericidal function of Paneth cells, evaluated as a reduced transcription of genes coding for cryptdins. However, we also observed a reduction in the bactericidal capacity of the whole epithelium, represented by a reduced expression of RegIIIγ in villi. In addition, we observed more intermediate cells in the proliferation zone of the Lieberkühn crypts in mice treated with CSC, and an increase in the percentage of immature granules in the Paneth cells. These results suggest that the damage caused by CSC stimulates a reparative process, which could be contributing to the functional alterations of the epithelium. In fact, it has been shown that after a chronic damage, Paneth cells can modify their transcriptional profile, expressing more markers to stimulate the proliferation of stem cells and the repair of the epithelium, reducing their bactericidal functions (34).

The impact of the cigarette on intestinal antimicrobial capacity incorporates in this scenario a third protagonist of the intestinal homeostasis: the intestinal microbiota. Our results suggest that the luminal arrival of CSC is capable of triggering

an imbalance in the bacterial population, and in this way, contribute to the alteration of the normal homeostasis of the intestine. This could be explained by the reduced expression of the bactericidal peptides, but also by a possible direct effect of the luminal arrival of the CSC. For example, it was possible to observe a marked increase of the family Rysipelotrichaceae in mice treated with CSC. It has been described that this family is sensitive to α-defensins (35), and therefore the reduced production of bactericidal peptides due to CSC could contribute to their proliferation. In addition, the increased abundance of this family was mainly due to the increase of the genus *Allobaculum*. In fact, the most affected genus by the treatment with CSC was *Allobaculum*. It is reported that this genus increases in response to Cadmium (29), and therefore, the high abundance of Cadmium in the CSC (36) could directly explain this effect. On the other hand, we observed a significant decrease of the Rikenellaceae family in mice treated with CSC. These results are consistent with a study in which patients with CD and a decreased production of bactericidal peptides (due to a mutation in NOD2) presented a lower abundance of this family (37).

In addition, we observed that mice exposed to CSC are more susceptible to inflammation after an inoculation with a strain of *S.* Typhimurium that is sensitive to antimicrobial peptides. These results support the notion that CSC treatment could impair the intestinal bactericidal capacity, as a result of alterations in Paneth cells and the whole epithelium. This way, it contributes to the development of dysbiosis and increase the susceptibility to suffer gastrointestinal infections.

Previous studies have suggested that loss of intestinal homeostasis and increased contact with intestinal bacteria can trigger an exacerbated inflammatory response in this tissue (9). In fact, the results obtained in IL-10$^{-/-}$ mice suggest that the loss of the intestinal homeostasis after CSC exposure can result in a symptomatic enterocolitis with a pathological inflammation in genetically susceptible individuals, as occurs in patients with CD. Even when IL-10$^{-/-}$ mice spontaneously develop a colitis, sharing features with colonic IBD (31), various studies have support that the enterocolitis of these mice represents a model of CD. A study performed by Berg et al. has shown that inflammatory changes first appear in the colon of IL-10$^{-/-}$ mice, but as the disease progresses, some mice also develop inflammation in the small intestine (38). Moreover, the enterocolitis exhibited by IL-10$^{-/-}$ mice has been associated to an uncontrolled Th1 response that leads transmural lesions in aged mice (31, 38). In addition, recent results from our laboratory showed that IL-10$^{-/-}$ mice have alterations in their Lieberkühn crypts even under SPF conditions, making them susceptible to an ileal noxa (39). This observation is consistent with a study describing that the exposure of IL-10 mice to cigarette smoke accelerated the development of colitis and increased the expression of interferon gamma in the small intestine (40).

In conclusion, the results obtained in this work support the participation of cigarette components in the loss of intestinal homeostasis and in the reduction of bactericidal capacity. Moreover, these results highlight once again the importance of the microbiota-host interaction in the origin of inflammatory pathologies, such as CD.

## DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in SRA files were deposited to NCBI database, Accession Number PRJNA525412.

## ETHICS STATEMENT

All the experiments using mice were conducted in agreement with the ethical standards and according to the local animal protection law. All experimental protocols were reviewed and approved by the Scientific Ethical Committee for Animal and Environment Care and the Scientific Ethical Committee for Research Safety of the Pontificia Universidad Católica de Chile (Protocol #170329009). The committee declared that this project complies with the basic principles set forth in Chilean Law 20,380 on Animal Protection (2009), the Terrestrial Animal Health Code of the World Organization for Animal Health (OIE, 24th Edition, 2015), the European Directive 2010/63 / EU and the Guide for the Care and Use of Experimental Animals (NRC, 8th Edition, 2011), documents to which this institution ascribes.

## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication. Specifically, LB designed and performed the experiments. In addition, she wrote the manuscript with the help of SB and MÁ-L, LB, CP-R, and FS-E carried out the manipulation of the mice. GS and LB carried out the experiments with *S.* Typhimurium. JM analyzed the microbiota data. GR carried out the implementation of the immunofluorescence assays. JC participated in the staining and analysis of intestinal sections. CP-R analyzed the histological damage of the sections. AK contributed to the interpretation of the results and to the supervision of the project. SB and MÁ-L conceived the study and were in charge of overall direction and planning.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02289/full#supplementary-material

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# β-Defensin 129 Attenuates Bacterial Endotoxin-Induced Inflammation and Intestinal Epithelial Cell Apoptosis

*Kunhong Xie[1,2†], Hongmei Xie[3†], Guoqi Su[1,2], Daiwen Chen[1,2], Bing Yu[1,2], Xiangbing Mao[1,2], Zhiqing Huang[1,2], Jie Yu[1,2], Junqiu Luo[1,2], Ping Zheng[1,2], Yuheng Luo[1,2] and Jun He[1,2*]*

[1] Institute of Animal Nutrition, Sichuan Agricultural University, Chengdu, China, [2] Key Laboratory of Animal Disease-resistant Nutrition, Chengdu, China, [3] Shandong Vocational Animal Science and Veterinary College, Weifang, China

Defensins have attracted considerable research interest worldwide because of their potential to serve as a substitute for antibiotics. In this study, we characterized a novel porcine β-defensin (pBD129) and explored its role in alleviating bacterial endotoxin-induced inflammation and intestinal epithelium atrophy. The *pBD129* gene was cloned and expressed in *Escherichia coli*. A recombinant pBD129 protein was also purified. To explore its role in alleviating the endotoxin-induced inflammation, mice, with or without lipopolysaccharide (LPS) challenge were treated by pBD129 at different doses. The recombinant pBD129 showed significant antimicrobial activities against the *E. coli* and *Streptococcus* with a minimal inhibitory concentration (MICs) of 32 μg/mL. Hemolytic assays showed that the pBD129 had no detrimental impact on cell viabilities. Interestingly, we found that pBD129 attenuated LPS-induced inflammatory responses by decreasing serum concentrations of inflammatory cytokines, such as the IL-1β, IL-6, and TNF-α ($P < 0.05$). Moreover, pBD129 elevated the intestinal villus height ($P < 0.05$) and enhanced the expression and localization of the major tight junction-associated protein ZO-1 in LPS-challenged mice. Additionally, pDB129 at a high dose significantly decreased serum diamine oxidase (DAO) concentration ($P < 0.05$) and reduced intestinal epithelium cell apoptosis ($P < 0.05$) in LPS-challenged mice. Importantly, pBD129 elevated the expression level of Bcl-2-associated death promoter (Bcl-2), but down-regulated the expression levels of apoptosis-related genes such as the B-cell lymphoma-2-associated X protein (Bax), BH3-interacting domain death agonist (Bid), cysteinyl aspartate-specific proteinase-3 (Caspase-3), and caspase-9 in the intestinal mucosa ($P < 0.05$). These results suggested a novel function of the mammalian defensins, and the anti-bacterial and anti-inflammatory properties of pBD129 may allow it a potential substitute for conventionally used antibiotics or drugs.

**Keywords: endotoxemia, inflammation, porcine β-defensin 129, intestinal epithelium, apoptosis**

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***Correspondence:**
*Jun He*
*hejun8067@163.com*

*[†]These authors have contributed equally to this work*

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## INTRODUCTION

Endotoxemia induced by bacterial endotoxins involves a series of responses, including secretion of pro-inflammatory mediators, expression of adhesion molecules, and multiple organ dysfunctions (1). Previous studies have indicated that endotoxemia usually caused destruction of tight junction integrity and intestinal epithelium apoptosis (2, 3), which subsequently led to disruption of

intestinal homeostasis and damage of the intestinal barrier functions (4, 5). The intestinal epithelium barrier not only contributes to absorption of nutrients, but also contributes to preventing pathogens and toxins from the intestinal lumen from entering circulation (6, 7). Damage of the intestinal epithelium barrier resulted in exposure of submucosa to a variety of pathogens, which subsequently activated the innate immune response and produced a large number of pro-inflammatory cytokines, such as the interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) (8). These pro-inflammatory cytokines not only caused elevated intestinal permeability, but also induced intestinal epithelial cell apoptosis (9).

The defensins, expressed in a variety of epithelial cells, are classified into alpha, beta, and theta forms based on the intramolecular disulfide bond patterns between six cysteines (10, 11). These proteins are a well-characterized group of small, disulphide-rich, cationic peptides that are highly diverse in their sequences and structures (12). Previous studies indicated that the β-defensins possess multidirectional biological properties, including antiviral, antibacterial, and anti-inflammatory effects (13, 14). However, evidence is accumulating to show that β-defensins can also play a role in regulating innate immunity and maintaining intestinal health. For instance, the β-defensin 2 was reported to attenuate inflammation and mucosal lesions during the pathological process of dextran sodium sulfate (DSS)-induced colitis (15). Moreover, the β-defensin 3 significantly decreased production of pro-inflammatory cytokines by macrophages upon *Porphyromonas gingivalis* lipopolysaccharide challenge (16). The porcine β-defensin 129 (pBD129), a newly isolated porcine β-defensin, was first identified in reproductive tissues and was found to be overexpressed in wild boars infected by mycobacteria (17, 18). Although numerous evidence indicates that multiple β-defensins can serve as a critical regulator for diverse biological events including immune responses (15–18), the involvement of pBD129 in regulating the inflammatory responses is just beginning to be explored.

In the present study, we explored the effect of pBD129 on inflammatory responses and intestinal epithelium barrier functions by using a mouse model. The *pBD129* gene was cloned and expressed in *Escherichia coli*, and a recombinant pBD129 protein was purified and characterized *in vitro*. To explore its role in regulating the endotoxin-induced inflammation, mice, with or without LPS challenge were treated by the recombinant pBD129 at different doses. Our study suggests a novel function of the mammalian defensins, and will assist in rational target selection, alleviating the endotoxemia-induced inflammation and damage of the intestinal epithelium barriers.

# MATERIALS AND METHODS

## Synthesis, Expression, and Purification of PBD129

The porcine β-defensin 129 gene (GenBank accession No. NM_001129975.1) was synthesized and cloned into the Sac 1/Hind III sites of pET32a(+) by Tsingke Biological Technology Co., Ltd. (Chengdu, China). The resulting plasmid [pET32a(+)-pBD129] was transformed into *E. coli* BL21(DE3). Cultivation of the *E. coli* BL21(DE3) was performed at 37°C in LB medium supplemented with ampicillin (100 μg $mL^{-1}$) at 200 rpm. After incubation to mid-log growth (OD600 of 1.0), 1 mM isopropyl-l-thiogalactopyranoside (IPTG) were added to induce the expression of pBD129 protein. Cells were harvested by centrifugation at 8,000 × g for 20 min at 4°C, and lysed by sonication in ice-water bath after suspending in Binding buffer (20 mM Tris-HCI, 0.5 M NaCl, 10 mM imidazole, pH 7.9). The supernatant of the cell lysate resulting from centrifugation at 8,000 × g for 30 min was applied to a Ni-NTA column (Shenggong, Shanghai). After washing to baseline absorbance with Binding buffer, the column was washed with Elution Buffer (20 mM Tris-HCI, 0.5 M NaCl, 500 mM imidazole, pH 7.9) at a flow rate of 1 mL/min. The fractions were collected and applied to 12% SDS-PAGE. The protein concentration was determined by the BCA assay (Beyotime, Shanghai, China). After dialyzing with sterile saline solution (0.09% [wt/vol] NaCl in distilled water), the purified protein pBD129 was stored at −80°C for further use.

## Mass Spectrometry Analysis

The expressed protein band was excised from gel for LC-MS/MS mass spectrometry analysis. Briefly, after the gel plug was digested with trypsin, 10 μL of the peptide mixture was separated at a flow rate of 400 nL/min on a C18-reversed phase column. A prominent nano 2D chromatography system (Shimadzu Corp., Kyoto, Japan) was attached to the mass spectrometer micrOTOF-QII (Bruker Corporation, Billerica, MA, USA). The data was collected using Bruker Daltonics micrOTOF control software 3.2 (Bruker Corporation) with the conditions 50–2,200 m/z scan range, 1,500 V capillary voltages, and 150°C drying argon gas temperature. Finally, the selected peptide masses were analyzed using Data Analysis software 4.1 (Bruker Corporation) and searched using the Mascot search engine version 2.3.01.

## Assays of the Antibacterial and Hemolytic Activities

Three Gram-positive species (*Streptococcus dysgalactiae* ATCC 12394, *Staphylococcus aureus* CICC23656, and *Bacillus subtilis*), three Gram-negative bacterial species (*E. coli* DH5α, *E. coli* $K88^+$, and *Salmonella typhimurium* CICC14028), and *Pichia pastoris* X33 were used for the measurement of the antibacterial activity. The minimum inhibitory concentration (MIC) was determined by the method as previously described (19). The bacteria were grown overnight at 37°C; the culture was then diluted using medium to a concentration of $1 \times 10^5$ CFU/mL and seeded into a 96-well plate at a density of 100 μL/well. Recombinant pBD129 was serially diluted from 512 μg/mL by a factor of 2, and 100 μL/well was added to the 96-well plate. The same volume peptide solutions (100 μL) without bacteria were used as negative controls. The reaction system was incubated at 37°C for 24 h. The OD600 nm was measured to calculate the MIC. The experiments were done in triplicates on the same plate. Moreover, hemolytic activity measurements were performed according to a previous study (20). Briefly, 10 mL whole porcine blood was centrifuged

at 1,500 × g for 10 min at room temperature. The porcine blood cells were washed three times with PBS buffer (150 mM NaCl; 10 mM Na2HPO4/NaH2PO4, pH 7.4) and resuspended in PBS buffer (in a 25-fold diluted concentration of erythrocytes compared to blood). Subsequently, 150 μL aliquots were added to 150 μL peptide solutions (final concentration 0–256 μg/mL pBD-129) in polypropylene 96-well microtiter plates, and the mixture was incubated for 1 h at 37°C. After incubation, the plate was centrifuged for 5 min at 1,500 × g and 150 μL supernatant of each well was transferred to a new 96-well plate. Extinction was measured at 450 nm with UV-1100 spectrophotometer (ShangHai, China) and the percentage hemolysis was calculated by comparison with the control samples containing no peptide or 1% Triton X-100.

## Animal Trial

The animal trial was approved by the Animal Welfare Committee of Sichuan Agricultural University (No. 20180718). Sixty male ICR mice (4 weeks old) were purchased from Chengdu Da Shuo laboratory animal Co., Ltd. (Chengdu, China), and used for a 3 × 2 factor design ($n = 10$). The mice were intraperitoneally injected by three doses of pBD129 (0, 4, and 8 mg/kg), and challenged by sterile saline or LPS. All animals were individually housed at 22 ± 2°C with a cycle of 12 h light/12 h dark, and free access to food and water. The injections of pBD129 were carried out for 6 days (once a day) via 1 ml insulin syringe (Braun, Melsungen, Germany). At 7 d, mice were either challenged (intraperitoneal injection) by sterile saline or LPS (*Escherichia coli* O55:B5; Sigma-Aldrich, SL, USA) at a dose of 10 mg/kg. Five hours after challenge, the mice were anesthetized via 20-s exposure to carbon dioxide and subjected to cardiac blood sampling. Duodenum, jejunum, and ileum samples were taken immediately after dislocation of the neck. A portion of the sample was fixed in formaldehyde solution for morphological observation and the other portion was rapidly frozen in liquid nitrogen and stored at −80°C until analysis. Blood samples were centrifuged at 3,000 × g for 15 min at 4°C, after which the serum was separated and stored at −20°C for further analysis.

## Serum Parameter Measurements

Serum diamine oxidase (DOA) assays were performed with commercially available kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). Mouse tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and IL-6 enzyme-linked immunosorbent assay (ELISA) kits were obtained from Beijing Sizhengbai Biotechnology Co., Ltd (Beijing, China). In addition, the 3100-type automatic biochemical analyzer (Hitachi Co., Tokyo, Japan) was used to determine the concentrations of Immunoglobulin G (IgG), Urea, Creatinine (Cre), C-reactive protein (CRP), and Alanine transaminase (ALT) in serum samples.

## Histopathological Assays

Samples taken from the duodenum, jejunum, and ileum were used for histological analysis. The samples were fixed overnight in 4% paraformaldehyde and then dehydrated with different concentrations of ethanol. After dehydration, samples were embedded in paraffin and were subsequently cut into 4-μm thick sections. The prepared tissue sections were stained with hematoxylin and eosin (H&E) and sealed with a neutral gum. Villus height and crypt depth were determined by using an image processing and analysis system (Image-Pro Plus 6.0, Media Cybernetics, Inc., Bethesda, MD, USA), and a previously described calculation method were adopted (21).

## Immunofluorescence Analysis

The jejunal tissue section was deparaffinized and rinsed with distilled water for 5 min. Tissue sections were then subjected to antigen retrieval by ethylenediaminetetraacetic acid (EDTA, 1 mol/L, pH 9.0, Gooddbio Technology Co., Ltd., Wuhan, China). Before overnight incubating at 4°C with rabbit anti-ZO-1 polyclonal antibody (Gooddbio Technology Co., Ltd., Wuhan, China), sections were blocked with 3% bovine serum albumin. The sections were washed three times with PBS (pH 7.4) for 5 min each time, and then goat anti-rabbit IgG-FITC secondary antibody (Gooddbio Technology Co., Ltd., Wuhan, China) was added thereto, followed by incubation at room temperature for 50 min in the dark. Then, sections were washed three times with PBS (PH = 7.4), and the nuclei were stained with 4′-6-diamidino-2-phenylindole (DAPI, Gooddbio Technology Co., Ltd., Wuhan, China) for 10 min at room temperature in the dark. Finally, the fluorescence of the sections was visualized by a confocal scanning microscope (NIKON ECLIPSE TI-SR), and the images were taken using NIKON DS-U3 software.

## Detection of the Cell Apoptosis

The proportion of apoptotic cells in isolated jejunal mucosal cells was determined by flow cytometry (CytoFlex, Beckman Coulter, Inc., Brea, CA, USA) using PE Annexin V Apoptosis Detection Kit I (Becton, Dickinson and Company, BD Biosciences, San Jose, CA, USA). First, the jejunum was dissected, the jejunal mucosa was scraped, and then filtered through a grind and a mesh to form a cell suspension. After washing twice with ice-cold PBS, the cell sample was made into a single cell suspension of $1 \times 10^6$ cells/mL. One hundred microlitre of the single cell suspension was centrifuged at 1,300 × g for 15 min to remove the supernatant, then the cells were stained with 5 μL of Annexin-V-FITC fluorescent dye at 4°C in the dark. After 10 min, add 5 μL of PI staining for 5 min at 4°C in the dark. Finally, detection of apoptotic cells was completed within 1 h after the addition of 400 μL Annexin V binding buffer (1x).

## RNA Extraction and Real-Time PCR

Total RNA was extracted from duodenal, jejunal, and ileal samples using TRIzol Reagent (TaKaRa, Dalian, China). The concentration and purity of total RNA were assayed by spectrophotometer (Beckman Coulter, DU800) at 260 and 280 nm. The ratio of absorption (260/280 nm) of samples was between 1.8 and 2.0. Then, each RNA sample was reverse-transcribed into cDNA using reverse transcriptase (Takara, Tokyo, Japan) after detection of RNA concentration and purity by spectrophotometer (Beckman Coulter, DU800). The PCR primer sequences were designed using Primer Premier 5.0 and are listed in **Supplementary Table 1**. Briefly, quantitative PCR

was performed by QuanStudio 6 Flex Real-Time PCR detection system (Applied Biosystems, Foster City, CA, USA), with a total of 10 μL of assay solution containing 5 μL SYBR Green mix (TaKaRa, Dalian, China), 0.2 μL Rox, 3 μL deionized $H_2O$, 1 μL cDNA template, and 0.4 μL each of forward and reverse primers. The comparative Ct value method was used to quantify mRNA expression relative to β-actin expression (22).

## Determination of Cysteinyl Aspartate-Specific Protease Activity

The activity of caspase-3 and caspase-9 were determined using the Cysteinyl aspartate-specific protease activity kit (Beyotime, Shanghai, China). To evaluate the caspase-3 and caspase-9 activity of the small intestine, tissue lysates were prepared after their respective treatment with various designated treatments. Assays were performed on 96-well microtiter plates by incubating 50 μL protein of tissue lysate per sample in 50 μL reaction buffer (1% NP-40, 20 mM Tris-HCl (pH 7.5), 137 mM Nad and 10% glycerol) containing 10 μL caspase-3 substrate (Ac-DEVD-*p*NA) (2 mM) or 10 μL caspase-9 substrate (Ac-LEHD-*p*NA) (2 mM). Lysates were incubated at 37 °C for 2 h. Samples were measured with the UV-1100 spectrophotometer (Shanghai, China) at an absorbance of 405 nm and 1 μg Cysteinyl aspartate-specific protease hydrolyzes Ac-DEVD-*p*NA or Ac-LEHD-*p*NA within 1 h to produce 1 nmoL of *p*NA represents U/μg.

## Statistical Analysis

The individual mouse was used as the experimental unit, and all data were expressed as mean ± standard error (SEM). Statistical analysis was carried out using two-way ANOVA followed by Bonferroni's multiple comparisons test using GraphPad Prism software (Version 7. GraphPad Software Inc., CA, USA).

# RESULTS

## *In vitro* Assays for the Antibacterial Activity of pBD129

pBD129 (Porcine β-defensin 129) was expressed in *E. coli* BL21 (DE3) and purified by using the Ni-NTA agarose column (**Supplementary Figure 1**). The purity of recombinant pBD129 was analyzed with Image Lab (Bio-Rad), and the results showed that the purity of recombinant pBD129 was 90%. The purified protein was identified by mass spectrometry (LC-MS/MS). After searching the amino acid sequence of pBD129 in the NCBI database (Accession No. NP_001123447.1), we found that the sequence coverage of the two protein sequences was more than 82%, indicating that the purified protein was porcine β-defensin 129 (**Supplementary Figure 2**). The MIC assays were carried out to evaluate the antimicrobial activity of pBD129. As shown in **Table 1**, pBD129 showed significant antimicrobial activities against the *E. coli* and *Streptococcus* with a minimal inhibitory concentration (MICs) of 32 μg/mL. Moreover, we measured the hemolytic activity of the pBD129 by using whole pig blood, and found that the recombinant pBD129 had no detrimental effect on the erythrocytes at all concentrations (0–256 μg/mL) (**Figure 1**).

**TABLE 1 |** Minimal inhibition concentration (MIC) of porcine β-defensin 129[a].

| Strain | pBD-129 (ug/mL) |
|---|---|
| Gram-negative bacteria | |
| *E.coli* DH5α | 32 |
| pathogenic *E.coli* K88+ | >512 |
| *Salmonella typhimurium* CICC14028 | >512 |
| Gram-positive bacteria | |
| *Streptococcus dysgalactiae* ATCC 12394 | 32 |
| *Staphylococcus aureus* CICC23656 | >512 |
| *Bacillus subtilis* | >512 |
| Fungi | |
| *Pichia pastoris* X33 | >512 |

[a]*Values are the means of 3 replicates per treatment.*

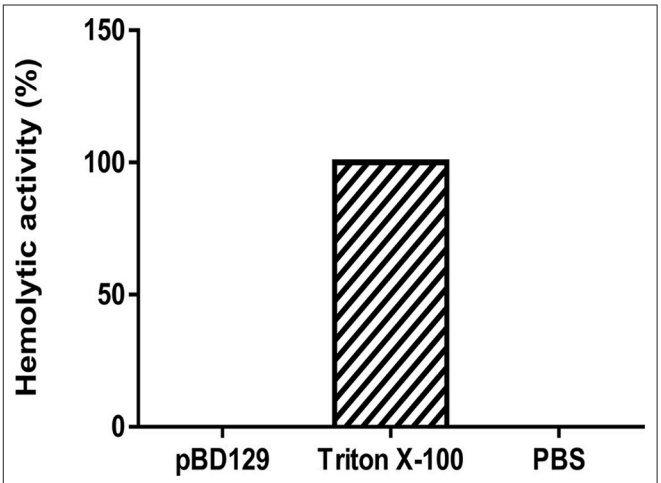


**FIGURE 1 |** The hemolytic activity of porcine β-defensin 129. Triton X-100 and phosphate buffer saline (PBS) were selected as positive and negative controls, respectively, $n = 3$.

## Effect of pBD129 on Serum Biochemical Parameters in Mice Upon LPS Challenge

The serum parameters are presented in **Table 2**. LPS challenge significantly increased the serum concentrations of inflammatory cytokines such as the IL-1β, IL-6, and TNF-α ($P < 0.05$). However, pBD129 injection significantly decreased the serum concentrations of these inflammatory cytokines upon LPS challenge ($P < 0.05$). No significant changes of serum inflammatory cytokines were observed in mice without being LPS challenged ($P < 0.05$). Additionally, LPS challenge significantly increased the serum concentrations of ALT, CRP, Cre, and urea ($P < 0.05$). Amongst the LPS-challenged groups, pBD129 injection at a high dose (8 mg/kg) significantly decreased the serum concentrations of ALT, CRP, Cre, and urea ($P < 0.05$). Moreover, pBD129 injection at a lower lose (4 mg/kg) can also decrease the serum concentrations of urea and Cre ($P < 0.05$). Interestingly, mice with LPS challenge showed an acute reduction in serum IgG concentration ($P < 0.01$), but pBD129

**TABLE 2 |** Porcine ionsBACTERIAf pameliorated the Biochemical Parameters of Serum during Bacterial Endotoxin-induced pathology[1].

| Item[2] | Treatment[3] | | | | | | *P*-value[4] | | |
|---|---|---|---|---|---|---|---|---|---|
| | Control | L-129 | H-129 | LPS | L-129 + LPS | H-129 + LPS | B | V | B*V |
| IL-1β (pg/mL) | 29.98 ± 9.76$^{b}$ | 37.13 ± 10.12$^{b}$ | 32.02 ± 12.48$^{b}$ | 198.86 ± 18.52$^{a}$ | 35.40 ± 6.15$^{b}$ | 38.88 ± 6.54$^{b}$ | <0.0001 | <0.0001 | <0.0001 |
| IL-6 (pg/mL) | 23.25 ± 0.72$^{c}$ | 24.71 ± 1.14$^{c}$ | 24.02 ± 0.83$^{c}$ | 1934.21 ± 13.16$^{a}$ | 199.11 ± 39.29$^{b}$ | 85.01 ± 12.79$^{c}$ | <0.0001 | <0.0001 | <0.0001 |
| TNF-α (pg/mL) | 28.89 ± 0.74$^{b}$ | 32.84 ± 1.11$^{b}$ | 31.62 ± 2.31$^{b}$ | 73.32 ± 5.56$^{a}$ | 40.79 ± 1.67$^{b}$ | 35.68 ± 1.40$^{b}$ | <0.0001 | <0.0001 | <0.0001 |
| ALT (mmol/L) | 43.25 ± 2.21$^{b}$ | 43.50 ± 6.08$^{b}$ | 37.50 ± 1.19$^{b}$ | 83.50 ± 5.06$^{a}$ | 80.00 ± 6.87$^{a}$ | 36.75 ± 2.75$^{b}$ | <0.0001 | <0.0001 | 0.0004 |
| CRP (mg/L) | 0.66 ± 0.22$^{b}$ | 0.75 ± 0.09$^{b}$ | 0.41 ± 0.16$^{b}$ | 3.83 ± 0.66$^{a}$ | 3.25 ± 0.45$^{a}$ | 1.38 ± 0.15$^{b}$ | 0.0029 | <0.0001 | 0.0175 |
| Cre (mmol/L) | 7.33 ± 0.08$^{c}$ | 7.70 ± 0.16$^{bc}$ | 8.86 ± 0.09$^{bc}$ | 11.00 ± 0.72$^{a}$ | 8.99 ± 0.34$^{b}$ | 9.08 ± 0.19$^{b}$ | 0.0675 | <0.0001 | 0.0003 |
| urea (mmol/L) | 7.99 ± 0.13$^{d}$ | 7.95 ± 0.25$^{d}$ | 8.10 ± 0.26$^{d}$ | 20.05 ± 0.18$^{a}$ | 10.81 ± 1.14$^{c}$ | 13.28 ± 0.22$^{b}$ | <0.0001 | <0.0001 | <0.0001 |
| IgG (g/L) | 0.58 ± 0.05$^{b}$ | 0.66 ± 0.08$^{ab}$ | 0.88 ± 0.06$^{a}$ | 0.18 ± 0.05$^{c}$ | 0.44 ± 0.07$^{bc}$ | 0.55 ± 0.03$^{b}$ | <0.0001 | <0.0001 | 0.2915 |
| DAO (U/L) | 24.15 ± 0.86$^{b}$ | 25.07 ± 0.88$^{b}$ | 27.82 ± 1.61$^{ab}$ | 31.44 ± 0.60$^{a}$ | 27.46 ± 1.52$^{ab}$ | 25.46 ± 0.18$^{b}$ | 0.3584 | 0.0159 | 0.0025 |

*Different lowercase letters indicate statistically significant differences from each other ($P < 0.05$).*

*[1]Values of the IgG, Cre, CRP, ALT, and UREA are 4 replicates per treatment; Values of the IL-1β, IL-6, and TNF-α are 5 replicates per treatment. Values of the DAO is 3 replicates per treatment.*

*[2]IgG, Immunoglobulin G; Cre, creatinine; CRP, C-reactive protein; ALT, Alanine transaminase; DAO, diamine oxidase; IL-1β, interleukin-1β; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α.*

*[3]Control, 200 μL sterile saline; LPS, 200 μL Lipopolysaccharide; L-pBD129, 200 μL of 0.6 mg/ml porcine β-defensin; H-pBD129, 200 μL of 1.2 mg/mL porcine β-defensin 129; L-pBD129 + LPS, 200 μL of 0.6 mg/mL pBD129 pretreated followed by LPS treated; H-pBD129 + LPS, 200 μL of 1.2 mg/mL pBD129 pretreated followed by LPS treated.*

*[4]B is the main effect of porcine β-defensin 129; V is the main effect of LPS infection; B*V is the interaction effect of the two main factors.*

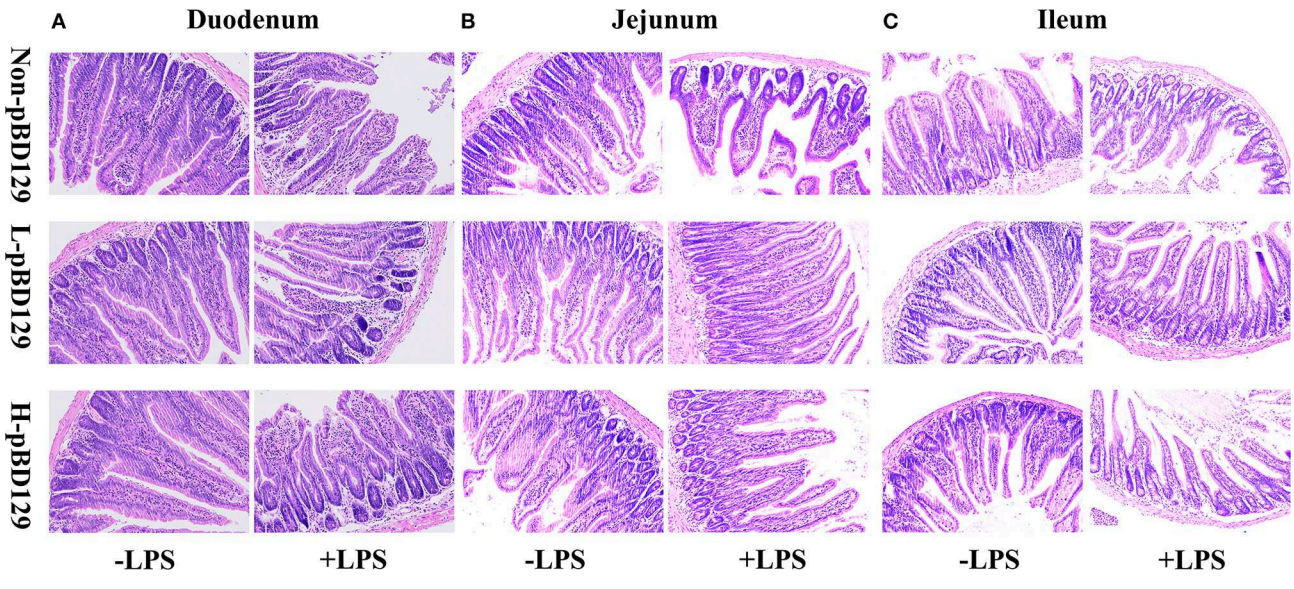


**FIGURE 2 |** Histological evaluation of small intestine tissue after exposure to pBD129 (H&E; × 200). **(A)** Representative H&E stained sections from the duodenum. **(B)** Representative H&E stained sections from the jejunum. **(C)** Representative H&E stained sections from the ileum. Non-pBD129, 200 μL Sterilized saline; L-pBD129, 200 μL of 0.6 mg/ml porcine β-defensin 129; H-pBD129, 200 μL of 1.2 mg/ml porcine β-defensin 129, $n = 3$/group.

injection at 8 mg/kg significantly increased the serum IgG concentration ($P < 0.01$).

## Effect of pBD129 on Intestinal Morphology, Permeability, and Distribution of the Major Tight Junction-Associated Protein ZO-1

LPS challenge resulted in atrophy of the intestinal mucosa (**Figure 2**). As compared to the control group (challenged by sterile saline), the LPS-challenged mice have a shedding epithelium and shortened villi in the small intestine (**Table 3**). However, the villus height in the jejunum and ileum were significantly elevated by pBD129 in the LPS-challenged mice ($p < 0.05$). Moreover, pBD129 significantly decreased the crypt depth and elevated the ratio of villus height/crypt depth in the small intestine ($p < 0.05$). To investigate the intestinal permeability, the serum DAO concentrations were determined (**Table 2**). We show that LPS challenge acutely increased the serum DAO concentrations ($p < 0.01$). However, pBD129 treatment at a higher dose (8 mg/kg) significantly

**TABLE 3 |** Effects of Porcine β-defensin 129 on the intestinal morphology of mice[1].

| Item[2] | Treatment[3] | | | | | | *P*-value[4] | | |
|---|---|---|---|---|---|---|---|---|---|
| | Control | L-129 | H-129 | LPS | L-129+LPS | H-129+LPS | B | V | B*V |
| Duodenum | | | | | | | | | |
| VH, μm | 478.10 ± 2.54$^{a}$ | 466.27 ± 1.68$^{a}$ | 471.79 ± 6.25$^{a}$ | 413.88 ± 17.25$^{b}$ | 445.34 ± 7.10$^{ab}$ | 428.28 ± 16.51$^{ab}$ | 0.6529 | <0.0001 | 0.1510 |
| CD, μm | 136.88 ± 5.20$^{b}$ | 140.10 ± 2.77$^{b}$ | 138.85 ± 0.81$^{b}$ | 181.04 ± 3.30$^{a}$ | 153.03 ± 3.30$^{b}$ | 146.39 ± 4.02$^{b}$ | 0.0005 | <0.0001 | 0.0001 |
| VH/CD | 3.51 ± 0.12$^{a}$ | 3.33 ± 0.08$^{a}$ | 3.40 ± 0.06$^{a}$ | 2.29 ± 0.09$^{c}$ | 2.92 ± 0.09$^{b}$ | 2.92 ± 0.05$^{b}$ | 0.0113 | <0.0001 | 0.0002 |
| Jejunum | | | | | | | | | |
| VH, μm | 398.03 ± 9.50$^{a}$ | 370.33 ± 11.47$^{ab}$ | 392.49 ± 11.46$^{a}$ | 253.60 ± 13.07$^{c}$ | 342.34 ± 13.07$^{ab}$ | 325.02 ± 14.04$^{b}$ | 0.0254 | <0.0001 | 0.0005 |
| CD, μm | 117.51 ± 2.08$^{b}$ | 114.73 ± 2.84$^{b}$ | 114.86 ± 1.69$^{b}$ | 142.62 ± 6.95$^{a}$ | 125.35 ± 3.42$^{ab}$ | 118.64 ± 2.83$^{b}$ | 0.0059 | 0.0004 | 0.0302 |
| VH/CD | 3.39 ± 0.07$^{a}$ | 3.23 ± 0.09$^{a}$ | 3.42 ± 0.12$^{a}$ | 1.79 ± 0.14$^{c}$ | 2.73 ± 0.08$^{b}$ | 2.74 ± 0.10$^{b}$ | 0.0003 | <0.0001 | <0.0001 |
| Ileum | | | | | | | | | |
| VH, μm | 224.83 ± 3.22$^{ab}$ | 221.24 ± 3.70$^{ab}$ | 228.64 ± 2.99$^{a}$ | 168.30 ± 6.82$^{c}$ | 208.01 ± 3.76$^{ab}$ | 207.70 ± 1.55$^{b}$ | <0.0001 | <0.0001 | <0.0001 |
| CD, μm | 86.04 ± 2.72$^{c}$ | 93.24 ± 1.86$^{c}$ | 93.82 ± 1.17$^{c}$ | 120.91 ± 2.02$^{a}$ | 105.09 ± 0.54$^{b}$ | 103.85 ± 1.00$^{b}$ | 0.0247 | <0.0001 | <0.0001 |
| VH/CD | 2.62 ± 0.12$^{a}$ | 2.38 ± 0.07$^{a}$ | 2.44 ± 0.05$^{a}$ | 1.39 ± 0.06$^{c}$ | 1.98 ± 0.05$^{b}$ | 2.0 ± 0.03$^{b}$ | 0.0150 | <0.0001 | <0.0001 |

*Different lowercase letters indicate statistically significant differences from each other ($P < 0.05$).*
*[1]Values are the means of 3 replicates per treatment.*
*[2]VH villus height, CD crypt depth, VH/CD the ratio of villus height and crypt depth.*
*[3]Control, 200 μL sterile saline; LPS, 200 μL Lipopolysaccharide; L-pBD129, 200 μL of 0.6 mg/ml porcine β-defensin; H-pBD129, 200 μL of 1.2 mg/mL porcine β-defensin 129; L-pBD129 + LPS, 200 μL of 0.6 mg/mL pBD129 pretreated followed by LPS treated; H-pBD129 + LPS, 200 μL of 1.2 mg/mL pBD129 pretreated followed by LPS treated.*
*[4]B is the main effect of Porcine β-defensin 129; V is the main effect of LPS infection; B*V is the interaction effect of the two main factors.*

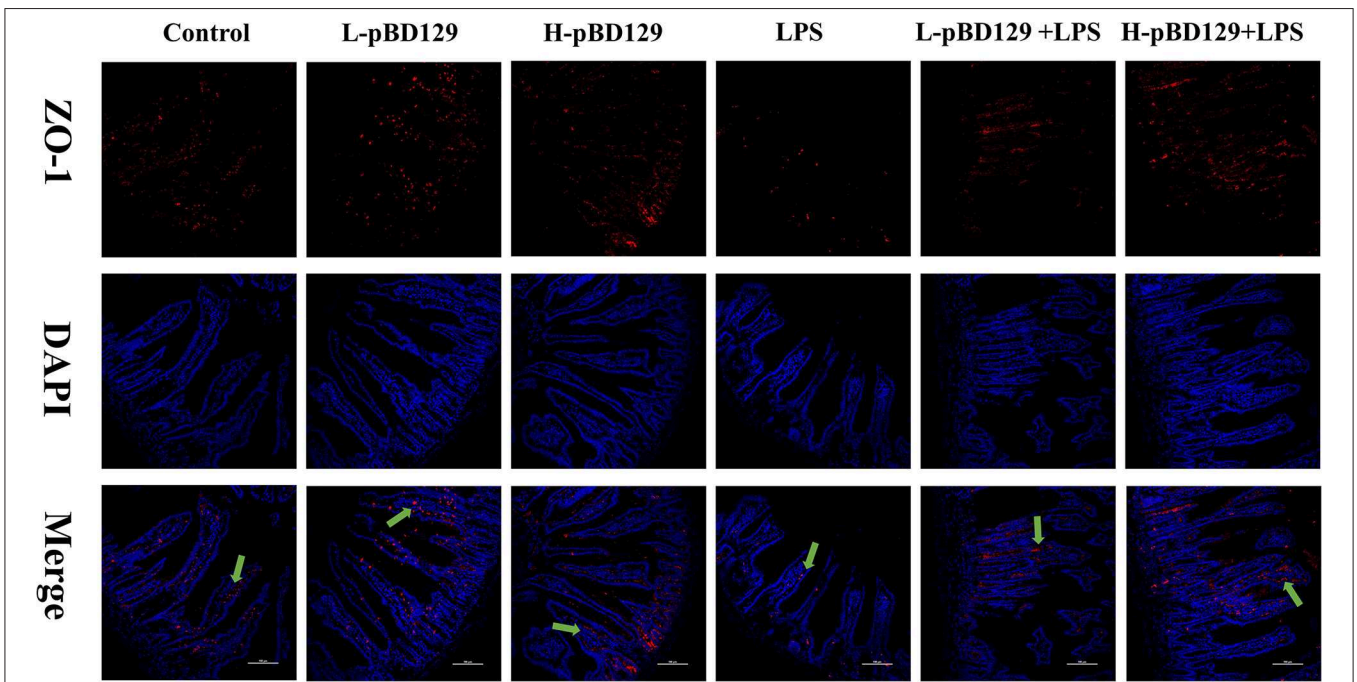


**FIGURE 3 |** Evaluation of the localization of ZO-1 and DAPI (DNA) in the jejunum of mice by immunofluorescence. ZO-1 protein (red), DAPI staining (blue), and pooled ZO-1 protein and DAPI are provided. The scale bar represents 100 μm. The green arrows mark jejunum positive for ZO-1 expression. Control, 200 μL sterile saline; LPS, 200 μL Lipopolysaccharide; L-pBD129, 200 μL of 0.6 mg/ml porcine β-defensin; H-pBD129, 200 μL of 1.2 mg/mL porcine β-defensin 129; L-pBD129 + LPS, 200 μL of 0.6 mg/mL pBD129 pretreated followed by LPS treated; H-pBD129 + LPS, 200 μL of 1.2 mg/mL pBD129 pretreated followed by LPS treated.

decreased the serum DAO concentration in LPS-challenge mice ($P < 0.05$). Importantly, we explored the distribution of the major tight junction-associated protein ZO-1 in jejunum by immunofluorescence analysis, and found that the localization of ZO-1 protein in the jejunum was significantly changed after LPS challenge (**Figure 3**). As compared to the control group, LPS challenge has resulted in decreased abundance of ZO-1 protein in the tight junction region, indicating the disruption

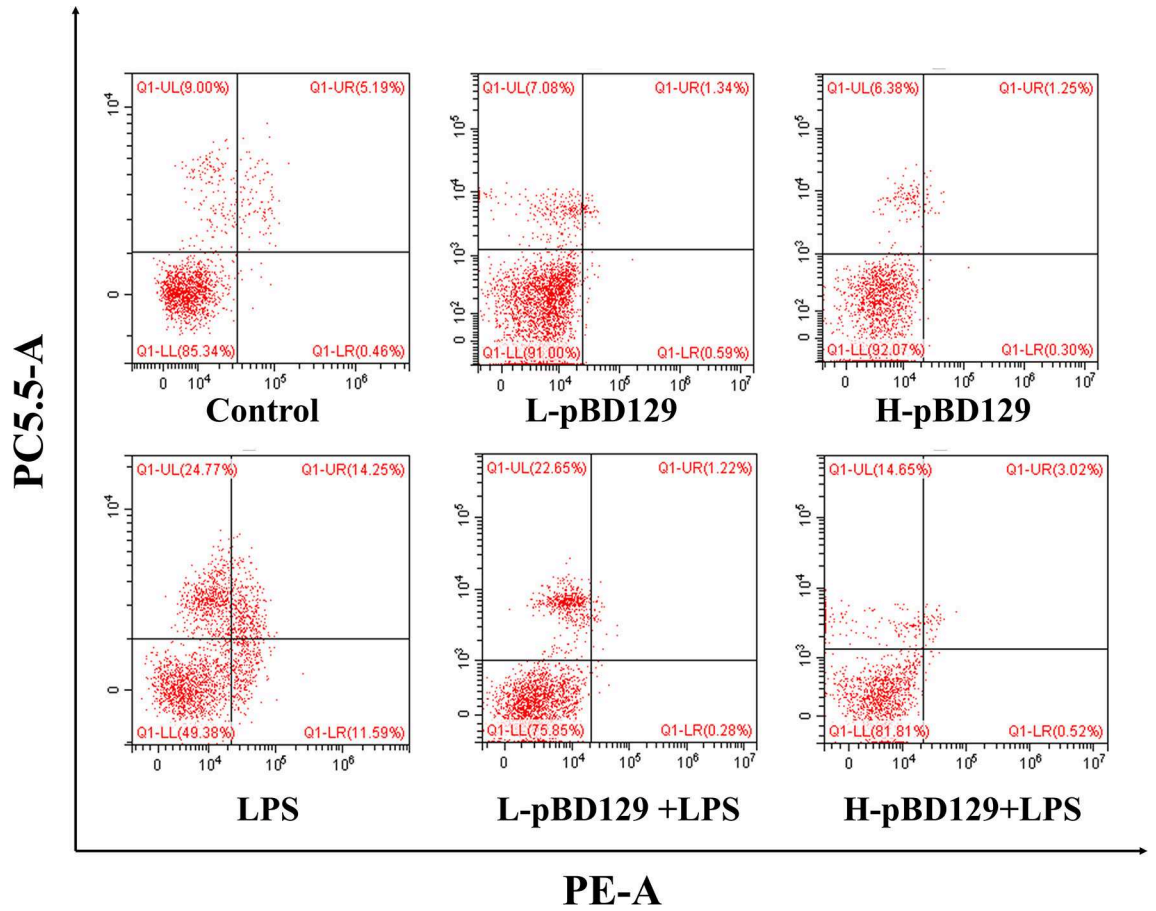


**FIGURE 4 |** Percentage of apoptotic cells in the jejunal mucosal of mouse. Frames were divided into four quadrants: Q1–UL represents necrotic cells. Q1–UR represents late-stage apoptotic cells. Q1–LL represents normal cells. Q1–LR represents early-stage apoptotic cells. Control, 200 μL sterile saline; LPS, 200 μL Lipopolysaccharide; L-pBD129, 200 μL of 0.6 mg/ml porcine β-defensin; H-pBD129, 200 μL of 1.2 mg/mL porcine β-defensin 129; L-pBD129 + LPS, 200 μL of 0.6 mg/mL pBD129 pretreated followed by LPS treated; H-pBD129 + LPS, 200 μL of 1.2 mg/mL pBD129 pretreated followed by LPS treated. $n = 3$/group.

of the tight junction. In contrast, the abundance of ZO-1 protein was significantly elevated and localized to the apical intercellular region of the intestinal epithelium in mice treated by pBD129.

## Effect of pBD129 on Intestinal Epithelium Cell Apoptosis

We found that necrotic apoptosis in the intestinal mucosa was significantly changed after LPS challenge (**Figure 4**). As compared to the control group, LPS challenge has resulted in elevated necrotic apoptosis in the intestinal mucosa. In contrast, the necrotic apoptosis was significantly decreased in the intestinal epithelium in mice treated by pBD129. In addition, as shown in **Figure 4** and **Table 4**, LPS challenge significantly increased the percentage of the early-stage apoptotic cells and the total apoptotic cells in the intestinal mucosa ($P < 0.05$). However, pBD129 significantly reduced the percentage of the early-stage apoptotic cells and the total apoptotic cells in the LPS-challenged mice ($P < 0.05$). Interestingly, the caspase-3 and caspase-9 activities in the small intestine were measured and, as shown in **Table 5**, LPS challenge significantly increased the activity of caspase 3 and 9. However, pBD129 reduced their activities in the LPS-challenged mice ($P < 0.05$).

## Effect of pBD129 on Critical Genes Related to Inflammatory Response, Intestinal Barrier Functions, and Cell Apoptosis

As shown in **Figure 5**, LPS challenge significantly elevated the expression levels of inflammatory cytokines such as the IL-6, IL-1β, and TNF-α in the small intestine ($P < 0.01$). However, pBD129 significantly decreased their expression levels in the LPS-challenged mice ($P < 0.01$). The expression levels of critical tight junction proteins such as the ZO-1, Occludin, and Claudin-2 were determined. As shown in **Figure 6**, LPS challenge has resulted in down-regulation of ZO-1 and Occludin in the small intestine ($P < 0.01$). However, pBD129 significantly elevated their expression levels in the duodenum and jejunum mucosa. In contrast, pBD129 decreased the expression level of Claudin-2 in the small intestine ($P < 0.01$). We also investigated the expression levels of critical apoptotic-related

**TABLE 4 |** Effects of Porcine β-defensin 129 on the Jejunal mucosal apoptosis of mice[1].

| Item[2] | Treatment[3] | | | | | | *P*-value[4] | | |
|---|---|---|---|---|---|---|---|---|---|
| | Control | L-129 | H-129 | LPS | L-129+LPS | H-129+LPS | B | V | B*V |
| EP | 0.92 ± 0.26$^{b}$ | 1.60 ± 0.63$^{b}$ | 0.21 ± 0.05$^{b}$ | 7.81 ± 2.22$^{a}$ | 1.28 ± 0.91$^{b}$ | 0.51 ± 0.12$^{b}$ | 0.0056 | 0.0177 | 0.0071 |
| LP | 5.70 ± 0.26 | 2.07 ± 1.33 | 1.36 ± 0.06 | 7.81 ± 2.22 | 2.91 ± 1.33 | 4.63 ± 1.32 | 0.0132 | 0.0768 | 0.6580 |
| TP | 6.62 ± 0.51$^{b}$ | 3.67 ± 1.26$^{b}$ | 1.58 ± 0.03$^{b}$ | 21.23 ± 2.49$^{a}$ | 4.18 ± 1.37$^{b}$ | 5.15 ± 1.22$^{b}$ | <0.0001 | 0.0001 | 0.0006 |

*Different lowercase letters indicate statistically significant differences from each other ($P < 0.05$).*
*[1]Values are the means of 3 replicates per treatment.*
*[2]EP, Early-stage apoptotic cell percentage; LP, Late-stage apoptotic cell percentage; TP, Total apoptotic cell percentage.*
*[3]Control, 200 μL sterile saline; LPS, 200 μL Lipopolysaccharide; L-pBD129, 200 μL of 0.6 mg/ml porcine β-defensin; H-pBD129, 200 μL of 1.2 mg/mL porcine β-defensin 129; L-pBD129 + LPS, 200 μL of 0.6 mg/mL pBD129 pretreated followed by LPS treated; H-pBD129 + LPS, 200 μL of 1.2 mg/mL pBD129 pretreated followed by LPS treated.*
*[4]B is the main effect of Porcine β-defensin 129; V is the main effect of LPS infection; B*V is the interaction effect of the two main factors.*

**TABLE 5 |** Effects of Porcine β-defensin 129 on the intestinal cysteinyl aspartate-specific protease activity of mice[1].

| Item[2] | Treatment[3] | | | | | | *P*-value[4] | | |
|---|---|---|---|---|---|---|---|---|---|
| | Control | L-129 | H-129 | LPS | L-129+LPS | H-129+LPS | B | V | B*V |
| Duodenum | | | | | | | | | |
| Cas-3, U/μg | 178.84 ± 9.49$^{c}$ | 177.81 ± 9.98$^{c}$ | 187.37 ± 0.74$^{c}$ | 555.12 ± 26.42$^{a}$ | 463.69 ± 33.86$^{ab}$ | 377.76 ± 4.54$^{b}$ | <0.001 | 0.002 | 0.001 |
| Cas-9, U/μg | 292.39 ± 26.81$^{c}$ | 282.09 ± 58.13$^{c}$ | 273.20 ± 43.58$^{c}$ | 831.71 ± 22.22$^{a}$ | 761.70 ± 55.09$^{ab}$ | 569.64 ± 50.40$^{b}$ | <0.001 | 0.02 | 0.05 |
| Jejunum | | | | | | | | | |
| Cas-3, U/μg | 701.59 ± 30.64$^{b}$ | 688.20 ± 17.36$^{b}$ | 813.20 ± 83.49$^{ab}$ | 1268.81 ± 229.91$^{a}$ | 779.77 ± 82.94$^{ab}$ | 736.09 ± 33.63$^{ab}$ | 0.05 | 0.08 | 0.03 |
| Cas-9, U/μg | 466.25 ± 10.51$^{b}$ | 494.21 ± 16.43$^{b}$ | 516.11 ± 15.09$^{b}$ | 708.94 ± 41.81$^{a}$ | 530.32 ± 53.65$^{b}$ | 503.62 ± 13.17$^{b}$ | 0.004 | 0.04 | 0.003 |
| Ileum | | | | | | | | | |
| Cas-3, U/μg | 312.39 ± 62.77$^{bc}$ | 193.70 ± 19.08$^{c}$ | 189.52 ± 7.00$^{c}$ | 630.45 ± 53.94$^{a}$ | 459.95 ± 40.12$^{ab}$ | 291.49 ± 38.29$^{bc}$ | <0.001 | <0.001 | 0.06 |
| Cas-9. U/μg | 652.58 ± 77.00$^{bc}$ | 427.55 ± 34.24$^{c}$ | 479.06 ± 53.22$^{c}$ | 1038.46 ± 92.64$^{a}$ | 848.28 ± 31.35$^{ab}$ | 694.09 ± 27.73$^{bc}$ | <0.001 | 0.002 | 0.21 |

*Different lowercase letters indicate statistically significant differences from each other ($P < 0.05$).*
*[1]Values of the Cas-3 and Cas-9 are 3 replicates per treatment.*
*[2] Cas-3, Cysteinyl aspartate-specific protease-3; Cas-9, Cysteinyl aspartate-specific protease-9.*
*[3]Control, 200 μL sterile saline; LPS, 200 μL Lipopolysaccharide; L-pBD129, 200 μL of 0.6 mg/ml porcine β-defensin; H-pBD129, 200 μL of 1.2 mg/mL porcine β-defensin 129; L-pBD129 + LPS, 200 μL of 0.6 mg/mL pBD129 pretreated followed by LPS treated; H-pBD129 + LPS, 200 μL of 1.2 mg/mL pBD129 pretreated followed by LPS treated.*
*[4]B is the main effect of porcine β-defensin 129; V is the main effect of LPS infection; B*V is the interaction effect of the two main factors.*

genes. As shown in **Figure 7**, LPS challenge down-regulated the expression of Bcl-2, but significantly elevated the expression levels of apoptotic genes such as the Bad, Bid, and Bax in the small intestinal mucosa ($P < 0.05$). However, pBD129 not only elevated the expression of Bcl-2, but also down-regulated the expression levels of the three critical apoptotic genes ($P < 0.05$). Moreover, LPS challenge resulted in up-regulation of caspase-3 and caspase-9 in the small intestine ($P < 0.05$). However, pBD129 significantly decreased their expression levels in the LPS-challenged mice ($P < 0.05$).

## DISCUSSION

Apoptosis of intestinal epithelial cells induced by pathogens disrupts intestinal barrier functions (23). In recent years, the β-defensins has attracted considerable research interest since it has been reported to play a critical role in the modulating the adaptive immunity and improving the intestinal barrier functions (24, 25). The pBD129 is a newly discovered porcine beta-defensin, which is highly expressed in the epithelial cells of the gastrointestinal mucosa (26, 27). In this study, we explored the role of pBD129 in regulating the inflammatory responses and intestinal epithelium barrier functions in mice.

The pBD129 was successfully expressed in *E. coli* BL21 (DE3) and the soluble proteins in the periplasmic space were purified. A significant degree of overlap (82%) was observed between the proteins identified in the LC-MS/MS data sets, indicating that the purified protein was porcine β-defensin 129. Antimicrobial activity assays showed that pBD129 has significant antimicrobial activity against the gram-positive bacteria (*Streptococcus*) and gram-negative bacteria (*E. coli* DH5α). The result is also consistent with previous studies on the porcine β-defensins (28, 29). Both indicated that the porcine β-defensins has a broad antibacterial spectrum. Moreover, we found that the pBD129 has a weak hemolytic activity, indicating that it is harmless to humans and animals, and may be tentatively used as a substitute for conventionally used antibiotics.

Lipopolysaccharide (LPS) is an important structural component of the outer membrane of gram-negative bacteria which triggers the systemic inflammation and induces damage of target organs such as kidneys, liver, and intestinal mucosa (30).

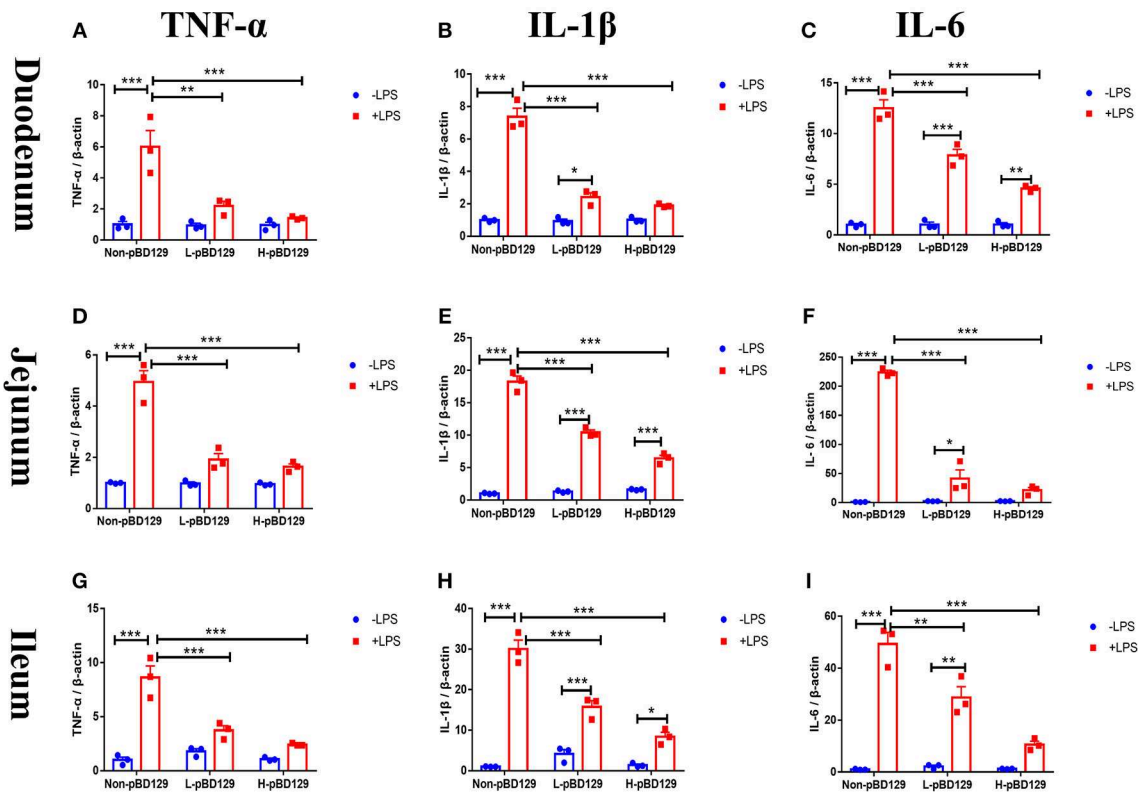


**FIGURE 5 |** Real time PCR analysis of TNF-α **(A)**, IL-1β **(B)**, IL-6 **(C)** mRNA abundance in Duodenum; Real time PCR analysis of TNF-α **(D)**, IL-1β **(E)**, IL-6 **(F)** mRNA abundance in Jejunum; Real time PCR analysis of TNF-α **(G)**, IL-1β **(H)**, IL-6 **(I)** mRNA abundance in Ileum. IL-1β, interleukin-1β; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α. Non-pBD129, 200 μL Sterilized saline; L-pBD129, 200 μL of 0.6 mg/ml porcine β-defensin 129; H-pBD129, 200 μL of 1.2 mg/ml porcine β-defensin 129. A-I, $n = 3$/group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Results are given as means ± SEM. Two-way ANOVA followed by Bonferroni's multiple comparisons test.

In the present study, the serum concentrations of inflammatory cytokines such as the IL-1β, IL-6, and TNF-α were both elevated upon LPS challenge, indicating the success of model construction. Interestingly, pBD129 treatment significantly decreased the serum concentrations of these inflammatory cytokines which suggested the β-defensins may act as a negative regulator for inflammatory responses. This result is consistent with previous studies on a variety of animal species (31, 32). Moreover, pBD129 treatment at a high dose (8 mg/kg) significantly decreased the serum concentrations of ALT, CRP, Cre, and urea, which has been widely used as biological markers of kidney and hepatic functionality (33). Additionally, pBD129 treatment (8 mg/kg) significantly increased the serum IgG concentration, which is consistent with previous findings that the β-defensins can act as a positive immune regulator for animals (34).

The intestinal epithelium provides a protective barrier, preventing both pathogenic, and commensal bacteria from escaping from the intestinal lumen. But some enteric pathogens can induce permeability defects in gut epithelia by altering tight junction proteins, which allows the translocation of toxins via the mucosa to access the whole body, subsequently destroying the intestinal mucosal homeostasis (35, 36). Disruption of the intestinal epithelium impairs the nutrient digestion and absorption (37). In the present study, LPS challenge significantly decreased the villus height in the small intestine. However, pBD129 significantly elevated the villus height in the LPS-challenged mice. This is probably due to the reduced inflammatory cytokines, since the IL-1β, IL-6, and TNF-α were found to induce atrophy of intestinal mucosa and disruption of intestinal functions (38–40). The DAO is a catalytic enzyme which is mainly synthesized in the digestive tract and involved in the metabolism, oxidation, and inactivation of histamine and other polyamines such as putrescine and spermidine in animals (41). Importantly, the serum DAO concentration has been widely used as a biomarker of the intestinal permeability since it can be released into the blood circulation (42). In the present study, LPS challenge significantly elevated the serum DAO concentration, indicating the disruption of the intestinal epithelium barriers. However, pBD129 treatment at 8 mg/kg significantly decreased the serum DAO concentration in LPS-challenge mice, indicating a protective effect of the β-defensins on intestinal mucosal integrity.

The intestinal epithelial cells (IECs) are connected in the lateral membrane by forming the tight junction (TJs) (43). TJs are mainly composed of cytoplasmic scaffold proteins such as ZO-1,

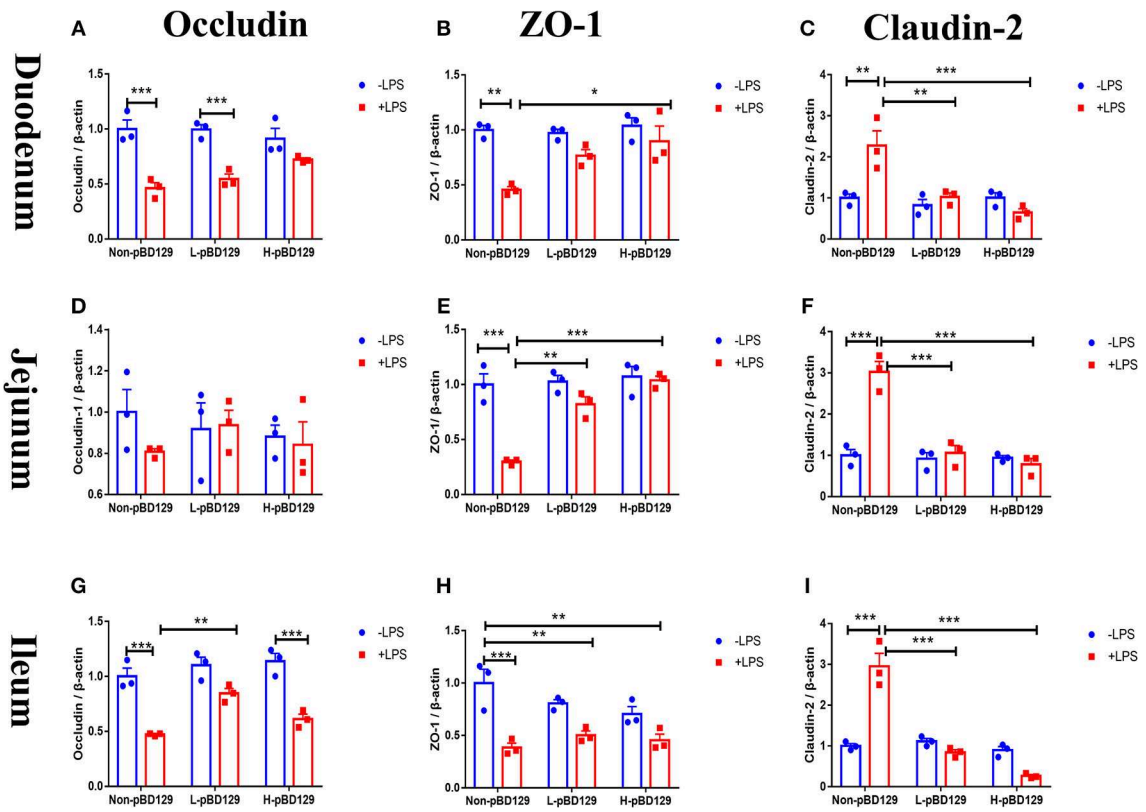


**FIGURE 6 |** Determination of relative changes in gene expression of TJ proteins in duodenum by real-time PCR analysis. **(A)** Occludin, **(B)** ZO-1, **(C)** Claudin-2. Determination of relative changes in gene expression of TJ proteins in Jejunum by real-time PCR analysis. **(D)** Occludin, **(E)** ZO-1, **(F)** Claudin-2; Determination of relative changes in gene expression of TJ proteins in Ileum by real-time PCR analysis. **(G)** Occludin, **(H)** ZO-1, **(I)** Claudin-2. ZO-1, Zonula occludens-1. A-I, $n$ = 3/group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Results are given as means ± SEM. Two-way ANOVA followed by Bonferroni's multiple comparisons test.

transmembrane proteins including claudins, and attachment adhesion molecules (JAM) (5), which controls the paracellular permeability of small molecules (44). Previous studies have indicated that inflammatory stress (i. LPS challenge) significantly decreased the abundance of TJ proteins (45, 46). A similar result was observed in the present study. However, we found that the abundance of ZO-1 protein was significantly elevated and localized to the apical intercellular region of the intestinal epithelium in mice after pBD129 treatment. The result is consistent with a previous study on porcine beta-defensin-2 (PBD-2) in DSS-treated mouse model.

Apoptosis is a form of physiological cell death that is important for the renewal of intestinal mucosa cells. In severe intestinal pathology, breakdown of intestinal mucosa via accelerated apoptosis increases intestinal permeability (47, 48). Previous studies have indicated that infections or stresses can increase intestinal epithelial cell apoptosis (49, 50). In the present study, LPS challenge increased the percentage of the apoptotic cells in the intestinal mucosa. However, pBD129 treatment significantly reduced the percentage of the early-stage apoptotic cells and the total apoptotic cells in the intestinal mucosa from LPS-challenged mice. This is also probably due to the decreased inflammatory cytokines after pBD129 treatment, since the IL-1β and TNF-α were found to induce apoptosis via intrinsic mitochondrial apoptotic pathway (51–53).

To gain insights into the mechanisms behind the pBD129 modulated intestinal barrier functions, we explored the expression levels of some critical molecules involved in the regulation of inflammatory response and apoptosis. Interestingly, the pBD129 was found to significantly decrease the expression levels of several critical inflammatory cytokines (i.e., IL-1β, IL-6, and TNF-α) and tight junction proteins (i.e., ZO-1 and Occludin) in the intestinal mucosa. The result is consistent with previous studies using different animal species (54, 55). The Bcl-2 is localized to the outer membrane of mitochondria, where it plays a critical role in promoting cellular survival and inhibiting the actions of pro-apoptotic proteins (56). In the present study, pBD129 treatment significantly elevated the expression levels of Bcl-2 and down-regulated the expression levels of critical apoptotic genes (Bad, Bid, Bax, caspase-3, and caspase-9) in the intestinal mucosa of LPS-challenged mice. The Bad, Bid, and Bax contributed to programmed cell death by inducing mitochondrial cytochrome *c* release, which activates caspase-9 and then caspase-3 (57, 58). The caspase-3 and caspase-9 are responsible for executing cell death during the demolition phase of apoptosis (59, 60), and we also found that pBD129 treatment significantly

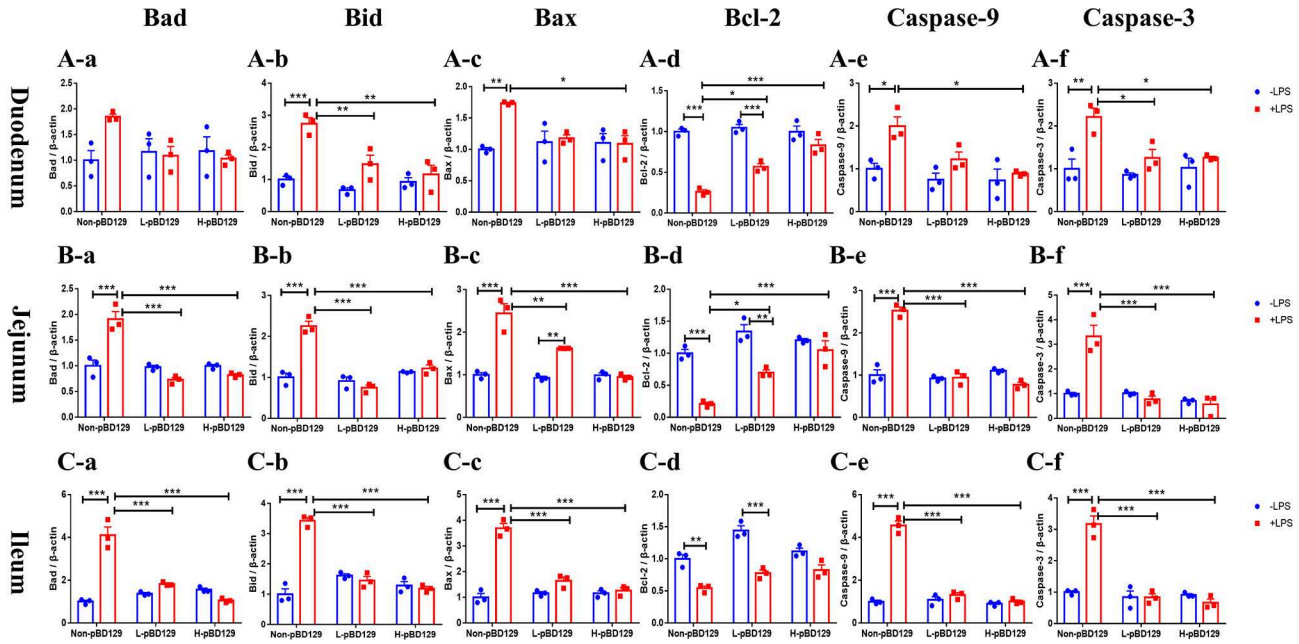


**FIGURE 7 |** Real time PCR analysis of Bad **(A-a)**, Bid **(A-b)**, Bax **(A-c)**, Bcl-2 **(A-d)**, Caspase-9 **(A-e)**, Caspase-3 **(A-f)** mRNA abundance in duodenum; Real time PCR analysis of Bad **(B-a)**, Bid **(B-b)**, Bax **(B-c)**, Bcl-2 **(B-d)**, Caspase-9 **(B-e)**, Caspase-3 **(B-f)** mRNA abundance in jejunum; Real time PCR analysis of Bad **(C-a)**, Bid **(C-b)**, Bax **(C-c)**, Bcl-2 **(C-d)**, Caspase-9 **(C-e)**, Caspase-3 **(C-f)** mRNA abundance in Ileum. Bad, Bcl-2 antagonist of cell death; BAX, B-cell lymphoma-2-associated X protein; Bid, BH3-interacting domain death agonist; BCL2, B-cell lymphoma-2; caspase-3, cysteinyl aspartate-specific proteinase-3; caspase-9, cysteinyl aspartate-specific proteinase-9. **(A-a)–(C-f)**, $n = 3$/group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Results are given as means ± SEM. Two-way ANOVA followed by Bonferroni's multiple comparisons test.

reduced the caspase-3 and caspase-9 activities in the small intestine of LPS-challenged mice. For all gene expression experiments (**Figures 5**–7), pBD129 has no dose-dependent effect, probably because the range of dose selection is not large, and a broad range of doses could be considered in the further study. In the present study, pBD129 significantly decreased the expression levels of caspase-3 and caspase-9 in the intestinal mucosa of LPS-challenged mice. These results offer a molecular basis for the pBD129 mediated cell apoptosis in the intestinal mucosa.

In conclusion, the pBD129 attenuates bacterial endotoxin-induced inflammatory responses and intestinal mucosa atrophy by reducing the secretion of inflammatory cytokines and the apoptosis of intestinal epithelial cells. Our results suggested a novel function of the mammalian defensins, and the anti-bacterial and anti-inflammatory properties of pBD129 may allow it a potential agent to prevent or alleviate the LPS-induced inflammation and damage of the intestinal epithelium barriers.

## DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the manuscript/**Supplementary Files**.

## ETHICS STATEMENT

This study was approved by the Animal Welfare Committee of Sichuan Agricultural University (No. 20180718).

## AUTHOR CONTRIBUTIONS

KX and HX performed most of the experiments. GS conducted the preparation of the pBD129 protein experiment. KX was also in charge of preparing the manuscript. JH contributed to study design and revised the manuscript. DC, BY, XM, ZH, JY, JL, PZ and YL contributed to the sample collection.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02333/full#supplementary-material

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# Maturation of Gut Microbiota and Circulating Regulatory T Cells and Development of IgE Sensitization in Early Life

*Terhi Ruohtula[1], Marcus C. de Goffau[2], Janne K. Nieminen[1], Jarno Honkanen[1], Heli Siljander[3], Anu-Maaria Hämäläinen[4], Aleksandr Peet[5], Vallo Tillmann[6], Jorma Ilonen[5], Onni Niemelä[7], Gjalt W. Welling[2], Mikael Knip[1,3,8,9,10], Hermie J. Harmsen[2] and Outi Vaarala[1]**

[1] Clinicum, University of Helsinki, Helsinki, Finland, [2] Department of Medical Microbiology, University Medical Center Groningen, University of Groningen, Groningen, Netherlands, [3] Children's Hospital, Helsinki University Hospital, University of Helsinki, Helsinki, Finland, [4] Department of Pediatrics, Jorvi Hospital, Helsinki University Hospital, Espoo, Finland, [5] Immunogenetics Laboratory, Institute of Biomedicine, University of Turku, Turku, Finland, [6] Department of Pediatrics, Tartu University Hospital, University of Tartu, Tartu, Estonia, [7] Department of Laboratory Medicine and Medical Research Unit, Seinäjoki Central Hospital and University of Tampere, Seinäjoki, Finland, [8] Research Programs Unit, Diabetes and Obesity, University of Helsinki, Helsinki, Finland, [9] Folkhälsan Research Center, Helsinki, Finland, [10] Tampere Center for Child Health Research, Tampere University Hospital, Tampere, Finland

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**Edited by:**
Julien Diana,
Institut National de la Santé et de la Recherche Médicale (INSERM), France

**Reviewed by:**
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United States Department of Agriculture, United States
Knut Rudi,
Norwegian University of Life Sciences, Norway

***Correspondence:**
Outi Vaarala
outi.vaarala@helsinki.fi

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Recent studies suggest that the cross-talk between the gut microbiota and human immune system during the first year of life is an important regulator of the later development of atopic diseases. We explored the changes in the gut microbiota, blood regulatory T cells, and atopic sensitization in a birth-cohort of Estonian and Finnish children followed from 3 to 36 months of age. We describe here an infant Treg phenotype characterized by high Treg frequency, the maturation of Treg population characterized by a decrease in their frequency accompanied with an increase in the highly activated Treg cells. These changes in Treg population associated first with the relative abundance of Bifidobacterium longum followed by increasing colonization with butyrate producing bacteria. High bifidobacterial abundance in the neonatal microbiota appeared to be protective, while colonization with *Bacteroides* and *E. coli* was associated with later risk of allergy. Estonian children with lower risk of IgE mediated allergic diseases than Finnish children showed an earlier maturation of the gut microbiota, detected as earlier switch to an increasing abundance of butyrate-producing bacteria, combined with an earlier maturation of Treg cell phenotype and total IgE production. The children with established allergic diseases by age 3 showed a decreased abundance of butyrate producing *Faecalibacterium*. These results suggest that as well as the maintenance of a bifidobacterial dominated gut microbiota is important during the first weeks of life, the overtake by butyrate producing bacteria seems to be a beneficial shift, which should not be postponed.

**Keywords: regulatory T-cells, bifidobacteria, gut microbiome, atopic diseases, IgE**

## INTRODUCTION

Our understanding of the cross-talk between the gut microbiota and human immune system during the time when allergic sensitization develops is limited, although accumulating evidence indicates that the gut microbiome plays a pivotal role in the regulation of allergic immune responses (1–8). The composition of the gut microbiome is determined by our microbial environment, dietary factors, and genetic background (9). The evidence of the mechanisms of the gut microbiome as a regulator of the immune response is largely based on studies in animal models showing e.g., that the gut microbiome directly or indirectly via metabolites is able to modulate the permeability of the intestinal epithelium (10–12), and the differentiation and function of effector and regulatory T cells (Treg cells) (13–21), which could further influence the development of immune-mediated diseases, including allergic, inflammatory, and autoimmune diseases. In prospective studies of children, indirect *in vitro* based evidence suggests that metabolites of the gut microbiome from the infants with high risk of atopic diseases could indeed modulate Treg cell phenotype (6). Arrieta et al. showed that reduced levels of fecal acetate and dysregulation of enterohepatic metabolites was accompanied with the gut microbiome changes predicting the risk of asthma in CHILD cohort (5). Thus, although the altered gut microbiota composition has been associated with allergic responses in children, the understanding of the mechanisms linking the gut microbiota and altered immune deviation is limited in humans.

To investigate the relationship between the development of the intestinal microbiota, circulating Treg cells, and IgE sensitization against environmental allergens, we obtained repeated blood and feces samples during the first 3 years of life from a cohort of infants living in Estonia or Finland, the neighboring countries with distinct differences in the standard of living[1,2] and incidence of allergic diseases (e.g., 12 month prevalence of asthma 9.3 vs. 19.0 %) (22, 23). We found that the composition of the neonatal gut microbiota associated with later risk of allergic sensitization and allergic diseases. Our study further shows that the maturation of the circulating Treg cells included an increase in the highly activated Treg cells, which was associated with the relative abundance of *Bifidobacterium longum* and colonization with butyrate producing bacteria, and this maturation process of the gut microbiota and Treg cells was delayed in Finnish children with a higher risk of IgE mediated allergic sensitization and diseases in comparison to Estonian children.

## MATERIALS AND METHODS

### Study Subjects

We studied a subgroup of Estonian and Finnish children participating in the DIABIMMUNE (Pathogenesis of type 1 diabetes: testing the hygiene hypothesis) study (Estonia; $n = 85$; 43/42 and Finland; $n = 76$; 42/34 male/female). These index cases carried HLA conferred genetic risk for type 1 diabetes and celiac disease as previously described (24) High-risk genotype (DRB1*03-DQA1*05-DQB1*02 and DRB1*0401/2/4/5-DQA1*03-DQB1*03:02 haplotypes) was found in 12, moderate-risk genotypes in 52 (either one of the high-risk haplotypes or, DRB1*04:01/2/5-DQA1*03-DQB1*03:02 with a neutral haplotype), slightly increased risk genotypes in 92 (either the DRB1*04:04-DQA1*03-DQB1*03:02 or DRB1*03-DQA1*05-DQB1*02 haplotype with a neutral haplotype), and neutral haplotypes in 5 children. Neutral haplotypes are all other except the protection associated haplotypes DQB1*02, 03:01 or 06:02, or any of the risk haplotypes. Of all the infants 74 Estonian and 71 Finnish children (38/36; 40/31 male/female) were born vaginally. The present study was conducted according to the guidelines of the Declaration of Helsinki, and was approved by the ethical committees of both study centers, and written informed consent was obtained from the children's parents. The number of study subjects and samples analyzed in the different assays are given in **Supplementary Table 1**.

### Flow Cytometry Analysis of Circulating Regulatory T-Cells

We analyzed the phenotype of peripheral blood regulatory T-cells in fresh heparinized blood samples obtained at the age of 3, 6, 12, 24, and 36 months (**Supplementary Table 1**). At least $1 \times 10^6$ events were acquired from each sample on a FACSCalibur™ and analyzed with the FlowJo™ software. The samples were compensated post acquisition with FlowJo™ software. The monoclonal antibodies are shown in **Supplementary Table 2**. To assess the number of circulating CD4+CD25highFOXP3+ T cells in the samples, we gated first CD4+ cells, and then the CD25+CD127−/lo population. The expression of FOXP3 protein was analyzed in these cell populations. CD4+CD25highFOXP3+ expression was quantified as median fluorescence intensity (MFI) in arbitrary units (AU) after subtraction of the negative-control antibody intensity. Intensity values over the 97.5 percentile of the negative-control antibody were regarded as positive. Intensities were calibrated to a set of particles containing known amounts of fluorescein isothiocyanate.

### Gene Expression Based Phenotypic Analysis of Circulating Regulatory T-Cells

We performed RT-qPCR analysis of enriched circulating regulatory T-cells at 6, 18, and 36 month samples (**Supplementary Table 1**). The product information for the reagents used is shown in **Supplementary Table 2**. Enrichment of Treg cells was made according to the manufacturer's instructions using MACS CD25+CD49d− magnetic beads with the exception that the magnetic labeling of CD25+ regulatory T cells, which was titrated to 2.5 µl of CD25 MicroBeads II per $10^7$ total cells for improved cell purity. In addition, the second enrichment elution with the MS column was omitted as it resulted in too high yield loss, resulting in mean 87.7% purity of the enriched Treg cells. An additional wash with PBS was added before the pellets were frozen at −70°C in RLT-lysis buffer for subsequent RT-qPCR analysis. Total RNA from purified Tregs was isolated with the Qiagen RNeasy Plus Micro kit according to the manufacturer's instructions. cDNA

[1]http://apps.who.int/nha/database
[2]http://wdi.worldbank.org

was synthesized using the random hexamer priming of the High Capacity cDNA Reverse Transcription kit according to the manufacturer's instructions. Real time PCR was performed with TaqMan Fast master Mix and StepOne Plus instrument. TaqMan Gene Expression Assays were used for real time PCR amplification of FOXP3, TGF-beta1, Helios, GATA-3, CTLA-4, IL-10, and IFN-γ. The endogenous reference used was the gene ribosomal 18S. To analyze the relative amount of mRNA of the gene of interest a comparative ΔΔ cycle threshold (Ct) method was used. An in-house control sample (calibrator sample) was used to control inter-assay variation. The calibrator sample was prepared from the phytohaemagglutinin-stimulated human PBMC derived RNA.

## Total and Allergen-Specific IgE Analysis

Total IgE and allergen-specific IgE concentrations were analyzed from serum samples (**Supplementary Table 1**) at the age of 6, 18, and 36 months by using the ImmunoCAP fluoroenzyme immunoassay (**Supplementary Table 2**). IgE to egg, cow's milk, house dust mite, cat, timothy grass, and birch, as well as total IgE concentrations were analyzed at the age of 6 months. Peanut was added to the panel at the age of 18 months and dog at the age of 36 months. The children were classified into four groups based on their clinical allergy diagnosis and allergen-specific IgE values: (A) No signs of allergy (no clinical diagnosis, no specific IgE responses); (B) IgE-sensitization during the study period, up to 36 months of age (no clinical diagnosis, at least one specific IgE response); (C) sensitized, up to the time point analyzed (no clinical diagnosis, at least one specific IgE response); (D) clinical non-IgE allergy [clinical diagnosis, no specific IgE response (25)]; and (E) clinical IgE allergy (clinical diagnosis and a specific IgE response to the same allergen).

## Analysis of Microbiota

Total DNA was extracted from a 0.25 g fecal sample using the repeated bead beating method described in detail by Yu and Morrison (26), with a number of modifications. In brief, four 3 mm instead of 0.5 mm glass beads were added during the homogenization step. Bead beating was performed using a Precellys 24 at 5.5 beats per millisecond in three rounds of 1 min each with 30 s pauses at room temperature in between. The incubation temperature after the bead beating was raised from 70 to 95°C. Importantly, protein precipitation with 260 μl of ammonium acetate was carried out twice instead of only once. Elution of DNA from the purification columns was done twice. Columns from the QiaAmp Stool Kit were replaced by those from the QIAamp DNA Stool Mini Kit. The V3–V4 region of the 16S rRNA gene was amplified from the fecal DNA by polymerase chain reaction (PCR) using modified 341F and 806R primers with a 6 nucleotide barcode on the 806R primer. The sequence of the 341F primer and the 806R primer was aatgatacggcg accaccgagatctacactctttccctacacgacgctcttccgatctNNNNCCTACG GGAGGCAGCAG & caagcagaagacggcatacgagat**CGTGAT**gtgact ggagttcagacgtgtgctcttccgatctGGACTACHVGGGTWTCTAAT, respectively, where lowercase letters denote adapter sequences necessary for binding to the flow cell, underlined lowercase are binding sites for the Illumina sequencing primers, bold uppercase highlight the index sequences as reported by Bartram et al. (27) and regular uppercase are the V3–V4 region primers (341F on for the forward primers and 806R for the reverse primers). The inclusion of four maximally degenerated bases ("NNNN") maximizes diversity during the first four bases of the run. Reaction conditions consisted of an initial 94°C for 3 min followed by 32 cycles of 94°C for 45 s, 50°C for 60 s, and 72°C for 90 s, and a final extension of 72°C for 10 min. An agarose gel confirmed the presence of product (band at ∼465 base pairs) in successfully amplified samples. The remainder of the PCR product (∼45 μl) of each sample was mixed thoroughly with 25 μl Agencourt AMPure XP magnetic beads and were incubated at room temperature for 5 min. Beads were subsequently separated from the solution by placing the tubes in a magnetic bead separator for 2 min. After discarding the cleared solution the beads were washed twice by resuspending the beads in 200 μl freshly prepared 80% ethanol, incubating the tubes for 30 s in the magnetic bead separator and subsequently discarding the cleared solution. The pellet was subsequently air dried for 15 min and resuspended in 52.5 μl 10 mM Tris HCl pH 8.5 buffer. Fifty microliter of the cleared up solution is subsequently transferred to a new tube. The DNA concentration of each sample was measured using a Qubit® 2.0 fluorometer and the remainder of the sample was stored at −20°C until library normalization. Library normalization was done the day before running samples on the MiSeq by making 2 nM dilutions of each sample. Samples were pooled together by combining 5 μl of each diluted sample. Ten microliter of the sample pool and 10 μl 0.2 M NaOH were subsequently combined and incubated for 5 min to denature the sample DNA. To this 980 μl of the HT1 buffer from the MiSeq 2 × 300 kit was subsequently added. A denatured diluted PhiX solution was made by combining 2 μl of a 10 nM PhiX library with 3 μl 10 mM Tris HCl pH 8.5 buffer with 0.1% Tween 20. These 5 μl were mixed with 5 μl 0.2 M NaOH and incubated for 5 min at room temperature. The resulting 10 μl were subsequently mixed with 990 μl HT1 buffer. One hundred and fifty microliter of the diluted sample pool is combined with 50 μl of the diluted PhiX solution and was further diluted by adding 800 μl HT1 buffer. Six hundred microliter of the prepared library was loaded into the sample-loading reservoir of the MiSeq 2 × 300 cartridge.

## Statistical Analysis

MiSeq sequencing pipeline and statistical analysis Software that was used to analyze the data received from Illumina paired-end sequencing, included PANDAseq (28), QIIME (29) and ARB (30). Paired-end reads that were shorter than 400 BP or longer than 500 BP were discarded by PANDAseq. Statistical analyses were performed on the family, genus and species level. QIIME identified sequences down to the Family and Genus level while ARB was used for most genera (31, 32), as is typically possible for most fecal bacteria using 16S (31, 32), to identify sequences down to the species level as earlier described by de Goffau et al. (33, 34) using the latest SILVA reference database (35). Importantly, species level resolution was achieved for species from the *Bifidobacterium* genus. Principal component analysis (PCA) was performed to find clusters of similar groups of samples or species.

PCA is an ordination method based on multivariate statistical analysis that maps the samples into a reduced number of relevant dimensions of variability. The hierarchical clustering analysis was performed with the Hierarchical Clustering Explorer 3.5. The Simpson index was used as a measure of microbial diversity. Non-parametric tests were used, as microbial abundances are rarely normally distributed.

The Mann-Whitney *U*-test, Spearman correlation analysis (rs), the Wilcoxon or $\chi^2$ tests were used as indicated. The use $\pm$ indicates that a standard deviation is given. All tests were two-tailed and a $p < 0.05$ was considered to indicate statistical significance. All statistical analyzes were performed using IBM® SPSS® Statistics 20.0. For the analysis of Flow cytometry data Graph Pad Prism 5 software was used.

# RESULTS

## Maturation Steps of Treg Cells During the First 3 Years of Life

To investigate the early maturation of Treg cells we followed the characteristics of blood Treg cells at the ages of 3, 6, 12, 24, and 36 months in the Estonian and Finnish children. During the first year of life, the numbers of blood Tregs (CD4+CD127−/loCD25high) as well as their FOXP3 protein expression was high, a drop occurred in the relative numbers of Tregs and their FOXP3 expression after 12 months of age (**Figures 1A,B**). At the same time when the frequency of blood Treg cells decreased, the proportion of highly activated TregFOXP3high cells increased (**Figures 1C,D**), particularly in the Finnish children (**Figure 1D**). Similarly, the expression of FOXP3, Helios, TGF-beta1, CTLA-4, and GATA-3 transcripts in Tregs decreased from 6 to 18 months and then increased to 36 months (**Figure 1E**). Our results revealed a two-step maturation process in the circulating Treg cells: a decrease in the proportion of Treg cells followed by an increase in the highly activated Treg cells, which are reported to show enhanced suppressive activity (36–40).

## Bifidobacterial Composition at 3 Months Shapes Treg Cells

We hypothesized that gut colonization could drive the changes in the Treg cell population. In 16S rDNA sequencing of fecal samples, bifidobacteria comprised the most dominant microbial group at 3 and 6 months (**Supplementary Figure 1**). Principal component 1 (PC1, variance of 51%) differentiated the children with a normal bifidobacteria dominated infant microbiota and those with an aberrant microbiota at 3 months (**Figure 2A**). A shift toward microbiota dominated by butyrate producers occurred subsequently (**Supplementary Figure 1**), and this correlated with the changes in the FOXP3 intensity in Treg cells (**Figure 2B**).

At 3 months of age, PC2 (16%), which is described by the abundance of *Bifidobacterium breve* minus the abundance of *B. longum,* showed a positive correlation with the relative numbers of Treg cells ($p = 0.006$; rs = 0.40). Also, the linear combination of these bifidobacterial species (*B. breve—B. longum*) correlated with the numbers of Treg cells at 3 months ($p = 0.002$; rs = 0.44), and further later at 6 and 12 months ($p = 0.015$; rs = 0.33; and $p = 0.018$; rs = 0.35, respectively) suggesting that the initial bifidobacterial composition at 3 months has a long-term effect on Treg cell numbers (**Figure 2C**).

The proportion of highly activated TregFOXP3high cells at 3 months showed an association with PC4 (12%) (**Figure 2D**), which was almost totally defined by the abundance of *B. longum* minus the amount of *B. pseudocatenulatum* ($p < 0.001$; rs = 0.80), and consequently *B. longum* minus *B. pseudocatenulatum* associated with the proportion of TregFOXP3high cells ($p = 0.007$; rs = 0.40).

According to our results, bifidobacterial species, as suggested by earlier studies (21, 41–43), play an important role in shaping the Treg cell population, and the relative abundance of *B. longum* in the neonatal microbiota seems to be a promoter of Treg cell maturation, such as the subsequent decrease in the Treg numbers and the increase in the relative numbers of highly activated TregFOXP3high cells.

## Delayed Maturation of Microbiome and Treg Cells in Finnish Children

We had a unique opportunity to compare the development of Treg cells between Estonian and Finnish children. At 3 months, Estonian children had more highly activated TregFOXP3high cells in comparison to Finnish children (**Figure 3A**) in whom an increase in activated TregFOXP3high cells occurred after 3 months of age (**Figure 1C**). Furthermore, a positive correlation between FOXP3 and CTLA-4 transcripts, which reflect functional activity of Tregs, was seen in Estonian children earlier, at the age of 6 months ($p = 0.008$; rs = 0.59 in Estonian children and $p =$ ns in Finnish children), while this kind of correlation between FOXP3 and CTLA-4 developed later in Finnish children and was seen at the age of 3 years ($p < 0.001$ for both at 36 months, rs = 0.58 and rs = 0.70 for Estonian and Finnish children, respectively). These findings suggest that the phenotype of blood Tregs remains longer immature during early life in Finnish children in comparison to Estonian children.

Also differences in the gut microbiota at 3 months of age was seen between Estonian and Finnish children, when the abundance of *B. breve* minus *B. longum* was higher in Finnish children ($p < 0.001$). At 18 months, PC2 (11%), which describes the abundance of well-established butyrate producers in relation to bifidobacteria, differentiates Estonian and Finnish children (**Figure 3B**). Estonian children had a lower bifidobacterial abundance than Finnish children (13% vs. 26%, $p = 0.02$), and a higher abundance of butyrate producing bacteria (40% vs. 29%, $p = 0.01$, **Figure 3B**) (for the butyrate producing bacteria see **Supplementary Table 3**). A drop in bifidobacteria took place only after 18 months in Finnish children. Despite of this, Estonian children scored higher at 36 months in PC1, which represents 67% of variation and primarily reflects the abundance of butyrate producers (**Figure 3C**). Consequently, a higher abundance of butyrate producers was found in Estonian children at 36 months (45% vs. 36%, $p = 0.02$) indicating a more mature microbiome composition. In addition, Finnish children showed a higher

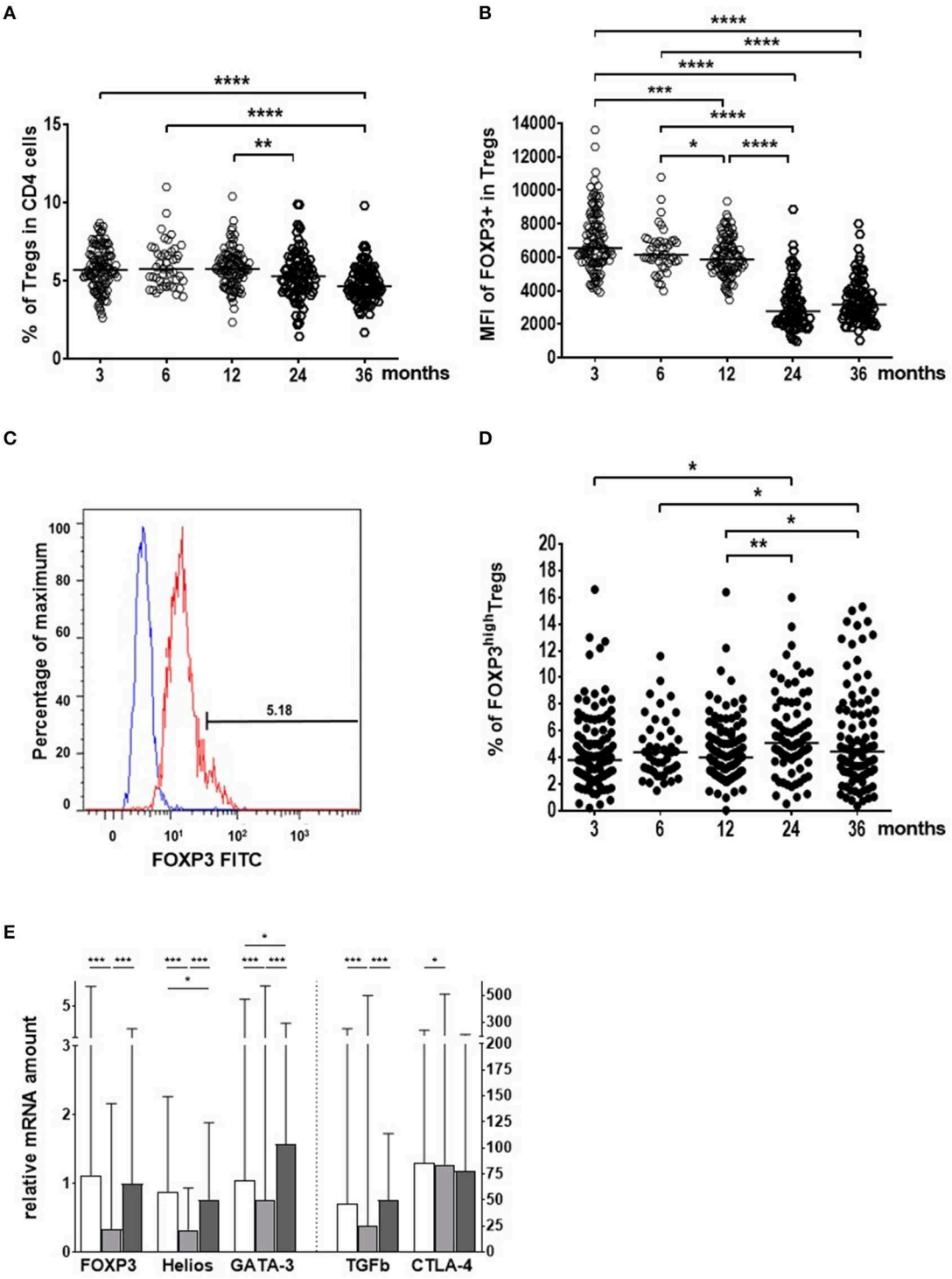


**FIGURE 1 |** Age-related changes in the blood regulatory T cell (Treg) population from the age of 3 to 36 months in Estonian and Finnish children. **(A)** The frequency (%) of Treg cells in the CD4+ cell population. **(B)** FOXP3 expression as the median fluorescence intensities (MFI) in Treg cells. **(C)** The gating for the identification of TregFOXP3High cells. **(D)** The proportion (%) of TregFOXP3High cells in the Treg population. Sample sizes for figures **(A,B,D)** are $n = 111$ at 3 mo; $n = 45$ at 6 mo; $n = 100$ at 12 mo; $n = 84$ at 24 mo; and $n = 92$ at 36 months of age. Medians are shown as horizontal lines **(E)**. The expression of Treg activation markers (mRNA) in Treg cells in all children studied. The columns are showing the medians with ranges. White columns indicate 6 month, light gray columns 18 month, and dark gray columns 36 month samples. The vertical dotted line indicates the use of two different scales on the y-axis. The age of the children (months), are marked on the x-axes in **(A,B,D)** and the different activation markers in **(E)** ($n = 33$ at 6 mo; $n = 96$ at 18 mo; and $n = 80$ at 36 months of age). Wilcoxon test (two-sided) was used for comparisons. *P*-values in the figure are shown as $^{****}p < 0.0001$; $^{***}p < 0.001$; $^{**}p < 0.01$ and $^{*}p < 0.05$.

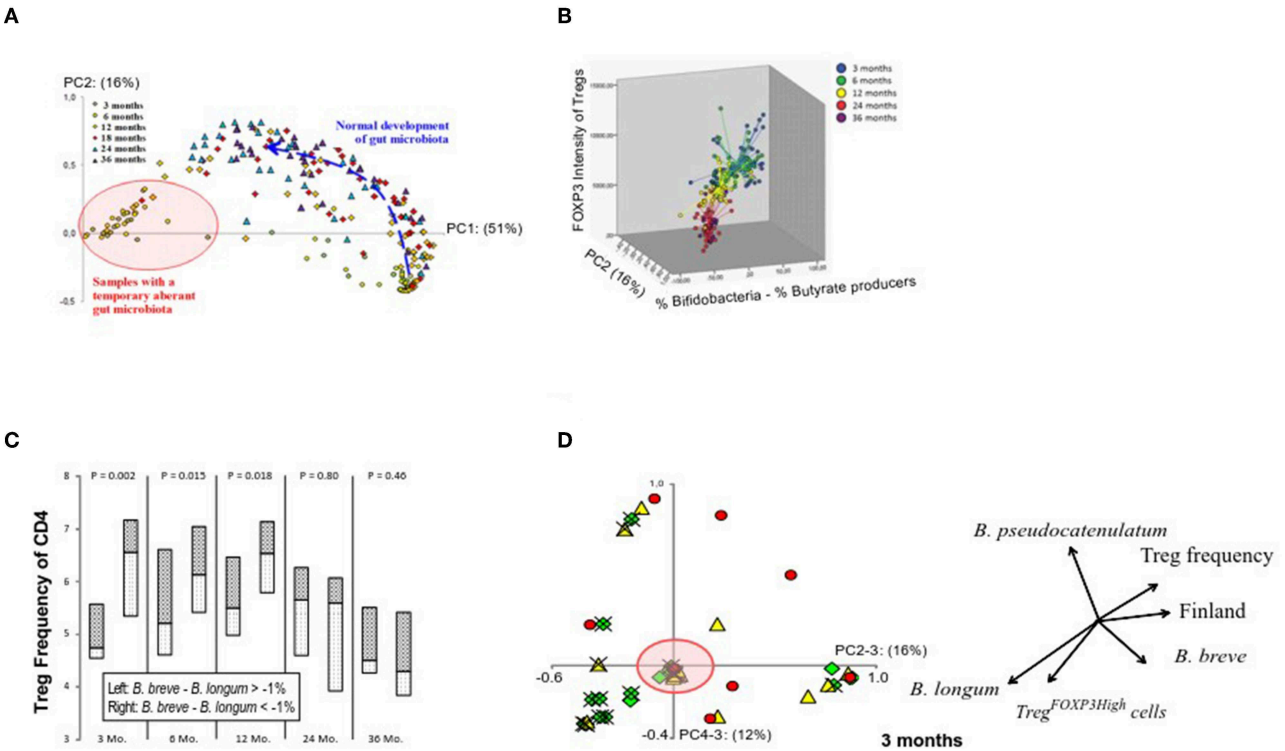


**FIGURE 2 |** Relation between the maturation of the gut microbiota composition and Treg phenotype. **(A)** Principal component analysis on all samples on the genus level shows the age-related maturation of gut microbiome. Principal component 1 on the x-axis (from 0 to 1) accounts for 51% of the variation in the data while principal component 2 on the y-axis accounts for 16%. Samples at 3 and 6 months are either found in the lower right corner (normal bifidobacteria-dominated microbiota) or in the left (red circle) representing an aberrant microbiota largely devoid of bifidobacteria and instead replaced by a combination of *Escherichia coli*, *Bacteroides*, *Ruminococcus gnavus,* and/or clostridia (pink circle). The normal bifidobacteria-dominated microbiota (lower right corner) usually is the basis for future development (blue arrow) into a more complex adult-like configuration. **(B)** A graphical representation of FOXP3 expression levels in the whole Treg population in relation to the gut microbiota development. **(C)** A graphical representation of the influence of the bifidobacteria composition at 3 months of age on Treg numbers at 3, 6, 12, 24, and 36 months. Children with a *Bifidobacterium breve—Bifidobacterium longum* abundance lower than −1% at 3 months are depicted on the left of each column and those with scores higher than −1% are depicted on the right, with median as a horizontal line in the boxes. *P*-values (top) were not based on the binary division of 1% but on the actual *B. breve—B. longum* abundance per sample and their corresponding Treg-value using a Spearman rho's correlation test. The value of −1% was chosen to specifically showcase those children who have *B. longum* in a non-insignificant amount and have more of it than *B. breve*. **(D)** The number of Treg cells with high expression of FOXP3 shows association with PC4, which is described by the relative abundances of *B. longum* and *B. pseudocatenulatum* at the age of 3 months and accounts for 12% of the variation. The arrows in the image are a direct representation of the Spearman correlation coefficient of each of the indicated factors with the respective principal components. The green diamonds (◊) represent TregFOXP3High frequencies >4.5%; yellow triangles (▲) 3%< TregFOXP3High >4.5%; and red circles (●) TregFOXP3High frequencies <3%. Estonian children are marked with an (×). The red circle indicates samples, which represent an aberrant microbiota composition, and hardly contain bifidobacteria as also illustrated in **(A)**.

abundance of *Bacteroides* at both 12 and 18 months of age (9.7% vs. 2.4%, $p = 0.003$ and 6.6% vs. 1.6%, $p = 0.008$).

## Neonatal Gut Microbiota Predicts Allergic Response

Next, we studied the association between the development of circulating allergen-specific IgE antibodies and their association with the composition of gut microbiota. We found that gut microbiota composition at 3 months of age, but not after that, showed an association with atopic sensitization, i.e., the development of allergen-specific IgE later in life. PC2 (genus level) shows association with atopic sensitization (**Figure 4A**) and it is nearly perfectly described by the abundance of bifidobacteria minus the abundances of *Bacteroides* and *E. coli* at 3 months of age ($p < 0.001$, rs = 0.96), which further shows an inverse association with atopic sensitization (**Figure 4B**) and represents a clear vector associated with the number of allergen-specific IgEs ($p = 0.012$), the age of the first allergen-specific IgE ($p = 0.039$) and also the diagnosis of allergies ($p = 0.012$). Furthermore, bifidobacteria and in particular the relative abundance of *B. longum* at 3 months showed an inverse association with atopic sensitization, the number of allergen-specific IgEs and the development of allergy ($p = 0.023$, $p = 0.026$, $p = 0.022$). As a conclusion, high abundance of *Bacteroides* and *E. coli* at the expense of the abundance of bifidobacteria in early infancy increased the risk of atopic sensitization and clinical allergy later in childhood.

At the age of 18 months, the expression of FOXP3, GATA-3, and CTLA-4 in Tregs was higher in the children with allergen-specific IgE sensitization without signs of clinical allergy when

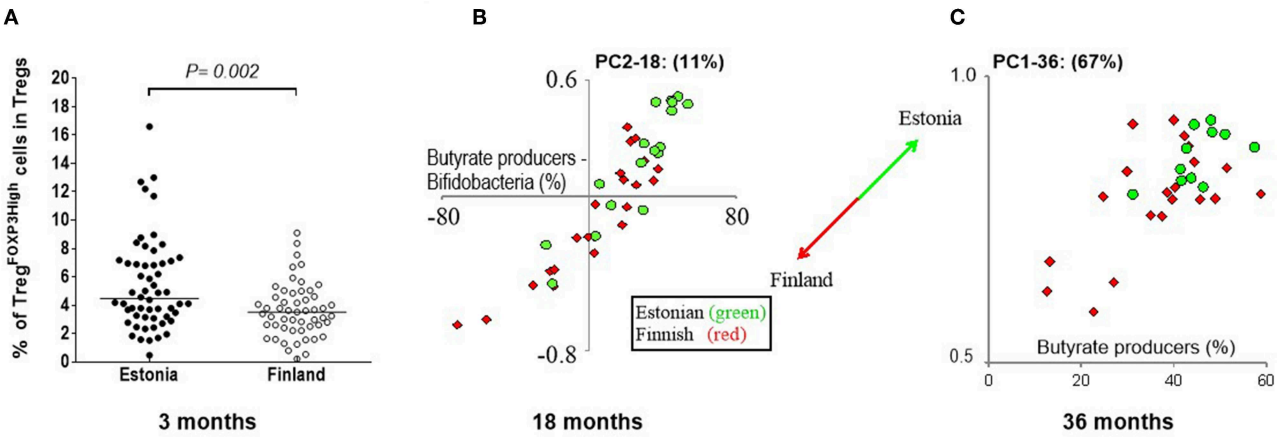


**FIGURE 3 |** The differences in Treg cells at 3 months and gut microbiome between Estonian and Finnish children at 18 and 36 months of age. **(A)** The frequencies of TregFOXP3high cells in the Treg population is higher at 3 months in Estonian ($n = 56$) than Finnish children ($n = 55$). Median values are shown with a horizontal line. The *P*-value was calculated with Mann-Whitney U (two-sided). **(B)** PC2 at 18 months is mostly driven by the amount of butyrate produces minus the amount of bifidobacteria (X-axis), as can be seen by the strong positive correlation. Estonian children score higher on PC2 (18 month samples) than Finnish children, which indicates that Estonians have more butyrate producers at 18 months than the Finnish children, while the Finnish children have more bifidobacteria. At 36 months **(C)** another pattern is seen, this time described by PC1 (67%). Estonian children on average score higher on PC1 (Y-axis), which in turn is driven mostly by the abundance of butyrate producers in the 36 month samples (X-axis). Estonian children have on average more butyrate producers than then Finnish at 36 months. The circles (•) show Estonian and diamonds (◊) Finnish children in these principal component plots.

compared to the children who did not develop clinical allergies (**Figure 4C**).

At the end of the follow-up, atopic sensitization correlated (rs = 0.57; $P < 0.001$) with PC1 on the genus level, which accounts for 67% of the variation at 36 months of age, and describes the abundance of a trophic network of butyrate producers. PC1 is inversely correlated with the number of allergen-specific IgEs (rs = −0.50; $P < 0.001$) (**Figure 5A**), and atopy (rs = −0.57; $P < 0.001$), diagnosis of food allergies (rs = −0.58; $P < 0.001$) and inhalation allergies (rs = −0.39; $P = 0.031$).

In children with food allergy, *Faecalibacterium* (*F. prausnitzii*) (**Figure 5B**), which is directly correlated with PC1 and is one of the most important butyrate producing groups, was decreased while the abundance of *Mogibacteriaceae* (**Figure 5C**) being inversely correlated with PC1, was increased.

## Increased Total IgE Production in Estonian Children

Because total IgE levels are considered as a predictive marker for development of later IgE-sensitization and allergies, we analyzed the associations between total IgE levels, gut microbiota composition and characteristics of Tregs. Interestingly, we found that total IgE levels were higher in Estonian than Finnish children in the blood samples collected at 6 months (median 8.4, range 2–311 vs. 5.2 range 2–311293.5 kU/L; $p = 0.02$) and 36 months (62.4; range 2.41–2956 vs. 25.9; range 1.26–456 kU/L; $p = 0.006$, respectively). In Estonian children, total IgE levels at 6 months did not correlate with later IgE-sensitization or allergies as they did in Finnish children ($p < 0.001$, rs = 0.40).

As the dynamic changes in GATA-3 and FOXP3 expression in Treg cells have been shown to regulate the function of Treg cells and Th2 immunity (44, 45), we next asked whether GATA-3 mRNA expression in Treg cells is associated with the different kinetics seen in the maturation of IgE production. Indeed, in Estonian children, GATA-3 expression in Treg cells at 6 months correlated positively with total IgE levels at 18 months ($P = 0.047$, rs = 0.56) while in Finnish children GATA-3 expression level in Treg cells at 6 months showed an inverse correlation with total IgE levels at 6 ($P = 0.001$, rs = −0.77) and later at 18 months ($P = 0.052$, rs = −0.55). Similarly, Helios expression (46, 47) in Treg cells at 6 months correlated negatively with the total IgE production in Finnish children at 6 and 18 months of age ($P = 0.002$, rs = −0.75 and $P = 0.021$, rs = −0.63).

# DISCUSSION

In the birth-cohort of Estonian and Finnish children followed from 3 to 36 months of age, we demonstrate that the composition of neonatal intestinal microbiota modulates the maturation of Treg population during the first year of life, and the risk of allergic diseases later in life. An earlier study in a US cohort identified a specific neonatal gut microbiota composition, which is characterized by a lower relative abundance of *Bifidobacterium*, *Akkermansia,* and *Faecalibacterium*, and a higher relative abundance of particular fungi, as a risk of atopy and asthma in a follow-up up to 4 years of age (6). That study also showed that fecal water extract from high-risk infants compromised Treg suppressive activity *in vitro* (6) suggesting that a defective function of Treg cells could be a factor contributing to the development of asthma. Those observations together with our findings support the view that neonatal microbiome is an important regulator of Treg cells and allergic sensitization. Our

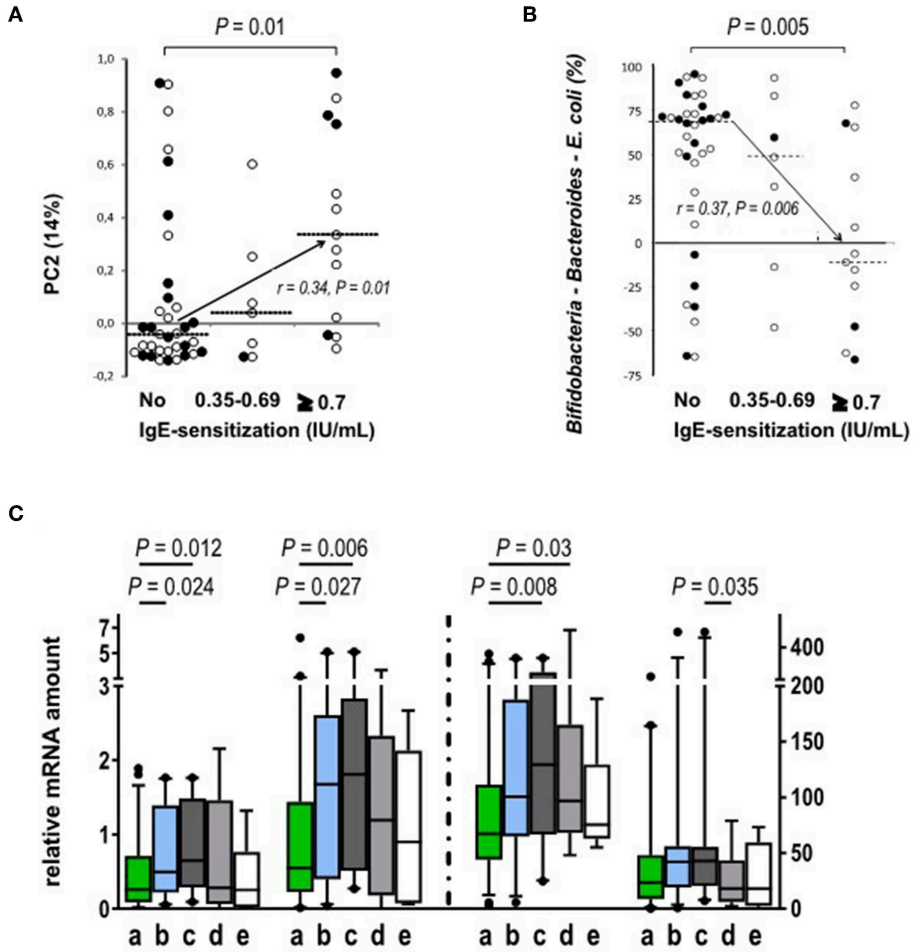


**FIGURE 4 |** Relation between atopic sensitization and the gut microbiota composition and Treg phenotype. **(A)** PC2, as derived from the genus level at 3 months of age, correlates positively with atopic sensitization during the study period ($p = 0.01$, rs = 0.34; No sensitization $n = 35$; sensitization between 0.35 and 0.69 $n = 7$; sensitization ≥0.7 $n = 13$). **(B)** The abundances of *Bacteroides* and *E. coli*. are individually significantly associated with the development of atopic sensitization while bifidobacteria are protective. When both Gram negative groups are subtracted from the abundance of bifidobacteria the resulting association inversely mirrors the association of PC2 with atopic sensitization ($p = 0.005$; rs = 0.37; No sensitization $n = 33$; Allergen specific IgE level between 0.35 and 0.69 $n = 7$; Allergen specific IgE level ≥ 0.7 $n = 12$). Open circles show Finnish (○) and black circles show Estonian (●) infants. **(C)** The expression (mRNA) of activation markers FOXP3, GATA-3, and CTLA-4 is increased in blood derived Treg cell population at the age of 18 months in the children with IgE sensitization but without clinical allergy. The children were classified into four groups based on their clinical allergy diagnosis and allergen-specific IgE values: (a) No signs of allergy (no clinical diagnosis, no allergen-specific IgE responses, $n = 46$ for FOXP3 and TGFb-1 and $n = 45$ for GATA-3 and CTLA-4); (b) IgE-sensitization developed during the study period (no clinical allergy diagnosis, at least one allergen specific IgE response ≥0.35 IU/mL, $n = 28$); (c) IgE-sensitized at the time of follow-up point (no clinical allergy diagnosis, at least one allergen specific IgE response ≥0.35 IU/mL, $n = 20$ for all other and $n = 21$ for TGFb-1); (d) clinical non-IgE allergy (clinical diagnosis of allergy, no allergen specific IgE response 45, $n = 16$); and (e) clinical IgE allergy (clinical diagnosis of allergy and a specific IgE response to the allergen related to the symptoms, $n = 6$). The different activation markers are marked on the x-axes. The vertical dotted line indicates the use of two different scales on the y-axis. Medians are marked with a horizontal line. Mann-Whitney *U*-test (two-sided) was used for comparisons.

findings together with earlier studies emphasize the importance of the intestinal colonization during the first weeks or months of life as a key determinant for the risk of atopic deviation, and particularly the maintenance of a bifidobacterial dominated microbiome (2–7). Altogether, these studies suggest that the window of opportunity for the primary prevention of atopic diseases leading to asthma may be narrow, from birth up to 3 months of age.

In addition, we found that Finnish children, having higher risk of IgE mediated allergic diseases, show signs of delayed maturation of the gut microbiota and Treg cell phenotype when compared to Estonian children. Immature phenotype of the gut microbiota was found to be a risk factor for later allergic asthma in a Danish COPSAC cohort study (7). Accordingly, as well as the maintenance of a bifidobacterial dominated gut microbiota is important during the first weeks of life, the overtake by butyrate producing bacteria seems to be a beneficial shift, which should not be postponed. Butyrate is the main energy source for colonic epithelial cells (48), and it regulates the assembly of tight junctions and gut permeability (49). Thus, the switch

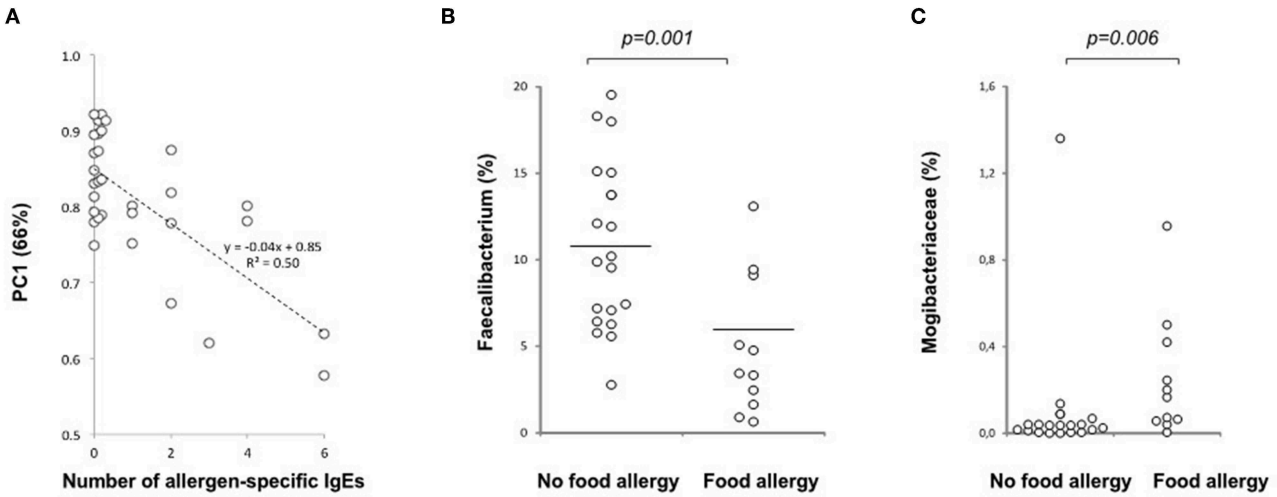


**FIGURE 5 |** Relation of the gut microbiota composition and allergy at 36 months of age. **(A)** A negative correlation is seen between PC1 and the number of allergen-specific IgEs at 36 months of age ($p < 0.001$, $n = 30$), **(B)** Decreased abundance of the butyrate producer *Faecalibacterium prausnitzii* in children with food allergy at 36 months of age ($p = 0.001$; No food allergy $n = 19$, Food allergy $n = 11$). **(C)** Increased abundance of *Mogibacteriaceae* in children with food allergy at the age of 36 months ($p = 0.006$, $n = 19$ and 11, respectively). Medians are marked with a horizontal line.

to an increasing abundance of butyrate producing bacteria may facilitate the maturation of the gut barrier mechanisms during the early life (50). Also in children with already established allergic diseases, we found a decrease in butyrate producing *Faecalibacterium*, which is in agreement with the findings in the American Gut Project (51). Recent studies suggest the importance of butyrate in the regulation of allergic airway inflammation by limiting eosinophilic inflammation (52) and type 2 innate lymphoid-cell proliferation and function (53).

According to our findings, remarkable age-related changes occur in the phenotype of the circulating Treg population during the first years of life, and these changes are tightly linked to the maturation of the intestinal microbiota. The neonatal Treg phenotype is characterized by the high frequency of circulating Treg cells, which is in agreement with an earlier study reporting increased numbers of Tregs in infants compared to adults (54). An increase in Treg cells after birth is characterized by a high frequency of naïve Treg cells (39, 40). The high ratio of *B. breve* vs. *B. longum* in the gut microbiota at 3 months of age showed association with high Treg numbers during the whole first year of life emphasizing the long-term effects of the neonatal gut microbiome. The following step of Treg maturation, i.e., the emergence of a highly activated Treg cell phenotype with high expression of FOXP3, TGF-beta1 and Helios showed instead an association with the high relative abundance of *B. longum*. Indeed, bifidobacteria species have been demonstrated to affect the induction of FOXP3 Treg cells (21). *B. longum* can activate Treg cells in newborn and adult mice, whereas *B. breve* shows an effect on regulatory T cells only in newborn mice. Furthermore, supplementation with *B. longum* protected against airway inflammation in an animal model (21). Also in humans, feeding a *B. longum* strain induced FOXP3 expressing regulatory T cells (55). The beneficial effects of *B. longum* 35624 has been suggested to be mediated by surface associated exopolysaccharide (41). A recent study showed that administration of *B. longum* changes the microbiota composition and thus metabolic mediators (42). Importantly, it seems that the effects of bifidobacteria on Tregs are dependent on species and strains, which should be considered in the design of possible probiotic treatments as suggested by others as well (43).

Whereas, the abundance of *B. longum* was an important determinant of Treg cell maturation during early infancy, the maturation of Treg cells after the first year of life, i.e., increasing numbers of highly activated Treg cells, showed a strong association with the increasing abundance of butyrate producing bacteria, and thus increasing diversity of the intestinal microbiome, which is likely contributed by changes in the diet. Our findings linking the temporal changes in the gut microbiome with Treg cell maturation are supported by an earlier study showing that Treg maturation between 18 and 36 months of age includes dynamic changes in the homing receptor pattern from a4b7-integrin, a gut homing receptor, to CCR4, which attract T-cells to non-gastrointestinal tissues (40). Accordingly, the early maturation of Tregs in the infants seems to be driven by intestinal antigens, such as diet and microbiome derived antigens. The effects of gut microbiome may be local in the intestine, such as reported for the extrathymic effects of butyrate on Treg differentiation (13, 19). On the other hand, changes in the gut microbiota and metabolites during colonization could affect the maturation process of Treg cells in thymus, as it has been shown that changes in the intestinal microbiota induced by antibiotic treatment can affect the thymus microenvironment, namely the T cell receptor repertoire of thymic Treg cells (56).

We had an opportunity to compare Finnish and Estonian children who live in a different environment regarding the risk for allergy and standard of living. We observed several signs of delayed maturation of Treg cells in Finnish children in comparison the Estonian children, such as a lower frequency of the highly activated Treg cells at 3 months of age, and later development of the correlation between FOXP3 and CTLA-4 transcripts regulating immune suppression activity in the Treg cell population. Related differences in the gut microbiota between Estonian and Finnish children were evident, i.e., higher ratio of *B. longum* vs. *B. breve* at 3 months, and an earlier switch from bifidobacteria dominated microbiota to a microbiota with increasing numbers of butyrate producers in Estonian children.

Finally, despite having less IgE mediated allergies, Estonian children had higher total IgE levels than Finnish children indicating an earlier maturation of IgE production, which takes place during the first 5 to 6 years of life when the IgE levels reach adult levels (57). Differential maturation of IgE production between populations has been observed earlier indicating the importance of environmental factors (58). In Estonian children high total IgE did not associate with IgE sensitization and the risk of allergic diseases like in Finnish children suggesting differential regulation of IgE responses. In Estonian children GATA-3 expression in Tregs at 6 months of age was associated with total IgE production and correlated with the expression of CTLA-4, which is a functional mediator of immune suppression. Interestingly, the expression of FOXP3, CTLA-4, and GATA-3 in Treg cells were increased in children with IgE sensitization who did not develop clinical allergy, which suggests that a transcriptional signature supporting Treg suppression activity, i.e., FOXP3 and CTLA-4, could provide homeostasis despite of up-regulation of Th2 transcription factor GATA-3 in Treg cells (59).

We recognize that our study has limitations due to its observational nature, and due to the possible confounding factors, such as reported higher frequency of infections, and use of antibiotics and antipyretic drugs in Finnish children (60). On the other hand, it is among the first studies, in which the immunological maturation and changes in the composition of the gut microbiota are monitored in healthy children at the same time during the first years of life. Similar kind of temporal changes in the gut microbiota as seen in our cohort were described recently in TEDDY cohort (61, 62). Also several observations we made here, linking the immunological changes with the maturation of gut microbiota, are mechanistically supported by the earlier literature showing more direct evidence, which has been possible to generate in animal models. In this longitudinal follow-up study, we were able to reveal that delayed maturation of the gut microbiota, Treg cell population and total IgE production are characteristics of children living in an environment with high risk of allergic diseases, which encourages treatments promoting healthy maturation of gut microbiota aimed at the prevention of allergies.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Hospital District of Helsinki and Uusimaa, Children's and Adolescents' Diseases and Psychiatry Ethical Committee, Tartu Ülikooli inimuuringute eetika komitee, and Ethics Review Committee (ERC) on Human Research of the University of Tartu. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

## AUTHOR CONTRIBUTIONS

OV, TR, MG, HH, JN, and MK planned, performed, and analyzed experiments. TR and MG performed data analysis and statistical analyzes. JN and JH were involved in data discussion and supervision of study design experiments. A-MH, AP, VT, ON, and HS collected and provided patient and/or control material and clinical characterization. JN, JH, TR, and MG planned experiments. JI, GW, and HH provided scientific and experimental input. MK was involved in study design, data discussion, and supervision of experiments. OV designed experiments, analyzed data, and supervised the study. OV, TR, and MG wrote the manuscript. All authors critically reviewed and approved the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02494/full#supplementary-material

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# Dietary SCFAs, IL-22, and GFAP: The Three Musketeers in the Gut–Neuro–Immune Network in Type 1 Diabetes

*Abhirup Jayasimhan*[1] *and Eliana Mariño*[2]*

[1] *Department of Immunology and Pathology, Monash University, Melbourne, VIC, Australia,* [2] *Infection and Immunity Program, Department of Biochemistry, Biomedicine Discovery Institute, Monash University, Melbourne, VIC, Australia*

Microbial metabolites have a profound effect on the development of type 1 diabetes (T1D). The cross-talk between the gut microbiota, the nervous system, and immune system is necessary to establish and maintain immune and gut tolerance. As quoted by Hippocrates, "All disease begins in the gut." Although this has been recognized for 2,000 years, the connection between the gut and autoimmune T1D is not yet well-understood. Here, we outline new advances supported by our research and others that have contributed to elucidate the impact of microbial metabolites on the physiology of the pancreas and the gut through their remarkable effect on the immune and nervous system. Among many of the mechanisms involved in the gut–beta-cell–immune cross-talk, glial fibrillary acidic protein (GFAP)-expressing cells are critical players in the development of invasive insulitis. Besides, this review reveals a novel mechanism for microbial metabolites by stimulating IL-22, an essential cytokine for gut homeostasis and beta-cell survival. The close connections between the gut and the pancreas are highlighted through our review as microbial metabolites recirculate through the whole body and intimately react with the nervous system, which controls essential disorders associated with diabetes. As such, we discuss the mechanisms of action of microbial metabolites or short-chain fatty acids (SCFAs), IL-22, and GFAP on beta-cells, gut epithelial cells, neurons, and glial cells via metabolite sensing receptors or through epigenetic effects. The fine-tuned gut–neuro–immune network may be profoundly affected by SCFA deficiency related to dysbiosis and diet alterations at very early stages of the initiation of the disease. Thus, dampening the initial immune response or preventing the perpetuation of the immune response by maintaining the integrity of the gut is among the alternative approaches to prevent T1D.

**Keywords: SCFA (short chain fatty acids), GFAP—glial fibrillary acidic protein, gut microbiota, glial cell, interleukin 22 (IL-22), ILC3s, beta cells, diabetes**

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*Jasenka Zubcevic,*
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*Gianluca Matteoli,*
*KU Leuven, Belgium*

***Correspondence:**
*Eliana Mariño*
*eliana.marino@monash.edu*

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## INTRODUCTION

Type 1 diabetes (T1D) is a chronic autoimmune disease in which T cells destroy the insulin-producing beta-cells of the pancreas (1–3). The beta-cell's attack happens when T cells recognize autoantigens such as glutamic acid decarboxylase (GAD), islet cell autoantigen 69 (ICA69), insulinoma-associated antigen 2 (IA2), islet-specific glucose-6-phosphatase catalytic

subunit–related protein (IGRP), and proinsulin, which are widely accepted as the initiating autoantigens in T1D (4, 5). Antigenic targets for T cell priming are not solely expressed in beta-cells, but also in multiple tissues distal to islets, and they can be found in food like insulin or produced by bacteria like GAD (6–8). This all leads to many questions. How antigen expression in other distal tissues to beta-cells control the invasive infiltration of immune cells into the pancreas? Is the gut an important compartment as a source of antigens that trigger T1D? Is the gut microbiota influencing T cell priming against beta-cells? The microenvironment regulates beta-cell function and maturity, in particular close interaction with endocrine cells, neuronal, immune, and vascular cells (9, 10). Pancreatic ducts are physiologically neighboring to the beta-cells, and their primary function is to deliver enzymes or pancreatic juices provided from the exocrine pancreas into the duodenum to help digestion. As such, the pancreatic beta-cells can be influenced by the gut, which is intimately connected not only through the pancreatic ducts but also by lymph ducts (11). Beyond the pancreas, there is hardly any tissue in the body that has not been somehow in contact with gut microbial SCFAs. From food fermentation, bacteria in the large bowel produce many metabolites that are used by the epithelial cells in the gut. The remaining amount is transported to the liver where they are metabolized and then released to systemic circulation. As such, SCFAs have a broad spectrum of remarkable beneficial properties that affect many systems, in particular under inflammatory conditions, regulating metabolic, and immune responses (12–14).

One example is the nervous system, which is also critical for the pancreas to function (15). Both the endocrine and the exocrine part of the pancreas are innervated by the sympathetic and the parasympathetic nervous system, as such pancreatic sensory neurons have been shown to play a critical role in controlling islet inflammation (16). Similarly, the enteric nervous system (ENS) via the enteric glial cells (EGCs) is vital to maintain gut and immune homeostasis (17, 18), given that diabetic animals and patients presented gastrointestinal motility disorders (19). In this review, we will discuss the gut–neuro–immune axis in T1D and its effect on beta-cell priming. In particular, we will focus on the role of GFAP-positive cell types as critical players in T1D and on the impact of the gut microbiota, SCFAs, and their mechanisms of action through interleukin 22 (IL-22).

## GFAP—NOT THE USUAL SUSPECT!

Beta-cells are involved in late T cell priming, suggesting that they are not required during the induction of T1D (20, 21). So, a critical consideration is—what might be driving the initiation of T1D? It has been shown in the pancreas that GFAP-expressing peri-islet Schwann cells (pSC or glial cells) can attract and recruit autoreactive cells, which precedes the attack on beta-cells. Two studies support the finding that immune responses to autoantigens expressed in pSCs precede the immune response to beta-cells (6, 7). In particular, they showed that early T cell attack on GFAP-expressing pSCs progressively results in the release of glial cell antigens, GFAP, and insulin (6, 7). GFAP epitopes for autoreactive T and B cells have now been identified in non-obese diabetic (NOD) mice and humans with T1D. Serum GFAP antibodies are now used as a predictive marker for the development of T1D, and it has been shown that administration of GFAP as a vaccine delayed the progression of T1D by regulating T cell differentiation (22, 23). GFAP-expressing glial cells of the peripheral nervous system require TRPV1 expression for their proper maturation, and studies have shown that depleting TRPV1-expressing cells reduced the development of insulitis in NOD mice (16, 24). It is fascinating that a cytoskeletal protein widely expressed in pancreatic ductal cells and also in pancreatic glial cells of the central and peripheral nervous system may work as an early autoantigen in T1D.

Exploring further this idea, Slattery's group has recently shown that ablation of autoantigen presentation in GFAP-expressing cell types reduced the development of invasive insulitis in NOD mice (25). We can speculate that reduction, but not total elimination of invasive infiltration, may be due to the absence of presentation of autoantigens other than insulin by GFAP-expressing cells, suggesting that Ag-derived ductal cell is one of the critical requirements in orchestrating the initiation of autoimmune responses to beta-cell antigens.

## THE SCFAS: MODULATOR OF GUT INFLAMMATION AND AUTOREACTIVITY

After many years of efforts and studies focusing on the destruction of the beta-cells in the pancreas, there is still no cure or method of prevention for T1D. So, it makes us wonder whether we have been losing the battle only because we are not looking beyond the walls of the pancreas. T1D can be viewed as an orchestrated autoimmune response originated in the gut. This is evident from the observation that in many autoimmune diseases including T1D, the integrity of the epithelial barrier is compromised, leading to a phenomenon termed as "leaky gut" (26, 27). Pathogens, microbial products, and food-derived antigens find the leaky gut as a route to encounter the resident immune cells. For example, Gram-negative bacteria produce lipopolysaccharides (LPS), an identified endotoxin that can induce immune responses via the toll-like receptor 4 (TLR-4) expressed on monocytes (28). Given the gut connects to the pancreas through pancreatic lymph nodes (PLNs) and mesenteric lymph nodes (MLNs), bacterial and food products can hyperactivate resident T and B autoreactive cells in the gut or the gut-associated MLNs (29). Alternatively, it has been shown that gut microbial products can reach PLNs and locally modify the presentation of pancreatic self-antigens (30). Therefore, excess of food, chemicals, and microbial antigens can skew the intestinal immune system toward a perpetually pro-inflammatory state that may trigger T1D. Newly-diagnosed children with T1D present autoantibodies to GAD, a pancreatic beta-cell autoantigen that is also produced by many bacterial species (31). For instance, T1D patients present antibodies against a heat shock protein from the *Mycobacterium avium* subspecies *paratuberculosis*, MAP Hsp65, which has a high

degree of homology with human GAD65, suggesting that cross-reactivity between MAP Hsp65 and GAD65 potentially could be a mechanism of triggering TID (32). Strong homology has been found between the islet-antigen IGRP and several gut- and oral-derived microbial peptides. These peptide sequences encode for magnesium transporter (Mgt), for hypothetical protein IEM_00289 and NAD synthetase, respectively, which activate NY8.3 $CD8^+$ T cells with comparable potency to IGRP native peptide (33). Thus, molecular mimicry between excess of gut microbial antigens and islet cell autoantigens may be a mechanism by which gut dysbiosis leads to T1D development.

T1D is a multifactorial condition; diet and environment play an inevitable role in disease modulation (1, 13). Human and murine studies have demonstrated that defects in the induction of central and peripheral tolerance checkpoints (34) also correlate with an altered gut microbiota (35–39), which are notable contributors to T1D pathology. Building on previously extensive reviews on the gut microbiota topic, we have firmly discussed that an altered microbiota and SCFA deficiency are primary causal factors triggering T1D (12–14, 40). The gut microbiota through the production of dietary SCFAs plays a significant role in host defense by modulating the immune system and metabolism. Studies conducted by our group have shown that the combination of a diet rich in acetate and butyrate protected 90% of the NOD mice against T1D, yielding exceptionally high levels of the corresponding SCFAs to the feces (35). In this study, SCFA-induced T1D protection happened via changes in gut/immune regulation-expanding regulatory T (Treg) cells and reducing pathogenic B cells, $CD4^+$, and $CD8^+$ T cells. Diet rich in SCFA acetate and butyrate not only reduced the levels of serum LPS and pro-inflammatory interleukin 21 (IL-21) but also increased the level of serum IL-22, an important cytokine that maintains a healthy commensal microbiota, gut epithelial integrity, and mucosal immunity and ameliorates metabolic disease (41–44). Alternatively, SCFAs can also reduce islet-specific immune responses by increasing the production of antimicrobial peptides (AMPs) in the beta-cells (45). As it has been shown, C-type lectin regenerating islet-derived protein IIIγ (REGIIIγ) and defensins disrupt surface membranes of bacteria, thus enabling a broad regulation of commensal and pathogenic bacteria in the gut (46–48). Diana's group showed that insulin-secreting beta-cells produced the cathelicidin-related antimicrobial peptide (CRAMP), which was defective in NOD mice. Intraperitoneal administration of SCFA butyrate stimulates the production of CRAMP on pancreatic beta-cells via G protein-coupled receptors (GPCRs), which also correlated with the conversion of inflammatory immune cells to a regulatory phenotype (45). Likewise, another study has shown that microbial SCFAs contribute to the increasing concentrations of serum IL-22 (35) required for beta-cell regeneration by up-regulating the expression of Regenerating Reg1 and Reg2 genes in the islets (49).

There are pieces of evidence of compromised gut integrity, dysbiosis, and associated inflammation of the gastrointestinal tract (GI) in NOD mice and patients with T1D (50–55), similar to what has been shown in other inflammatory or autoimmune gut diseases (i.e., infection, celiac disease, IBD). The gut microbiota and the ENS play a critical role in diabetic gastrointestinal motility disorders, as individuals with diabetes suffer from symptoms such as nausea, heartburn, vomiting, diarrhea, abdominal pain, and constipation (56, 57). For example, it is known that slow motility of the GI leads to alterations of the gut microbiota that favors pathogenic bacterial overgrowth and subsequently diarrhea (58, 59). As such, the abundance and diversity of bacteria needed to maintain the integrity of the gut were significantly lower in children with T1D compared to healthy controls (60). On the other hand, animal studies have suggested that accelerated colonic transit time, relative to constipation, could be caused by autonomic neuropathy and diabetes-induced denervation of sympathetic nerve terminals (56, 61). Diet and/or deficiency of dietary SCFAs can also modulate the intestinal motility and survival of enteric neurons by miRNAs, which are involved in energy homeostasis, lipid metabolism, and proliferation and development of GI smooth muscles. miRNAs have been vastly studied in organ damage caused by diabetes, and one study has shown in mice that high-fat diets delay the GI transit, partly by inducing apoptosis in enteric neuronal cells, and this effect was shown to be mediated by Mir375 associated with reduced levels of Pdk (62). There is still too much to understand about the intrinsic mechanisms underlying the connection between the gut microbiota and the ENS and how this affects the course of T1D. Particularly high-fiber or specialized acylated starch diets that boost the microbial production of SCFAs are effective in the control of gut infections and diarrhea, as it has been shown to promote commensal acetate-producing bacteria (63).

## IL-22 AND ENS TAKE CONTROL OF T1D

Activation of IL-22 through microbial SCFAs contribute to the maintenance of gut homeostasis by the close connection between the intestinal-resident innate lymphoid cell 3 (ILC3) and EGCs. IL-22 is expressed by ILC3, which lies close to EGCs (64), but its role in T1D is still elusive (14). ILCs sustain appropriate immune responses to commensals and pathogens at mucosal barriers by potentiating adaptive immunity and regulating tissue inflammation (65, 66). Likewise, EGCs have critical roles in maintaining gut homeostasis, as they can sense the pathogenic bacteria through toll-like receptors (TLRs). EGCs surround neurons and also connect with blood vessels and lymphatics (67), which allowed EGC-derived signaling molecules to modulate mucosal immunity. As such, EGCs sense environmental stimuli and extend their stellate projections into the ILC3 aggregates within the crypto-patches of the intestinal lamina propria and release neurotrophic factors that stimulate IL-22 secretion from ILC3s (68). The notion that gut microbiota affects the development and maturation of EGCs was shown in germ-free (GF) mice, which present a defective influx of EGCs into the intestinal mucosa (69). This occurs via expression of the neuroregulatory receptor (RET), as ablation of RET in ILC3 leads to reduced IL-22 production and compromised epithelial protection in colon inflammation mouse models (69).

Aligned with this idea, does the early autoreactivity to GFAP observed during insulitis originate in the gut? This is possible to the connections between the pancreas, the ENS, and the gut. The fine-tuned neuro–beta-cell cross-talk is more likely to be broken by the pathological changes occurring in a perturbed gut. Alterations of the gut microbiota, referred to as dysbiosis, decrease epithelial permeability, causing inflammation, and associated tissue damage that exposes numerous self-antigens harbored in the gut and associated enteric neuronal tissues. Gut microbial products can also sense enteric neurons and EGCs partly by pattern recognition receptors, such as TLRs. Indeed, pathogenic and commensal SCFA-producing bacteria up-regulate differentially toll-like receptor 2 (TLR2) expression on human EGCs (70). Expression of TLR2 on enteric neurons and EGCs controls $nNOS^+$ neurons and acetylcholine-esterase-stained fibers in the myenteric ganglia. For example, *Escherichia coli* promoted expression of MHC II molecules on EGCs and significantly induced S100B protein overexpression and nitric oxide (NO) release from EGC, which was counteracted by pre-treatment with TLR and S100B inhibitors (70). As such, the myenteric plexus of TLR2Ko mice presented reduced expression of glial markers, GFAP, and S100B. Overexpression of GFAP has been observed to correlate with inflammatory responses in the gut (71). S100B is considered as a neurotrophin, due to its either tropic or toxic effects depending on the concentration in the extracellular milieu. Excess amount of S100B acts on RAGE (receptor for advanced glycation end-products), leading to the phosphorylation of mitogen-activated protein kinases (MAPK) and subsequent activation of the nuclear factor κB (NF-κB) and the associated release of NO. Excess NO causes damage to the tissue, resulting in inflammation and reduced integrity of the guts (72, 73). The protective role of EGCs in the maintenance of the gut epithelial integrity has been demonstrated in mice lacking GFAP-positive (+) glia that presented fatal hemorrhagic jejuno-ileitis (74).

During chronic tissue inflammation, significantly increased expression of GFAP on glial cells after stimulation with LPS and pro-inflammatory cytokines has been shown (75), similar to what has been seen in Crohn's disease (CD) and necrotizing enterocolitis (NEC). On the other hand, the presence of MHC class II expression on activated EGCs suggests that these cell types can present antigens (76, 77) derived from multiples sources, including microbes and host. EGCs, with the help of their stellate projections, sample microbial antigens crossing the epithelial barrier and activate diabetogenic T cells. This is given under dysbiosis, predominant in T1D and many autoimmune diseases, and the release of microbial antigens such as LPS may break the tolerance of EGCs leading to overexpression of glial cell markers GFAP and S100B. Thus, GFAP-expressing glial cells may have a protective role in maintaining the integrity of the gut, but under uncontrolled inflammatory conditions, it may lead to autoreactivity. As such, glial cell-derived protein GFAP is now an identified autoantigen in T1D and autoantibodies to GFAP has been detected in NOD mice and humans with T1D (23), thus showing the relevance of the microbiota–EGC pathways in T1D.

One study has shown that SCFA butyrate can induce increasing excitatory choline acetyltransferase (ChAT) neurons through the butyrate transporter monocarboxylate transporter (MCT), which is expressed by enteric neurons (78). However, it is still unknown what factors control neuronal MCT2 expression. Further studies will be necessary to determine how SCFAs regulate MCT2 expression and control the activity of intestinal neural circuits. SCFAs exert their function through two mechanisms, via metabolite sensing GPCRs or inhibition of histone deacetylase (HDAC) activity (13, 35, 79, 80). There are three receptors for SCFA acetate, butyrate, and propionate, namely GPR43 (FFA2), GPR41 (FFA3), and GPR109a. GPR43 is activated by SCFAs with varying potency—acetate > propionate > butyrate. GPR43 is expressed on gut epithelial cells and certain immune cells (81). GPR109a is expressed on a variety of immune cells, as well as adipocytes, hepatocytes, gut and retinal epithelium, vascular endothelium, and neuronal tissue (82). GPR109a is primarily activated by both niacin and butyrate ligands. While niacin levels are not high enough to activate the receptor under normal physiological conditions, levels of butyrate, obtained from the gut environment, and its oxidized form, β-hydroxybutyrate, are sufficient to stimulate a response (82). Similarly, GPR41 has been reported to be expressed on EGCs and enteric neurons (83, 84). GPR41 also binds the three major SCFAs, but with differing affinities (85).

Similar to the effects exerted through the GPCRs, SCFAs can influence the function and development of immune cells directly through epigenetic regulation of gene expression such as inhibition of HDACs (13, 86). HDACs allow the conversion of repressive chromatin structures, which takes place on lysine residues on N-terminal tails of histones 3 and 4, to increase gene transcription. HDACs are a group of 18 known enzymes that remove acetyl groups from the histones tails that bind DNA (87). Although little is known about the effects of SCFAs on EGCs through epigenetic modifications, it has been shown that butyrate treatment increases acetylation of the H3K9 in primary enteric neurons and the EGC *in vitro* (84).

SCFAs can also modulate gut motility by the production of serotonin by epithelial enterochromaffin cells (ECs) (88, 89). For instance, GF mice present gut dysmotility that was reversed by inoculation with SCFA-producing bacteria. Tested in human-derived EC cell lines, SCFAs increased serotonin (5-hydroxytryptamine [5-HT]) by up-regulating THER expression of tryptophan hydroxylase 1 (Tph1) (89) and by the serotonin-selective reuptake transporter (SERT), which is expressed by intestinal epithelial cells (90). Another critical role of SCFAs on the ENS is evidenced by the conversion of primary bile acids synthesized *de novo* into secondary bile acids in the liver (91). Aside from their role in dietary fat absorption, secondary bile acids can activate several GPCRs and nuclear hormone receptors, including the G-protein-coupled bile acid receptor 1 (TGR5) and farnesoid X receptor (FXR), highly expressed in enteric neurons and enteroendocrine L cells that improved intestinal inflammation and glucose tolerance in HFD-fed mice (92). TGR5 also affect peristalsis that is mediated partly by serotonin 5-HT (93), implicating its potential for the treatment of constipation and diarrhea. Altogether, this suggests the relevance of the gut–neuro–immune axis in T1D (**Figure 1**).

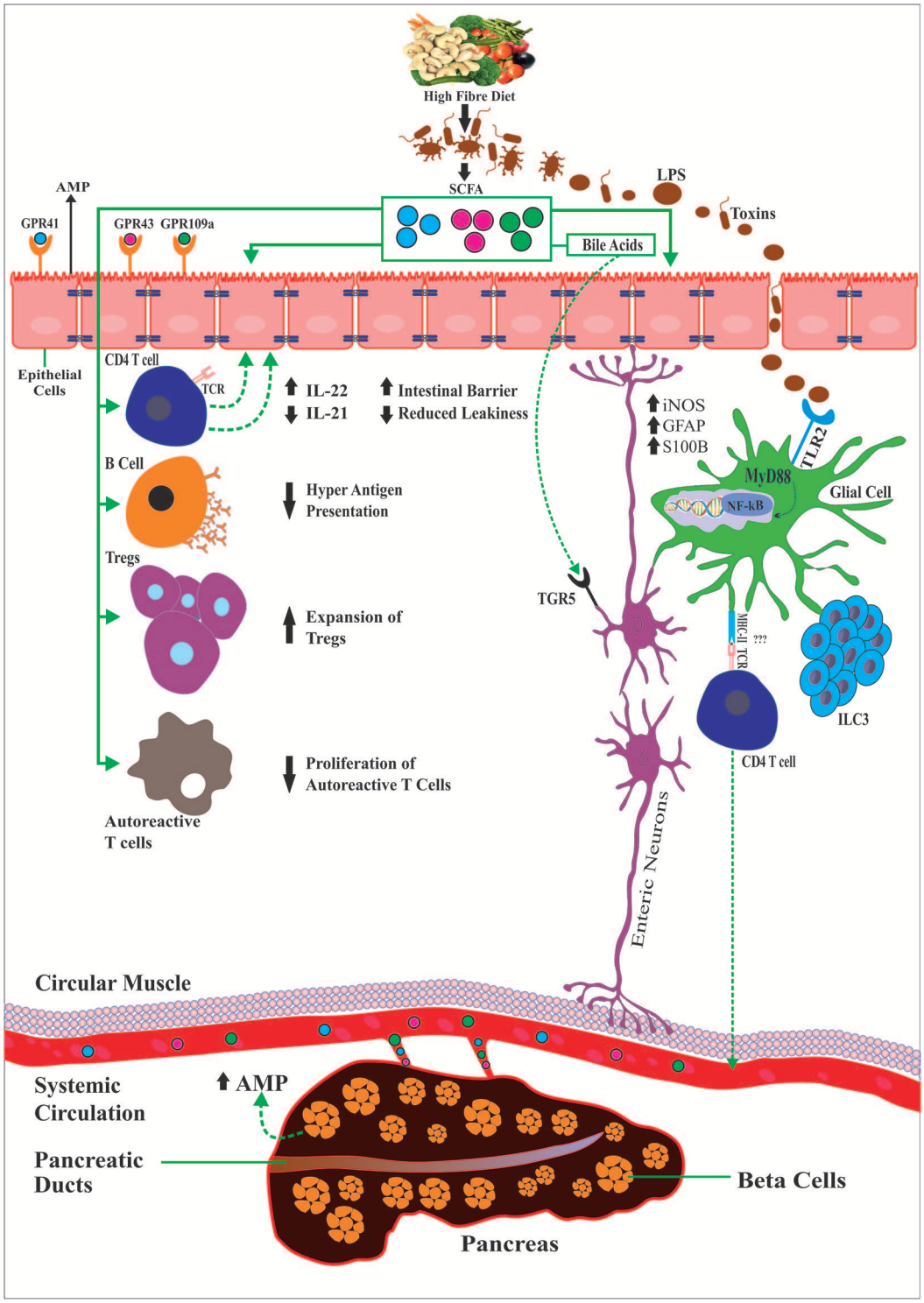


**FIGURE 1 |** Diet and gut microbiota through the production of dietary SCFAs exert anti-inflammatory effects by controlling the activity of multiple immune cell types, outside or locally in the intestinal mucosa, the enteric glial cells and neurons but also glial cells in the pancreatic islets and the beta-cells. As such SCFAs promote IL-22 production $CD4^+$ T cells or by supporting ILC3 cells, the major producers of IL-22. Also SCFAs can reduce production of pro-inflammatory cytokines IL-21, LPS, induce beta cell regeneration via AMPS, regulate GFAP in the gut and beta-cells, modulate the expansion of regulatory T and reduction of autoreactive CD8 T cells and reducing B cell hyperactive antigen presentation capacity. Activation of GPRCs (GPR41 and GPR43) on enteroendocrine cells of the intestinal epithelium and TLR signaling (e.g., TLR2 and TLR4) maintains subsets of enteric neurons resulting changes in gut motility, conversion of primary bile acids into secondary bile acids, which activate TGRS expressed by enteroendocrine cells and enteric neurons among many others.

## CONCLUDING REMARKS

Among the described effects that SCFAs have on modulating the immune system, beta-cell biology, and gut homeostasis, we have uncovered a novel role for SCFAs by modulating the ENS in the gut, central for the control and prevention of T1D. Overall, an immune response to antigens presented not only by GFAP-expressing pSCs in the pancreas but also by GFAP-expressing EGCs in the gut is a novel finding involved in the initiation of the autoimmune process. Could it trigger antigen-experienced autoreactive cells to move up the gut and reach the ductal and beta-cells, and break the GFAP-expressing neuronal mantle of the islets? This is an unexplored field and requires further research. Given the close location and connection between the gut and the pancreas and their intrinsic dependence from the nervous system, this fine-tuned immuno–neuro-islet cross-talk may be profoundly affected by perturbed gut homeostasis at very early stages of the initiation of the T1D. Dampening the initial immune response or preventing the perpetuation of the islet-specific immune response by maintaining the integrity of the gut is among the possible therapeutic approaches to reprogram T1D (12, 14). Thus, any hope for a cure may lie in methods that can halt immune-mediated beta-cell damage by maintaining or improving gut–immune tolerance.

## AUTHOR CONTRIBUTIONS

EM developed the conceptual idea, wrote and edited the manuscript. AJ wrote and edited the manuscript.

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# Norovirus Changes Susceptibility to Type 1 Diabetes by Altering Intestinal Microbiota and Immune Cell Functions

*James A. Pearson[1], Ningwen Tai[1†], Dilrukshi K. Ekanayake-Alper[2,3†], Jian Peng[1], Youjia Hu[1], Karl Hager[4], Susan Compton[3], F. Susan Wong[5*], Peter C. Smith[3*] and Li Wen[1*]*

[1] Endocrinology, Internal Medicine, School of Medicine, Yale University, New Haven, CT, United States, [2] Colombia Center for Transplant Immunology and Institute of Comparative Medicine, Columbia University Medical Center, Colombia University, New York, NY, United States, [3] Department of Comparative Medicine, School of Medicine, Yale University, New Haven, CT, United States, [4] Department of Lab Medicine, School of Medicine, Yale University, New Haven, CT, United States, [5] Diabetes Research Group, Division of Infection and Immunity, School of Medicine, Cardiff University, Cardiff, United Kingdom

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**Edited by:**
Julien Diana,
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***Correspondence:**
Li Wen
li.wen@yale.edu
Peter C. Smith
peter.smith@yale.edu
F. Susan Wong
wongfs@cardiff.ac.uk

[†] These authors have contributed equally to this work

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Environmental factors contribute to Type 1 diabetes (T1D) susceptibility. The gut microbiome, which includes bacteria, viruses, and fungi, contributes to this environmental influence, and can induce immunological changes. The gut viral component of the microbiome, related to T1D has mostly focused on coxsackieviruses and rotavirus. The role of norovirus, another common enteric virus, in susceptibility to T1D was hitherto unknown. Norovirus is highly infectious and encountered by many children. We studied the mouse norovirus 4 (MNV4), related to human noroviruses, in the Non-obese diabetic (NOD) mouse model, to determine its role in influencing susceptibility to T1D. We infected MNV-free NOD mice with MNV4 by exposing the mice to MNV4-positive bedding from an endemically-infected mouse colony to mimic a natural infection. Control MNV-free NOD mice were exposed to MNV-free bedding from the same colony. Interestingly, MNV4 infection protected NOD mice from the development of T1D and was associated with an expansion of Tregs and reduced proinflammatory T cells. We also found MNV4 significantly modified the gut commensal bacteria composition, promoting increased α-diversity and Firmicutes/Bacteroidetes ratio. To elucidate whether T1D protection was directly related to MNV4, or indirectly through modulating gut microbiota, we colonized germ-free (GF) NOD mice with the MNV4-containing or non-MNV4-containing viral filtrate, isolated from filtered fecal material. We found that MNV4 induced significant changes in mucosal immunity, including altered Tuft cell markers, cytokine secretion, antiviral immune signaling markers, and the concentration of mucosal antibodies. Systemically, MNV4-infection altered the immune cells including B cell subsets, macrophages and T cells, and especially induced an increase in Treg number and function. Furthermore, *in vitro* primary exposure of the norovirus filtrate to naïve splenocytes identified significant increases in the proportion of activated and CTLA4-expressing Tregs. Our data provide novel knowledge that norovirus can protect NOD mice from T1D development by inducing the expansion of Tregs and reducing inflammatory T cells. Our study also highlights the importance of distinguishing the mucosal immunity mediated by bacteria from that by enteric viruses.

**Keywords: norovirus, type 1 diabetes, non-obese diabetic mice, gut microbiota, Tuft cells, Treg**

## INTRODUCTION

Type 1 diabetes (T1D) is a T cell mediated autoimmune disease resulting from the destruction of insulin-producing pancreatic β-cells. The incidence of T1D is increasing worldwide, at a rate too fast to be associated with genetic changes alone. Increasing evidence suggests that environmental factors can contribute to the risk of T1D development. Studies in both animal models and humans have identified changes in the bacterial component of the gut microbiota composition in individuals with T1D or "at-risk" compared to controls (1–11). In addition, enteric viruses, such as the Coxsackieviruses and Rotavirus have been associated with the development of T1D in both humans and mouse models (12–21). Interestingly, a coxsackievirus B vaccine has shown to protect Non-obese Diabetic (NOD) mice from the development of virus-induced diabetes (22) and a recent human study suggests that rotavirus vaccination of infants has contributed to the decreased incidence of T1D in Australian children (23). However, little is known about other viruses including norovirus in relation to T1D development.

Norovirus, a common enteric virus belonging to the family *Caliciviridae*, is highly contagious and responsible for the majority of non-bacterial gastroenteritis outbreaks, especially in the winter, and infections have often been referred to by the non-scientific name "stomach flu" (24). In most individuals, norovirus infections are short-lived but the infection can have severe complications in young children, the elderly and immunocompromised individuals (25–27). Transmission occurs by the fecal-oral route, either through direct contact with infected individuals or indirectly through exposure to contaminated food or water, and by infectious aerosols generated by vomiting (28–31). Given that the majority of individuals diagnosed with T1D are children, and the incidence appears to peak in winter (32) when it is also high season for norovirus infection, it is extremely important to investigate the role of norovirus in mediating susceptibility to T1D. It is likely norovirus infection is an additional stressor of the immune system prior to diagnosis and is not the cause of T1D, as many people infected with norovirus do not develop T1D.

Murine norovirus (MNV), a non-enveloped single-stranded RNA virus related to human noroviruses, is often used to study the role of norovirus infections in mouse models. MNV was first described following the investigation of high mortality in Rag/Stat-1 double-deficient mice (33). MNV has been shown to alter susceptibility to inflammatory bowel disease (IBD) and food allergies (34–36), by altering other components of the gut microbiota composition and gut immunity. Furthermore, the bacterial components of gut microbiota also affect the host susceptibility to norovirus infection, supported by the observation that antibiotic treatment prevented norovirus persistence due to changing the gut bacterial composition (37). Thus, MNV can alter both gut microbiota composition and immune responses of the host.

Our previous studies and those of other investigators have demonstrated that susceptibility to T1D development in NOD mice depends on immune signaling in response to the microbiota (8, 9, 38–41) and to coxsackievirus (42, 43) or rotavirus (17, 19). However, it is unknown if MNV alters the susceptibility to T1D development in NOD mice. We hypothesize that MNV infection will affect T1D susceptibility in NOD mice by changing gut commensal bacteria and hence local, as well as systemic, immune responses of the hosts. The current study was to test this hypothesis and to fill our knowledge gap regarding the role of norovirus infection in T1D development.

## MATERIALS AND METHODS

### Mice

Specific pathogen-free (SPF) NOD mice were purchased from the Jackson Laboratory and housed under SPF conditions in individually-ventilated filter-topped cages at the Yale Animal Resource Center (YARC). These mice were reported free of murine norovirus, ectromelia virus, murine rotavirus, lymphocytic choriomeningitis virus, mouse hepatitis virus, mouse parvovirus, minute virus of mice, pneumonia virus of mice, reovirus, Sendai virus, *Mycoplasma pulmonis*, *Helicobacter* spp., pinworms, fur mites, and opportunistic bacteria (https://www.jax.org/strain/001976). Germ-free (GF) NOD mouse breeders were generously provided by Alexander Chervonsky (University of Chicago, USA) and have been bred and maintained at the gnotobiotic facility of YARC. All the mice received autoclaved food (Global 2018S, Envigo) and hyperchlorinated (4–6 ppm) water *ad libitum* and were maintained on 12-h light/dark cycles. The use of mice in this study was approved by the Institutional Animal Care and Use Committee at Yale University.

### MNV Detection and Infection

The SPF mouse housing room in which this study was conducted was screened for MNV infection by PCR of fecal samples. Fecal pellets were homogenized in PBS and DNA was isolated using a DNeasy kit (Qiagen) according to the manufacturer's instructions. PCR amplification was performed using a PCR Core kit (Roche) and primers specific for the MNV non-structural gene (see **Supplementary Table 1**). The strain of MNV identified by sequencing was consistent with MNV4. The MNV4-positive cage bedding was collected and introduced to the cages that housed MNV-free NOD mice (4–5 week of age). The cages had half the bedding replaced with autoclaved clean bedding, weekly. As a control, another set of MNV-free NOD mice were introduced to MNV-free bedding from different cages within the same housing room. To avoid cross-contamination, the control NOD mice were housed in a different room in the same facility. All the mice were screened by PCR for the presence of MNV4 in the fecal material and by an immuno-fluorescence assay for the presence of anti-MNV antibodies in the serum. Briefly, microscope slides were mounted with monolayers of MNV-infected RAW 264.7 cells, a mouse macrophage cell line. Serum samples (1:10 dilution) were added to the slides and the binding of MNV antibodies was detected with fluorescein-conjugated goat anti-mouse antisera. All MNV+ mice continued to actively shed virus throughout the study. Only mice exposed to MNV had anti-MNV antibodies in the serum. All control (MNV4-free) mice remained free of MNV infection.

## Diabetes Incidence

MNV-infected and control NOD mice were monitored for glycosuria weekly, for 25 weeks. Glycosuria was confirmed by two blood glucose measurements, 24-h apart, of over 250 mg/dl (>13.9 mmol/L).

## Histology

Pancreata from 12-week old MNV-free (control) and MNV-infected NOD female mice were formalin-fixed and embedded in paraffin. Tissues were stained with hematoxylin and eosin. Insulitis was scored under light microscopy. 150–200 islets from 4 to 5 mice were individually scored.

## 16s rRNA Sequencing of Gut Microbiota

Fecal samples were collected from MNV-free (control) and MNV-infected mice and resuspended in 300 μl TE buffer containing 0.5% SDS and 200 μg/ml Proteinase K. Bacterial DNA was extracted as previously described (44). The V4 region of the 16S rRNA gene was amplified from each DNA sample using a bar-coded, broadly conserved, bacterial forward, and reverse primer as previously published (8). Bacterial DNA samples were used for pyrosequencing with Ion Torrent PGM sequencing system (Life Technologies). The results were analyzed using QIIME 1.8. α-diversity, a measure of the number of bacteria, and β-diversity, a measure of the composition of the microbiota were both analyzed and β-diversity was plotted using a principal coordinate analysis (PCoA) plot.

## Flow Cytometry and Intracellular Staining

Immune cells were incubated with an Fc-blocking antibody at 4°C for 15 min. Post-incubation, cells were stained for surface markers using antibodies (conjugated with different fluorochrome) against CD4, CD8, CD11b, CD11c, CD19, CD21, CD23, CD39, CD69, CD86, CTLA4, CXCR3, KLRG1, TCRbeta, IgA, IgD, IgM, CCR6, CCR7, CCR9, and a viability dye (all from BioLegend), for 30 min at 4°C. For Treg staining, cells were stained for surface markers prior to fixation for 1 h at room temperature and subsequent permeabilization (buffers purchased from Tonbo Bioscience). The cells were then incubated with an Fc-blocking antibody at 4°C for 15 min prior to staining with anti-FoxP3 (eBioscience), incubated for 30 min, at 4°C; following by washing. For intracellular cytokine staining, cells were incubated at 37°C in the presence of PMA (Sigma), Ionomycin (Sigma) and Golgi Plug (BD) for 4 h prior to washing and surface staining as outlined above. After surface staining, cells were fixed (20 min, room temperature) and permeabilized (buffers purchased from Sigma) and incubated with an Fc-blocking antibody at 4°C for 15 min prior to staining with anti-cytokine antibodies (30 min, 4°C) and washing. Cells were analyzed on a BD LSR II flow cytometry followed by analysis using Flowjo software.

## Infection of Germ-Free NOD Mice

Fecal pellets were pooled from 6 individual 12-week old MNV4+ donors and resuspended in sterile PBS. Pellets were homogenized using a bead beater machine and large fecal materials were removed by centrifugation (500 rpm, 3 min). Supernatant was further centrifuged at high speed (13,000 rpm, 5 min) followed by filtration through a 0.22 μM filter (Millipore). This filtered supernatant was then divided into two portions, with half of the solution exposed to UV light (20 min, room temperature) to destroy the virus (virus –) and the other half left unexposed to UV light (virus +). GF NOD mice (∼4 weeks-old) were gavaged with 200 μl of the UV-treated or non-UV treated solution. GF NOD mice were regularly assessed for viral presence in the fecal sample and antibodies in the serum, before and after inoculation, as described earlier. Mice were terminated 8-weeks after gavage for the study and the experimental design is shown in **Supplementary Figure 1**.

## qPCR

RNA was extracted from the distal small intestine of colonized GF NOD mice, using a Qiagen RNAeasy kit, prior to cDNA synthesis following the manufacturer's instructions (Bio-Rad). qPCR was performed using a qPCR cycler (iQ5; Bio-Rad Laboratories), according to the manufacturer's instructions with the specific primers listed in **Supplementary Table 1**. The relative gene expression was determined using the $2^{-\Delta\Delta CT}$ method by normalization with GAPDH housekeeping gene.

## Antibody Measurements

Serum and cecal wash were collected from the mice studied at termination. Antibody concentrations were determined by ELISA, using the reagents purchased from Southern Biotech, following the protocol previously described (8). Samples were diluted (serum 1:50–1:100; cecal samples 1:2–1:10) before the antibody measurements. Antibody concentrations were converted based on each of the standard curves.

## Treg Suppression Assay

Treg cells were purified from spleen of colonized GF NOD mice using magnetic bead isolation kits (EasySep TM Mouse CD4+CD25+ Regulatory T cell isolation kit, Stemcell Technologies). BDC2.5 CD4 T cells were isolated from BDC2.5 T cell receptor (TCR) transgenic NOD mice by negative selection using hybridoma supernatants to deplete MHCII+ (10.12.16) antigen presenting cells (APCs) and CD8 T cells (TB105). Hybridoma supernatants were kindly provided by the late Charles Janeway Jr. (Yale University). Isolated Tregs were co-cultured with BDC2.5 CD4 T cells in a 1:2 (Treg:Teff) ratio in the presence of irradiated APCs and different concentrations of BDC2.5 mimotope peptide. The cells were cultured for 4 days prior to pulsing with $^{3H}$-thymidine, for a further 18 h. Data were presented as corrected counts per minute (Δcpm) after subtracting from background (Tregs + BDC2.5 CD4 T cells without antigenic peptide).

## *In vitro* Culture

Splenocytes from colonized GF NOD mice ($2 \times 10^6$) were cultured in the presence or absence of non-UV treated (MNV4-containing) fecal material, prepared as described above. Cells were stimulated for 16 h with the final 4 h in the presence of PMA, Ionomycin (both Sigma) and Golgi Plug (BD), prior to surface and intracellular staining as outlined above. Another set

of cells was stimulated for 16 h with UV-treated and non-UV treated (MNV4-containing) fecal material for RNA isolation after removing the stimulators. The cultured cells were also used for T cell and APC isolation by negative selection using monoclonal antibody supernatants. For T cell isolation, 10.2.16, HB198 and N418 were used to remove MHC II+ cells by magnetic beads. For APC isolation, Y19 was used to remove Thy1+ T cells by complement. All the mAb supernatants were provided by the late Charles Janeway Jr. (Yale University).

## Adoptive Transfer

Splenocytes were cultured for 12 h, in the presence or absence of non-UV treated (MNV4-containing) fecal material, prepared as described above. After thorough washing, the stimulated splenocytes were adoptively transferred into Rag-deficient NOD mice ($4 \times 10^6$/donor). Tissues were harvested 1 week post-transfer.

# RESULTS

## MNV4 Infection Protects NOD Mice From the Development of Type 1 Diabetes

To determine whether norovirus can alter the susceptibility of NOD mice to development of type 1 diabetes, MNV-free NOD mice were exposed at 4–5-weeks of age to bedding from either MNV- or MNV+ cages, of mice housed in the same facility. All MNV-infected mice were confirmed as virus positive by PCR in fecal samples, 2-weeks post-exposure, and the majority of the mice continued to shed virus throughout the study. All non-infected control mice remained virus-free. The mice from 10-weeks of age were tested weekly for glycosuria and followed longitudinally to determine the incidence of spontaneous diabetes in norovirus-infected and control mice. We found that norovirus exposure significantly delayed and reduced the incidence of diabetes development, compared to the control mice, by 25-weeks of age (**Figure 1A**). The delay was more striking at 14–20 weeks of age when only ∼30% of infected mice developed diabetes by 20 weeks, compared to over 85% of control mice ($p = 0.006$). To determine, whether norovirus infection also reduced the immune cell infiltration in the pancreatic islets, pancreata from 12-week old pre-diabetic mice were studied for the severity of islet infiltration. In agreement with the diabetes incidence, we found norovirus-infected mice also had less insulitis compared to norovirus-free control NOD mice (**Figure 1B**).

## MNV4 Infection Promotes the Expansion of Regulatory T Cells and Reduces Inflammatory Cytokines and T Cell Activation

As β-cell damage in T1D is an immune-mediated process we investigated changes of the immune system that could contribute to diabetes protection, in response to norovirus infection. We demonstrated that norovirus infection specifically enhanced the total number and proportion of regulatory T cells in the pancreatic draining lymph nodes (PLN) compared to uninfected control mice (**Figures 2A,B** and **Supplementary Figure 2**). We also evaluated the cytokine secretion profile from CD4 T cells and our results showed a significant reduction in IFNγ-secreting CD4 T cells, specifically in the PLN and Peyer's patches (PP), while the proportion of IL-10, TNFα, or IL-17a-secreting CD4 T cells remained unchanged (**Figure 2C** and **Supplementary Figures 3A–D**). Whilst we found no differences in CD4 T cell activation (CD69, **Supplementary Figure 3E**), we observed a significant reduction in CD8 T cell activation, particularly in the PLN (**Figure 2D** and **Supplementary Figure 3F**). We found no significant differences in IFNγ, TNFα, or IL-10 secretion from CD8 T cells (**Supplementary Figures 3G–I**). We also assessed changes in B cells, macrophages and dendritic cells in different lymphoid tissues and did not observe significant differences between the two groups (**Supplementary Figure 4**). Thus, the protection in

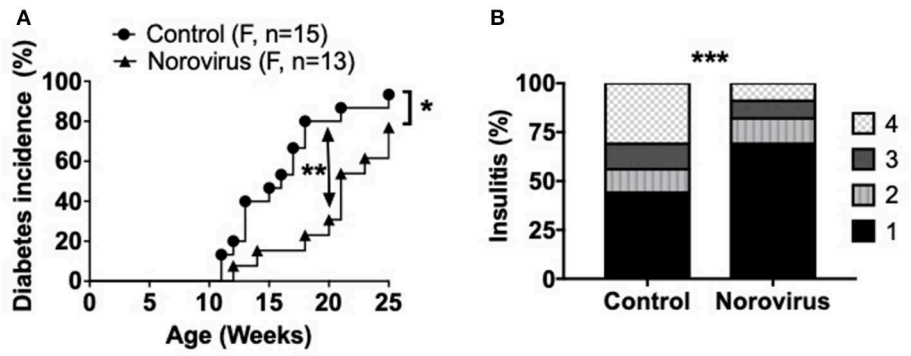


**FIGURE 1 |** Norovirus infection protects NOD mice from the development of T1D. **(A)** Diabetes incidence was observed in MNV-infected (norovirus group) or MNV-negative (control) NOD mice, $n = 13$–15. Mice were monitored for glycosuria weekly for 25 weeks. Diabetes was confirmed by 2 blood glucose measurements, 24-h apart, of over 250 mg/dl (13.9 mmol/L). **(B)** 12-week old NOD mice infected or free of norovirus were harvested for their pancreata and assessed for insulitis by light microscopy, $n = 5$–6. Islets were graded using the following scale: 1: No insulitis, 2: >25% insulitis, 3: 25–50% insulitis, 4: >50% insulitis. Data were analyzed for significance using a log-rank test for survival at 20 and 25 weeks of age **(A)** or a chi-square test **(B)**. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Data are representative of one of two experiments.

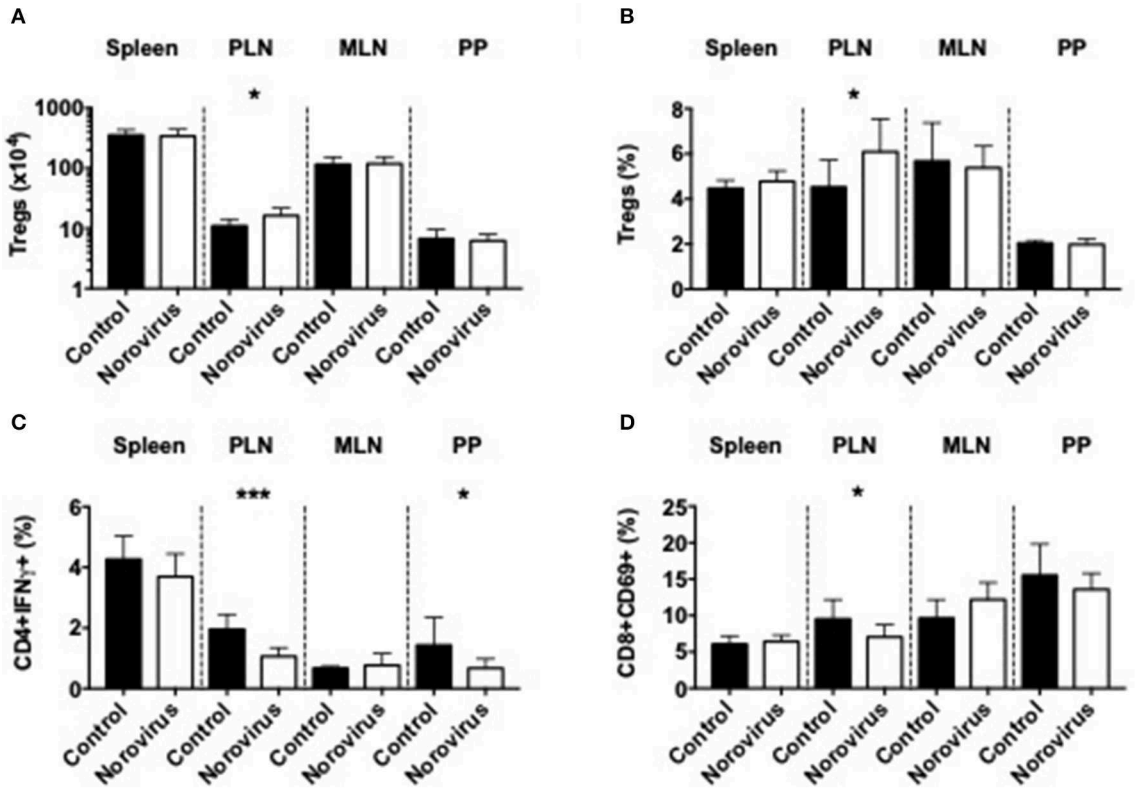


**FIGURE 2 |** Norovirus infection expands Tregs and reduces inflammatory T cells. The number **(A)** and proportion **(B)** of CD4+FoxP3+ T cells were investigated from the spleen, pancreatic lymph node (PLN), mesenteric lymph node (MLN), and Peyer's patches (PP) of norovirus-free and norovirus-infected NOD mice. Tregs were gated on live single TCRbeta+CD4+CD8- T cells prior to FoxP3 gating. **(C)** Cells were stimulated for 4-h in the presence of PMA, Ionomycin, and Golgi Plug prior to surface and intracellular staining. IFNγ-secreting CD4 T cells were gated from live, single TCRbeta+CD4+CD8- T cells prior to gating on IFNγ. **(D)** The proportion of CD69+ CD8 T cells gated from live single TCRbeta+CD8+CD4- T cells prior to gating on CD69. Data were analyzed for significance using a Student's *T*-test. $^{*}P < 0.05$, $^{***}P < 0.001$. Data represents pooled data from two independent experiments; $n = 9$–11 mice.

the NOD mice infected with norovirus was associated with the expansion of Tregs and reduction of IFNγ-producing CD4 T cells and activation of CD8 T cells.

## MNV4 Infection Alters the Composition of Gut Commensal Bacteria

Our previous studies and those of others have shown that the changes in composition of gut commensal bacteria influence susceptibility to T1D in both NOD mice and humans (1, 3, 6, 8, 9, 11, 45). As norovirus is an intestinal virus and can alter the overall gut commensal bacteria composition in IBD (35), we investigated whether gut commensal bacterial composition was altered in MNV4-infected mice by 16S rRNA sequencing. We found that norovirus infection increased the intestinal microbial α-diversity and Firmicutes/Bacteroidetes ratio compared to the control mice that are more susceptible to diabetes (**Figures 3A,B**). Both of these changes have been reported to be associated with protection from the development of T1D in humans (1). To determine whether gut commensal bacterial composition was significantly different between uninfected control mice and norovirus-infected NOD mice, we conducted a principal component analysis of β-diversity. Our results revealed that the presence of norovirus significantly altered the composition of gut commensal bacteria (**Figure 3C**). Furthermore, we found significant increases in the relative abundance of *Akkermansia muciniphilia* and non-identifiable species of *Ruminococcus*, *Mogibacteriaceae*, *Lachnospiraceae*, and *Anaerostipes* in uninfected NOD mice compared to norovirus-infected NOD mice (**Figure 3D**). However, norovirus-infected mice had increased relative abundances of *RF32*, *Mucispirillum schaedleri* and *YS2* species compared to uninfected control mice. Together, our data demonstrate that MNV4 infection can alter the composition of gut commensal bacteria in NOD mice, suggesting a role in diabetes protection in the MNV4-infected NOD mice.

## MNV4 Infection of Germ Free (GF) NOD Mice Significantly Alters Intestinal Immunity

In the above experiments using SPF NOD mice, we infected the mice naturally with MNV+ bedding from the cages housing MNV-infected mice and the control mice had MNV- bedding. However, it was not clear whether diabetes protection and Treg cell expansion were the direct effect of norovirus or an indirect

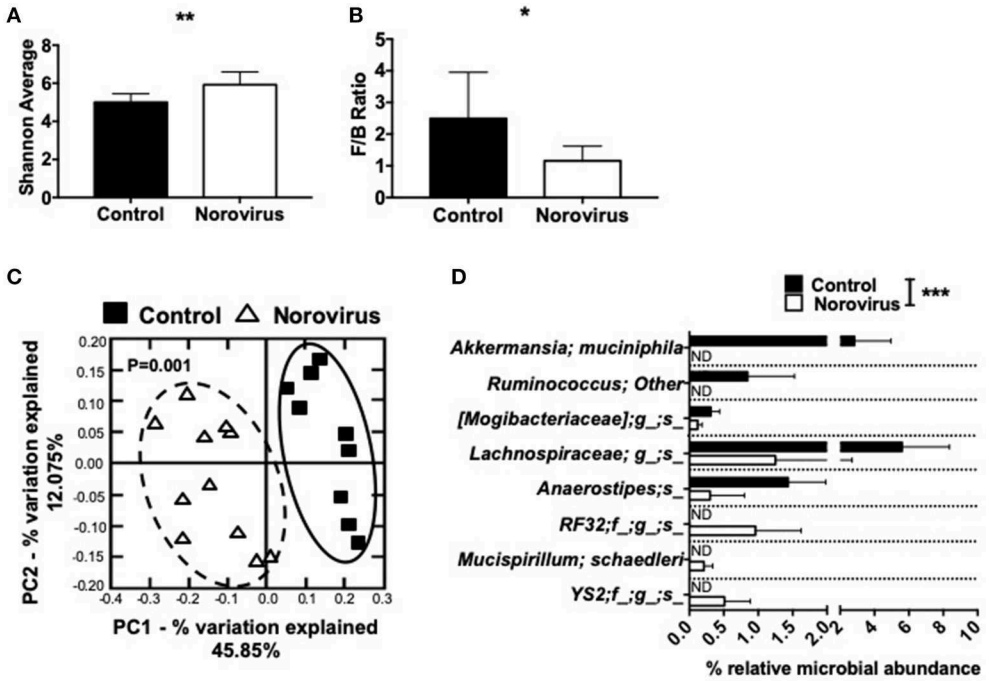


**FIGURE 3 |** Norovirus infection alters the gut commensal bacterial composition in NOD mice. Fecal pellets were collected from 12-week old NOD mice that were MNV-free (control) or MNV-infected (norovirus). **(A)** α-diversity was assessed using the Shannon diversity index. **(B)** The Firmicutes/Bacteroidetes ratio were calculated from the phylogenetic data. **(C)** β-diversity was investigated using a principal component analysis plot, with significant species differences plotted **(D)**. Data were assessed for significance using a Student's *T*-test **(A,B)**, ANOSIM **(C)** or multiple *T*-test and FDR correction **(D)**. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Data shown are pooled from two experiments ($n = 9$–11 mice). ND, not detectable.

effect through virally-induced gut commensal bacterial changes. To elucidate the specific impact of norovirus on the immune system, we studied Germ-Free (GF) NOD mice. We infected GF NOD mice with pooled and filtered [removing the bacteria (46)] donor fecal material from mice infected with MNV4 (see Material & Methods and **Supplementary Figure 1**). As a control we UV-treated the same material to destroy the virus. All control mice remained negative for MNV4 (virus–) by PCR following colonization, and also negative for MNV-specific antibodies in serum, whereas the MNV4 infected mice (virus+) were positive for both virus in the feces and MNV-specific antibodies in the serum. Furthermore, we investigated the composition of gut microbiota from the infected mice but did not find significant changes between the virus-infected or non-virus-infected group (**Supplementary Figure 5**). Thus, the changes observed in the mice are predominantly associated with the presence/absence of norovirus.

To investigate the effect of MNV infection on intestinal immunity, we measured various cytokines in the cecal wash from virus– and virus+ ex-GF mice. We found significant increases in the concentration of IFNα, IL-4, IL-10, IL-17a, and IFNγ but no differences in TGFβ in virus+ mice compared to virus– mice (**Figure 4A**). Different MNV strains have different cell tropisms, and have been shown to infect both immune cells (47, 48) and specialized intestinal epithelial cells (IECs) called Tuft cells (49). To determine whether MNV4 were present in IECs, we isolated the IECs from the small intestine of ex-GF mice and detected MNV4 by qPCR. We found that the MNV4 gene was highly expressed in the mice exposed to MNV4 compared to the uninfected controls (**Figure 4B**). This suggests that IECs are most likely a target for MNV4 infection. Next, we assessed the Tuft cell-related gene expression in the small intestine of the ex-GF mice by qPCR, given MNV1 has been reported to infect Tuft cells (49). Interestingly, we found significant increases in Tuft cell-related genes including dlck1, IL-25, and succinate receptor (binding of which with succinate can activate Tuft cell responses) (50), in virus-infected mice compared to controls (**Figures 4C–E**). We also detected significant increases in the expression of single-stranded RNA-recognizing toll-like receptors (TLRs) 7 and 8, the IFNα receptor, IFNλ and antiviral signaling genes including Rig1, Stat1, NFκB, and IFN regulatory factors (IRF) 1 and 3 in virus+ mice when compared to virus- mice (**Figures 4F–N**). We did not observe any significant changes in the gene expression of tight junction (zonulin and claudin 2), the gut permeability markers; neither antimicrobial peptides or TLR3, which recognizes double-stranded RNA, between the two groups (**Supplementary Figures 6A–H**). Together, our data suggest that MNV significantly alters the intestinal immune responses resulting in altered cytokine secretion and activation of both Tuft cell and antiviral responses.

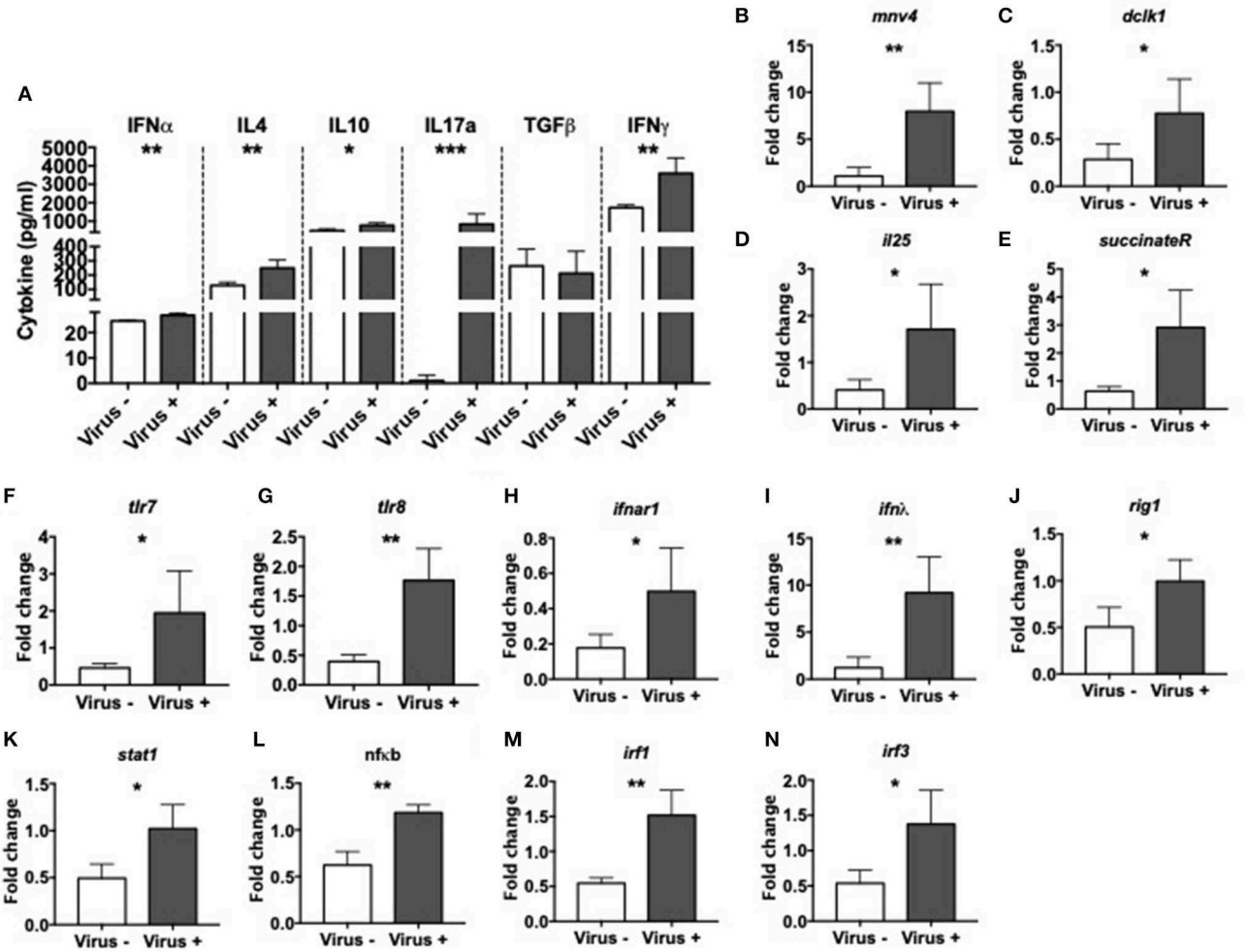


**FIGURE 4 |** Altered intestinal immunity in norovirus infected NOD mice. GF NOD mice were orally gavaged at ∼4 weeks of age with pooled and filtered norovirus-enriched fecal filtrate from norovirus-infected NOD mice at 12-weeks of age that was UV-treated (virus–) or non-UV-treated (virus+). Only mice gavaged with the non-UV-treated filtrate were infected with norovirus and developed anti-MNV antibodies. **(A)** Cytokines in cecal flushes from infected/non-infected mice were measured by ELISA. **(B–N)** RNA from the distal small intestine immediately adjacent to the cecum was extracted and equal concentrations of cDNA synthesized. cDNA was then subject to qPCR for MNV4, genes associated with Tuft cells [doublecortin-like kinase 1 (dclk1), IL25, succinate receptor 1; **C–E** respectively], with Toll-like receptor (TLR) genes (tlr7 and tlr8; **F,G** respectively) and antiviral immune signaling genes [Interferon α receptor 1 (ifnar1), interferon lamda (ifnλ), retinoic inducible gene I (rigI), signal transducer and activator of transcription 1 (stat1), nuclear factor kappa-light-chain-enhancer of activated B cells (nfκb), interferon regulatory factor (IRF) 1 and 3; **H–M** respectively]. The relative expressions of these genes were determined using the $2^{-\Delta\Delta CT}$ method by normalization with GAPDH. Data were assessed for significance using a Student's *T*-test **(A–N)**. Data shown are representative of one of two experiments with $n = 4$ per group/experiment. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

## MNV4 Infection of GF NOD Mice Significantly Alters APCs

To further investigate the effect of MNV infection on immune cells, we focused on the antigen presenting cells (APCs). We first assessed the effect on mucosal B cells by evaluation of immunoglobulins in the gut lumen of infected and non-infected ex-GF mice. Our results revealed significant reductions in the concentrations of IgG2a and IgG2b subclasses in the intestine of MNV-infected mice compared to controls (**Figure 5A**); however, these changes were not present in the serum of the same mice (**Supplementary Figure 7A**). To probe if the mucosal antibody changes were due to changes of local B cells, we investigated the phenotype of B cells in PP and PLN. We found a reduced population of follicular CD21+CD23+ B cells but an increase in marginal zone CD21+CD23- B cells in the PP of virally-infected mice compared to the controls (**Figures 5B,C** and **Supplementary Figure 7B**). We also observed significant reductions in immature IgD+IgM- B cells, while IgD+IgM+ mature B cells were increased in virally-infected mice compared to the uninfected controls in the PP (**Figures 5D,E** and **Supplementary Figure 7C**). There was a similar trend in the PLN, although the changes were not statistically significant. There were no changes in IgD-IgM+ B cells or IgA+ B cells (**Supplementary Figures 7D,E**). Next, we investigated the effect

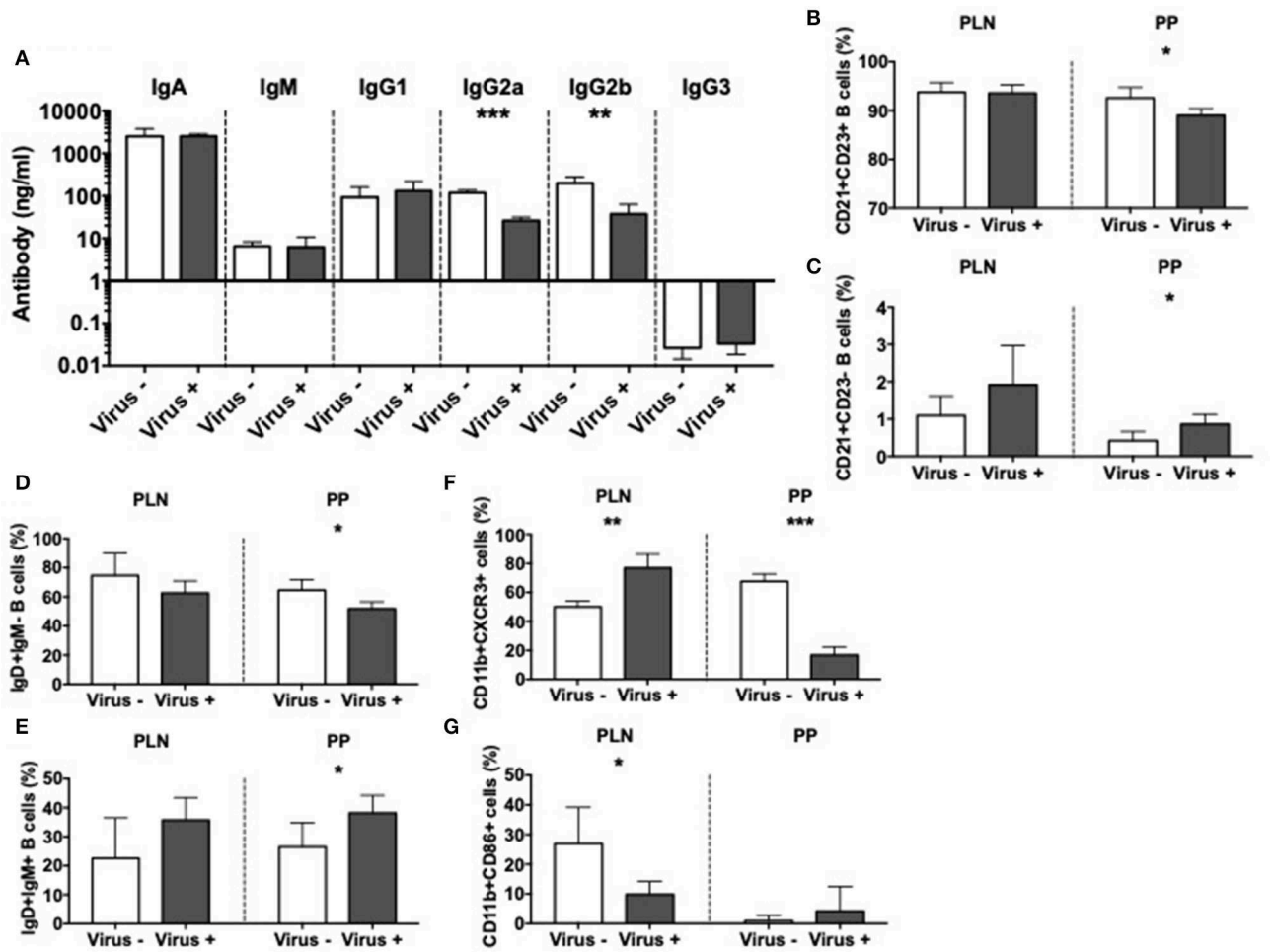


**FIGURE 5 |** Altered APC immunity in norovirus infected NOD mice. GF NOD mice were orally gavaged at ∼4 weeks of age with pooled and filtered norovirus-enriched fecal filtrate from norovirus-infected NOD mice at 12-weeks of age that was UV-treated (virus–) or non-UV-treated (virus+). Only mice gavaged with the non-UV-treated filtrate were infected with norovirus and developed anti-MNV antibodies. **(A)** Cecal antibody concentrations were determined by ELISA. **(B–G)** The proportion of cell subsets was determined by flow cytometric analysis. **(B,C)** The proportion of follicular (CD21+CD23+; **B**) and marginal zone (CD21+CD23-; **C**) B cells from the pancreatic lymph nodes (PLN) and Peyer's patches (PP) were gated from live, single CD19+TCRbeta- cells prior to gating on CD21/CD23. **(D,E)** The proportion of naïve (IgD+IgM-) and mature (IgD+IgM+) B cells were gated as in **B,C** prior to gating on IgD/IgM. **(F)** The proportion of CXCR3+ macrophages, gated from live, single CD19-TCRbeta-IA$^{g7}$(MHCII)+CD11b+CD11c- cells prior to gating on CXCR3. **(G)** The proportion of CD86+ macrophages gated as in **F** prior to gating on CD86. All data were assessed for significance using a Student's *T*-test. Data shown are representative of one of two experiments with $n = 4$ per group/experiment. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

of MNV infection on other APC populations. Our results showed the reduction of CXCR3+ macrophages in the PP but increased in the PLN of virally infected mice compared to the controls (**Figure 5F** and **Supplementary Figure 8A**). This suggested an enhanced recruitment of macrophages to the PLN, whereas the macrophage recruitment in PP was reduced; there were no changes in any other tissues studied (**Supplementary Figure 8B**). Interestingly, we observed significant reductions in CD86+ macrophages in the PLN, suggesting a reduced ability to activate the T cells in the PLN (**Figure 5G** and **Supplementary Figure 8C**). We did not find significant differences in CD11b+CD11c+ cells or CD11b-CD11c+ dendritic cells (**Supplementary Figures 8D–G**). Taking together, our data indicate that MNV4 infection predominantly alters mucosa-associated B cell responses as well as influences macrophage recruitment and costimulation locally.

## MNV4 Infection of GF NOD Mice Significantly Alters T Cells

Having identified significant differences in the APCs related to the viral infection, we asked if MNV infection also affects T cells. In line with our results in MNV-infected SPF mice, presented earlier (**Figure 2A**), we also discovered that MNV infection of GF mice significantly increased the number of Tregs in the spleen and PLN of ex-GF mice compared to the uninfected control ex-GF mice (**Figure 6A**). Interestingly the T cells from infected ex-GF mice showed increased

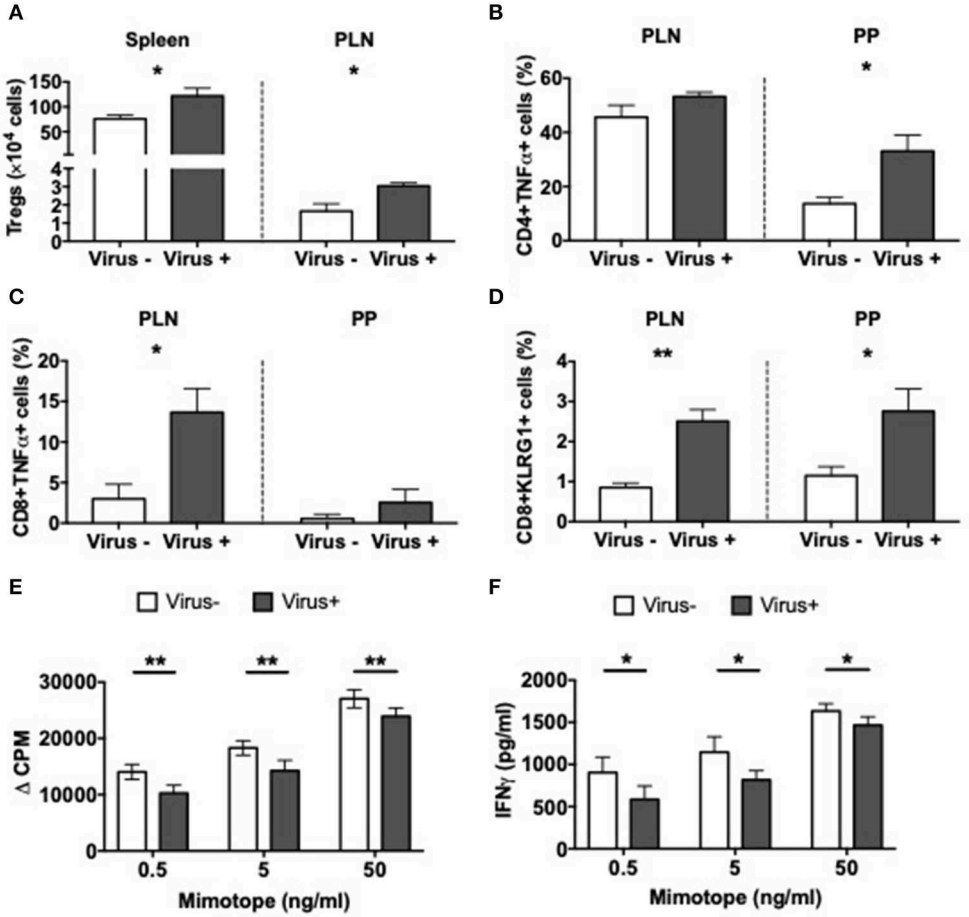


**FIGURE 6 |** Altered T cell immunity in norovirus colonized NOD mice. GF NOD mice were orally gavaged at ∼4 weeks of age with pooled and filtered norovirus-enriched fecal filtrate from norovirus-infected NOD mice at 12-weeks of age that was UV-treated (virus–) or non-UV-treated (virus+). Only mice gavaged with the non-UV-treated filtrate were infected with norovirus and developed anti-MNV antibodies. **(A)** The number of CD4+FoxP3+ Tregs were calculated from live single TCRbeta+CD4+CD8- T cells prior to FoxP3 gating in the spleen and pancreatic lymph node (PLN). **(B,C)** Cells were stimulated for 4-h in the presence of PMA, Ionomycin, and Golgi Plug prior to surface and intracellular staining. TNFα-secreting CD4 **(B)** or CD8 **(C)** T cells were gated from live single TCRbeta+ cells prior to CD4 or CD8 gating then subsequent gating on TNFα. Cells shown are from the PLN and Peyer's patches (PP). **(D)** The proportion of KLRG1+ CD8 T cells gated from CD19-TCRbeta+CD4-CD8+ T cells prior to gating on KLRG1+ cells in the PLN and PP. **(E)** Proliferation of BDC2.5 CD4+ T cells to mimotope peptide in the presence of Tregs (1:2) from virus- or virus+ mice. Background proliferation (APCs + BDC2.5 + Tregs without peptide) was subtracted from proliferation with peptide. Data are shown as change in counts per minute (ΔCPM). **(F)** IFNγ measured by ELISA from Treg suppression culture supernatants in **E**. All data were assessed for significance using a Student's *T*-test. Data shown are representative of one of two experiments with $n = 4$ per group/experiment. $^{*}P < 0.05$, $^{**}P < 0.01$.

TNFα-secreting CD4 and CD8 T cells in the PP and PLN, respectively (**Figures 6B,C** and **Supplementary Figures 9A,B**). However, we did not observe any significant differences in IL-10-, IL-17a-, or IFNγ-secreting T cells in either the PP or PLN (**Supplementary Figures 9C–H**). We also found increased proportions of KLRG1-expressing CD8 T cells in virus-infected mice vs. the controls (**Figure 6D** and **Supplementary Figure 9I**), suggesting an increase in the antigen-experienced memory CD8 T cells. To test whether MNV4 infection affected the function of the Treg cells, we performed antigen-specific Treg suppression assays. We found that Tregs from MNV4-infected mice showed significantly greater suppression of the proliferation of BDC2.5 CD4+ T cells and secretion of IFNγ upon recognition of the antigenic peptide, compared to the Tregs from the uninfected controls (**Figures 6E,F**). Interestingly, we did not find any differences in IL10 or TGFβ production in the culture supernatants of the Treg suppression assays (**Supplementary Figures 9J,K**). To further confirm that MNV4, not the altered gut microbiota, was responsible for the Treg expansion, we colonized GF NOD mice with the gut bacteria from SPF NOD mice in the presence or absence of MNV4. We found that only when MNV4 was present was there an expansion of Tregs in the PLN and PP (**Supplementary Figure 9L**). Thus, our data suggest MNV4 predominantly promotes immune responses both in the intestinal tissue and the PLN, with the expansion and enhanced function of Treg cells in PLN, providing protection against T1D development in the infected mice.

## MNV4-Containing Fecal Filtrate Modulates T Cell Response Differently Dependent on Whether the T Cells Have Previously Been Exposed to MNV4

To determine the response of immune cells to direct exposure to MNV4, splenocytes from norovirus+ and norovirus- ex-GF NOD mice were cultured overnight with the fecal filtrate collected from the ex-GF norovirus+ mice. There was minimal T cell activation (determined by CD69 expression) without exposure to the viral positive fecal filtrate regardless of the source of splenocytes (**Figures 7A,B** and **Supplementary Figures 10A,B**). However, upon exposure to the virus-positive fecal filtrate, T cells from MNV4 naive ex-GF mice were highly activated, whereas T cells from MNV4 infected ex-GF mice showed significantly reduced activation (**Figures 7A,B** and **Supplementary Figures 10A,B**). While we did not observe obvious changes in Tregs, we found that MNV4 exposure promoted more activation and increased expression of CTLA4 on Tregs in splenocytes from virus-naïve ex-GF mice compared to virus-experienced ex-GF mice (**Figures 7C,D** and **Supplementary Figures 10C–E**). This suggests that MNV could modulate Tregs. Again, there was no difference in IL-10- secreting CD4 T cells (**Supplementary Figure 10F**). Direct exposure of splenocytes to MNV-containing fecal filtrate induced significantly more IFNγ-secreting CD8 T cells and TNFα-secreting CD4 and CD8 T cells, respectively, from cells taken from MNV-experienced ex-GF mice compared to the cells from naïve ex-GF mice (**Figures 7E–G** and **Supplementary Figures 10G–J**). Interestingly, direct exposure of MNV-containing fecal filtrate did not have much effect on macrophages, dendritic cells or B cells from both MNV-experienced and MNV-naïve ex-GF mice (**Supplementary Figure 11**). To determine whether MNV4 infected T cells and/or APCs directly, we assessed MNV4 gene expression in purified splenic T cells and APCs by qPCR. Interestingly, we found that T cells but not APCs were infected by MNV4 (**Figures 7H,I**), suggesting that MNV has direct effect on T cells. Finally, to confirm whether exposure to MNV4 affected the Treg recruitment *in vivo*, we first cultured splenocytes from MNV4-naïve NOD mice *in vitro* in the presence or absence of MNV4 for 12 h, followed by adoptive transfer of the MNV-exposed T cells into Rag-deficient NOD mice. Treg expansion was observed in the recipients as early as a week after the transfer (**Figure 7J**). These Tregs, specifically in the PLN also expressed more CD39 (**Figure 7K** and **Supplementary Figure 12A**) when compared to the Tregs from the recipients that were transferred with non-MNV4-exposed cells. As CD39 positive Tregs have better suppressive function (51), this may explain the enhanced Treg suppression (**Figure 6E**) and protection from T1D (**Figure 1A**) observed in our study. Moreover, we demonstrated that the MNV4-exposed Tregs had increased expression of CCR6, CCR7, and CCR9, compared to the Tregs without exposure to MNV4 (**Figures 7L–N**, respectively, and **Supplementary Figures 12B–D**), however, only the increase in CCR7 expression was restricted to the PLN. This may suggest these Tregs are preferentially recruited via CCR7 to the pancreatic lymph nodes in response to chemokine expression (CCL19/CCL21).

## DISCUSSION

Viral infections, particularly enteric viruses, have been linked to the development of T1D (12–15, 18, 20, 43, 52, 53); however, most of these studies have focused on the coxsackievirus or rotavirus, little is known the role of norovirus in T1D development. Using the NOD mouse model of human T1D, we investigated the effect of noroviral infection on the natural history of T1D development. We identified four novel findings in this study. First, unlike the studies in coxsackievirus or rotavirus, which promote T1D development (17, 54), norovirus infection protects mice from T1D development. Second, we discovered that norovirus infection induces an expansion of regulatory T cells but reduces proinflammatory T cells, specifically in pancreatic draining lymph nodes, potentially as a direct effect of viral infection. Third, we found that noroviral infection alters the composition of gut commensal bacteria in the hosts, which adds another plausible mechanism for T1D protection. This is different from an IL-10 deficient IBD mouse model, in which noroviral infection triggered gut microbiota-driven IBD development (35). Last but most importantly, we verified our findings by infection of GF NOD mice, which tested the viral effect more directly. Using GF mice, we identified that norovirus infection alters the phenotype of mucosal B cells and macrophage and inhibits mucosal IgG2a and IgG2b-producing B cells. Thus, our study demonstrated that noroviral infection protects NOD mice from T1D development, and this was associated with the expansion, recruitment and enhanced function of Treg cells, more evident in pancreatic draining lymph nodes, alteration of β-diversity in gut commensal bacteria and reshape mucosal B cells and macrophages.

Many studies including our own have shown that the gut microbiota can modify the immune system in NOD mice (2, 5, 8–11, 38, 40, 44, 55, 56) and the composition of gut microbiota is altered in individuals with pre-diabetes and human individuals with T1D (6, 7, 57–59). However, it is not clear if the effects are directly due to the gut commensal bacteria or enteric viruses, as many endogenous (bacteriophages) and exogenous (from the environment) viruses are present in the gut ecosystem. Furthermore, some enteric viruses are harmless whereas others are pathogenic, with the latter causing damage to the enterocytes and alteration of gut microbiota (60). It is also important to note that the gut microbiota composition can also alter the susceptibility of the virus to persist. Thus far, most of the studies in relation to viral infection and T1D development, in mouse and man, have been focused on the immunopathogenic effect; the knowledge of immune-regulatory effect of enteric viral infection is largely unknown. By probing the role of murine norovirus infection in T1D development in NOD mice, we discovered that MNV4 infection has beneficial effects in preventing T1D development in NOD mice. MNV4 infection promotes the expansion of Treg cells and the suppressive function of Tregs; and alters the mucosal B cell response. Further, MNV4 infection

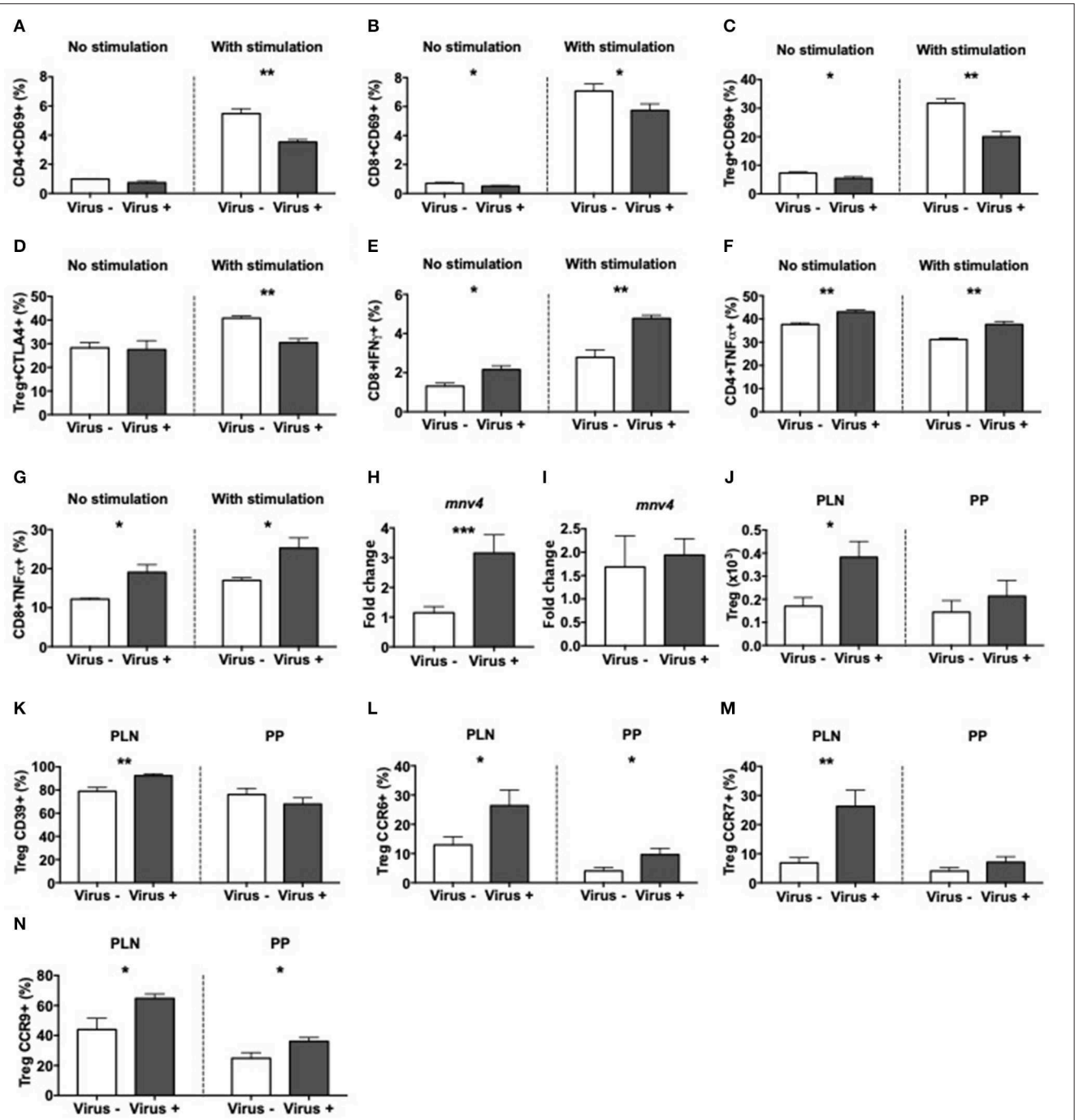


**FIGURE 7 |** Altered T cell immunity after exposure to MNV4 *in vitro*. Fecal pellets from MNV4-infected 12-week old GF NOD were homogenized and filtrate was cultured with splenocytes from MNV4- or MNV4-colonized GF NOD mice. Cells were stimulated for 12 h prior to the addition of PMA, Ionomycin and GolgiPlug for a further 4 h preceding the surface and intracellular staining. As a control, splenocytes were not stimulated with MNV4-containing fecal filtrate but were stimulated with PMA, Ionomycin and GolgiPlug. **(A,B)** The proportion of CD69+ CD4 T cells **(A)** and CD8 T cells **(B)** were gated from live, single TCRbeta+CD19- cells prior to gating on CD4 or CD8 respectively and then CD69. **(C,D)** CD4+FoxP3+ Tregs were investigated for CD69+ **(C)** and CTLA4+ **(D)** cells. Tregs were gated as in B, prior to FoxP3 gating and then CD69 or CTLA4 gating. **(E–G)** The proportion of IFNγ-secreting CD8 T cells **(E)** and TNFα-secreting CD4 T cells **(F)** and CD8 T cells **(G)** were gated as in **A,B** prior to subsequent gating on IFNγ or TNFα. RNA from cultured T cells or APCs was isolated following exposure to UV-treated or UV-untreated (MNV4-containing) fecal material. Equal concentrations of cDNA were synthesized and then subjected to qPCR for MNV4 **(H,I)**. The relative expressions of these genes were determined using the $2^{-\Delta\Delta CT}$ method by normalization with GAPDH. MNV4-exposed or MNV4-naive splenocytes were adoptively transferred into Rag-deficient NOD mice ($4 \times 10^6$/donor). Seven-days later mice were sacrificed and Tregs were investigated by flow cytometric analysis. Tregs were gated from live, single cell, CD4+TCRbeta+Foxp3+ T cells. Treg number **(J)** and the proportion of CD39- **(K)**, CCR6- **(L)**, CCR7- **(M)** and CCR9-expressing **(N)** Tregs are shown. All data were analyzed for significance using a Student's *T*-test. Data shown are representative of one of two experiments, averaged from experimental duplicates with $n = 4$ per group/experiment. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}p < 0.001$.

affected the overall richness of the gut bacteria and changed the β-diversity of the gut commensal bacteria. To distinguish virus-dependent gut commensal bacterial effects, we took a novel approach using GF NOD mice and reconstitute with a fecal filtrate preparation with or without norovirus, from which gut bacteria were removed. With this approach in GF mice, we are able to better identify the immune responses directly related to the noroviral infection. Importantly, we were able to recapitulate Treg expansion in infected ex-GF mice. Using a DSS induced colitis model, Kernbauer et al. showed that MNV infection replaced the immune-beneficial function of gut microbiota mediated by group 2 innate lymphoid cells (ILC2) and type 1 interferon signaling (61). Similarly, we found an increased gene expression of IL-25 in response to norovirus infection, which has been reported to induce and regulate ILC2 cells (62). We also found increased IL-4 in the intestinal wash of norovirus–infected mice, suggesting that there may be a role for IL-4-producing ILC2s in mediating some of the intestinal changes observed in our study. Although we did not find obvious changes in IL-5, IL-13, or Th2 cell markers, which are associated with ILC2 (62, 63), we did, however, observe changes in both the maturity of B cells and the proportion of follicular B cells in the PP, accompanied with alterations of IgG2 antibody production in the gut lumen but not in the circulation. Moreover, our results also demonstrated elevated soluble IFNα in the gut lumen and highly up-regulated genes encoded for IFNα receptor and Stat-1 (33) in the intestine tissue of norovirus infected ex-GF mice. This suggests that the norovirus induces a stronger local immune response.

It has recently reported that a strain of norovirus infects Tuft cells in the intestine (49). To determine whether MNV4 was present in IECs, we determined MNV4 gene expression by qPCR from isolated IECs of ex-GF mice. We found that MNV4 was highly expressed in the infected mice, suggesting that MNV4 can infect IECs. To investigate if MNV4 infection affects Tuft cells, we evaluated the Tuft cell markers (49) in the distal small intestine, by qPCR. We found enhanced expression of Tuft-cell related genes in our MNV4 infected mice, supporting the interactions of MNV-Tuft cell/IEC. It should be noted, however, that the tropism for MNV4 is not yet known, therefore, whether MNV4 directly infects Tuft cells, as does CR6 (49), or if the Tuft cells are indirectly affected by MNV4 infection remains to be determined.

In addition to Tuft cells, gut epithelial cells express an array of Toll-like receptors (TLRs). Noroviruses are single-stranded RNA (ssRNA) viruses and TLR7 and TLR8 are the receptors for ssRNA. Not surprisingly, both genes encoding TLR7 and TLR8 are highly up-regulated in the small intestinal tissue of the virally-infected ex-GF mice. Interestingly, gene expression of RIG-I, the receptor for double-stranded RNA (dsRNA), was also significantly up-regulated in the same tissue from the norovirus infected mice. It is possible that dsRNAs are derived from either replicating viral genomes or self-RNAs released upon either infection-mediated cell lysis or the physiological turnover of gut epithelial cells or a combination of all of the above.

As insulin-producing β-cell destruction is T cell mediated in T1D, norovirus infection will most likely impact the T cells directly or indirectly to affect the disease susceptibility. It is intriguing that MNV4 infection promotes the expansion of regulatory T cells *in vivo*, which in turn can suppress the ongoing β-cell destruction and prevent the mice from T1D development. This is supported by the reduction of proinflammatory cytokine-secreting T cells in the PLN of the infected mice. Interestingly, direct exposure of norovirus+ fecal material to splenocytes from naïve ex-GF mice resulted in a higher proportion of activated Tregs and increased CTLA-4 expression on Tregs, whereas the direct exposure of splenocytes from virally-experienced ex-GF mice to norovirus+ fecal filtrate induced fewer activated Tregs but more inflammatory T cells. We also observed that MNV4-exposed Tregs, following adoptive transfer into Rag-deficient NOD mice, expressed higher CD39 and were preferentially recruited to the PLN. This suggests that primary norovirus infection promotes an immune suppressive stage whereas secondary or persistent infection could lead to an inflammatory state. This may partially explain the rise of diabetes development after 20-weeks of age in the infected NOD mice as MNV4 actively replicates in the infected mice.

In our study we infected mice at ∼4 weeks of age, leading to diabetes protection. It is possible that the age of the mice may influence either the ability of norovirus to modulate the immune system to promote tolerance over diabetogenicity or the type of host immune response to noroviral infection or both. In rotavirus studies, Graham et al. found that rotaviral infection in young NOD mice led to diabetes protection while infection in adulthood accelerated the development of T1D (16, 17). We will test this possibility in the future studies.

It is important to note that norovirus is a common mouse pathogen in animal facilities, causing persistent infections (64). Eradication of MNV from animal facilities requires rederivation in addition to strict observance of stringent standard operating protocols (65–67). Our study shows that MNV can protect NOD mice from developing T1D; however, it is possible that MNV infection may result in negative effects in different disease models using different mouse strains. Thus, it is recommended that more specific pathogens should be tested and reported in research studies.

In summary, our findings demonstrate that norovirus infection promoted protection from T1D development in NOD mice by increasing the number of Tregs, increasing intestinal immunity (including Tuft cells), changing the composition of gut commensal bacteria and local immune cell phenotype and function. Given the high virulence of norovirus in humans, it is likely to be encountered by individuals at risk of developing T1D, particularly as children are more susceptible. Our study not only highlights the importance of virus-commensal bacteria-immune interactions in T1D but also suggests that some enteric viral infections may be beneficial in preventing autoimmune disease such as type 1 diabetes.

## DATA AVAILABILITY STATEMENT

All relevant data are contained within the manuscript and the raw sequencing data are available on request.

## ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee at Yale University.

## AUTHOR CONTRIBUTIONS

JP, NT, DE-A, JP, YH, KH, and SC conducted experiments. JP, NT, DE-A, YH, PS, and LW designed the experiments and analyzed the results. FW consulted the study. JP, NT, DE-A, and LW wrote the manuscript. FW and SC edited the manuscript. PS and LW conceived and supervised the study.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02654/full#supplementary-material

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# Induction of Intestinal Th17 Cells by Flagellins From Segmented Filamentous Bacteria

*Yanling Wang[1], Yeshi Yin[2], Xin Chen[1], Yongjia Zhao[1], Yichen Wu[1], Yifei Li[1], Xin Wang[3], Huahai Chen[2]* and Charlie Xiang[1]**

[1] State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, National Clinical Research Center for Infectious Diseases, Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, The First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou, China, [2] Key Laboratory of Comprehensive Utilization of Advantage Plants Resources in Hunan South, College of Chemistry and Bioengineering, Hunan University of Science and Engineering, Yongzhou, China, [3] State Key Laboratory of Breeding Base for Zhejiang Sustainable Pest and Key Laboratory for Food Microbial Technology of Zhejiang Province, Zhejiang Academy of Agricultural Sciences, Hangzhou, China

T-helper-17 (Th17) cells are a subset of CD4+ T cells that can produce the cytokine interleukin (IL)-17 and play vital roles in protecting the host from bacterial and fungal infections, especially at the mucosal surface. These are abundant in the small intestinal lamina propria (SILP) and their differentiation are associated with the colonization of the intestinal flora. Segmented filamentous bacteria (SFB) drew the attention of researchers due to their unique ability to drive the accumulation of Th17 cells in the SI LP of mice. Recent work has highlighted that SFB used microbial adhesion-triggered endocytosis (MATE) to transfer SFB antigenic proteins into small intestinal epithelial cells (SI ECs) and modulate host immune homeostasis. However, which components of SFB are involved in this immune response process remains unclear. Here, we examined the roles of SFB flagellins in Th17 cells induction using various techniques, including ELISA, ELISPOT, and RNA-seq *in vitro* and *in vivo*. The results show that the immune function of SFB flagellins is similar to SFB, i.e., induces the appearance of CD4+ T helper cells that produce IL-17 and IL-22 (Th17 cells) in the SI LP. Furthermore, treatment of mice with SFB flagellins lead to a significant increase in the expression of genes associated with the IL-17 signaling pathway, such as IL-6, IL-1β, TNF-α, IL-17A, IL-17F, and IL-22. In addition, SFB flagellins have an intimate relationship with intestinal epithelial cells, influencing the expression of epithelial cell-specific genes such as Nos2, Duox2, Duoxa2, SAA3, Tat, and Lcn2. Thus, we propose that SFB flagellins play a significant role in the involvement of SFB in the induction of intestinal Th17 cells.

**Keywords: segmented filamentous bacteria, flagellin, Th17 cells, IL-17A, SI EC**

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*Ivan Monteleone, University of Rome Tor Vergata, Italy*
*Nicolaas Adrianus Bos, University Medical Center Groningen, Netherlands*

***Correspondence:**
*Huahai Chen*
*chenhuahai2008@163.com*
*Charlie Xiang*
*cxiang@zju.edu.cn*

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## INTRODUCTION

The gastrointestinal tract of vertebrates is colonized by a diverse array of microorganisms, that maintain a mutually beneficial relationship with the host (1). The important link of this relationship is based on the perception of specific bacterial species, that trigger responses required for maintaining homeostasis between microbiota and host (2). It is well-recognized that several individual bacterial species can affect the development and function of various immune cells

and immune responses both in the gut and system (3). For instance, *Clostridium* can inhibit intestinal inflammation and IgE production through Foxp3+ regulatory T cells (4), and segmented filamentous bacteria (SFB) can induce Th17 cells differentiation in the small intestine (5, 6).

SFBs are spore-forming gram-positive bacteria with a segmented and filamentous morphology and primarily colonize the distal ileum of mice and rats (7). These bacteria tightly adhere to small intestinal epithelial cells (SI ECs), influencing the immune responses (5, 8). In particular, SFB induces the differentiation of Th17 cells that are characterized by the production of IL-17A, IL-17F, and IL-22. Th17 cell differentiation is controlled by the expression of RAR-related orphan receptor gt (RORgt) (9, 10). To date, the cytokines that can promote the differentiation of Th17 cells have been well-defined *in vitro* (9). IL-6, TGF-β, and IL-21 promote the differentiation of Th17 cells (11, 12). The coordinated activities of IL-1 and TNF can accelerate this process (13). In addition, cytokine IL-23 is not sufficient to generate Th17, but maintains the expansion and pathogenicity of Th17 cells (14).

At steady state, numerous Th17 cells are found in the small intestinal lamina propria (SILP), where they accumulate only in the presence of luminal commensal microbiota such as SFB (10). It has been suggested that the production of ATP and serum amyloid proteins induced by intestinal microorganisms could contribute to the generation of intestinal Th17 cells (5, 15). Furthermore, a recent report revealed that the microbiota could induce the production of IL-1β and that stimulation of IL-1β-IL-1R signaling is essential in promoting the differentiation of Th17 cell (16). The mechanisms by which SFB mediate the differentiation of intestinal Th17 cells have been elucidated. Unlike invasive pathogens, SFB tightly adhere to the IECs of the ileum and do not penetrate the IEC cytosol. Simultaneously, SFB use microbial adhesion-triggered endocytosis (MATE) to transfer T cell antigens into the SI ECs (17) and induce the secretion of SAAs, which act on CD11c+ cells to induce the production of IL-1β and other cytokines that shape the tissue microenvironment to potentiate the induction of Th17 cells (5, 18).

It is clear that SFB can promote the differentiation of Th17 cells, but which components of SFB are involved in this immune response process remains unclear. In addition, the difficulty to successfully isolate and culture SFB *in vitro* has hindered thorough investigations. Until recently, the complete genome sequence of mouse SFB and rat SFB has been published (19, 20). However, one major question remained: How does the microbiota induce Th17 cells? Most reported microbiota-immune effects are mediated by the recognition of microbes by PRRs such as Toll-like receptors (TLRs) (21). The microbial ligands recognized by TLRs are not unique to pathogens, however, and are produced by both pathogenic and commensal microorganisms. It is well-known that the bacterial flagella gene is an important functional gene that affects bacterial colonization and host immune regulation (22). When flagellin adheres to the base of the intestinal epithelium, it initiates an innate immune response and the flagellin-mediated proinflammatory response (23). In addition, studies have shown that bacterial flagellin are recognized by Toll-like receptor 5 (TLR5) (24). TLR5 detects flagellin via MyD88, resulting in the induction of proinflammatory cytokines, antimicrobial defenses, and antiapoptotic effects (25). The flagellin of *Salmonella enterica serovar* is encoded by the *fliC* and *fliB* genes, of which *fliC* is the primary gene (26). In addition, studies shown that *Salmonella* FliC could result in the production of cytokines and the activation of dendritic cell (DC) (27, 28). In addition, immunization of mice with *Salmonella* FliC causes a robust activation of immune cells (29).

The complete genome sequence of mouse SFB showed that SFB encoded more than 40 (3% of total) putative chemotaxis- and flagella-related proteins, and a complete set of genes for flagellar assembly was identified, although they have lost many enzymes for completing pathways essential for their growth and survival (20, 30). Furthermore, the contribution of SFB flagellins to the immune system due to its non-observability in electron microscope analysis remains unclear. Thus, our research group has been prompted to extensively study SFB flagellins. Furthermore, we previously reported that SFB widely express the flagellin protein and encode four types of flagellin, of which three, FliC2, FliC3, and FliC4, are capable of binding to the TLR5 receptors (31), as earlier described (19). Based on the findings of recent studies, we think it is necessary to further investigate the contribution of SFB flagellins. By studying the action of SFB flagellins on the intestinal tract, we found that SFB flagellins promote the production of cytokines, such as IL-17, IL-21, and IL-22, and activate IEC to secrete SAAs. In addition, the induction of Th17 cells by SFB is affected by these cytokines, which ultimately promote the production of Th17 cells (8). Therefore, we believe that SFB flagellins are likely to be important proteins for the adhesion of SFB to host cells and are recognized as antigens by host epithelial cells or SILP CD11c+ cell surface receptors and initiate subsequent signaling pathways, thus playing key roles in the induction of Th17 cells.

# MATERIALS AND METHODS

## Animals

Five-week-old male C57BL/6J (B6) mice were obtained from the SLRC Laboratory Animal Center (Shanghai, China). These were randomly divided into control and experimental groups and housed under SPF conditions at the Laboratory Animal Center of Zhejiang University. All animal experiments were approved by the Ethics Committee of the First Affiliated Hospital, Zhejiang University.

## Heterologous Expression, Extraction, and Purification of the Flagellin Proteins

Heterologous expression, extraction, and purification of SFB-mFliC3, SFB-rFliC3, SFB-m5i-FliC3, and sal-FliC3 were performed as described elsewhere (31). Briefly, the QIAamp Fast DNA Stool Mini Kit (Qiagen, Germany) was used to extract rat and mouse bacterial genomic DNA. The SFB-specific PCR primers 779F and 1008R were used to detect SFB DNA (32). Furthermore, on the basis of the conserved region in the SFB flagellin gene sequences obtained in our previous study, we designed a pair of SFB fliC3-specific primers, fliC3 F and

fliC3 R. SFB fliC3 genes were subcloned into the pET-28a vector. Then, IPTG (Sangon Biotech, China) was added to overexpress FliC3 proteins in chemically competent BL21(DE3) cells (Transgen Biotech, Beijing, China). BugBuster master mix (Merck Millipore, Germany) was used to extract total bacterial proteins, a His Bind purification kit (Merck Millipore, Germany) was employed to purify SFB FliC3 proteins, and Pierce™ High Capacity Endotoxin Removal Spin Columns (Thermo Scientific™, USA) were used to eliminate endotoxins in the protein samples. A bicinchoninic acid (BCA) protein assay kit (Beyotime Biotechnology, China) was used to determine the protein concentrations.

## LP Cell Isolation and *in vitro* Co-culture Experiments

Lamina propria lymphocytes (LPLs) were isolated as previously described, with minor modifications (33). Briefly, intestines were opened longitudinally and washed in ice-cold HBSS medium. Then, the intestinal tissues were cut into 1.5-cm segments and shaken twice for 25 min at 37°C in HBSS medium with 5% FBS (Gibco), 5 mM EDTA, and 1 mM DTT. After removal of the epithelial cells, the pieces were washed, minced, and shaken once for 45 min at 37°C in RPMI-1640 with 5% FBS, 1.5 mg/mL type VIII collagenase (Sigma-Aldrich), and 100 KU/l DNase I (Sigma). Then, the supernatants from the digestion were washed and resuspended in 40% Percoll (GE Healthcare, USA) and overlaid onto 80% Percoll in a 15-mL Falcon tube followed by centrifugation at 2,000 rpm for 20 min at room temperature. The LPLs were collected at the interphase of the Percoll gradient, washed, and resuspended in T cell medium. The cells were used immediately for subsequent experiments. LP CD4+ T cell subsets were first enriched by magnetic-activated cell sorting beads (MACS; Miltenyi Biotec) and then further purified with a FACSAria II (BD, USA). Approximately $5 \times 10^4$ purified CD4 T cells were cocultured in 96-well U-bottom plates with $1 \times 10^5$ MACS purified splenic CD11c+ cells as APCs in the presence or absence of flagellin proteins. After 24, 48, 72, and 96 h, the cell supernatant was collected, and IL-17A protein production was measured by ELISA (R&D Systems, USA).

## Cell Viability Assay

Cell viability was determined using a CCK-8 assay kit (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. Briefly, $5 \times 10^4$ CD4 T cells and $1 \times 10^5$ splenic CD11c+ cells in 100 μL of culture media were plated to a 96-well U-bottom plates in the presence or absence of flagellin proteins. After 0, 24, 48, 72, and 96 h, CCK-8 (10 μL per well) reagent was added, and the reaction system was incubated for 1 h under the same incubator conditions. The relative viability of cells stimulated with different flagellin proteins was determined by measuring the absorbance of each well at a wavelength of 450 nm.

## IL-17A ELISPOT Assay

IL-17A ELISPOT was performed using a Mouse IL-17A FluoroSpot kit (Mabtech, Sweden). Briefly, an antibody solution was added to the plate and coated overnight at 4°C. Then, $5 \times 10^4$ of purified CD4 T cells then were cocultured in pre-coated plates with $1 \times 10^5$ MACS purified splenic CD11c+ cells as APCs in the presence or absence of flagellin proteins and incubated at 37°C in a humidified incubator with 5% $CO_2$ for 72 h. In addition, spot analysis was performed with an automated fluorospot reader equipped with filters for the fluorophores used. The number of cells responding to flagellin protein stimulation was compared to the number of cells spontaneously secreting cytokines, which was determined by incubating the same number of cells in the absence of flagellin proteins.

## Flagellin Administration

Approximately 30 μg of purified SFB-mFliC3, SFB-rFliC3, SFB-m5i-FliC3, sal-FliC3, anti-CD3, or PBS were intraperitoneally injected into C57BL/6 mice (all mice were injected at a dose of 200 μL) (34). After 2 and 24 h, the ileal tissues and serum were collected for subsequent experiments or stored at −80°C.

## Cytokine Analysis by ELISA

The 1.5-cm small intestine tissues were cut from the distal ileum of each mice, opened longitudinally, washed in ice-cold PBS immediately, and weighed. Then, the tissues were lysed with T-PER™ Tissue Protein Extraction Reagent (Thermo Scientific™, USA) supplemented with protease inhibitors (Yeasen, China), followed by centrifugation at 1,500 *g* for 20 min at 4°C. The supernatants from the histiocyte lysates were collected and used immediately for subsequent experiments or stored at −80°C. IL-17A and IL-6 concentrations were measured using Mouse IL-17 Quantikine ELISA Kit and Mouse IL-6 Quantikine ELISA Kit (R&D Systems, USA). Cytokine concentrations in the tissues were expressed as amount per gram of tissue. Cytokine concentrations in the serum were expressed as amount per mL of serum.

## RNA-Seq and qPCR Analyses

Total RNA was isolated from small intestinal tissues and spleens using RNeasy Mini Kit (Qiagen, Germany), and total RNA was isolated from cocultures of CD4+ T cells and CD11c+ cells using iScript™ RT-qPCR Sample Preparation Reagent (Bio-Rad, USA). For real-time qPCR analysis, a PrimeScript™ RT reagent Kit (Takara, Japan) was used to synthesize cDNA, and qPCR was performed using SYBR® Premix Ex Taq™ (Tli RNaseH Plus) (Takara, Japan) on a 7500 Fast Real-Time PCR System (ABI). The primers used in the experiments are described in **Supplementary Table 1**. For RNA-seq analysis, RNA library preparation was performed using an Illumina TruseqTM RNA sample prep Kit. After assessing the library quality using TBS380 Picogreen, sequencing was conducted on an Illumina HiSeq2000. The sequence reads were mapped to the mouse reference genome using Tophat2 and Hisat2 software and normalized to Fragments Per Kilobase per Million mapped reads (FPKM) values. The raw RNA-seq datasets used in this study have been deposited in the NCBI Sequence Read Archive (http://www.ncbi.nlm.nih.gov/sra) as accession number PRJNA531884.

## Cell Culture and Flagellin Protein Stimulation of Epithelial Cell Lines

To detect stimulation of SFB flagellin proteins on a epithelial cell line, a method similar to a recent report (35, 36) was used. The mouse SI epithelial cell line MODE-K (BeNa Culture Collection, China) was maintained in RPMI-1640 with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin solution. Purified SFB-mFliC3, SFB-rFliC3, SFB-m5i-FliC3, and sal-FliC3 (1 μg/mL) (37, 38) and phosphate-buffered saline (PBS) were added separately in MOED-K grown in 24-well plates and then incubated at 37°C for 24 h. The mRNA expression levels of SAAs and CCAAT-enhancer-binding protein (C/EBPD) were evaluated by qPCR.

## Western Blotting

Purified CD4 T cells, CD11c+ cells, and MODE-K cells were lysed in ice-cold RIPA lysis buffer (Beyotime Biotechnology, China) supplemented with protease inhibitor (Beyotime Biotechnology, China). BCA protein assay kit (Beyotime Biotechnology, China) was used to determine the protein concentrations. Equal amounts of total protein (25 μg) were separated by SDS-PAGE and the protein was transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, USA). Following blocking with 0.5% bovine serum albumin, the membranes were incubated overnight with antibodies to GAPDH and TLR5 (Abcam, USA). Then the membranes were incubated with the secondary antibodies coupled to horseradish peroxidase. The Tanon-4500 gel imaging system was used to detect the protein bands (Tiangen, China).

## Application of Neutralizing Antibody

To neutralize TLR5, purified CD4 T cells, and CD11c+ cells were pre-incubated with an anti-TLR5 monoclonal antibody or the same concentration of monoclonal Rat IgG (InvivoGen, USA) for 1 h based on the manufacturer's directions. Cells were then treated with SFB flagellin proteins at 37°C for 3 days. The conditioned media were collected and analyzed for IL-17 using an ELISA kit as described above.

## Statistical Analysis

All of the statistical analyses were performed using GraphPad Prism and SPSS Software (version 22.0; SPSS Inc., USA) with two-tailed unpaired student's *t*-test or one-way ANOVA, followed by the appropriate *post-hoc* test. $P < 0.05$ were considered to be statistically significant.

# RESULTS

## Induction of Th17 Cells by SFB Flagellins *in vitro*

Studies have shown that bacterial flagellins play a key role in the process of bacterial colonization and host immune regulation (22). To investigate whether SFB flagellins stimulate immune cells and participate in host immune regulatory response, CD4+ T cells, which were purified from small intestinal lamina propria, were cocultured with splenic CD11c+ cells as APCs and stimulated *ex vivo* either with anti-CD3 or with purified SFB-mFliC3, SFB-rFliC3, SFB-m5i-FliC3, and sal-FliC3. Cytokine expression was analyzed after stimulated for 24, 48, 72, and 96 h. As shown in **Figure 1A**, although less efficient than anti-CD3, all of these SFB flagellins and sal-FliC3 induced IL-17 expression in a significant number of CD4+ cells. In the case of SFB-mFliC3, the concentration of IL-17A gradually increased with time and the activation effect of CD4+ T cells was the best at about 72 h. Furthermore, the addition of SFB flagellins or sal-FliC3 to cultures with only T cells did not promote the production of Th17 cell cytokines. We conclude that the presentation of SFB flagellin-derived antigens by DCs such as CD11c+ cells is necessary and promotes the generation of Th17 cells. However, liquid chromatography-tandem mass spectrometry (LC-MS/MS) showed that a small amount of contaminating *E. coli* proteins from chemically competent BL21(DE3) cells were in the purified FliC3 (data not shown). To prove that FliC3, rather than contaminating *E. coli* proteins, induce Th17 cell cytokines, we used the same method to purify the empty BL21(DE3) cells to obtain the contaminating *E. coli* proteins. The co-culture system of the SILP CD4+ T cells and CD11c+ cells were then stimulated with purified contaminating *E. coli* proteins. We found that contaminating *E. coli* proteins do not promote the differentiation of Th17 cells (**Supplementary Figure 1A**).

Next, to estimate the cytotoxicity of SFB flagellins and sal-FliC3 over time, a CCK-8 assay was performed to quantify the cell viability of SFB flagellins and sal-FliC3-treated T cells. **Supplementary Figure 1B** shows the cell viability of different SFB flagellins and sal-FliC3-treated T cells after incubation for 0, 24, 48, 72, and 96 h. No significant differences in the relative viability of different SFB flagellins and sal-FliC3-treated T cells to control cells were observed. These results suggested that SFB flagellins had no obvious cytotoxic effects on these cells using our experimental conditions. To more accurately observe the expression of IL-17A at the single-cell level, an IL-17A ELISPOT assay was used to quantify the percentage of Th17 responding to these flagellin antigens (**Figure 1B**). Our results further indicated that SFB flagellins could stimulate immune cells, promoting the differentiation of CD4+ T cells. Anti-CD3 was assumed to activate 100% CD4 T cells, and we found that SFB-mFliC3 activated 30–40% of the CD4 T cells, and SFB-rFliC3, SFB-m5i-FliC3, and sal-FliC3 activated 20–30% of the CD4 T cells. We also assessed the influence of SFB flagellins on Th17 cell differentiation *in vitro* by quantitative RT-PCR. The addition of SFB flagellins to cocultures of CD4+ T cells and CD11c+ cells promoted the differentiation of Th17 cells, including IL-17A, IL-17F, IL-22, and RORgt. In addition to the increase in Th17 cell effector cytokines, the mRNA levels of the IL-1β and AhR were also highly upregulated in SFB flagellin-treated cells (**Figure 1C**). However, no remarkable changes were observed in relation to the mRNA levels of IL-21, IL-23, and TGF-β, which were defined to promote the differentiation of Th17 cells (**Supplementary Figure 1C**). In addition, the expression of Th1 cell-related cytokines in SFB flagellin-treated cells such as IFN-γ and T-bet, were not significantly enhanced (**Supplementary Figures 1D,E**).

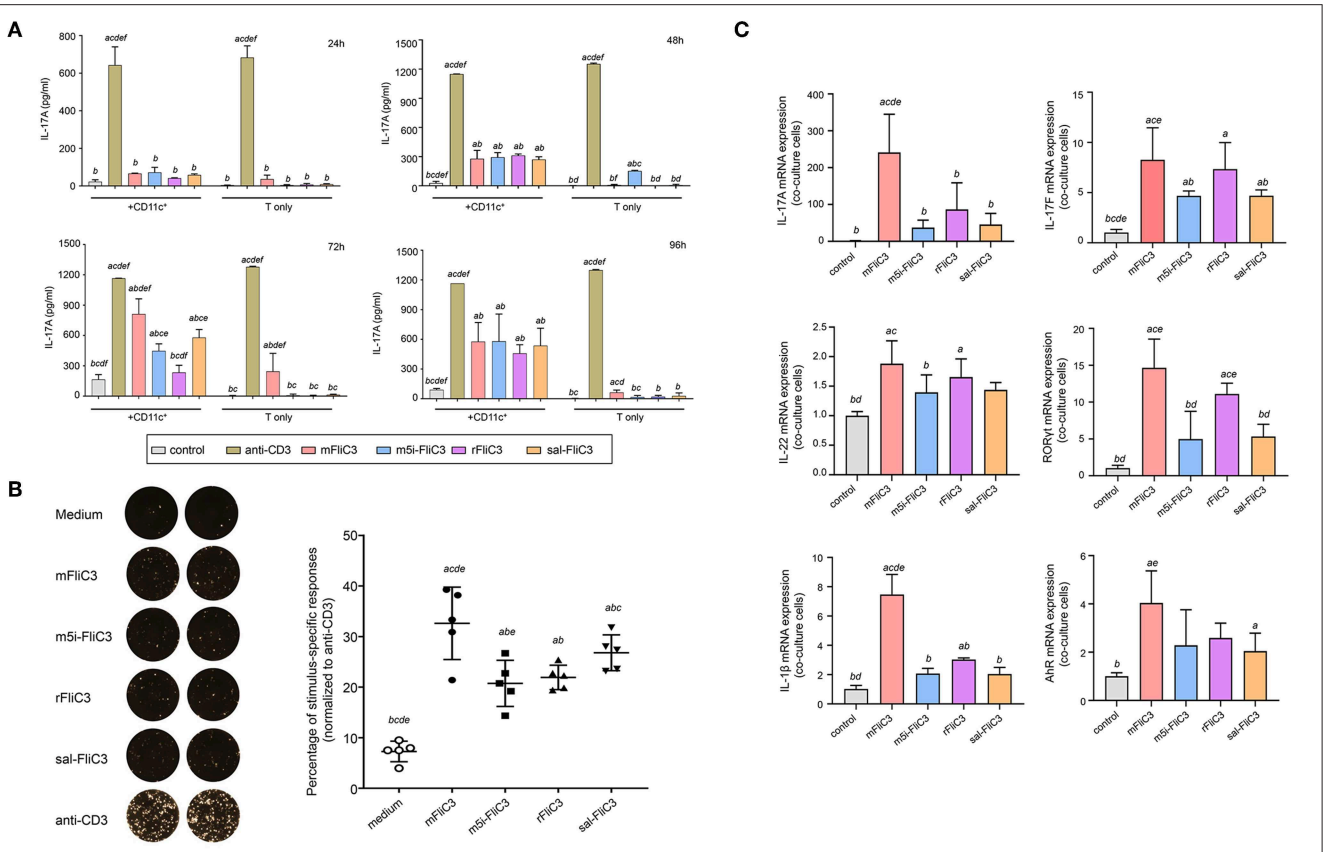


**FIGURE 1 |** Cytokine IL-17A Production by Small Intestinal Lamina Propria Cells after stimulated *ex vivo* with SFB flagellins and other antigens. **(A)** Activation of SILP CD4+ T cells by SFB-mFliC3, SFB-rFliC3, SFB-m5i-FliC3, sal-FliC3, anti-CD3, or PBS. IL-17A ELISA assay was evaluated after 24, 48, 72, and 96 h. (a–f) Represent statistical significance relative to the control group; anti-CD3 group; mFliC3 group; m5i-FliC3 group; rFliC3 group; sal-FliC3 group, respectively. **(B)** IL-17A ELISPOT assay of SILP CD4+ T cells from WT mice treated with SFB flagellins and other antigens. (Left) Representative ELISPOT images. (Right) Compilation of results from multiple animals. Each symbol represents cells from a separate animal. **(C)** The mRNA expression of IL-17A, IL-17F, IL-22, RORγt, AhR, and IL-1β relative to Gapdh in a co-culture system stimulated with SFB flagellins. Data are expressed as the mean ± SEM of three independent experiments. Error bars indicate median values. (a–e) Represent statistical significance relative to the control group; mFliC3 group; m5i-FliC3 group; rFliC3 group; sal-FliC3 group, respectively.

## Induction of Th17 Cells by SFB Flagellins *in vivo*

To further prove that SFB flagellins can induce Th17 cells differentiation *in vivo*, 5-week-old male mice were intraperitoneally injected with SFB-mFliC3, SFB-rFliC3, SFB-m5i-FliC3, sal-FliC3, anti-CD3, or PBS. IL-17A and IL-6 concentration was analyzed after stimulation for 2 and 24 h. The cytokine concentration of mice immunized with SFB flagellins, compared with mice intraperitoneally injected with PBS, had significant changes, under the same feeding conditions. We found that when mice were intraperitoneally injected with SFB flagellins for 2 h, the concentration of IL-17A significantly increased in both the small intestine and serum (**Figure 2A**). Consistent with this result, the IL-6 concentration, which was well-acknowledged as a factor of contributing to the Th17 differentiation, was also significantly increased in the small intestine and serum (**Figure 2B**). However, although IL-6 and IL-17A concentration was still at a high level in mice injected with anti-CD3 for 24 h, either in the small intestine or in the serum, they all returned to normal level in the mice with flagellin administration (**Supplementary Figures 2A,B**). Meanwhile, the transcription of IL-17A and IL-6 in the small intestine was then monitored. As we saw, flagellin administration for 2 h triggered about a 15- to 20-fold increase of IL-17A mRNA levels and the mRNA level of IL-6, which promotes Th17 cell differentiation was significantly upregulated by 40- to 50-fold (**Figure 2C**). However, cytokine levels in the small intestine of the mice that received flagellins for 24 h returned to normal levels (**Supplementary Figure 2C**). In conclusion, we further evidenced the new point that SFB flagellins can promote the differentiation of Th17 cells by animal *in vivo* experiments and *in vitro* cell co-culture experiments.

## Influence of SFB Flagellins on the Intestinal Gene Expression Profiles of Mice

To determine the influence of SFB flagellins host gene expression, we compared the gene expression profiles in the ileum of C57BL/6 mice after administration of SFB-mFliC3, SFB-rFliC3, SFB-m5i-FliC3, and sal-FliC3 with PBS. We found that administration of mice with SFB-mFliC3, SFB-m5i-FliC3,

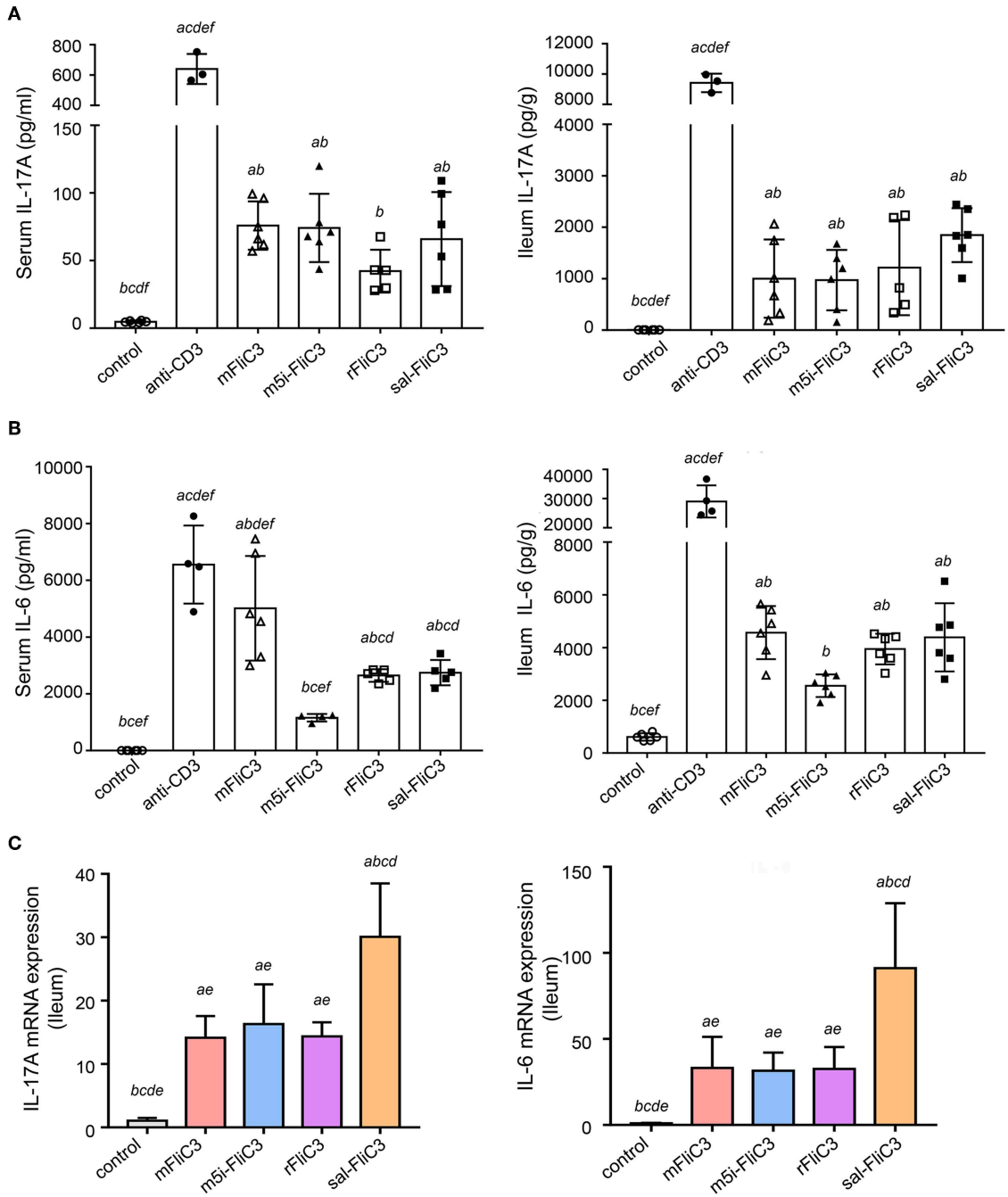


**FIGURE 2 |** SFB flagellins promote a swift, transient, Th17-related response *in vivo*. Mice ($n = 6$) were i.p. injected with SFB flagellins or other antigens. The distal ileum and serum cytokine were assayed 2 h later. **(A)** Cytokine IL-17A concentrations detected in serum and supernatants of the distal ileum isolated from either mouse, which were i.p. with SFB flagellins or other antigens for 2 h ($n = 6$). **(B)** Serum and intestinal IL-6 concentrations in conventional and experimental mice that received i.p. immunizations for 2 h ($n = 6$). See statistical significance in **Figure 1A**. **(C)** The mRNA expression of IL-17A, IL-6 relative to Gapdh in intestinal of the indicated mice. Data are expressed as the mean ± SEM of three independent experiments. Error bars indicate median values. See statistical significance in **Figure 1C**.

SFB-rFliC3, or sal-FliC3 induced a significant change in the expression of 937, 1,140, 938, and 1,277 genes, respectively. In addition, these four gene sets showed fraction overlap, which contained 594 genes (**Figures 3A,B**). Among them, we emphatically distinguished genetic profiles differences between SFB-mFliC3 treatment group and PBS control group. As shown in **Figure 3C**, numerous (>70%) of these differentially expressed genes were upregulated and a handful of (>30%) were downregulated in SFB-mFliC3 treatment group compared to the PBS control group. GO and KEGG enrichment analyses of

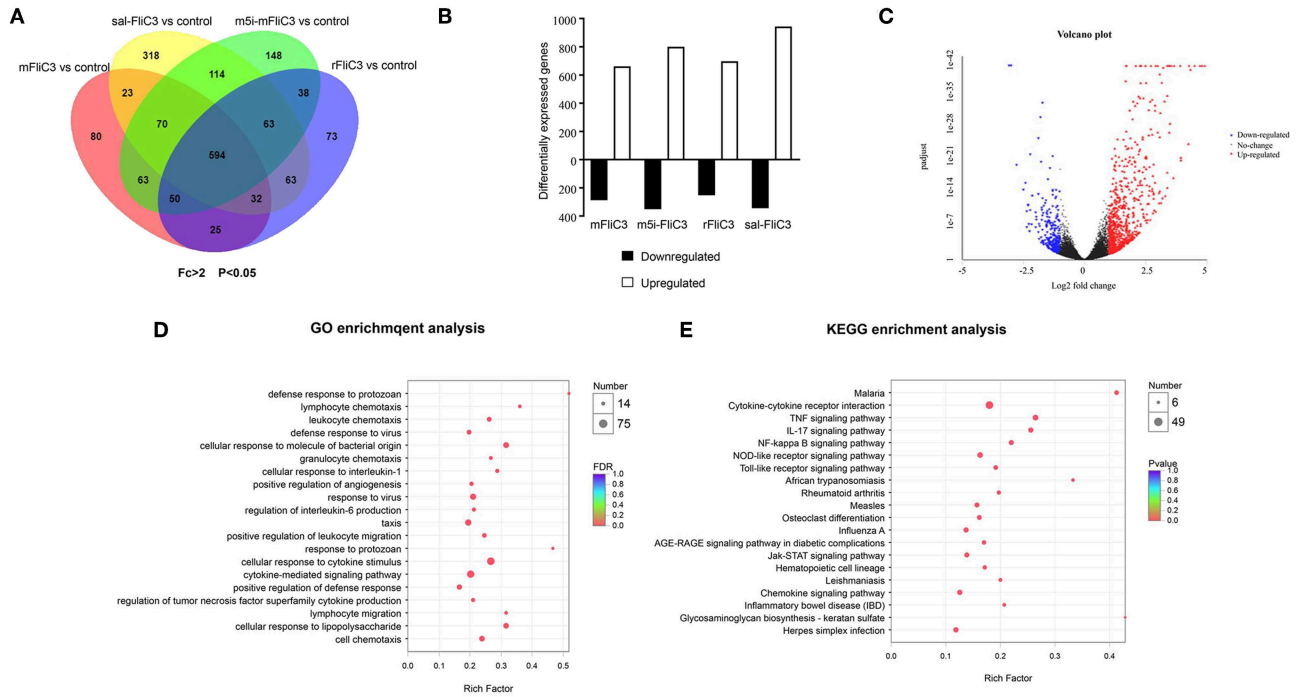


**FIGURE 3 |** Transcriptional programs induced by SFB flagellins. **(A)** Venn diagrams showing the overlap between genes as influenced by either SFB-mFliC3, SFB-rFliC3, SFB-m5i-FliC3, or sal-FliC3 stimulation of mice ($n$ = 3/group, $p < 0.05$). **(B)** RNA-seq analysis of differentially expressed genes in SFB-mFliC3, SFB-rFliC3, SFB-m5i-FliC3, and sal-FliC3 stimulation of mice relative to conventional mice ($n$ = 3/group). Bar graphs represent number of genes higher expressed (open bar; fold-change > 2, $p < 0.05$) and lower expressed (black bar; fold change <-2, $p < 0.05$) in each treatment relative to the control group after 2 h of stimulation. **(C)** Volcano plot shows gene expression differences between SFB-mFliC3 and controls. Each point represents a gene from the uniquely common set of 51,912 genes between platforms. Volcano plot distributions of fold change (log2 [fold change]) (X-axis) and student's $t$-test $p$-values (–log10 [$p$-value]) (Y-axis) **(D,E)**, GO and KEGG enrichment analyses between small intestinal in SFB-mFliC3 stimulated mice vs. wild-type (WT) mice (2 h after inoculation).

upregulated genes showed that SFB-mFliC3 significantly induced the immune system pathways. Furthermore, the chemokine signaling pathway, Toll-like receptor signaling pathway, and NF-kappa B signaling pathway, which indirectly promote the immune regulatory response and induce the differentiation of intestinal Th17 cells, had significantly changed (**Figures 3D,E**). We also found that SFB-mFliC3 administration of mice induced a significant change in expression of 118 immune system-related genes (**Supplementary Figure 3**). To assess significant changes associated with Th17 cells induced by SFB-mFliC3, we focused on the expression of genes specific for IL-17-mediated signaling (**Figure 4A** and **Supplementary Figure 4**). Gene profiling indicated that the expression of genes specific to IL-17-mediated signaling, including Th17-promoting cytokines, chemokines, and antimicrobial molecules, such as HAMP were significantly enhanced. Furthermore, the expression of the chemokine CCL20 was also significantly upregulated. However, no remarkable changes in the expression of IL-23 and AhR were observed (**Supplementary Figure 5A**). To verify the reliability of the intestinal gene expression profiles of the mice, the expression of Th17-promoting cytokines and Th17 effector cytokines mRNAs, including TNF-α, TGF-β, IL-1β, IL-21, IL-17F, and IL-22, were confirmed by quantitative RT-PCR (**Figures 4B,C**). Concordant with the gene expression profiles, the expression of TNF-α, IL-1β, IL-17F, and IL-22 were significantly upregulated, and the expression of IL-21 and TGF-β slightly changed. However, the mRNA levels of Th17-promoting cytokine and Th17 effector cytokine mRNAs returned to baseline levels after 24 h (**Supplementary Figure 5B**). Thus, we inferred that the immune regulatory response induced by SFB flagellins was temporary. We also verified the expression of Th17 cell-related cytokines, including IL-17A, IL-17F, IL-22, IL-6, IL-21, and TNF-α, in the spleen (**Supplementary Figure 5C**). The mRNA levels of Th17 cell-related cytokines were not significantly enhanced in the spleen. In summary, SFB flagellins promote the production of the innate cytokines IL-17A, IL-17F, and IL-22 in the small intestine, a pattern that resembles a Th17 related innate response.

## SFB-Flagellins Activate SI EC

Studies have confirmed that SFB induced Th17 cell differentiation in the intestine, mainly due to the interaction between SFB and the IECs, thus generating an environment conducive to Th17 cell differentiation (35). To examine whether SFB flagellins were involved in these unique signaling pathways, we examined the effects of SFB flagellins on SI EC gene expression profiles (**Figure 5A**). The expression of ROS-generating enzyme dual oxidase 2 (Duox2), its maturation factor Duoxa2 and nitric oxide synthase 2 (Nos2), were highly

**A**

| Fold Change | | | | gene symbol | gene title |
|---|---|---|---|---|---|
| mFliC3/control | m5i-FliC3 /control | rFliC3/control | sal-FliC3 /control | | |
| 30.358 | 49.453 | 45.223 | 86.642 | CXCL-5 | chemokine (C-X-C motif) ligand 5 |
| 26.760 | 24.201 | 26.723 | 23.818 | CXCL-10 | chemokine (C-X-C motif) ligand 10 |
| 22.848 | 31.801 | 7.150 | 30.782 | LCN2 | lipocalin 2 |
| 19.603 | 15.856 | 35.704 | 52.237 | CCL20 | chemokine (C-C motif) ligand 20 |
| 15.391 | 20.635 | 13.105 | 24.319 | IL-17F | interleukin 17F |
| 15.338 | 24.403 | 27.684 | 55.561 | CXCL-2 | chemokine (C-X-C motif) ligand 2 |
| 10.440 | 11.664 | 8.225 | 11.893 | MMP13 | matrix metallopeptidase 13 |
| 6.212 | 8.515 | 7.316 | 11.142 | TNF-α | tumor necrosis factor-alpha |
| 5.844 | 9.863 | 6.413 | 18.649 | MMP3 | matrix metalloproteinases 3 |
| 5.441 | 4.763 | 3.640 | 4.371 | CXCL-9 | chemokine (C-X-C motif) ligand 9 |
| 4.857 | 3.975 | 5.460 | 4.317 | CXCL-11 | chemokine (C-X-C motif) ligand 11 |
| 4.840 | 5.641 | 4.643 | 6.807 | MMP10 | matrix metallopeptidase 10 |
| 4.744 | 4.698 | 4.380 | 7.260 | HAMP | hepcidin antimicrobial peptide |
| 4.371 | 4.344 | 3.717 | 4.787 | IL-1β | interleukin 1 beta |
| 3.818 | 4.817 | 5.363 | 10.275 | IL-6 | interleukin 6 |
| 3.737 | 6.635 | 3.585 | 7.934 | IL-22 | interleukin 22 |
| 1.000 | 5.419 | 8.926 | 21.333 | S100A9 | S100 calcium binding protein A9 |

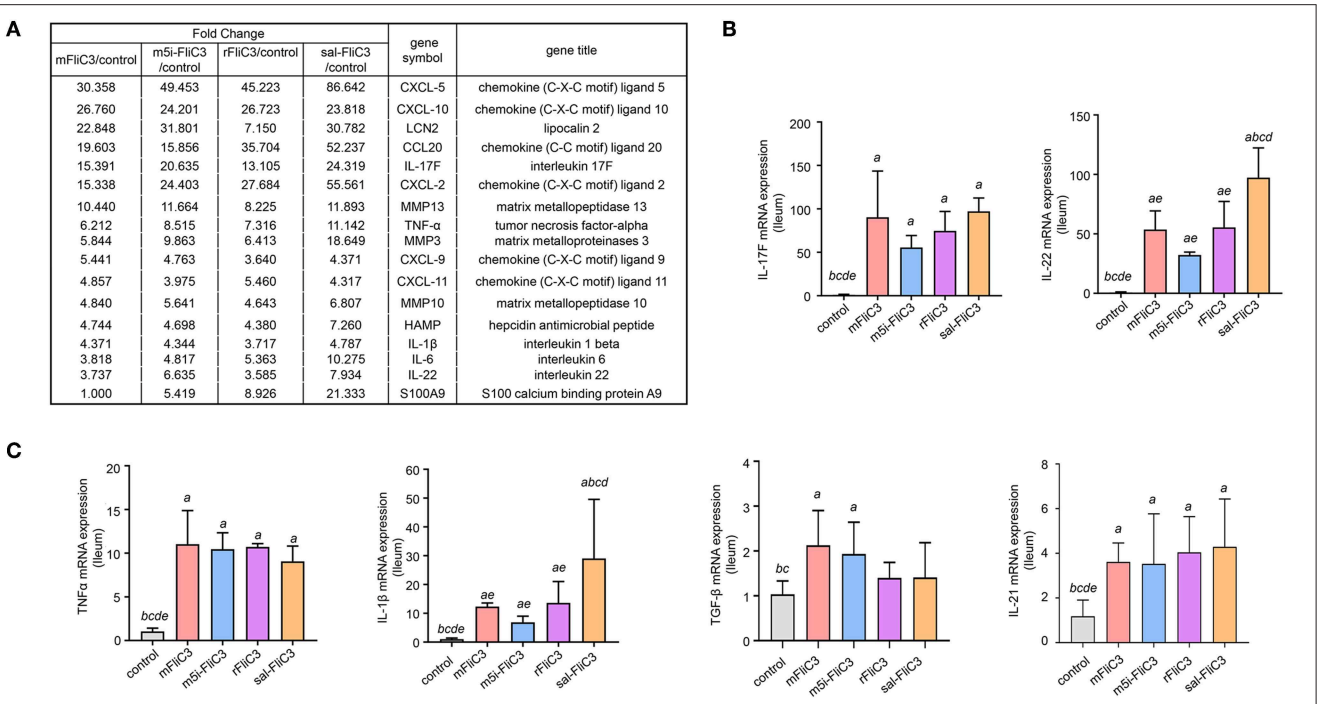


**FIGURE 4 |** Enteric IL-17 signaling activation mediated by SFB flagellins. **(A)** The comparisons of gene expression specific for Th17-related response arranged by fold change in control and experimental groups after RNA-seq. **(B,C)** qPCR for IL-17F, IL-21, IL-22, TGF-β, TNF-α, and IL-1β relative to Gapdh in the intestines of the indicated mice that were i.p. injected with SFB flagellins or other antigens for 2 h ($n = 3$). Error bars indicate median values. See statistical significance in **Figure 1C**.

upregulated in SI ECs of mice intraperitoneally injected with SFB flagellins compared to conventional mice. In addition, the mRNA levels of these genes were verified by qPCR analysis (**Figure 5B**). Studies have shown that SFB colonization induces the secretion of SAAs, which act on CD11c+ cells to induce the production of IL-1β and other cytokines that shape the tissue microenvironment to potentiate the induction of Th17 cells (5, 35). Interestingly, two subtypes of serum amyloid A (SAA2 and SAA3) were significantly upregulated in the intestines of mice intraperitoneally injected with SFB flagellins (**Figure 5C**). However, there was no significant change in the expression of SAA1. A recent study suggested that C/EBPD could interact with both regulatory regions of SAAs (35). All results indicated that the mRNA levels of C/EBPD were highly upregulated (**Figure 5C**). We next examined the expression of SAAs and C/EBPD in a mouse SI EC line (MODE-K) stimulated with SFB flagellins *in vitro*. In line with RNA-seq, the co-induction of SAA3 and C/EBPD expression was observed (**Figure 6**). However, no differences in the expression of SAA1 and SAA2 were observed (**Supplementary Figure 6**).

## DISCUSSION

SFBs play a significant role in promoting the differentiation of intestinal Th17 cells (6, 39). Here, a variety of experimental methods were used to prove that SFB flagellins can significantly induce mucosal expression of Th17-related cytokines, such as IL-17 and IL-22. Studies have indicated that *Salmonella* FliC could lead to the induction of cytokine and the activation of dendritic cell (DC). In addition, immunization of mice with sal-FliC causes a robust activation of immune cells (29). In some published studies, purified *Salmonella* flagellins were intraperitoneally injected into mice, then the spleen, ileal tissues, and serum were collected after 2, 4, 6, 8, and 24 h. The expression of Th17-related cytokines at different time points showed that the immune response induced by *Salmonella* flagellin was temporary and peaked at 2 h, and cytokine expression levels returned to baseline after 24 h (40, 41). Interestingly, in this study, we found that similar to the immunization of mice with sal-FliC, mice immunized with SFB flagellins promote the swift, intense, transient production of the factors controlling Th17 differentiation and Th17-related cytokines, such as IL-17A, IL-17F, and IL-22. And SFB flagellin administration significantly triggered the upregulation of activator protein 1 transcription factor mRNA level, which contributes to the differentiation of Th17 cells (42). However, there were no significant changes in the expression of genes encoding IL-23, IL-21, and TGF-β in our *in vitro* cell co-culture experiments, and the expression of IL-21 and TGF-β slightly changed in our *in vivo* experiments. Cytokines which promoted the differentiation of Th17 cells have been well characterized *in vitro*. IL-6, TGF-β, and IL-21 are known to promote the differentiation of Th17 cells. The coordinated activities of IL-1 and TNF can accelerate this process. However, studies have proved that according to the location and timing of the immune response, the collaboration of pro-inflammatory cytokines such as IL-21, TNF, and IL-1 with TGF-β, might play

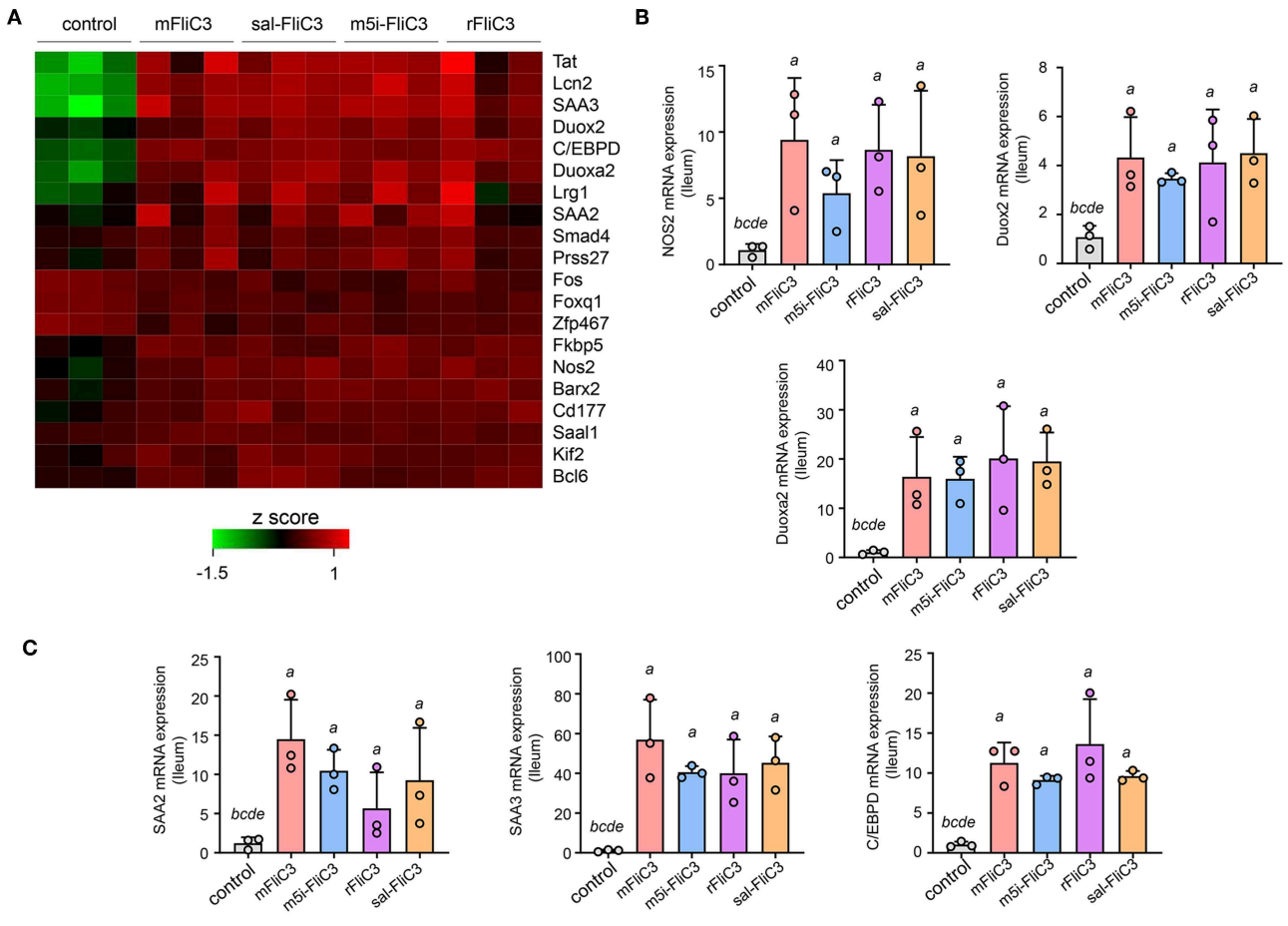


**FIGURE 5 |** SFB flagellin-mediated EC activation. **(A)** Heat map showing the relative abundance for gene transcripts significantly expressed in flagellin-treated mice vs. conventional mice. **(B,C)** qPCR for the selected genes relative to Gapdh. Error bars indicate median values. See statistical significance in **Figure 1C**.

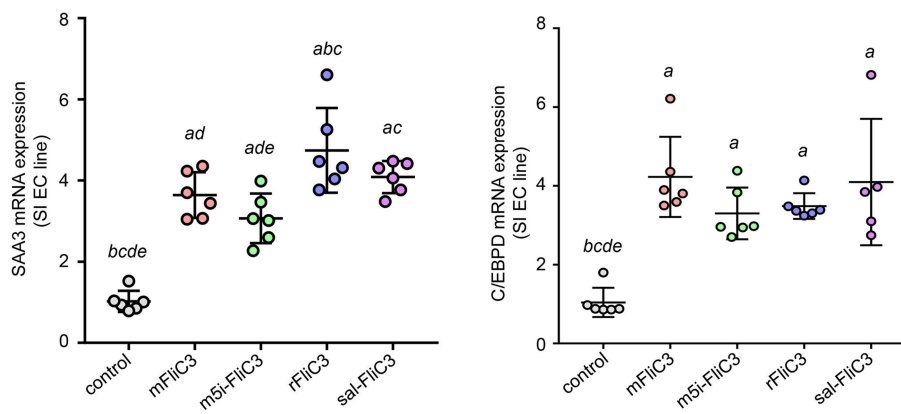


**FIGURE 6 |** A unique gene expression pattern of IEC stimulated by SFB flagellins. MODE-K cells were stimulated by SFB flagellins for 24 h. The mRNA levels of SAAs and Cebpd were evaluated by qPCR. Error bars indicate median values. See statistical significance in **Figure 1C**.

contradictory pro- or anti-inflammatory roles (43, 44), which also explains the inconsistent expression of some cytokines *in vivo* and *in vitro*. Interestingly, among the Th17-conducting cytokines, IL-1β was upregulated, whether in cocultures of CD4+ T cells and CD11c+ cells stimulated with SFB flagellins or in the gut of mice treated with SFB flagellins, which is concordant

with the findings of previous reports (16, 35). IL-1β induced by commensal microbiota is vital for the development of TH17 cells (16). Atarashi et al. (35) showed that the secretion of SAAs induced by SFB adhesion acted on CD11c+ cells to stimulate the significant production of IL-1β and other cytokines, which then caused constitutive accumulation of Th17 cells. Inadequate IL-1β production is part of the reason for the reduction in Th17 cell accumulation.

A recent study has shown contradictory results on the effect of flagellin (45), suggesting that flagellin can increase the production of Treg cells and may inhibit a Th17-like immune response. However, this study was based an experiment in which flagellin doses were much higher than those used in the present study, and instead of SFB, their flagellin originated from *Vibrio vulnificus*, in which the role of flagellins remains unclear. In addition, low and high doses of LPS, the TLR4 ligand, promote Th2 and Th1 responses, separately (46). Thus, we also speculate that the different influences of flagellin might be depend on different doses administered to the mice. Although the role of bacterial flagellins in shaping the host immune response has also been reported (47), the protein sequence similarity between SFB and other bacterial flagellins is very low and information on the contribution of SFB flagellins to the immune system is limited due to its difficulty in electron microscope analysis. Our research group was the first to demonstrate that SFB flagellins are expressed in the ileum mucosa (31). In this study, we proved that SFB flagellins could promote the differentiation of Th17 cells and assessed its regulatory network. Consistent with SFB, SFB flagellins have an intimate relationship with IECs, reflected in the SI EC gene expression profiles induced by SFB flagellins administration. The expression of several SI EC specific genes and inflammatory response genes were highly upregulated, including Duox2, Duoxa2, SAA3, Tat, and Lcn2. Studies have shown SFB promoted the production of reactive oxygen species (ROS), which can suppress the activity of Rho GTPase family and then affect actin reorganization through adhesion to IECs (35, 48). In this study, SFB flagellins administration caused a marked increase expression of Duox2 and Duoxa2. Thus, we believe that SFB flagellins may be involved in the reorganization of actin. In addition, SFB induce SI EC to produce SAAs and then evoke the induction of IL-17. Interestingly, SFB flagellins elicited increase mRNA levels of SAA2 and SAA3, but not SAA1. However, we found that the mRNA levels of C/EBPD was up-regulated significantly. Consistent with this result, the co-induction of SAA3 and C/EBPD expression was observed in MODE-K cells stimulated by SFB flagellins *in vitro*. However, host specificity of SFB flagellins in immune stimulation was not observed either *in vivo* or *in vitro*.

Although SFB flagellins were not detected by electron microscope analysis, the expression of SFB flagellins in the intestine has been proven by a variety of technologies. In addition, the genome sequences showed that SFB encode more than 40 (3% of total) putative chemotaxis- and flagella-related proteins (19, 30). In addition, comparative genomic analysis showed that SFB genomes were similar to *Clostridial* genomes and several *Clostridial* genomes also encoded flagellar assembly proteins (20). Surprisingly, only SFB flagellin proteins have the TLR5 binding sites (30). Our previous results constitute evidence, which is consistent with another study (19), that SFB encode four types of flagellin, three of which, FilC2, FliC3, FliC4, were recognized by Toll-like receptor 5. In this study, western blot analysis showed that CD4+ T cells, splenic CD11c+ cells, and a mouse SI EC line (MODE-K) all expressed TLR5, with the SI EC line showing the highest expression (**Supplementary Figure 7A**). Next, we investigated whether TLR5 in CD4+ T cells and splenic CD11c+ cells, as classic costimulatory receptor, play an important role in promoting T cell activation. We stimulated T cells alone with SFB flagellins to determine whether there is an interplay between TLR5 and TCR during gut T cell activation. We found that the addition of SFB flagellins to cultures with only T cells, did not promote the differentiation of Th17 cells. Then, the anti-TLR5 blocking antibody was introduced into the cocultures of CD4+ T and CD11c+ cells. Surprisingly, the effect of T cell activation was not significantly attenuated when the anti-TLR5 blocking antibody was added (**Supplementary Figure 7B**). These findings suggest that SFB flagellins promote T cell activation and this effect may not or at least not only correlate with TLR5 activation. Most importantly, studies have shown only a slight reduction in the percentage of Th17 cells in the lamina propria of both MyD88- and TRIF-deficient mice, demonstrating that TLR signaling may be dispensable for Th17 cell differentiation in the SI LP (39). So we speculate that the TLR5 signaling pathway may be one, but not the only one, of the unique signaling pathways elicited by SFB colonization. Recent work has highlighted that SFB tightly adheres to SI ECs and transferred SFB antigenic proteins into SI ECs by microbial adhesion-triggered endocytosis (MATE), thus participating in host immune response (17). However, it is still unclear which components of SFB may be involved in this process. 3D reconstruction of SFB hook-like holdfast revealed membrane vesicles originate at the tips of holdfast (17). One speculated that the tail-like structures might be, in fact, flagella (49). However, the involvement of SFB flagellins in endocytosis of SFB by epithelial cells and its occurrence in MATE vesicles remain elusive.

SFB have been extensively studied due to their unique ability to drive the generation of intestinal Th17 cells. However, functionally analogous microbes have not been identified to date. A recent report showed that a human symbiont bacterial species called *Bifidobacterium adolescentis* could induce the accumulation of intestinal Th17 cells in mice (50). Interestingly, another study also showed that a coalition of 20 symbionts from an IBD patient could promote the production of Th17 cells in mice (35). This suggests alternative pathways of promoting Th17 cell accumulation. In this study, we demonstrated for the first time that SFB flagellins as SFB antigens could promote the differentiation of Th17 cells. However, another investigation has shown that SFB protein P3340 as antigen could also promote Th17 cell differentiation (6). This thus suggests that, rather than a single bacterial component, multiple bacterial components such as SFB flagellins and SFB protein P3340 coordinate to facilitate Th17 cell differentiation.

In summary, while flagellins are well-acknowledged as a potent activator of a broad range of cell types involved in innate and adaptive immunity, little is known about the contribution

of SFB flagellins in immunity. In this study, we found that SFB flagellins have the similar immune function as SFB, influencing the expression of the same genes, including Th17-related cytokines, such as IL-6, IL-1β, TNF-α, IL-17A, IL-17F, and IL-22, and epithelial cell-specific genes, such as Duox2, Duoxa2, SAA3, Tat, and Lcn2. Thus, by linking SFB flagellins to the expression of these genes, we present a new viewpoint in which SFB flagellins, as a previously unappreciated component, are a key of the immune response to SFB. However, a critical question remains: How do SFB flagellins promote the differentiation of Th17 cells? Further investigations of such questions may have a tremendous impact on our understanding of the molecular and cellular mechanisms that SFB regulates in intestinal T cell homeostasis.

## DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in NCBI Sequence Read Archive, https://www.ncbi.nlm.nih.gov/bioproject/PRJNA531884/.

## ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. The protocol was approved by the Laboratory Animal Care and Usage Committee of Zhejiang University.

## AUTHOR CONTRIBUTIONS

YWa and YY designed the experiments, performed most of the experiments, and analyzed the data. YWa analyzed the data and wrote the manuscript. YZ, XC, and YWu helped in some of the mouse experiments. XW and YL helped with the statistical analyses of some data. CX and HC conceived and supervised this study, provided critical suggestions and discussions throughout the study, and revised the manuscript. All authors have read and approved the final manuscript.

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## SUPPLEMENTARY MATERIAL

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# Fungal Dysbiosis and Intestinal Inflammation in Children With Beta-Cell Autoimmunity

*Jarno Honkanen[1], Arja Vuorela[1], Daniel Muthas[2], Laura Orivuori[1], Kristiina Luopajärvi[3], Mysore Vishakante Gowda Tejesvi[4], Anton Lavrinienko[4,5], Anna Maria Pirttilä[4], Christopher L. Fogarty[1], Taina Härkönen[3,6], Jorma Ilonen[7], Terhi Ruohtula[1], Mikael Knip[3,6], Janne J. Koskimäki[4†] and Outi Vaarala[1*†]*

[1] Clinicum, Faculty of Medicine, University of Helsinki, Helsinki, Finland, [2] Translational & Experimental Medicine, Early Respiratory, Inflammation and Autoimmunity, BioPharmaceuticals R&D, AstraZeneca, Gothenburg, Sweden, [3] Children's Hospital, University of Helsinki and Helsinki University Hospital, Helsinki, Finland, [4] Ecology and Genetics Research Unit, University of Oulu, Oulu, Finland, [5] Department of Biological and Environmental Science, University of Jyväskylä, Jyväskylä, Finland, [6] Research Program for Clinical and Molecular Metabolism, Faculty of Medicine, University of Helsinki, Helsinki, Finland, [7] Immunogenetics Laboratory, University of Turku, Turku, Finland

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**Edited by:**
Julien Diana,
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Janet Marie Wenzlau,
University of Colorado Anschutz Medical Campus, United States

***Correspondence:**
Outi Vaarala
outi.vaarala@helsinki.fi

[†]These authors share senior authorship

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Although gut bacterial dysbiosis is recognized as a regulator of beta-cell autoimmunity, no data is available on fungal dysbiosis in the children at the risk of type 1 diabetes (T1D). We hypothesized that the co-occurrence of fungal and bacterial dysbiosis contributes to the intestinal inflammation and autoimmune destruction of insulin-producing beta-cells in T1D. Fecal and blood samples were collected from 26 children tested positive for at least one diabetes-associated autoantibody (IAA, GADA, IA-2A or ICA) and matched autoantibody-negative children with HLA-conferred susceptibility to T1D (matched for HLA-DQB1 haplotype, age, gender and early childhood nutrition). Bacterial 16S and fungal ITS2 sequencing, and analyses of the markers of intestinal inflammation, namely fecal human beta-defensin-2 (HBD2), calprotectin and secretory total IgA, were performed. Anti-Saccharomyces cerevisiae antibodies (ASCA) and circulating cytokines, IFNG, IL-17 and IL-22, were studied. After these analyses, the children were followed for development of clinical T1D (median 8 years and 8 months). Nine autoantibody positive children were diagnosed with T1D, whereas none of the autoantibody negative children developed T1D during the follow-up. Fungal dysbiosis, characterized by high abundance of fecal *Saccharomyces* and *Candida*, was found in the progressors, i.e., children with beta-cell autoimmunity who during the follow-up progressed to clinical T1D. These children showed also bacterial dysbiosis, i.e., increased Bacteroidales and Clostridiales ratio, which was, however, found also in the non-progressors, and is thus a common nominator in the children with beta-cell autoimmunity. Furthermore, the progressors showed markers of intestinal inflammation detected as increased levels of fecal HBD2 and ASCA IgG to fungal antigens. We conclude that the fungal and bacterial dysbiosis, and intestinal inflammation are associated with the development of T1D in children with beta-cell autoimmunity.

**Keywords: mycobiome, dysbiosis, gut, inflammation, *Candida*, *Saccharomyces*, type 1 diabetes**

## INTRODUCTION

Type 1 diabetes (T1D) is an immune-mediated disease in which autoimmune mechanisms are considered to be responsible for the destruction of insulin-producing pancreatic beta cells. While the triggers of the disease process remain open, the development of local inflammation in the pancreatic islets and formation of autoantibodies against beta-cell antigens are early events in the development of T1D (1–4). Autoantibodies emerge against various beta-cell antigens, such as insulin, glutamate decarboxylase, islet antigen 2, and zinc transporter 8, several years before the clinical disease manifestation, and the risk of T1D correlates with the number of beta-cell autoantibodies. Other immunological aberrancies in T1D include up-regulation of IFNG and IL-17 pathways (5–9). We have previously shown that children with beta-cell autoimmunity have a decreased abundance of butyrate-producing bacteria and an increased abundance of bacteria belonging to the phylum Bacteroidetes in their gut microbiota (10, 11). Similar alterations in the bacterial community in children with beta-cell autoimmunity have been confirmed in several later studies (12–14). Intestinal inflammation has been associated with T1D as demonstrated by up-regulated expression of HLA class II molecule and cytokines IFNG, TNFA and IL-4 mRNA in jejunal biopsies (15).

The role of gut microbiota as a regulator of autoimmune diabetes is well-established in animal models of T1D, in which modulation of the microbiota affects the disease development (16). To date, the studies of the microbiome in relation to T1D have focused on the bacterial community of the gut microbiota, however, human microbiome is a complex ecosystem composed of bacteria, fungi, archaea, and viruses. Several fungal species have been identified in the human gastrointestinal tract (17, 18), representing 0.1–1.0% of the intestinal microbiota (commonly referred to as *mycobiota*). The fungal cells are outnumbered by the bacterial ones, but as eukaryotic organisms, fungi have substantially more diverse biochemical pathways than bacteria (19). Thus, when the bioactive capacity of the intestinal microbiota is considered, the role of mycobiota is of major importance with a remarkable potential to modulate host cellular functions. That said, the current knowledge of the involvement of mycobiota in the perturbations of the microbial communities and host health is limited. The role of mycobiota as a regulator of intestinal inflammation and inflammatory diseases has been emphasized by recent studies in inflammatory bowel disease, allergy, and asthma (20–22).

Moreover, the changes in the bacterial microbiota may be linked to the alterations of the mycobiota, which are likely disrupting the interkingdom interactions within the microbiome, as seen in Crohn's disease (21, 22). Indeed, intestinal mycobiota can modulate the composition of the bacterial compartment either by direct interactions with bacteria, or via the immune system of the host (18, 23).

In the current study, we analyzed the composition of the fungal and bacterial gut microbiota, as well as markers of intestinal inflammation, in a cohort of islet autoantibody positive and negative children carrying HLA-conferred genetic susceptibility to T1D. We then followed the cohort for the development of T1D for median of 8 years and 8 months. Combining the fungal and bacterial data, the children with genetic risk of T1D were grouped into three major clusters defined by the relative abundance of *Saccharomyces*, Clostridiales, and Bacteroidales (Firmicutes and Bacteroidetes phyla, respectively). An increased ratio of Bacteroidales to Clostridiales was found in autoantibody positive children while the children who during the follow-up also progressed to clinical T1D, showed high abundance of *Saccharomyces* and *Candida*, as well as signs of intestinal inflammation, i.e., increased levels of fecal HBD2 and circulating ASCA IgG. Our results indicate that dysbiosis of fungal and bacterial gut microbiota as well as intestinal inflammation are associated with the development of T1D.

## MATERIALS AND METHODS

### Study Subjects

Experimental design of the current study is presented in **Figure 1**. Here we collected fecal and blood samples from 52 children with HLA-conferred susceptibility to T1D (**Table 1**) and followed them for the development of T1D for a median of 8 years and 8 months (range 8 years and 2 months−9 years and 1 month). The children studied for fecal microbiome were recruited from the participants of the nutritional intervention studies (24–26). We identified 26 children tested positive for at least one T1D-associated autoantibody (IAA, GADA, IA-2A, or ICA) (cases), and selected autoantibody-negative healthy control children matched for age, sex, HLA-DQB1 genotype and early life nutrition. At the start of the follow-up, fecal samples were collected (between February 2009 and February 2010) using stool collection vials and immediately stored in home freezers (−20°C). The frozen samples were delivered to the study center, and the samples were stored at −80°C until processing. At the time of fecal sample collection, the study subjects did not have gastroenteritis and had not received antibiotic treatment during the past 3 months. Nine children developed T1D during the follow-up. The control children remained non-diabetic and negative for all four autoantibodies analyzed. The study was approved by the ethics committees of the participating hospitals and the families and/or the children taking part in the study gave their written informed consent.

### Autoantibody Assays

Biochemically defined autoantibodies IAA, IA-2A, and GADA were analyzed with specific radio-binding method and ICAs with standard immunofluorescence method as previously described in (24–26). The cut-off levels used were 2.80 relative units (RU) for IAA, 5.36 RU for GADA and 0.78 RU for IA-2A determined as the level above 99 percentiles in more than 350 non-diabetic Finnish children. Islet cell antibodies were measured using an indirect immunofluorescence method using a cut-off value of 2.5 Juvenile Diabetes Foundation units.

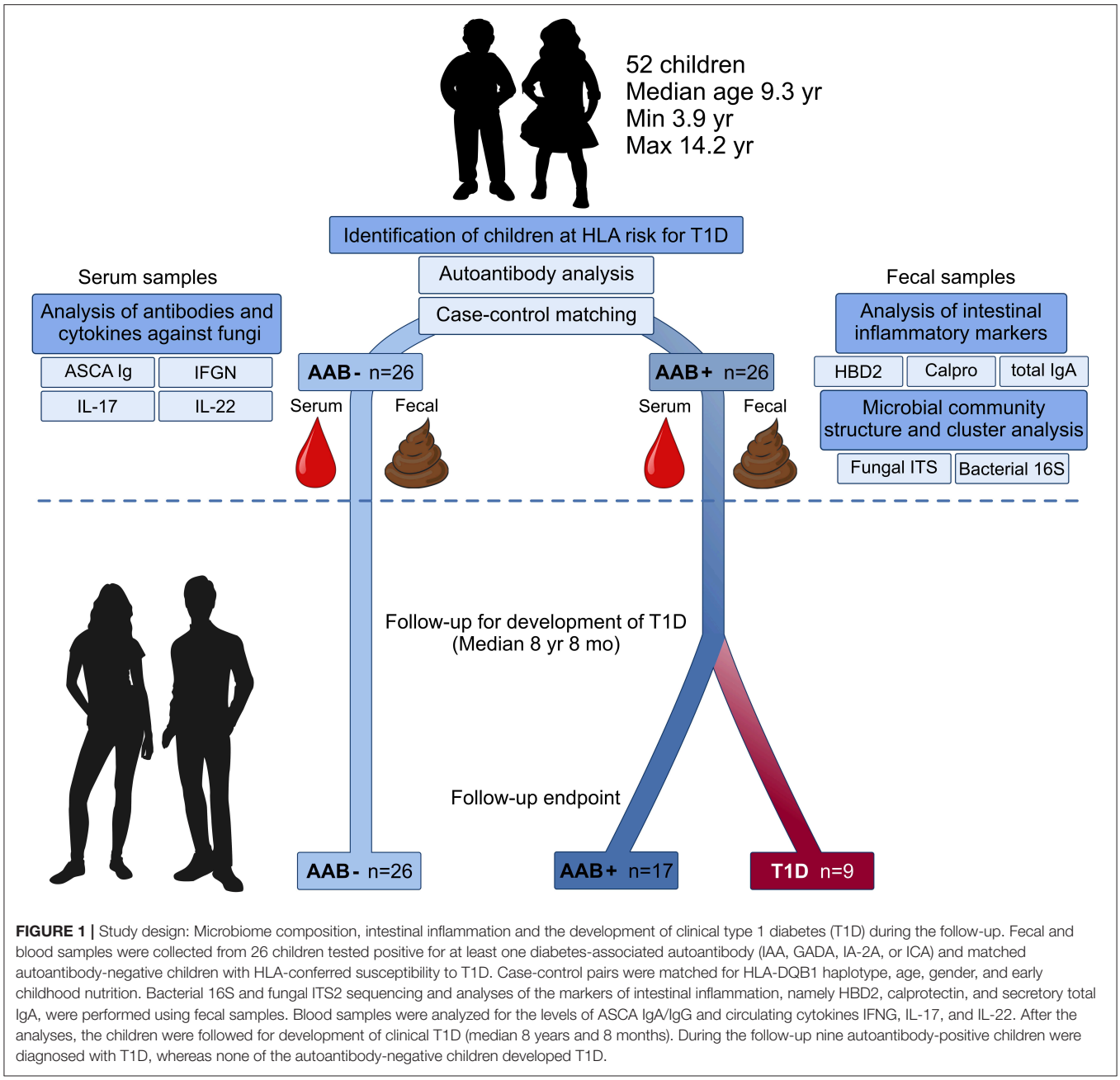


**FIGURE 1 |** Study design: Microbiome composition, intestinal inflammation and the development of clinical type 1 diabetes (T1D) during the follow-up. Fecal and blood samples were collected from 26 children tested positive for at least one diabetes-associated autoantibody (IAA, GADA, IA-2A, or ICA) and matched autoantibody-negative children with HLA-conferred susceptibility to T1D. Case-control pairs were matched for HLA-DQB1 haplotype, age, gender, and early childhood nutrition. Bacterial 16S and fungal ITS2 sequencing and analyses of the markers of intestinal inflammation, namely HBD2, calprotectin, and secretory total IgA, were performed using fecal samples. Blood samples were analyzed for the levels of ASCA IgA/IgG and circulating cytokines IFNG, IL-17, and IL-22. After the analyses, the children were followed for development of clinical T1D (median 8 years and 8 months). During the follow-up nine autoantibody-positive children were diagnosed with T1D, whereas none of the autoantibody-negative children developed T1D.

## HLA Genotyping

Screening of HLA-risk alleles was performed as previously described (24–26). The initial HLA-DQB1 typing for risk-associated (DQB1*02, DQB1*03:02) and protective (DQB1*03:01, DQB1*06:02, and DQB1*06:03) alleles was complemented with DQA1 typing for DQA1*02:01 and DQA1*05 alleles in those with DQB1*02 without protective alleles or the major risk allele DQB1*03:02.

## DNA Extraction

DNA was extracted from fecal samples by using QIAamp Fast DNA Stool Mini Kit (Qiagen, Germany). In short, fecal samples (180–220 mg) were thawed in 1 ml of InhibitEX Buffer, vortexed for 1 min and incubated at 95°C for 10 min to enhance the lysis of hard-to-lyse taxa. After centrifugation, 200 µl of the supernatant was transferred to a new tube with proteinase K and Buffer AL and vortexed thoroughly. The lysate was incubated at 70°C for 10 min followed by addition of 0.3 vol. of absolute ethanol. Then, samples were vortexed, pipetted to the QIAamp spin column and centrifuged at 20,000 × g for 1 min. The column was washed with AW1 and AW2 Buffers, and the pure DNA was eluted in 200 µl of Buffer ATE and stored at −20°C. The quantity and quality of DNA was determined by using NanoDrop ND-1000

**TABLE 1 |** Characteristics of the study subjects. AAb+ are children positive for at least one diabetes-associated autoantibody and AAb- children are negative for beta-cell autoantibodies. The study subjects were participants in the TRIGR and FINDIA pilot studies.

| Characteristics | AAb+ children (*N* = 26) | AAb– children (*N* = 26) |
|---|---|---|
| Female/male | 9/17 | 9/17 |
| **Age (years)** | | |
| TRIGR pilot study | 13.3 (11.7–14.2) | 12.7 (11.9–13.6) |
| FINDIA pilot study | 5.1 (4.0–6.1) | 5.3 (3.9–7.0) |
| **HLA-DQB1 genotype** | | |
| *02:0302 | 7 | 7 |
| *03:02/x | 12 | 11 |
| *02(DQA1*05)/y | 5 | 7 |
| *02(DQA1*03)/y | 1 | 0 |
| *02(DQA1*02:01) | 1 | 1 |

*Data are number (N) or medians (with range). X not DQB1*02, DQB1*03:01, or DQB1*06:02, y not DQA1*02:01-DQB1*02, DQB1*03:01, DQB1*03:02, DQB1*06:02, or DQB1*06:03.*

spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

## Amplification of Bacterial 16s rRNA and Fungal ITS2 Region

The bacterial hypervariable regions V4-V5 of 16S rRNA gene were amplified using primers F519 (5′-CAGCMGCCGCGGTAATWC-3′) and R926 (5′-CCGTCAATTCCTTTRAGTTT-3′). The F519 primer contained an Ion Torrent pyrosequencing adapter sequence A (Thermo Fisher Scientific, USA), 9-bp unique barcode sequence and one nucleotide linker. The R926 primer contained an Ion Torrent adapter trP1 sequence. For fungal analysis, the ITS2 region was amplified using fITS7 (5′-GTGARTCATCGAATCTTTG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) primers including the Ion Torrent pyrosequencing adaptor with a 10-bp barcode sequence to the ITS4 primer (27). PCR reactions were performed in three replicates, each containing 1x Phusion GC buffer, 0.4 μM of forward and reverse primers, 200 μM dNTPs, 0.5 U of Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific) and 50 ng of genomic community DNA as the template and molecular grade water in a total reaction volume of 50 μl. For the bacteria, PCR cycling conditions were as follows: initial denaturation at 98°C for 3 min, 35 amplification cycles of 98°C for 10 s, 64°C for 10 s, and 72°C for 20 s, followed by a final extension step of 72°C for 7 min. For the fungi, the annealing temperature was adjusted to 56°C, while other PCR cycling conditions were kept unchanged. After the amplification, PCR products from the pooled triplicate reactions were purified with Agencourt AMPure XP beads (Agencourt Bioscience, MA, USA) and quantified with Agilent 2,100 Bioanalyzer (Agilent Technologies, CA, USA). The amplicons from each sample were then combined in equimolar concentrations to generate sequencing libraries. Sequencing was performed at Biocenter Oulu Sequencing Center with Ion Torrent PGM System on 316v2 chip using 400 bp chemistry (Thermo Fisher Scientific, USA).

## Bioinformatics Analysis

The bacterial and fungal sequencing data were processed using QIIME v.1.9.1 (28). The read data were quality controlled using the usearch quality filter pipeline, thus potential chimeric sequences were identified and removed with uchime (29). After filtering out low-quality and chimeric reads, the bacterial dataset consisted of 1.202 million reads across the 52 samples, with a mean of 23,120 reads per sample. The respective final fungal dataset comprised of 130,000 high-quality, chimera-free reads from the 52 samples, with a mean of 2,501 reads per sample. The sequences were clustered into the operational taxonomic units (OTUs) by a similarity threshold of 97% with usearch (30). Low-abundance OTUs (represented with <5 reads) were removed across the datasets. The taxonomy was assigned using the Greengenes 16S rRNA gene reference database (31) for bacteria (v.13_8) and UNITE ITS database for fungi (2019 release, v.8) (32). Prior to downstream analysis, the bacterial and fungal OTU tables were rarefied to 5,800 and 327 reads/sample, respectively, to avoid biases caused by variation in sequencing depth among samples (33). All the raw sequencing data were deposited in the NCBI-SRA database with an accession number SUB3267498.

We estimated beta diversity using the unweighted and weighted UniFrac distances (as well as non-phylogenetic Bray Curtis dissimilarity) between samples. Both UniFrac metrics incorporate phylogenetic distances between taxa, yet while the unweighted UniFrac compare microbial communities based on the presence/absence information, the weighted UniFrac also consider the differences in taxon abundance (34). Differences in the fungal and bacterial gut microbiota structure among children were visualized by Principal Coordinate analysis (PCoA) using EMPeror (35).

## Measurements of Fecal HBD2, Total IgA, and Calprotectin

Thawed fecal samples were mixed with extraction buffer and vortexed thoroughly. Then, the supernatant was collected and stored at −20°C until the analysis of total IgA, HBD2, and calprotectin levels. Total IgA concentrations were analyzed as previously described (36). HBD2 analyses, were performed with a commercial ELISA Kit according to the manufacturer's instructions (Immunodiagnostik AG, Bensheim, Germany). Fecal calprotectin levels were determined using Calprolab calprotectin ELISA test according to the manufacturer's instructions (Calpro AS, Lysaker, Norway).

## ELISA Analysis of Serum ASCA IgA/IgG Levels

Serum ASCA IgA and IgG concentrations were analyzed with a commercial ELISA kit according to the manufacturer's instructions (Demeditec, Germany), with the exception that 1:10 dilution of the serum samples were used. The samples below the lower limit of detection (LOD) were given an arbitrary value of 50% of the LOD being 0.5 U/ml for both ASCA IgA and IgG.

## Serum Cytokine Analysis

The serum concentrations of IFNG, IL-17A and IL-22 were analyzed using the Milliplex MAP Kit (HTH17MAG-14K) according to the manufacturer's recommendations (Merck-Millipore Corp., Billerica, MA, USA). Quantification of the markers was performed with a Bio-plex 200 Luminex-instrument and Bio-Plex Manager software (Bio-Rad, Sweden). The samples below Minimum detectable concentration (MinDC) DC were given an arbitrary value of 50% of MinDC.

## Statistical Analyses

The Graph Pad Prism 6.04 (Graph Pad Inc., La Jolla, California, USA), SPSS 22 (SPSS, Chicago, Illinois, USA) and JMP 13.0.0 statistical softwares were used for the statistical analyses, unless otherwise noted. Non-parametric Mann–Whitney *U*-test was used for comparisons between two groups. Groupwise comparisons were performed with the Kruskal–Wallis test. The correlations between the variables were analyzed with the non-parametric Spearman correlation test. Fisher's' exact test was used to analyze the distribution of autoantibody-positive children and disease progressors in different clusters. All statistical analyses were performed two-tailed. $P < 0.05$ was considered statistically significant. Despite of the matching of the autoantibody positive and negative children for age, T1D risk genotype and sex, the pairs were considered independent in the statistical analyses.

For the hierarchical clustering analysis, the relative abundance data was imported into JMP 13.0.0 (SAS Institute Inc. Cary, North Carolina, USA). All abundance values were treated as numerical values and Ward's hierarchical clustering was performed using standardized data with default settings. Statistical significance of samples grouping for beta diversity analysis was determined using the permutational multivariate analysis of variance (PERMANOVA) and the analysis of similarities (ANOSIM) (999 permutations) implemented by the *adonis* and *anosim* functions in the vegan R package (37).

# RESULTS

## Study Design and Clinical T1D During the Follow-Up

In this study, we analyzed the gut microbiome of autoantibody positive and negative children with HLA conferred risk of T1D (**Figure 1**). We combined sequencing of the (1) bacterial 16S ribosomal RNA (rRNA) gene (2) and fungal internal transcribed spacer 2 (ITS2) region and coupled this with the (3) analysis of the markers of intestinal inflammation, namely fecal HBD2, secretory total IgA, and calprotectin. Blood samples were screened for the levels of ASCA IgA/IgG and circulating cytokines IFNG IL-17 and IL-22. Finally, the children in a cohort were followed for the development of clinical T1D (median of 8 years and 8 months). During the follow-up time, nine autoantibody positive children were diagnosed with T1D, and none of the autoantibody negative children developed T1D or autoantibodies.

## Fungal and Bacterial Dysbiosis in Children With Beta-Cell Autoimmunity

The gut mycobiota was composed of two fungal phyla, Ascomycota and Basidiomycota, but was dominated by Ascomycota (average 93%) at the phylum level and by *Saccharomyces* at the genus level (average 43%) (**Figures 2A,B**). All 52 study subjects were positive for Ascomycota, and 29 of them (56%) positive for Basidiomycota (11 of 26 autoantibody positive and 18 of 26 negative individuals, 42 and 69%, respectively). Children with autoantibodies had marginally increased abundance of Ascomycota and decreased levels of Basidiomycota (**Figure 2A**). The most frequently observed genera were *Saccharomyces* (found in all 52 individuals), *Candida* (found in 9 of 26 autoantibody positive and 13 of 26 autoantibody negative individuals, 35 and 50%) and *Debaryomyces* (found in 9 of 26 autoantibody positive and 4 of 26 autoantibody negative individuals, 35 and 15%). Children with autoantibodies had increased abundance of *Debaryomyces* and decreased abundance of *Malassezia* (**Figures 2B–D**). The number of children positive for *Debaryomyces* or *Malassezia* was, however, low, and 25.0 and 23.1% of the studied children were positive for *Debaryomyces (13/52)* and *Malassezia (12/52)*, respectively. The autoantibody-positive children who developed clinical T1D during the follow-up had significantly decreased abundance of genus *Verticillium* compared to children with or without autoantibodies (**Figure 2E**). *Verticillium* positivity was found in 16 of 26 (62%) autoantibody positive and 17 of 26 (65%) autoantibody negative children. We did not observe differences in the number of OTUs between children with or without autoantibodies (**Figure 2F**). Children who developed T1D had decreased fungal diversity (Shannon) compared to children with multiple autoantibodies (**Figure 2G**). Principal coordinate analysis based on weighted and unweighted UniFrac distances did not show clear differences between autoantibody-negative and autoantibody-negative children (**Supplementary Figures 1C,D**). The fungal community composition is consistent with the gut mycobiome communities previously reported for humans (18, 38, 39). List of the most abundant fungal species shared among the autoantibody-negative and positive children is presented in **Supplementary Table 1**.

Next, we addressed the whole microbiota and performed a combinational analysis of fungal and bacterial communities. Our hierarchical clustering analysis based on the combined fungal and bacterial data revealed the presence of three major clusters as defined by different combinations of fungi, belonging to the phylum Ascomycota, and the bacterial phyla Bacteroidetes and Firmicutes (**Figures 3A–C**). The fungal and bacterial community structures differed among children assigned to distinct clusters ($p < 0.001$, PERMANOVA) (see the **Supplementary Table 2**). Cluster 1 ($n = 17$, 33%) was characterized by high abundance of Clostridiales and low abundance of Bacteroidales in combination with high abundance of *Saccharomyces* (**Figures 3B,C** and **Supplementary Figure 2A**). Conversely, Clusters 4 ($n = 22$, 42%) and 5 ($n = 8$, 15%) were characterized by high abundance of Bacteroidales and low abundance of Clostridiales, and Cluster 4 also showed high abundance of *Candida* compared to Cluster

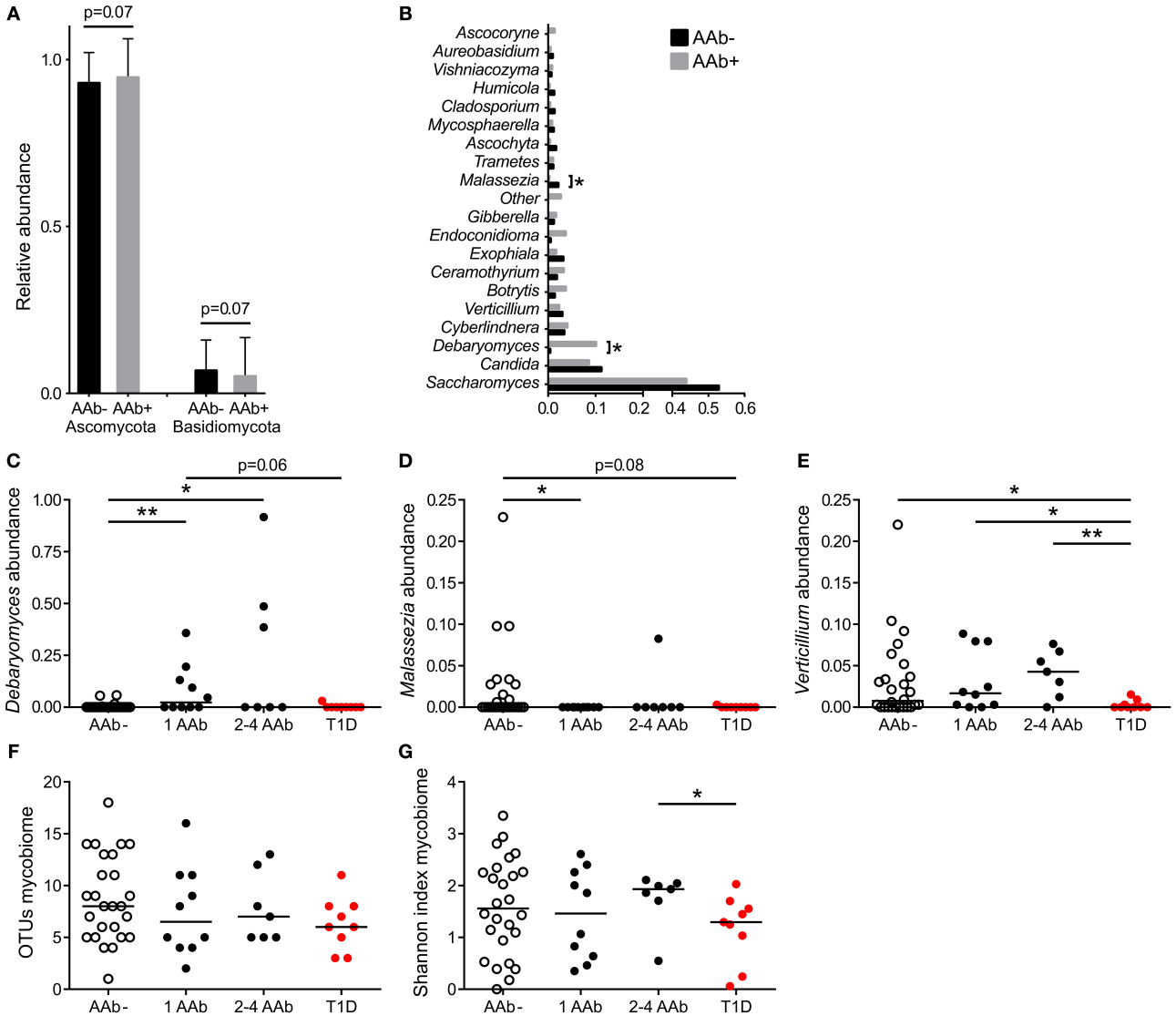


**FIGURE 2 |** Fungal community characteristics in children with or without T1D-associated autoantibodies. **(A)** Relative abundance of fungal phyla Ascomycota (left columns) and Basidiomycota (right columns). Children without autoantibodies: black columns and children with autoantibodies: gray columns. **(B)** Relative abundance of 20 most abundant fungal genera in children with (gray bars) and without autoantibodies (black bars). **(C–E)** The relative abundances of *Debaryomyces* **(C)**, *Malessezia* **(D)**, and *Verticillium* **(E)** in healthy children without autoantibodies, in children with a different number of autoantibodies and in children who have progressed from autoantibody-positive state to clinical T1D. **(F,G)** Number of OTUs and Shannon diversity index in healthy children without autoantibodies, in children with a different number of autoantibodies and in children who have progressed from autoantibody-positive state to clinical T1D. Healthy children without autoantibodies are marked with open circles, children with 1–4 autoantibodies with black circles and children who have progressed to clinical disease with red circle. *p*-values were calculated with the Mann–Whitney *U*-test. $^{*}p < 0.05$, $^{**}p < 0.01$.

1 (**Figures 3B,C** and **Supplementary Figure 2B**). Although, abundance of Ascomycota was high in Clusters 1 and 4, the abundance of *Saccharomyces* and *Candida* differed significantly between the clusters (**Figure 3B**, **Supplementary Figures 2A,B** and **Supplementary Tables 2**, **3**). Relative abundances of *Debaryomyces*, *Malassezia* and *Verticillium* in Clusters 1, 4 and 5 are shown in **Supplementary Figures 2C–E**. The relative abundances of *Saccharomyces* and *Candida* in children with or without autoantibodies are shown in **Supplementary Figures 2F,G**. Analysis of similarities of fungal and bacterial communities between different clusters, revealed that Clusters 4 and 5 differed from Cluster 1 for both fungal and bacterial communities (**Supplementary Table 3**). Host gender had no statistically significant ($p > 0.05$, PERMANOVA) explanatory effect on the fungal and bacterial communities (**Supplementary Table 2**). Children age and HLA-risk class made a statistically significant contribution to the total variation in the bacterial community ($p = 0.02$, $R^2 = 0.08$, $p < 0.001$, $R^2 = 0.14$, for the host age and HLA-risk class, respectively), albeit with a relatively low coefficient of determination, and non-significant ($p > 0.05$) contribution of both factors to the mycobiome community structure (**Supplementary Table 2**).

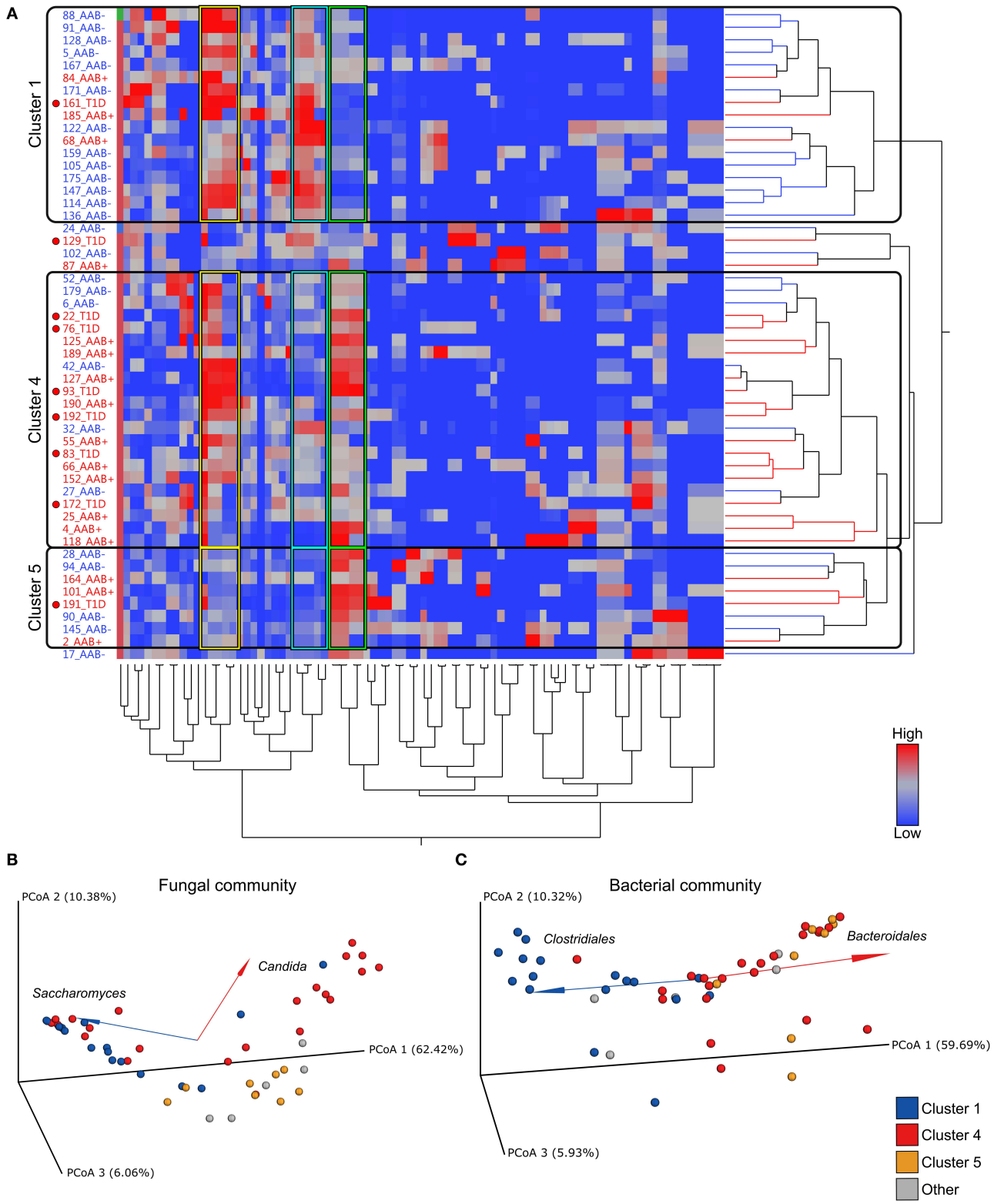


**FIGURE 3 |** Hierarchical clustering over bacterial and fungal taxa and PCoA plots of fungal and bacterial communities. **(A)** Heatmap showing the clustering of the relative abundances. The clustering resulted in three major clusters (Clusters 1, 4, and 5) defined by differential abundance of fungi belonging to the phylum Ascomycota and the bacterial phyla Firmicutes and Bacteroidetes. Clusters 1, 4, and 5 are encircled with black borders. In the heatmap children with autoantibodies are denoted with AAb+ (red font) and autoantibody-negative children with AAb (blue font). Children who have progressed from autoantibody-positive state to clinical

*(Continued)*

**FIGURE 3 |** disease are denoted with T1D and a red circle in the heatmap. Color intensity of the heatmap increases with the taxa relative abundance from low (blue) to high (red). **(B,C)** Principal coordinate analysis (PCoA) biplots (incorporate taxonomy information) on weighted UniFrac distances between the fungal **(B)** and bacterial **(C)** gut microbial communities profiles of children at risk of the type 1 diabetes development. Each point represent a single sample and is colored according to the major taxa clusters (Clusters 1, 4, 5, and other), as defined by the hierarchical clustering analysis. Order and genus-level taxonomy displayed by biplot arrows illustrates that the abundance of **(B)** the *Saccharomyces* and *Candida* contribute to the separation of Cluster 1 and Cluster 4, and **(C)** Clostridiales and Bacteroidales contribute to the distinct clustering patterns of Cluster 1 in comparison to Clusters 4 and 5. *p*-values were calculated with the Mann–Whitney *U*-test. $*p < 0.05$, $**p < 0.01$. Grouping significance was determined using the PERMANOVA (999 permutations) with the *adonis* function in the vegan R package ($p < 0.001$).

**TABLE 2 |** Distribution of autoantibody-negative children, children with one or multiple autoantibodies and disease progressors in the different Clusters.

| | Cluster 1 | Cluster 4 | Cluster 5 |
|---|---|---|---|
| AAb- | 13 (76.5%) | 6 (27.2%) | 4 (50%) |
| 1 AAb | 1 (5.8%) | 7 (31.8%) | 2 (25%) |
| 2-4 AAbs | 2 (11.7%) | 3 (13.6%) | 1 (12.5%) |
| T1D progressors | 1 (5.8%) | 6 (27.2%) | 1 (12.5) |
| Total | 17 | 22 | 8 |

*Relative proportions of different study groups within the Clusters are presented in the parentheses.*

Next, we analyzed the distribution of autoantibody positive and negative children in these three major microbiome clusters. The children with beta-cell autoimmunity were enriched in Clusters 4 and 5 (Cluster 1 vs. Cluster 4, $p = 0.004$, Cluster 1 vs. Cluster 5, $p =$ ns and Cluster 4 vs. Cluster 5, $p =$ ns, Fisher's exact test), while the children negative for beta-cell autoimmunity, and thus considered healthy children, were enriched in Cluster 1 (shown in **Figure 3A** and in **Table 2**). By the end of the follow-up, 6 out of 22 children in Cluster 4 (27%) had developed T1D, one of eight children in Cluster 5 (13%), and just one of 17 children in Cluster 1 (6%) were diagnosed for T1D (Cluster 4 vs. 5, $p = 0.64$ and Cluster 4 vs. 1, $p = 0.11$, respectively). One child who was not assigned in any of the three major clusters developed T1D.

## Intestinal Inflammation in Children With Fungal and Bacterial Dysbiosis

To address the relation between the composition of the intestinal microbiota and host inflammatory response, we analyzed fecal concentrations of HBD2, which is an antimicrobial peptide secreted by the epithelial cells in response to microbial stimulus and IL-17/IL-22 pathway activation (40). Interestingly, the children in Cluster 4 had higher fecal HBD2 levels compared to children in Clusters 1 and 5 (**Figure 4A**), and consequently autoantibody positive children had higher levels of fecal HBD2 than autoantibody-negative children (**Figure 4B**). However, the autoantibody-positive children in Cluster 4 had higher levels of fecal HBD2 than those in Cluster 5 (**Supplementary Figure 3**). Notably, the children with only one autoantibody showed higher levels of fecal HBD2 compared to autoantibody-negative children (**Figure 4C**). Fecal calprotectin levels did not differ between the autoantibody-negative or positive children or between the species clusters (**Supplementary Figures 4D–F**).

Since the high abundance of order Saccharomycetales (the most abundant genera in the data set belonging to the Saccharomycetales were: *Saccharomyces, Candida,* and *Debaryomyces*) was a key feature of the children in Cluster 1 and 4, we measured the levels of serum ASCA shown earlier to be associated with fungal dysbiosis in Crohn's disease (41, 42). ASCA IgG levels were significantly higher in children belonging to Clusters 1 and 4 compared to the children in Cluster 5 (**Figure 4D**), suggesting that the ASCA IgG production is indeed induced by high abundance of Saccharomycetes, which was observed in children in the Clusters 1 and 4. ASCA IgG levels did not, however, correlate with the abundance of Saccharomycetes or *Saccharomyces* in either the children in Cluster 1 or 4 ($p = 0.33$ and $p = 0.73$). Indeed, the highest ASCA IgG levels were observed in the children who progressed to clinical T1D, irrespective of the assigned Cluster (**Figures 4E,F**). Moreover, ASCA IgG levels showed a tendency of positive correlation with the duration of autoantibody positivity in the Cluster 4 children (**Figure 4G**). ASCA IgA levels did not differ significantly between the clusters, but it should be noted that the majority of individuals studied had ASCA IgA levels below the lower limit of detection (**Supplementary Figures 4G–I**). We also observed a positive correlation between fecal total IgA levels and Saccharomycetes (and also *Saccharomyces*) abundance in Cluster 4, suggesting a local intestinal immunostimulatory effect of *Saccharomyces* in the children in Cluster 4 (**Figures 4H,I**).

## Bacteroidetes vs. Firmicutes Ratio as a Regulator of Systemic Low-Grade Inflammation

Given that Th1 and Th17 immunity have been earlier associated with fungal dysbiosis (43) and T1D (6, 9, 44), we measured concentrations of the circulating cytokines IFNG, IL-17A, and IL-22, in the serum samples of the study participants. In Cluster 1, enriched with the autoantibody negative children and representing thus healthy children, both IFNG and IL-17A concentrations correlated positively with the abundance of Bacteroidetes (**Figures 5A,D**) and inversely with the abundance of Firmicutes (**Figures 5B,E**). In agreement with this, we found that the children in Cluster 5, characterized by increased Bacteroidetes to Firmicutes ratio, showed increased levels of circulating IFNG and IL-17A (**Figures 5C,F**). In Cluster 4, enriched with the autoantibody positive children with intestinal inflammation, no correlations were observed between circulating cytokines and microbiota composition. No correlation was found between the relative abundance of *Saccharomyces* and circulating cytokines (**Figures 5G,H**) in Cluster 1. Serum IFNG and IL-17A concentrations in children with or without beta-cell autoimmunity are shown in **Supplementary Figure 5**.

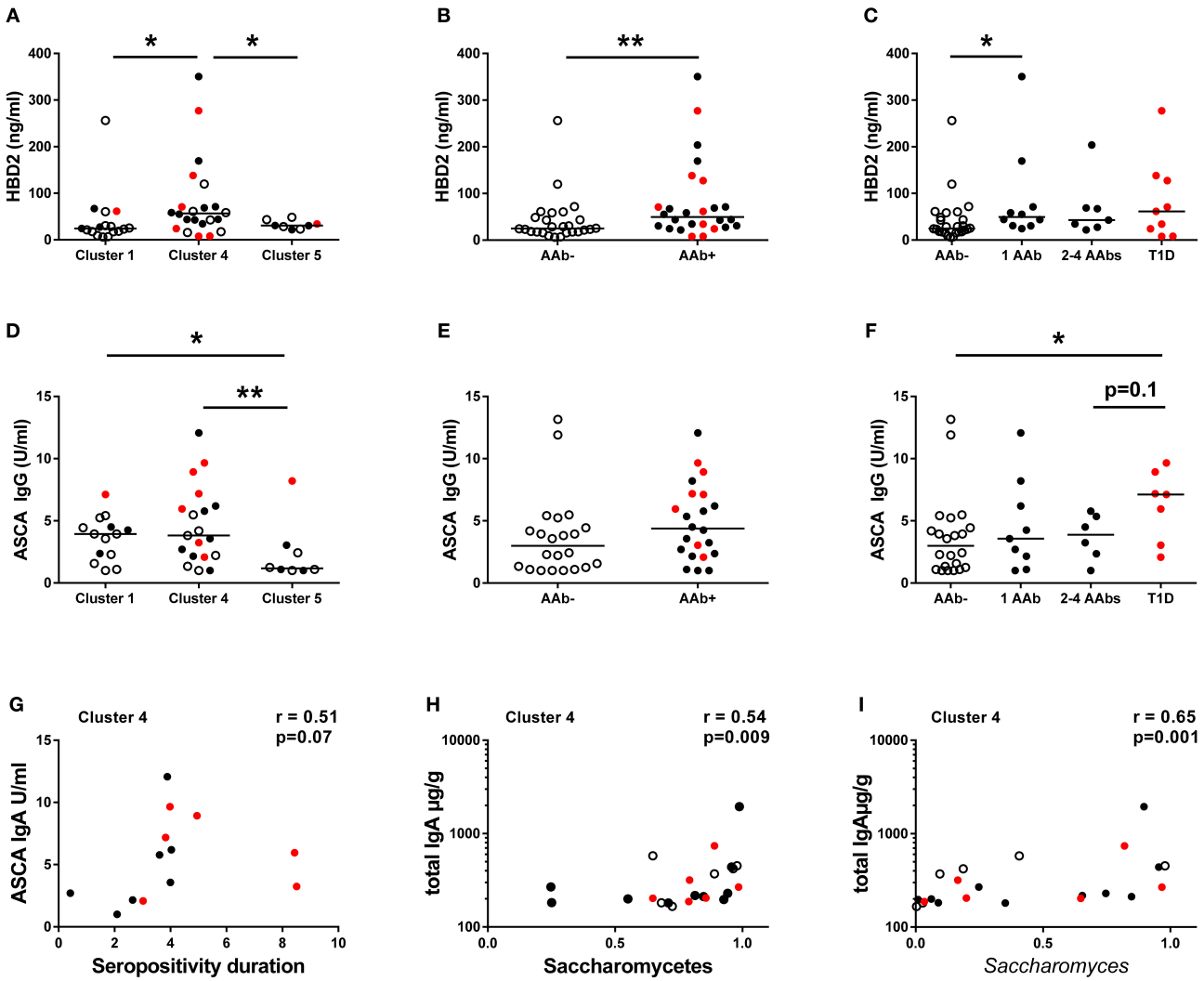


**FIGURE 4 |** Inflammatory markers in different clusters and in children with or without beta-cell autoimmunity. **(A)** Fecal concentrations of HBD2 in the Clusters 1, 4, and 5. HBD2 levels were higher in the species Cluster 4 compared to Clusters 1 and 5. **(B)** Children with autoantibodies had elevated levels of fecal HBD2 compared to autoantibody-negative children. **(C)** Children with only one autoantibody had elevated levels of fecal HBD2 compared to autoantibody-negative children. **(D)** Serum ASCA IgG concentrations were significantly higher in the Clusters 1 and 4 compared to the Cluster 5. **(E)** Serum ASCA IgG concentrations in AAb- and in AAb+ children. **(F)** The autoantibody-positive children who have progressed to clinical disease had elevated serum ASCA IgG antibodies compared to autoantibody-negative children. **(G)** The relationship between serum ASCA IgA levels and seropositivity duration in the species Cluster 4. **(H)** The relationship between fecal total IgA and relative abundance of Saccharomycetes in the Cluster 4. **(I)** The relationship between fecal total IgA and relative abundance of *Saccharomyces* in the Cluster 4. Healthy children without autoantibodies are marked with open circles, children with 1–4 autoantibodies with black circles and children who have progressed to clinical disease with red circle. Horizontal lines represent median values. *p*-values were calculated with the Mann–Whitney *U*-test. Correlations were calculated with the Spearman rank correlation test.*$p < 0.05$, **$p < 0.01$.

## DISCUSSION

In a prospective study including 52 children at risk of T1D, we show that intestinal dysbiosis is associated with the later development of T1D, and is characterized by altered fungal and bacterial communities and intestinal inflammation. Signs of intestinal inflammation and increased permeability have been earlier associated with clinical T1D (15, 26, 45–47). Bosi et al. (48) have shown increased intestinal permeability also in pre-diabetes (48). To our knowledge, however, these kinds of associations between the composition of the intestinal microbiome, intestinal inflammatory markers, and their potential contribution to the disease progression have not been reported earlier in children at risk of T1D.

The combined hierarchical clustering analysis of fungal and bacterial taxa provided the separation of the autoantibody positive children into two groups which differed in the progression to T1D during the follow-up. Long-term follow-up studies are rare, but show that nearly all children positive for multiple autoantibodies and genetic risk of

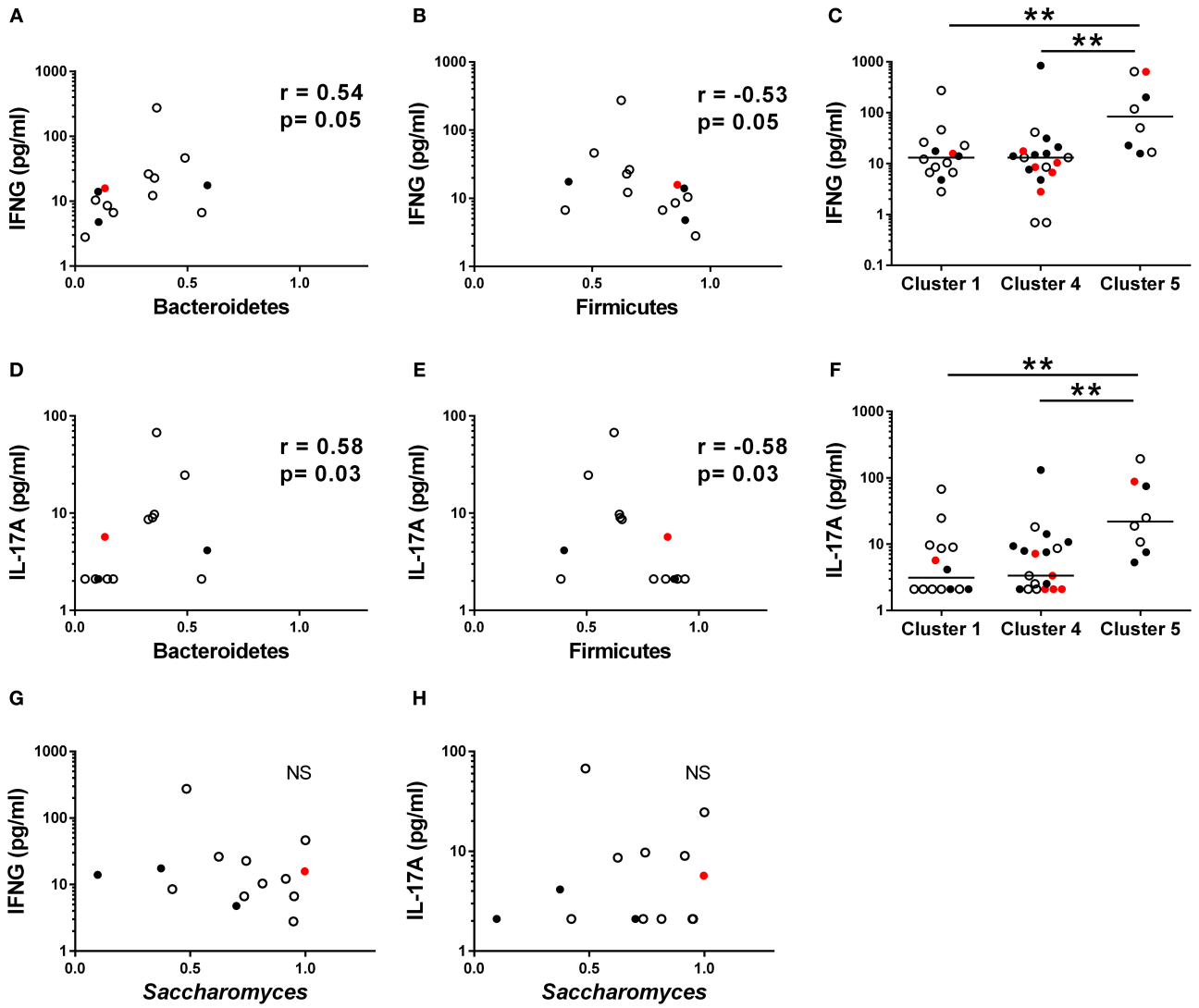


**FIGURE 5 |** Serum cytokine concentration in different clusters. **(A,B)** The relationships between serum IFNG and relative abundance of fecal Bacteroidetes and Firmicutes in the Cluster 1. **(D,E)** The relationships between serum IL-17A and relative abundance of fecal Bacteroidetes and Firmicutes in the Cluster 1. **(C,F)** IFNG and IL-17A levels were significantly higher in the Cluster 5 compared to the Clusters 1 and 4. **(G,H)** The relationships between serum IFNG and relative abundance of fecal Bacteroidetes and Firmicutes in the Cluster 1. Horizontal lines represent median values. *p*-values were calculated with the Mann–Whitney *U*-test. Correlations were calculated with the Spearman rank correlation test.*$p < 0.05$, **$p < 0.01$.

T1D develop the clinical disease in 15–20 years (49). Thus, the children who developed T1D in our cohort can be considered as rapid progressors (Cluster 4) in comparison to the autoantibody positive children who remained healthy (Cluster 5). The levels of fecal HBD2 indicating epithelial intestinal inflammation were the highest in the children with rapid disease progression (i.e., Cluster 4) suggesting that intestinal inflammation is a marker of disease progression. The altered bacterial community, which was seen in Clusters 4 and 5, is likely associated with the development of beta-cell autoimmunity as such. We did not find significant differences in fecal calprotectin levels between the clusters, or children with or without beta-cell autoimmunity, suggesting that neutrophil activation is not mediating intestinal inflammation. The levels of fecal calprotectin in our study cohort were comparable to the levels reported earlier in healthy children (50).

Our mycobiome data suggest that fungal dysbiosis could play a role in the disruption of intestinal homeostasis and development of subclinical low-grade intestinal inflammation, which associate with the disease progression.

Altered abundances of fungi assigned to *Malassezia* and *Debaryomyces* taxa were found in children with beta-cell autoimmunity, and a decreased abundance of fungi assigning to *Verticillium* genus was observed in children who later progressed to clinical T1D. Fecal *Debaryomyces*

and *Malassezia* have been occasionally reported in human studies, but currently there is no consensus whether these fungal taxa are permanent residents of human intestinal microbiota (17). *Verticillium* has been reported very rarely in humans (51, 52). In line with a recent comprehensive review on human gut mycobiota (18), *Saccharomyces,* and *Candida* genera were the most frequently observed fungal taxa with the highest relative abundances among the children in our cohort.

Importantly, the high relative abundance of *Candida* was characteristic to the fungal dysbiosis that separated the autoantibody positive children with rapid disease progression from the rest of the autoantibody positive children who did not develop T1D, and from the autoantibody negative children. Thus, the increased colonization with *Candida* could be an important factor contributing to the intestinal inflammation and further progression to T1D.

*Candida* is a member of the healthy intestinal microbiome, and the degree of *Candida* colonization is regulated by host related factors, such as epithelial integrity and IL-17/IL-22 immunity, and by the composition of the commensal bacterial community (43, 53–56). Commensal bacteria interfere with fungal colonization and compete for surface and nutrients, and bacteria-produced short chain fatty acids (SCFAs) can inhibit *Candida* virulence by preventing yeast-hyphal transition (57). Bacteria can also modulate epithelial barrier function and integrity by their SCFA metabolites, such as butyrate, and by regulation of the production of mucus, IL-22, and anti-microbial peptides (54, 55, 58). Thus, the low relative abundance of Clostridiales and butyrate-producing bacteria found in the autoantibody positive children could contribute to the increasing colonization by *Candida*. Low abundance of butyrate-producing bacteria has been reported also in autoantibody positive children in earlier studies (10, 59). However, despite of the low abundance of Clostridiales in both Clusters 4 and 5, increase in *Candida* was only seen in Cluster 4 including the rapid progressors. In humans, efficient control and eradication of *Candida* requires the activation of IL17A and IFNG-producing Th17 cells (60, 61). It is thus possible that the observed high levels of circulating IL-17 and IFNG in Cluster 5 could provide resistance to fungal colonization. Indeed, the abundance of *Saccharomyces* was significantly decreased in Cluster 5 with the highest levels of circulating IL-17 and IFNG. The increase in IL-17 and IFNG in Cluster 5 can actually be a consequence of the bacterial dysbiosis characterized by low abundance of Firmicutes and high abundance of Bacteroidetes, similarly as seen in the autoantibody negative children in Cluster 1, who showed a positive correlation with circulating IL-17 and IFNG and a high Bacteroidetes to Firmicutes ratio. Instead, in Cluster 4, the children did not respond to bacterial dysbiosis with IL-17 and IFNG upregulation, which could provide a niche for *Candida* colonization, and finally to the local mucosal inflammation in the intestine. When we analyzed the relationship between relative abundances of *Saccharomyces* or *Candida* and circulating cytokines, we did not observe significant correlations in children in Cluster 1 underlining the importance of Bacteroidetes and Firmicutes in regulation of IFNG and IL-17 responses in a healthy state.

The human intestinal microbiota is a dynamic system of bacteria, fungi, protists and viruses that co-exist and thus, may converge in response to various external or internal stimuli. Interkingdom associations between bacteria and fungi within gut microbiome have been previously reported in Crohn's disease, where different fungal genera were positively correlated with several bacterial taxa (21, 22). The potency of the mycobiota to regulate the bacterial compartment is suggested by animal studies showing that restoration of the bacterial compartment after antibiotic depletion of bacteria was strongly influenced by colonization with *C. albicans* (62). In a mouse model of liver injury, administration of *Saccharomyces boulardii* changed the composition of intestinal bacterial compartment by increasing the relative abundance of Bacteroidetes and decreasing the relative abundance of the bacteria belonging to the Firmicutes (63). Interestingly, Enterobacteriaceae, such as *Escherichia coli* has been shown to cooperate with yeast to favor their colonization and inflammatory properties in the intestine in an animal model of ulcerative colitis (64). The implication of these data is that intestinal fungal and bacterial communities can regulate each other, but the understanding of the ecological network and its cross-talk with the host remain largely unknown.

We recognize that our study has limitations, such as the relatively low number of studied individuals and the lack of longitudinally collected fecal and blood samples during the follow-up. Although we observed the alterations in the bacterial and fungal communities and in the markers of intestinal inflammation in the samples collected already years before the signs of clinical T1D, longitudinal sampling and mechanistic studies would have strengthened the study, which is currently descriptive in nature. Temporal relationship of bacterial and fungal dysbiosis linked to the development of intestinal inflammation, beta-cell autoimmunity and T1D needs further prospective and mechanistic studies, and as always, the results should be to be replicated in independent cohorts before the findings can be generalized.

There is an urgent need for new biomarkers that could be used for the identification of the individuals with increased risk of beta-cell autoimmunity and for the prediction of the progression from autoantibody positivity to T1D. It is tempting to speculate that an increased *Candida* abundance and associated intestinal inflammation, measured by increased levels of ASCA and HBD2 levels, could provide new tools for the more accurate prediction of T1D.

Longitudinal studies are needed to provide information on the sequential order of the changes in gut microbiota, intestinal inflammation and peripheral immunity leading to beta-cell autoimmunity and clinical T1D. Despite the limitations in the current study, our findings show that intestinal mycobiota is diverse and can be analyzed in pediatric fecal samples. Our results emphasize the importance of the fungal dysbiosis, in addition to bacterial dysbiosis, in shaping intestinal homeostasis and inflammation preceding T1D.

## DATA AVAILABILITY STATEMENT

All microbiome data was uploaded to NCBI BioProject database with accession number PRJNA420169 & PRJNA420171. The other datasets are available on request to the first author (JH).

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the Hospital District of Helsinki and Uusimaa. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

## AUTHOR CONTRIBUTIONS

JH, JK, and OV conceived the original idea. JH and OV wrote the manuscript. JK, AL, and MT were responsible for DNA analyses and bioinformatics of the microbiological studies. DM was responsible for clustering analyses. AV performed serum ASCA Ig and cytokine analyses. JH, LO, JK, AL, MT, AV, CF, and DM analyzed the data. TR and KL coordinated the study subject recruitment and sample collection. MK contributed to the study subject recruitment and edited the manuscript. JK, AL, and AP contributed to the writing and critically reviewed the manuscript. MK and TH were responsible for the autoantibody analyses. JI was responsible for HLA typing. LO performed the HBD2, total IgA, and calprotectin analyses. OV was responsible for the study design.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00468/full#supplementary-material

**Supplementary Figure 1 |** Diversity of the fungal communities. **(A)** Rarefaction curves showing the alpha diversity in the fungal community across all the samples, and **(B)** in children with 1-4 β-cell autoantibodies (red curve) and autoantibody negative samples (blue curve). Each curve is showing an average number of OTUs found in the given number of sampled sequences after rarefaction at the depth of 327 sequences per sample. Principal coordinate analysis (PCoA) plots based on the weighted **(C)** and unweighted **(D)** UniFrac distances between the fecal fungal communities in children with (red dots) or without (blue dots) autoantibodies.

**Supplementary Figure 2 |** Relative abundances of fungal genera *Saccharomyces*, *Candida*, *Debaryomyces*, *Malassezia* and *Verticillium* in different Clusters and in children with or without T1D-associated autoantibodies. Relative abundances of *Saccharomyces* **(A)** and *Candida* **(B)** in major Clusters 1, 4, and 5. **(C–E)** relative abundances of *Debaryomyces*, *Malassezia* and *Verticillium* in fecal samples from children in major Clusters. **(F,G)** Healthy children without autoantibodies are marked with open circles, children with 1–4 autoantibodies with black circles and children who have progressed to clinical disease with red circle. *P*-values were calculated with the Mann–Whitney *U*-test. $*p < 0.05$, $**p < 0.01$.

**Supplementary Figure 3 |** Intestinal inflammation in children with autoantibodies in Clusters 4 and 5. AAb+ children in the Cluster 5 had significantly lower fecal HBD2 levels compared to AAb+ children in the Cluster 4. Horizontal lines represent median values. Children with 1–4 autoantibodies are marked with black circles and children who have progressed to clinical disease with red circle. Dotted line represents the highest observed value in the Cluster 5. 81% of the individuals in the cluster 4 had HBD2 level higher than the highest value in the Cluster 5. *p*-values were calculated with the Mann–Whitney *U*-test. $*p < 0.05$.

**Supplementary Figure 4 |** Inflammatory markers in different clusters and in children with or without beta-cell autoimmunity. **(A)** Fecal concentration of total IgA in the major species Clusters 1, 4, and 5. **(B)** Fecal total IgA concentrations in AAb+ children and AAb- children. **(C)** Fecal concentration of total IgA in AAb-children, in children with a different number of autoantibodies and in children who have progressed to clinical T1D. **(D)** Fecal concentration of calprotectin in the major species Clusters 1, 4, and 5. **(E)** Fecal calprotectin concentrations in AAb+ children and AAb- children. **(F)** Fecal concentration of calprotectin in AAb-children, in children with a different number of autoantibodies and in children who have progressed to clinical T1D. **(G)** Serum ASCA IgA antibody levels in the different species Clusters. **(H)** Serum ASCA IgA levels in AAb- and AAb+ children. **(I)** Serum ASCA IgA concentrations in AAb- children and in children with only one or multiple autoantibodies and in those children who have progressed to clinical disease. **(J)** Serum IL-22 levels in different species clusters. **(K)** IL-22 levels in AAb- and AAb+ children. **(L)** IL-22 levels in AAb- children and in children with different numbers of autoantibodies.

**Supplementary Figure 5 | (A,B)** Serum IFNG and IL-17A concentration in children with or without beta-cell autoimmunity.

**Supplementary Table 1 |** The top 25 most abundant fungal species (based on the observed sequences) shared among the autoantibody-negative children and children with autoantibodies.

**Supplementary Table 2 |** Summary of the permutational multivariate analysis of variance (PERMANOVA). PERMANOVA statistical tests were performed on the weighted UniFrac distances and Bray Curtis dissimilarity between the (1) fungal and (2) bacterial gut microbial communities profiles of children at risk of the type 1 diabetes development. Statistical tests were run using the adonis function in R package vegan (with 999 permutations). The significant (<0.05) values shown in bold.

**Supplementary Table 3 |** Summary of the analysis of similarities (ANOSIM). ANOSIM statistical tests were performed on the weighted UniFrac distances and Bray Curtis dissimilarity between the (1) fungal and (2) bacterial gut microbial communities profiles of children at risk of the type 1 diabetes development. Statistical tests were run using the anosim function in R package vegan (with 999 permutations). The Benjamini-Hochberg False Discovery Rate (FDR) correction for multiple testing was used to calculate q-values. The significant (<0.05) values shown in bold.

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**Conflict of Interest:** DM was employed by the company AstraZeneca, but AstraZeneca did not have a role in the study.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Psychological Stress, Intestinal Barrier Dysfunctions, and Autoimmune Disorders: An Overview

*Hanna Ilchmann-Diounou and Sandrine Menard**

*Neuro-Gastroenterology and Nutrition Team, Toxalim (Research Centre in Food Toxicology), Université de Toulouse, INRAE, ENVT, INP-Purpan, UPS, Toulouse, France*

Autoimmune disorders (ADs) are multifactorial diseases involving, genetic, epigenetic, and environmental factors characterized by an inappropriate immune response toward self-antigens. In the past decades, there has been a continuous rise in the incidence of ADs, which cannot be explained by genetic factors alone. Influence of psychological stress on the development or the course of autoimmune disorders has been discussed for a long time. Indeed, based on epidemiological studies, stress has been suggested to precede AD occurrence and to exacerbate symptoms. Furthermore, compiling data showed that most of ADs are associated with gastrointestinal symptoms, that is, microbiota dysbiosis, intestinal hyperpermeability, and intestinal inflammation. Interestingly, social stress (acute or chronic, in adult or in neonate) is a well-described intestinal disrupting factor. Taken together, those observations question a potential role of stress-induced defect of the intestinal barrier in the onset and/or the course of ADs. In this review, we aim to present evidences supporting the hypothesis for a role of stress-induced intestinal barrier disruption in the onset and/or the course of ADs. We will mainly focus on autoimmune type 1 diabetes, multiple sclerosis and systemic lupus erythematosus, ADs for which we could find sufficient circumstantial data to support this hypothesis. We excluded gastrointestinal (GI) ADs like coeliac disease to privilege ADs not focused on intestinal disorders to avoid confounding factors. Indeed, GIADs are characterized by antibodies directed against intestinal barrier actors.

**Keywords: intestinal permeability, psychological stress, type 1 diabetes, multiple sclerosis, systemic lupus erythematosus, microbiota, immune response**

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***Edited by:***
*Julien Diana,*
*Institut National de la Santé et de la Recherche Médicale (INSERM), France*

***Reviewed by:***
*Gianluigi Giannelli,*
*National Institute of Gastroenterology S. de Bellis Research Hospital (IRCCS), Italy*
*Federica Facciotti,*
*European Institute of Oncology (IEO), Italy*

****Correspondence:***
*Sandrine Menard*
*sandrine.menard@inrae.fr*

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## INTRODUCTION

Autoimmune disorders (ADs) are multifactorial diseases involving, genetic, epigenetic, and environmental factors. In the past decades, there has been a continuous rise in the incidence of ADs, which cannot be explained by genetic factors alone. Changes in our lifestyle including diet, hygiene, exposure to social adversity, or pollutants have been suggested to be risk factors for ADs. ADs are associated with defect of the intestinal barrier; and besides nutrition, another environmental factor well described to impair the intestinal barrier is psychological stress. The aim of this review is to compile evidences highlighting a relationship between stress, intestinal barrier disruption, and occurrence of ADs. Even though no causative role of stress-induced intestinal barrier defect on AD onset has been demonstrated so far, the goal of this manuscript

is to combine evidences on the basis of a review of the literature and offer a new field of research and perspectives on ADs. We will focus on three of the most studied ADs—autoimmune type 1 diabetes (T1D), systemic lupus erythematosus (SLE), and multiple sclerosis (MS)—for which we could find sufficient evidence supporting our hypothesis, that is, role of psychological stress and defect of intestinal barrier functions.

This review is based on epidemiological and preclinical studies. Numerous excellent and recent reviews treating either ADs and stress, or stress and the intestinal barrier will be quoted to support this hypothesis.

## STRESS

Stress, firstly described in 1936 by Selye, is defined as a real (physical) or perceived (psychological) threat to homoeostasis, to which the organism has to react by an adaptive response (1).

Life time window, length, and frequency of exposure to stress play pivotal roles in their consequences on the individual pathophysiology. Indeed, acute and chronic stress exposure could occur in early life in a still maturating organism or at adulthood in mature organism. Traumatic experiences can lead to so-called post-traumatic stress disorder (PTSD), a condition in which the patient suffers from anxiety, depression, and flashbacks long after the traumatic experience (2). Persisting stress or inadequate response can lead to harmful maladaptive reactions depending on the kind of stress that will be discussed below. In this review, psychological stress is used as a general term that encompasses several psychological aspects (i.e., anxiety, depression, etc.).

The stress response is orchestrated by hypothalamic–pituitary–adrenal (HPA) axis and sympathetic nervous system (SNS). The neuroendocrine and autonomous responses are mediated by hormones such as epinephrine, norepinephrine, corticotropin-releasing hormone (CRH), adrenocorticotropic hormone (ACTH), glucocorticoids (cortisol in human and corticosterone in rodents) (3, 4). In the past, special attention has been paid to glucocorticoid in stress response and immune regulation. Endogenous glucocorticoids, part of the endocrine stress response, have ubiquitous functions in the development, metabolism, and inflammation. In general, glucocorticoids have been described to dampen immune response all along the inflammation process [for review, see (5)]: they attenuate signaling pathways of many pattern recognition receptors (6, 7), diminish leukocyte transmigration by reducing adhesion molecules (8), decrease the production of chemoattractants (9), program macrophages to anti-inflammatory M2c subtype (high expression of scavenger receptors and secretion of anti-inflammatory cytokines) (10), and decrease T cell response (11, 12), preferentially Th1 and Th17 by promoting Th2 and Treg (13, 14). Owing to their immunosuppressive effects, glucocorticoids have been used to treat various immune-related disorders like ADs.

The literature of the past 60 years has focused on immunosuppressive properties of glucocorticoids, but glucocorticoids can also enhance inflammation and immunity [for review, see (5)]. We will not go into the details of the diverging effects of glucocorticoid on immune response, but part of the explanation might reside in the diversity of glucocorticoid-receptors in different tissues, the presence or absence of 11βHSD, an enzyme inactivating cortisol, the time of glucocorticoid exposure (before or after tissue injury/inflammation) (15), and the dose (16). All those factors might explain that stressful events inducing glucocorticoids release could play a role in AD occurrence that can be treated by exogenous glucocorticoids. As an example, in humans, childhood maltreatment is associated with modified methylation of the glucocorticoid receptor gene NR3C1 in adults in brain and in leucocytes (17–19).

## ROLE OF STRESS IN AUTOIMMUNE DISORDERS (20)

The onset of at least 50% of autoimmune disorders has been attributed to unknown trigger factors. Many retrospective studies observed that most of patients suffering from AD report uncommon emotional stress before disease onset (21). This is obviously a vicious cycle as AD causes stress in patients (22, 23). This review will focus on three of the most studied ADs that will provide sufficient evidence to support the hypothesis of a role of stress-induced intestinal barrier defect on AD onset, that is, T1D, SLE, and MS. T1D is characterized by a defect of insulin production by pancreas owing to an autoimmune response against host pancreatic β-cells (24). SLE is an AD characterized by severe and persistent inflammation that leads to tissue damage in multiple organs (25, 26). MS is a chronic disease affecting the central nervous system and characterized by a defect of the blood–brain barrier and demyelination of the neurons of the central nervous system due to infiltration of auto-reactive T cells (27, 28). The most widely used preclinical MS model is experimental autoimmune encephalomyelitis (EAE).

A potential association between stressful events and T1D has been highlighted already a long time ago when Thomas Willis links, in 17th century, T1D onset to prolonged sorrow (29). Early life stress seems to be of particular risk for T1D development (30, 31). This is in accordance with literature highlighting neonatal maturation of pancreas as critical and vulnerable to stressors (32). Stress in adult has been described to increase incidence of SLE (33) and is able to exacerbate SLE symptoms (physical pain, sleep disturbances, and unemployment) (34). Around 70% of MS patients reported unusual amount of stress before the onset of the disease (35, 36).

Those epidemiological studies suggest that stress could be involved in both triggering and exacerbating ADs. Whether it is dependent on the kind of stress or ADs involved is unknown, and it would be interesting to conduct both retrospective epidemiological studies and preclinical studies to better document the role of stress in ADs. However, some interventional studies suggest that stress management could benefit to AD patients. Indeed, escitalopram (antidepressant) decreases the risk of MS relapsing in women (37). Diazepam (tranquilizer) decreases EAE incidence and histological signs

associated with this disease in a mouse model (38). A meta-analysis of 21 trials showed that in the 10 studies of children and adolescents with supportive or counseling therapy, cognitive behavioral therapy and family system therapy reduced glycosylated hemoglobin and as such improved diabetes control (39). Interestingly, in the 11 studies in adults, no beneficial effect of stress management could be observed on T1D (39).

# CONSEQUENCES OF STRESS ON INTESTINAL BARRIER AND SYSTEMIC IMMUNE RESPONSE

Stress can affect various physiological processes. Already Selye observed and others confirmed that the gastrointestinal tract and the immune system are particularly responsive to stress no matter the origin of the stress (1).

## Actors of Intestinal Barrier and Function

Intestinal epithelium is the mammalian organism's biggest surface in contact with the environment. Therefore, intestinal barrier functions are highly diverse and well developed. The intestinal barrier has to fulfill conflicting functions. Indeed, the intestinal barrier allows the transport of nutrient but at the same time filters and defends the organism from harmful luminal content (pathogens, toxins, etc.). Among the main actors of the intestinal barrier we can quote, intestinal microbiota, intestinal epithelium, and immune response (innate and adaptive). All those actors are in close relationship and regulate one another [for review, see (40)]. Intestinal microbiota not only participates in the protection against pathogens colonization but also contributes to maturation of intestinal epithelium and immune system and provides various nutritional compounds (41). The intestinal epithelium is formed by distinct cell types distributed along the crypt–villus axis. Although they all derive from a common stem cell progenitor located in the crypts, their morphology and roles differ [for review, see (42)]. The intestinal epithelium is renewed every 5 days, and this constant renewing confers high plasticity and protection to the intestinal barrier because defective cells are removed rapidly (43). Intestinal permeability is the ability of intestinal epithelium to allow the selective entrance of luminal antigens into the organism (44). Another actor of the intestinal barrier is the intestinal immune system. Gut-associated lymphoid tissue (GALT) represents the inductive site for B and T cells of mucosal intestinal barrier and includes the Peyer patches (PPs), the appendix, and isolated lymphoid follicles (ILFs). The humoral response in the intestines can be divided into four stages: predominant IgA induction in mucosal B cells, recirculation of IgA plasma blasts and homing into the intestinal mucosa, terminal B cell differentiation to plasma cells with local IgA production, and export of IgA through the intestinal epithelial layer [for review, see (45)]. Most intestinal T cells mature in peripheral lymphoid organs where they acquire the expression of intestinal homing receptors to migrate to the effector site of the intestines, that is, the mucosal epithelia and the *lamina propria*. Intestinal lymphocytes are continuously exposed to food and microbial antigens. These lymphocytes help to maintain the integrity of the intestinal barrier and immune homeostasis. Owing to their close location to luminal antigens, they have dual functions: regulatory functions (i.e., maintaining tolerance toward food antigens and commensal microbiota) and effector functions (i.e., prevention of pathogenic invasion) [for review, see (46)]. Innate lymphoid cells (ILCs) are lymphocytes that do not express the type of diversified antigen receptors expressed on T cells and B cells. ILCs are largely tissue-resident cells participating in tissue homeostasis [for review, see (47)]. A defective intestinal barrier will lead to inappropriate intestinal but also systemic immune response leading to gastrointestinal disorders and to extra-intestinal diseases like autoimmune diseases (48–50).

Intestinal barrier homeostasis is highly regulated, and a defect in microbiota composition could lead to intestinal hyperpermeability and intestinal inflammation. Intestinal inflammation not only will contribute to intestinal hyperpermeability (51) but also will favor microbiota colonization by pathobionts (52). Microbiota, intestinal permeability, and immune response mutually regulate one another making it difficult to define their respective role as cause or consequence in complex established pathologies.

## Psychological Stress Impairs Intestinal Barrier

Stress plays a role in the course of gastrointestinal disorders like irritable bowel syndrome (IBS) (53–55) and inflammatory bowel disease (IBD) (56). IBS is a very interesting model to study the consequences of stress on the intestinal barrier. Indeed, the occurrence of stressful events is considered as a contributing factor triggering and/or maintaining IBS (57, 58), suggesting that dysfunctional interactions in the brain–gut axis contribute to the pathophysiology of the disease (59) and as such justifying its new classification as a disorder of the brain–gut interaction (60). In this review, we will focus on the consequences of psychological stress on the intestinal barrier and its consequences on systemic immune response (**Figure 1**).

### Microbiota Dysbiosis

Stress is modifying microbiota in animal and human. Neonatal maternal separation induces microbiota dysbiosis in mice at different ages (61, 62). Limited nesting stress alters microbiota in rat pups (63). In adults, chronic water avoidance stress increases susceptibility to indomethacin-induced hyperpermeability in mice, and the effect is transferable *via* fecal microbiota transfer (64). Germ-free (GF) mice have exaggerated HPA stress response after restraint stress (65), showing the role of microbiota in the regulation of stress response. Mice exposed to social disruption stress have increased circulating IL-6 and MCP-1 levels; these effects were totally abolished by antibiotic treatment showing the importance of microbiota in the induction of stress effects (66).

In humans, decreased total abundance of Actinobacteria, Lentisphaerae, and Verrucomicrobia is associated with PTSD in South African individuals (67). Microbiota dysbiosis has been described in IBS patients [for review, see (68)].

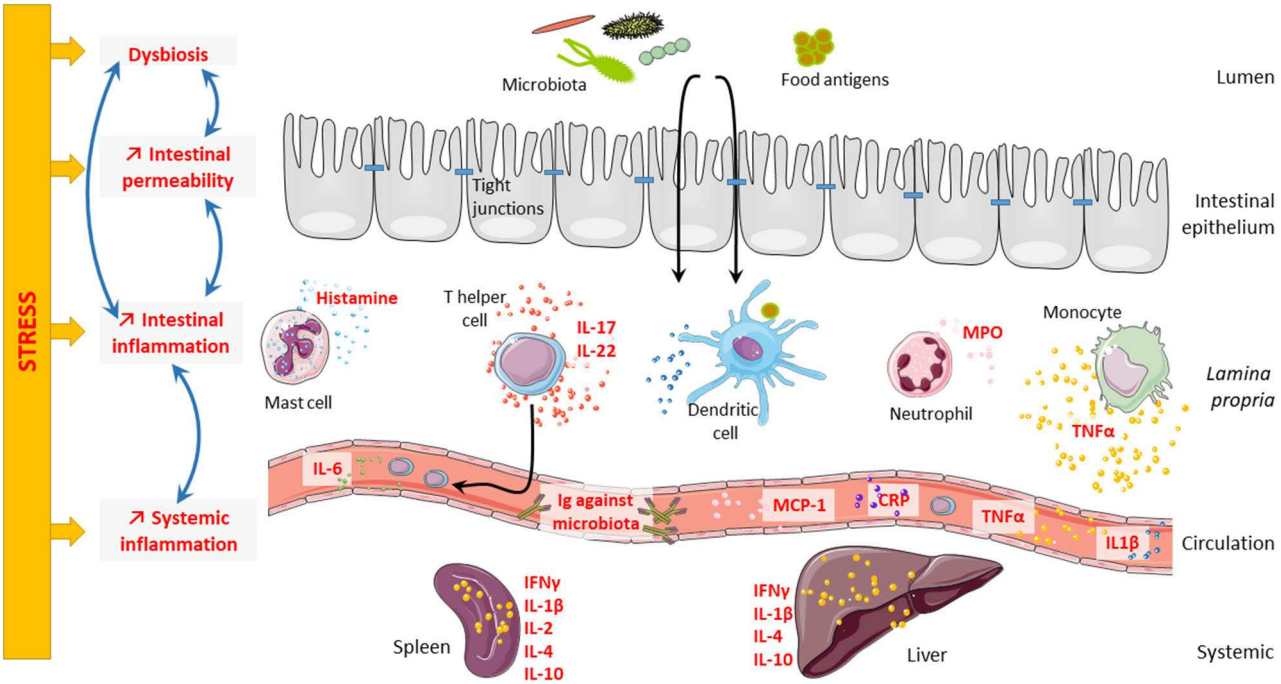


**FIGURE 1 |** Consequences of stress on intestinal barrier and systemic inflammation. Psychological stress can impair intestinal barrier at different levels. Indeed, stress can lead to microbiota dysbiosis, intestinal hyperpermeability, and intestinal inflammation. Interestingly, all these elements are highly connected and regulate one another. Microbiota dysbiosis can trigger intestinal hyperpermeability and intestinal inflammation; and in contrast, both intestinal hyperpermeability and intestinal inflammation can induce microbiota dysbiosis. Finally, stress can also induce systemic inflammation that might be related to intestinal inflammation.

## Stress Is Associated With Intestinal Hyperpermeability

In preclinical models and epidemiological studies, stress has been associated with an increase of intestinal permeability. Chronic water avoidance stress increases intestinal permeability and decreases tight junction protein expression in colon of adult rat (69) and overall intestinal permeability in mice (70). Chronic neonatal maternal separation, a model of early life stress, also increases intestinal permeability in rat (63, 71, 72) and mice (73). Maternal separation applied just for one time (acute stress) increases intestinal permeability in rats (74). Combination of different stressors [subacute (isolation, limited movement) and chronic crowding stress] also decreases tight junction mRNA expression in rats (75). In a mouse model of social disruption, a social stressor, bacterial RNA (*Lactobacillus* spp.), is increased in spleen, which indicates bacterial translocation (76).

In human, acute psychological stress like public speaking has also been shown to induce intestinal hyperpermeability (77). Intestinal hyperpermeability has also been described in IBS patients (78). The stress hormones cortisol (human) and corticosterone (mice) have been shown to mediate stress increased intestinal hyperpermeability as administration of the GR agonist dexamethasone mimics the intestinal hyperpermeability (69, 74).

## Stress Exacerbates Intestinal and Systemic Inflammation

Chronic neonatal maternal separation in rats increases cytokine expression, myeloperoxidase activity, and mast cell numbers in colonic tissue and exacerbate TNBS-induced colitis (71). Neonatal maternal separation in mice increases TNFα expression by intestinal tissue in young adult (61) and lipopolysaccharide (LPS)-stimulated TNFα secretion of isolated *lamina propria* immune cells in aging (62). Acute restraint stress augments histamine release by mast cells (79). Acute acoustic stress increases intestinal IL-17 and IL-22 expression in mice (80).

In human, stress aggravates IBD symptoms including higher release of pro-inflammatory effectors (56). In IBS, an increased state of activation of immune cells has been described even though this observation is under debate (81).

Not only the intestinal immune system is influenced by psychological stress, but there is also evidence for modified systemic immune response without direct proof that inflammatory immune cells were activated in the gastrointestinal tract. Neonatal maternal-deprived rats have increased cytokine expression in liver and spleen (71). Humoral immune response against microbiota is increased in neonatal maternal-deprived mice (62, 73). Social disruption stress in mice increases bacterial translocation and induces circulating IL-6 and MCP-1 (66, 76).

Stress is associated with an increase in pro-inflammatory response as described in PTSD patients (82). A meta-analysis of several studies showed that IL-6, TNFα, and IL-1β secretion are increased in response to acute stress in human (83). Childhood victimization is associated with elevated C-reactive protein (CRP) levels in young adult (84, 85).

## DEFECT OF INTESTINAL BARRIER IN AUTOIMMUNE DISORDERS (86)

We provided evidence that stress might play a role in onset or course of ADs, and we reviewed the well-documented deleterious role of stress in intestinal barrier functions. We will now summarize the data regarding the defect of the intestinal barrier in ADs. Indeed, the observed defect of the intestinal barrier in ADs is an interesting lead that largely contributes to the rise of the hypothesis, suggesting a contribution of stress-induced intestinal barrier defect in ADs (**Figure 2**).

### Microbiota in Autoimmune Disorders

Microbiota is known to contribute to intestinal mucosal permeability and induction of innate defenses and as such represent a risk factor for ADs (87, 88). A growing body of evidence suggests that intestinal microbiota can affect the incidence and/or severity of immune-mediated extra-intestinal diseases (89). Aberrant microbiota has been described in patients suffering from T1D (90), SLE (88), and MS (91). Knowledge regarding microbiota dysbiosis in AD patients and animal models will be summarized here, and interventional studies, which help to understand the role of microbiota in those diseases, will be discussed at the end of the paragraph.

Increased microbial diversity and low level of butyrate have been observed in feces of pediatric T1D patients (92, 93). In the BABYDIET cohort, early development of islet auto-antibodies is associated with alteration in the composition of mucin-degrading bacteria, that is, increase of Bacteroides and decrease of *Akkermansia* (94). A reduction of microbial diversity is more pronounced before the time of diabetes onset (95). Fecal transplantation of NOD diabetic microbiota in NOD-resistant mice induced insulitis, suggesting a diabetogenic gut microbial community (96, 97). Antibiotic treatment accelerates disease development (98, 99), suggesting a protective role of microbiota colonization in T1D.

Microbiota dysbiosis has been observed in relapsing–remitting MS patient compared with healthy control (100–103) with no consensus on the involvement of a particular bacterial species. In contrast, another study comparing 16S RNA profiles of feces from MS and healthy patients has not shown any differences (101). Demyelination initiates after colonization with feces of specific pathogen-free mice (104). Microbiota depletion by non-absorbable antibiotics delays the development of EAE by reducing the number of mesenteric Th17 cells (105). GF mice present attenuated symptoms in both spontaneous and induced EAE models (104, 106), suggesting a deleterious role of microbiota colonization in MS. Furthermore, microbiota shapes and predicts the course (chronic-progressive or relapsing–remitting) of EAE in a mouse model (107).

Only a few studies on human SLE microbiome in small cohorts report microbial dysbiosis (108–110), but they are confirmed by preclinical studies in mouse models (110). A study performed in a larger and diversified cohort of SLE patient showed that the severity of disease is associated with more severe microbiota dysbiosis (111).

#### Interventional Studies: What Do They Tell Us?

Here, we will focus on direct supplementation by living bacteria like probiotic and fecal microbiota transplantation (FMT) treatment but not on indirect interventions like prebiotics or nutritional compounds produced by bacteria, as short chain fatty acids, for example, which may involve indirect effects. Once ADs are diagnosed, the production of antibody against self-antigen will remain and will still damage tissues, but this process could be delayed or reduced. Probiotics are living microorganisms that confer a health benefit to the host (112). Probiotics are known to have beneficial effects on the intestinal barrier (113, 114) and anti-inflammatory properties (115–119) and as such represent an interesting tool to delay, reduce, or even prevent ADs.

Animal studies suggest beneficial effects of probiotics supplementation on EAE *via* a stimulation of IL-10 production (106, 120–124). Clinical studies showed that a mixture of probiotics improves expanded disability status score and decreased inflammatory markers (125). Regarding T1D, probiotic treatment delays the onset of T1D in an experimental rat model and improves the intestinal barrier (126). Probiotics also protect NOD mice from T1D by reducing intestinal inflammation (127). In humans, it has been demonstrated in the TEDDY (The Environmental Determinants of Diabetes in the Young) cohort that early probiotic supplementation is associated with a decreased risk of islet autoimmunity as compared with late or missing supplementation (128). In animal models for lupus nephritis, probiotic administration lowers inflammatory response in the kidney and intestines in female and castrated males but not in non-castrated males (129).

FMT with microbiota from different diabetes resistant mouse strains delays the onset of T1D in NOD diabetes-prone mice (97). Few studies investigate to role of FMT on AD symptoms, and most of the time the recommendation for FMT treatment was to target associated gastrointestinal troubles. Neurological symptoms are improved and MS progression is paused in three MS patients who underwent FMT treatment for chronic constipation (130). Unfortunately, no data are available on intestinal barrier functions of probiotics and FMT treatments in parallel to beneficial effects on ADs.

#### Mimicking

Antigens from infectious agents and myelin proteins can share structural similarities called molecular mimicry. This molecular mimicry can be responsible for activation of naïve autoreactive T cells recognizing peptides from infectious agents but also from self-antigens myelin proteins. Cross-reactivity could occur when

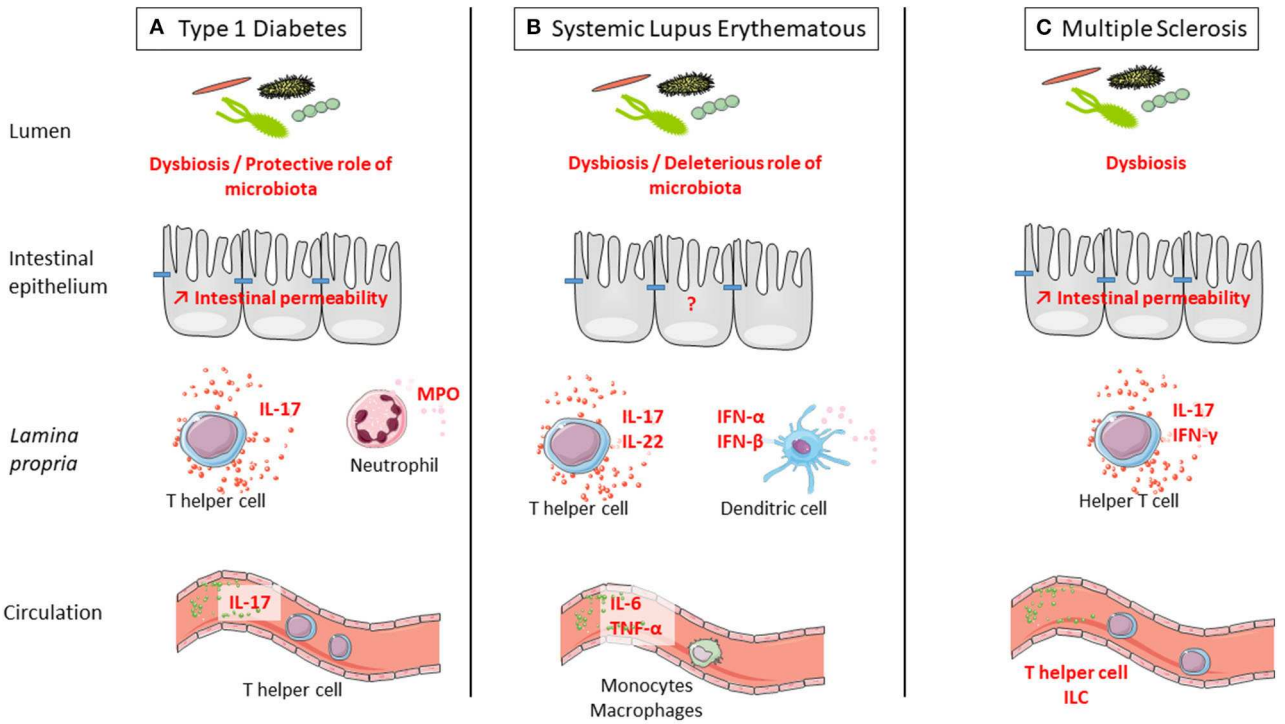


**FIGURE 2 |** Defect of intestinal barrier and systemic immune response is observed in three examples of autoimmune disorders (ADs): type 1 diabetes (T1D), systemic lupus erythematosus (SLE), and multiple sclerosis (MS). **(A)** T1D is associated with microbiota dysbiosis, intestinal hyperpermeability, increased IL-17 secretion in the intestine and at systemic level, and increased myeloperoxidase (MPO) in the intestine. Interestingly, colonization by a complex microbiota is protective from type 1 diabetes. **(B)** SLE is associated with microbiota dysbiosis and increased secretion at the intestinal level of IL-17 and IL-22 by T cells and IFN-α and IFN-β by dendritic cells. At the systemic level, there is a higher secretion of IL-6 and TNF-α by monocytes and macrophages in SLE. Interestingly, colonization by a complex microbiota is deleterious for SLE onset. **(C)** MS is associated with microbiota dysbiosis, intestinal hyperpermeability, and increased secretion at the intestinal level of IL-17 and IFN-γ by T cells. At the systemic level, the number of innate lymphoid cell (ILC) population was observed in MS. Interestingly, colonization by a complex microbiota is deleterious for MS onset.

important motifs are conserved and overall structures of TCR–peptide–MHC interaction are similar, suggesting that cross-reactivity may happen frequently (131). Myelin basic protein, the immunodominant autoantigen of MS, cross react with Epstein–Barr virus (EBV), influenza A virus, herpes simplex virus, human papilloma virus (132), or human herpesvirus-6 (133). Regarding EBV, MS patients seem to have increased antibody titers against certain antigens of the virus than have control subjects even before the onset of MS (134). Despite cross-reactivity, infectious agents can impair self-antigen tolerance by indirect activation (135). It has been showed that an integrase expressed by intestinal Bacteroides encodes a low-avidity mimotope of the pancreatic β-cell autoantigens and as such might participate to T1D onset. Colonization of GF mice with Bacteroides promotes the recruitment of diabetogenic $CD8^+$ T cells to the gut (136).

## Intestinal Hyperpermeability in Autoimmune Disorders

In ADs, intestinal hyperpermeability has been described, resulting in an increased entry of luminal antigens derived from food and/or intestinal microbiota or pathogens. The associated inflammation has been suggested to participate in AD onset and/or exacerbation.

Even though it is still unclear whether intestinal hyperpermeability is a trigger or a consequence of T1D progression (93, 137, 138), epidemiological and preclinical studies demonstrated that intestinal hyperpermeability occurs before disease onset (139, 140). Reversion of intestinal hyperpermeability by treatment with a zonulin 1 (intestinal homolog of a *Vibrio cholerae* enterotoxin, which reversibly increases intestinal permeability) inhibitor ameliorates T1D manifestation in rat model (141). Microbial translocation in pancreatic lymph nodes activates NOD2, and IL-17 production in pancreatic lymph nodes and pancreas which contributes to T1D development (142).

Intestinal hyperpermeability precedes EAE onset and increases while disease progresses (143). In this model, increased intestinal permeability is associated with the increase of crypt depth and mucosa thickness in jejunum and ileum, as well as with an overexpression of zonulin 1 (143) as observed for T1D (141, 144).

Intestinal barrier defect and subsequent exposure to microbial products play an important role in the pathology of SLE

(145, 146). sCD14, lysozyme, and CXCL16 are markers of antimicrobial response found increased in SLE subject attesting to a defect of the intestinal barrier (147).

### Intestinal Inflammation

T1D is associated with increased intestinal myeloperoxidase activity and goblet cell (producing mucus) density, supporting the idea that early intestinal inflammation might lead to intestinal hyperpermeability (148, 149). Many studies suggest that the increased number of Th17 cells is involved in the pathogenesis of autoimmune diabetes. Higher numbers of IL-17 secreting cells are detected in recent-onset T1D-promoting inflammatory response to β-cells (150, 151). Th17 is increased in the peripheral blood of children with T1D (151, 152). *In vitro* IL-17 potentiates inflammatory and proapoptotic responses on human islets cells (151). Anti-IL-17 treatment reduces islet T cell infiltrates and GAD65 autoantibodies in NOD mice (153). Neutrophil extracellular traps (NETs) might contribute to the generation of ADs by exposing autoantigen (154). The role of NET has been studied particularly in T1D. Indeed, degradation of NETs in the gut prevents immune infiltration of pancreatic islet preserving β-cell mass and systemic inflammation (155).

In a mouse model of SLE developing severe nephritis, α4β7 expressing T cells is increased in PPs and pro-inflammatory cytokines (IL-17, IL-22, IFNα, and β) are much more expressed in distal ileum (156). Furthermore, intestinal monocytes/macrophages of SLE patients have an altered expression of type 1 interferon-stimulated genes, HLA-DR, and Fcγ receptors (157, 158). Monocytes isolated from plasma of SLE patients release higher pro-inflammatory cytokines in response to LPS than do healthy patients (159). More generally, higher production of pro-inflammatory cytokines by monocytes/macrophages has been described in SLE patients [for review, see (160)].

In MS, elevated Th1 and Th17 pro-inflammatory responses are observed in *lamina propria*, PPs, and mesenteric lymph nodes (143). GF EAE animals produce lower levels of IFNγ and IL-17 in the intestines associated with a higher number of Treg cells (104). Monocolonization of GF animals with segmented filamentous bacteria, IL-17 inducer in gut (161, 162), induces EAE and shows that microbiota can affect neurologic inflammation by recirculation of Th17 to the brain, causing inflammation (106). Autoreactive T cells from gut could migrate in different organs depending on pathologies, to brain in the case of MS, to liver in the case of autoimmune cholestatic liver disease (163, 164), or to the kidney in the case of SLE (165). Interestingly, not only T cells seem to be involved in MS but also circulating ILC. Indeed, a higher number of ILC have been observed in MS patients (166).

## CONCLUSION

As a conclusion, compiling evidences highlight the importance of both intestinal barrier defect and stress in ADs. Stress is well known to have long-lasting deleterious consequences on the intestinal barrier. A transversal research on ADs, stress, and intestinal barrier function would be of great interest and would bring new understanding in the pathophysiology of ADs. Identifying stress-induced intestinal barrier dysfunction as an actor of ADs could bring new possibilities for therapeutic targets and especially preventing strategies toward the spreading epidemic of ADs. Therapeutic strategies suggest that probiotics and FMT treatment might improve AD symptom, but preventive strategies in an at-risk population still need to be explored. In this review, we did not mention autoimmune thyroid diseases (AITDs) that are the most frequent ADs (167). It is difficult to study AITDs by themselves, as they are often observed together with other ADs, which are named polyautoimmunity (168). Then, even though there are sufficient data supporting the role of stress in AITD onset (20), evidences for a defect of intestinal barrier functions in AITD are sparse, and only two studies are available regarding microbiota dysbiosis (169, 170). For those reasons, we did not use AITD to illustrate the hypothesis of this review, supporting a role of stress-induced intestinal barrier disruption in the onset and/or the course of ADs. However, we wanted to mention the case of AITD as data on intestinal barrier function would be of great interest in the future.

## AUTHOR CONTRIBUTIONS

HI-D and SM reviewed the literature, wrote and corrected the manuscript, and drew the figures. All authors contributed to the article and approved the submitted version.

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