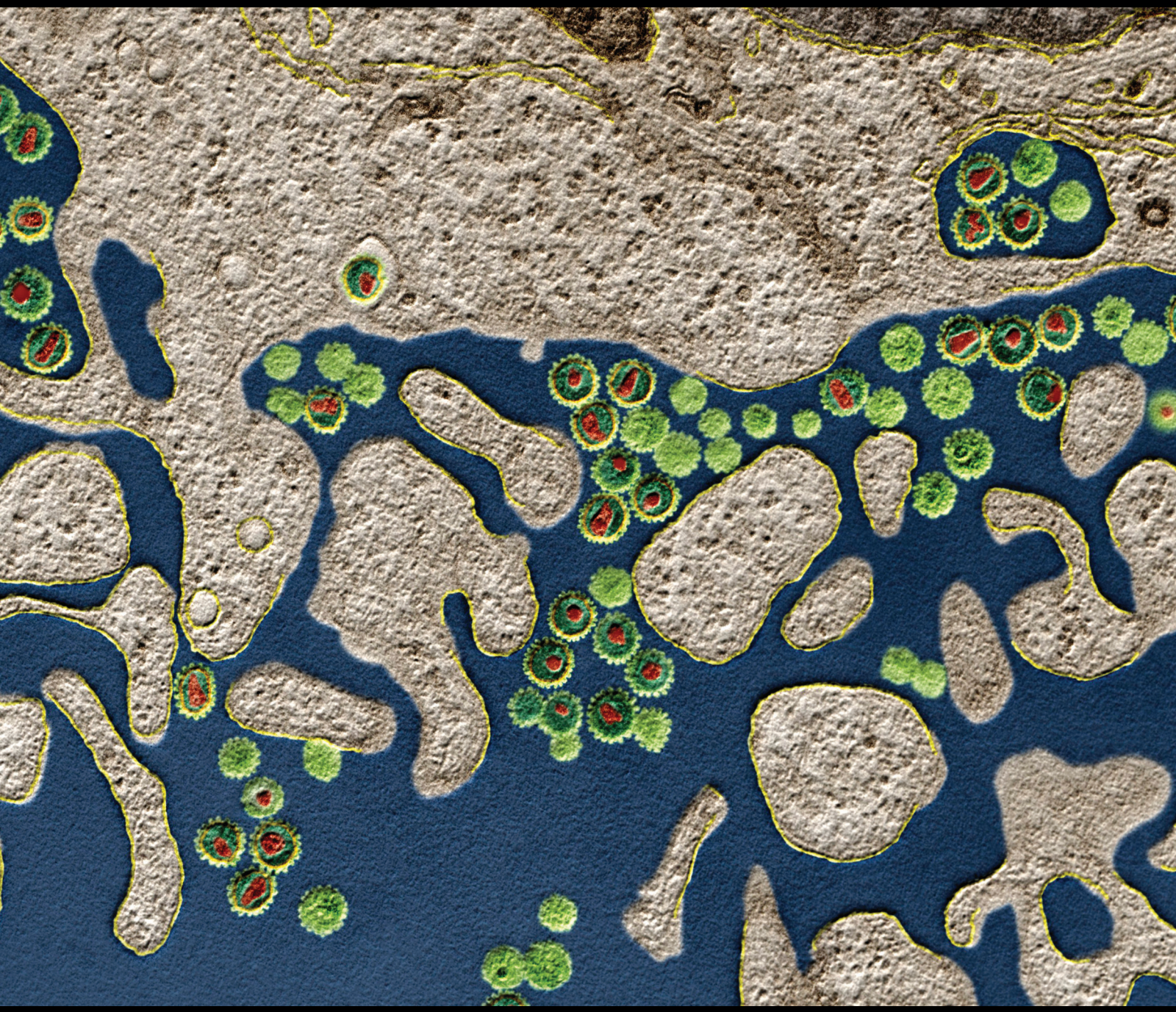
Journal of Immunology Research

Intestinal Microbiota as Modulators of the Immune System

Guest Editors: Borja Sánchez, Miguel Gueimonde, Amado Salvador Peña, and David Bernardo



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Editorial Board

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Editorial

Intestinal Microbiota as Modulators of the Immune System

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The gastrointestinal immune system is exposed to a large amount of different products mainly innocuous (derived from "friendly" bacteria and/or food antigens) but sometimes also dangerous and infectious (as invading bacteria or viruses). Despite that, it is effective in discriminating between them and hence maintaining immune tolerance against the natural inhabitants of our gut, but initiating immune responses against the harmful invading microorganisms [1, 2]. In the last decades, the western lifestyle has seen an increase in the prevalence of immunoregulatory disorders which has been linked to changes in the microbiota composition due to the increased use of antibiotics and the absence of intestinal parasites as proposed in the "hygiene hypothesis." Indeed, the immune system has become more dependent upon the microbiota and the natural environment [3]. However, recent data indicate that helminth-induced immunomodulation occurs independently of changes in the microbiota [4].

The commensal microbiota plays a central role modulating the outcome of immune responses in the gastrointestinal tract keeping immune homeostasis in health [5]. Indeed, germ-free animals have an immature immune system and can develop inflammation which is reverted once the microbiota is conventionalized [6]. The commensal microbiota also has the capacity to modulate several aspects of the host including its physiology and/or nutritional status contributing therefore to several diseases affecting not only the gut but also distant organs [7–9]. Therefore, not surprisingly the gut microbiota modulation (via pre/probiotics or through faecal microbiota transplants) appears a very promising area of research aiming to modulate the outcome of the immune system looking for an impact in the clinics. In this context, several clinical trials are underway to assess the true efficacy of faecal microbiota transplantations [10] as well as their long term effects [11, 12]. In agreement with that, there are still many factors regarding the host/microbiota cross talk which remain obscure and that have to be addressed to further our understanding about how the microbiota can modulate the outcome of the immune responses in the host.

In this special issue, we have therefore aimed to gain depth into the current understanding of immune processes in the human gastrointestinal tract in health and disease by selecting work in progress of active investigators in the field.

The study by C. M. C. Maranduba et al. reviews the most recent advances on intestinal microbiota and its role in the maintenance of the host homeostasis. The review describes, in a detailed manner, the interaction of the microbiota with the intestinal immune system and the mechanisms involved in such interaction. The authors also discuss the latest results on a current hot topic in microbiota research: the interaction with the nervous system and the impact upon the gut-brain axis [13]. This research area is attracting increasing attention for elucidating the impact of the microbiota beyond the classically studied intestinal interactions, which promises to

expand our understanding on the role of the microbiota on human biology in broad terms.

In a similar context, C. Ferreira et al. discuss the impact of microbes on the gut-associated immune system function and, moreover, on the onset and development of inflammatory disorders. An exhaustive revision of metagenomic and animal data in the framework of different diseases, mainly inflammatory bowel disease, asthma, and obesity, showed deep alteration on the gut-associated microbiota profiles, as well as deficiencies in the immune response. Alterations in the intestinal microbiota composition promote systemic inflammation that is a hallmark of obesity and subsequent insulin resistance [14].

Pre- and probiotics have been extensively used for improving the balance of the intestinal microbiota and immune response modulation [15, 16]. A human target group that may benefit very much from strategies aimed at the modulation of the gut microbiota and the stimulation of the immune system is that of premature newborns. It is known that in these infants both the microbiota establishment process and the immunity are altered. In this issue L. Moles et al. report the results of a pilot study on the effects of the administration of two probiotic strains, isolated from human milk, to preterm infants. The authors evaluated several microbiological and immunological markers observing the ability of the strains to modulate the microbiota and to survive the gastrointestinal passage. Moreover, a reduction of fecal calprotectin, an inflammatory marker, was observed throughout the probiotic treatment in agreement with previous observations [17, 18].

Continuing with the relevance of probiotics, P. Carasi et al. administered the strain *Lactobacillus kefiri* CIDCA 8348 to healthy mice during 21 days. This strain was chosen in a previous study for its ability to induce chemokine CCL20 gene expression, an attractant of immune cells. The overall impact of *L. kefiri* on the mouse gut-associated immune system varied from an increase in fecal IgA to the reduction of several proinflammatory mediators in Peyer patches. Overexpression of interleukin 10 and mucin 6 genes in the ileum and the ability of *L. kefiri* to reduce the proinflammatory effects of lipopolysaccharide make strain CIDCA 8348 a candidate to be included in functional foods targeting inflammatory bowel disorders.

We have also selected manuscripts which discuss the role of the microbiota not only in immune homeostasis, but also in different diseases like HIV and *Helicobacter pylori* infection. The gastrointestinal tract has been recently described as the main HIV reservoir in the human body. While in healthy controls there is a reciprocal cross talk between the commensal microbiota and the host, HIV infection can dramatically affect both the microbiome and the host's immune system adding therefore a third factor to the dialogue in these patients. In this special issue, K. Vyboh et al. have reviewed the impact of HIV infection in the gastrointestinal immune system and how that can lead to changes not only in the microbiota composition and function, but also in the mucosal permeability resulting in microbial translocation from the lumen. As a consequence, viral and microbial factors work together in the patients creating a positive feedback mechanism which enhances HIV progression leading to a vicious cycle of immune activation [19, 20].

Helicobacter pylori infection is one of the most common causes of chronic gastritis. In this issue, L. A. Cherdantseva et al. performed a histological examination of the gastric mucosa during development of chronic gastritis in these patients. Their findings confirmed that *H. pylori* infection causes an increase in the number of infiltrating immune cells, including macrophages and lymphocytes which also had an enhanced capacity to secrete proinflammatory nitric oxide synthase which may allow an accumulation of free radicals in the tissues leading to an aggravation of the inflammatory process with impaired regeneration processes.

Moving towards more immunologically related studies, the role of intestinal dendritic cells (DCs) in the gastrointestinal compartment cannot be avoided as they are specialized antigen-presenting cells with the ability to extend their dendrites between epithelial cells and directly sample bacteria from the intestinal content [21, 22]. In a former study, M. Wiese et al. [23] selected *Lactobacillus* strains active against *H. pylori*. In their new research, the authors used monocyte-derived DCs for assessing the immunomodulatory abilities of those previously selected strains in the presence or absence of *Helicobacter pylori*. Both lactobacilli species were able to increase the maturation of DCs and to induce the production of IL23. However, the strains differed in their ability to induce IL-10 leading to different IL-10/IL-12p70 ratios. Altogether, the results presented suggest that the *H. pylori*-induced DCs tolerogenic phenotype may be overcome by the presence of certain lactobacilli.

Regulatory T-cells (Tregs) are essential to maintain immune homeostasis as they are critical for prevention of spontaneous inflammation. While development of Tregs requires the presence of TGFβ at the time of the antigen presentation elicited by DCs, the presence of IL-6 promotes T-cell differentiation towards a Th17 proinflammatory profile as seen in several autoimmune diseases including rheumatoid arthritis (RA). In their review, R. Rogier et al. discuss the mechanisms by which the intestinal microbiota can influence the Th balance in the lamina propria. To that end, the authors have reviewed the background information about RA being a Th17 disease, how the intestinal microbiota can modulate the outcome of immune responses, and the evidence linking, in both in vivo and animal models, the commensal microbiota with RA development likely via TLR recognition by the host.

Continuing with Tregs, information regarding their development in the neonatal liver is scarce. In their study, A. Maria et al. describe how Treg can be already found on the third day after birth in the murine thymus, spleen, and liver. However, by the first week of life the frequency of liver Treg cells exceeds that of the spleen by 1.5–2-fold in a transient manner since 6 weeks after birth frequency of liver Tregs was reduced. Given that conventionalization of germ-free animals usually leads to a rapid expansion and mucosal Tregs, and considering that the liver receives most of its blood flow via the intestinal portal vein, the authors hypothesized and proved that the transient increased in neonatal liver Tregs was controlled by the intestinal microbiota as differences between frequency of liver and spleen Treg were abrogated

in MyD88 knockout animals. This study expands our current knowledge on how the intestinal microbiota can also modulate the immune properties of tissues where they do not get direct access and also about the mechanisms of liver tolerance development.

As stated before, the intestinal immune system and the beneficial microorganisms within the lumen of the intestinal host communicate extensively to eliminate pathogens and markers to activate the innate and acquired immune response are necessary. In this issue, K. Radulovic and J. H. Niess review the role of CD69 which is highly expressed in intestinal T-cells. They propose that not only is this molecule just an activation marker but also it is essential in the regulation of intestinal inflammation. They review the evidence about how microbial-derived factors recognized by pattern recognition receptors could contribute to the CD69 expression on the surface of colonic T-cells and may be involved in lymphocyte migration in particular in inflammatory bowel disease. Although the authors are fully aware that most of the data come from mice research, they propose that since the intestinal microflora also regulates this marker in intestinal inflammation it may be a good target molecule for the treatment of inflammatory bowel disease.

Finally, an excellent review by M. J. B. Silva et al. covering many of the aspects described earlier in this editorial and the mechanisms involved in the modulation of host-microbe interactions has also been selected. It summarizes the possible effects of the breakdown of the homeostatic association that can lead to intestinal inflammation and pathology.

It has been a pleasure to select the work presented in these areas by experts in the respective fields. We hope that their findings will help to enrich the knowledge of the mediators of inflammation of the human gastrointestinal tract and will form the basis for new approaches to the treatment of common infections and those conditions that although rare have such a bad prognosis.

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Research Article

Impact of Kefir Derived *Lactobacillus kefiri* on the Mucosal Immune Response and Gut Microbiota

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The evaluation of the impact of probiotics on host health could help to understand how they can be used in the prevention of diseases. On the basis of our previous studies and *in vitro* assays on PBMC and Caco-2 ccl20:luc reporter system presented in this work, the strain *Lactobacillus kefiri* CIDCA 8348 was selected and administrated to healthy Swiss mice daily for 21 days. The probiotic treatment increased IgA in feces and reduced expression of proinflammatory mediators in Peyer Patches and mesenteric lymph nodes, where it also increased IL-10. In ileum IL-10, CXCL-1 and mucin 6 genes were upregulated; meanwhile in colon mucin 4 was induced whereas IFN-γ, GM-CSF, and IL-1β genes were downregulated. Moreover, ileum and colon explants showed the anti-inflammatory effect of *L. kefiri* since the LPS-induced increment of IL-6 and GM-CSF levels in control mice was significantly attenuated in *L. kefiri* treated mice. Regarding fecal microbiota, DGGE profiles allowed differentiation of experimental groups in two separated clusters. Quantitative PCR analysis of different bacterial groups revealed only significant changes in *Lactobacillus* population. In conclusion, *L. kefiri* is a good candidate to be used in gut inflammatory disorders.

1. Introduction

Interactions between commensal bacteria, intestinal epithelial and immune cells play a crucial role in the maintenance of gut homeostasis [1, 2]. Microbial recognition through pattern-recognition receptors induces the expression and release of many different immune mediators, such as chemokines and pro- or anti-inflammatory cytokines which contribute to orchestrating both the innate and the adaptive immune response [3, 4]. The use of probiotics to modulate immune responses at mucosal and systemic level constitutes a very interesting alternative regarding the prevention and treatment of infectious diseases [5, 6] and different immunopathologies such as inflammatory bowel diseases and allergies [7–9] or metabolic disorders [10, 11].

Kefir grains are constituted by a complex symbiotic microbiota, and they are used to obtain fermented milks named "kefir" [12]. Several health-promoting properties such as immunological, antimicrobial, antitumoral, and hypocholesterolemic effects have been associated with kefir-consumption [13–17] and the study of the beneficial properties attributed to kefir-isolated microorganisms constitutes a field of great interest for the development of functional foods.

Immunomodulatory properties have been reported for different yeasts and bacteria isolated from kefir grains. Among kefir yeasts, *Kluyveromyces marxianus* CIDCA 8154

and *Saccharomyces cerevisiae* CIDCA 8112 downregulate intestinal epithelial innate response through a mechanism dependent on NF-kB modulation [18]. In the case of lactic acid bacteria retrieved from kefir, *L. kefiranofaciens* has been proven to ameliorate colitis in a DSS-induced murine model [19] and to produce antiasthmatic effects on ovalbumin-allergic asthma mice [20]. On the other hand, Carey and Kostrzynska [21] showed that *L. kefiri* attenuates the proinflammatory response in intestinal epithelial cells induced by *Salmonella* Typhimurium and Hong et al. [22] showed its influence on Th1 and proinflammatory cytokines production on macrophages.

One of the most important lactobacilli retrieved from kefir is *Lactobacillus kefiri* [23–26]. In previous studies, our workgroup has demonstrated that secretion products and surface proteins from *L. kefiri* exert a protective action against the invasion of *Salmonella enterica* serovar Enteritidis to Caco-2 cells [27] and also against the cytotoxic effects of clostridial toxins on Vero cells [28]. Moreover, *L. kefiri* strains have been proven to be safe [29] and to adhere to gastrointestinal mucus [30]. On the other hand, *L. kefiri* strains preserve a high percentage of viability after both spray-drying [31, 32] and freeze-drying procedures [33]. All the mentioned properties show the potentiality of *L. kefiri* as probiotic microorganism.

The study of the mechanisms underlying probiotic effect on the host on nonpathological conditions may be helpful for evaluating safety and further application of beneficial microorganisms in the prevention and treatment of different diseases. Taking into account the potentiality of *L. kefiri* as a novel probiotic, we propose to evaluate the immunomodulatory properties of kefir-isolated *L. kefiri* strains by *in vitro* and *in vivo* assays, along with changes in gut microbiota composition induced by *L. kefiri* administration.

2. Materials and Methods

2.1. Bacterial Strains and Growth Conditions. *Lactobacillus kefiri* CIDCA 83111, 83113, 83115, 8321, 8325, 8345, and 8348 were isolated from kefir grains [12]. *L. kefiri* JCM 5818 was obtained from the Japanese Collection of Microorganisms (Reiken, Japan). Previously, *L. kefiri* CIDCA 83115, 8321, 8345, and 8348 were characterized as aggregating strains; meanwhile *L. kefiri* CIDCA 83111, 83113, and JCM 5818 were described as nonaggregative strains [34]. Lactobacilli were cultured in MRS-broth (DIFCO, Detroit, USA) 37°C for 48 h in aerobic conditions. Frozen stock cultures were stored at −80°C in skim milk until use.

2.2. Stimulation Assay with Caco-2 ccl20:luc Reporter System. The experiments were performed as described previously [35]. Briefly, Caco-2 cells stably transfected with a luciferase reporter construction under the control of CCL20 promoter (Caco-2 ccl20:luc) [36] were cocultured 2 h with a suspension of the *L. kefiri* strains (10^7 CFU per well) to be tested (multiplicity of incubation = 100). Then, cells were stimulated using flagellin from *Salmonella enterica* ser. Typhimurium (FliC) ($1\,\mu g\,mL^{-1}$) for 6 h. Luciferase activity was measured in a Labsystems Luminoskan TL Plus luminometer (Thermo Scientific, USA) using a luciferase assay system (Promega, Madison, WI, USA). Luminescence was normalized and expressed as the percentage of the mean of stimulated control (NAL).

2.3. PBMC Stimulation Experiments. Peripheral blood samples pretested for the absence of HIV or hepatitis virus infections were obtained from healthy volunteers (EFS Aquitaine, Bordeaux Blood Bank). Human PBMCs were isolated by centrifugation on Ficoll-Hypaque gradients. After washing, 2×10^6 cells/well were cultured in 12-well plates in RPMI-1640 medium supplemented with $2\,g\,L^{-1}$ $NaHCO_3$, $300\,mg\,L^{-1}$ L-glutamine, $100\,\mu g\,mL^{-1}$ streptomycin, $100\,IU\,mL^{-1}$ penicillin (Sigma Chemical Co., St. Louis, MO, USA) and 10% FBS.

L. kefiri stimulation experiments on PBMC were performed coculturing 2×10^7 bacteria per well (MOI = 10) during 24 h at 37°C in an atmosphere of 95% air and 5% CO_2. Culture supernatants were collected and kept at −80°C until cytokines analysis. Experiences were realized in triplicate. Cell viability was not affected after 24 h of coincubation with bacteria (data not shown).

2.4. Quantification of Cytokine Levels in Culture Supernatants. Profiles of cytokines were analyzed after *L. kefiri* strain stimulation of PBMC using the Human Th1/Th2 11plex FlowCytomix Kit (eBioscience). It was designed to measure human IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 p70, TNF-α, and TNF-β. Analysis was performed in a flow cytometer BD Accuri C6 (BD Biosciences). TGF-β was measured using the eBioscience human/mouse TGF beta 1 Ready-SET-Go! ELISA Kit (minimum detectable concentration 8.0 pg/mL).

2.5. Mice. Male Swiss albino mice, 4-week-old (Janvier, Le Genest St Isle, France), were quarantined 2 weeks after arrival and were housed under standard laboratory conditions with free access to food and water. The temperature was kept at 22°C and a 12-hour light/dark schedule was maintained. All procedures were performed according to the guidelines of the local ethics committee and in strict accordance with the guidelines issued by the European Economic Community "86/609." Mice were randomly divided into two groups (n = 12/group) and received by gavage 10^8 CFU of *L. kefiri* CIDCA 8348 (Lk group) or PBS (control group) daily for 7 days and 21 days; at each time point 6 mice of each group were sacrificed.

2.6. Tissue and Stool Sampling. Stools were collected at days 7, 14, and 21 and stored at −80°C until analysis. At the end of the experimental protocol, day 7 or 21, ileum and colon samples were collected and were preserved at −20°C in RNAlater (QIAGEN, Hilden, Germany) until RNA extraction. On day 21 Peyer Patches (PP) and mesenteric lymph nodes (mLN) were also removed and preserved at −20°C in RNAlater for expression analysis, and ileum and colon explants were collected in RPMI medium and processed immediately in order to analyze cytokines' secretion.

2.7. Quantification of Gene Expression in Tissue Samples by qRT-PCR

2.7.1. RNA Extraction. Total RNA was isolated using the RNeasy Mini Kit (QIAGEN, Hilden, Germany) with an additional DNase treatment (Turbo DNA-free, Ambion, Inc.) according to the manufacturer's instructions.

2.7.2. cDNA Synthesis. One μg of total RNA was reverse-transcribed using the Maxima Reverse Transcriptase (Fermentas, France) with anchored-oligo (dT) 18 primer, according to manufacturers' instructions.

2.7.3. Quantitative PCR. Quantitative real-time PCR analyses were performed using a CHROMO 4 System (Bio-Rad). The reaction mixture comprised Maxima SYBR Green/ROX qPCR Master Mix (Fermentas, France), 0.5 μmol L^{-1} of each primer, and the respective standardized cDNA as a template. Target gene copy numbers were normalized against the housekeeping genes hypoxanthine phosphoribosyltransferase (HPRT) and β2 microglobulin (B2m). Cytokine and chemokine genes evaluated were *il1b, il6, il10, il12p70, il17a, il23, ifng, tnfa, tgfb, cxcl1, baff, april, gmcsf*; the transcription factors studied were *foxp3* and *rorgt;* epithelial barrier and IgA related genes were *zo-1, occludin,* and *pIgR*; mucin genes were *muc1, muc2, muc3, muc4, muc6,* and *muc13*. Primer sequences and PCR conditions are available upon request (E-mail: maria.urdaci@agro-bordeaux.fr). A negative control reaction without template was included for each primer combination.

2.8. Evaluation of Cytokine Secretion by Ileum and Colon Explants. Ileum and colon explants were cultured in RPMI medium supplemented with 10% fetal bovine serum (Gibco-Invitrogen, Carlsbad, CA, USA), 100 μg mL^{-1} streptomycin and 100 IU mL^{-1} penicillin G, 100 μg mL^{-1} gentamycin or RPMI complete medium with addition of 10 μg mL^{-1} of LPS from *E. coli* as a stimulus (all from Sigma Chemical Co., St. Louis, MO, USA) for 24 h at 37°C in an atmosphere of 95% air and 5% CO_2 [37]. Supernatants were collected, centrifuged, and frozen for later cytokines (IL-6, IL-4, IL-10, IL-17A, IFN-γ, and GM-CSF) measurements (Ready-SET-Go! ELISA Kit, eBioscience, France). All assays were performed according to the manufacturer's instructions. The minimum detectable concentrations were 4.0 pg mL^{-1} (IL-6, IL-4, and GM-CFS), 15 pg mL^{-1} (IFN-γ), and 30.0 pg mL (IL-10 and IL-17A).

2.9. Determination of Total IgA in Stools. At 7, 14, and 21 days after *L. kefiri* treatment the level of total IgA in stools was measured by ELISA according to the technique described by BD Pharmigen. Briefly, Maxisorp Nunc plates were coated overnight with purified rat anti-mouse IgA (BD 556969). The plates were washed with PBS containing 0.05% v/v Tween 20 (PBS-T) and blocked with FBS 10% v/v in PBS. Plates were incubated for 2 h at room temperature with purified mouse IgA kappa (BD 553476) or fecal samples. Plates were revealed using biotin rat anti-mouse IgA (BD 556978), streptavidin horseradish peroxidase (BD 554066), and trimethylbenzidine (TMB substrate reagent set BD OptEIA 555214). Using a Mutliscan FC microplate reader (Thermo Scientific) absorbance was read at 450 nm. All determinations were performed in triplicate.

2.10. Microbiota Population Analysis in Feces by q-PCR. Microbiota population analysis in feces was performed on the day 21 of the experience. DNA extraction was performed using the NucleoSpin Soil Genomic DNA isolation kit (Macherey-Nagel) according to the manufacturer's instructions except the feces solubilisation step. Quantification of bacterial populations was carried out using primers synthesized by Biomers (France). PCR reactions were performed on a CHROMO 4 System (Bio-Rad) using Maxima SYBR Green/ROX qPCR Master Mix (Fermentas, France). Twenty ng DNA and 0.2 μmol L^{-1} of each primer were used in PCR mix. A negative control reaction without template was included for each primer combination. Melting curve was conducted from 70°C to 90°C read every 0.5°C during 2 s. The resulting data were collected and analyzed using Opticon Monitor. Standard curves were made with pure cultures of appropriate strains extracted using the same protocol as feces. Primers sequences are able on Table 1.

2.11. Qualitative Analysis of Fecal Microbiota by PCR-DGGE. HDA1 and HDA2-GC (GC clamp required for DGGE analysis [38], targeting the V2-V3 region [39]) were used to assess microbial diversity in each sample. The PCR products were separated in 8% polyacrylamide gels (37.5 : 1 acrylamide : bisacrylamide) with a range of 30–50% denaturing gradient (100% denaturant consisted of 7 M urea and 40% deionized formamide) cast with Bio-Rad's Model 475 gradient delivery system (BioRad, Hercules, CA, USA). The electrophoresis was performed in TAE 0.5X buffer for 5 h at a constant electric current of 125 mA and a temperature of 60°C with the DCode Mutation Detection System (Bio-Rad, Hercules, CA, USA). Clustering analysis was performed using the UPGMA (unweighted pair group method with arithmetic mean clustering algorithm) to calculate the dendrograms.

2.12. Statistical Analysis. Statistical comparisons for significant differences were performed according to Student's t-test. Differences with $P < 0.05$ were considered significant.

3. Results

3.1. Cytokines Profile of PBMC Cocultured with L. kefiri Strains. A preliminary screening of the eight *L. kefiri* strains was carried out using PMBC. PBMC and bacteria coculture assays were performed and profiles of cytokines secreted during incubation with the strains were analyzed. The levels of IL-2, IL-4, IL-5, TNF-β y TGF-β1 were under the lower range of reliable detection. Meanwhile a significant increase in IL-1β, IL-6, IL-10, TNF-α, IL-8, and IL-12 p70 concentrations was observed for all tested microorganisms (Table 2). In an attempt to predict the type of Th response they could promote, we analyzed the TNF-α/IL-10 and IL-10/IL-12 ratios (Table 3).

Table 1: Sequences of oligonucleotide primers.

Population	Forward and reverse primers (5′-3′)	Reference
Total bacteria (HDA)	ACTCCTACGGGAGGCAGCAG GTATTACCGCGGCTGCTGGCAC	[39]
Lactobacillus group	AGCAGTAGGGAATCTTCCA ATTYCACCGCTACACATG	
Firmicutes	GGAGYATGTGGTTTAATTCGAAGCA AGCTGACGACAACCATGCAC	[66]
Bacteroidetes	GGARCATGTGGTTTAATTCGATGAT AGCTGACGACAACCATGCAG	
Faecalibacterium prausnitzii	AGATGGCCTCGCGTCCGA CCGAAGACCTTCTTCCTCC	[67]
Escherichia coli	CATGCCGCGTGTATGAAGAA CGGGTAACGTCAATGAGCAAA	[68]
Prevotella group	CACCAAGGCGACGATCA GGATAACGCCYGGACCT	[69]
Clostridium leptum group	GCACAAGCAGTGGAGT CTTCCTCCGTTTTGTCAA	[70]
Enterococcus spp.	CCCTTATTGTTAGTTGCCATCATT ACTCGTTGTACTTCCCATTGT	[71]
Clostridium coccoides group	CGGTACCTGACTAAGAAGC CTTCCTCCGTTTTGTCAA	
Bifidobacterium spp.	TCGCGTCYGGTGTGAAAG CCACATCCAGCRTCCAC	
Bacteroides fragilis group	CTGAACCAGCCAAGTAGCG CCGCAAACTTTCACAACTGACTTA	[72]
Segmented filamentous bacteria	GACGCTGAGGCATGAGAGCAT GACGGCACGGATTGTTATTCA	[73]
Lactobacillus murinus	GTGGCGAACGGGTGAGTAA GCACCTGTTTCCAAGTGTTATCC	[74]
Akkermansia muciniphila	CAGCACGTGAAGGTGGGGAC CCTTGCGGTTGGCTTCAGAT	[75]

Table 2: Cytokine production after exposing PMBCs for 24 h to *L. kefiri* strains. Cytokines concentrations in culture cell supernatant (pg mL^{-1}) were measured using Flow Human Th1/Th2 11plex FlowCytomix Kit (eBioscience). The results are expressed as mean ± SD of experiments performed with three different donors.

L. kefiri	IL-1β	IL-6	IL-8	IL-10	IFN-γ	TNF-α	IL-12p70
CIDCA 8321	1294 ± 526	1552 ± 709	5771 ± 1284	205 ± 76	131 ± 22	10436 ± 3785	312 ± 72
CIDCA 8325	2050 ± 75	2571 ± 94	4824 ± 531	313 ± 11	59 ± 38	16169 ± 45	572 ± 94
CIDCA 8345	1655 ± 8	2033 ± 15	4399 ± 106	230 ± 3	85 ± 20	15368 ± 1075	449 ± 21
CIDCA 8348	1936 ± 10	2719 ± 13	3855 ± 40	435 ± 90	83 ± 4	13551 ± 198	502 ± 121
CIDCA 83115	1023 ± 60	1778 ± 12	3621 ± 34	192 ± 9	49 ± 2	8613 ± 500	738 ± 206
CIDCA 83111	604 ± 83	2401 ± 81	3806 ± 167	253 ± 1	103 ± 23	9908 ± 175	815 ± 189
CIDCA 83113	1148 ± 26	1722 ± 95	3920 ± 202	201 ± 11	53 ± 22	7514 ± 427	475 ± 59
JCM 5818	591 ± 103	919 ± 40	4228 ± 12	84 ± 2	62 ± 13	6872 ± 1647	246 ± 94
Nonstimulated PBMC	35 ± 2	71 ± 6	418 ± 202	21 ± 1	15 ± 11	175 ± 5	41 ± 13

The highest TNF-α/IL-10 ratio was observed for the nonaggregating strain *L. kefiri* JCM 5818 and the lowest for the autoaggregative strain *L. kefiri* CIDCA 8348. In agreement with these results, *L. kefiri* CIDCA 8348 showed the highest IL-10/IL-12 ratio while *L. kefiri* JCM 5818 was, among other strains such as CIDCA 83111, 83113, and 83115, in the opposite ratio, expecting a poor anti-inflammatory effect.

3.2. Regulation of Caco-2 ccl20:luc Reporter System by L. kefiri Strains. The ability of the eight strains of *L. kefiri* to modulate intestinal innate response to proinflammatory stimuli such as flagellin (FliC) was studied using a Caco-2 ccl20:luc reporter system [18, 36]. Only three strains (CIDCA 8348, 83111, and JCM 5818) downregulated cell activation induced by FliC (Figure 1), suggesting their potential anti-inflammatory properties.

Table 3: TNF-α/IL-10 and IL-10/IL-12 ratio determined after *in vitro* PBMC stimulation with *L. kefiri* strains. Means with the same letter for each parameter are not significantly different.

L. kefiri	TNF-α/IL-10	IL-10/IL-12
CIDCA 8321	$50.9 \pm 11.4^{c,d}$	$0.66 \pm 0.24^{d,e,f}$
CIDCA 8325	51.7 ± 0.1^{d}	0.55 ± 0.06^{e}
CIDCA 8345	66.8 ± 4.7^{e}	0.51 ± 0.02^{e}
CIDCA 8348	31.2 ± 0.5^{b}	0.87 ± 0.18^{f}
CIDCA 83115	44.9 ± 2.6^{c}	0.26 ± 0.01^{b}
CIDCA 83111	39.2 ± 0.7^{c}	0.31 ± 0.02^{c}
CIDCA 83113	37.4 ± 2.1^{c}	0.42 ± 0.02^{d}
JCM 5818	81.8 ± 19.6^{f}	0.34 ± 0.03^{c}
Nonstimulated PBMC	8.3 ± 0.2^{a}	0.005 ± 0.002^{a}

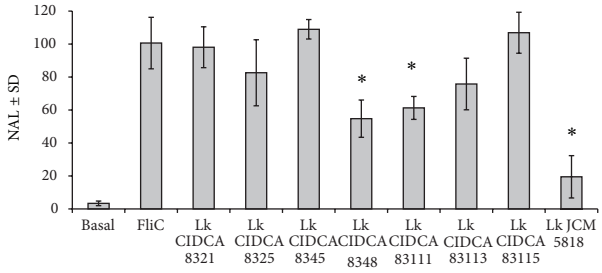

Figure 1: Modulation of proinflammatory response in Caco-2 ccl20:luc reporter system by *L. kefiri* strains. NAL: normalized average luminescence expressed as percentage of activity induced with flagellin stimulation; FliC: *Salmonella*-isolated flagellin; Basal: without any stimulation. Results are expressed as mean ± standard deviation and are representative of at least three independent experiments. $^{*}P < 0.01$.

L. kefiri CIDCA 8348 was chosen to perform *in vivo* studies on Swiss mice since parameters associated with safety and other beneficial properties have been previously demonstrated [29]. Moreover, *L. kefiri* CIDCA 8348 is an aggregative strain. This is an important property for probiotics since it has been proposed that aggregation represents a mechanism by which gastrointestinal commensals adhere to each other and it could allow them to colonize persistently in biofilms on the host's mucosa [40].

3.3. Kinetics of Fecal IgA Response after Oral Administration of L. kefiri CIDCA 8348 in Swiss Mice. Stool suspensions were assayed for total IgA by ELISA to evaluate the induction of mucosal IgA (Figure 2). An induction was observed after 14 days of probiotic administration and the levels continue rising after 21 days. Even though no differences in IgA secretion were observed after 7 days of treatment between groups, flow cytometry quantified IgA^{+} cells were significantly higher in mLN from Lk group (data not shown).

3.4. Effect of L. kefiri Administration on Gene Expression of Gut Mucosa. The expression of cytokines, chemokines, mucins, and epithelial barrier genes as well as IgA related genes was studied by qRT-PCR in ileum and colon after 7 and 21 days of oral administration of *L. kefiri* CIDCA 8348.

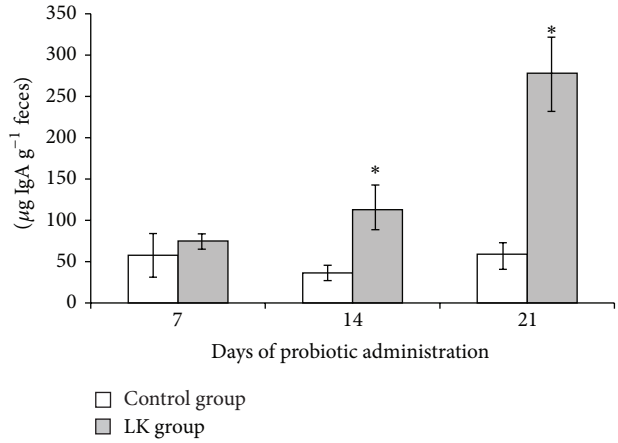

Figure 2: IgA quantification from fecal samples taken on day 7, 14, or 21 from control mice and *L. kefiri* treated mice (Lk). Results are expressed as mean ± standard deviation. $^{*}P < 0.05$.

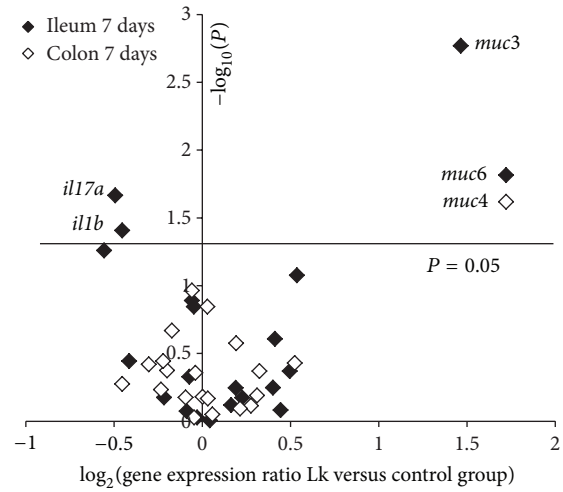

Figure 3: Gene expression ratio in ileum (black) and colon (white) of Lk group *versus* control group after 7 days of *L. kefiri* administration. The x-axis of the plot represents $\log_2$ relative expression level of the gene and the y-axis displays the $-\log_{10} P$ (statistical significance). The names of the genes which displayed significant differences are included.

As shown in Figure 3, a seven-day treatment significantly downregulated IL-1β and IL-17A gene expression in ileum; meanwhile mucin 3 and mucin 6 were upregulated. In contrast, in colon only gene expression of mucin 4 was modified.

The administration of *L. kefiri* for a longer period, 21 days, produced higher expression levels of IL-10, CXCL-1, and mucin 6 genes in ileum (Figure 4(a)). In colon, downregulation of IFN-γ, GM-CSF, and IL-1β genes was observed together with the upregulation of mucin 4 (Figure 4(a)).

The effect of *L. kefiri* treatment for 21 days on gene expression was also evaluated in Peyer patches (PP) and

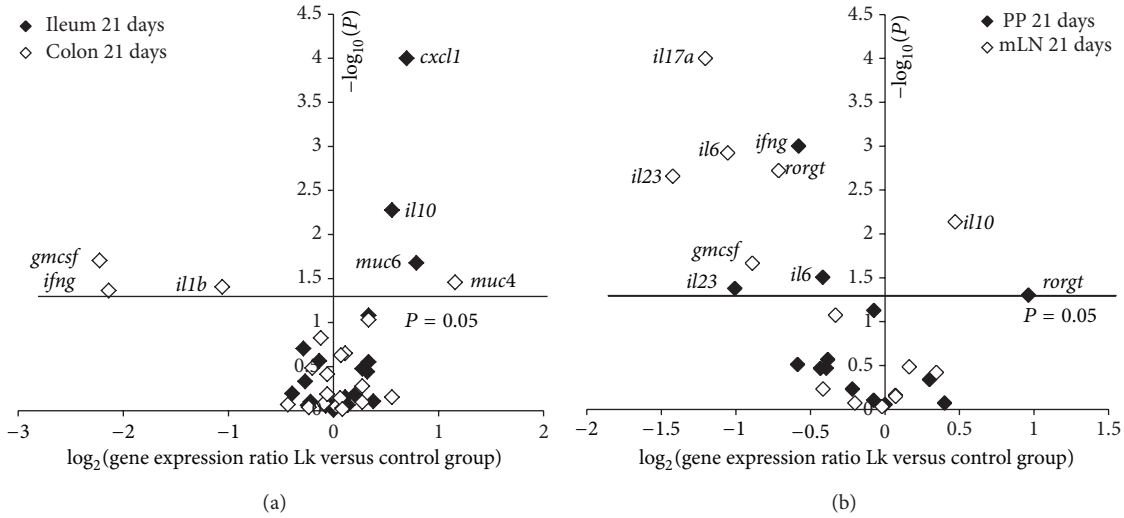

Figure 4: Gene expression ratio of Lk group *versus* control group after 21 days of *L. kefiri* administration. The x-axis of the plot represents $\log_2$ relative expression level of the gene and the x-axis displays the $-\log_{10} P$ (statistical significance). The names of the genes which displayed significant differences are included. (a) Expression in ileum (black) and colon (white). (b) Expression in PP (black) and mLN (white).

mesenteric lymph nodes (mLN) (Figure 4(b)). In PP the expression of IL-23, IFN-γ, and IL-6 was downregulated. Interestingly, in mLN not only proinflammatory mediators (IL-6, IL-23, IL-17A, and GM-CSF) and RORγt transcription factor were downregulated but also IL-10 gene expression was increased.

3.5. Ex Vivo Mice Intestinal Explants to Study Mucosal Anti-Inflammatory Effect of L. kefiri. To analyze the ability of *L. kefiri* treatment to modulate the mucosal immune response in a proinflammatory environment, *ex vivo* experiments were performed stimulating ileum and colon explants with LPS from not treated (control) and 21-day *L. kefiri* treated mice. LPS stimulation induced an increment of IL-6 and GM-CSF levels in control mice (Figures 5(a) and 5(b)). These increments were significantly attenuated in both ileum and colon explants of *L. kefiri* treated mice (Figures 5(a) and 5(b)). Moreover, in colon explants from Lk group a higher secretion of IL-10 was observed in LPS stimulated samples (Figure 5(b)). The levels of IL-4, IL-17, IFN-γ, and TNF-α were undetectable in both Lk and control mice explants.

3.6. Effect of L. kefiri Administration on Fecal Microbiota. The qualitative profile of fecal microbiota was determined by PCR-DGGE (Figure 6(a)). Microbial diversity was assessed by the number of amplification bands generated from each sample. There were no differences between control and Lk group (32 ± 3 and 30 ± 2, resp.). However, changes in the microbial community composition were produced since the cluster analysis based on the Pearson product-moment correlation coefficient and UPGMA linkage allowed differentiation of the experimental groups in two clusters (Figure 6(b)).

As expected, an increment in *Lactobacillus* population was observed by qPCR but quantitative differences were not observed in the two major phyla, Firmicutes or Bacteroidetes (Figure 6(c)). Moreover, no significant changes were detected in other evaluated bacterial populations (Table 1).

4. Discussion

In the last years, an increasing number of *in vitro* and *in vivo* experiments have supported the idea that probiotic microorganisms confer their health benefits to the host by interacting with the immune system, particularly through establishing and maintaining a balance between pro- and anti-inflammatory cytokines [41, 42]. In kefir, bacteria and yeasts exist in symbiotic association and contributed to beneficial properties. Several authors have demonstrated the ability of kefir to modulate the mucosal immune response in mice and suggest that a Th1 response was controlled by Th2 cytokines [15, 16]. Some immunological effects were attributed to the formation of bioactive peptides during milk fermentation and also to production of exopolysaccharides as kefiran [13]. However, features regarding the effects of bacteria remain very important. It has been recently described that one strain of *L. kefiranofaciens* protects mice in a model of allergy [20] and also in an experimental model of colitis [19], but to our knowledge, our work constitutes the first report of the *in vivo* immunomodulatory activity of *L. kefiri*.

In the present work we demonstrated that *L. kefiri* strains induced the secretion of proinflammatory Th1 mediators such as IL-1β, IFN-γ, IL-6, IL-12p70, and TNF-α in PBMC as well as the production of the Th2 cytokine IL-10. These findings are not surprising, since several authors have reported the upregulation of these proinflammatory cytokines by probiotic bacteria on PBMC [6, 43–45] or in mice macrophages by *L. kefiranofaciens* [22]. However, we found that *L. kefiri* strains stimulate immune cells to produce different ratios of cytokines, suggesting that they could possess different T cell polarizing abilities.

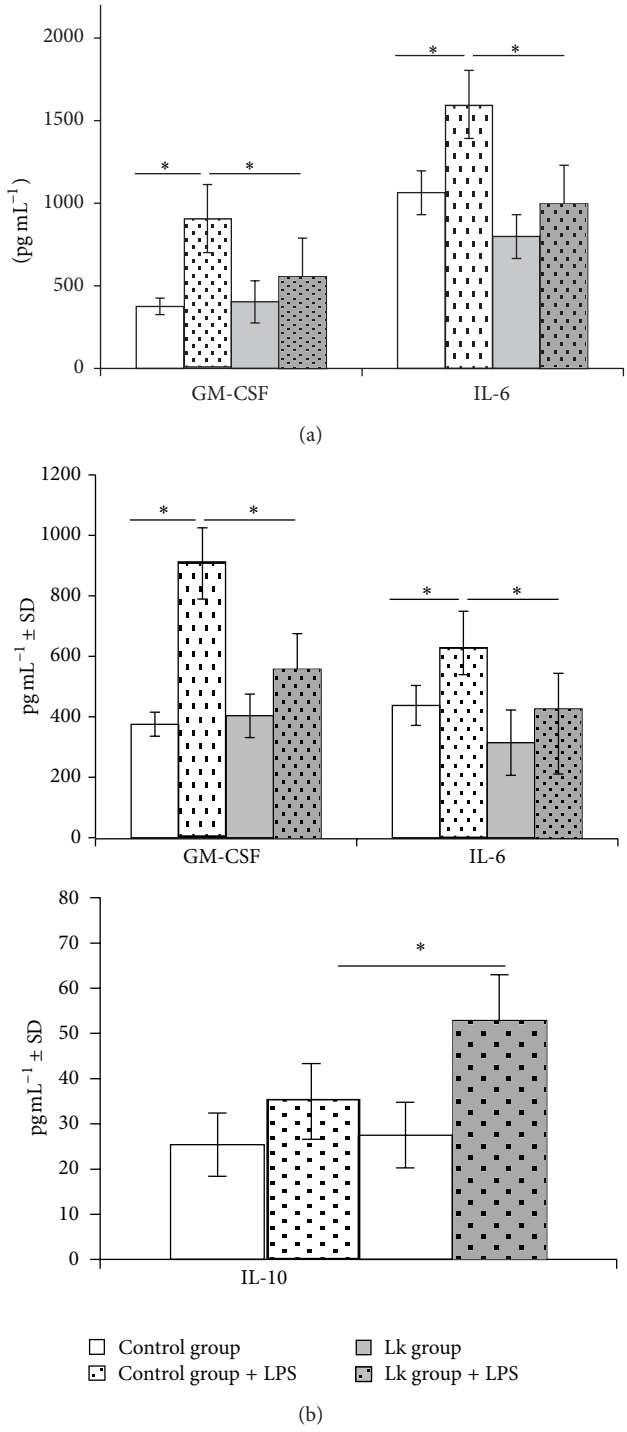

Figure 5: Cytokine's release in supernatants of (a) ileum and (b) colonic explants cultured for 24 h in the presence of LPS. Results are expressed as mean ± standard deviation. $^{*}P < 0.05$.

Cytokines are mutually regulated molecules; thus the balance between them influences CD4+ T-cell differentiation towards Th1, Th2, or Th17 cells. IL-12 induces Th1-mediated responses; meanwhile the anti-inflammatory cytokine IL-10 suppresses the production of IL-12 among other Th1 cytokines. The observed differences in the production of IL-12, IL-10, and TNF-α could contribute to understanding the type of response a strain may promote [45, 46]. JCM 5818 showed the highest TNF-α/IL-10 ratio whereas CIDCA 8348 presented the lower ratio. Moreover, CIDCA 8348 showed also the highest IL-10/IL-12 ratio which presupposes that it is a good anti-inflammatory candidate [47]. In concordance with these results, the strain CIDCA 8348 was also capable, along with other two *L. kefiri* strains, of eliciting an anti-inflammatory response on flagellin-stimulated intestinal epithelial cells (Caco-ccl20 reporter system) which has been previously reported for several probiotic bacteria [48] and yeasts [18, 49]. Curiously, JCM 5818 strain that presented the most anti-inflammatory capacity using Caco-ccl20 reporter system presented the most proinflammatory profile using PBMC. It might be interesting in the future to study the *in vivo* anti-inflammatory properties of this strain.

Although *in vitro* research using PBMC from healthy donors or intestinal epithelial cells can be used to screen the immunomodulatory activity of probiotic strains candidates, while reducing considerably the use of animals for screening purposes, they could not always be a good indicator of *in vivo* effect [4, 46, 47]. In consequence, to better understand the immunomodulatory ability of *L. kefiri*, the strain CIDCA 8348 was selected to be administered orally to mice in order to analyze the effect on different aspects of mucosal immune response and microbiota modulation.

CIDCA 8348 strain occasioned an increment in IgA+ B cells in mLN and it correlated with an increase of IgA in fecal samples of *L. kefiri*-treated mice. These findings are in agreement with results reported for some lactobacilli-based probiotics [50, 51] or even for the administration of kefir-fermented milk [16, 52]. SIgA, the predominant immunoglobulin in secretions, is a key element in maintaining gut homeostasis and in the protection of mucosal surfaces against pathogens [53]. Expression of molecules involved in class switch to IgA, expansion of IgA-expressing B cells, and their differentiation to IgA secreting plasma cells was studied. Even though no changes in the expression of APRIL, BAFF, and TGFβ1 genes in PP, mLN, ileum, or colon were observed, IL-10 was significantly induced in both ileum and mLN. It has been described that this cytokine induces IgA production, either through induction of TGFβ within the target B cell itself or through enhancement of the postswitch maturation [54]. Nevertheless, a downregulation of the expression of proinflammatory cytokines (IL-1β and IL-17A) was observed in ileum tissue at 7th day of administration of *L. kefiri*. This effect became more evident after 21 days of treatment, when a significant decrease of several proinflammatory mediators was determined in Peyer's patches (IL-6, GM-CSF, IL-17A, and IFN-γ), mesenteric lymphoid nodes (IL-6, GM-CSF, and IL-17A), and colon (GM-CSF, IFN-γ, and IL-1β) showing the anti-inflammatory ability of this *L. kefiri* strain *in vivo*. This kind of results, which support the suppression of proinflammatory immunity by probiotics, was reported for different nonpathogenic and probiotic bacteria by other authors in healthy [55] or disease models [47], but this is the first report for *L. kefiri* isolated from kefir. Moreover, the anti-inflammatory cytokine IL-10 was increased in ileum as well

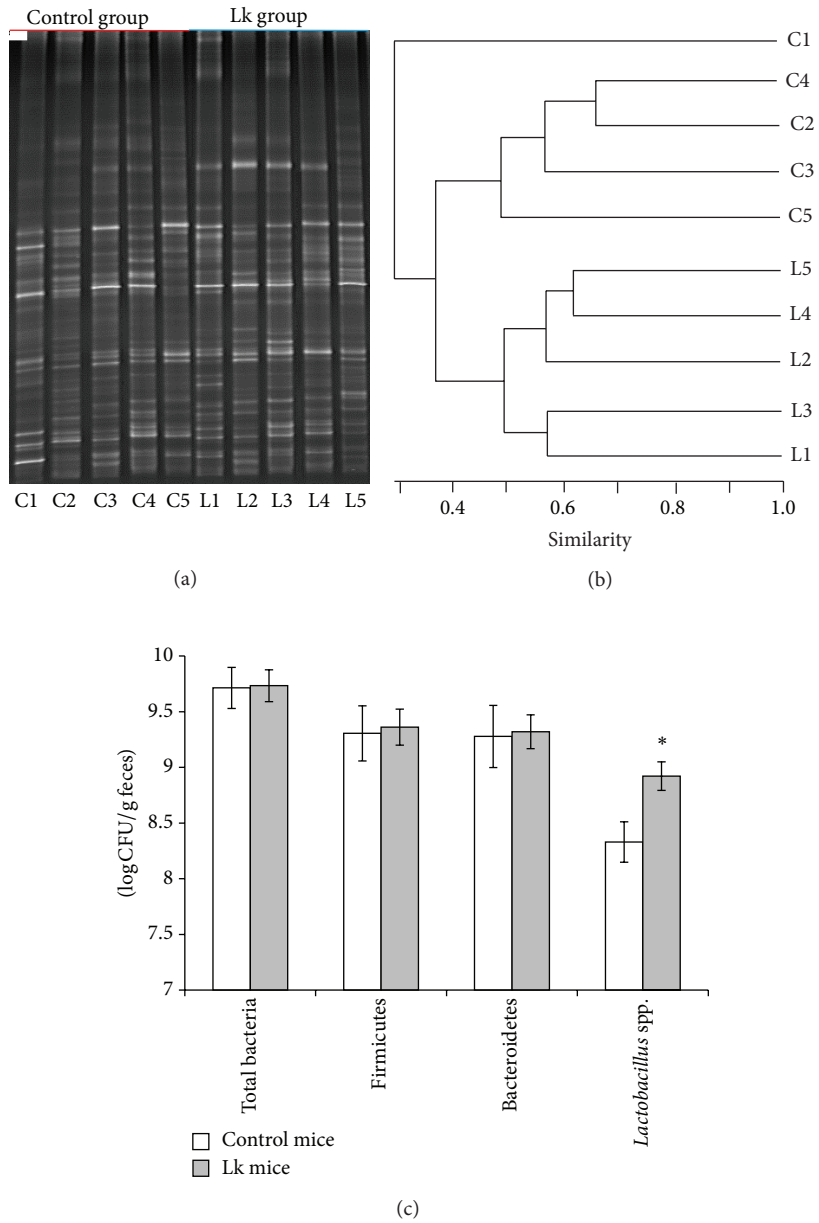

Figure 6: Evaluation of microbiota on fecal samples taken on the 21st day of trial from control and Lk groups. (a) Total bacteria DGGE profiles of five mice from control group (lanes C1 to C5) and five from Lk group (lanes L1 to L5). (b) Dendrogram for the total bacterial DGGE profiles. Clustering analysis was performed using the UPGMA linkage. (c) qPCR quantification of total bacteria, *Firmicutes, Bacteroidetes,* and *Lactobacillus* spp. Results are expressed as mean ± standard deviation. $^{*}P < 0.05$.

as the chemokine CXCL-1. This interesting chemoattractant, analogous in function to human IL-8, is an important regulator of neutrophil recruitment from the lamina propria to the epithelium and has been shown to be essential in protection against DSS-induced colitis [56].

On the other hand, intestinal explants from *L. kefiri*-treated mice showed a downregulation of IL-6 and GM-CSF after *in vitro* stimulation with a proinflammatory mediator such as LPS in comparison with control mice. Taken together, all these experiments allowed us to confirm the anti-inflammatory phenotype associated with *L. kefiri* CIDCA 8348 administration.

Regarding another feature on mucosal physiology, we studied the effect of *L. kefiri* administration on the expression of mucin genes. Mucins are the main component of the mucus layer and it has been described that their secretion could be modified by changes in host microbiota, infections, and probiotic or antibiotic treatments [57–59]. Only a few authors have evaluated the effect of probiotic administration in healthy lab animals. Particularly, Dykstra et al. [60] observed differential induction of *muc1, muc2,* and *muc3* in ileum and colon after administration of *Lactobacillus plantarum* 299 v to Sprague-Dawley rats. In addition, studies performed in Swiss mice revealed that administration of

L. plantarum L91 induced *muc2* in colon [61]; meanwhile Jiang et al. [62] reported that *L. rhamnosus* GG-treated C57BL/6NHsd mice overexpressed *muc3* without changes in m*uc1, muc2,* or *muc4*. In *L. kefiri*-treated mice *muc3* and *muc6* increased their expression in the ileum after 7 days of treatment whereas at 21 days only *muc6* was increased. In colon, at 7 and 21 days *muc4* expression was increased in *L. kefiri*-treated mice. These changes could be associated with the presence of *L. kefiri* in the gut or with the modifications in microbiota populations induced by it [63]. Moreover, differences in the quantity and composition of the local microbiota [64] as well as the characteristics and thickness of the mucus layer [58, 65] could have an impact in the way *L. kefiri* interacts with the epithelium or its effect on microbiota.

5. Conclusion

In this study, we demonstrated that *L. kefiri* strains isolated from kefir stimulated the production of different ratios of pro/anti-inflammatory cytokines *in vitro*. We proved that the administration of *L. kefiri* CIDCA 8348 to mice not only downregulates expression of proinflammatory mediators but also increases anti-inflammatory molecules in gut immune system inductive and effector sites. Likewise, the increment in IgA production together with mucin induction and the impact in microbiota demonstrate the importance of this probiotic in the regulation of intestinal homeostasis. Thus, it is a good candidate to be used in gut inflammatory disorders.

Abbreviations

PBMC:	Peripheral blood mononuclear cells
GM-CSF:	Granulocyte macrophage colony-stimulating factor
CXCL-1:	Chemokine (C-X-C motif) ligand 1
IL:	Interleukin
IFN:	Interferon
TNF:	Tumor Necrosis Factor.

Conflict of Interests

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

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Review Article

Toll-Like Receptor Mediated Modulation of T Cell Response by Commensal Intestinal Microbiota as a Trigger for Autoimmune Arthritis

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In autoimmune diseases, a disturbance of the balance between T helper 17 (Th17) and regulatory T cells (Tregs) is often observed. This disturbed balance is also the case in rheumatoid arthritis (RA). Genetic predisposition to RA confers the presence of several polymorphisms mainly regulating activation of T lymphocytes. However, the presence of susceptibility factors is neither necessary nor sufficient to explain the disease development, emphasizing the importance of environmental factors. Multiple studies have shown that commensal gut microbiota is of great influence on immune homeostasis and can trigger the development of autoimmune diseases by favoring induction of Th17 cells over Tregs. However the mechanism by which intestinal microbiota influences the Th cell balance is not completely understood. Here we review the current evidence supporting the involvement of commensal intestinal microbiota in rheumatoid arthritis, along with a potential role of Toll-like receptors (TLRs) in modulating the relevant Th cell responses to trigger autoimmunity. A better understanding of TLR triggering by intestinal microbiota and subsequent T cell activation might offer new perspectives for manipulating the T cell response in RA patients and may lead to the discovery of new therapeutic targets or even preventive measures.

1. Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease, which is characterized by chronic inflammation and progressive cartilage and bone destruction in multiple joints. A world-wide prevalence of about 1% ranks RA among the most-common autoimmune disorders [1]. Current therapy of RA is based on a choice, or often a combination, of nonsteroidal anti-inflammatory drugs (NSAIDs), disease-modifying antirheumatic drugs (DMARDs), glucocorticoids, and recently the so-called Biologicals targeting specific cytokines or certain immune cells.

The etiopathology of RA is complex, because cells of the innate and adaptive immune system as well as joint resident cells such as fibroblasts and chondrocytes contribute to the development and progression of RA [2]. The production of proinflammatory cytokines such as tumor necrosis factor (TNF) α and interleukin (IL)-1 and activation of lymphocytes are considered to play important roles in RA pathogenesis [3, 4]. A specific subset of T cells, known as T helper 17 (Th17) cells, is considered to be a major pathogenic mediator in RA [3, 5, 6].

Although the exact etiology remains unclear to date, RA is generally considered a multifactorial disease in which both genetic and environmental factors play a role [7]. Epidemiological studies have revealed that the largest genetic risk factors for RA are certain alleles of the HLA-DR gene [8]. In addition, polymorphisms in protein tyrosine phosphatase N22 (PTPN22), peptidyl arginine deiminase type IV (PADI4), signal transducer and activator of transcription 4 (STAT4), and TNF receptor-associated factor 1/complement C5 (TRAF1/C5) were found associated with RA [8]. However, the presence of susceptibility factors is neither necessary nor sufficient to explain the disease development, underlining a critical role for environmental factors.

Meta-analysis has shown that smoking is one of the environmental factors associated with RA pathogenesis [9]. In addition to smoking, periodontal pathogens such as

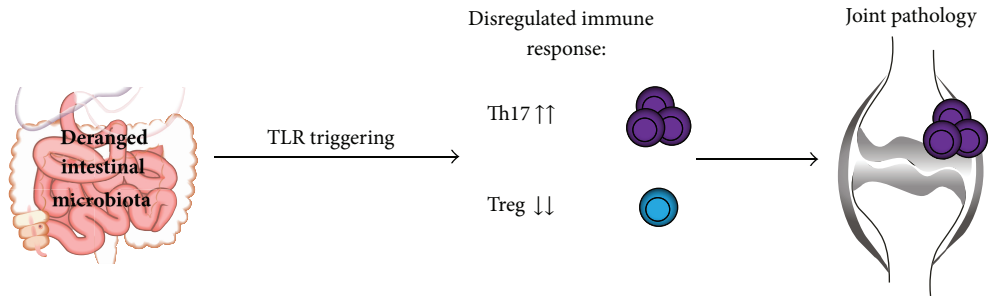

Figure 1: Exposure to deranged intestinal microbiota or a disregulated immune response to microbiota drives rheumatoid arthritis by promoting Th17 and deranging Treg cells.

Porphyromonas gingivalis and the induced periodontal disease have been implicated in the etiology of RA [10, 11]. Besides infectious bacteria, commensal bacteria have been implicated in the pathogenesis of RA [12]. In addition, there is strong evidence that Toll-like receptors (TLRs), which recognize microbial products, contribute to RA progression [13–15].

Most of the polymorphisms associated with RA are involved in regulating T cell activation [16]. The genetically altered T cells are *potentially* autoreactive, that is, they may react to self-antigens in the joint and cause autoimmunity; nevertheless, the "naïve" T cells (called Th0) first need to become activated and acquire a pathogenic phenotype in order to be harmful. Exposure to (deranged) intestinal microbiota may be a critical factor. The aim of this review is to discuss the role of intestinal bacteria in the development of RA in the context of T cell modulation and the possible role that TLRs play in this process (Figure 1).

2. Th17 Cells and Rheumatoid Arthritis

Th17 cells protect against bacterial and fungal infections; however they also promote the development of autoimmune diseases such as multiple sclerosis, inflammatory bowel disease, psoriasis, and RA [17–22]. Regulatory T cells (Tregs) downregulate inflammation and serve to prevent tissue damage and autoimmunity. The balance between Th17 cells and Tregs is strictly regulated, and imbalance is thought to promote autoimmunity [23]. In RA, increased percentages of Th17 cells have been found in peripheral blood mononuclear cells (PBMCs) of patients [22]. These Th17 cells were shown to be potent inducers of matrix metalloproteinases and proinflammatory cytokines upon interaction with synovial fibroblast, thereby contributing to joint damage [22].

Other studies found increased levels of Th17 cells and decreased levels of Tregs in peripheral blood of patients with active RA [24, 25]. Furthermore, RA patients have Tregs with decreased suppressive activity [26]. Transforming growth factor (TGF) β is a key factor involved in maintaining the Th17/Treg cell balance: TGFβ in combination with IL-6 or IL-21 promotes Th17 differentiation, but when TGFβ is present in combination with IL-2, it will induce differentiation of Tregs [27, 28]. Inhibition of IL-6 function was shown to correct the Th17/Treg cell imbalance in RA patients [24]. Targeting the Th17 pathway in autoimmune diseases such as RA is very promising [29]. However, factors promoting Th17 differentiation are poorly understood. Since specific intestinal microbiota greatly promotes Th17 differentiation in intestinal mucosa, exposure to (deranged) intestinal microbiota may be a critical factor in autoimmune arthritis.

3. Intestinal Microbiota and Regulation of the Immune Response

Large numbers of commensal microorganisms inhabit the gastrointestinal tract of mammals. It has been shown that this commensal microbiota is essential for a proper development of the immune system, as GF mice possess an underdeveloped mucosal immune system [30]. GF mice have decreased numbers of lamina propria $CD4^+$ cells, hypoplastic Peyer's patches, and greatly reduced immunoglobulin A (IgA) producing plasma cells [30, 31]. In addition, also spleen and lymph nodes are underdeveloped in GF mice, as they contain poorly formed B and T cell zones [30]. Introduction of *Bacteroides fragilis* into GF mice has been shown to induce correct development of the immune system [32].

Ivanov et al. showed that the introduction of SFB in GF mice resulted in an increase of Th17 cells in the intestinal lamina propria [33]. In the murine gut, the presence of SFB has been shown to promote the development of Th17-mediated autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE), colitis, and arthritis [34–36]. Colonization of mice with *B. fragilis*, a human commensal, induces Tregs and prevents development of 2,4,6-trinitrobenzene sulfonic acid- (TNBS-) induced colitis [37]. In addition, oral treatment of mice with polysaccharide A (PSA), a molecule expressed by *B. fragilis*, induced IL-10 producing Tregs and protected against EAE [38]. Another study showed that colonization of mice with microbiota belonging to the *Clostridium* species also resulted in the induction of Tregs [39]. In addition, colonization of young mice with mix of *Clostridium* species resulted in resistance to dextran sodium sulfate- (DSS-) induced colitis [39]. These studies suggest that intestinal microbiota plays an important role in maintaining the balance between pro- and anti-inflammatory T cells, thereby preserving intestinal homeostasis.

A recent study elegantly demonstrated the specific labeling and tracking of intestinal leukocytes [40]. It was shown

that intestinal leukocytes migrate to and from the intestine at steady state [40]. In addition, the migration of intestinal Th17 cells in arthritic K/BxN mice was studied and showed that gut derived Th17 cells end up in the spleen [40]. The fraction of gut-derived Th17 cells present in the spleen correlated with serum level of pathogenic auto antibodies [40]. This is the first study which shows that gut-derived Th17 cells can contribute to autoimmune arthritis [40].

Taken together, it is conceivable that a disturbed balance in the composition of microbiota, the so-called dysbiosis, could result in disruption of intestinal and systemic immune homeostasis. A link between intestinal microbiota and autoimmune deficiencies such as RA seems therefore plausible [41].

4. Rheumatoid Arthritis and Microbiota

Treatment with tetracycline antibiotics, in particular minocycline, was shown to significantly reduce disease activity in seropositive RA patients with disease duration of <1 year [42]. Moreover, the commonly used DMARD sulfasalazine is known to have both anti-inflammatory and antimicrobial properties [43]. Using a small set of oligonucleotide probes detecting broad groups of bacteria, intestinal microbiota of RA patients was found different from that of fibromyalgia (FM) patients [44]. The authors did not include healthy control subjects in the study; however a group of patients with FM, having a comparable age and sex distribution and receiving similar treatment with NSAIDS drugs, were included as controls. This study showed that RA patients had significantly less *bifidobacteria* species, bacteria of the *Bacteroides-Porphyromonas-Prevotella* group, *Bacteroides fragilis* subgroup, and the *Eubacterium rectal-Clostridium coccoides* group, when compared to FM patients [44].

A recent study using 454 pyrosequencing of 16S rRNA of intestinal microbiota in stool samples found a strong correlation between the presence of *Prevotella copri* with disease in new-onset untreated RA patients [45]. Abundance of *P. copri* in this study was inversely correlated with the presence of HLA-DRB-1 risk alleles, suggesting requirement of intestinal microbial signals in the absence of genetic predisposition factors for one to develop the disease. Another study demonstrated that fecal microbiota of RA patients contained significantly more *Lactobacilli* compared to healthy controls [46]. Altogether, the efficacy of oral antibiotic treatment and recent findings on disturbed composition of intestinal microbiota in early RA supports the involvement of microbiota in the development of RA.

5. Experimental Evidence on the Role of Commensal Microbiota in Arthritis

The critical role of commensal microbiota in the development of arthritis has been shown in at least three spontaneous autoimmune models of arthritis. These studies showed that spontaneous disease in mice with T cell-activating genetic modifications is greatly diminished under germ-free (GF) or specified pathogen-free (SPF) conditions [13, 36, 47]. Another study showed that oral treatment with enrofloxacin, a broad-spectrum antibiotic, exacerbates collagen induced arthritis (CIA) in mice by influencing production of a number of proinflammatory cytokines such as IL-6 and IL-17 [48].

IL-1 receptor antagonist (IL-1Ra) deficient mice spontaneously develop autoimmune arthritis due to excessive IL-1 signaling [49]. Development of autoimmune arthritis in this mouse model is dependent on microbial flora, as arthritis was strongly attenuated in GF IL-1Ra$^{-/-}$ mice [13]. Colonization *with Lactobacillus bifidus* resulted in arthritis with incidence and severity scores comparable to those observed in conventionally housed mice [13]. The GF status IL-1Ra$^{-/-}$ mice resulted in a notable decrease in IL-17 and IL-1β production by splenocytes upon CD3 as well as TLR2 and TLR4 stimulation, suggesting abolishment of Th17 differentiation [13].

SKG mice have a mutation in the gene encoding an SH2 domain of ZAP-70, a signal transduction molecule in T cells. The aberrant ZAP-70 is thought to change the thresholds of T cells to thymic selection, which results in the positive selection of otherwise negatively selected autoimmunity T cells [50]. SKG mice develop chronic autoimmune arthritis under conventional conditions; however in strictly controlled SPF environment arthritis failed to develop [47]. Arthritis in SKG mice was shown to be accompanied with high sera levels of IL-6, known to be important in Th17 induction. However, in sera from SKG mice kept in SPF conditions IL-6 was undetectable [47].

T cells of K/BxN mice express a transgenic T cell receptor which recognizes a self-peptide derive from glucose-6-phosphate isomerase (GPI). These autoreactive T cells stimulate GPI-specific B cells to produce high amounts of GPI autoantibodies. Th17 cells seem to be driving arthritis in this model, as neutralization of IL-17 blocked the development in SPF-housed K/BxN mice [36]. Intriguingly, GF K/BxN mice have an almost complete deficiency of Th17 cells and are protected from severe arthritis [36]. Moreover, oral treatment of K/BxN mice with vancomycin or ampicillin inhibited the development of arthritis, while in neomycin-treated mice disease was aggravated [36]. Introduction of segmented filamentous bacteria (SFB), a gut-residing bacteria, in GF K/BxN mice resulted in an increase of Th17 cells in the lamina propria and in onset of arthritis [36]. These results suggest that certain intestinal microbiota is able to trigger an imbalance in the T cell response which leads to the development of autoimmune arthritis in a genetically predisposed host.

6. TLR-Mediated Interactions between Bacterial Antigens and the Immune System

Although the mechanism by which commensal intestinal microbiota triggers the development of autoimmune diseases remains poorly understood to date, TLRs are most likely involved. TLRs recognize microbe-associated molecular patterns (MAMPs), which are shared by many microorganisms [51]. Each TLR recognizes certain MAMPs; for

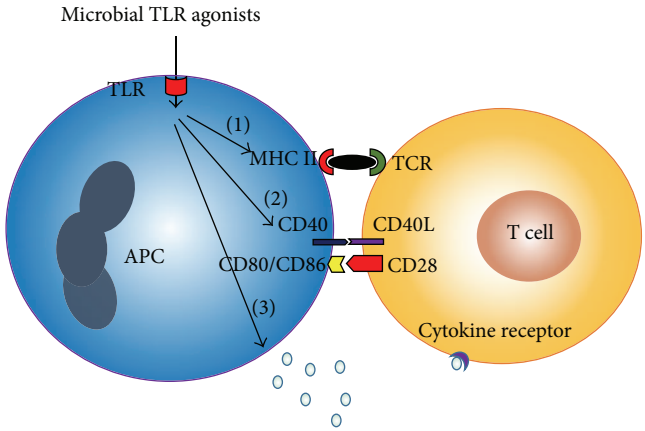

FIGURE 2: Toll-like receptor (TLR) activation on antigen presenting cells (APCs) enhances the antigenic signal to T cells. TLR activation induces the upregulation of MHC II (1), costimulatory molecules such as CD80, CD86, and CD40 (2), and release of cytokines (3).

instance, TLR2, TLR4, TLR5, and TLR9 recognize peptidoglycans, lipopolysaccharides (LPS), flagellin, and unmethylated CpG motifs in bacterial DNA, respectively [52]. TLRs are expressed by a number of immune cells, including dendritic cells (DCs), macrophages, neutrophils, T cells, and B cells, but TLRs have also been found on resident cells, such as epithelial and endothelial cells [53].

Antigen presenting cells (APCs) such as DCs and macrophages are known to express TLRs, and activation of TLRs induces the upregulation of MHC class II molecules and thereby may substantially influence the strength of the antigenic signal to T cells in the "immunological synapse" [54] (Figure 2). Furthermore, activation of TLRs induces upregulation of costimulatory molecules such as CD80, CD86, and CD40, which provide the second signal for T cell activation (Figure 2). The third signal for T cell activation and differentiation, the cytokine environment, is dramatically affected by the type and the extent of TLR activation (Figure 2). For instance, activation of TLR4 and TLR9 is thought to skew T cell differentiation toward the Th1 phenotype through induction of IL-12 production by DCs, whereas TLR2 activation might induce a Th2-biased immune response through production of IL-10 and IL-13 [55–61]. TLR4-induced IL-23 contributes to the expansion and survival of Th17 cells [62]. In addition, conditioned medium from TLR4-stimulated DCs or PBMCs induces Th17 differentiation and IL-17 production, a process potentiated by TGFβ [63].

In addition to the type of TLR activation, the extent of TLR triggering also seems to determine the type of immune response generated. For instance, it was demonstrated that a high dose of LPS triggers a Th1 response via TLR4 while a low LPS dose results in a Th2 response to an inhaled antigen [64]. Besides APC-mediated T cell activation, some TLRs such as TLR2, 5, and 7/8 are functionally expressed on T cells and directly cause T cell activation and proliferation upon stimulation [65–67]. Others (TLR3 and TLR9) can enhance survival of activated $CD4^+$ T cells [68].

Also joint resident cells are known to functionally express TLRs. RA synovial fibroblasts (RASF) for instance are known to express TLR2, TLR3, TLR4, and TLR9 [69]. Stimulation of RASF with TLR2, TLR3, and TLR4 antigens (peptidoglycans, polyinosinic:polycytidylic acid, and LPS, resp.) results in high production of inflammatory cytokines, MMPs, and vascular endothelial growth factor and results in exacerbation of the Th1 and Th17 response [69].

A study with TLR deficient IL-$1Ra^{-/-}$ mice demonstrated that TLRs play distinct roles in the regulation of the T cell balance. In this study it was shown that Th17 differentiation is reduced in TLR4 deficient IL-$1Ra^{-/-}$ mice, while $TLR2^{-/-}$ deficiency results in a shift in T cell balance from Th2 and Treg towards Th1 cells [13]. In addition, it was shown that IL-$1Ra^{-/-}$ $TLR2^{-/-}$ mice develop a more severe arthritis compared to IL-$1Ra^{-/-}$ $TLR2^{+/+}$ mice [13]. In contrast, TLR4 deficiency in IL-$1Ra^{-/-}$ mice resulted in protection against severe arthritis [13]. This study shows that sensing of microbiota by TLRs plays a critical role in maintaining T cell balance and arthritis development.

7. Intestinal TLR Triggering

Commensal bacteria normally do not cross the epithelial barrier. A specific population of CX3CR1 expressing cells in lamina propria has been shown to sample the lumen and interact with commensal bacteria in the lumen [70]. Although, these cells were first identified as DCs, recent studies demonstrated that CX3CR1 expressing cells in the gut are more similar to macrophages than DCs [71, 72]. This is based on the observation that CX3CR1 expressing in the intestinal lamina propria are nonmigratory and cannot prime naïve T cells [71, 72]. However, another study identified $CD103^-$ $CD11b^+$ DCs which also express CX3CR1; these cells lacked macrophage markers such as F4/80 or CD64 [73]. CX3CR1 expressing cells were thought to be nonmigratory; however a recent study showed that these cells do migrate to mesenteric lymph nodes after antibiotic-induced dysbiosis and in the absence of MyD88 [74]. Despite this finding, it is believed that the $CD11b^+$ $CD103^+$ classical DC subset is mainly responsible for presentation of bacterial antigen to naïve $CD4^+$ T cells and Th17 differentiation in the intestinal lamina propria [74–76]. Stimulation of $CD11b^+$ $CD103^+$ cells with flagellin, a TLR5 ligand, resulted in the expression of high amounts of IL-23 [76]. A recent study identified a subset of CCR2-expressing $CD103^-$ $CD11b^+$ DCs, in lamina propria which were able to drive IL-17 production in vitro [77]. These DCs produced IL-12 and IL-23p40, and production of these cytokines increased in response to TLR4 stimulation with LPS. These DCs were found in murine as well as human lamina propria [77].

A recent study showed that luminal bacteria stimulate the recruitment of $CD103^+$ DCs to the epithelium, where these DCs can also sample the lumen [78]. Recruitment of the DC to the epithelium was shown to be depending on chemokines and TLR signaling [78]. Another study showed that TLR5 is highly expressed in DCs in the intestinal mucosa, but not in splenic DCs [79]. This same study showed that $TLR5^{-/-}$

mice had increased Treg levels in the intestinal lamina propria, which suggests that TLR5 plays a role in regulating the intestinal Th17/Treg cell balance [79]. Another study demonstrated that TLR5 is expressed by $CD11c^{hi}$ $CD11b^{hi}$ DCs in lamina propria of mice [80]. These intestinal DCs induce the differentiation of Th1 and Th17 cells in response to flagellin [80]. In addition, TLR9 deficient mice were shown to have more Tregs and reduced Th1 and Th17 cell levels in the intestine [81].

Besides DCs also intestinal epithelial cells (IECs) in the gut are known to express TLRs. TLR 1, 2, 3, 4, 5, and 9 are known to be expressed by IECs in human small intestine, and TLR1-9 have been shown to be present on IEC in the colon [82]. In the mouse TLR1, 2, 3, 4, 5, 9, and 11 have been detected in the small intestine, and in the colon TLR2, 3, 4, and 9 were shown to be present [82]. The expression of TLRs in the gut seems to be regulated by commensal bacteria, as it was shown that the expression of TLR2, 3, 4, and 5 was higher in colonic epithelial cells of specific pathogen-free mice when compared to GF mice [83]. An in vitro study showed that TLR4 and basolateral TLR9 stimulation on IECs drives an inflammatory response [84]. However, apical TLR9 activation resulted in the production and secretion of galectin-9, which was shown to support the development of Tregs [85].

TLR signaling on IEC is also important in maintaining the epithelial barrier; for instance, TLR2 activation on epithelial cells protects against barrier disruption by upregulating the expression of zonula occludens, while TLR4 signaling results in increased intestinal permeability through upregulation of membrane protein kinase C activity [86, 87]. Translocation of bacteria across the membrane will result in an inflammatory response in the intestinal lamina propria. It has been hypothesized that intestinal barrier function, in particular the intercellular tight junctions modulated by zonulin among others, may be impaired in autoimmune disease [88, 89]. However, it is not yet clear whether this is indeed the case in individuals with autoimmune diseases such as RA.

As mentioned before a shift in the Th17/Treg cell balance is considered to be an important aspect of autoimmunity. The studies discussed here indicate an important role of intestinal TLR triggering in shaping the T helper cell subsets. This makes microbial recognition in the intestine interesting in the context of autoimmune diseases such as RA. The studies quoted here are mainly in mice. The role of intestinal TLR triggering in shaping the T cell response in humans remains mainly unclear and warrants thorough future investigation.

8. Specific Bacteria Shape the Intestinal Immune Response

Round et al. showed that polysaccharide A (PSA) of *B. fragilis* activated TLR2 directly on Tregs, which resulted in activation of these Tregs [90]. However, *B. fragilis* lacking PSA induces a Th17 response, which suggests that PSA induces an anti-inflammatory response through activation of TLR2 [90]. In addition, it was shown that PSA of *B. fragilis* prevents TNBS-induced colitis by inducing IL-10 producing Tregs. However, PSA induced protection was absent in $TLR2^{-/-}$ mice indicating that TLR2 signaling is required for PSA-induced protection [37]. Another study showed that *B. fragilis* is able to release PSA in outer membrane vesicles which are sensed by DCs through TLR2 resulting in induction of Tregs and IL-10 production [91].

A recent study showed that presentation of SFB antigens by $MHCII^{+}$ $CD11c^{+}$ intestinal DCs is required for mucosal Th17 cell differentiation [92]. In MHCII deficient mice, no SFB-induced Th17 differentiation was observed; however recovery of MHCII expression on only $CD11c^{+}$ cells was able to rescue Th17 induction [92]. In mice lacking peripheral lymph nodes and gut-associated lymphoid tissue, SFB induced Th17 priming was unaffected, suggesting that SFB-induced T cell priming takes place in the lamina propria [92]. It is likely that the presence of SFB also triggers TLR signaling. SFB encode four types of flagellin, three of which are recognized by TLR5 [93]. In the mouse gut TLR5 is expressed by $CD11c^{hi}$ $CD11b^{hi}$ DCs in lamina propria which induce the differentiation of Th1 and Th17 cells in response to flagellin [80]. This suggests that SFB skew T cell differentiation via TLR5 triggering. Involvement of TLRs in bacteria-induced mucosal T cell responses and the subsequent systemic autoimmunity seems therefore plausible.

9. Conclusion

Results of multiple studies show that commensal intestinal microbiota affect the Th17/Treg cell balance in the lamina propria and that intestinal Th17 cells can promote experimental arthritis [33, 36, 37, 39]. In addition, studies with experimental models of arthritis suggest that recognition of intestinal microbiota is required for the onset of autoimmune arthritis [13, 36, 47]. It is likely that TLRs mediate the effects of intestinal microbiota on Th cell differentiation in lamina propria. Multiple studies have shown that TLR activation plays an important role in shaping the intestinal T cell subsets [80, 84, 85, 90]. In addition, the study with IL-1Ra/TLR2 and IL-1Ra/TLR4 double gene deficient mice points toward an important role of these TLRs in T cell mediated autoimmune arthritis [13]. It remained unclear how microbiota-induced Th17 cells exactly contribute to systemic autoimmunity in RA. Cross-reactivity of bacteria-specific Th17 cells to endogenous (joint-derived) antigens is a possible mechanism. Another possibility is that microbiota induced T cells promote the differentiation of self-reactive Th17 cells by changing the cytokine environment. Migration of intestinal Th17 cells to the joint and subsequent production of proinflammatory mediators is another possible mechanism. A better understanding of these yet unexplored areas and the involvement of TLR triggering by intestinal microbiota in the gut in systemic autoimmunity might offer new perspectives for manipulating the T cell response in RA patients and may lead to the discovery of new therapeutic targets or even preventive measures.

Conflict of Interests

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

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Research Article

Administration of *Bifidobacterium breve* PS12929 and *Lactobacillus salivarius* PS12934, Two Strains Isolated from Human Milk, to Very Low and Extremely Low Birth Weight Preterm Infants: A Pilot Study

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The preterm infant gut has been described as immature and colonized by an aberrant microbiota. Therefore, the use of probiotics is an attractive practice in hospitals to try to reduce morbidity and mortality in this population. The objective of this pilot study was to elucidate if administration of two probiotic strains isolated from human milk to preterm infants led to their presence in feces. In addition, the evolution of a wide spectrum of immunological compounds, including the inflammatory biomarker calprotectin, in both blood and fecal samples was also assessed. For this purpose, five preterm infants received two daily doses (~10^9 CFU) of a 1 : 1 mixture of *Bifidobacterium breve* PS12929 and *Lactobacillus salivarius* PS12934. Bacterial growth was detected by culture-dependent techniques in all the fecal samples. The phylum *Firmicutes* dominated in nearly all fecal samples while *L. salivarius* PS12934 was detected in all the infants at numerous sample collection points and *B. breve* PS12929 appeared in five fecal samples. Finally, a noticeable decrease in the fecal calprotectin levels was observed along time.

1. Introduction

The gut microbiota of preterm infants is usually described as aberrant when compared to that of healthy term infants. Very often, the former is characterized by a notably lower bacterial diversity, a lower presence of bifidobacteria, and a higher concentration of potentially pathogenic bacteria [1–7]. This may have short-, medium-, and long-term health consequences since early colonizing organisms interact with the intestinal mucosa to shape the developing immune system [8, 9].

In fact, interactions with different components of the microbiota are crucial to the establishment and development of T-cell subsets, including NK, Treg, and Th17 cells, in the appropriate proportions to achieve homeostasis [10].

Many preterm infants lack an important part of transplacental transfer of maternal antibodies since this process occurs mainly in the last third of pregnancy; in addition, they have an impaired pattern-recognition receptor function and a reduced leukocyte endothelial adhesion and extracellular bacterial elimination [11]. Together, these alterations in the microbial colonization pattern and in the maturation of immune system, together with their stay in a hospital environment and other factors, predispose preterm infants to infections and/or to diseases such as necrotizing enterocolitis (NEC) [12–15].

The administration of probiotics to preterm neonates often leads to a decrease in the morbidity and mortality rates, in those of NEC and, in some cases, even in those of sepsis [16–22]. Additional benefits associated with probiotic supplementation in preterm neonates include earlier achievement of full enteral feeding [22], a lower colonization by *Enterobacteriaceae* [23], and a better neurological and immunological evolution [22, 24]. For these reasons, the number of institutions including probiotic supplementation in routine preterm care is increasing rapidly although the safety of probiotics in very low and extremely low birth weight infants is still a matter of debate [25], the mechanisms backing such effects are not well known yet [10], and global conclusions are difficult to establish because different studies usually make use of different probiotic strains, dosages, and/or treatment period.

Human milk is acknowledged as the best feeding option to preterm infants [26, 27] because its use decreases the incidence of many negative outcomes of prematurity, such as late onset sepsis or NEC [28–30]. In addition, human milk seems to be an important source of potentially beneficial bacteria to the infant gut and some strains may find future applications as probiotics for preterm infants [31–36]. In this context, the objective of this exploratory study was to assess early gut colonization in a short cohort of preterm neonates receiving a combination of two probiotic strains isolated from human milk. Furthermore, a wide variety of blood and fecal immunological parameters were assessed in order to elucidate their utility in future studies involving a larger cohort.

2. Materials and Methods

2.1. Study Design and Sampling. Five preterm infants were enrolled in this study within 2 days after their birth. All of them met the following inclusion criteria: birth weight < 1,300 g, gestational age at birth < 29 weeks, and absence of any malformation or metabolic disease at birth. The most relevant demographic and clinical variables from mother-infant pairs were compiled by the Medical Staff of the Service of Neonatology of the Hospital Universitario La Paz (Madrid, Spain). The Ethical Committee on Clinical Research of the Hospital Universitario La Paz of Madrid approved all study protocols (code number: 3551). Samples and clinical information were obtained after written informed consent by the infants' parents. This trial is registered with ClinicalTrials.gov identifier NCT02192996.

After spontaneous meconium expulsion (between the second and the fourth days of life), a mixture of *Bifidobacterium breve* PS12929 and *Lactobacillus salivarius* PS12934, containing ~1×10^9 colony-forming units (CFU) of each strain, was suspended in a sterile saline solution and administered twice a day to the infants through an enteral feeding system. Meconium samples were collected prior to probiotic administration and, later, fecal ($n = 14$) and blood ($n = 10$) samples were collected weekly for up to 28 days. Fecal samples were aliquoted and stored at −80°C or −20°C until microbiological or immunological analysis, respectively. Blood samples were collected in ethylenediaminetetraacetic acid (EDTA) tubes; subsequently, the plasma was obtained within 4 h after extraction and stored at −20°C until analysis.

2.2. Microbiological Analysis. Adequate dilutions of five meconium and fourteen stool samples were spread onto Kanamycin Aesculin Azide Agar (KAA; Oxoid) for *Enterococcus* species isolation; de Man, Rogosa and Sharpe (MRS; Oxoid, Basingstoke, UK) supplemented with L-cysteine (0.5 g/L) (Sigma, St. Louis, USA) (MRScys) for isolation of lactic acid bacteria; MacConkey (MCK; BioMérieux, Marcy l'Etoile, France) for isolation of *Enterobacteriaceae;* Sabouraud Dextrose Chloramphenicol (SDC, BioMérieux) for isolation of yeasts; TOS-Propionate (TOS; Merck, NJ, USA) for isolation of bifidobacteria; and Columbia Nalidixic Acid Agar (CNA, BioMérieux) as a general medium for isolation of other bacterial groups. Plates were aerobically incubated at 37°C for up to 48 h, with the exception of MRScys and TOS plates that were anaerobically incubated (85% nitrogen, 10% hydrogen, and 5% carbon dioxide) in an anaerobic workstation (Mini-MACS Don Whitley Scientific Limited, Shipley, UK) at 37°C for 48 h. Bacterial counts were recorded as the CFU/g of meconium or feces and transformed to $\log_{10}$ values before statistical analysis.

At least one representative of each different colony type obtained from each sample was isolated. Approximately 140 isolates were analyzed by optical microscopy and identified by MALDI-TOF mass spectrometry in a Vitek-MS instrument (BioMérieux, Marcy l'Etoile, France) in the facilities of Probisearch S. L. (Tres Cantos, Spain).

Pulsed-field gel electrophoresis (PFGE) genotyping of all the isolates identified as *L. salivarius* or *B. breve* was carried following a protocol previously described [37]. The profiles were compared to those of *L. salivarius* PS12934 and *B. breve* PS12929, respectively.

2.3. Immunological Analysis. The concentration of 18 cytokines, chemokines, and growth factors, including interleukin (IL) IL-1_β, IL-6, IL-12 (p70), interferon-γ (INF-γ), tumor necrosis factor-α (TNF-α), IL-2, IL-4, IL-10, IL-13, IL-17, IL-8, growth related oncogene-α (GRO-α), macrophage-monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1_β (MIP-1_β), IL-5, IL-7, granulocyte colony stimulating factor (G-CSF), and granulocyte-macrophage colony stimulating factor (GM-CSF), was determined in 5 meconium, 14 feces, and 10 plasma samples by using a Bio-Plex 200 system instrument (Bio-Rad, Hercules, CA) and the Bio-Plex Pro Human Cytokine, Chemokine and Growth Factor Assays (Bio-Rad). Parallel, the concentration of immunoglobulin (Ig) IgG_1, IgG_2, IgG_3, IgG_4, IgM, and IgA was determined using the Bio-Plex Pro Human Isotyping Assay Kit (Bio-Rad).

Before analysis, 0.1 g of meconium and fecal samples was diluted in 0.9 mL of peptone water, homogenized, and centrifuged for 15 min at 14,000 ×g at 4°C; then, supernatants (≥200 μL) were collected. Plasma samples were defrosted and properly diluted immediately before the immunological assay. Analyses were carried out in duplicate following the

manufacturer's protocol and standard curves were performed for each analyte. Lower limit of quantification (LLOQ) was different for each one of the parameters, ranging from 0.02 to 11.74 ng/L for cytokines and from 0.01 to 2 ng/L for immunoglobulins.

Additionally, calprotectin levels (LLOQ: 8 ng/L) were determined in 5 meconium, 14 feces, and 8 plasma samples using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Calpro, Lysaker, Norway) according to the manufacturer's instructions. The standard curve of calprotectin was obtained from triplicates of each assayed concentration and fit to a 4-parameter curve model.

2.4. Statistical Analysis. The statistical analysis was performed using R 2.15.3 (R-project, http://www.r-project.org). When data were not normally distributed, medians and interquartile ranges (Q1 and Q3) were calculated for all sampling times, and means and 95% confidence interval (95% CI) were used for normally distributed data. The richness and diversity of meconium and fecal microbiota were determined by calculating the Shannon-Weaver diversity index, which takes into account the number and evenness of the bacterial species. The Kruskal-Wallis test for nonnormal data or one-way ANOVA test, when data were normally distributed, was used to evaluate the differences between sampling times, in all measured variables, in plasma samples and for the comparison of immunological variables between plasma and fecal samples. The nonparametric Friedman test or one-way ANOVA test, when data were normally distributed, was used in fecal samples to evaluate the differences between sampling times in all measured variables. In all cases, P values of <0.05 were considered to be significant. Redundancy analysis (RDA) was used for exploration of whole data sets and evaluation of the possible relationship between gut colonization and immunological parameters with the clinical status of the participants. Finally, heatmaps of plasma and fecal samples were plotted. To do this, calculation of Kendall's correlation coefficients was performed and Ward agglomeration methods were used to obtain the clustering of the variables and cases matrix.

3. Results

3.1. Demographic and Clinical Characteristics of the Participants. The clinical and demographic data of the mothers and infants who participate in this study are summarized in Table 1. Although five preterm infants were included in this study, there were 2 sets of twins (infants 1 and 2; infants 3 and 4) and, therefore, data were collected from three mothers (Table 1).

All the infants were female and were born by Cesarean section with a mean gestational age of 28 weeks and 2 days. The mean birth weight was 1,020.4 g and the mean height and head circumference were 34.5 cm and 25.0 cm, respectively. These parameters showed Z-scores < 0. Infants stayed in the NICU a mean time of 30.6 days with a mean age at discharge of 65.4 days, which represented a mean corrected gestational age of 37 weeks and 5 days (Table 1). Additional information of clinical features is provided as supplemental information (Supplemental Information 1; see Table S1 of the Supplementary Material available online at http://dx.doi.org/10.1155/2015/538171).

3.2. Microbiological Analysis. Bacterial growth was detected in one meconium sample and in all the fecal samples. Differences in the bacterial counts of fecal samples were evaluated by nonparametric Friedman test on days 7, 14, 21, and 28 (data not shown).

Globally, the phylum *Firmicutes* predominated in all the fecal samples except in those belonging to infant 5 where *Proteobacteria* was present in a similar proportion (Figure 1(a)). On the other hand, *Proteobacteria* dominated at the 14th day of intervention in fecal samples of the siblings 3 and 4. The phylum *Actinobacteria*, mainly represented by the genus *Bifidobacterium*, was isolated from day 7 although not in all the fecal samples (Figure 1(a)).

Among the *Firmicutes*, the genera *Enterococcus* and *Lactobacillus* were isolated from all the fecal samples except in that of infant 2 at day 21 where *Lactobacillus* could not be detected. The bacterial counts of *Enterococcus* decreased significantly from day 7 to day 21 of treatment ($P = 0.043$) from 10.00 to 8.30 log CFU/g. In contrast, *Lactobacillus* counts increased from 6.60 log CFU/g after 7 days of probiotic treatment to 8.32 log CFU/g at the end of the intervention; in this case, the differences were not statistically significant due to both the individual variability and the small cohort. The genus *Staphylococcus* was mainly isolated in the first weeks of the study from meconium and 7-day fecal samples (Figure 1(b)) with median counts of 4.30 and 9.44 log CFU/g, respectively.

In relation to *Proteobacteria*, the genus *Enterobacter* was isolated from all the fecal samples except from two from infant 2 (days 7 and 21) and from one of infant 3 at day 28 (Figure 1(b)). Similarly, the genus *Klebsiella* was isolated from all fecal samples except from two collected at day 7 (siblings 3 and 4) and one at day 21 (infant 2). Bacterial counts of these two genera were significantly different at every sampling day ($P = 0.007$ and 0.046 for *Enterobacter* and *Klebsiella*, resp.) and a decrease was observed in *Klebsiella* median counts (from 10.19 log CFU/g at day 7 to 8.48 log CFU/g at day 28).

Finally, the *Bifidobacterium* median counts oscillated between 7.98 and 9.98 log CFU/g in the 6 fecal samples where this genus was detected (Figure 1(b)).

The SDI of the fecal samples fluctuated during the study probably due to the different antibiotic treatments that the infants received (Figure 1(c)).

In order to detect the presence of *L. salivarius* PS12934 and *B. breve* PS12929 in fecal samples, all the fecal isolates belonging to such species were PFGE genotyped. This technique revealed that *L. salivarius* PS12934 was present in all the infants at numerous sampling points while *B. breve* PS12929 could be detected after day 14.

The heatmap obtained from the fecal samples at different sampling times of all the infants is shown in Figure S1. The dendrogram resulted after Kendall correlation coefficient calculation highlights the similar species profile of fecal

Table 1: Epidemiological and clinical relevant data from the mother-infant pairs of this study.

Mothers	1		2		3
Age (years)	30		18		28
Fever	No		Yes		No
Leukocytosis (>15,000 leukocytes/μL)	No		Yes		Yes
C-reactive protein (mg/L)	26		7.6		40
Antenatal antibiotics treatment	Yes		Yes		Yes
Antenatal corticosteroids treatment	Complete		Uncomplete		Complete
Chorioamnionitis	No		Yes		Yes
Type of delivery	C-section		C-section		C-section
Multiple delivery	Yes		Yes		No
Infants	1	2	3	4	5
Rupture of fetal membranes (h)	672	0	0	0	432
Twin position	1	2	2	1	1
Sex	F	F	F	F	F
Gestational age (wk)	28 + 5	28 + 5	28 + 6	28 + 6	27 + 2
Birth weight (g) (Z-score)	1070 (−0.71)	980 (1.01)	1082 (−0.66)	1200 (−0.26)	770 (−1.02)
Birth height (cm) (Z-score)	36 (−1.3)	36 (−1.3)	36 (−1.3)	36 (−1.3)	32 (−1.8)
Birth head circumference (cm) (Z-score)	26 (−0.8)	26 (−0.8)	25.5 (−1.1)	26 (−0.8)	24 (−0.8)
Apgar score at 1 min	8	9	8	5	7
Apgar score at 5 min	9	9	9	7	8
Revival	Ventilation	No	Ventilation	Ventilation	Ventilation
PDA	Yes	No	Yes	Yes	No
Meconium spontaneous expulsion	Yes	Yes	Yes	Yes	Yes
Meconium expulsion (h)	24	9	48	36	14
Probiotic starting age (d)	2	2	2	2	4
Probiotic treatment length (d)	18	18	31	19	25
NICU stay (d)	18	8	14	64	49
Age at discharge (d)	51	51	60	64	101
Corrected gestational age at discharge (wk)	36	36	37	38	42
Death	No	No	No	Yes	No

PDA: patent ductus arteriosus; NICU: neonatal intensive care unit.
Antenatal corticosteroid treatment was uncompleted or complete when mother received one or two doses of betamethasone, respectively, within one week and 24 h before delivery.
Apgar test ranged from 1 to 10: less than 5 means risk; up to 7 means normal.
Twin position means the position at birth, 1 being the infant who was nearest to the cervix.

samples of infant 2 at different sampling times and the almost identical species profile of fecal samples from days 7 and 14 of twins 3 and 4.

3.3. Immunological Analysis. A wide range of immune compounds were analyzed in plasma and fecal samples of the preterm infants throughout the study. An exploratory screening, using a principal component analysis (PCA) to detect outliers, revealed that the 7th day fecal sample from infant 4 was very different from the rest of the sample sets (data not shown). This infant was suffering a gastric bleeding at this sampling time and, therefore, this sample was excluded from the results of data sets.

Median values of the immune compounds concentrations in meconium and, also, in fecal samples at 7th and 14th days of probiotic supplementation are shown in Table 2. In general, the values obtained for all the immune factors showed a high interindividual variability in both detection frequencies and amounts. The levels of some immune compounds changed throughout the study; those of IgG_2 and MCP-1 decreased progressively ($P = 0.074$ and $P = 0.076$, resp.) while that of IgA increased (>50 times) from meconium to fecal samples obtained at day 7 after birth ($P = 0.074$) (Table 2). However, only the inflammatory biomarker calprotectin decreased significantly along sampling time ($P = 0.041$).

Plasma concentrations of the immune compounds are shown in Table 3 and, as it can be observed, no significant changes were found. Globally, chemokines and proinflammatory compounds tended to decrease, with the exception of IL-12 and TNF-α. The levels of the latter and those of the anti-inflammatory compounds remained very constant along time. Plasma immunoglobulins also showed a high individual variability although all decreased, with the exception of IgG4 and IgM (Table 3).

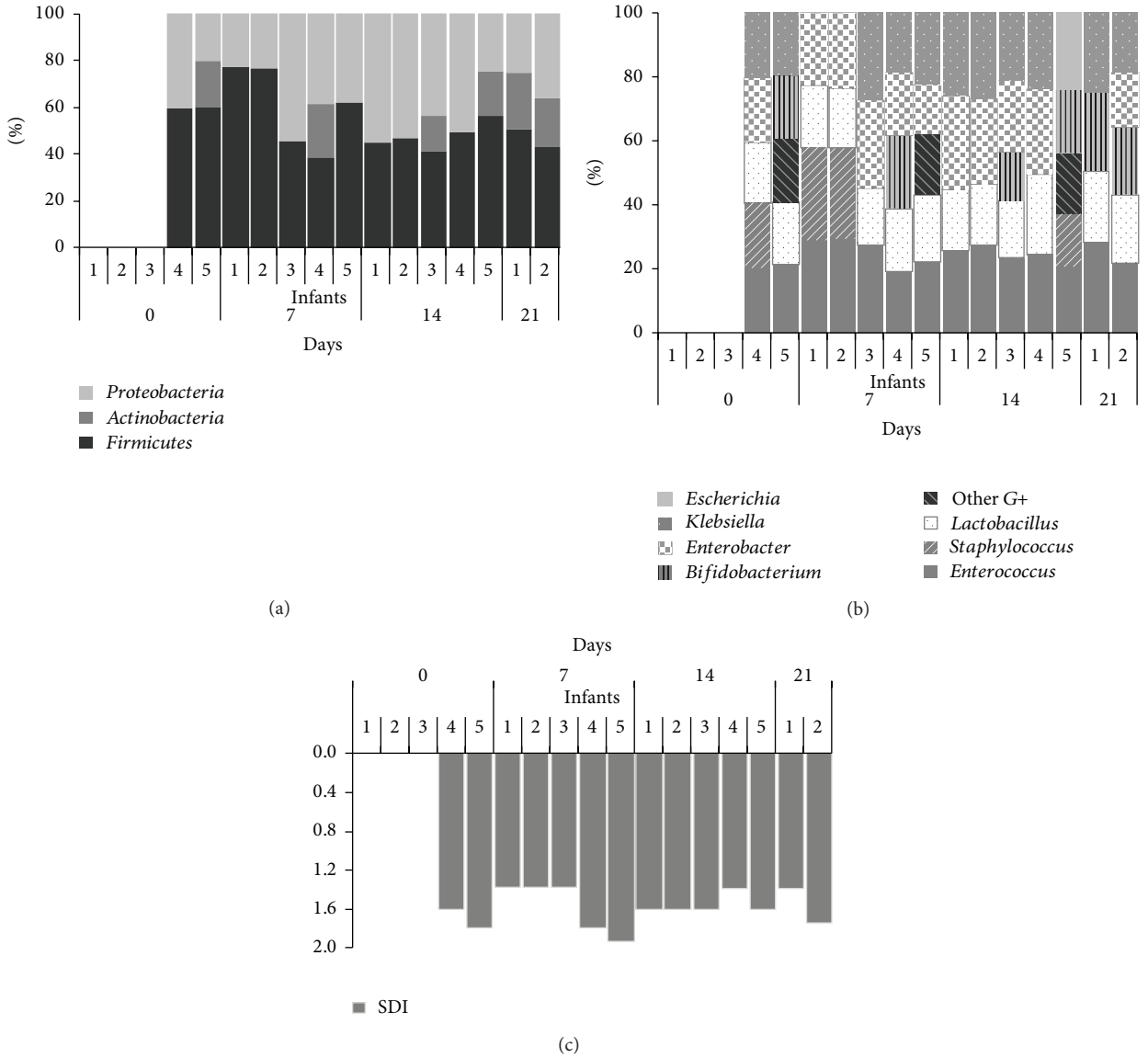

Figure 1: Phyla (a), genera (b), and bacterial diversity assessment by the SDI (c) of the microbiota of the meconium and fecal samples analyzed in this study. The relative contributions of the phyla and genera to the microbiota of the infant's gut and the SDI values were labeled per case and sampling time.

The plasma concentrations of the different immune compounds were compared with their respective fecal values. All the immunoglobulins, with the exception of IgA, were significantly different in both types of samples. Among the remaining immune parameters, calprotectin, IL-10, GRO-α, and GM-CSF were significantly higher in feces ($P = 0.000$, $P = 0.045$, $P = 0.048$, and $P = 0.000$, resp.) while IL-8, MCP-1, and MIP-1_β were more abundant in plasma ($P = 0.012$, $P = 0.000$, and $P = 0.001$, resp.) (Table S2).

3.4. Multivariate Analysis of the Studied Population. A multivariate analysis was performed for investigating the possible relationship between clinical features and the immunological and microbiological profiles of fecal and plasma samples. The clinical variables considered were the following: antibiotherapy (Antibiotics); air way resume (AWResume) including ventilation, caffeine, and surfactant treatment; C-RP; hemoglobin amounts (Hb); hematocrit percentage (Hcte); ibuprofen treatment (Ibu.T); ibuprofen doses (Ibu.doses); number of stools per day (N°.stools); nutrition resuming the median feeding type (Nutrition); patent ductus arteriosus (PDA); Sepsis; spontaneous stools (Spont.stools); Transfusion; and Weight.

The redundancy analysis (RDA) of the above-mentioned variables for fecal samples is shown in Figure 2. The obtained model explains the 33% of the variability and the ANOVA test of the model was statistically significant ($P = 0.020$). The meconium samples were located opposite to microbial growth and in coincidence with the constrained antibiotic

Table 2: Frequency and concentration of immune compounds in fecal samples ($N = 14$) along time.

	Day 0 ($N = 5$)		Day 7 ($N = 4$)		Day 14 ($N = 5$)		P value*
	n (%)	Median (IQR) (mg/kg)	n (%)	Median (IQR) (mg/kg)	n (%)	Median (IQR) (mg/kg)	
Immunoglobulins							
IgG_1	5 (100)	3.95 (1.23–6.36)	4 (100)	0.45 (0.23–0.80)	5 (100)	1.26 (0.47–2.43)	0.819
IgG_2	5 (100)	23.82 (23.19–24.17)	4 (100)	2.98 (2.46–3.97)	5 (100)	2.66 (2.60–3.62)	0.074
IgG_3	4 (80)	0.02 (0.01–0.02)	1 (25)	0.01 (0.01–0.01)	2 (40)	0.22 (0.11–0.32)	0.424
IgG_4	5 (100)	0.03 (0.02–0.14)	4 (100)	0.02 (0.01–0.03)	5 (100)	0.03 (0.00–0.06)	0.449
IgM	4 (80)	2.72 (0.19–8.73)	3 (75)	1.10 (0.87–6.00)	5 (100)	2.79 (0.44–10.02)	0.819
IgA	5 (100)	3.57 (0.88–21.73)	4 (100)	201.23 (35.09–356.74)	5 (100)	7.49 (2.96–7.78)	0.074
		(ng/kg)		(ng/kg)		(ng/kg)	
Proinflammatory							
Calprotectin†	5 (100)	309.50 (282.00–343.90)	4 (100)	144.80 (132.30–180.40)	5 (100)	38.42 (34.16–63.96)	0.041
IL-1_β‡	1 (20)	31.47	3 (75)	41.34 (8.00–74.68)	3 (60)	39.20 (−36.24–114.64)	0.937
IL-2	1 (20)	8.47	1 (25)	8.18	0 (0)	—	0.368
IL-6	0 (0)	—	0 (0)	—	1 (20)	27.44	0.368
IL-12 (p70)	2 (40)	29.07 (28.82–29.32)	2 (50)	37.13 (36.38–37.89)	1 (20)	82.98	0.926
IL-17	2 (40)	72.94 (62.76–83.11)	2 (50)	66.08 (64.46–67.71)	2 (40)	69.31 (65.15–73.48)	1.000
IFN-γ	4 (80)	214.90 (190.40–238.30)	4 (100)	299.80 (255.40–320.80)	4 (80)	248.10 (215.80–265.50)	0.449
TNF-α	1 (20)	20.87	0 (0)	—	0 (0)	—	0.368
		(ng/kg)		(ng/kg)		(ng/kg)	
Anti-inflammatory							
IL-4	3 (60)	2.74 (2.43–3.48)	4 (100)	2.63 (2.49–2.85)	3 (60)	2.12 (2.06–2.26)	0.268
IL-5	0 (0)	—	0 (0)	—	0 (0)	—	—
IL-10	1 (20)	25.62	2 (50)	37.21 (35.85–38.57)	3 (60)	39.20 (38.66–53.87)	0.319
IL-13	0 (0)	—	0 (0)	—	0 (0)	—	—
		(ng/kg)		(ng/kg)		(ng/kg)	
Chemokines							
IL-8	4 (80)	20.94 (19.00–23.82)	3 (75)	16.16 (15.56–17.20)	2 (40)	17.05 (16.45–17.64)	0.128
GRO-α‡	5 (100)	206.30 (117.04–295.57)	3 (75)	222.10 (−77.61–521.80)	4 (80)	263.50 (261.05–265.88)	0.763
MCP-1	5 (100)	20.08 (15.02–28.89)	2 (50)	18.37 (16.82–19.93)	3 (60)	16.98 (14.21–17.34)	0.076
MIP-1_β	5 (100)	53.79 (52.03–68.66)	4 (100)	58.16 (46.46–66.42)	4 (80)	49.60 (35.16–69.89)	0.449
		(ng/kg)		(ng/kg)		(ng/kg)	
Haematopoietic stimuli							
IL-7	0 (0)	—	0 (0)	—	0 (0)	—	—
G-CSF	1 (20)	28.99	0 (0)	—	0 (0)	—	0.368
GM-CSF	5 (100)	1729.00 (1086.00–2312.00)	4 (100)	1830.00 (1648.00–2010.00)	4 (80)	1879.00 (1783.00–1920.00)	0.819

Levels of immune compounds were expressed as median and interquartile range (IQR) when data were not normally distributed and as mean and 95% confidence interval (95% CI) when they were. *Friedman test was used to determine the differences between fecal samples along time when data were not normally distributed and one-way ANOVA when they were. †Concentration was expressed as ng/Kg of feces for all the proinflammatory parameters with the exception of calprotectin whose units were mg/Kg. ‡Normally distributed.

vector. Although the rest of fecal samples showed a less clear separation, the evolution of microbial colonization can be observed along the RDA1 axis in coincidence with the constrained vectors for AWResume, Nutrition, Spont.stools, PDA, and Transfusion and in opposite not only with the antibiotics and C-RP vectors, but also with the coordinates of proinflammatory compounds, such as calprotectin, MCP-1, MIP-1_β, TNF-α, and IL-8 (Figure 2).

The RDA of plasma samples (Figure 3) explains the 70% of the variability and the ANOVA test of the model was statistically significant ($P = 0.010$). The bidimensional plot shows two points clearly separated from the others: infant 4 at day 19 and infant 5 at day 7. Three different situations were observed; on the one hand coordinates from infants 1, 2, and 3 did not change among sampling times, while on the other infant 5 showed a normalization far away of proinflammatory variables and hematological parameters coordinates; and finally infant 4 that initially was close to her corresponding twin and the rest of participants appeared at day 19, in the positive RDA1 and RDA2 coordinates, related to

Table 3: Frequency and concentration of immune compounds in plasma samples ($N = 8$) along time.

	Day 7 ($N = 3$)		Day 14 ($N = 5$)		P value*
	n (%)	Median (IQR) (mg/L)	n (%)	Median (IQR) (mg/L)	
Immunoglobulins					
IgG_1	3 (100)	2159.80 (2075.95–2174.30)	5 (100)	1727.30 (1205.50–2029.60)	0.297
IgG_2	3 (100)	1135.20 (796.03–1147.50)	5 (100)	741.24 (683.84–930.95)	0.456
IgG_3	3 (100)	52.54 (46.75–64.53)	5 (100)	43.91 (41.35–48.14)	0.297
IgG_4	3 (100)	23.25 (22.02–67.30)	5 (100)	44.66 (10.59–49.08)	0.655
IgM	3 (100)	263.75 (176.71–934.18)	5 (100)	335.18 (261.41–366.78)	0.882
IgA	3 (100)	27.03 (18.20–40.41)	5 (100)	4.44 (4.00–14.31)	0.101
		(ng/L)		(ng/L)	
Proinflammatory					
Calprotectin†	3 (100)	0.86 (0.47–1.11)	5 (100)	0.37 (0.37–0.63)	0.456
IL-1_β‡	1 (33)	15.81	0 (0)	—	—
IL-2	2 (67)	35.41 (19.34–51.48)	3 (60)	9.70 (6.54–11.23)	1.000
IL-6	3 (100)	24.14 (15.99–65.65)	5 (100)	17.06 (10.10–19.24)	0.297
IL-12 (p70)	3 (100)	27.55 (19.89–91.22)	5 (100)	28.35 (22.71–29.16)	0.882
IL-17	1 (33)	167.20	2 (40)	35.66 (34.65–36.67)	0.221
IFN-γ	2 (67)	670.07 (371.30–968.83)	4 (80)	150.06 (67.73–225.91)	0.643
TNF-α	3 (100)	15.06 (11.35–66.01)	5 (100)	13.14 (11.83–20.40)	0.764
		(ng/L)		(ng/L)	
Anti-inflammatory					
IL-4	3 (100)	1.95 (1.57–7.96)	5 (100)	1.99 (1.69–2.90)	0.882
IL-5	1 (33)	39.43	1 (20)	9.65	0.317
IL-10	3 (100)	11.80 (11.10–69.56)	3 (60)	20.11 (16.02–22.68)	0.513
IL-13	1 (33)	11.27	1 (20)	5.06	0.317
		(ng/L)		(ng/L)	
Chemokines					
IL-8	3 (100)	31.69 (24.45–85.28)	5 (100)	29.76 (22.37–30.79)	0.655
GRO-α‡	2 (67)	204.44 (−1859.94–2268.82)	3 (60)	55.42 (45.11–65.73)	0.306
MCP-1	3 (100)	193.91 (123.14–204.59)	5 (100)	88.62 (60.77–192.54)	0.456
MIP-1_β	3 (100)	234.90 (210.30–292.20)	5 (100)	174.60 (150.00–250.80)	0.297
		(ng/L)		(ng/L)	
Haematopoietic stimuli					
IL-7	2 (67)	28.14 (17.72–38.57)	3 (60)	10.48 (8.84–12.50)	0.564
G-CSF	3 (100)	30.89 (23.21–96.81)	5 (100)	47.27 (41.84–51.46)	0.655
GM-CSF	3 (100)	248.80 (191.00–299.30)	4 (80)	132.35 (114.97–151.83)	0.157

Levels of immune compounds were expressed as median and interquartile range (IQR) when data were not normally distributed and as mean and 95% confidence interval (95% CI) when they were. *Kruskal-Wallis test was used to determine the differences between blood samples along time when data were not normally distributed and one-way ANOVA test when they were. †Concentration was expressed as ng/L of plasma for all the proinflammatory parameters with the exception of calprotectin whose units were mg/L. ‡Normally distributed.

constrained variables vectors corresponding to C-RP, Sepsis, and PDA reflecting the clinical worsening of this infant at this moment.

Those clinical categorical variables explained by the fecal and plasma RDAs were used, together with the microbiological, immunological, and clinical parameters, to create two heatmaps, one for each type of samples (Figure 4). The results from all the available fecal samples of the 5 infants were used to perform the heatmap showed in Figure 4(a). The samples' dendrogram shows two arms which clearly separate meconium and feces. The variables' dendrogram, obtained after samples clustering, shows two principal arms. The lower one is divided into two: the first of them that included clinical variables, some bacterial genera such as *Escherichia*, *Staphylococcus*, *Bifidobacterium*, and *Paenibacillus,* immunoglobulins IgG_3 and IgG_4, and cytokines IL-4, IL-13, and IL-2 and the second one that included antibiotherapy, IgG_1, IL-5, IL-6, and IL-7. The upper arm is also divided and included the rest of the bacterial genera and immunological parameters together with the weight of the infants. The results obtained for all the

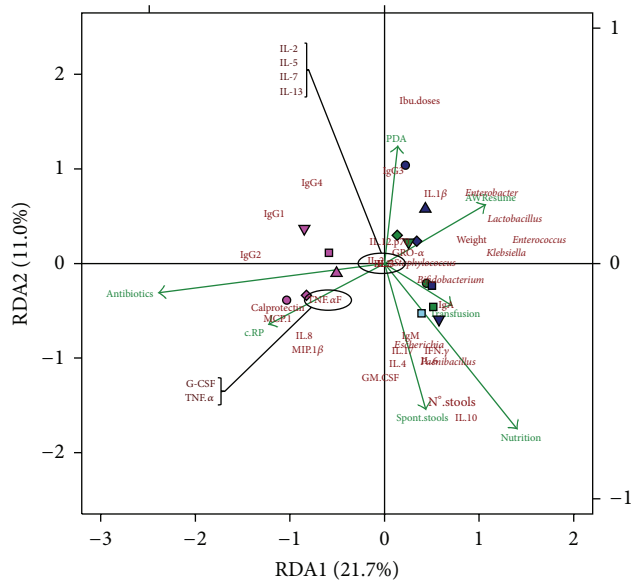

FIGURE 2: Redundancy analysis of the fecal samples obtained at different sampling times from the preterm infants. Cases were represented with points and then labeled per infant (1: circle, 2: square, 3: diamond, 4: triangle, and 5: inverted triangle) and sampling time (0: medium violet red, 7: green, 14: midnight blue, and 21: sky blue) Quantitative variables matrix, including the hematological and immunological parameters, ibuprofen doses (Ibu.doses), number of stools per day (N°.stools), and weight, was represented with each variable name or abbreviator in dark red color; clinical categorized observations vectors matrixes were used as constrained variables (airway resume (AWResume), antibiotherapy (Antibiotics), C-RP, ibuprofen treatment (Ibu treatment), nutrition type (Nutrition), patent ductus arteriosus (PDA), Sepsis, spontaneous stools (Spont.stools), and Transfusion) and represented as vectors in green color. The bidimensional RDA plot explains the 33% of the variability and showed a P value of 0.020 after 299 permutations when ANOVA test of the model was performed.

available plasma samples from the 5 participants were used to perform the heatmap showed in Figure 4(b). The plasma samples' dendrogram shows two groups, in one of them 2 samples of the infant 2 cluster together with her twin at day 14 and samples of infant 5 clusters together with sample of infant 1 at day 7. In the second arm, siblings 3 and 4 at day 14 of probiotic supplementation initiate the clustering, which ends with sample of day 7 of infant 5 and sample of day 19 of infant 4 as previously observed in Figure 3. The dendrogram related to variables, obtained after infants clustering, showed two principal arms: one of them included clinical variables, hematological parameters, calprotectin, IL-1$_\beta$, IL-4, IL-13, immunoglobulins IgA and IgG$_3$, ibuprofen doses, and Hb and the second principal arm also divided including most of the cytokines, chemokines, and growth factors, the rest of the immunoglobulins, the birth weight, and the Hcte.

4. Discussion

In this pilot study, the bacterial composition of fecal samples obtained from five preterm infants supplemented with a probiotic mixture of two strains isolated from human milk during their earlier days of life at the NICU was assessed. In addition, a wide range of cytokines, chemokines, growth factors, and immunoglobulins were determined in all plasma, meconium, and fecal samples in order to describe their immunological profiles, their changes over time, and their potential relationship with bacterial colonization and clinical features.

The results obtained in this study suggest that the administration of *B. breve* PS12929 and *L. salivarius* PS12934 to preterm infants may increase the levels of *Lactobacillus* and *Bifidobacterium* in their feces. In fact, *L. salivarius* PS12934 could be isolated from the fecal samples of the preterm infants from day 7 of intervention and its presence remained constant throughout the study. *B. breve* PS12929 was also isolated from fecal samples after day 14 of intervention and, since then, it had increasing presence in the fecal samples. The higher frequency and concentration of *Lactobacillus* and *Bifidobacterium* in the feces analyzed should be considered a positive outcome of this study because the pattern of gut colonization in this specific infant population is usually characterized by a dominance of opportunistic pathogens and a reduced (or even absent) population of lactobacilli and bifidobacteria [7, 15, 38]. In fact, the SDI values of the fecal samples were higher than those previously described in a similar cohort that did not receive probiotics [7]. The intensive use of antibiotics at the NICU has been related to a dramatic reduction in microbial diversity and to increased presence of *Enterobacter* [39]; however, the administration of the probiotic strains in this study seemed to, somehow, compensate the antibiotic side effects.

Up to the present, there has been a complete lack of studies focused on fecal immunological parameters among preterm infants. As a consequence, there are no reference values for this population and, therefore, this study may constitute a starting point for future investigations. Although scarce, there are some studies dealing with blood immune

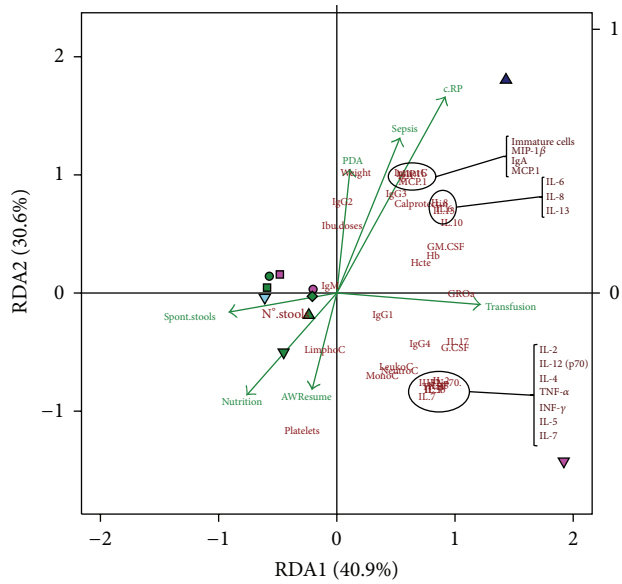

FIGURE 3: Redundancy analysis of the blood samples obtained at different sampling times from the preterm infants. Cases were represented with points and then labeled per infant (1: circle, 2: square, 3: diamond, 4: triangle, and 5: inverted triangle) and sampling time (0: medium violet red, 7: green, 14: midnight blue, and 21: sky blue) Quantitative variables matrix, including the hematological and immunological parameters, ibuprofen doses (Ibu.doses), number of stools per day (N°.stools), and weight, was represented with each variable name or abbreviator in dark red color; clinical categorized observations vectors matrixes were used as constrained variables (airway resume (AWResume), antibiotherapy (Antibiotics), C-RP, ibuprofen treatment (Ibu treatment), nutrition type (Nutrition), patent ductus arteriosus (PDA), Sepsis, spontaneous stools (Spont.stools), and Transfusion) and represented as vectors in green color. The bidimensional RDA plot explains the 71% of the variability and showed a P value of 0.010 after 199 permutations when ANOVA test of the model was performed.

compounds in preterm babies. Globally, they show that there are differences in the blood immune profiles depending on the infant gestational age [40–42]. It is important to note that the volume of the blood samples that are usually extracted from preterm neonates for clinical purposes is usually very low. Therefore, multiplex technologies, as the one used in this study, are required in order to be able to simultaneously analyze a high number of immune compounds [42, 43].

The results obtained in this study must be interpreted with caution due to three relevant limitations: the absence of a control group, a very small population size, and the scarcity of previous studies dealing with the immunological features of very low or extremely low weight birth infants and how they may be affected after a probiotic treatment. In this context, the levels of IL-8 found in a previous work focused on term neonates [44] were lower than those obtained in this study while those of IL-4 and IL-6 were similar; in contrast, the values of the remaining immunological parameters were higher in all the sampling times. This may illustrate the immune immaturity of these preterm infants. Similarly, levels of IL-2, IL-6, IL-8, IL-10, IL-13, IL-17, TNF-α, IFN-γ, and MCP-1 were lower in preterm infants born at 30–32 weeks than in those born after 36 weeks, indicating a lower stimulation or activation of Th1 cells and antigen-presenting cells in preterm babies as the gestational age decreases [42]. In the present work, the concentrations of the chemokines IL-8 and MCP-1 and those of the cytokines IL-4, IL-10, and IL-13, which are related to anti-inflammatory processes, were higher than those reported for preterm neonates born at 30–32 weeks and similar to those found in older infants (>36 weeks) [42]. This suggests that the administration of the probiotic strains may exert a modulatory effect on the immune system of these infants.

In addition, very low or extremely low weight birth infants usually require a strong and highly individualized medical intervention (antibiotics, oxygen, corticoids, ibuprofen, transfusions, etc.) for, at least, the first days of life due to a wide variety of life-threatening conditions. Such conditions, together with their corresponding treatments, may alter the microbial gut colonization process and, also, the infants' immune responses. Therefore, it is very difficult to obtain a homogeneous VLBW or ELBW infant population even in cohorts with a high number of infants. This is another important limitation that interventional studies, such as probiotic administration, must face when dealing with such infant subpopulations.

Despite all the limitations cited above, it is also true that a significant reduction of the inflammatory marker calprotectin in feces was observed throughout the probiotic treatment, which is in agreement with previous studies [3, 45, 46]. This is a promising outcome that must be reassessed in the future in a placebo-controlled intervention involving a large cohort.

The increase in IgA observed at day 7 may be due to the microorganisms colonizing the preterm gut, which triggers the production of this Ig by the gut-associated lymphoid tissue (GALT) [47]. IgA has the ability to penetrate the gut mucosal surface in conjunction with antigens and, as a

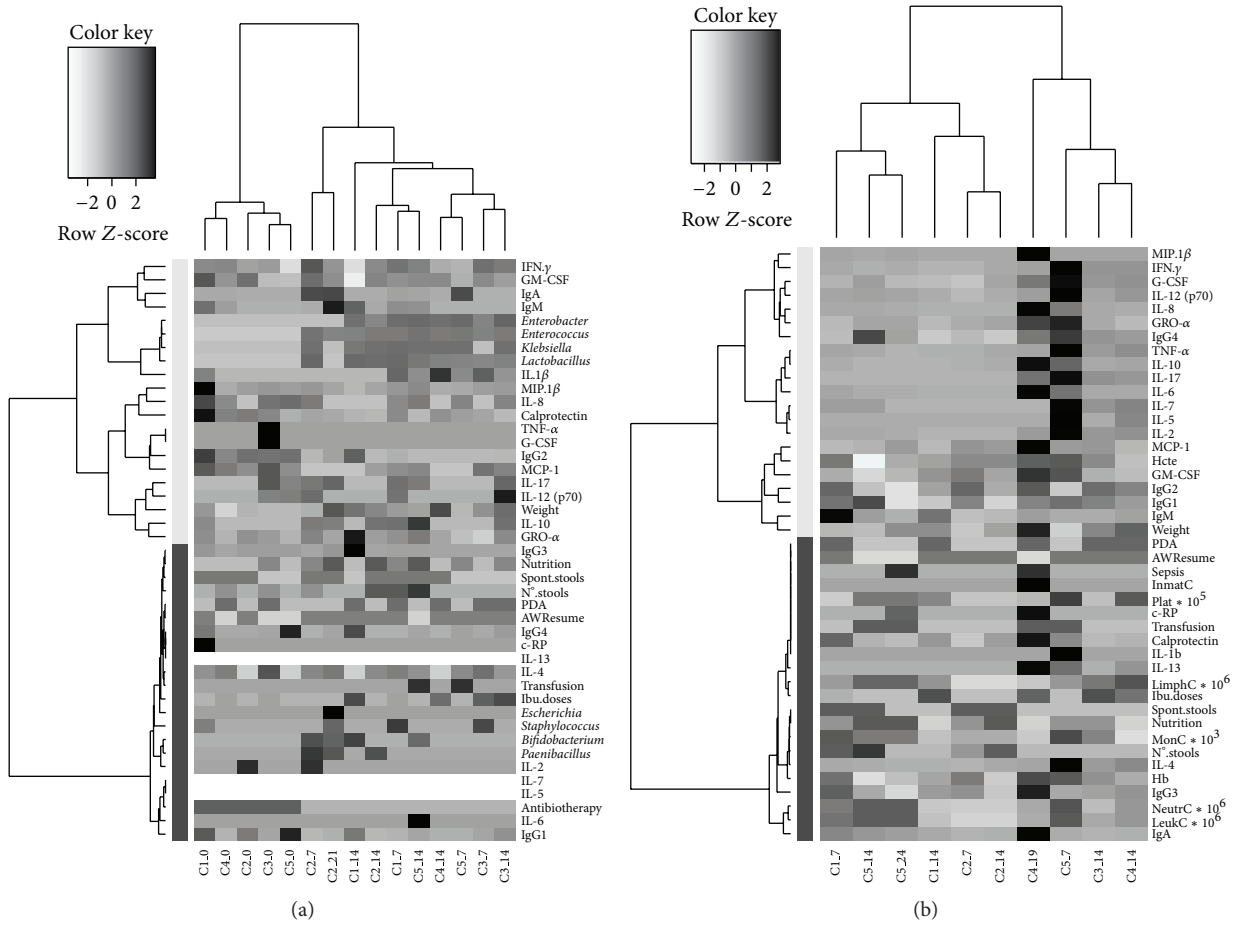

Figure 4: Heatmaps of fecal (a) and plasma (b) samples matrixes, considering all the quantitative variables measured and the categorized variables that were explained in the correspondent RDA, were performed. Clustering functions were applied to samples and variables after scaling the whole data set. In order to represent as much information as possible in the plot, the heatmaps were plotted using the measured data matrix scaled per variable and columns were labeled per infant and sampling time.

consequence, to induce effector immune responses, playing a key role in the maintenance of intestinal microbiota and immune homeostasis [48].

The multivariate analysis applied to all the available plasma and fecal samples from the five preterm infants revealed a clear relation between the parameters assessed in this work and the clinical evolution of the infants. In the fecal-related RDA, microbial colonization acted as the principal agent opposed to the levels of certain proinflammatory immunocompounds and in agreement with the clinical variables associated with an improvement of the infants' health. Since bacterial species coordinate coefficients had positive values in the RDA1 axis, calprotectin and other proinflammatory parameters, such as IL-8, MIP-1$_\beta$, MCP-1, G-CSF, or TNF-α, showed negative values. RDA1 axis coordinate coefficients for IgG_1, IgG_2, and IgG_4 were negative while those for the secretory IgA and IgM immunoglobulins were positive. Although these findings must be taken with caution due to the inherent limitations of this work and to the high number of potential interactions and confusing factors, it should be noted that an abnormal gut microbial colonization predisposes the neonatal intestine to inflammation and to a cascade of proinflammatory and anti-inflammatory cytokines responses [49]. On the other hand, the evolution of the infants' microbiota was different than that observed in other preterm infants devoid of probiotic treatment [7] but similar to that of preterm neonates that received probiotics [23].

Finally, the dendrograms obtained for samples and variables represented in the heatmaps (Figure 4) seem to reinforce the hypothesis that probiotic strains may contribute to the development of a normal gut bacterial colonization and that this process is essential to reduce the health burden associated with prematurity [50, 51]. Although the present cohort was very small, a promising influence of the probiotic supplementation on gut colonization was observed, including an increase in bacterial diversity and in the presence of lactobacilli and bifidobacteria at relatively high levels.

Although multicenter, randomized clinical trials involving bigger cohorts and longer intervention times with

these strains will be required to determine their efficacy in the prevention of sepsis or NEC, the results of this work may provide useful information for future studies dealing with probiotic gut colonization and, particularly, with the detection and quantification of fecal and blood immunocompounds in preterm infants.

Conflict of Interests

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

Authors' Contribution

Laura Moles, Esperanza Escribano, and Javier de Andrés contributed equally to this work.

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Research Article

Lactic Acid Bacteria Strains Exert Immunostimulatory Effect on *H. pylori*-Induced Dendritic Cells

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The aim of this study was to find out if selected lactic acid bacteria (LAB) strains (antagonistic or nonantagonistic against *H. pylori in vitro*) would differ in their abilities to modulate the DCs maturation profiles reflected by their phenotype and cytokine expression patterns. *Methods*. Monocyte-derived DCs maturation was elicited by their direct exposure to the LAB strains of *L. rhamnosus* 900 or *L. paracasei* 915 (antagonistic and nonantagonistic to *H. pylori*, resp.), in the presence or absence of *H. pylori* strain *cagA*+. The DCs maturation profile was assessed on the basis of surface markers expression and cytokines production. *Results*. We observed that the LAB strains and the mixtures of LAB with *H. pylori* are able to induce mature DCs. At the same time, the *L. paracasei* 915 leads to high IL-10/IL-12p70 cytokine ratio, in contrast to *L. rhamnosus* 900. *Conclusions*. This study showed that the analyzed lactobacilli strains are more potent stimulators of DC maturation than *H. pylori*. Interestingly from the two chosen LAB strains the antagonistic to *H. pylori*-*L. rhamnosus* strain 900 has more proinflammatory and probably antibactericidal properties.

1. Introduction

Treatment of *H. pylori* infection is a long-term and not always efficient process. Antibiotic therapy leads to eradication of this pathogen in approximately 60–90% of the cases. However, even the efficiently treated individuals are still at risk of reinfection [1, 2]. Administration of selected strains of lactic acid bacteria (LAB), a component of intestinal microbiota, is an established factor improving efficiency of *H. pylori* eradication [3–5]. Some LAB strains prevent *H. pylori* colonization of gastric mucosa, thus decreasing the number of these bacteria in the stomach. The principle mechanism behind this effect of LAB is synthesis of lactic acid, which alters gastric pH and inactivates urease, a pivotal enzyme for *H. pylori* viability [6, 7]. The antagonism between LAB and *H. pylori* can be also associated with synthesis of other antibacterial compounds, for example, bacteriocins, autolysins, or thermostable proteins [7]. Apart from the bacterial antagonism, recent studies center around potential immunological mechanisms through which LAB can support eradication of *H. pylori* and attenuate inflammation of gastric mucosa. These include influence of LAB on enhanced local synthesis of IgA, modulation of specific IgG levels [8, 9], and induction of pro- and anti-inflammatory cytokine profiles [10].

Acute inflammation observed during an early phase of *H. pylori* infection is characterized by enhanced production

of proinflammatory Th1/Th17 cytokines, presence of cell-mediated cytolysis, plasma cell infiltration, and synthesis of specific antibodies in the stomach and duodenum [11–14]. In turn, chronic inflammation associated with long-term gastrointestinal colonization by this pathogen is reflected by suboptimal Th1 response observed at later stages of the infection, as well as by an increase in Treg lymphocyte count [15–18]. The type of immune response is to a large extent determined by the activity of antigen-presenting cells (APCs), especially dendritic cells (DCs) which constitute a "link" between the nonspecific and specific responses [19–21]. Acute *H. pylori* infection is associated with migration of DCs to the antral mucosa [22–24]. The increased inflow of DCs during an early phase of inflammation results mainly from their ability to induce immune response against *H. pylori*. However, it is not reflected by elimination of this microorganism; phenotypic and functional changes of DCs result in development of chronic inflammation and tolerance of these cells to *H. pylori* antigens [15, 25, 26]. Therefore, two questions arose regarding whether this process could be modulated by intestinal microbiota, namely, by selected LAB strains, and whether antagonism between the latter bacteria and *H. pylori*, associated with release of antibacterial compounds, might modulate activity of the immune system. Moreover, still little is known on the immunological mechanisms associated with the development of *H. pylori* infection in presence of various strains of commensal bacteria [5, 7].

2. Material and Methods

2.1. Bacteria and Their Selection. The studied LAB strains were selected from among 29 strains of *Lactobacillus casei, Lactobacillus rhamnosus, Lactobacillus paracasei,* and *Lactobacillus plantarum*. The strains were identified by the sequencing of ribosomal RNA-encoding genes [27]. All the strains originated from the Pure Culture Collection of Industrial Microorganisms at the Technical University of Lodz (ŁOCK). The activity of interstrain antagonism was investigated using the agar slab method [28]. The method was based on the observation of parallel growth of the strains under study (the indicator—*H. pylori cagA*+ strain 95 and one of the LAB strains). Agar slabs of 10 mm in diameter were aseptically cut off from the de Man, Rogosa and Sharpe medium (MRS, Oxoid) overgrown with a lawn of LAB strain incubated for 24 h at 37°C, 5% CO_2, and placed on plates with Wilkins-Chalgren Anaerobe Agar (Oxoid) inoculated with the indicator strain (10^5-10^6 CFU/mL). After 5 days of incubation in anaerobic conditions at 37°C, the diameters of growth inhibition zones around the agar slabs were measured. The results are given in mm, minus the agar slab diameter (Table 1).

Finally, the study included human strains of two Gram-positive bacteria, *L. rhamnosus* 900 and *L. paracasei* 915 (kindly provided by the Institute of Technology Fermentation and Microbiology, Faculty of Biotechnology and Food Sciences, Technical University of Lodz), and Gram-negative *H. pylori cagA*+ strain 95 (obtained from the Department of Microbiology and Clinical Immunology, The Children's Memorial Health Institute). The isolated live bacterial strains or their combinations were used as stimulating agents in all the experiments.

Table 1: Antagonistic activity of lactic acid bacteria strains.

Species	Strain	Growth inhibition zone [mm]
Lactobacillus casei	ŁOCK 899	1.7
	ŁOCK 901	0
	ŁOCK 902	0
	ŁOCK 903	0
	ŁOCK 904	0
	ŁOCK 905	1.7
	ŁOCK 906	2.1
	ŁOCK 907	0
	ŁOCK 908	4.8
	ŁOCK 909	0
	ŁOCK 910	0
	ŁOCK 911	2.5
Lactobacillus rhamnosus	ŁOCK 900*	5.21
Lactobacillus paracasei	ŁOCK 912	0
	ŁOCK 913	2.9
	ŁOCK 914	0
	ŁOCK 915*	0
	ŁOCK 916	2.9
	ŁOCK 917	1.6
	ŁOCK 918	2.7
	ŁOCK 919	3.6
	ŁOCK 920	2.2
	ŁOCK 921	1.7
	ŁOCK 922	0
	ŁOCK 923	0
	ŁOCK 924	0
Lactobacillus plantarum	ŁOCK 862	2.3
	ŁOCK 864	0
	ŁOCK 943	1.6

*The selected strains.

2.2. Generation of Human Monocyte-Derived Dendritic Cells. Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coat of healthy volunteers (from the Blood Centre in Bydgoszcz, Poland) by means of Lymphocyte Separation Medium 1077 (LSM, PAA) gradient centrifugation. Monocyte-derived DCs were generated from monocytes ($CD14^+$ cells) isolated with an aid of CD14 beads (Becton Dickinson, positive selection), as previously described [29–32]. The purity of the cells was greater than 95%. Subsequently, the isolated cells (1×10^6/mL) were cultured in RPMI 1640 (PAA) with 2% human serum (AB, Rh+ serum from the Blood Centre in Bydgoszcz, Poland) at 37°C and 5% CO_2 for 6 days. IL-4 (50 ng/mL, R&D) and granulocyte-macrophage colony-stimulating factor (GM-CSF, 100 ng/mL, R&D) were

Table 2: Expression of chosen receptors (CD14, HLA-DR, CD80, CD83, CD86, CD40, and CD11c) on monocyte-derived DCs surface.

Receptor type	CD14	HLA-DR	CD80	CD83	CD86	CD40	CD11c
iDC							
%	1.98 [0.38–1.99]	97.11 [89.30–99.55]	4.99 [2.88–5.73]	1.27 [0.89–3.32]	98.55 [97.25–99.70]	26.4 [12.10–37.95]	98.7 [97.50–99.00]
GFI	183 [146–220]	336 [274–363]	181 [170–188]	150 [145–160]	463 [350–581]	160 [130–190]	676 [650–793]

DCs: dendritic cells; iDCs: immature DCs; %: the percentage of DCs expressing the analyzed receptor; GFI: geometric mean fluorescence intensity of the analyzed receptor in DCs population exhibiting its expression; values are expressed as the medians of six independent experiments and range of lower quartile-upper quartile [Q1–Q3].

added to the culture medium in order to stimulate DCs development.

2.3. Dendritic Cells Stimulation. The DCs (1×10^6/mL) were suspended in 1 mL of RPMI 1640 (PAA) supplemented with 2% human serum (AB, Rh+ serum provided by the Blood Centre in Bydgoszcz, Poland) and incubated at 37°C and 5% CO_2 in presence of *H. pylori, L. rhamnosus* 900, *L. paracasei* 915, *L. rhamnosus* 900 + *H. pylori,* or *L. paracasei* 915 + *H. pylori*. The DCs were incubated with bacteria or medium alone (control DCs) for 24 h. The DC to bacterial cell ratio was 1 : 10. The live bacteria at concentrations providing optimal maturity and viability of DCs (not shown) were used as stimulating agents in all the experiments. The cells were collected by gentle pipetting and centrifuged at 250 ×g for 10 min. The culture supernatant was collected and stored at −80°C until cytokine analysis. The cells were resuspended in PBS, and trypan blue exclusion test showed that the culture contained 90% of viable cells.

2.4. Cell Surface Phenotype Expression. Subsequently, the cells were stained for CD14, CD11c, CD80, CD86, and CD40 (all from Becton Dickinson) using mouse anti-human monoclonal antibodies conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), or peridinin-chlorophyll proteins (PercP). A total of 20 000 events were collected according to the manufacturer's procedure that was described elsewhere [33]. The cells were subjected to flow cytometric analysis with FACScan flow cytometer (Becton Dickinson), and the cytometric data were analyzed using FlowJo version 7.6.1 software (Tree Star). The percentage of cells showing expression of the studied receptors and the average receptor density expressed as the geometric mean of fluorescence intensity (GFI) were analyzed in a population of DCs.

2.5. Cytokine Assay. Cytokine concentrations in DCs cell culture supernatants were estimated following 24 h of bacterial or medium alone (control DCs) stimulation. The cytokine levels were measured by means of commercially available ELISA kits: DuoSet, BD Bioscience (IL-12p70, IL-10, and TNF-α), and R&D Systems (IL-23), according to the manufacturer's instructions. Before performing the tests, the supernatant samples were diluted according to each kit's protocol and the final results were obtained by appropriate multiplication. The protein level in the diluted sample was calculated from a reference curve generated for a given assay by using reference standards containing known concentrations of appropriate protein. Results were expressed as pg per mL. The range of cytokine detection was as follows: from 7.8 to 500 pg/mL for IL-12p70, IL-10, TNF-alfa and from 125 pg/mL to 8000 pg/mL for IL-23.

2.6. Statistics. Statistical analysis was conducted with Statistica 9.0 software (StatSoft). The normal distribution was checked using the Shapiro-Wilk test. Due to the nonnormal distribution of the data, Mann-Whitney U test was performed. Statistical significance was considered at $P < 0.05$.

3. Results

3.1. The Antagonistic Spectrum of LAB Strains. Antagonistic effect of LAB strains was tested against *H. pylori cagA*+ strain 95. The antagonistic activity of *Lactobacillus* spp. was examined with the agar slab method, which is based on analysis of simultaneous growth of the indicator strain (*H. pylori cagA*+ strain 95) and a tested strain (LAB). The results of the slab culture constituted the basis for selection of the studied strains of LAB. The strongest antagonistic effect against *H. pylori*, manifested by a 5.21 mm zone of inhibition, was documented in the case of *L. rhamnosus* 900. Finally, two strains of LAB were selected for further analyses: *L. rhamnosus* 900, antagonistic to *H. pylori,* and the nonantagonistic *L. paracasei* 915.

3.2. Phenotype of Monocyte-Derived DCs. Monocyte-derived DCs were analyzed for surface phenotype by flow cytometry. Cells grown in GM-CSF and IL-4 alone after 6 days were immature, as defined by lack expression of CD14, relatively to stimulated DCs poor expression of CD83 and CD80 (Table 3) and lower expression of CD40, HLA-DR, and CD86. Almost all monocyte-derived DCs had expression of CD11c, characteristic marker for myeloid DCs (Figure 1, Table 2).

3.3. Phenotype of Bacteria-Stimulated DCs. Differences in the expression of DCs surface molecules were analyzed after one day of the bacterial stimulation (LAB strains: *L. rhamnosus 900*, *L. paracasei* 915; *H. pylori*; mixture: *L. rhamnosus* 900 + *H. pylori* and *L. paracasei* 915 + *H. pylori*) (Table 3).

Compared to the unstimulated DCs (control DCs), bacteria-stimulated DCs (irrespective of their variant) were

Table 3: Effect of the examined bacteria and their mixtures on the expression of DCs markers (CD14, HLA-DR, CD80, CD83, CD86, and CD40).

Receptor type	Control	*L. rhamnosus* 900	*L. paracasei* 915	*H. pylori*	*L. rhamnosus* 900 + *H. pylori*	*L. paracasei* 915 + *H. pylori*
CD14						
%	0.83 [0.47–0.87]	1.57 [0.38–2.83]	0.89 [0.50–2.67]	1.29 [0.85–2.18]	1.63 [0.43–1.92]	0.85 [0.41–4.70]
GFI	172 [147–221]	217 [165–230]	198 [162–256]	162 [149–179]	206 [157–211]	232 [169–258]
HLA-DR						
%	95.42 [94.80–99.21]	96.21 [94.90–98.11]	98.21 [95.60–98.50]	98.70 [98.60–99.15]	96.85 [95.09–97.30]	96.75 [94.30–99.11]
GFI	335 [294–360]	778* [700–1806]	765* [632–1423]	1504* [1040–1811]	777* [717–1989]	839* [713–1436]
CD80						
%	4.20 [3.91–5.18]	15.50*† [12.40–28.20]	37.80*† [25.80–47.60]	3.71 [2.53–11.90]	18.5*† [13.10–29.10]	36.65*†‡ [28.10–56.20]
GFI	154 [140–181]	152 [147–160]	216*†‡ [166–224]	154 [149–162]	149 [148–170]	177‡ [161–185]
CD83						
%	1.56 [0.99–1.94]	14.7* [8.95–18.88]	39.35*†‡§ [27.27–45.00]	6.27* [3.94–13.01]	13.35* [9.52–18.43]	30.65*†§ [18.77–50.98]
GFI	160 [146–207]	155 [132–239]	152 [141–157]	147 [140–159]	155 [155–181]	143 [136–152]
CD86						
%	97.25 [89.11–99.61]	96.45 [95.18–99.10]	96.30 [95.66–99.43]	99.00 [95.44–99.33]	97.45 [95.71–99.55]	97.12 [94.96–99.35]
GFI	554 [373–608]	2740* [1118–3390]	2079.5* [899–3143]	3388* [1514–3742]	3447* [3048–3667]	2496* [2002–3226]
CD40						
%	35.51 [17.33–40.41]	30.26 [26.34–39.89]	23.70 [23.46–27.78]	21.05 [16.95–28.25]	30.45 [24.79–53.67]	26.65 [12.17–45.45]
GFI	145 [105.2–175]	145 [141–153]	175 [132–219]	131 [123–140]	146 [127–167]	145 [142–149]

DCs: dendritic cells; %: the percentage of DCs expressing the analyzed receptor; GFI: geometric mean fluorescence intensity of the analyzed receptor in DCs population exhibiting its expression; values are expressed as the medians of six independent experiments and range of lower quartile-upper quartile [Q1–Q3]; statistically significant differences: *stimulators *versus* control (unstimulated DCs), †stimulators *versus H. pylori*, ‡stimulators *versus L. rhamnosus 900*, and §stimulators *versus L.rhamnosus* 900 + *H. pylori*; $P < 0.05$.

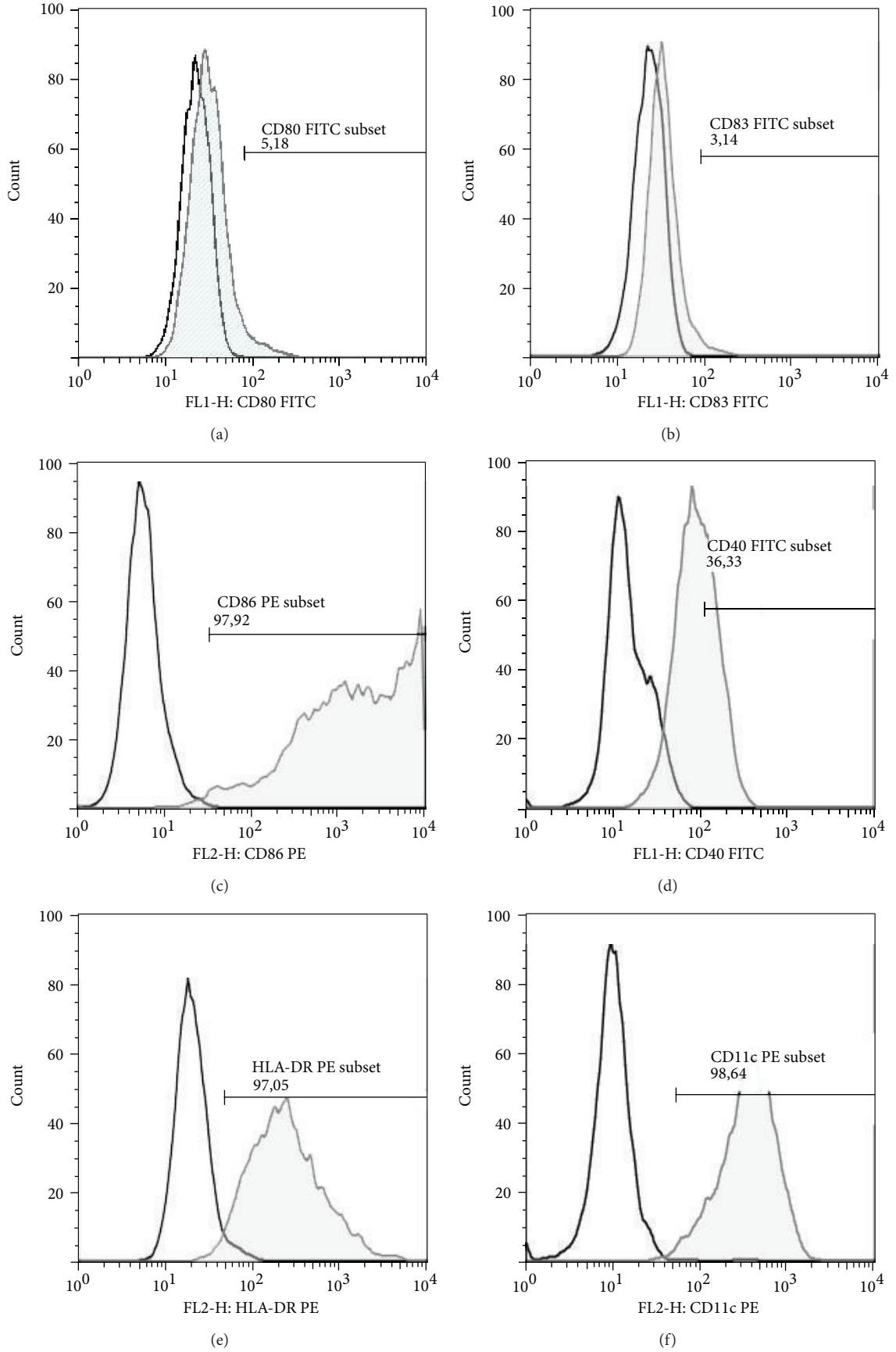

Figure 1: Phenotype of immature DCs. Histograms of representative cytometric data illustrate the following: (a) percentage of $CD80^+$ DCs; (b) percentage of $CD83^+$ DCs, (c) percentage of $CD86^+$ DCs; (d) percentage of $CD40^+$ DCs; (e) percentage of HLA-DR^+ DCs; and (f) percentage of $CD11c^+$ DCs. DCs: dendritic cells; stimulated DCs are represented by filled curves; isotype controls are represented by empty curves.

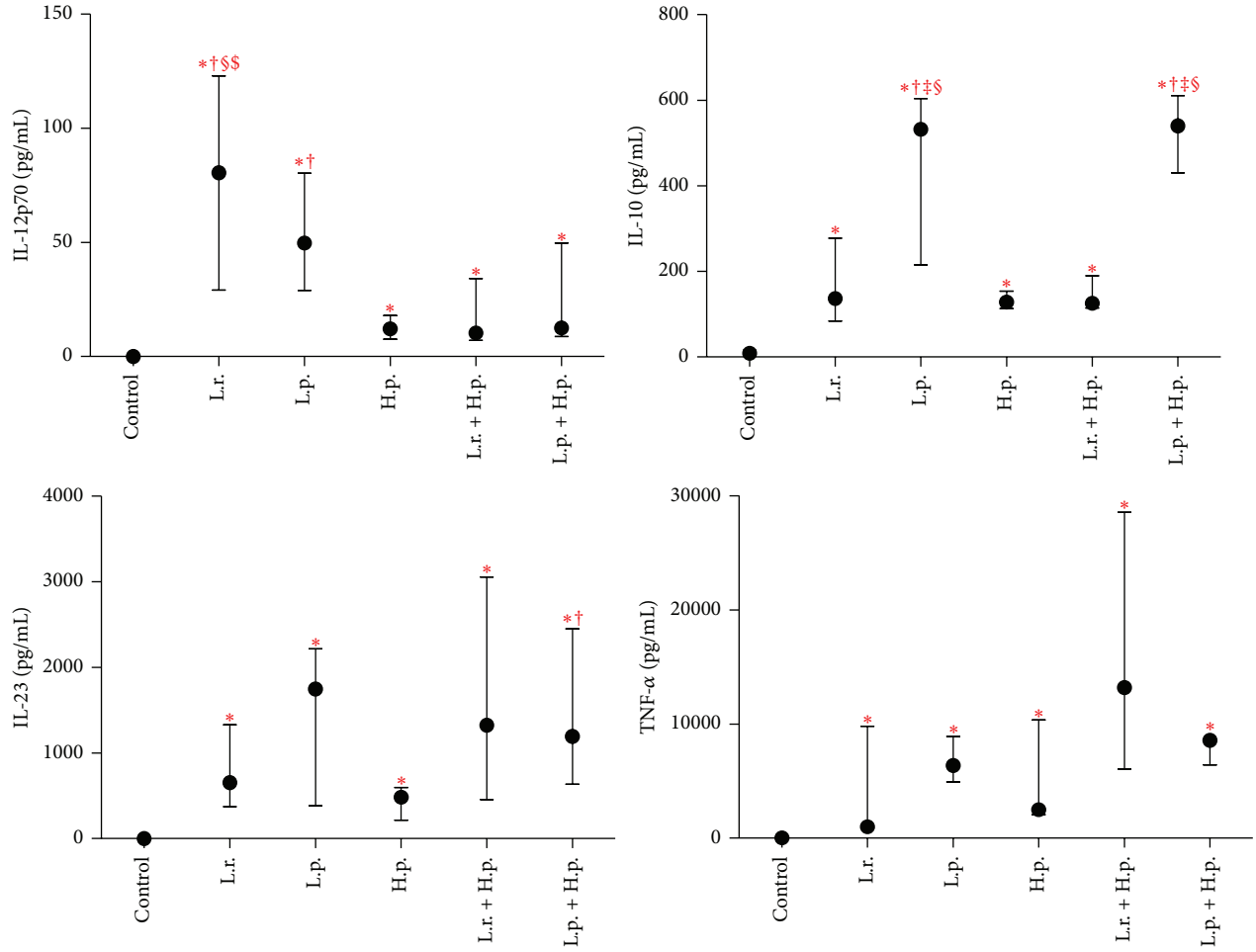

Figure 2: Effect of the examined bacteria and their mixtures on the production of cytokines by DCs population. Values expressed as medians from six independent experiments and interquartile ranges [Q1–Q3]; control: unstipulated DCs; L.r.: *L. rhamnosus* 900; L.p.: *L. paracasei* 915; H.p.: *H. pylori*; statistically significant differences are given as follows: ∗: stimulators *versus* control (unstimulated DCs), †: stimulators *versus* *H. pylori,* ‡: stimulators *versus L. rhamnosus* 900, §: stimulators *versus L. rhamnosus* 900 + *H. pylori,* and $: stimulators *versus L. paracasei* 915 + *H. pylori*; $P < 0.05$; DCs: dendritic cells.

reflected by a significant increase in HLA-DR and CD86 receptor densities on DCs (GFI for CD86 and GFI for HLA-DR).

Furthermore, the stimulation with either single bacterial strain caused a significant increase in the percentage of CD83-positive cells but the highest percentage of these cells was observed after stimulation with *L. paracasei* 915. A mixture of *L. paracasei* 915 + *H. pylori* turned out to exert stronger stimulatory effect on the expression of CD83-positive DCs than *H. pylori* alone or the mixture of *L. rhamnosus 900* and *H. pylori*.

A significant increase in the percentage of CD80-positive DCs was observed solely after exposure of DCs to LAB strains alone or in combination with *H. pylori.* In turn, *H. pylori* alone turned out to be significantly weaker inducer of the CD80-positive cells than the LAB strains and their mixtures. Moreover, we showed that exposure to *L. paracasei* 915 was reflected by significantly higher increase in density of CD80 receptor (GFI for CD80 receptor) than in the case of stimulation with *H. pylori*. Both *L. paracasei* 915 alone and in the mixture with *H. pylori* caused significantly greater increase in GFI for CD80 than did *L. rhamnosus* 900.

3.4. Comparison of Cytokine Levels after Bacterial Stimulation. The DCs were stimulated for 24 h with live bacteria, either a single strain or a mixture of two bacterial strains (Figure 2). All the stimulators effectively induced cytokine synthesis (IL-10, IL-12p70, IL-23, and TNF-α) when compared with control DCs (unstimulated DCs).

L. rhamnosus 900 alone turned out to be stronger inducer of IL-12p70 than *H. pylori* alone and mixtures of *H. pylori* + LAB. Also another analyzed LAB strain, *L. paracasei* 915, proved to be better stimulator of IL-12p70 synthesis than *H. pylori.*

Furthermore, the stimulation with either *L. paracasei* 915 alone or its combination with *H. pylori* was reflected by significantly more enhanced synthesis of IL-10 than the exposure to *L. rhamnosus* 900, *L. rhamnosus* 900 + *H. pylori,* and *H. pylori* alone.

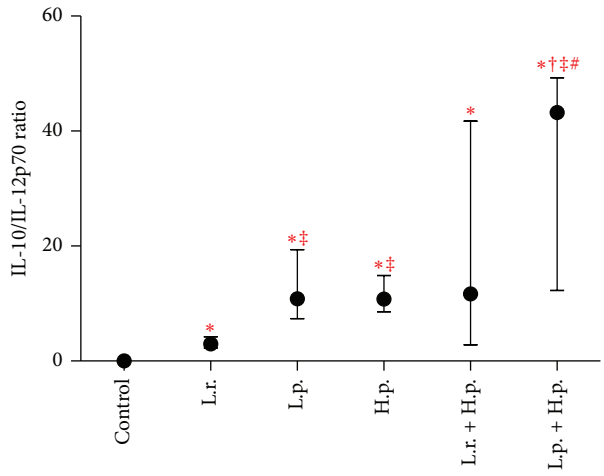

FIGURE 3: The cytokine IL-10/IL-12p70 ratio. Values expressed as medians from the ratios from six independent experiments and interquartile ranges [Q1–Q3]; control: unstipulated DCs; L.r.: *L. rhamnosus* 900; L.p.: *L. paracasei* 915; and H.p.: *H. pylori*; statistically significant differences are given as follows: ∗: stimulators *versus* control (unstimulated DCs), †: stimulators *versus H. pylori,* ‡: stimulators *versus L. rhamnosus* 900, stimulators *versus L. paracasei* 915; $P < 0.05$; DCs: dendritic cells.

Finally, stimulation of DCs with any of bacterial strains or their mixtures caused an increase in the synthesis of IL-23. However, *H. pylori* alone turned out to be a weaker stimulator of IL-23 *versus L. paracasei* 915 + *H. pylori* ($P < 0.05$) and *L. rhamnosus* 900 + *H. pylori* ($P < 0.1$).

Stimulation with all the bacteria and their mixtures resulted in a significant increase in TNF-α concentration, but without statistical differences.

Next, we calculated the IL-10/IL-12p70 ratios obtained from these studies (Figure 3). These allowed the ranking of the strains from an "anti-inflammatory" to a "proinflammatory" profile. The strains *L. paracasei* 915 and *H. pylori* were classified as more anti-inflammatory. *L. rhamnosus* 900 showed a slightly proinflammatory profile with a very low IL-10/IL-12p70 ratio. The mixture *L. paracasei* 915 + *H. pylori* showed strong anti-inflammatory capability. In contrast, despite the rather high ratio of IL-10/IL-12p70, the mixture *L. rhamnosus* 900 + *H. pylori* did not show differences between stimulators.

4. Discussion

In this study, we provided evidence for the immunostimulatory effect of LAB strains on *H. pylori*-induced DCs. We also reported for the first time that the LAB strains induce more mature phenotype of DCs than *H. pylori* alone (as shown by greater percentage of $CD80^+$ DCs). Thus, our findings point to potential application of some of these bacteria as a component of *H. pylori* infection treatment.

There are three consecutive stages of DC maturation: immature DCs (iDCs), semimature DCs (smDCs), and mature DCs (mDCs). The cells representing these phenotypes can be distinguished on the basis of cytometric analysis of HLA-DR, CD80, CD86, CD83, and CD40 receptor expressions and profile of secreted cytokines, such as IL-10, IL-12p70, IL-23, and TNF-α [34–36]. Activation of iDCs with foreign antigens, for example, bacterial Ag, results in their transformation to smDCs or mDCs. The phenotype of semimature DCs does not differ from that of mDCs: their ability to synthesize cytokines is limited as shown by markedly lower concentrations of proinflammatory cytokines and moderate level of IL-10 in culture supernatant. In contrast, the fully mature DCs cause activation of T cell response and synthesize an array of cytokines, for example, IL-12p70, IL-12p40, IL-6, and TNF-α [34, 35, 37]. It is noteworthy that all DCs constitutively express CD86 and HLA-DR on their surfaces [38, 39]. Therefore, we identified iDCs, mDCs, and smDCs on the basis of percentage of cells expressing CD83 and CD80 and fluorescence intensity of these receptors on DC surface as well as cytokine production.

The increase in the percentage of $CD83^+$ cells, observed after stimulation with either all the analyzed bacteria (*H. pylori*, LAB) or their mixtures (LAB with *H. pylori*), likely reflected the process of DC maturation [31, 40]. *L. paracasei* 915, that is, the strain nonantagonistic to *H. pylori*, turned out to be the most potent activator of DC maturation among all the analyzed variants, as shown by the most pronounced increase in the percentage of $CD83^+$ cells and density of CD80. Analysis of the expression of CD80 receptor, responsible for late activation of DCs [38], showed that *H. pylori* was the only bacterium that did not stimulate an increase in the percentage of $CD80^+$ cells. Therefore, the analyzed strain of *H. pylori* stimulated maturation of DCs to a lesser extent, which likely corresponded to development of smDCs with tolerogenic phenotype [41, 42]. In contrast, the mixtures of *H. pylori* with the LAB strains stimulated differentiation of CD80-positive DCs. Therefore, the analyzed lactobacilli likely enhanced the process of DCs maturation despite the presence of *H. pylori*. This phenomenon may directly affect the following: (a) presentation of Ag to antigen-naive lymphocytes T, (b) profile of secreted cytokines, and (c) characteristics of T-dependent response (e.g., predominance of Th1, Th2, or Th17 response).

It is commonly known that the effective response of T lymphocytes requires two types of activation signal: (a) interaction between Ag presented by MHC I/II and the TCR/CD3 receptor and (b) interaction between receptors, such as CD80, CD86, and CD28 or CTLA-4. Too weak second signal leads to anergy of T lymphocytes and resultant apoptosis thereof [38, 43]. The abovementioned process involves a number of molecules supporting the presentation, such as CD40 and CD83 participating in activation of T lymphocytes [44, 45]. Although we documented an increase in the percentage of $CD83^+$ cells in DC population, both relative and absolute numbers of $CD40^+$ cells remained unchanged. Moreover, it should be stressed that all the analyzed strains and the mixtures thereof exerted similar effect on CD86 and HLA-DR expressions. These findings suggest that we did not obtain fully mature DCs since, as mentioned previously, the presence of the latter needs to be confirmed by secretion of specific cytokines to culture supernatant. Apart from maturation of DCs, also polarization of these cells toward DC1 or

DC2 function constitutes equally important component of response to *H. pylori* infection; the process of polarization can be analyzed on the basis of concentrations of selected cytokines, especially IL-12p70 and IL-10. The fact that the level of biologically active form of IL-12 after stimulation with *H. pylori* alone was lower than after the exposure to the analyzed LAB strains may reflect immunosuppressive effect of *H. pylori* or polarization of DC towards Th2 response [15]. It should be emphasized that the mixtures of analyzed LAB strains (*L. rhamnosus* 900 and *L. paracasei* 915) and *H. pylori* induced secretion of IL-12p70 at a similar level as did *H. pylori* alone, which suggests that the latter bacterium might inhibit the LAB-induced immune response. This hypothesis is supported by the results of a previous study in which *H. pylori* was shown to release a factor that inhibited secretion of IL-12 by DCs [24, 46]. However, despite the fact that IL-23 belongs to the family of IL-12, similar effects were not observed. We showed that the level of IL-23 after stimulation with *L. paracasei* 915 and *H. pylori* mixture was significantly higher than in the case of exposure to *H. pylori* alone. In turn, the concentration of IL-23 in the culture of DCs stimulated with the mixture of *L. rhamnosus* 900 and *H. pylori* turned out to be similar as in the case of DCs induced with *L. paracasei* 915 and *H. pylori*. High level of IL-23 corresponds to proinflammatory function of activated DCs and can be associated with induction of Th17 response [47, 48]. The DCs stimulated with the bacterial mixtures seemed to be more effective and their phenotype resembled that of mDCs to a larger extent than the phenotype of the cells exposed to *H. pylori* alone. Enhanced synthesis of IL-23, involved in the control of Th17 response, may be beneficial in the case of *H. pylori*-induced inflammation as previous studies showed that it improves the antibacterial potential [13, 22]. Apart from IL-23, DCs synthesize an array of other proinflammatory cytokines, for example, TNF-α [49]. Both LAB and *H. pylori*, as well as their mixtures, enhanced synthesis of TNF-α; however, the levels of this cytokine did not differ significantly between the analyzed culture variants. Previous studies showed that bacterial stimulation of DCs is reflected by enhanced synthesis of TNF-α; this cytokine exerts pleiotropic effects [50–55], determined by duration of the exposure. Moreover, high level of TNF-α was shown to be a marker of DC maturation. It is interesting that mature DCs can also synthesize these cytokines that act antagonistically to proinflammatory cytokines, for example, IL-10 [56]. Both *L. paracasei* 915 strain and the mixture thereof with *H. pylori* turned out to be the strongest inductors of IL-10 synthesis. These findings confirm that a nonantagonistic strain can stimulate tolerogenic response associated with activation of type-2 polarized DCs. The concentration of IL-10 documented after stimulation with *H. pylori* was markedly lower, similar to that observed after exposure to *L. rhamnosus* 900 alone or in mixture with *H. pylori.* As mentioned above, low level of this cytokine may be characteristic for smDCs [34], which further confirms that stimulation with *H. pylori* promotes tolerogenic phenotype of DCs. Low levels of both IL-10 and IL-12p70 in *H. pylori*-induced culture may also point to the lack of DC polarization and result in the lack of their reactivity with T lymphocytes. However, the hereby presented findings suggest that such dysregulation of immune response may be at least partially counterbalanced by LAB strains, as shown by increased expression of DC surface markers (CD80 and/or CD83) and higher concentration of IL-23 in culture supernatant.

The fact that LAB stimulated maturation of DCs suggests that these bacteria may normalize immune mucosal function during symptomatic *H. pylori* infection. However, we could not unambiguously distinguish which of the LAB strains, antagonistic or nonantagonistic one, was a stronger enhancer of antibacterial reaction associated with activation of T-dependent (Th1, Th17) response. On one hand, we documented a marked increase in CD80 expression solely on the surface of DCs stimulated with *L. paracasei* 915 and its mixture with *H. pylori*, which points to greater potential of the nonantagonistic LAB strainas a stimulator of DC maturation. On the other hand, the same LAB strain proved to be a strong inductor of IL-10 synthesis. In turn, this cytokine is known to stimulate response of Treg lymphocytes [57], and percentage of these latter cells increases in the course of *H. pylori* infection, being tightly associated with the activity and phenotype of DCs. In contrast, elimination of Tregs may promote eradication of *H. pylori* [58]. Therefore, lower mucosal counts of Tregs will be reflected by stronger immune response (Th1 or Th17 response) and resultant elimination of *H. pylori*. Understanding the profile of T lymphocyte in the coculture of these cells with LAB/*H. pylori*-stimulated DCs is warranted (actually under study). However, *L. rhamnosus* 900 in contrast to *L. paracasei* 915 shows reduced IL-10/IL-12p70 ratio. Therefore, it seems that nonantagonistic strain may be more supressive/tolerogenic. It should be noted also that the mixture *L. paracasei* 915 + *H. pylori* was also strongly anti-inflammatory. Taking together, *L. rhamnosus* 900 proved to be a weaker stimulator of DC maturation, the polarization of cellular response induced by this bacterium could be more beneficial in the context of *H. pylori* infection.

5. Conclusions

First, the LAB strains used here were much more potent DC maturation agents than *H. pylori*. Second, *H. pylori*-induced DCs tolerogenic phenotype was at least partially overcome by the LAB strains. Third, the *L. rhamnosus* strain 900 (antagonistic to *H. pylori*) proved to be more effective than *L. paracasei* strain 915 (nonantagonistic to *H. pylori*) in DCs protection against tolerogenic action of *H. pylori*.

Conflict of Interests

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

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Review Article

Intestinal Microbiota as Modulators of the Immune System and Neuroimmune System: Impact on the Host Health and Homeostasis

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Many immune-based intestinal disorders, such as ulcerative colitis and Crohn's disease, as well as other illnesses, may have the intestines as an initial cause or aggravator in the development of diseases, even apparently not correlating directly to the intestine. Diabetes, obesity, multiple sclerosis, depression, and anxiety are examples of other illnesses discussed in the literature. In parallel, importance of the gut microbiota in intestinal homeostasis and immunologic conflict between tolerance towards commensal microorganisms and combat of pathogens is well known. Recent researches show that the immune system, when altered by the gut microbiota, influences the state in which these diseases are presented in the patient directly and indirectly. At the present moment, a considerable number of investigations about this subject have been performed and published. However, due to difficulties on correlating information, several speculations and hypotheses are generated. Thus, the present review aims at bringing together how these interactions work—gut microbiota, immune system, and their influence in the neuroimmune system.

1. Introduction

The human body is colonized by a vast number of microbes, collectively referred to as the human microbiota. The link between these microbes and our health is the focus of a growing number of research initiatives, and new insights are emerging rapidly. The fact that the number of microbial cells composing the human microbiota surpasses that of own body cells allows us to foresee the existence of an intertwined relationship between the biology of the human host and such microorganisms, which has been moulded by millennia of evolution. Studies regarding the understanding of the various aspects of the conjunct of unicellular organisms carried in the human body rely on molecular biology tools in order to unravel the species that are present as well as the genes found to be operating the host-microorganism interaction [1]. Over the past few years, next-generation DNA sequencing has allowed substantial fulfilment of the efforts directed at

clarifying aspects related to our whole microbiota, concerning mainly its composition and the inherent variability, which may occur interpersonally and in a single individual in the course of one day or due to aging. Besides, the cited variability may occur as a response to certain illnesses; taking advantage of it, this variability can constitute a powerful diagnostic tool and give important clinical correlations [2–4].

Considering that humans, as well as other multicellular organisms, have evolved in an environment where unicellular organisms have always been ubiquitous, it is intuitive to think that the composing elements of our microbiome started to be selected much earlier in our evolutionary history. The implication is that both our metabolic traits and those of the organisms we host have been forged by evolution in a mutualistic fashion, so that the presence of certain microorganisms is connected to physiological functioning, and variations of the microbial composition of our bodies may be linked to metabolic alterations in various sites on the human body [5]. Here, we are going to focus on the alterations that may occur in the gut microbiota.

Gut microbiota gives individual-specific milieu for ingested food, and host intestine provides unique genetic background for the growth of specific bacteria. The human gastrointestinal tract is inhabited by 1×10^{13} to 1×10^{14} microorganisms and from 500 to 1,000 species [6, 7] and more than 7,000 strains [8]. The balance between this complex community of gut bacteria, food nutrients, intestinal genomics, and physiological site is increasingly recognized as a major contributor to human health. In certain disorders where environmental factors are implicated, an imbalance between commensal bacteria with pathogenic potential (which we term pathobionts) and commensal bacteria with beneficial potential (symbionts) has a role in pathogenesis.

Arumugam et al. [26] have highlighted the advances made on understanding the gut microbiota by summarizing and adding data from metagenomic sequencing of stool samples. The intestinal microbiota has bacteria as its virtually sole component. Bacteroidetes, which is an abundant phylum, together with Firmicutes, correspond to 90% of the intestinal gut pool of microorganisms [6]. There are also efforts to determine the enterotypes: clusters in which the levels of three genera among the whole gut microbiome varied in a similar way. Enterotype 1 was identified by the variation of *Bacteroides* and enterotype 2 displayed altered levels of *Prevotella*, both of them components of the Bacteroidetes phylum; and enterotype 3, *Ruminococcus*, belongs to the Firmicutes phylum. These enterotypes have been shown to be highly robust and were not restricted to region, country, or continent. The bacterial genera enriched in each of the enterotypes appear to be connected to the mechanism by which the intestinal microbiota degrades fermentable substrates in the colon [26]. Enterotypes clusters depend on long-term diets whose changes can be detectable 24 hours after diet change and remained stable after 10 days [27]. However different, the enterotypes could not be connected to any of the host features measured nationality, gender, age, or body mass [26]. Nevertheless, de Fellipo et al. have found that African children who have diet high in fiber compared to European children showed a significant enrichment in Bacteroidetes and depletion in Firmicutes as well as the microbial biodiversity [28].

Although the gut microbiome is highly variable, the summation of genomes comprised in it tends to be quite conserved when considered the microbial metabolic pathways [29], being particularly relevant when discussing the gut microbiome, in order to understand the underlying mechanisms involved in the host-microbiota relationship both in healthy individuals and in those suffering from intestinal or metabolic diseases [3, 30].

The microbial metabolism is seen as a complement to the host metabolism. Thereby, alterations in this metabolism, due to alterations either in microbiota composition or in diet or some other modifications, can happen and have been specifically related to diseases, among which are irritable bowel syndrome (IBS) [9–11]; inflammatory bowel disease (IBD), as ulcerative colitis and Crohn's disease [12–14]; colorectal cancer [15, 16]; obesity [31]; type 1 diabetes [32]; and type 2 diabetes [18].

Although those are multifactorial conditions, they seem to be connected to the intestinal microbiota, in a relationship not yet fully understood. Studies showed that altered balance between the two major enteric bacterial phyla, the Bacteroidetes and the Firmicutes, has been associated with clinical states, and microbial and nutrient lifetime changes, from early metabolic programming to late age immunity decline, may have major impact on health and well-being [33]. The microbial alterations apparently involved in the pathogenesis of some specific diseases are displayed in Table 1.

Concerned with finding answers to understand the connection of gut microbiota and disease, studies have already perceived the influence of human gut microbiota and its perturbations on homeostasis, as already cited, nutrition and behaviour, due to the connection of these microbes to the availability of nutrients, and modulation of the immune, neuronal, and endocrine systems [34, 35]. Thus, the gut microbiota in fact participates in the regulation of physiological and metabolic pathways. In the next topics, the major and current interactions of gut microbiota and other systems will be related. All metabolic and physiologic forms of alterations influenced by the gut microbiota or influencing its composition reflect systemic-wide alteration of balance. The best-described host-microbiota interaction to date is that involving the intestinal epithelium and the immune system, with increasing knowledge about neuroimmune interaction.

2. Gut Microbiota and Immune System

The human gastrointestinal tract is constantly in contact with an overwhelming antigenic load in the form of commensal bacteria and dietary antigens. The system must be able to discriminate pathogens that require a protective immune response, from normal microbiota or food antigens, where a dynamic unresponsiveness state is necessary [36].

The gastrointestinal tract (GI) is inhabited by several types of microorganisms (bacteria, virus, protozoan, etc.)—the gut microbiota. Commensal bacteria, the most frequent

Table 1: Profile of alterations in the gut microbiota in IBS, IBD, colorectal cancer, obesity, and type 2 diabetes.

Disease	Microbial alteration	Reference
Irritable bowel syndrome	Increased presence of Firmicutes, specifically *Ruminococcus* sp., *Clostridium* sp., and *Dorea* sp.; reduction in *Bifidobacterium* and *Faecalibacterium* spp.; decrease of *Bacteroides* in afflicted children; increased presence of *Dorea* sp., *Ruminococcus* sp., *Haemophilus* sp. and *parainfluenzae* sp. *in paediatric patients.*	[9–11]
Inflammatory bowel disease	Reduced complexity of Firmicutesand Bacteroidetes, *with decrease in the abundance of Clostridium leptum* and *Clostridium coccoides;* increase in bacteria of the Gammaproteobacteriaclass*;* presence of adherent and invasive *Escherichia coli;* decreased presence of *Faecalibacterium prausnitzii;* altered abundance of members of the families Enterobacteriaceae, Ruminococcaceae, and Leuconostocaceae,with increased presence of *Clostridium* and reduced presence of *Roseburia* and *Phascolarctobacterium.*	[12–14]
Colorectal cancer	Members of the genus *Fusobacterium* appear increased on colorectal cancerous tissue; reduction in bacteria of the phyla Firmicutesand Bacteroidetes; alterations in number of butyrate producing bacteria (*Coprococcus* spp.; *Eubacterium rectale*; *Roseburia* spp.; *and Faecalibacterium prausnitzii),* related to the protective effect of butyrate for the enterocytes.	[15, 16]
Obesity	Decreased presence of Bacteroidetes; increased presence of Actinobacteria.	[3, 17]
Type 2 diabetes	Overall alterations of the microbiota; increased presence of *Clostridium* spp.; *Akkermansia muciniphila; Bacteroides* spp.; and *Desulfovibrio* spp.	[18]
Ulcerative colitis	Decreased presence of Firmicutes, Lentisphaerae, and Verrucomicrobia*;* increased presence of Proteobacteria, Fusobacteria, and Spirochaetes.	[19]

microorganisms in intestinal environment, are beneficial for the host, while pathogenic bacteria are able to cause problems, such as gut inflammation and invasiveness. The symbiosis process happens when there is a favourable balance between commensal bacteria and pathogenic bacteria over a period of time [37]. In this process, the interaction of microbiota, intestinal epithelium, and mucosal immune system results in a local and systemic homeostasis. However, in a dysbiosis process, the interaction between commensal and pathogenic bacteria is altered, resulting in homeostasis disruption [38]. This breakdown of homeostasis can result from local infection and inflammation to complications that can affect several other human systems like the central nervous system and endocrine system [39]. In the next paragraphs, we will describe, briefly, how intestinal immune system is formed and how it interacts with microbiota.

2.1. Intestinal Barrier. Basically, the spatial interaction between microbiota and intestinal immune system can be divided into three layers. The first layer, facing to the intestinal lumen, is composed mainly by mucus and can be divided into another two sublayers: the outer sublayer, less dense, is highly colonized by microbiota, while the inner mucous layer is composed of high concentration of bactericidal antimicrobial peptides (AMPs) and secretory IgA (SIgA) specific for commensals microorganisms. Due to these components, the inner dense layer is virtually impervious to microbes [39–41].

The second layer is composed of a monolayer of intestinal epithelial cells (IECs) that are in touch with the lamina propria (LP) in their basolateral surface and with the mucous layer in their apical surface. The IECs are composed by several cellular types, like goblet cells which produce mucin (forming mucus); absorptive enterocytes and enteroendocrine cells, both producing cholecystokinin and ghrelin (which regulate appetite); Paneth cells, the leading producer of AMPs; and M cells, involved in capturing antigens to present them to immune system [42, 43]. IECs have a very important role in separating the internal body organs from the outside environment through the formation of tight junctions and secretion of mucus and AMPs (such as defensins, lysozymes, cathelicidins, phospholipase-A2, and C-type lectins) [42]. Furthermore, ECs express pattern-recognition receptors (PRRs), which include Toll-like receptors (TLRs), Nod-like receptors (NLRs), and Rig-I like receptors [44]. Interestingly, the production of some types of AMPs, like regenerating islet-derived protein 3γ (REGIIIγ), REGIIIβ, and angiogenin-4, is influenced by commensal microorganisms in a TLR/MyD88 dependent way. However, other AMPs like lysozyme, phospholipase-A2, and defensins seem not to be influenced by microbiota [42]. A very important cell type present in IECs layer is the M cells. These cells work directly with the immune system, sampling antigens from lumen and carrying them in a unidirectional way to antigen presentation cells localized under the epithelium [42]. Enteroendocrine cells also act in gut barrier protection by producing enteroendocrine peptide glucagon-like peptide-2 (GLP-2), which is regulated by the nutritional status of the host, such as short-chain fatty acids production. The main characteristics in gut barrier function of GLP-2 are inducing intestinal epithelial cell proliferation; increasing the expression of intestinal tight junction proteins;

and regulating the innate immune system by controlling the expression of antimicrobial peptides produced by Paneth cells [45].

The third layer, under the IECs, is formed by lamina propria and mesentery. The elements of the local immune system denominated gut-associated lymphoid tissues (GALT) are located within this layer. In the lamina propria, mature isolated lymphoid follicles (ILFs), which are formed from crypt patches (prenatal) and Peyer's patches (PPs), can be found. Microbe-associated molecular patterns (MAMPs) derived from colonizing bacteria are sensed by PRRs on IECs or dendritic cells (DCs) that recruit and activate T and B cells in ILFs. PPs, under IECs, receive antigens through M cells and pass them to DCs, which interact with T and B cells. In PPs and ILFs there are several plasma cells that normally produce and release IgA. DCs that sample antigens from LP or through IECs migrate to mesenteric lymph node to induce differentiation of effector T cells that traffic to the lamina propria [46].

2.2. Gut Microbiota and Intestinal Immune System Interaction. The functional interaction between microbiota and intestinal immune system begins with commensal bacteria that promote an anti-inflammatory environment (this process is summarized in Figure 1 and in the text below). In a symbiosis context, MAMPs continuously stimulate IECs to secrete regenerating REGIIIγ into the lumen, thymic stromal lymphopoietin (TSLP), IL-33, IL-25, and tumor growth factor-β (TGF-β) under epithelium. These immunological mediators induce the development of tolerogenic macrophages and tolerogenic DCs [39, 46]. Tolerogenic DCs produce TGF-β and retinoic acid (RA) that stimulate the development of T regulatory cells. Thus, through Treg cells (that use diverse mechanisms of regulation), macrophages (that produce IL-10), and tolerogenic DCs, the gut immune system is able to establish and maintain an anti-inflammatory environment. In addition to essential regulatory roles of TGF-β, this cytokine is associated with other epithelial-derived substances (such as B-cell activating factor (BAFF) and proliferation-inducing ligand (APRILL)), in order to induce development of IgA-producing cells (plasma cells) [47]. This immunoglobulin is able to prevent the binding of commensal bacteria on host epithelium and is thus involved in the formation of the gut microbiota [48].

In a dysbiosis context, the presence of the pathogens can disrupt this regulated anti-inflammatory environment. When enteric pathogens overcome commensal bacteria, the imbalance between commensal and pathogenic bacteria causes a significant liberation of MAMPs. This increase in MAMPs can induce IECs, activated DCs, and macrophages to secrete inflammatory cytokines like IL-1β, IL-6, IL-12, and IL-23. These cytokines stimulate the development of effector $CD4^+$ T helper 1 (TH1) cells and TH17 cells (that produce IL-17A, IL-17F, and IL-22) resulting in chronic inflammation [39]. In this context, the IL-22 cytokine has a crucial role. This molecule, produced by TH17 cells and by innate immunity cells (like NK-cells and $\gamma\delta$T cells), acts on intestinal epithelial cells by inducing the expression of several AMPs as REGIIIγ and REGIIIβ that directly affects the microbiota. Interestingly, activated proinflammatory cells seem to work both in symbiosis and in dysbiosis; however, in case of symbiosis, the proinflammatory cells are kept under control by regulatory mechanisms (tolerogenic DCs and macrophages and T regulatory cells) and contribute by releasing IL-22, which promote production of REGIIIγ by IECs and help to protect the epithelial barrier [39].

Although the mechanisms above described are already well established and despite of the existence of a vast literature about the subject, many aspects of microbial/immune system relationship still need to be elucidated. Furthermore, recent studies have added further evidence that demonstrate how the microbiota and immune system can interact to maintain homeostasis. Thus, the next paragraphs will describe some of the new evidences supporting this idea.

2.3. New Evidences about Gut Microbiota and Intestinal Immune System. Other recent studies have addressed the interactions between the gut microbiota and the immune system. These interactions may be related to maintaining the balance between the gut microbiota and immune system axis, both local and systemic.

Masahata et al. [49] showed the existence of a relationship between the IgA-secreting cells and the microbiota composition. In this study, to assess the importance of appendix associated lymphoid tissue (called caecal patches) in IgA-secreting cells generation, germ-free mice were appendectomized and colonized with bacteria. These authors found a decrease in IgA-secreting cells in large intestine, as well as a reduction of faecal IgA levels. Concomitantly, a significant reduction in the number of faecal bacterial species in appendectomized mice was noticed. However, in a very interesting way, these differences in the number of IgA-secreting cells and bacterial community disappeared after eight weeks of colonization. This normalization of colonic IgA-secreting cells correlates to increasing and enlargement of the solitary intestinal lymphoid tissues. Thus, these results suggest that IgA-secreting cells are involved with the maintenance of microbial homeostasis in the large intestine and contribute to shaping of the normal microbial community. Moreover, these findings demonstrate that development of immune system and microbiota are in a close accordance.

Forkhead box P3 (FoxP3) regulatory T cells (Tregs) perform an important role in gut homeostasis, mainly by controlling the function and proliferation of effector T cells. Several works have already demonstrated that germ-free mice are defective in these cells, proving a crucial role of the microbiota on Treg induction [50, 51]. Recently, Cording et al. [52] evaluated the commensal microbiota influence in proliferation of T $CD4^+$ cells and Treg cells in animals submitted to long-term antibiotic treatment. These studies showed a significant reduction in the number of Treg cells on mesenteric lymph nodes and Peyer patches after treatment. Treg cells proliferation was also reduced in these tissues but not in the spleen and peripheral lymph nodes. Interestingly, the microbial reduction affected the proliferation of conventional T $CD4^+$ cells in all analysed tissues (mesenteric lymph nodes, Peyer patches, peripheral lymph nodes, and spleen). Thus, the authors conclude that microbial stimulus locally

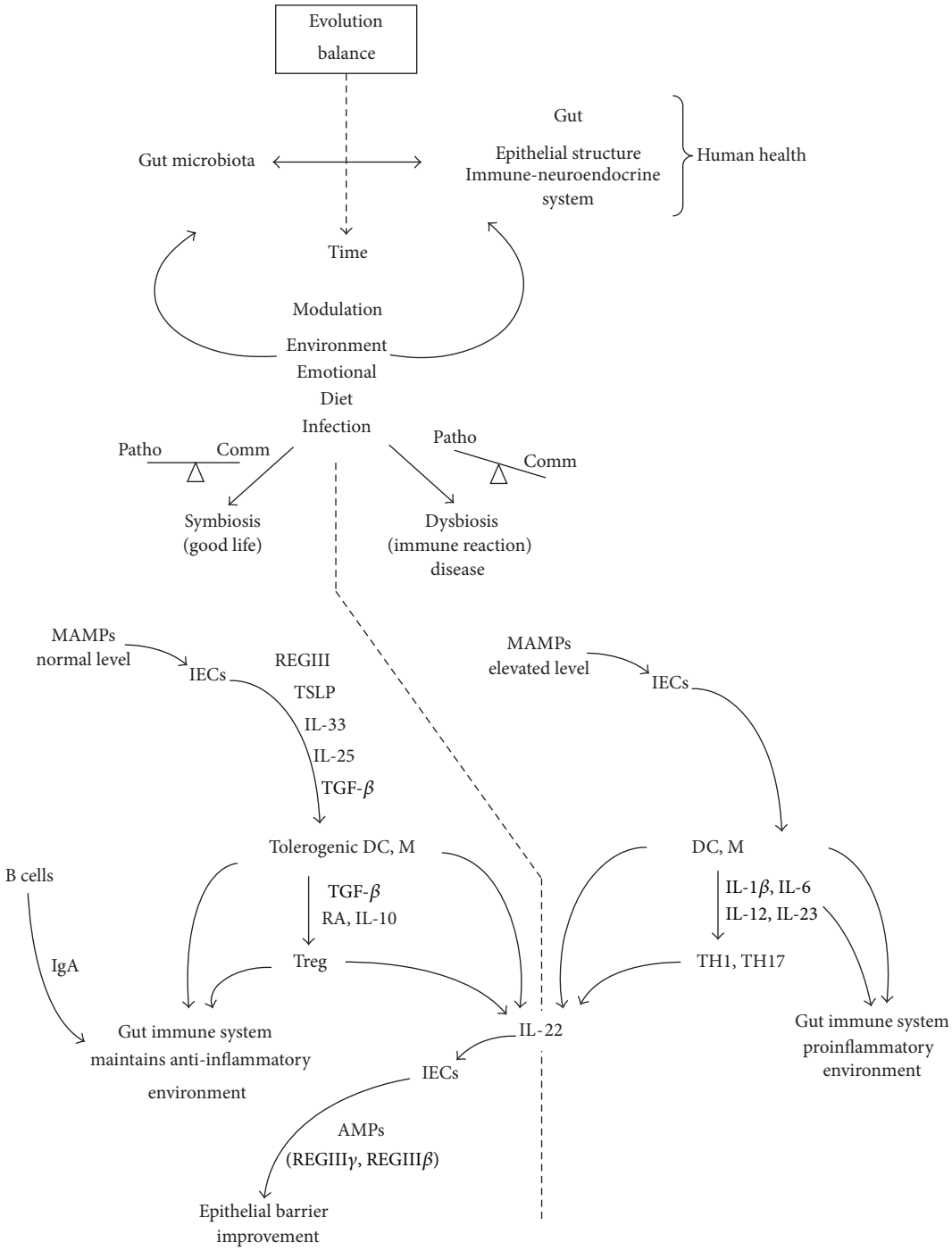

Figure 1: The functional interaction between microbiota and intestinal immune system. The evolutionary balance is formed over time, being modulated by the environmental pressure. Gut microbiota and gut environment are developed together, fitting for the benefit of both or tolerating each other. The immune system monitors the interaction to ensure homeostasis and contributes to symbiosis. However, the unbalance caused when dysbiosis is installed may cause the immune system reaction. Symbiosis and dysbiosis depend on balance between commensal and pathogenic bacteria. Commensal bacteria promote an anti-inflammatory environment. In a symbiosis context, MAMPs continuously stimulate IECs to secrete molecules that act protecting the epithelium and producing a tolerogenic environment. In dysbiosis, there is a significant liberation of MAMPs that can induce IECs, activated DCs, and macrophages to secrete inflammatory cytokines. Consequently, a development of immune effectors is generated. IL-22 is produced in both situations, but its contribution to epithelial barrier improvement is controlled by immune regulation. M: macrophage; Comm: commensal bacteria; Patho: pathogenic bacteria.

affects the Tregs proliferation while conventional $CD4^+$ T cells are affected systemically. This study, together with several others, confirms microbiota influence in homeostasis through Treg formation.

Despite the undoubted influence of microbiota in the regulatory cells, the mechanisms by which bacterial population induces the development of Treg cells remain poorly understood. To unravel this mystery, Obata et al. [53] inoculated germ-free mice with commensal microbiota and monitored the changes in IL-2 expressing-$CD4^+$ T cells and $FoxP3^+$ Treg cells population in lamina propria. The results showed an increase on IL-2^+ $CD4^+$ T cells that peaked in day 3 of bacterial colonization and returned to basal frequency around day 7. However, the analyses of the kinetics of Treg cells expansion demonstrated that, different from IL-2^+CD4^+ T cells, Treg cells continued to expand and became the most abundant $CD4^+$ T cells in colon. This expansion was dependent on early IL-2, considering that treatment with neutralizing antibody to IL-2 abrogates this event. These findings suggest that microbiota stimulated the Treg cells development in an IL-2 dependent manner.

After determining the importance of IL-2, this study compared the genes that are upregulated in Treg cells responsive to IL-2. These comparisons allowed selecting the Uhrf1 gene ("ubiquitin-like, with pleckstrin-homology and RING-finger domains 1") that was upregulated in colonic Treg cells. In agreement, *Uhrf1* knockout mice showed a defective accumulation of colonic Treg cells that was associated with spontaneous development of colitis. Thus, the authors suggest that colonizing bacteria can elicit, through antigen presentation cells, an early IL-2 production by effector $CD4^+$ T cells. This IL-2 provides a signal for Tregs proliferation and to induce upregulation of *Uhrf1* gene. This last event supports the continuous proliferation of Treg cells that are able to prevent excessive immune response against microbiota.

Attempting to determine how commensal microbes can regulate host intestinal immunity and promote homeostasis, Mortha et al. [54] performed a very interesting and important work that established an axis between microbiota, innate immunity, and regulatory cells. Evaluating the role of granulocyte-macrophage colony-stimulating factor (GM-CSF)—renamed colony-stimulating factor 2 (Csf2)—in intestinal homeostasis, the authors observed that Csf2 knockout mice ($Csf2^{-/-}$) presented a significant reduction in the frequency, number, and proliferation of regulatory cells ($CD45^+$ $TCR\beta^+$ $CD4^+$ $FoxP3^+$) in the colon. These alterations in Tregs number were associated with a significant reduction in the frequency and number of IL-10- and IL-2-producing cells and with an increase of colonic IFN-γ producing T cells. Moreover, in $Csf2^{-/-}$ mice were found reduced numbers of colonic dendritic cells (DCs) and macrophages besides a significant reduction in production of regulatory mediators (retinoic acid, IL-10, and TGF-β) important to Treg cells generation. These results demonstrate that Csf2 is involved in colonic homeostasis influencing the number, frequency, and function of DCs and macrophages and, thereafter, in Treg differentiation.

Once the importance of Csf2 for homeostasis is known, the study showed that $ROR\gamma t^+$ type 3 innate lymphoid cells (ILC3) (reviewed in [55]) localized in isolated lymphoid follicles (ILFs) are the main producers of Csf2 and this production is stimulated by macrophage-derived IL-1β. Finally, using antibiotic treated mice and MyD88 knockout mice, this work determined that the microbiota is able to stimulate the macrophage-derived IL-1β production in a MyD88 dependent way. Collectively, these results revealed that commensal bacteria are sensed by macrophages that produce IL-1β. This cytokine stimulates the release of Csf2 by ILC3, which in turn controls the production of regulatory mediators by DCs and macrophage, to maintain colonic Treg homeostasis. Disturbance in this relationship induces homeostasis breakdown and can result in impairment of oral tolerance to dietary antigens [54].

Several works are trying to identify metabolites of the microbiota able to influence the immune system and induce homeostasis. In this context, Smith et al. [56] demonstrated that germ-free mice have significant reduction on the concentration of three of the most abundant types of short-chain fatty acids (SCFA: acetic acid, propionic acid, and butyric acid) suggesting a relation between these molecules and the immunological problems faced by this kind of mice. To clarify this question, germ-free mice were treated with SCFA (individually or in combination) for 3 weeks. As expected, these mice showed increase in frequency and number of colonic Treg cells, which do not happen with TH1 or TH17 cells. The SCFA treatment was also able to induce increase of *FoxP3* and *IL-10* gene expression and IL-10 production, suggesting that SCFA can induce specifically $FoxP3^+$ IL-10-producing Treg cells. Moreover, the SCFA treatment was able as well to reduce the symptoms of T cell-transfer model of colitis. Collectively, these results demonstrate that SCFA play an important role in maintaining homeostasis through Treg cells.

The actions of microbiota-derived metabolites on Treg cells (mainly SCFA) were also confirmed by other studies conducted by Furusawa et al. [57] suggesting that these compounds can subvert the adaptative immunity, diminishing the effector response and contributing to health.

The consequences of losing the intestinal immunologic control are not merely local but do reflect in a systemic manner. The lack of homeostasis may lead to invasion of immunogenic molecules derived from the cell wall of Gram-negative bacteria to the bloodstream, in a condition named endotoxemia. Changes in gastrointestinal barrier function, caused by diet change, can also develop endotoxemia [58]. The increase in gut permeability can be caused by alterations in the gut microbiota; alterations in the expression, localisation, and distribution of tight junction proteins (claudin, ZO-1, and occludin); decrease in intestinal alkaline phosphatase activity leading to a decrease in LPS detoxification; and, recently observed, overactivation of the CB1 receptor (discussed later) [59]. During dysbiosis, the gut microbiota may produce high levels of endotoxins, which once in the bloodstream cause mild and continuous induction of proinflammatory mediators, resulting in low-grade systemic inflammation. This inflammatory state contributes to the progression of

many human diseases, including obesity, type 2 diabetes, liver and cardiovascular diseases, and inflammatory bowel diseases.

In order to visualize how the microbiota influences the immunologic status as a whole, IBD is given as an instance, as it is one of the most studied diseases and one of the most aggressive conditions related to the gut microbiota and immune system. Numerous studies have correlated the reduction in *Faecalibacterium prausnitzii* (which belongs to the phylum Firmicutes and is the major bacterium of the *Clostridium leptum* group) to IBD. Cao et al. [60] by meta-analysis (with a total of 1180 patients analyzed) revealed that IBD patients have a significant reduction of *F. prausnitzii*. The authors suggest a possible protective benefit of *F. prausnitzii* against the development of IBD and recommend the use of prebiotics and probiotics so as to augment the levels of this species. Table 2 summarizes more examples of the immune alterations which happen due to alterations in the levels of specific bacteria.

As demonstrated by the studies described above, the intestinal microbiota and the immune system interact continuously to the establishment of a complex dynamic equilibrium that maintains the host health. Despite numerous papers that address this issue, many gaps remain to be elucidated and several other strategies will be needed to answer these questions. Nevertheless, a complete understanding of the immunity/microbiota relationship may be the key to treatment of several important diseases that affect humans.

3. Gut Microbiota and Neuroimmune System Interaction

Microbiota can alter behavior, humor, and anxiety in stress response [61]. These alterations can be achieved through the pituitary-adrenal axis (HPA) system. Several researches have demonstrated by distinct methodologies, such as germ-free mice [62], pathogenic bacteria infection [63], antibiotic use [64], vagotomy [65], and measurement of excitation by vagal afferents [66], a role for enteric nervous system (ENS) and vagus nerve, which belongs to the autonomic nervous system (ANS), as pathways for modulating the central nervous system (CNS) by microbiota. Inversely, they also demonstrate how CNS or ANS influence microbiota via intestinal secretion and motility, besides the soluble molecules in the lumen and internally below the gut epithelial layer. In addition to this, there is hormone releasing by epithelial cells and secreted microbial products that induce the epithelial releasing of molecules that modulates the neural system [69]. To understand this systemic communication branch, it is necessary to understand the two main gut-brain axes: the HPA and the ANS.

HPA axis initiates with the secretion of corticotropin-releasing hormone (CRH) by neurons in the paraventricular nucleus of the hypothalamus. CRH reaches the anterior portion of pituitary gland, which secretes adrenocorticotropin hormone (ACTH) into the bloodstream reaching the adrenal glands and inducing cortisol release that will act throughout the body via glucocorticoid receptor (GR). This phenomenon was named adaptive stress response [67, 68].

ANS is divided into sympathetic, parasympathetic, and enteric systems. To detect the signals generated in the gut, the ANS make use of sensory neurons that are divided into intrinsic ones localized inside the tissular intestinal structure, as the intrinsic primary afferent neurons (IPANs), which are located in the myenteric nervous system, and extrinsic ones that comprise the vagal and spinal extrinsic primary afferent neurons, which are out of the tissue structure of the intestine and project dendrites to form synapses with the enteric neurons. To complete the neuronal intestinal network, sympathetic neurons communicate with the myenteric plexus, by innervating each other [69].

Vagus nerve provides information from intestines to the brain by solitary tract and sends information that can alter behavioural responses by activation of the interaction between locus coeruleus, also considered a major site for integrating stress responses, and forebrain, to produce corticotropin-releasing factor (CRF) [70, 71]. In addition, vagus nerve talks to the hypothalamus interfering in the HPA axis [72]. This chronically activated pathway promotes neural alterations leading to anxiety, panic disorders, and depression [73]. This view brings ideas for investigating the cross talk between gut bacteria and the CNS via vagus nerve and HPA axis.

3.1. Gut Microbiota and HPA Axis. It has been reported that HPA axis prevents massive damage to the inflammatory sites. Once the stress response is activated, cortisol secretion negatively regulates inflammation and immune response. Overactivation of HPA axis by chronic stressors may explain its detrimental effects on immune cells [67].

For example, while in mast cells cortisol inhibits the release of histamine, which reduces eosinophil recruitment, in T cells the glucocorticoid receptors regulate the expression of IL-4, IL-5, and IL-13 when exposed to allergens [74, 75]. It has been proved that not only the brain but also immune cells are sources of neuropeptides. Kavelaars et al. [76] showed that corticotropin-releasing factor and arginine vasopressin can induce secretion of beta-endorphin in mononuclear cells. Moreover, Westly et al. [77] provided strong evidence that immune cells can synthesize proopiomelanocortin. In addition, glutamate is known to be produced by dendritic cells (DCs) in the context of antigen presentation [78]. Literature has increased when regarding neuroactive products being endogenously produced by immune cells (Table 3) [78–86].

Most importantly, little is known about the idea of microorganisms or their products to be responsible for triggering the neuroactive components release by immune cells. Indirectly, it has been demonstrated that microbiota can program central responses. While germ-free mice had an overstressed response that could be reversed by microbiota reconstitution with faeces or with *Bifidobacterium infantis* [86], enteropathogenic *Escherichia coli* were capable of enhancing the response to stress.

Ait-Belgnaoui et al. [87] suggested that microbiota might alter gut permeability and lead to lipopolysaccharides (LPS)

Table 2: Gut microbiota microorganisms and correlated immune state, disease, or symptoms.

Gut microbiota microorganism	Model system studied	Associated physiopathological condition	References
Bifidobacterium lactis (LAFTI B94)	Rat	Decrease in the levels of TNF and iNoS in rats with colitis induced by TNBS	[20]
Bifidobacterium infantis (35624)	Mouse	Induction of Treg and inhibition of NF-κB in mice with enteric *Salmonella*-induced enteritis	[20]
Escherichia coli (Nissle 1917)	Human and mouse	Diminishing of TLR2- and TLR4-induced inflammation of the colon in humans and mice with ulcerative colitis and colitis induced by DSS	[20]
Lactobacillus rhamnosus (Lr32 and GG)	Mouse and rat	Induction of Treg in mice and rats with colitis induced by TNBS associated with hLA-B27	[20]
Lactobacillus salivarius (Ls33)	Mouse	Decrease of colonic inflammation of mice with colitis induced by TNBS	[20]
Lactobacillus reuteri (strain not specified)	Mouse	Upregulation of NGF and decrease of IL-8 and TNF levels in IL-10 deficient mice	[20]
Lactobacillus plantarum (299V)	Mouse	Decreased levels of IFN-γ and IL-12p40 in IL-10 deficient mice	[20]
Lactobacillus fermentum (CECT5716)	Rat	Lower levels of TNF and iNoS in the colon of rats with colitis induced by TNBS	[20]
Lactobacillus casei (LAFTI L26)	Rat	Decreased levels of cyclooxygenase 2 in the colon of rats with TNBS-induced colitis	[20]
Lactobacillus acidophilus (NCFM)	Human	Prevention of the loss of insulin sensibility in individuals with glucose intolerance and/or diabetes mellitus	[21]
Lactobacillus gasseri (SBT2055)	Human	Weigh, BMI, circumference of waist and hip, and visceral and subcutaneous fat reduction in individuals with BMI from 24,2 to 37 km/m^2 and visceral fat accumulation	[21]
Bacteroides thetaiotaomicron (strain not specified)	Rat	Decrease in the levels of IL-8 and TNF in rats with enteritis induced by enteric *Salmonella*	[20]
Bacteroides fragilis (wild type)	Mouse	Production of IL-10 derived of T CD4+ in mice with colitis induced by TNBS	[20]
Fusobacterium nucleatum (ATCC 25586)	Human	Occurrence of colon-rectal carcinoma	[22]
Faecalibacterium prausnitzii (DSM 17677 in mouse and wild type in humans)	Human and mouse	Decrease in the levels of NF-κB, IL-8, and TNF and increase in the production of IL-10 in mice with TNBS-induced colitis; protection against development of IBD in humans	[20]
Helicobacter pylori (absent or present in low levels—wild type)	Human	Paediatric asthma and reflux esophagitis occurrence	[22, 23]
Akkermansia muciniphila (ATCC BAA-835)	Mouse	Improved metabolic disorders in diet-induced obese mice and counteracted diet-induced colon mucosal barrier dysfunction	[24, 25]

DSS, sodium dextran sulphate; IFN-γ, interferon-γ; IL, interleukin; iNoS, inducible nitric oxide synthase; NF-κB, nuclear factor κB; NGF, neural growth factor; TGFβ, transforming growth factor-β; TLR, Toll-like receptors; TNBS, trinitrobenzenesulfonic acid; TNF, tumor necrosis factor; Treg, regulatory T cells.

transmigration into the blood, increasing neuroendocrine response to stress. Probiotic treatment attenuated HPA response by enhancing the intestinal-epithelial barrier, thus reducing circulating LPS. It leads to the conclusion that gut bacteria have an important role in altering HPA response by acting directly with part of its structure or indirectly by protecting gut permeability. However, the underlying mechanisms remain unclear.

The opposite way also occurs. For example, mice exposed to a social stressor called social disruption presented significantly changed community structure with decreased abundance of *Bacteroides* spp. and increased *Clostridium* spp. In addition, increased circulating levels of IL-6 and the chemokine CCL2 (also known as MCP1) were shown, which is indicative of immune reaction [88].

3.2. Gut Microbiota and Development and Regulation of CNS. As we have seen, the gut microbiota influence is not restricted to the gastrointestinal tract, and studies show the close relationship between the microorganisms and the development and regulation of the nervous system [62, 75, 87]. This influence is due to the fact that microbes are capable of releasing products that act upon the development and function of the nervous system [61, 67, 68, 70]. In this context it is necessary to elucidate the beneficial and deleterious effects of the gut microbiota in the nervous system [43].

Table 3: Cellular sources of neuroactive products in the immune cells.

Cellular source	Hormone/neurotransmitters
Lymphocytes	Acetylcholine, melatonin
B lymphocytes	ACTH, endorphins, GH, IGF-1
T lymphocytes	5-HT, ACTH, endorphins, TSH, chorionic gonadotropin, GH, PRL, parathyroid-hormone-related protein, IGF-1, VIP
Macrophages	ACTH, endorphins, GH, substance P, IGF-1, atrial natriuretic peptide
Dendritic cells	Glutamate, dopamine
Splenocytes	LH, FSH, CRH, adrenaline, endomorphins
Thymocytes	CRH, LHRH, AVP, OT, adrenaline
Mast cells	VIP, somatostatin
Neutrophils	VIP, somatostatin
Megakaryocytes	Neuropeptide Y

5-HT, 5-hydroxytryptamine (serotonin); ACTH, adrenocorticotropic hormone (corticotropin); AVP, arginine vasopressin; CRH, corticotropin-releasing hormone; FSH, follicle-stimulating hormone; GH, growth hormone; IGF-1, insulin-like growth factor 1; LH, luteinizing hormone; LHRH, luteinizing-hormone-releasing hormone; OT, oxytocin; PRL, prolactin; TSH, thyroid-stimulating hormone; VIP, vasoactive intestinal peptide.

Recent studies demonstrated that morphological and functional abnormalities of the enteric nervous system (ENS), the complex neuronal network that autonomously regulates most gastrointestinal functions, also could be related with microbiota and immune system. Using TLR2 knockout mice (TLR2$^{-/-}$), Brun et al. [89] detected a significant reduction in the number of enteric glial and neuronal cells in these mice, suggesting that the development of these cell types is dependent on TLR2 signalling. In addition, alteration of neurochemical profile (reduction of neuronal nitric oxide synthase—nNOS), increase of the frequency and amplitude of spontaneous contraction, elevation of intestinal traffic, and reduced levels of glial cell line derived neurotrophic factor (GDNF) in smooth muscle cells were observed. All these changes in TLR2 mice were completely reversed by administration of exogenous GDNF, confirming that these abnormalities on enteric nervous system are TLR2/GDNF dependent.

To investigate the influence of gut microbiota on ENS integrity and function, wild type mice (C57BL/6j) were depleted from microbiota through treatment with broad-spectrum antibiotics. These depleted mice presented reduced expression of neuronal peripherin, nNOS, and glial S100β proteins, similarly to TLR2$^{-/-}$. All these alterations were associated with a reduction of GDNF expression and, again, the supplementation with GDNF was able to reverse these abnormalities. In a very interesting way, these defects presented by microbiota-depleted mice were also partially restored when these mice received TLR2 agonist. Thus, this work confirms that ENS integrity and functionality are dependent on gut microbiota and TLR2/GDNF pathway. Moreover, these results showed that microbiota stimulated-TLR2 not only represents an immunological role, but also influences directly ENS integrity and is very important to preserve gut homeostasis [89].

IPANs, in the myenteric plexus of the enteric nervous system, provide the intestinal mucosa with sensory fibers that innerves the gut velocities [90, 91]. In this regard, IPANs are neurons cells prone to respond to probiotics and commensal bacteria and alter the gastrointestinal physiology [69]. As they are also sensitive to bioactive bacteria and to neurotransmitters released by microbes, Kunze et al. [92] verified that rats fed with *Lactobacillus reuteri* displayed increased excitability and number of action potentials in IPANs. Other studies showed the analgesic activities promoted by species from the *Lactobacillus* genus, which was obtained from the inactivated microorganism and conditioned media used [93, 94].

The *Lactobacillus reuteri* CRL1098 and JCM1112, isolated from the human intestine and other animals, can produce vitamin B12, an important vitamin for the nervous system, and its deficiency could induce neuropathies [62]. Wall et al. [95] demonstrated in their study that when *Bifidobacterium breve* strains, a commensal group, were used as probiotic, the mice brain fatty acid composition was changed, showing increase in concentration of arachidonic and docosahexaenoic acid, both important in neurogenesis and neurotransmission, when compared to the nonsupplemented group.

Taylor and Feng [96] showed that circulating substances in the blood, such as tryptophan (an important precursor of the neurotransmitter serotonin), are changed with the presence or absence of intestinal microbiota. Treatment of Sprague-Dawley rats with *Bifidobacterium infantis* shows an increase in plasma tryptophan and decrease in frontal cortex 5-hydroxyindole acetic acid (5-HIAA) levels, which suggests that there may be happening reduced serotonin degradation in this brain area. Moreover, the supplementation with *Bifidobacterium infantis* was capable of reducing the inflammatory mediators (IL-6, TNF-α, and IFN-γ), demonstrating the influence of gut microbiota also on the immune system [97]. The increase of tryptophan is consistent, once IFN-γ has been shown to be a potent stimulus in the activation of indoleamine (2,3)-dioxygenase (IDO), the enzyme involved in the conversion of tryptophan to kynurenine [96].

3.3. Gut Microbiota and Experimental Autoimmune Encephalomyelitis Model. Taking into account the relationship between the nervous system, the immune system, and the gut microbiota, it is important to highlight studies that relate the influence of these microorganisms in the development of autoimmune diseases, as multiple sclerosis, directly related to the CNS [43].

Experimental autoimmune encephalomyelitis (EAE) is an experimental model used to study multiple sclerosis, an autoimmune demyelinating disease of the central nervous system. Although the cause of the disease remains unknown, studies have reported the involvement of environmental factors associated with a genetic predisposition. The immune response in EAE is mainly characterized by T helper 1 and T helper 17 cells [98]. Segmented filamentous bacteria (SFB) present in the intestine are related to the induction of Th17

and are indicated to be associated with autoimmune diseases with such cellular profile [99].

In order to verify the influence of intestinal microbiota on the development of EAE, induced animals were treated with antibiotics to reduce the intestinal microbiota; the results showed reduction of clinical signs of EAE in animals with compromised gut microbiota; this reduction was accompanied by a decrease of IFN-γ, MIP-1α, MIP-1β, MCP-1, IL-17, and IL-6 associated with increased IL-10 and IL-13 release [69]. Ochoa-Repáraz et al. [100] relate the action of B $CD5^+$, regulatory B cells, to this improvement of clinical signs in microbiota altered by antibiotics.

The use of germ-free mice also demonstrates the importance of the intestinal microbiota in the development of EAE. Clinical signs of EAE in germ-free animals are attenuated when compared to conventionally colonized animals. These animals showed reduction in the production of proinflammatory cytokines in the CNS, accompanied by increase in number of regulatory cells. Lee et al. [101] induced EAE in germ-free animals and observed a reduction of the inflammatory cytokines (IFN-γ, Il-17A) together with an increase in the regulatory T cells $CD4^+CD25^+$ $FoxP3^+$ (Tregs) not only in the gut, but also in the spinal cord when compared to the wild type mice. Additionally, deficiency was found in dendritic cells to promote differentiation of TH17 cells in germ-free mice [69, 101].

Research in this area is still very incipient and not conclusive. As above described, evidences from works involving EAE indicate that the benefits brought by the microbiota do not apply to improving symptoms of this model. However, recent findings showed that specific microorganisms of the intestinal microbiota could improve the clinical signs of EAE. In this case, these strains of lactobacilli can enhance the immune-regulatory activity both by increased production of IL-10 and by increased rate of B and T regulatory cells. In this study it was found that, of the three strains used, monostrain oral treatment failed therapeutically in EAE, and mixture of lactobacilli strains suppressed the progression and reversed the clinical and histological signs [102].

Thus, the interaction of gut microbiota, immune system, and nervous system is not fully understood with many points remaining to be clarified, which justifies the development of new studies.

3.4. Gut Immune System and Nervous Cannabinoid Signaling. Recently a novel signalling pathway correlating gut immune system and nervous cannabinoid receptors has been investigated. As well known, cannabinoids receptors, composed by CB1 and CB2 receptors, are present in immune and neural cells [103–105]. Recently CBs were found in the luminal surface of the epithelial microvilli, Peyer's patches, ganglionic cells of the myenteric plexus, and smooth muscle of the blood vessels walls [106]. The localization of these receptors in the intestinal epithelium, immune system cells, and nervous system brings new perspective on treatments of disorders related to those systems. From what is already known, CB2 receptors have been connected to analgesic and anti-inflammatory functions in several experimental models of colitis [107, 108].

Such field has gained attention since Rousseaux et al. [94] showed increased mRNA expression of receptors CB2 *in vitro* and in epithelial cells in the colonic section after oral administration of *Lactobacillus acidophilus* NCFM. This result was accompanied by decrease in normal visceral perception. The improvement of visceral pain was attributed to direct contact of NCFM with epithelial cells able to induce CB2 expression, through the NF-κB pathway. Recently, Aguilera et al. [109], after causing dysbiosis by stress and antibiotic treatment, showed increased CB2 receptor mRNA expression in colonic tissues of mice. During the investigation, the authors found increased CB2 expression to be positively correlated with *Lactobacillus* spp. counts and negatively correlated with *Clostridium* spp. counts. Those observations indicated that intestinal endocannabinoid system might modulate visceral pain response and the presence of a bacterial group as a pathogenetic component [109].

Karmaus et al. [110] verified that CB1 activation, by gut microbiota, increased gut permeability. This permeability is caused by altering the distribution of tight junction proteins which elevates endotoxemia. The use of prebiotics for regulating gut microbiota or antagonist of CB1 in obese mice models regulated gut permeability with improved distribution and localisation of tight junction proteins.

Once CBs receptors of intestinal tissue are activated by cannabinoids ligands, it may also activate CBs receptors of other local systems. These data begin to become interesting when they are crossed with studies about cannabinoid system of immune cells. Karmaus et al. [110] demonstrated that the CB1 and CB2 knockout DC presented augmentation of activation-related molecules, such as MCH I, MHC II, CD80, and CD86, after contact with LPS. Chiurchiù et al. [105] verified that the treatment with anandamide, an endocannabinoid, on DCs isolated from healthy donors and multiple sclerosis patients, led to a decrease on production of TNF-α and IL-6. In the same study, it was also shown that treating the DCs with anandamide also decreased their ability of inducing Th1 and Th17. An increase in CB2 expression accreted of decrease of fatty acid amide hydrolase (anandamide degrading enzyme) was also noted in the multiple-sclerosis-patient DCs in comparison to DCs isolated from healthy donors. Such evidences support the DCs to be immunomodulated by cannabinoids. Furthermore, the immunomodulation of DCs by eCBS follows stimulus and polarization of the T cells. Thus, a new possible interaction between gut microbiota and immune system can be perceived through regulation by the endocannabinoid system, having as an initial aim multiple sclerosis studies, as well as an opportunity to understand the interaction comprised in the axis gut-immune-brain.

4. Conclusion

The intestinal microbiota has drawn progressively more attention from the scientific community due to the association of its role in the human physiology and in the development of diseases following dysbiosis. It is known to be associated with regulation of digestion, absorption of nutrients,

biochemistry processes, immune modulation of the mucosa, and the production of toxins substances, autonomous nervous system interaction, and nervous development. In order to advance in the understanding of this complex interaction, the screening of the possible interactions of metabolic pathways is made necessary. Taking a beneficial view of prebiotic and probiotic, mapping the microbiome in agreement with nutrigenomics and nutrigenetics may give rise to the construction of nutritional metabolic collections. These research areas might potentially aid in unraveling several hypotheses related to ambient factors that may lead to disorders of unknown etiology such as the hygiene hypothesis, which postulates that decreased microbial exposure has, in part, driven immune deregulation. Further studies are still needed in order to clarify the interaction between gut microbiota and neuroimmune system, as well as with endocrine system, so as to create nutrigenetic profiles that may aid in reaching individual homeostasis.

Conflict of Interests

The authors certify that they have no commercial or associative interest that represents a conflict of interests in connection with the paper.

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Review Article

CD69 Is the Crucial Regulator of Intestinal Inflammation: A New Target Molecule for IBD Treatment?

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CD69 has been identified as an early activation marker of lymphocytes. However, recent work has indicated that CD69 plays an essential role for the regulation of inflammatory processes. Particularly, CD69 is highly expressed by lymphocytes at mucosal sites being constantly exposed to the intestinal microflora (one of the nature's most complex and most densely populated microbial habitats) and food antigens, while only a small number of circulating leukocytes express this molecule. In this review we will discuss the role of CD69 in mucosal tissue and consider CD69 as a potential target for the development of novel treatments of intestinal inflammation.

1. Introduction

CD69 is commonly used as the marker of activated cells, most often lymphocytes and natural killer (NK) cells. But this molecule is much more than a simple activation marker; it is an important regulator of immune responses in the intestine. The primary role of the intestine is absorption of nutrients. Assisting in the digestion and producing essential vitamins and hormones, trillions of commensal bacteria live in the intestinal lumen [1, 2]. The intestinal immune system has to enable the coexistence of these beneficial microorganisms with the host, but also the efficient elimination of pathogens. To achieve these specific tasks, the mucosal immune system of the intestine developed very specific characteristics.

CD69 is highly expressed by lymphocytes at mucosal sites that are separated by a single layer of intestinal epithelial cells from the lumen. Together with the overlying mucus the intestinal epithelium forms a complex and dynamic mucosal barrier that physically prevents the access of luminal bacteria to the deeper sterile tissues [3]. The cells of the mucosal barrier actively participate in the elimination of pathogens by secreting mucus and antimicrobial peptides, presenting microbial derived antigens to T cells, providing tolerogenic signals (mucus proteins) to dendritic cells (DC) and shaping innate and adaptive immune responses by secretion of cytokines and chemokines [4–8].

However, many pathogens are able to avoid these defensive mechanisms and penetrate the mucosal barrier. The complex network of innate and adaptive immune cells underlying the intestinal epithelium is developed to protect host from penetrating pathogens. High proportions of intestinal lymphocytes are effector memory cells to ensure the fast elimination of pathogens that have passed the mucosal barrier [9–11].

On the other side the regulation of overwhelming immune responses to intestinal microorganisms and pathogens is important in the gut to prevent abnormality and tissue destruction that can lead to diseases, such as inflammatory bowel disease (IBD). Regulatory T cells secreting immunosuppressive cytokines, such as transforming growth factor- (TGF-) β and interleukin- (IL-) 10, limit overwhelming immune responses to pathogens

and are essential for the development of tolerance toward the commensal microflora. Several types of regulatory T cells (Treg) have been described in the gut. Foxp3$^+$ Treg are necessary for the development of tolerance in intestine [12] and are the best studied Treg cells. Tr1 and Th3 cells can be induced in oral tolerance models. Tr1 and Th3 cells have regulatory properties depending on the cytokines IL-10 and TGF-β [10, 13, 14]. IL-10 and TGF-β are also produced by intestinal macrophages and DC, which also contribute to oral tolerance [13, 15].

Both memory T cells and regulatory T cells express CD69 in the gut. In contrast to any other body compartment, intestinal T lymphocytes express high levels of CD69, while only a small number of circulating leukocytes express this molecule in healthy individuals [16, 17]. CD69 is a transmembrane glycoprotein with a C-type lectin domain (CTLD) [18–20]. This molecule is not expressed in detectable levels on naïve leukocytes, but its surface expression is induced promptly upon activation [17–19, 21]. In human diseases, CD69 expression is increased on leukocytes at the site of inflammation [22–25]. Furthermore, early *in vitro* studies described CD69 as a proinflammatory molecule whose engagement with Abs induced intracellular Ca^{2+} influx, lymphocyte proliferation, and the production of proinflammatory mediators, such as IL-2, tumor necrosis factor- (TNF-) α, and nitric oxide (NO) [26–30]. CD69 is also necessary for the cell-contact dependent stimulation of macrophages by T cells [31]. However, recent *in vivo* studies with transgenic mice showed that CD69 can limit the immune response and proposed a regulatory function of CD69. CD69 has been shown to have a role in leukocyte migration, in the function of regulatory T cells and resident tissue memory T cells. In contrast to *in vitro* data, *in vivo* studies reported no role of CD69 in the lymphocyte proliferation [32] and T cell priming, therefore excluding the possibility that CD69 serves as a costimulatory molecule [21]. In different murine disease models, including asthma [33, 34], arthritis [35–37], myocarditis [38], pathogen clearance [39], tumor immunity [40, 41], and IBD [42–44], absence of CD69 expression deeply affected the disease course by exacerbating the disease severity in most cases.

Because CD69 is highly expressed by memory T cells and regulatory T cells in the gut, which play an essential role (i) in eliminating pathogens and (ii) in regulating potential harmful immune responses in the gut, we consider CD69 not as a simple activation marker but as a molecule involved in the regulation of immune responses at mucosal sites. We searched http://www.ncbi.nlm.nih.gov/pubmed database (search terms CD69 or inflammation or inflammatory bowel disease) for the studies on CD69 and intestinal inflammation. We found numerous research articles and reviews dealing with the topics of genetic and molecular structure of CD69 and its functional characteristics. In this review we will summarize the current knowledge about the role of CD69 in regulation of mucosal immune system responses in the intestine of mice and humans. Particularly, we will discuss the potential signals driving CD69 expression in the gut, the role of CD69 for the differentiation of regulatory T cells in the gut and review the possible potential of CD69 for the development of novel target therapies for intestinal inflammation.

2. How Is the Gene Coding for CD69 Organized and What Is the Molecular Structure of CD69 Protein?

Before we will discuss the relevance of CD69 for the regulation of intestinal immune responses we will briefly summarize the genetic organization of the gene cluster coding for CD69 and the molecular structure of CD69. CD69 (a type II C-lectin transmembrane homodimer protein that consists of disulfide-linked subunits [18–20]) is encoded in the NK gene cluster on chromosome 6 in the mouse and on chromosome 12 in the human genome [19, 22]. When the murine gene locus is compared with the human genome, a 58% homology between them can be identified [17]. The NK gene cluster contains the genes coding for NK cell activating and inhibiting receptors, such as CD94 and NKG2, required for the recognition of the target cells by NK cells. Though being structurally homologous with CD94 and NKG2, CD69 is not involved in target cell recognition by NK cells [17, 19, 45]. Upstream of the transcriptional start site in the mouse *CD69* gene putative binding sites for the inducible transcriptional factors nuclear factor (NF)-κB, erythroblast transformation-specific related gene-1 (ERG-1), and activator protein- (AP-) 1 are located [22].

The *CD69* gene exists in a single copy. The transcription of *CD69* leads to the formation of 22.5 kDa polypeptide which can be differentially glycosylated to form 28 or 32 kDa subunits (Figure 1) [17]. These subunits can be randomly combined to form 28-28, 28-32, or 32-32 kDa receptors [17, 28, 46, 47]. Each subunit consists of an extracellular CTLD domain connected by the short neck region to the single transmembrane domain and short cytoplasmatic tail (Figure 1) [17, 18, 22, 48]. Subunits are connected with the disulfide bridge in the extracellular neck region (Figure 1) [17].

Because the extracellular subunits of CD69 form a CTLD, it is likely that a yet not identified ligand binds to CD69. Most members of the CTLD family bind bacterial cell surface carbohydrates in a Ca^{2+}-dependent manner. Many members of the CTLD family, such as the asialoglycoprotein DC-SIGN, are expressed by macrophages and DC and serve as pattern recognition receptors (PRRs) [17, 49, 50]. The multi-CTLD endocytic receptor CD23 (FcεRII) is the low affinity receptor for IgE and binds the glycosylated Fc fragment of IgE [51]. CD72 (expressed by B cells) binds the glycoprotein CD5 expressed by T cells, a process required for the costimulation of T cells [52]. Members of the CTLD family hence bind microbial derived cell surface carbohydrates or glycoproteins leading to speculations that CD69 might bind carbohydrates or glycoproteins. When the extracellular domain of CD69 was analyzed by crystallography these studies demonstrated the absence of classical C-type lectin Ca^{2+}-binding residues in the extracellular CTLD domain of CD69 [18]. Since classical C-type lectin Ca^{2+}-binding residues are missing, carbohydrates are likely not the ligand for CD69 [18, 53]. In order to

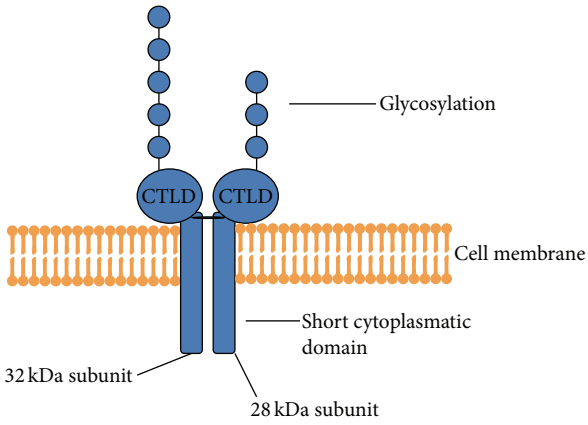

Figure 1: The structure of CD69 molecule. CD69 is membrane-bound protein, a homodimer of two (28 and 32 kDa) differentially glycosylated subunits. Each subunit consists of extracellular C-type lectin domain (CTLD) connected by the short neck region with the single spanning transmembrane domain and short cytoplasmatic tail. Subunits are connected with the disulfide bridge in the extracellular neck region.

generate appropriate signal transduction pathways by CD69 both extracellular CD69 CTLD domains are required to be cross-linked indicating that CD69 may rather bind cell associated glycoproteins than soluble molecules as a ligand [17, 36, 40]. Further investigations are needed to identify the physiological ligand of CD69.

After cross-linking the extracellular CTLD domains, the cytoplasmatic tail of CD69 generates an intracellular signal transduction pathway [17, 18, 22]. The signaling cascade activated by CD69 is not defined in detail. Recent studies showed that the cytoplasmatic domain of this molecule is associated with the Janus family kinase (Jak)3, which then activates the transcriptional factor STAT5 (Figure 2) [33]. Jak/STAT signaling pathway is evolutionary conserved [54] and regulates central cellular processes, such as development and growth. Its disruption can lead to the development of cancers and/or immune deficiencies. Also, the Jak/STAT signaling pathway regulates immune processes, such as the production of interferons and interleukins. The activation of the Jak3/STAT5 pathway indicates the importance of CD69 for the regulation of cellular processes and the immune system.

3. Is CD69 an Early Activation Marker?

Although constantly expressed by monocytes, platelets, Langerhans cells, and a small population of resident lymphocytes in the thymus and secondary lymphoid organs (SLO), CD69 is not found on resting circulating lymphocytes in humans [18, 55–57]. *In vitro* cell activation, using various activators, showed rapid induction of CD69 on human T and B lymphocytes, NK cells, macrophages, neutrophiles, and eosinophiles but also on murine T cells and DC [17–19, 21, 55, 56, 58]. Studies in mice showed that *in vivo* type I interferons (IFN-I) strongly upregulate CD69 expression [42, 59, 60]. Furthermore, our group demonstrated that oral administration of a defined antigen to T cell receptor-transgenic mice induces CD69 expression by CD4 T cells in the colonic lamina propria (LP) within 24 h after the feeding [42]. This was not the case with the other activation marker of lymphocytes CD25 [42]; CD25 induction is reported at the late time points after cell activation [61]. Therefore, CD69 is the first activation-induced protein that can be detected on the surface of lymphocytes [61, 62]. Already at 2 h after the stimulation, this receptor can be found on the surface of human lymphocytes, but its expression is transient as it peaks 18–24 h after stimulation and decreases then [17]. One early study on human peripheral blood mononuclear cells (PBMCs) demonstrated that such a rapid induction of CD69 is due to the presence of this molecule in the cytoplasm of resting lymphocytes as its induction was independent of RNA and protein synthesis [58]. This is why CD69 is widely used in studies for the identification of recently activated leukocytes, especially lymphocytes and NK cells, but the role of CD69 in regulating immune processes has not been intensively studied.

4. Does the Intestinal Microbiota Regulate the Expression of CD69?

About half of all murine intestinal CD4 T lymphocytes express CD69 in homeostatic conditions [42], indicating their activated state. A constant antigen challenge could lead to high CD69 expression by T cells. Since T cells of the gut are exposed to a high antigen load derived from the intestinal microflora and food the homeostatic balance between inflammatory and immunosuppressive immune processes has been considered as a state of physiological inflammation at mucosal sites [63]. When murine T cells isolated from the gut were compared to T cells isolated from the spleen the proportion of CD69-expressing CD4 T cells was lower in spleen as compared to the gut [42]. When T cells were isolated from the colonic LP of OT-II x $RAG^{-/-}$ mice, a high proportion of CD4 T cells expressed CD69, which was not observed on T cells isolated from the small intestine [42]. CD4 T cells of OT-II x $RAG^{-/-}$ animals kept in specific pathogen-free (SPF) conditions are considered naïve as they specifically recognize chicken ovalbumin (OVA) protein that is not found in food or water of SPF mice facilities. It is surprising that these cells express an activation marker. Possible antigen-independent signals may drive CD69 expression by T cells. Microbial-derived factors recognized by pattern recognition receptors could contribute to the CD69 expression on the surface of colonic T cells [42].

The presence of commensal microorganisms is the crucial factor that contributes to high CD69 expression by intestinal lymphocytes. Reduced surface expression of CD69 by intestinal LP CD4 T cells isolated from germ-free (GF) mice and from mice depleted of intestinal microflora by the treatment with broad-spectrum antibiotics has been reported [42].

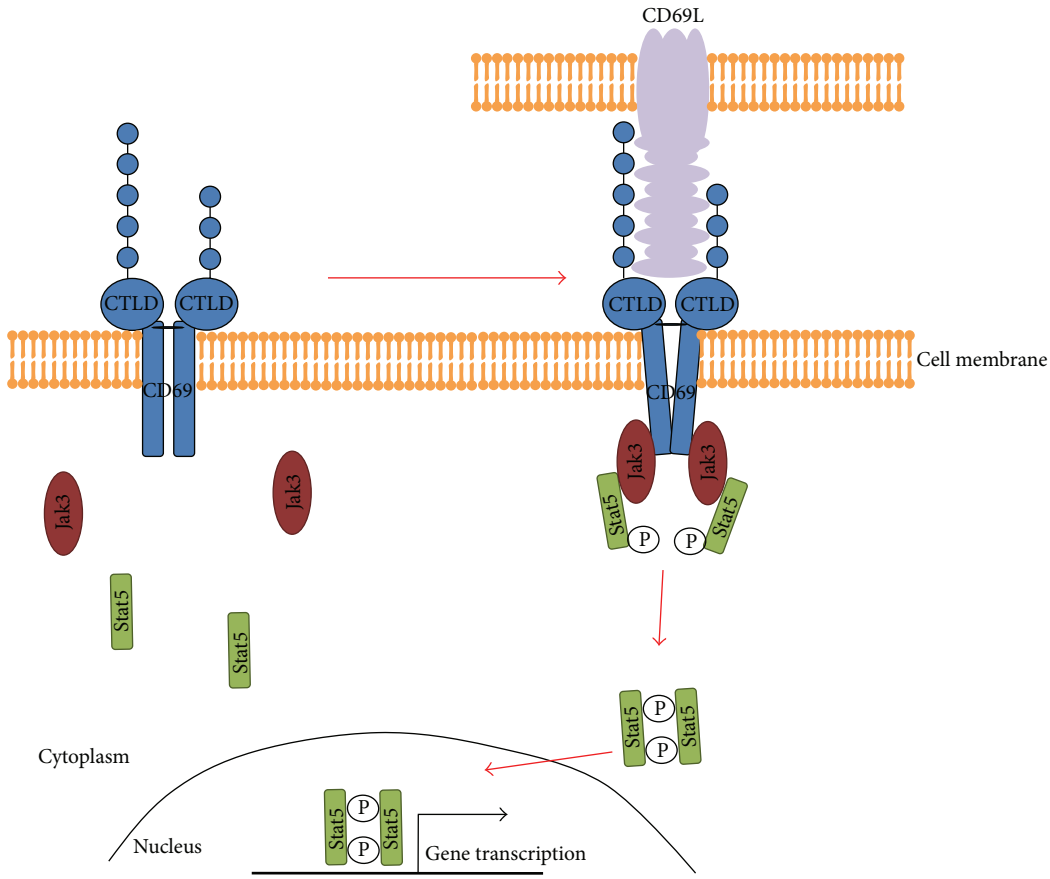

FIGURE 2: The proposed signalling pathway of CD69. After binding a putative ligand (CD69L) that is most probably a membrane bound protein, the cytoplasmatic tail of CD69 associates with Jak3 kinase. Jak3 recruits and phosphorylates the transcription factor Stat5. Phosphorylated Stat5 (Stat5-P) dimerizes in the active form and translocates to the nucleus where it can regulate the gene transcription.

In line with our findings, decreased expression of CD69 by intestinal intraepithelial TCR$\gamma\delta^+$ T cells after the ablation of the microflora has been reported in mice [64]. These results demonstrate that high CD69 expression by lymphocytes in the gut is the consequence of the close proximity of the microflora in intestinal immune compartment. Luminal microorganisms are of importance for the development of mucosal immune responses in the intestine highlighting that the development and function of the mucosal immune system in the intestine differ from the immune system in other body compartments. The induction of CD69 by the specific intestinal environment could play an essential role in shaping immune responses of the gut to protect the host from an uncontrolled invasion of luminal microorganisms. We will hence further discuss the role of CD69 in regulating lymphocyte migration, in controlling the function of resident memory T cells and the differentiation of regulatory T cells.

5. Does Lymphocyte Migration Depend on CD69?

CD69 is of importance for the retention of lymphocytes in lymphoid compartments. Activated lymphocytes express CD69, which leads to the retention of lymphocytes in lymph nodes possibly to obtain effector characteristics. Naïve immune cells constantly recirculate through the body, entering the SLO in their search for the potential pathogen-derived antigens and egressing back to the circulation if the specific antigen is not found [65]. The combination of addressins, chemokines, and receptors is tissue-specific to regulate the lymphocyte traffic to a defined tissue compartment. In the intestine, SLO express the chemokines CCL-19 and CCL-21 that bind to CD62L and CCR-7 expressed by naïve-cells, respectively [66–68]. In mice the egress of the lymphocytes from SLO is in general dependent from sphingosine-1 phosphate receptor type 1 ($S1P_1$) expression and its interaction with sphingosine-1 phosphate (S1P) from the circulation [59, 69–72]. After egress, lymphocytes that recognized intestine-derived antigens express CCR-9 and $\alpha 4\beta 7$ that can bind to CCL-25 and MadCAM-1 expressed specifically in the small intestine [66, 67].

Recently, studies in mice pointed at CD69 as one of the major regulators of lymphocyte migration throughout the body. Expressed on activated cells, this molecule captures the lymphocytes that recognized antigen in the SLO for a certain time period that allows them to become fully activated cells [59]. As shown in mice CD69 directly binds $S1P_1$ receptor

on the lymphocyte surface and mediates internalization of $S1P_1$, preventing the lymphocyte egress [59, 70]. Also, CD69 prevents the egress of T cells from the thymus as shown by transgenic overexpression studies in mice [73, 74]. A very recent study demonstrated that CD69 controls selectively the egress of activated antigen-experienced CD4 T cells from Peyer's patches (PP) during the *Salmonella* infection in mice [75]. The same study also showed the existence of $CD69/S1P_1$-independent pathway responsible for the global "shut-down" of lymphocyte egress from *Salmonella* infected PP [75]. This creates a need for further investigations of lymphocyte migration during inflammatory conditions in the intestine, as this process could be regulated by completely other molecules than the normal, homeostatic migration. This study also showed a particularly important role CD69 plays in the immune responses of intestinal CD4 T cells [75]. Supporting this, our study showed that CD4 T cell accumulation in the murine colonic LP during IBD is CD69-dependent [43]. Furthermore, the absence of CD69 deeply affected the pattern of chemokine expression and *in vitro* responses to chemokine stimuli by murine CD4 T cells [43]. Hence, CD69 regulates the traffic of intestinal CD4 T cells through complex mechanisms that include both $S1P_1$ and chemokines; these processes are of great relevance for the inflammation development in intestine. The importance of CD69 on the lymphocyte migration in human IBD needs to be further investigated.

6. Does CD69 Expression Indicate Resident Memory T Cells?

During the immune response the majority of the effector lymphocytes die by apoptosis but a certain number of them remain as long-living memory cells. Memory lymphocytes provide fast and efficient protection during the reexposure to the same antigen again. Different types of memory T cells exist in mice and humans depending on their location and migratory pattern [76]. These types can be distinguished based on the surface markers expression and the cytokine profile. All murine memory cells are expressing high levels of CD44, in contrast to naïve and effector lymphocytes [77, 78], while human memory T cells are usually characterized based on multiple marker expression. Central memory T cells (T_{CM}) of humans and mice migrate from the blood to the SLO and express the SLO-homing receptors CD62L and CCR7. T_{CM} cells secrete IL-2, but not effector cytokines [79, 80]. Effector memory T cells (T_{EM}) migrate from the blood into the peripheral nonlymphoid tissues as they express the inflamed tissue homing receptors and not CD62L and CCR7. Studies in humans showed that these cells very efficiently protect the peripheral tissues by production of effector cytokines such as IFN-γ and IL-4 [79–81]. Recently, the existence of tissue resident memory T cells (T_{RM}) has been reported not only in the human skin [82] and lungs [83, 84], but also in murine lungs [83, 84], central nervous system [85], bone marrow (BM) [86], and intestine [87, 88]. In mice CCR7-negative T_{RM} cells are retained in the periphery and do not migrate from the periphery to secondary lymphoid structures [87, 89]. The intestinal T_{RM} cells in mice are particularly well characterized. It is found that the major phenotypic characteristic of these cells is the expression of CD103 and CD69 [87, 89, 90]. TGF-β signaling promotes the expression of CD69 and CD103 and therefore is crucial for the formation and maintenance of T_{RM} cells in the murine gut [91]. CD69 is necessary for the formation of BM T_{RM} cells in mice as $CD69^{-/-}$ CD4 effector T cells fail to migrate to BM in the late phase of an immune response [92]. Most probably $CD69^+CD49b^+$ effector T cells are the precursors of BM T_{RM} cells as the blockade of their expression impairs the formation of murine T_{RM} CD4 lymphocytes [93]. Very recent study in mice reported the existence of recirculating memory T cells (T_{RCM}) that migrate from the peripheral tissues to the local SLO and then further in the systemic circulation [76]. These cells are characterized as $CCR7^+CD62L^{int}CD69^-CD103^{+/-}$ [76]. This confirms that CD69 is expressed by memory cell subset that is retained in the periphery. Furthermore, studies in humans showed that the expression of CD69 is the major characteristic of the intestinal resident memory T cells and that constant expression of CD69 distinguishes the tissue resident from circulating memory T cells [94]. Hence, CD69 emerges as the major factor that contributes to the immunological memory in the peripheral tissues, such as the intestine. Further studies need to elucidate if CD69 is just a marker indicating T_{RM} cells or if it is involved in regulation of the effector functions (beside retention in lymphoid tissues) of these cells.

7. Is the Differentiation of Regulatory T Cells CD69-Dependent?

Treg cells suppress the differentiation and/or proliferation of effector cells, thereby preventing the immune reactions against self-antigens (autoimmunity) and harmless antigens (e.g., commensal microflora). It is considered that Treg cells can be the powerful therapeutic tool for the treatment of inflammatory diseases. Indeed, adoptively transferred $Foxp3^+CD4$ Treg cells were able to suppress the disease development in murine models of colitis [95, 96], arthritis [97], and experimental autoimmune encephalitis [98]. Foxp3 Treg cells develop in thymus from positively selected $CD69^{hi}$ TCR^{hi} thymocytes [99]. These cells in both mice and humans can also be generated on the periphery from naïve $CD25^-CD4$ T cells in the presence of TGF-β and retinoic acid [100–102]. The mechanisms of suppression by $Foxp3^+$ cells are largely unknown, but the role of TGF-β in this process has been suggested [103]. Our group showed that CD69 affects the generation of murine peripheral $Foxp3^+$ Treg cell population as the fraction of these cells was reduced in the intestine of CD69-deficient mice [42]. This effect was especially strong after the oral administration of a specific small protein antigen (OVA) that in normal mice induces the differentiation of Foxp3 Treg cells [42]. Furthermore, naïve CD4 T cells from CD69-deficient animals had a reduced ability to differentiate in $Foxp3^+$ cells *in vitro* [42]. Supporting the role of CD69 in the development of Foxp3 Treg cells, several publications reported that crosslinking of CD69

induces the production of TGF-β by murine [40, 42, 104] and human cells [105].

Studies on mice and human cells have shown that CD69-expressing CD4 T cells have regulatory properties. In the murine model of spontaneous systemic lupus erythematosus $CD69^+CD4$ T cells suppressed the production of proinflammatory cytokines by $CD69^-CD4$ T cells [106]. Han et al. in their paper called murine $CD69^+CD4^+CD25^-$ tumor-induced T cells a new Treg cell subset as they observed suppressive properties of these cells mediated by membrane-bound TGF-β [107]. This novel regulatory cell type was also found among human peripheral blood cells and is characterized as $CD4^+LAP/TGF\text{-}\beta^+Foxp3^-TGF\text{-}\beta RII^+CD69^+$ cells showing TGF-β-dependent suppression of immune responses [108]. These cells accumulate in hepatocellular cancer patients and their number positively correlated with the tumor size [109, 110]. Also, priming the human DC with supernatant of apoptotic tumor cells imprinted the DC to induce $CD69^+$ Treg cells [111]. These data confirmed that the presence of Treg cells favors the growth of the cancer. On the other side, high frequency of $CD69^+CD4$ Treg cells decreased the risk of graft-versus-host disease after the transplantation of allogenic organs in humans [112].

It is postulated that stable expression of CD69 defines this novel CD4 Treg cell subset. Lymphocyte activation activates the canonical NFκB signaling pathway that controls early and transient expression of CD69 on recently activated human cells [113]. The late and stable expression of CD69 on human lymphocytes is controlled by the noncanonical NFκB pathway [113]. Activation of these different signaling pathways distinguishes activated and regulatory T cells. Confirming this hypothesis, a recent study reported that the anti-inflammatory drug curcumin induced the late phase CD69 expression connected with increased TGF-β production *in vitro* [114].

The existence of $CD69^+$ Treg cells in intestinal tissues and their possible role in the homeostasis and inflammation in humans has yet to be studied. Oral administration of a defined antigen to mice induced $CD69^+CD4$ T cells that are Foxp3-negative but LAP/TGF-β1-positive cells in colonic LP [42]. If the $Foxp3^-LAP/TGF\text{-}\beta1^+CD69^+$ cell is a precursor of fully matured peripheral $Foxp3^+$ Treg cells needs to be elucidated. To summarize we believe that CD69 regulates TGF-β production by T cells and may serve as a regulatory molecule in the immune system. To further discuss our hypothesis we review the role of CD69 in intestinal inflammation.

8. Does CD69 Regulate Intestinal Inflammation?

Recent studies in CD69-deficient mice showed that this molecule regulates immune responses in intestine [42–44]. As already discussed, CD69 expression on intestinal lymphocytes is regulated by the microflora. Furthermore, $CD69^{-/-}$ mice were not able to establish oral tolerance to the small food-derived protein OVA [42]. This could be due to the reduced regulatory cell-mediated responses in the absence of CD69. In several different models of experimental colitis, the deficiency of CD69 led to a very serious clinical picture of the disease. Transfer of $CD69^{-/-}$ naïve CD4 T cells into immunodeficient $RAG^{-/-}$ hosts induced a high body weight loss with rise in the systemic levels of proinflammatory cytokines IFN-γ, IL-17A, and TNF-α as compared to $RAG^{-/-}$ animals receiving T cells from wt animals [42]. In antigen-specific colitis models, the transfer of OVA-specific OT-II $CD69^{-/-}$ naïve CD4 T cells into $RAG^{-/-}$ animals followed by oral delivery of OVA protein resulted also in significant body weight loss and severe colitis [43]. The same was observed in a chemically induced colitis model when dextran sodium sulfate (DSS) was administrated to CD69-deficient mice. These animals develop severe disease with increased transcript levels of the proinflammatory chemokines and cytokines, such as IFN-γ [43]. In all these models, histopathological examination of the colonic tissue in mice revealed that absence of CD69 induce increased infiltration of leukocytes and serious damage of the mucosal colonic layer with loss of the Goblet cells and hyperplasia of the crypts [42, 43]. Intriguingly, the recent paper of Hasegawa et al. reported attenuated disease in $CD69^{-/-}$ mice in both acute and chronic DSS colitis models [44]. These contradictions could be the consequence of the different mice background used (B6 and Balb/c), different sources, and the compositions of DSS as well as the different protocols used for the disease induction. Furthermore, different housing conditions in mice facilities, such as the composition of water and food, could induce the alterations in intestinal microflora that can greatly influence the disease development, especially in IBD models.

$CD69^{-/-}$ mice showed increased susceptibility to infection with the food-derived intracellular pathogen *Listeria monocytogenes (Lm)* [39]. Although bacterial clearance capability was the same in wild type and $CD69^{-/-}$ macrophages, increased expression of type I and II IFNs and reduced number of *Lm*-specific T cells were observed in CD69-deficient mice [39]. These mice also showed pathological changes in spleen and liver [39], indicating that they could not control the infection and resolve it locally in the intestine. Furthermore, CD69 affects the disease course in murine models of asthma [33, 34], arthritis [35–37], myocarditis [38], and tumor immunity [40, 41] as demonstrated in CD69-deficient animals. This means that CD69 is not just an activation marker but also strongly involved in the regulation of inflammation.

9. Can CD69 Be Targeted for the Treatment of IBD?

We believe that CD69 regulates intestinal inflammation. CD69 is upregulated in patients with Crohn's disease treated with TNF antibodies [115]. How the CD69 pathway could be manipulated for the treatment of patients with IBD will be discussed in the following section. IBD, including Crohn's disease (CD) and ulcerative colitis (UC), is a chronic, progressive, and destructive inflammatory disorder of the gut [11, 116]. This relapsing and remising disease typically occurs in the second or third decade of life and severely

affects the patients' quality of life [117]. The disease symptoms include severe diarrhea, rectal bleeding, abdominal pain, fever, weight loss, and fatigue [118, 119]. CD is a patchy and segmental transmural inflammation that can affect any part of the gut, while UC represents the inflammation of mucosal layer that starts at rectum, but it can spread even to the whole colon in the uninterrupted pattern [120]. The progressive bowel damage in CD often leads to the formation of fistulae and granulomas [121]. IBD pathology is very complex and not yet fully understood. Multiple genetic, environmental, and immunological factors that contribute to the disease have been identified [116, 122]. The role of the intestinal microflora in the IBD development is well proven in experiments with mice raised under GF conditions [117, 120]. It is shown that disease severely affects the functions of intestinal epithelial cells inducing the cellular stress accompanied with impaired secretion of mucus and antimicrobial peptides [123, 124]. Both innate and adaptive immune system responses are altered in IBD [116, 120, 125]. In general CD is associated with excessive Th1/Th17 responses, while in UC the elevated levels of Th2- and NK cells-produced cytokines are described [11, 116, 121, 126].

For decades IBD is commonly treated with corticosteroids as an unspecific anti-inflammatory agent [127]. However, the broad palette of side effects and inability of long-term remission phase maintenance by corticosteroid therapy created a need for the novel treatment strategies. Advances in understanding the disease pathology enabled the use of the specific modulators of immune responses. Some of these new modulators affect the effector functions of the immune cells involved in IBD development. For now, the most effective were TNF inhibitors that were able to establish the long-term remission and although they may increase the risk of the opportunistic infections, serious complications are rarely observed [128]. Furthermore, early anti-TNF treatment induced complete mucosal healing [129]. Also anti-IL-6 and IL-6R Abs are showing very promising results in clinical trials [130]. Agents affecting the immune cell migration are also good candidates for IBD treatment. Anti-α4 Ab was efficient in the treatment of CD, but it highly increased the susceptibility to the systemic infections [98, 99]. Therefore, anti-$\alpha 4\beta 7$ Ab that specifically blocks the migration of the lymphocytes to the intestine is tested, proving to be successful in the treatment of UC patients [131]. Anti-CXCL-10 Ab as a cell-specific migration inhibitor that prevents the migration of activated Th1 cells to the periphery is also being tested as a possible treatment for IBD [132]. The blocking agents of CCR9, specific intestinal homing marker, could be beneficial for IBD patients, too. Recent study showed that removal of $CCR9^+$ cells by leukapheresis was efficient in IBD treatment, but more extensive studies on this are needed [133].

Most of the studies on the function of CD69 in the diseases are carried out in mice. Sometimes the results obtained from the same disease models are contradictory between different labs showing the need for worldwide standardization in animal breeding conditions and experimental procedures. Also in the context of intestinal immunology, it is known that there are differences between murine and human hut in the microbiota composition and mucosal immune responses. Therefore, studies conducted in mice cannot always be translated to humans. On the other hand, it is difficult to collect all the relevant *in vivo* data in humans. Most of the *in vitro* activation studies on CD69 are done with PBMCs, as there are not many opportunities to isolate cells from the human intestine. Studies in mice showed clearly that CD69 is very important in lymphocyte migration, but whether it has the same role in humans needs to be investigated. Still, the results of human studies on CD69 to date are highly complementary with the data obtained in mice, showing that CD69 has the same expression pattern during homeostasis and inflammatory diseases in mice and humans, being the marker of activated, resident memory or regulatory cells.

Based on the results in studies discussed in this review, the stable induction of CD69 expression should lead to the reduced lymphocyte migration to intestinal LP and to the generation of $CD69^+$ Treg cells. It has been shown that T cells isolated from the IBD patients are resistant to TGF-β and Treg suppression [134], but the possible role of CD69 in this effect is not known. The exact role of $CD69^+$ tissue resident memory cells in intestine should be analyzed in the future studies. Today we are still far away from the possible use of CD69 as a therapeutic agent. Very rigorous and detailed preclinical *in vivo* and *in vitro* studies are required before considering clinical use of CD69-dependent therapy on humans. It has already been observed that targeting a single molecule on lymphocytes can lead to serious complications in humans, while the side effects were absent in all preclinical studies [135]. CD69 targeting can affect the functions of different immune cell types (memory, regulatory and effector lymphocytes) and can modulate the production of both proinflammatory and regulatory cytokines and chemokines. Hence, extensive research on the possible side effects has to be done. Still, CD69 has a profound effect in the functioning of intestinal immune system and this molecule possesses a high potential as a target for the IBD treatment. Identification of the physiological ligand for CD69 receptor would be crucial for the clarification of its role in the immune system and the establishment of the possible therapeutic procedures in the treatment of human diseases.

10. Conclusion

CD69 has been for decades used as a simple marker of activated leukocytes without knowing any concrete role this receptor could play in the regulation of immune responses. The discovery that CD69 expression depends on the presence of the intestinal microflora opened new insight into the role CD69 has in immunity and inflammation in intestine. Induced by the specific antigen and/or intestinal microflora, CD69 regulates the essential processes such as the migration of lymphocytes, cytokine secretion, and generation of regulatory and memory T cells at the mucosal sites (Figure 3). CD69 directs the immune responses in the intestine toward the oral tolerance and regulatory responses (Figure 3) [48]. *In vivo* CD69 limited the intestinal inflammation proving to be one of the crucial negative regulators of the immune responses

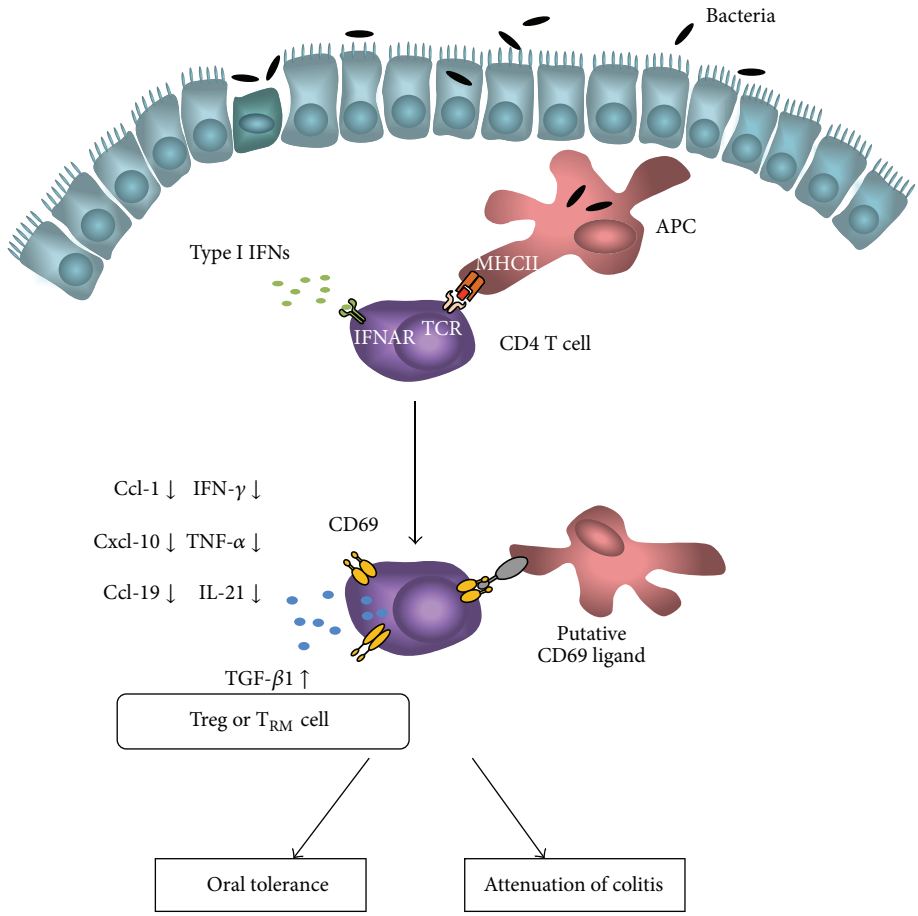

Figure 3: The role of CD69 in mucosal immunity. Activation of intestinal CD4 T cell by antigen recognition, type I interferons (IFN-I), or by presence of intestinal microflora leads to the upregulation of CD69 expression on the cell surface. After binding a ligand, CD69 activates the intracellular pathways that result in decreased production of proinflammatory cytokines (IFN-γ, TNF-α, and IL-21) and chemokines (Ccl-1, Cxcl-10, and Ccl-19) and increased production of regulatory cytokine TGF-β1. If the CD4 T cell establishes a stable expression of CD69, this cell can differentiate into CD69$^+$ regulatory T cell (Treg) or tissue resident memory T cell (T$_{RM}$). Therefore, upregulation of CD69 leads to the decreased migration of activated CD4 T cells to the intestine and to the increased regulatory responses, which ensures the establishment of oral tolerance and the attenuation of colitis severity.

in the gut. The activation of CD69 induces tolerogenic cytokines and immune-suppressive cells that could attenuate the inflammation in intestine. Therefore, we believe that CD69 represents a very good target molecule that should be tested for the treatment of IBD.

Conflict of Interests

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

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Review Article

The Multifaceted Role of Commensal Microbiota in Homeostasis and Gastrointestinal Diseases

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The gastrointestinal tract houses a complex and diverse community of microbes. In recent years, an increased understanding of the importance of intestinal microbiota for human physiology has been gained. In the steady state, commensal microorganisms have a symbiotic relationship with the host and possess critical and distinct functions, including directly influencing immunity. This means that recognition of commensal antigens is necessary for the development of complete immune responses. Therefore, the immune system must face the challenge of maintaining mucosal homeostasis while dealing with undue passage of commensal or pathogenic microbes, as well as the host nutritional status or drug use. Disruption of this fine balance has been associated with the development of several intestinal inflammatory diseases. In this review, we discuss the mechanisms involved in the modulation of host-microbe interactions and how the breakdown of this homeostatic association can lead to intestinal inflammation and pathology.

1. The Normal Microbiota

It has been estimated that trillions of microbes inhabit our gastrointestinal tract (GIT), most of which reside in the distal intestine, where they synthesize essential vitamins and process indigestible components of our diet, such as plant polysaccharides. Furthermore, these microbes influence both normal physiology and disease susceptibilities [1].

The first step towards understanding the relationship between the host and microbes is the characterization of the normal microbiota and the differences that are associated with disease. Moreover, it has been reported that age, genetics, environment, and diet can alter the relationship of intestinal microbiota and host [2].

Eckburg and colleagues [3] showed that in adults most of the intestinal bacteria belong to just a few phyla. Bacteroidetes and Firmicutes are usually dominant, which is consistent with recent studies [4, 5]. Actinobacteria, Proteobacteria, Fusobacteria, and Verrucomicrobia phyla are frequent but generally minor constituents [3–5]. Our microbiota also contains methanogenic archaea (mainly *Methanobrevibacter smithii*), eukarya (mainly yeasts), and viruses [6].

In recent years, our knowledge regarding species and functional composition of the human intestinal microbiome has increased rapidly, but very little is known about the composition of this microbiome around the world. Arumugam and colleagues [7] characterized variations in the composition of the intestinal microbiota in 39 individuals from four continents by analyzing the fecal metagenome. The phylogenetic composition showed that the Firmicutes and Bacteroidetes phyla constitute the majority of the human intestinal microbiota. The *Bacteroides* genus was the most abundant but also the most variable among individuals.

According to the variation between the microbiota, it was proposed that the intestinal microbial community could be stratified into three groups, called enterotypes. Each of these three enterotypes is identifiable by the variation in the levels of one of three genera: *Bacteroides* (enterotype 1), *Prevotella* (enterotype 2), and *Ruminococcus* (enterotype 3). Despite the stability of these three major groups, their relative proportions and the species present are highly variable between individuals.

Regarding bacterial stability another study analysis of fecal samples from 37 healthy adults showed that individual microbiota was notably stable over five years. Extrapolation of these data suggests that most of the bacteria present in the intestine were residents for decades. Bacteroidetes and Actinobacteria are significantly more stable than the average population [8]. Concerning the stability of Bacteroidetes, it was shown that these bacteria have evolved in species-specific physical interactions with the host that mediates stability, and the genetic locus commensal colonization factors (CFC) represents a novel molecular mechanism for symbiosis [9]. It is important to point out that the fecal microbiota differs from mucosal microbiota [3, 10]. Therefore, Siezen and Kleerebezem proposed a new term called "faecotypes" instead of "enterotypes," since it is known that the microbial abundance and composition changes dramatically throughout the GIT, and perhaps "enterotypes" may not reflect the microbial composition of the whole intestine [11].

Although the intestinal microbiota is stable in adulthood, it undergoes fluctuations during childhood and old age. In children, the type of bacteria colonizing the intestine is defined very early according to the type of childbirth. Normal delivery is an important source of intestinal Actinobacteria, especially *Bifidobacterium*, while cesarean delivery provides a bacterial community similar to that found on the skin surface, dominated by *Staphylococcus* and the colonization by *Lactobacillus*, *Bifidobacterium*, and *Bacteroides* [12, 13]. In elderly individuals, there is a decreasing quantity and diversity of species of *Bacteroides* and *Bifidobacterium* and an increase in facultative anaerobe bacteria such as *Fusobacterium*, *Clostridium*, and *Eubacterium* species. Increase of these bacteria genus is harmful to host since they present high proteolytic activity, which is responsible for putrefaction of large bowel [14].

The majority of the gut microbes are harmless or beneficial to the host. However, studies of human microbiota composition have discovered that alterations in the microbiome composition are present in obese individuals [15], as well as in individuals with a variety of other diseases such, as inflammatory bowel diseases (IBDs) [16] and cancer [17]. Furthermore, antibiotic administration impacts the human intestinal microbiota. These antimicrobial agents contribute to the decrease of colonization resistance of members of the commensal microbiota, which can lead the development of a range of diseases, as well as the emergence antimicrobial resistance. Moreover, it was believed that the commensal microbiota could normalize a few weeks after treatment discontinuation, but this is not true for some specific members that may be affected for long periods of time [18].

2. Gut Microbiota, Nutrition, and Metabolism

The microbiome is strongly influenced by diet. This factor was suggested to be more of a determinant than hygiene, climate, ethnicity, and geography in a study comparing the gut microbial composition between children from a rural African village and a city in Europe [19]. Further, there was no difference in terms of the prevalence of the four major phyla found in the human gut (Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria) when comparing a low-fat/high-fiber diet and a low-fiber/high-fat diet in different studies. However, there was a difference in terms of proportion between those phyla. More Actinobacteria and Bacteroidetes were found in low-fat/high-fiber diets, whereas more Firmicutes and Proteobacteria were found in low-fiber/high-fat diets [19, 20]. Another example of diet influencing human gut microbiota was shown by a study comparing populations in Russia and other countries. Russian subjects presented some specific populations of Firmicutes and Actinobacteria phyla, which were probably related to their diet, since those bacteria are specialized in starch metabolism, and starch-rich foods are typical in this country [21]. Furthermore, in a murine model, it was possible to relate specific components from the diet with the prevalence of different species of bacteria in the gut, which clearly shows the influence of diet in the composition of microbiota [22].

Diet administered to infants during the first six months of life is also important for the microbiota composition. Recent studies with infants in China showed different proportions of Actinobacteria and Bacteroidetes populations between breast-fed and formula-fed infants with a higher proportion of both types in the breast-fed diet [23]. Although the composition of microbiota is stable in healthy adults, diet can rapidly change the proportion of some bacterial populations in the gut, in less than 24 hours. Administration of a high-fat diet to humanized gnotobiotic mice increased the population of Firmicutes and decreased the Bacteroidetes population [24]. Interestingly, this change in human gut microbiota in response to an altered diet is faster in an animal-based diet than in a plant-based diet [25]. In addition, this effect varies for different populations of bacteria in the gut. Enterotypes are related to a long-term diet and thus were not affected in an experimental model until 10 days after the administration of a specific diet [20].

Evolution of the Western diet with the introduction of processed food and changes in nutritional characteristics of the human diet, especially in fiber, sugar, and fatty acid contents, have been proposed to be related to the increase of the incidence of chronic diseases [26, 27]. In this context, the composition of microbiota, which depends on the diet, is important because of the influence of bacteria metabolism for the production of important metabolites for the host [19, 24]. One relevant metabolite produced by fermentation of dietary fiber is the short-chain fatty acids (SCFAs). Acetate, butyrate, and propionate are the main SCFAs that result from fermentation of carbohydrates and amino acids in the diet [28]. The presence of these metabolites are microbiota-dependent, since rats and germ-free mice

showed a small amount of SCFAs in the intestine, which was probably coming from the diet [29]. Short-chain fatty acids have been described as important anti-inflammatory molecules. Administration of acetate in drinking water was enough to decrease inflammation in a colitis experimental model. The mechanism seems to be through reduction of production of proinflammatory chemokines and cytokines, such as macrophage inflammatory protein 1-alpha (MIP-1α) and tumor necrosis factor alpha (TNF-α). In this way, mice treated with acetate showed less migration of neutrophils into the gut. Furthermore, this SCFA is important in reducing inflammation in other sites, and not only in the intestine. The effect of acetate through its binding to the G-protein-coupled receptor 43 (GPR43) is also relevant to control inflammation in experimental models of arthritis and asthma [30]. In addition, mice fed with a low-fiber diet showed higher cell infiltration in allergic airway inflammation. Furthermore, treatment of mice with propionate induced protective effects in this disease through G-protein-coupled receptor 41 (GPR41) and not the GPR43 receptor [31]. Interestingly, this study showed that dietary fiber can change the gut and lung microbiota, another consistent example of how diet can change the microbiome and how this can be important for the host [31]. These studies demonstrate how diets rich in fiber could attenuate proinflammatory diseases [30, 31].

The microbiota has been described as an important factor in modulation of host energy metabolism and even in the level of some lipid classes in the serum. The amounts of 18 phosphatidylcholine species and nine triglyceride species in serum of conventional mice were reduced compared with levels in germ-free mice [32]. Recent studies have associated normal microbiota with obesity. Interestingly, conventional mice showed a higher percentage of total body fat than germ-free mice, and conventionalization of those mice with fecal microbiota increased their body fat within only 10 days after their colonization. This effect cannot be associated with differences in metabolic rate or in chow consumed by those mice. The authors suggested that gut commensals may inhibit the expression of FIAF (fasting-induced adipose factor), which can block the production of LPL, an important lipase [33]. Also, the simple transplantation of microbiota from obese mice can induce weight gain in a murine model [34]. Furthermore, another study showed an interesting alteration in the composition of the main phyla of bacteria in the gut of *ob/ob* mice which are, by spontaneous mutation, deficient in leptin which leads to an increase in food intake and obesity phenotype [35]. A higher frequency of Firmicutes and a lower frequency of Bacteroidetes were found in these mice, which develop obesity [36]. The same pattern was also found in humans. Obese people were found to have more Firmicutes than Bacteroidetes but, after a diet therapy, they presented an increased amount of Bacteroidetes [37]. Composition of microbiota, in association with genotype and lifestyle, is an important factor in obesity. The microbiota from obese humans can even influence the production of some metabolites, which are typical of this disorder, including the general metabolism of amino acids [38].

3. Commensal Intestinal Bacteria and the Immune System

Although microbes are frequently seen as pathogenic, it is well established that most of them live in symbiosis with humans. Most of the microbes that inhabit the human intestine have a highly coevolved relationship with the immune system, which leads to the maintenance of homeostasis between the host and resident microbes.

During development and into adulthood, intestinal bacteria contribute to the shape and function of the gastrointestinal immune system [39] and play an important role in both health and disease [40]. This partnership involves bacterial signals that are recognized by host immune cells to mediate beneficial outcomes for both microbes and humans.

Another way to prevent the growth of pathogenic microorganisms is through the activation of the immune cells, such as macrophages, neutrophils, innate lymphoid cells 3 (ILC3), and B and T cells, to release antimicrobial factors. Commensal bacteria can also lead to SCFA production, enhancing the intestinal barrier function and stimulating mucus and antimicrobial peptides production [41]. In the same way, pathogenic bacteria also have mechanisms to prevent the growth of commensal bacteria. For example, some Gram-negative pathogenic bacteria have a secretion system dedicated to the protein secretion, such as type VI secretion system (T6SS) that is implicated directly in its pathogenicity and ability to kill their commensal competitors [42].

Stimulation of pattern-recognition receptors (PRR) present in intestinal epithelial cells (IEC), such as Toll-like receptor (TLR), NOD-like receptor (NLR), and RIG-like receptor (RLR), by commensal bacteria results in thymic stromal lymphopoietin (TSLP) production by these cells. TSLP can enhance B cell-activating factor (BAFF) and a proliferating-inducing ligand (APRIL) production. Additionally retinoic acid produced by dendritic cells (DCs) can promote IgA class-switching in B cells, and also is an important cofactor for the differentiation of Foxp3+ Tregs and has been shown to inhibit the generation of Th17 cells. IgA that is produced by lamina propria B cells is secreted into the intestinal lumen (SIgA), where it is able to alter microbiota composition and function [40, 41, 43].

Another important immune regulatory cytokine produced abundantly by IEC in the intestine is transforming growth factor-beta (TGF-β). IEC-derived TGF-β in combination with TSLP and retinoic acid promotes the conditioning of a subset of DCs found in the intestinal lamina propria and mesenteric lymph nodes that express the integrin α chain CD103 ($CD103^+$ DCs) [44].

$CD103^+$ express CCR7 that mediates homing to secondary lymphoid organs, drive the expression of gut-homing receptors CCR9 and $\alpha4\beta7$ integrin on responding T cells, and induce differentiation of naive CD4+ T cells into $FoxP3^+$ regulatory T cells [44, 45]. This subset of DCs is also the one that preferentially receives delivery of intestinal antigens by goblet cells at steady state which is consistent with their tolerogenic properties [46].

Interleukin-10 produced by DCs and macrophages also have the potential to induce Foxp3$^+$ Tregs. The involvement of IL-10 in intestinal tolerance was confirmed in a model of experimental colitis. It has been shown that *B. fragilis* is able to prevent intestinal pathology by IL-10 production, and this cytokine is reduced within the gut-associated lymphoid tissue (GALT) of germ-free animals [47, 48]. A selected mixture of *Clostridia* species was shown to induce Tregs in the mouse colon, and oral administration of these species protected mice against colitis and allergic inflammation [49]. This indicates that commensal bacteria are involved in the promotion of FoxP3$^+$ regulatory T-cell differentiation and maintaining intestinal tolerance [50].

Recently, it has been demonstrated that, in order to promote intestinal homeostasis, the commensal microbiota depends on the crosstalk between macrophages and retinoic acid receptor-related orphan receptor-γt^+ (RORγt^+) ILC3. The microbiota stimulates macrophages to produce IL-1β that binds to the IL-1β receptor in ILC3s, promoting granulocyte-macrophage-colony stimulating factor (GM-CSF) release. ILC3-derived GM-CSF induces DCs and macrophages to produce regulatory molecules, such as IL-10 and retinoic acid [51].

In addition to its role in crosstalk with macrophages, RORγt^+ ILC3 acts directly in the maintenance of the intestinal homeostasis and in the defense against intestinal pathogens. RORγt^+ ILC3 are associated with IL-22 production, which can induce REGIIIγ (C-type lectin antimicrobial peptides regenerating islet-derived protein) production by IECs. REGIIIγ regulates the intestinal microbiota and contributes to the tolerance in the gut [52, 53]. At the same time, the commensal microbiota can induce IL-25 secretion by endothelial cells, which acts on lamina propria IL-17 receptor B (IL-17RB)$^+$ DCs and suppresses IL-22 production by RORγt^+ ILC3s [41]. It is a mechanism to ensure the maintenance of intestinal homeostasis.

Regarding adaptive immune response, the intestinal epithelium and underlying lamina propria contain T cells that play important role in maintaining intestinal homeostasis. T regulatory (Treg) cells are known to express the transcription factor forkhead box P3 (Foxp3) and suppress the activation, proliferation, and effector function of a wide range of immune cells, playing a key role in maintenance of intestinal homeostasis through anti-inflammatory cytokines such as IL-10 [54].

However, Treg cells are not homogeneous and terminally differentiated. A recent study demonstrated coexpression of RORγt and Foxp3 in Treg cells, which implies the conversion from Treg cells to Th17 cells, capable of producing IL-17. This is associated with a decreased suppressive function of Treg cells in patients with IBDs [55]. It was shown that Foxp3 is able to physically bind to RORγt and its transcriptional activity thereby blocking IL-17 production. But in the presence of appropriate inflammatory stimuli Treg cells display an IL17$^+$ Foxp3$^+$ CD4$^+$ phenotype and can produce IL-17 [54].

However, when alterations in the normal microbiota, termed dysbiosis, occur in the gut, they lead to failure of the immune system regulation by commensal microbiota, resulting in an inflammatory state, with a predominance of Th1 and Th17 profile responses [41]. Inflammation in the intestine diminishs the tolerogenic characteristics of CD103$^+$ DCs like the expression of the enzyme aldehyde dehydrogenase (ALDH) that participates in the conversion of retinal to RA and the expression of TGF-β. Conversion of Tregs is lower in this setting favoring a proinflammatory response with more production of the cytokine interferon-γ (IFN-γ) [56].

4. Resistance to Colonization by Commensal Microbes

As mentioned above, the microbiota is essential for modulating the immune system and some aspects of host metabolism. Therefore, changing the composition of the microbiota can be problematic for the host. Utilization of antibiotics as a treatment against bacterial infection has a huge impact in medicine [57–59]. Despite the benefits associated with antibiotic treatment, this therapy can change the microbiota for a long time. It has been reported that the combination regimen of amoxicillin, tetracycline, and metronidazole for two weeks induces an alteration in gut microbiota in patients with ulcerative colitis (UC) that lasts for three months [60]. In an experimental model, changes in the microbiota by metronidazole treatment were able to alter the integrity of the gut leading to exacerbation of *Citrobacter rodentium* infection [61]. In humans, hemorrhagic colitis can be associated with previous antibiotic treatment [62].

The fact that the presence of a normal microbiota inhibits the colonization of opportunistic pathogenic bacteria is called colonization resistance (CR) [63]. Colonization of the gut by pathogens such as *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Shigella flexneri*, and *Vibrio cholerae* is exacerbated by previous antibiotic treatment, showing the important role of the microbiota in inhibiting the attachment of these microorganisms to the intestine [64–66]. Interestingly, colonization of gnotobiotic mice with only one component of the microbiota is enough to control *Escherichia coli* colonization [67], and treatment with antibiotics can make conventional mice as susceptible as germ-free mice to colonization by *Salmonella* [68]. The mechanisms through which the microbiota can induce colonization resistance are not completely understood but may be associated with the systemic modulation of immune responses [69–71], and with the production of microbicidal substances [72–74]. Interestingly, the host immune response necessary to contain the pathogen could actually favor the growth of the pathogen and other harmful microbes by causing dysbiosis of the gut microbiome, and consequent impairment of colonization resistance mechanisms [75, 76].

5. Intestinal Dysbiosis

Breakdown of homeostasis in the gut environment causes dysregulation of intestinal immune responses and an imbalance of the normal intestinal bacteria called dysbiosis. The genetics of the host, as well as environmental perturbations such as antibiotic treatments, diet, or infections can influence

the structure of the microbial community. These disturbances can lead to loss of diversity of the microbiota with a reduction in the commensals that are beneficial to the host and an increase in microbes that are potentially pathogenic. The importance of maintenance of diversity within the gut microbiota to gain maximum health benefits comes primarily from evidence that shows that members of the microbiota have diverse and nonredundant effects on host health. For example, the human symbiont *Bacteroides fragilis* directs the development of regulatory T cells and suppresses Th17 responses [77], whereas segmented filament bacteria (SFB) are able to induce production of IL-17 in the gut [47]. Thus, a dysbiotic gut microbiota represents a shift in the stability of the microbial community that is characterized by quantitative and qualitative changes in the composition, as well as in the local distribution of its members.

Recent studies have demonstrated an association between changes in the gut microbiota and acute mucosal infections, suggesting that they could act as a trigger for subsequent gastrointestinal disorders such as IBDs. Loss of diversity of the intestinal microbial community with increased abundance of Enterobacteria can be observed in several intestinal infections, such as *Citrobacter rodentium* [78], *Salmonella typhimurium* [76], and oral models of *Toxoplasma gondii*. Besides changes in the microbial composition, an exacerbated response to commensal signals is thought to be a major cause of pathology in experimental infections with *T. gondii* [79]. In *T. gondii* infection the changes in the microbiota aggravate the intestinal immune response caused by the parasite. In contrast, in *S. typhimurium* infection it seems that the alterations in the microbiota are not the cause but a consequence of the inflammatory process generated by the pathogen.

Acute infection with *T. gondii* causes translocation of bacteria from the intestinal lumen to peripheral tissues such as the spleen, mesenteric lymph node, and liver [80]. Disruption of intestinal homeostasis can lead intestinal bacteria to reach systemic sites in different settings. Microbial translocation, which is the translocation of microbial products from the gut lumen into the systemic circulation, and subsequent immune activation are thought to determine disease progression during HIV infection. Levels of plasma lipopolysaccharide (LPS), a marker of bacterial translocation, are increased in HIV infected patients [81]. Impairment of intestinal barrier integrity early in acute retroviral infection and loss of intestinal Th17 cells are probable causes of translocation in HIV infected individuals [82]. Furthermore, a shift in the gut commensal community was observed in HIV-infected subjects with overgrowth of Proteobacteria, which are known to have proinflammatory potential. The changes in the microbiota were associated with dysregulation of immune responses and consequent chronic inflammation [83]. In a humanized mouse model, treatment of irradiated recombination activating gene 2 (RAG2) deficient mice, which lack mature lymphocytes due to the inability to initiate V(D)J recombination, reconstituted with human cord blood cells with dextran sodium sulfate (DSS) induced bacterial translocation to the spleen and mesenteric lymph nodes [84].

Recently, an association of a genetic defect of the host and changes in the composition of the microbiota with nonalcoholic fatty liver disease steatohepatitis severity has been demonstrated revealing a role for inflammasomes in intestinal dysbiosis [85]. Inflammasomes are multiprotein complexes of innate immunity capable of recognizing a diverse range of conserved molecular motifs unique to microbes as well as tissue damage signals. Inflammasomes drive caspase-1 cascade activation which promotes secretion of proinflammatory cytokines IL-1β and IL-18 [86]. Alterations in the microbial profile were observed in the gut of mice deficient in the inflammasomes NOD-like receptor pyrin domain containing 6 (NLRP6) or NOD-like receptor pyrin domain containing 3 (NLRP3). Microbiota dysbiosis resulted in accumulation and recognition of bacterial products in the portal circulation through TLR signaling leading to hepatic steatosis and inflammation. In fact, the liver has been shown to have an important role in maintenance of compartmentalization of commensal intestinal microbes, clearing bacteria that reach systemic vascular circuits. In both animal models and human patients with liver disorders, loss of this function leads to aberrant immune responses against gut commensals [87].

More recently, profiling studies of the microbiota have associated pathogenicity of inflammatory diseases with distinct shifts in the composition of the intestinal microbiota. Assessment of intestinal commensals in type II diabetes patients revealed a moderate degree of dysbiosis with a decrease in butyrate-producing bacteria and an increase in several opportunistic pathogens [88]. Studying the microbiome of a large pediatric cohort of Crohn's disease (CD) patients prior to treatment, Gevers and colleagues observed increased abundance of Enterobacteria and amplification of dysbiosis after antibiotic use [89]. These authors suggested that screening of the microbiota profile at an early stage of the disease could be a useful diagnostic tool for CD. Since diagnosis of IBD is particularly challenging in children due to variations in symptoms, enhanced technologies that could rapidly identify microbial patterns associated with development of the disease would be very important [90].

A common hallmark of intestinal microbiota dysbiosis is the outgrowth of opportunistic pathogens or also called pathobionts. This phenomenon could be explained by recent evidence that suggests that inflammation in the intestine establishes a nutritional local environment that is better suited for the growth of certain microorganisms. It is probable that these potentially pathogenic microbes are more capable of utilizing the nutrients that are generated by the inflammatory process [91]. Furthermore, bacteria might adapt to growth in dysbiotic conditions and acquire even higher pathogenic potential by horizontal gene transfer of virulence factors, indicating that disruption of the intestinal homeostasis and consequent changes in the microbial community could contribute to pathogen evolution [92]. Thus, preventing dysbiosis, especially in the hospital environment, may have an even more fundamental role for the control of emerging infectious diseases.

The homeostatic relationship between host and microbiota does not imply that microorganisms are not continually

sensed by the host immune system. Recognition of small numbers of commensal bacteria and their products that are probably continuously penetrating the intestinal epithelial cell layer and may result in protective adaptive immune responses being induced in the intestinal mucosa [93]. In fact, the stimulatory capacity of the microbiota has been shown to be important in maintaining responsiveness against pathogenic microbes [70, 94].

Disruption of intestinal homeostasis by intestinal inflammatory disorders such as IBDs or gastrointestinal infections has been previously linked with newly acquired responsiveness against antigens from normal gut bacteria. In fact, it has long been reported by several groups that the systemic adaptive immune system can indeed be primed against gut bacterial antigens [95–97]. Interestingly, commensal-specific responses are observed in healthy individuals, suggesting that commensal recognition is a common occurrence and, in most circumstances, is not associated with pathogenic responses [98]. Therefore, tolerance towards commensals is maintained in a healthy gut. Whether microbiota-specific responses could be detrimental in the context of dysregulation of the intestinal homeostasis is not known. Recent data suggest that acute infections may result in the disruption of tolerance to gut microbes. Experimental *T. gondii* ileitis leads to translocation of bacteria and generation of T cells specifically against commensal antigens. These cells are long-lasting and capable of proliferating and become activated upon antigen recognition [80]. Despite the clear association between commensal-specific responses and inflammatory disorders, whether acute mucosal infections could function as a trigger for the development of IBDs remains to be addressed. Gaining further insight of how recognition of bacteria in the gut influences immune responses could help understand how intestinal inflammatory disorders occur and may also permit the development of new strategies to prevent the onset of such syndromes.

6. The Role of the Intestinal Microbiota in Inflammatory Bowel Disease

Inflammatory bowel disease is an immune-mediated disorder that is characterized by chronic intestinal inflammation and which encompasses primarily ulcerative colitis and Crohn's disease (CD). Bloody, mucous diarrhea is the almost universal hallmark of UC [99]. Symptoms of CD are more subtle and varied, partly as a result of its diffuse and diverse anatomical location. The most common symptom is abdominal pain [100]. However, there are other associated symptoms, such as diarrhea, poor appetite, and weight loss. These symptoms are presented in nearly 80% of children and adolescents with IBDs.

Etiologic factors have been associated with different environmental aspects that contribute to inflammatory bowel diseases such as smoking and appendectomy. Vitamin D levels, diet, hormone use, and stress have also been postulated as risk factors for one or both main forms of IBDs, but these factors need to be further investigated [99, 101].

The critical function of adult gut performance is related to the metabolism of dietary components, such as cholesterol, intestinal motility, and immune system modulation [101, 102]. Preserving eubiosis, which is the state of equilibrium of the microbiota in the gastrointestinal tract, is relevant for maintaining the integrity of the intestinal epithelium and contributing to antimicrobial defenses [101]. Microbe-induced Treg cells that prevent potential inflammatory responses by both adaptive and innate immunity responses also promote homeostasis. Some problems in homeostasis may result in an anomalous activation of some innate receptors and subsequent tissue damage, leading to systemic inflammation that results in symptoms associated with IBDs. For example, IBD is related to a dysfunctional immune response and activates T-helper cells in the gut mucosa, probably because of the deregulation of the normally controlled immune response to commensal bacteria. It is important to note that the number of commensal bacteria is reduced in patients with IBD [102].

Several studies have shown protection of the gut against external bacteria by commensal microbes, supporting their function in the etiology of IBDs [101]. For example, CD was associated with a reduction in the antibacterial peptide expression. These factors can explain the association between maintenance of inflammatory responses to intestinal pathogens and loss of tolerance to commensal microbiota [101].

The NOD2 signaling pathway is presented and is important as a regulatory factor of proinflammatory proteins induced by NF-κB. After proinflammatory stimuli such as TNF-α and IFN-γ, the expression of NOD2 may be upregulated in epithelial cells, including those of the gastrointestinal tract. It has been postulated that the decrease in the function of NOD2 reduces the responsiveness of the host to pathogens, culminating in chronic intestinal inflammation. The impaired function of this receptor facilitates the invasion of bacteria and changes the mucosal immune responses against gut luminal antigens [103]. Taking the example of Crohn's disease (CD), genetic studies have begun to elucidate the loci associated with subphenotypes of the disease, as the location of the disease and clinical outcome. It has been suggested that patients with CD have mutations in NOD2 and thus poorly respond to bacterial antigens [104].

7. The Role of Gut Commensals in Colorectal Cancer

Several cancer types are associated with infectious agents. Well-known examples include cervical and gastric cancer, which can be caused by human papillomaviruses and the bacteria *Helicobacter pylori*, respectively [105, 106]. It is becoming increasingly evident that the gut bacterial population plays an important role in colon carcinogenesis [17].

Studies of fecal microbiota of 19 patients with colorectal cancer (CRC) and 20 healthy control subjects demonstrated differences in the fecal microbial composition between these two groups. The CRC group had a significant increase in the relative abundance of Fusobacteria phyla compared with the control group. Regarding Bacteroidetes and Firmicutes

phyla, no difference was observed in their relative abundance. However, a positive correlation between the abundance of *Bacteroides* species and CRC was observed [107].

Other studies have also demonstrated that the genus *Bacteroides* had higher rates of colonization in CRC patients [107, 108]. A possible mechanism could be through the release of enterotoxins, such as fragilysin, an oncogenic bacterial toxin [109]. Fragilysin-producing *B. fragilis*, termed enterotoxigenic *B. fragilis* (ETBF), found in colonic biopsy specimens has been demonstrated to have a significant correlation with the presence of active inflammatory bowel disease [110, 111]. Fragilysin is able to induce a gut inflammatory state. Fragilysin can stimulate IL-8 secretion by intestinal epithelial cells and stimulates expression of the neutrophil chemoattractant and activators epithelial cell-derived neutrophil attractant 78 (ENA-78) and growth related oncogene α (GRO-α) [112–114]. In addition to its inflammatory effects, fragilysin induces colonic epithelial cell proliferation, as well as expression of the oncogene c-Myc [115].

Gut microbial profiling of germ-free IL-10-deficient mice that develop spontaneous colitis revealed that intestinal inflammation induces changes in the composition of the microbiota with an overgrowth of Enterobacteria. Monoassociation with the commensal murine adherent-invasive *E. coli* NC101 contributed to the development of invasive tumors in germ-free IL-10-deficient mice treated with the colon-specific carcinogen azoxymethane (AOM). Deletion of the virulence factor polyketide synthase (Pks) genotoxic island of *E. coli* NC101 reduced numbers of tumors and invasion in mice, and presence of Pks^+ *E. coli* NC101 was associated with patients with IBD and CRC, suggesting that colitis-induced dysbiosis and expansion of virulence microbes can lead to tumorigenesis [116].

8. Intestinal Infections and the Microbiota

The gut flora usually contributes to a healthy environment. However, pathogenic and commensal bacteria are responsible for acute and chronic inflammation of the mucosa, influencing both the innate and adaptive immune responses [117].

8.1. Salmonella typhimurium. Members of the *Salmonella* genus are a diverse group of facultative intracellular gram-negative organisms that are responsible for a broad spectrum of enteric and systemic diseases found in humans and other vertebrates. *S. typhimurium* is a common pathogen found in humans and causes acute gastroenteritis [118]. Also, *Salmonella* causes invasive infections, such as enteric fever, septicemia, and osteomyelitis. The virulence of these bacteria depends on their serotypes, the state of the host, and the size of inoculum. Additionally, *Salmonella* has the ability to change the process of phagocytosis [119, 120]. Upon entry into the human host, *Salmonella* spp. must overcome the resistance to colonization mediated by the gut microbiota and the innate immune system. These bacteria successfully accomplish this by inducing inflammation and mechanisms of the innate immune defense. Many models have been developed to study *Salmonella* spp. interactions with the microbiota and these have helped to identify factors necessary to overcome colonization resistance and to mediate disease. Microbiota-produced butyrate and acetate can have dramatic effects on both the host and *Salmonella* spp. during infection [121].

Salmonella typhimurium has been shown to be unable to colonize the mouse intestine in the absence of inflammation, as the normal microbiota in the noninflamed state is able to effectively outcompete an avirulent (lacking inflammatory capacity) *Salmonella* intruder [76, 91]. Other studies have found that different antibiotics have variable effects on the total number and distribution of gut bacteria but that each antibiotic tested enhanced *Salmonella*-induced epithelial cell invasion and inflammation [122]. After antibiotic removal and some recovery of the microbiota, mice were still susceptible to *Salmonella*-induced enteritis, suggesting that the correct balance of microbial diversity and numbers is required for effective colonization resistance.

8.2. Pathogenic Escherichia coli and Citrobacter rodentium. Enteropathogenic *E. coli* (EHEC) and enterohemorrhagic *E. coli* (EPEC) are human diarrheal pathogens that cause much morbidity and mortality worldwide. Unlike the harmless commensal strains of *E. coli* that reside in the human intestine, pathogenic strains of *E. coli* are highly adapted enteric bacteria that have specific virulence determinants such as a pathogenicity island called the locus of enterocyte effacement (LEE) which leads to the formation of attaching and effacing (A/E) lesions. EHEC strains also are able to produce several cytotoxins [123]. EHEC causes inflammation in the large intestine, whereas EPEC affects mainly the proximal small intestine. *Citrobacter rodentium* is a natural pathogen found in mice that carries a homolog of the LEE pathogenicity island of EPEC and EHEC and, therefore, is used as a model to study the molecular basis of pathogenic *E. coli* infections. Unlike the harmless commensal *E. coli* that reside in the human intestine, pathogenic *E. coli* are highly adapted enteric bacteria that have evolved to use attaching and effacing (A/E) lesion formation as a major mechanism of infection [124].

Although the commensal microbiota has crucial roles in resistance to enteric pathogen infections, certain pathogens can use the microbiota to facilitate their infection. Commensals may have a direct role in controlling pathogenic bacteria. For example, *Bifidobacterium* species directly inhibit the growth of EHEC by acidification of the local environment [125]. Commensal *E. coli* can compete for nutrients against EHEC strains [126]. The microbiota is also involved in the ability of *C. rodentium* to colonize the intestine, since germ-free mice are unable to clear the bacteria. During the late phase of the infection, virulence factors of *C. rodentium* are downregulated and the bacteria are outcompeted by the microbiota [127]. Additionally, recent findings suggest that the microbiota is important for *C. rodentium* resistance mediated by the production of IL-22 [128].

8.3. Clostridium difficile. Clostridium difficile is an opportunistic pathogen of humans that causes intestinal infections

named CDI (*Clostridium difficile* infection). This infection is a major cause of diarrhea and antibiotic-induced colitis. There are classical manifestations associated with CDI, such as the progression of mild diarrhea to fulminant colitis and toxic megacolon. Infections caused by this microorganism are correlated with the decrease of commensal organisms in the gut [129]. Antibiotics are also linked with this pathogen, and an inappropriate and excessive use of antibiotics predisposes toward development of the infection [130].

Patients over 65 years hospitalized with recent antibiotic exposure present the highest risk of developing this infection. Studies showed that reduction of Bacteroides and Firmicutes phyla in the gut caused by antibiotics seems to be important in understanding *C. difficile* pathophysiology [119, 131].

One of the strategies to treat CDI, especially in recurrent cases, is fecal microbiota transplantation. This technique is based on the transplantation of a microbiota obtained from a healthy donor. The sample is processed and transplanted into patients with recurrent CDI. This is a successful technique that provides a >90% success rate. An example of its effectiveness is that symptoms of infection caused by *C. difficile* are mostly resolved after the procedure [129].

9. Effects of Probiotics

Probiotics are "live microorganisms which, when administered in adequate amounts, confer health benefits to the host" [132]. Therefore, to fulfill their objectives, these microorganisms should resist the adversities of the host organism, stomach pH, and bile salts, until they reach the intestine. Beneficial effects of these microorganisms and their safety to the host must be proved. In addition, they should be stable and viable from the start of production to consumption. The major microorganisms currently utilized as probiotics are bacteria of the genera *Lactobacillus* and *Bifidobacterium* and the yeast *Saccharomyces boulardii*. Probiotics are currently being consumed in supplemented foods, fermented milks, and yogurts [133–135], and also ingested with medicines, as discussed by Vieira and collaborators [136].

When they reach the gut, probiotics can act in several ways. One of them is in the intestinal lumen by stimulating mucin production, defensins, and bacteriocins [137, 138]. Other mechanisms of action include the ability to maintain and modulate intestinal homeostasis by enabling survival of cells during intestinal infections by pathogens, preventing bacterial translocation, competing with pathogens for space and nutrients, reducing intestinal permeability, and producing or inducing the production of lactate and acetate. In addition, they can affect the metabolism of the microbiota [125, 134, 139, 140]. Modulation of host immunity is another benefit of probiotics consumption. Probiotic microorganisms are able to stimulate the immune system, either the innate immune responses, by inhibiting signaling pathways, such as the MAPKs [138, 141] and NF-kB [94, 142] and by altering the profile of secreted cytokines [143, 144], or the adaptive immune responses, by stimulating T lymphocytes [145, 146].

Studies in animal models and human clinical studies have generated a positive outlook for the use of probiotics in the prevention and treatment of several diseases. The use of probiotics in murine models of IBD and clinical studies of this disease has not shown significant results, except for an improvement of symptoms in some cases [147, 148]. In murine cancer models, probiotics promoted inactivation of mutagenic compounds suppressing pre-cancerous lesions [149], inhibition of development of cancer cells [145, 150], and a reduction in the size and number of tumors [151]. Moreover, the use of probiotics in a human study showed a reduced risk of developing colorectal cancer [152]. *Saccharomyces boulardii* promoted a reduction in the duration of diarrhea in children without specific etiology [153], and administration of *Lactobacillus rhamnosus* GG reduced the duration of diarrhea caused by rotavirus in children [154]. Another study showed positive results for antibiotic-associated diarrhea when *Bifidobacterium lactis* and *Streptococcus thermophilus* were administered to children [155].

There are no reports in the literature of negative effects of probiotics in healthy people. All negative effects have been observed in critically ill, hospitalized or postoperative patients. Immunosuppression and prior antibiotic treatment were shown to be predisposition factors in cases of *Lactobacillus* bacteremia. Importantly, the consumption of *Lactobacillus* did not increase the incidence of bacteremia during a 10-year study [156]. Patients admitted to the intensive care unit (ICU) developed fungemia following use of *Saccharomyces boulardii* [157] and the same result was observed in neutropenic patients [158]. Children with short bowel syndrome developed sepsis associated with use of probiotic *Lactobacillus rhamnosus* GG [159], and acidosis due to the production of D-lactate during bacterial fermentation [160]. *Lactobacillus rhamnosus* GG induced sepsis in a patient who underwent a cardiac surgery [161]. Probiotics constitute a source of antibiotic resistance genes. *In vivo* transfer of these genes to bacteria in the gastrointestinal tract has been reported in mice and humans [162]. Evaluation of the transferability of resistance genes is important to determine the full safety of a probiotic strain.

Understanding the molecular mechanisms through which probiotics act in the gut, altering the host physiology and modulating the immune system, could lead to the development of more successful therapies for various disorders. Furthermore, it is important to characterize which microorganism presents the best results for a particular disease. Research with microorganisms is progressing, and the clinical safety and efficacy of the use of probiotics need to be confirmed.

10. Conclusion

The gastrointestinal tract is the primary site of interaction between the host immune system and commensal and pathogenic microbes. A large body of evidence has now been gathered confirming the fundamental role of gut commensal microbes in the maintenance of intestinal homeostasis. The gut microbiota is a complex community of symbiotic microorganisms that is highly susceptible to disturbances. Dysregulation of intestinal homeostasis leads to loss of

microbial diversity, overgrowth of pathobionts, and translocation of bacteria. Commensal dysbiosis and consequent abnormal sensing of commensal bacterial antigens is associated with the pathogenesis of various disorders. Although both genetic and environmental factors are involved, the molecular mechanisms responsible for triggering dysbiosis are still largely unknown. Furthermore, whether these changes are specific to each disease needs to be addressed. Probiotics have been successfully used as a strategy to regulate an altered microbiota and provide important signals to activate proper immune responses in several inflammatory disorders, gastrointestinal infections and cancer. A better understanding of how disturbances in the intestine can affect intestinal homeostasis resulting in atypical responsiveness against commensal bacteria could provide new important insights into the etiology of inflammatory diseases, such as IBD, and may contribute to the development of new strategies for prevention and therapy of these disorders.

Conflict of Interests

The authors declare that there is no significant financial, professional, or personal conflict of interests.

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Review Article

HIV and the Gut Microbiota, Partners in Crime: Breaking the Vicious Cycle to Unearth New Therapeutic Targets

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The gut microbiota plays a key role in health and immune system education and surveillance. The delicate balance between microbial growth and containment is controlled by the immune system. However, this balance is disrupted in cases of chronic viral infections such as HIV. This virus is capable of drastically altering the immune system and gastrointestinal environment leading to significant changes to the gut microbiota and mucosal permeability resulting in microbial translocation from the gut into the peripheral blood. The changes made locally in the gut have far-reaching consequences on the other organs of the body starting in the liver, where microbes and their products are normally filtered out, and extending to the blood and even brain. Microbial translocation and their downstream effects such as increased indolamine 2,3-dioxygenase (IDO) enzyme expression and activity create a self-sustaining feedback loop which enhances HIV disease progression and constitute a vicious cycle of inflammation and immune activation combining viral and bacterial factors. Understanding this self-perpetuating cycle could be a key element in developing new therapies aimed at the gut microbiota and its fallout after infection.

1. Introduction

The interplay between gut microbiota and the immune system is a complex balance to maintain health and immunity, notably in chronic inflammatory diseases. Here, we review the changes in gut microbiota during HIV infection and the factors which modulate gut microbiota in relation to inflammation in HIV patients. We also discuss the local and systemic impact of the changes in gut microbiota and microbial translocation from the gut into the periphery in HIV infection. Finally, we discuss the potential immunotherapeutic interventions targeting gut mucosal immunity and microbiota to reduce HIV-induced inflammation.

2. Gut Microbiota: A Fragile Long-Term Partnership

As humans, we tend to think of ourselves as independent entities; however we have coevolved with billions of microorganisms that have colonized our mucosal tissues and contribute to our host diversity. The interactions between host and microorganism have recently been identified as a two-way street, where host immune pressure and food intake impact the quality of mucosal-associated flora and in turn certain microbes tailor our local and systemic immune system. The oral-gastrointestinal (GI) tract which contains the largest population of microorganisms constitutes the digestive microbiota, better known as gut microbiota. The healthy gut microbiota is composed of a diverse and highly variable population of microbes that include bacteria, viruses, and over 50 genera of fungi [1, 2]. In physiological conditions, the gut microbiota exerts a predominantly positive effect on our immune defenses such as promoting immune cell maturation [3]. In return for providing a niche rich in nutrients, the microbiota provides for us by means of carbohydrate digestion and fermentation, by vitamin production, and

most notably by helping our bodies establish gut-associated lymphoid tissue (GALTs) [4].

One of the more common constituents of the gut microbiota is the multiple strains of Lactobacilli, a lactic acid-producing bacterium which is capable of producing lactacin B, a bacteriocidal compound [5]. Lactobacilli are commonly thought of as highly beneficial, so much so that strains tend to be added to different foods labeled as probiotic in hopes of positively affecting the gut microbiota composition. To look at a few examples, *L. acidophilus* interacts with dendritic cells (DCs) to induce production of interleukin-10 (IL-10), an anti-inflammatory cytokine [6]. In addition, *L. paracasei* works from the other end of the spectrum by means of a protease that it encodes which has the ability to degrade highly inflammatory interferon (IFN) γ-induced protein 10 (IP-10, CXCL10) [7]. Together, different strains of Lactobacilli are capable of decreasing inflammation in the GI.

The gut microbiota also has diverse effects on cancer development. One group proposed a "driver-passenger" model for colorectal cancer whereby naturally occurring gut microbiota may act as a "driver" creating DNA damage and driving genome instability leading to creation of tumors. "Passenger" or opportunistic bacteria may then take over leading to a dysbiosis of the gut [8]. Despite potentially triggering colorectal cancer, another group showed that the gut microbiota may also be key in cancer treatment [9]. Cyclophosphamide (CTX), a DNA-alkylating chemotherapy agent, is dependent on a healthy gut microbiota to properly impact the polarization of splenocytes into Th17 cells, which play a key role in maintaining the integrity of mucosal immunity by secretion of IL-17 [10]. Indeed, when CTX is used in germ-free mice, or mice on antibiotic treatments, a reduced elicitation of Th17 cells was found [11].

One of the more established functions of the gut microbiota is prevention of various diseases. By outcompeting pathogenic microorganisms for food and space, the gut microbiota is able to check pathogenic growth and prevent damage to the host [12, 13]. However, some viral infections have been known to use the microbiota to their advantage. Mouse mammary tumor virus (MMTV), a retrovirus, is capable of coating itself in lipopolysaccharides (LPS) derived from the gut microbiota and interacting with pattern recognition receptor toll-like receptor 4 (TLR4) on myeloid cells [14]. The subsequent production of IL-10 contributes to successful MMTV infection via induction of immune tolerance [14]. In addition, poliovirus uses the gut microbiota as well by binding to LPS to promote infection resulting in a more severe clinical course [15]. By using the gut microbiota to their own advantage, viruses such as poliovirus and MMTV are capable of circumventing immune detection and elimination in favour of enhanced replication.

3. HIV and the Digestive Tract: A Land of Opportunity

The GALT, in particular CD4+ T cells residing in the GALT, is one of the main sites in HIV infection which constitute a long-term reservoir site even in patients receiving successful antiretroviral therapy (ART) [16]. Whatever the route of infection, mucosal regions house a rich microbiota which alters the infectivity of the target cells. Once infection has occurred, HIV rapidly depletes CD4+ T cells from the GALT as a larger percentage of these cells express elevated level of CCR5, the coreceptor for cellular entry, compared to peripheral blood [17]. Indeed, in an experimental infection of macaques by simian immunodeficiency virus (SIV), a rapid decrease of 90% of CD4+ T cells in the GALT was observed within 2 weeks of infection [18].

The other hallmark of HIV infection is persistent immune activation which makes CD4+ T cells more susceptible to infection, thus creating a vicious cycle by increasing production of IFN-γ [19], IL-6 [20], IP-10 [21], and indoleamine2,3-dioxygenase (IDO) [22]. CD4+ T cell destruction associated with immune activation in the gut leads to high levels of CD8+ T cell infiltration and epithelial cellular damage. In addition, HIV-infected cells are known to display an altered expression of microRNAs (miRNAs) in which multiple miRNAs are downregulated [23]. As miRNAs in the GI can also be affected by the microbiota [24, 25], it is entirely likely that HIV creates changes to the GI miRNA profile as well. In the GI tract, Mucosal barrier damages disrupt the integrity of the epithelial tissue and favor microbial translocation into the circulating blood [26]. This "leaking GALT" in addition to HIV has been linked to the development of acquired immunodeficiency syndrome (AIDS) [27]. ART has the ability to partially reconstitute this loss of CD4+ T cells in the gut, but only to roughly 50% when compared to noninfected controls [28]. One of the most significant consequences to the GALT caused by HIV is the drastic decrease of Th17 cells. There is also an increase in immunosuppressive regulatory T cell (Treg) frequency in the GALT which is influenced by the levels of IDO [22]. This shift in the balance of Treg and Th17 cells in favor of Tregs leads to increased mucosal permeability and microbial translocation and therefore further fuels immune activation [29].

4. The Importance of the Tryptophan Pathway: A Crossroad between Microbes and Host

IDO is an immunomodulatory enzyme found in dendritic cells (DC) and macrophages which breaks down Tryptophan (Trp) into Kynurenine (Kyn) [30–32]. IDO is known to be induced by IFN-γ in response to inflammatory signals [33]. In addition, Tryptophan 2,3-dioxygenase (TDO), a hepatic enzyme, is highly similar to IDO, which also acts on the Kyn pathway [34, 35]. TDO may also be found in the placenta, testis, and brain after stimulation [35–37]. Enhanced immunosuppressive Kyn production by IDO and/or TDO plays a harmful role in cancers and viral infections including HIV infection [22, 29, 38, 39]. Kyn inhibits T cell proliferation [40, 41] while another IDO catabolite, quinolinic acid, is linked to neurodegenerative diseases including AIDS dementia complex [42]. It is known that monocyte derived-DCs specifically expressing IDO promote Treg expansion and that the IDO induction in these DCs can be achieved by the HIV

transactivator protein Tat [43, 44]. Furthermore, our team has recently shown that increased IDO enzyme activity and Kyn production are linked to the imbalance of Th17/Treg and microbial translocation in chronic HIV infection [29]. In untreated HIV infection, IDO levels were found to be elevated and were correlated with the high levels of immune activation. After several years of continuous successful ART, these levels decreased, approaching what is seen in healthy subjects [29]. Interestingly, an enrichment of a gut microbiota subset which has the capacity of catabolizing Trp through the IDO pathway was found in HIV-infected subjects [45].

5. HIV and Gut Microbiota: Partners in Crime Enhancing Immune Activation in a Stepwise Process

5.1. Local Effects

5.1.1. The Gastrointestinal Tract. The alteration of Th17/Treg balance in the GALT induced by HIV leads to microbial translocation of commensal and pathogenic bacterial products into the blood stream resulting in a generalized and persistent immune activation [46]. However, there were also changes to the types and amounts of bacteria that comprise to microbiota. An in-depth analysis of the changes in microbiota of HIV-infected patients was assessed by Vujkovic-Cvijin et al. In their study, the total bacterial load and amount of diversity appeared to be similar across infected and uninfected groups; however HIV viremic patients had microbiota communities distinctly enriched in Proteobacteria, most notably of the family Enterobacteriaceae which includes known pathological microbes such as *Salmonella, Escherichia,* and *Shigella* [45]. In fact, these pathological microbes tend to be the cause of bacteremia in advanced HIV-infected patients [47]. Viremic patients also displayed a decrease in *Bacteroides* and *Alistipes,* which are depleted in inflammatory bowel disease [48]. The particular enrichments and depletions in viremic HIV patients were found to be linked to a decrease in Th17 cells in gut biopsies as well as an increase in immune activation and correlation with IDO activity and IP-10 plasma levels as a trustable marker of HIV disease progression [45]. The link between IDO activity and the microbiota appears to be a self-sustaining feedback loop, which encourages pathological microbe growth. Multiple bacteria enriched in HIV viremic patients in Vujkovic-Cvijin's et al. study have enzymatic homologs of IDO, which are capable of producing Kyn from Trp. The initial assault from HIV to the gut causes inflammation, which may in turn create a microenvironment more suitable to pathologic bacteria. This bacterial community may be capable of outcompeting its beneficial counterpart by way of Kyn production through IDO, and, once established, they are capable of producing Kyn which further fuels their growth.

However, some ART-treated patients exhibited microbial communities highly similar to viremic patients, while others were much more similar to healthy subjects. The diversity may be an indication of clinical outcome or could indicate that the microbiota recovery time is variable. In line with this hypothesis, a recent study by Lozupone et al. looked at bacterial variance during ART [49]. They examined HIV-infected patients who were untreated or had been on ART for varying lengths of time. The study showed that genera of bacteria that are elevated in HIV-infected patients *versus* healthy subjects such as *Peptococcus* decreased over time spent on ART to levels approaching that of healthy subjects [49]. Pérez-Santiago et al. showed related results in a cohort of HIV infected men on successful ART [50]. Indeed, they demonstrated an association between enriched levels of *Lactobacillales* and preserved immune function as indicated by decreased microbial translocation, lower T cell proliferation, and higher percentages of CD4+ T cells in the gut and periphery [50].

Lactobacilli are clearly important for regulating and maintaining physiological gut immunity, a concept which was explored by Zelante et al. in a mouse model [51]. Indeed, Lactobacilli, specifically *L. reuteri*, are capable of catabolizing Trp into indole-3-aldehyde (IAld) when there is an excess of nutritional Trp and IDO activity is low. IAld is then capable of stimulating natural killer (NK) cells via aryl hydrocarbon receptor (AhR) to produce IL-22 which controls the gut microbiota, ensuring a diverse ecosystem [51]. However, in cases where IDO activity is elevated due to the migration of IDO-expressing DCs to gut mucosa, Trp is preferentially broken down into immunosuppressive Kyn. Higher levels of Kyn and the subsequent expansion of Tregs create a tolerogenic environment where normal commensals like *Candida albicans* can become pathogenic creating candidiasis. Interestingly, the same study showed that administration of oral IAld to mice with mucosal candidiasis restored IL-22 production by NK cells and decreased the candidiasis [51]. This distinctive use of Trp by Lactobacilli may in part account for its association with better clinical outcomes in HIV by way of limiting Kyn production and may represent an important strategy for future treatments.

5.1.2. The Liver Firewall. Recently, Balmer et al. helped elucidate the role of the liver in the control of microbial translocation using a mouse model [52]. In their study, livers of healthy mice did not show any signs of containing microbes. However, once the gut epithelial cells were breached, microbes gained access to underlying vasculature, which drains directly into the hepatic portal vein [52]. Mice challenged with *E. coli* alone did not have any detectable bacteria in the liver but after inducing experimental intestinal inflammation, *E. coli* was consistently found in the liver. Once microbial products reach the liver, Kupffer cells, specialized hepatic macrophages, are capable of clearing the bacterial challenge. In the case of liver tissular insults, mice showed a drastic reduction in bacterial clearance [52].

Since the initiation of ART treatment, patients have shown increased survival and that survival has led to a rise in non-AIDS conditions all related to immune activation that affects kidney, cardiovascular organs, and liver [53]. HIV induces hepatic damages via multiple mechanisms. First, the damage can occur directly through infection of Kupffer cells [54]. The liver is further damaged by inflammation, favored by microbial translocation. LPS in the portal vein

system is capable of activating Kuppfer cells, leading to a release of inflammatory cytokines and perpetuating the continued inflammation and therefore hepatic damages [53]. Liver damage can further be exacerbated by alcohol abuse, obesity, metabolic syndrome, and ART hepatotoxicity. HIV is also capable of accelerating the development of liver cirrhosis in patients coinfected with HCV [55]. Under viral infection conditions that increase immune system inflammation, the increased microbial translocation is linked to a decrease in the liver's ability to clear bacteria [56]. Epidemiological evidence indicates that a cohort of patients displaying nonalcoholic fatty liver disease or steatohepatitis showed evidence of serum IgG and IgA against intestinal commensal microbes which signifies that compartmentalization of the gastrointestinal microbiota is compromised in liver disease due to the failure of the hepatic vascular firewall [52].

5.2. Systemic Effects

5.2.1. Circulating Blood. HIV infection is a major cause of microbial translocation where bacterial products egressing the gut by the portal vein cannot be fully cleared by the Kupffer cells in the liver leading to microbes and their products being present in peripheral blood. Levels of microbial translocation can be measured by sCD14, the soluble form of CD14, released into the circulation by monocytes upon microbial product stimulation [57]. In HIV viremic patients, sCD14 is elevated but, once patients are treated with ART, these levels decrease to a level similar to healthy individuals [58]. IDO enzymatic activity also follows this trend [29]. Another soluble inflammatory marker which is linked to IDO activity is soluble CD40 ligand (sCD40L) as CD40-CD40L signaling is known to be key in IDO induction. sCD40L is mainly produced by activated T cells, platelets, and B cells and its plasma levels are increased in chronic HIV infection [59]. As part of the TNF-receptor superfamily, engagement of CD40 and CD40L, in the presence the HIV envelope protein gp120, can sensitize DCs for apoptosis [60]. Our group has recently reported that sCD40L is able to stimulate Treg expansion and differentiation, and, most notably, production of Kyn through IDO resulting in microbial translocation [61].

IDO can also be used to predict disease outcomes independently of viral load and CD4+ T cell counts. In a Ugandan cohort of HIV-infected patients, higher IDO activity was strongly associated with higher HIV RNA copies and low CD4+ T cell counts in absence of ART. Following ART initiation, IDO levels remained predictive of low CD4+ T cell recovery and increased mortality [62]. Furthermore, the same group identified IDO levels to be associated with neurocognitive disorders [63].

5.2.2. The Brain. IDO produced at local gut mucosal sites and circulated in the peripheral blood affects multiple organs in multiple ways, including the brain. Activated monocytes are capable of trafficking the virus into the central nervous system where the infection is mainly perpetuated by infected macrophages [64]. In fact, it is not only infected cells that can cause complications. Blood-brain barrier endothelial cells can synthesize Kyn after immune activation [65]. In mice, activation of IDO leads to inflammation-associated depression. This induction is mediated in part through the viral protein Tat which synergizes with IFN-γ already present due to inflammation [66]. Furthermore, high circulating levels of IDO in HIV patients are associated with depression [67] and are found in HIV-associated dementia [68]. In line with this, it has been shown that sCD40L is also involved in cerebral inflammation and dementia in HIV-infected patients [36, 37, 69, 70].

6. Perspective for New Immunotherapeutic Targets

The human gut microbiota is complex and deeply intertwined with the immune system, which makes it one of the many factors involved in HIV infection. During HIV infection, the microbiota is affected on a local level in the gastrointestinal tract, which creates changes to our immune system. The alterations to immune system favoring inflammation lead to increased microbial translocation which is normally cleared in the liver, except in cases of liver damage or when this translocation persists for long periods of time. The systemic effects of the microbiota can be explained by the production of IDO, which occurs at the level of the gut and also at multiple sites including the brain. IDO and its immunosuppressive catabolites are further capable of altering the immune system by enhancing Treg populations and downregulating Th17 populations, creating a vicious circle (Figure 1). The topic of intervention in relation to the microbiota is not new and includes targeting the GI biological, immune, and mechanical barrier [64]. However, targeting factors outside of the GI tract may also be beneficial.

All this makes IDO, TDO, CD40L (an upstream inducer of IDO), and the microbiota targets during HIV treatment to improve the immune system as summarized in Table 1. Such attempt used 1-methyl-tryptophan (1-MT) in the brain of CX3CL1−/− mice after challenges of LPS [71]. CX3CL1−/− mice normally display persistent neural inflammation and depressive-like behavior upon LPS challenge but with 1-MT, a competitive inhibitor of IDO, these effects were abrogated only 72 H after challenge [71]. Similar results were seen in another mouse model using 1-MT to promote clearance of HIV-infected macrophages in the brain, an environment simulating HIV encephalitis, where administration of the drug caused infected macrophages to decrease by almost 90% [72]. 1-MT was also used in an SIV model using rhesus macaques on ART. Although Kyn levels remained high, suggesting 1-MT was not fully effective against IDO activity, macaques with unsuccessful ART displayed reduced viral load [73]. However, in a more recent study of 1-MT in rhesus macaques with SIV on ART treatment, there was no effect found on inflammation, viral RNA in blood, or gastrointestinal tissue [74].

IDO may be a potential target not only to treat HIV infection, but also in the prevention of infection. In HIV-exposed seronegative female commercial sex workers, cervical mononuclear cells were shown to have much lower levels of IDO than HIV-infected individuals [75]. One possible

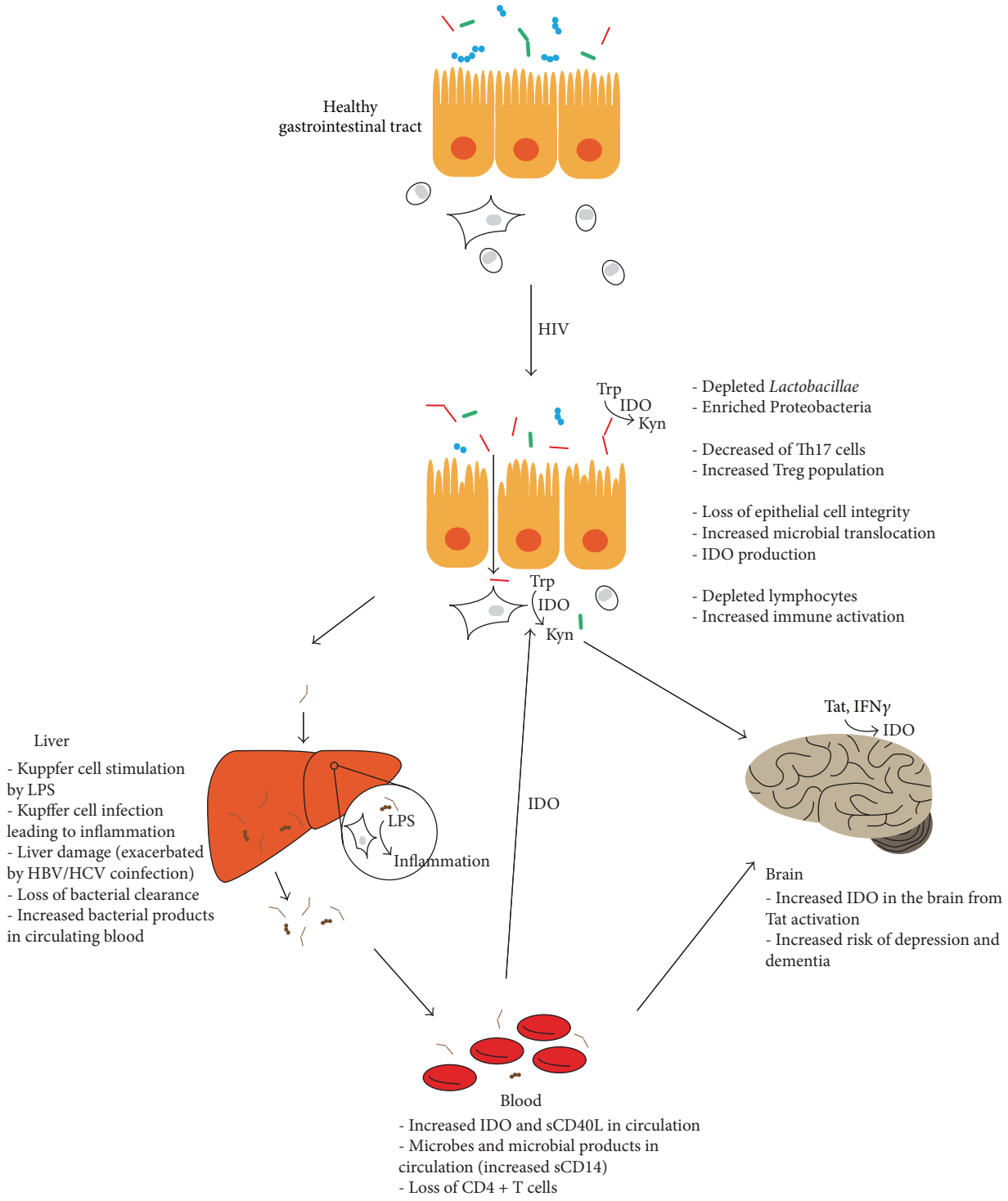

Figure 1: The vicious cycle of HIV infection. HIV infection has immediate effects in the gut where lymphocytes are depleted and damage to the endothelium allows for microbial translocation. Microbiota, when not cleated by the liver, go on to have systemic effects most notably through IDO production which is capable of creating a vicious cycle of inflammation.

avenue in HIV prevention may lie in understanding the "immune quiescent" nature of seronegative sex workers immune system [76].

Another treatment possibility is to act on the microbiota directly. Probiotics and prebiotics help support and grow the microbiota and have been used in different diseases with gastrointestinal inflammation [77]. Probiotics consist of microorganisms, frequently Lactobacilli, that are taken with the aim of positively influencing the host microbiota and therefore health. Prebiotics on the other hand are

Table 1: Selected studies targeting the gut microbiota or subsequent downstream effects.

Therapeutic target	Drug	Study details	Result	Reference
IDO	1-MT	CXCL1−/− mice	Decreased activation in the brain and decreased depressive behaviour	[71]
	1-MT	Mice with injections to the brain of HIV-infected macrophages	Increased CD8+/IFN-γ+ T cells in the periphery and an 89% decrease in HIV-infected macrophages in the brain	[72]
	1-MT	SIV in rhesus macaques on ART	No change to T cell counts or activation, viral load, or Trp metabolism	[74]
	1-MT	SIV in rhesus macaques on ART	Only partial effect on IDO activity and significant drop in viral load for macaques with unsuccessful ART	[73]
Pro-/prebiotics	Pro and prebiotics	SIV in pigtail macaques on ART	Enhanced reconstitution and functionality of CD4+ T cells and increased frequency of GI tract APCs	[78]
	Bifidobacterium lactis	Meta-analysis of formula supplementation in HIV-infected infants (>6 months)	Improved infant growth and protection against CD4+ T cell loss	[79]
Sevelamer	Sevelamer	Acute SIV infection in pigtail macaques	Drug bound LPS in the gut, drastically decreased inflammation and immune activation, and slightly decreased viral replication	[81]
	Sevelamer	HIV patients not receiving ART	No significant change to microbial translocation, inflammation, or immune activation, but significant decrease in LDL cholesterol	[82]
IL-7	Recombinant human IL-7	HIV patients on successful ART	Increased CD4+ and CD8+ T cells, increased a4b7 T cells, and decreased sCD14	[84]

indigestible food ingredients such as inulin that aim to promote microbiota associated with good health. When used together, one study found that pro- and prebiotics increased CD4+ T cell reconstitution and functionality while a meta-analysis found that probiotics improved infant growth and protected against CD4+ T cell loss [78, 79]. New studies have also begun looking into Sevelamer as a treatment for microbial translocation and subsequent inflammation. Sevelamer is a phosphate-binding drug already shown to decrease blood levels of LPS in cases of chronic kidney disease [80]. Like 1-MT, Sevelamer has conflicting results. Indeed, in an SIV model, Sevelamer decreased microbial translocation while also decreasing inflammation and immune activation [81]. However a study on nontreated HIV-infected patients showed a lack of any significant changes to microbial translocation, inflammation, or immune activation [82].

Although not directly aimed at the microbiota, our group was instrumental in a study using IL-7 as a treatment meant to restore gut immunity and integrity. IL-7 is known to induce gut epithelial cells to produce IL-7, while an absence of gut microbiota is known to decrease IL-7 [83]. After IL-7 administration, Patients showed increased CD4+ and CD8+ T cells, as well as an increase in gut-homing lymphocytes ($\alpha 4\beta 7$+ T cells). Patients also displayed a decrease in sCD14 indicating an improvement in the gut barrier integrity [84].

7. Concluding Remarks

Research has only begun to scratch the surface of how our microbiome fully influences HIV infection. However, it is clear that a complex interplay between gut microbiota and altered immune system mediated by the virus contributes to disease progression and immunodeficiency. Therefore, design and implementation of new and combinatory immunotherapeutic strategies which target both gut microbiota and host immunosuppressive mechanisms could represent novel additions to current ART treatments to reduce generalized immune activation and inflammation as a consequence of HIV/microbiota partnership.

Conflict of Interests

No conflict of interests to declare.

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Review Article

The Central Role of the Gut Microbiota in Chronic Inflammatory Diseases

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The commensal microbiota is in constant interaction with the immune system, teaching immune cells to respond to antigens. Studies in mice have demonstrated that manipulation of the intestinal microbiota alters host immune cell homeostasis. Additionally, metagenomic-sequencing analysis has revealed alterations in intestinal microbiota in patients suffering from inflammatory bowel disease, asthma, and obesity. Perturbations in the microbiota composition result in a deficient immune response and impaired tolerance to commensal microorganisms. Due to altered microbiota composition which is associated to some inflammatory diseases, several strategies, such as the administration of probiotics, diet, and antibiotic usage, have been utilized to prevent or ameliorate chronic inflammatory diseases. The purpose of this review is to present and discuss recent evidence showing that the gut microbiota controls immune system function and onset, development, and resolution of some common inflammatory diseases.

1. Introduction

Commensal microbiota consists of many microorganisms that cover all host mucosal surfaces, but most reside in the gastrointestinal tract, which is the subject of this review. Amazingly, although the human body is composed of approximately 100 trillion cells, only 10 trillion are human cells while 90 trillion are microbes. The genes of these microorganisms form our metagenome, known as our second genome [1]. Thus, it is not surprising that this large arsenal of gene products has a relevant role in body homeostasis [2, 3]. The relationship between the gut microbiota and its host plays a key role in immune system maturation, food digestion, drug metabolism, detoxification, vitamin production, and prevention of pathogenic bacteria adhesion [4]. One of the most important roles of the microbiota is the maturation of the immune system in the postnatal period. The first appearance of adaptive immunity in humans coincides with acquisition of a complex diet and microbiota, which suggests that mucosal immunity in the intestines has evolved to tolerate diverse microbes and food antigens.

Colonization of the gastrointestinal tract begins after birth, despite the fact that some researchers have discovered a small community of bacteria living in the placenta [5]. However, there is no convincing evidence demonstrating that such bacteria normally reach the fetus through the placenta. It

is known that colonization initiates from maternally acquired bacteria during birth [6] and breastfeeding and continues throughout our life [7–9]. Over the lifetime of the individual, or at least until stabilization of colonizing microbiota in adulthood, there is a change in the profile of the predominant phyla in the gastrointestinal tract, migrating from a community dominated by Actinobacteria and Proteobacteria to one dominated by Firmicutes and Bacteroidetes [10]. The metagenome of an infant gut is characterized by an enrichment of genes required for the breakdown of simple sugars, such as lactose and galactose, while the weaned infant microbiota is enriched in genes for polysaccharide breakdown and vitamin production [11, 12]. Most bacterial species in the human and mouse gut belong to the phyla Bacteroidetes and Firmicutes, but less abundant bacterial phyla, such as Actinobacteria, Proteobacteria, and Verrucomicrobia, as well as methanogenic archaea, mainly *Methanobrevibacter smithii*, are also present [13, 14].

The composition of the microbiota is influenced by environmental factors such as diet, antibiotic therapy, and environmental exposure to microorganisms. Additionally, it can vary according to sex, age, and geographical origin of the individual [15]. An overgrowth of pathogenic microbial colonies causes an imbalance known as dysbiosis. Antibiotic therapy, alcohol misuse, and inappropriate diet are factors that can lead to dysbiosis [16–18].

The normal relationship between the gut microbiota and the immune system is established by bacteria, cells, and receptors of both the innate and adaptive immune systems. Microbes are held in the intestinal lumen through the combined efforts of the epithelial barrier, mucus layer, antimicrobial peptides, and antibodies. Controlling the intestine's metabolic products is also important for the maintenance of a mutually beneficial relationship between the microbiota and the immune system. When this connection is broken and fails to resolve itself, an inflammatory response is initiated.

Here, we review the mechanisms by which the gut microbiota contributes to the development of asthma, bowel disease, and obesity, highlighting the regulatory role of the gut microbiota in immune system function.

2. Mechanisms Linking the Microbiota and Its Products to the Immune System

Recently, several studies have shown possible links between the gut microbiota and the immune system. Here, we summarize some of the "sensors" that are involved in this interaction and describe related pathological conditions.

Innate immune cells such as macrophages, neutrophils, and dendritic cells, as well as other cell types including epithelial cells, which form the interface between the body and the external environment and are in close contact with the microbiota, express several membrane and intracellular proteins that sense microbial molecules. Examples of these sensors include pattern-recognition receptors such as Toll-like receptors (TLRs), C-type lectin, nucleotide oligomerization domain (NOD) receptors (NLRs), and retinoic acid inducible gene (RIG)-I-like receptors (RLRs), which are activated by microbial molecules including flagellin, lipopolysaccharide, lipoteichoic acid, peptidoglycans, N-acetylglucosamine, and double stranded-RNA. Considering the types of ligand that activate these receptors, it is not surprising that some participate in the microbiotic regulation of the immune system and serve as regulators themselves. These receptors also play a role in shaping the microbiota. For example, in the absence of TLR5, a receptor activated by bacterial flagellin, mice present changes in microbiota composition that have been associated with the development of metabolic syndrome in these animals [19].

NLR proteins are expressed in a wide variety of both immune and nonimmune cells and detect microbial and endogenous signals released from these cells. These proteins consist of three domains: a central nucleotide-binding domain termed NACHT (referred to as the NOD domain) and both amino- and carboxy-termini consisting of leucine rich repeats (LRR domains). These latter two domains are important, respectively, for interaction with other proteins that initiate a signaling cascade and for recognition of molecules that activate a family of receptors comprising 22 different human proteins. These proteins are classified based on their N-terminal domain, which includes a caspase recruitment domain (CARD) on Nod1, Nod2, and NLRC3, 4 and 5; a pyrin domain (PYD) on NLRP1-14; an acidic transactivating domain on NLRA; or a baculovirus inhibitor repeat (BIR) on NAIP [20]. Several studies have shown that changes in the expression of these intracellular sensors lead to modifications in the composition (both qualitative and quantitative) of the microbiota and the immune system and have been associated with the development of conditions including colitis, bacterial infection, obesity, and insulin resistance [21].

Another class of sensors that detects molecules derived from the microbiota is the G protein-coupled receptors (GPCRs). These receptors will be discussed in detail below. At least three GPCRs have been identified that bind to short chain fatty acids (SCFAs) produced by gut bacteria: GPR41, GPR43, and GPR109A. GPR41 (i.e., FFAR3) and GPR43 (i.e., FFAR2) are both highly expressed on immune cells such as polymorphonuclear cells and macrophages [22]. Additionally, other cells and tissues including adipose tissue, enteroendocrine cells and the cells of the sympathetic nervous system have also been shown to express these receptors and to mediate some of their biological effects [23]. G protein-coupled receptors are activated by SCFA; butyrate binds to GPR41 with high affinity, but acetate and propionate have a greater affinity for GPR43 [24–26]. Some of the effects associated with SCFA depend on the activation of GPR43 and include reactive oxygen species (ROS) production and neutrophil chemotaxis. More recently, it has been shown that via this receptor, SCFA modulates the number of T regulatory cells (Tregs) in the colon, an effect that will be further described in last section of this review. GPR41 activation has been associated with regulation of metabolism and energy expenditure.

GPR43 is reported to activate both Gi/o and Gq, while GPR41 signals via Gi/o only. Both receptors induce intracellular calcium mobilization and inhibit cAMP accumulation.

The MAPKs ERK1/2, JNK, and p38 are activated by SCFAs through binding to the GPCRs [27]. GPR41 and GPR43 activation of ERK1/2 is dependent on Gi/o, because the inhibition of this G protein by the pertussis toxin abolishes the stimulatory effect of SCFAs on this pathway in cells expressing only the GPR41 and reduces it in more than 50% in cells expressing GPR43 alone [27]. In neutrophils, ERK1/2, p38, and PKB are activated by SCFAs through a pertussis toxin sensitive pathway and are important for GPR43-dependent chemotaxis. A recent study has demonstrated a chemotactic of SCFAs through a mechanism involving PI3Kγ and the small G protein Rac2 [23]. Recently, it has also been shown that SCFAs induce chemokine and cytokine expression in colonic epithelial cells in a GPR41- and GPR43-dependent manner. In this study, the authors demonstrate the involvement of Gi/o, ERK, p38, and the transcription factor ATF2 in this SCFA-induced expression [28].

GPR109A, also known as hydroxy-carboxylic acid 2 receptor or HM74a, is a receptor for nicotinate. Additionally, this protein binds to the ketone body β-D-hydroxybutyrate and to the SCFA butyrate [29, 30]. This receptor is expressed on hematopoietic-derived cells, white and brown adipocytes, keratinocytes, colonocytes, and hepatocytes [29, 30].

3. Asthma

Asthma is a chronic airway disease characterized by excessive contraction of airway smooth muscle (termed airway hyperresponsiveness or AHR), exacerbated mucus production, eosinophilia, and elevated Th2 cytokine production [31]. Asthma affects approximately 300 million people worldwide, and it is estimated that in 2025, more than 100 million people will be diagnosed with this pathology [32, 33]. Current treatment is based on anti-inflammatory therapies, which do not cure asthma. Furthermore, AHR may persist even in the absence of inflammation. The treatment of asthma is complex because there are many asthma phenotypes, and its effectiveness depends on environmental and genetic factors [34]. Asthma prevalence is increasing in Western countries due to lifestyle modifications including excessive hygiene (i.e., little exposure to microbes) and use of antibiotics and a high-fat diet [35–40]. Epidemiological studies have shown that exposure to microbes early in life is a critical factor in the induction of allergic diseases, leading to the development of the hygiene hypothesis [35–39]. Briefly, this theory proposes that excessive cleaning and reduced pathogen exposure leads to an inadequate immune response [41]. Likewise, the use of antibiotics early in life is also associated with allergic sensitization and AHR [42]. Thus, exposure to microbes early on has a great influence on immune function later in life. Moreover, the intestinal microbiota, our largest collection of microorganisms, modulates the pathophysiological processes of asthma. Several groups have noted that the hygiene hypothesis should be rewritten to include the role of the intestinal microbiota and thus renamed as the "microflora hypothesis." The "microflora hypothesis," initially discussed by Noverr and Huffnagle [43], postulates that perturbations in the gastrointestinal microbiota, resulting from reduced microbial exposure due to changes in diet and antibiotic use [44], lead to an underdeveloped microbiota. This "immature" microbiota delays proper maturation of the immune system. The sequence of events that promotes the development of immunological tolerance is disrupted, leading to allergic hypersensitivity [43].

Epidemiologic studies have identified associations between alterations in the composition of gut bacterial communities and the development of allergies [45, 46]. Children with asthma have a different intestinal microbiota compared to nonasthmatic children. Asthmatic children have a high prevalence of certain species of *Clostridium difficile* (bacterium with pathogenic characteristics) and low *Bifidobacterium* (nonpathogenic bacteria) in their intestinal microbiota [45, 47]. Clinical trials have indicated that feeding *Lactobacillus rhamnosus* GG and *Lactobacillus fermentum* to mothers in the prenatal and early postnatal periods may be effective in the treatment and prevention of early atopic disease in children [47, 48].

Studies in animal models have also shown that gut bacteria modulate experimental asthma [22, 49]. Researchers have employed three main strategies to interfere with gut colonization and showed its effects beyond the local gut immune response. These strategies include maintaining germ-free (GF) mice (devoid of microbiota) in a sterile environment, microbiota depletion/perturbation by antibiotic therapy, and alteration of the microbiota composition through modification of the host's diet.

The mechanisms by which the innate immune system recognizes the commensal-derived signal that regulates Th2 inflammation is currently being studied. Dendritic cells (DCs), basophiles, and invariant natural killer T (iNKT) cells are part of this mechanism. DCs are the primary antigen-presenting cells responsible for the antigen-specific activation of naive T cells. Microbes in the intestine are sampled by DCs either directly from the lumen or through the gut-associated lymphoid tissue (GALT). A combination of signals from microbes results in phenotypic changes in the DCs, which leads to the differentiation of Th1, Th2, and Treg cells (Figure 1). One phenotypic change is the increased production of IL-10 by these cells. DCs expressing high levels of IL-10 drive the generation of CD4+FOXP3 Tregs and the establishment of tolerance. Tolerance can be established by the activation of Th1 and Treg cells. This regulatory mechanism plays a key role in the immunoregulatory action of many probiotics. In this way, the intestinal microbiota may induce Treg cells in the GALT that then spread to the airways in response to allergen exposure. This idea is supported by the finding that oral treatment with *Lactobacillus reuteri* results in an increase in Treg cells in the draining lymph nodes of the lung. Additionally, *L. rhamnosus GG* has been shown to reduce the murine allergic airway response through associated increases in FOXP3T cells only when bacteria are administered in the neonatal period [50, 51]. The generation of Treg cells is only one mechanism; other mechanisms may also account for the effects of microbiota in immune regulation, as discussed below.

Germ-free mice exhibited an exaggerated number of airway eosinophils, increased production of Th2 cytokines,

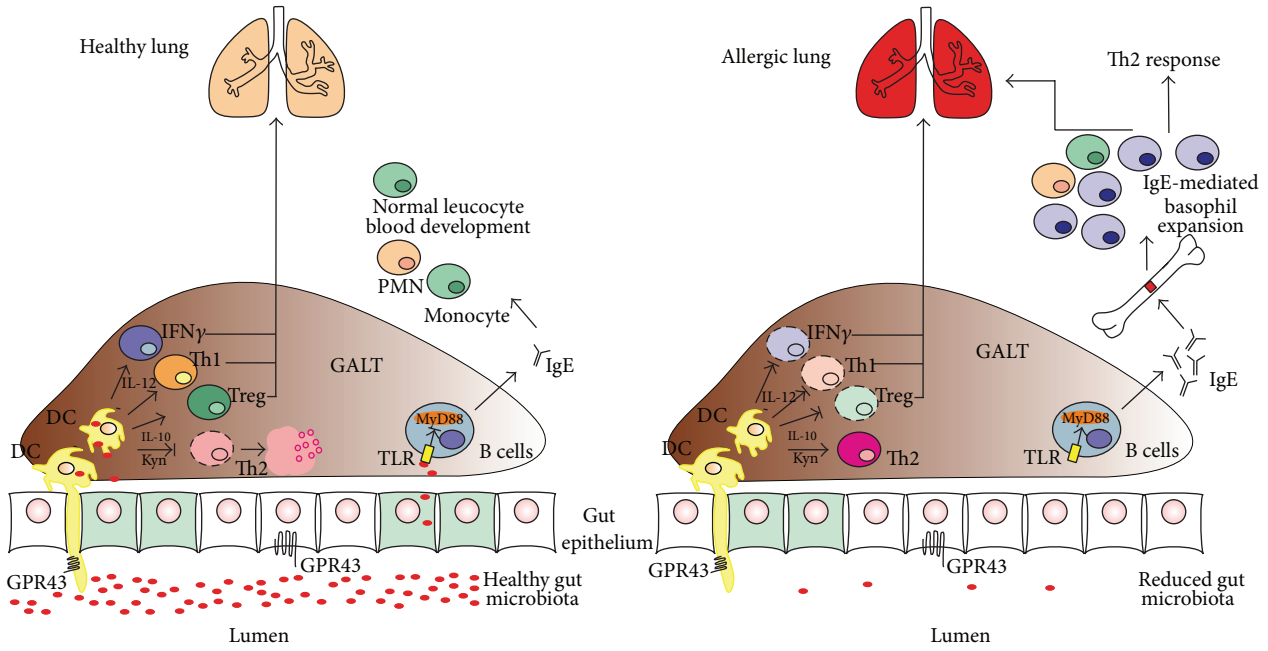

Figure 1: Schematic representation of the pulmonary allergic response induced by gastrointestinal (GI) immune cells and two microbiota-related conditions (a healthy gut microbiota and a reduced gut microbiota following antibiotic treatment). Microbes in the intestines are sampled by Toll-like receptors (TLRs) on DCs either directly in the lumen or in the gut-associated lymphoid tissue (GALT). In the healthy gut microbiota, polymorphonuclear development (PMN) is normal and DCs become regulatory DCs (DCr) that promote development of Tregs and/or Th1 cells and natural killer (NK) cells. These NK cells inhibit Th2 inflammation. Antibiotic treatment kills a large proportion of healthy microbiota, leading to a reduced gut microbiota and an inflammatory environment without DCs, Th1 cells or NK cells. In this environment, an unhealthy microbiota elevates serum immunoglobulin E (IgE) levels, increases circulating basophil populations, and exacerbates basophil-mediated Th2 responses (adapted from Forsythe [110]).

elevated immunoglobulin E (IgE) production and an altered number and phenotype of DCs when sensitized and challenged with ovalbumin (OVA). This phenotype was abolished by recolonization of germ-free mice with the complex commensal flora of specific pathogen-free mice [52]. Interestingly, Tregs were unaffected in GF mice, although the number of basophils was increased. Moreover, depletion or deletion of bacterial communities was associated with elevated serum IgE concentrations, an increased circulating basophil population, exaggerated Th2 cells responses, and allergic inflammation [53] (Figure 1). Additionally, the exaggerated Th2 response was reduced upon depletion of basophils. Thus, basophils are an important link between the gut microbiota and allergic inflammation. Recently, investigators have discovered a mechanism by which commensal bacteria regulate basophil functions, interfering with the susceptibility of the Th2 immune response. They found that treatment with oral antibiotics increased serum IgE concentrations by increasing the level of circulating basophils and inducing an exaggerated Th2 inflammation. B cell-intrinsic expression of MyD88 is an important step in increasing serum IgE and basophil levels. When expression of MyD88 is blocked by a healthy microbiota, there is no development of allergic airway inflammation (Figure 1) [52].

Treg cells and basophils are not the only cell types affected by microbiota in mouse models. GF mice contain an increased number of iNKT cells compared to specific pathogen free (SPF) mice [49]. iNKT cells secrete abundant levels of IL-4, IL-12, and IFN-γ upon activation, resulting in increased susceptibility to allergic inflammation. Moreover, greater Th2-mediated airway inflammation was observed in GF mice than in SPF mice when mice were sensitized with OVA. Asthma in GF mice was CD1-d dependent, because depletion of these cells decreased allergic inflammation. Interestingly, researchers also observed that colonization of neonatal, but not adult, GF mice with conventional microbiota protected the animals from mucosal iNKT accumulation and asthma [49]. Thus, microbial contact early in life is critical for the establishment of mucosal iNKT cell tolerance to antigens in exposed airways.

In addition to innate immune cells, other elements related to microbiota may be important in the regulation of Th2-mediated airway inflammation. SCFAs are the major end products of bacterial metabolism in the human large intestine. The fermentation of complex plant polysaccharides leads to the production of SCFAs such as propionate, butyrate, and acetate. As described above, SCFAs have been reported to show anti-inflammatory properties such as leukocyte recruitment, leukocyte chemotaxis, and chemokine production [54, 55]. Animals deficient in a receptor coupled to GPR43 that binds to SCFAs, including acetate, have an exaggerated inflammatory response in models of colitis, arthritis, and asthma. OVA-sensitized GPR43 KO mice have a greater inflammatory infiltrate in the airways and lung

tissue compared to littermate mice [22]. Moreover, Trompette et al. [56] found that fermentable dietary fiber content changed the composition of mouse gut and lung microbiota by altering the ratio of Firmicutes to Bacteroidetes bacteria, which consequently increased the concentration of SCFAs, specifically propionate. Mice fed a high-fiber diet were protected against allergic inflammation in the lungs by increased DC phagocytic function, although the DCs also displayed an impaired ability to mediate Th2 airway inflammation. Altogether, these studies suggest that SCFAs are important in controlling allergic pulmonary inflammation. However, there are only a few studies showing SCFA modulation of immune system function. The mechanism by which SCFA reduces AHR remains unknown. All SCFAs have the same effect on airway inflammation and lung function. The question, of which microbiota is more important for immunological responses in the airways, lung microbiota or gut microbiota, remains unanswered.

As a whole, the gut microbiota has a significant effect on airway immunity. Therefore, it is relevant to consider the composition of the host microbiota with the same level of importance as genetic polymorphisms and environmental factors when diagnosing and treating asthma.

4. Inflammatory Bowel Disease

The incidences of inflammatory bowel disease have risen rapidly over the last several years. Crohn's disease and ulcerative colitis are the main inflammatory bowel diseases (IBDs) and are characterized by a chronic and exacerbated inflammation of the intestinal mucosa [57]. In addition to genetics, several factors contribute to the high incidence of IBDs such as lifestyle and the intestinal microbiota. Commensal microbiota plays an important role in the pathogenesis of inflammatory bowel disease [58, 59] because experimental colitis has been successfully treated with an antibacterial agent [60] and antibodies against microbial antigens in IBD patients [61]. In experiments, GF mice were more susceptible to colitis induced by dextran sodium sulfate (DSS) [22]. Recolonization of GF mice with feces from conventional mice reversed this phenotype, showing that microbiota plays a beneficial role in colitis [22]. It is clear that dysbiosis results in a lack of immune regulation and breakdown of tolerance to commensal microorganisms. Dysbiosis allows outgrowth of more pathogenic microorganisms and promotion of the exacerbated inflammation underlying IBD [62]. Abnormal gut colonization has been observed in subsets of Crohn's disease and ulcerative colitis patients [63]. Patients with IBD, compared to healthy controls, have fewer bacteria with anti-inflammatory properties and/or more bacteria with proinflammatory properties [64]. In addition, an abnormal microbiota can cause IBD by expansion of colitogenic strains that initiate development of colitis [65]. The molecular mechanisms involved in the indirect effects of the microbiota on the host intestine in inflammatory bowel disease are described below.

The host develops a complex mucosal immune system composed of epithelial and hematopoietic cells in order to avoid ongoing inflammatory reactions to the microbiota and preserve its ability to react to pathogenic insults. When such interactions are perturbed, an exacerbated inflammation occurs, leading to the development of IBD [55]. Recent findings have focused on the molecular mechanisms involved in the interaction between the gut microbiota and epithelium cells [66–69] (Figure 2). The intestinal epithelium is more than a single layer of cells working as a physical barrier; it has developed mechanisms to protect itself from uncontrolled inflammatory responses and to prevent bacterial dissemination to other organs. The epithelial responses against the gut microbiota highlight the importance of a self-limiting or noninflammatory cellular immune response scenario in the antigen-rich intestinal environment. The reestablishment of intestinal barrier integrity regulates the inflammatory response in IBD [65, 68, 70]. GF mice recolonized with gut microbiota have shown a marked reduction in inflammation. The exacerbated response in colitis was related to a lack of bacterial colonization of the gut that provides beneficial effects in IBD [22]. Bacteria likely protect against IBD by directly or indirectly enhancing the intestinal environment via increased production of molecules such as SCFA by beneficial bacteria. SCFAs, mainly acetate, propionate, and butyrate, which are produced by bacteria of the Bacteroidetes and Firmicutes phyla after fermentation of dietary fiber, show anti-inflammatory properties in IBD [22, 71, 72]. Patients with colitis and/or Crohn's disease have reduced levels of these bacteria in the colon [63]. The SCFAs carry out many functions in the gut such as serving as fuel for the intestinal epithelium cell, regulating gut epithelium cell proliferation, differentiation, and gene expression, and initiating anti-inflammatory effects on intestinal mucosa [22, 73–76].

Butyrate elicits biological effects on intestinal epithelial cells by binding to GPR109A, a G protein-coupled receptor, which is highly expressed in the colon [29]. Activation of the GPR109A receptor by butyrate leads to a decrease in intracellular levels of cAMP and this reduction controls electrolyte and water absorption to reduce the incidence of diarrhea in IBD [77]. SLC5A8, known as SMCT1 (sodium-coupled monocarboxylate transporter 1), is a butyrate transporter in a Na^+-dependent electrogenic process and is highly expressed in the colon. Butyrate has the ability to influence gene expression in the colon through histone deacetylase (HDAC) inhibition [78]. Interestingly, the expression of SLC5A8 and GPR109A in the gut is influenced by bacteria colonization. In the intestines of GF mice, the absence of the microbiota and consequently the absence of SCFAs leads to marked suppression of SLC5A8 and GPR109A expression [79]. In contrast, colonization of GF mice leads to expression of these genes to levels comparable to those of normal conventional mice [80]. Thus, lack of expression of these genes in GF mice could render them more susceptible to developing experimental colitis and Crohn's disease. Reduction in the intracellular availability of butyrate in colonocytes may decrease its protective effects in IBD patients. Butyrate, through a different mechanism, has also been shown to be protective against colonic inflammation and colon cancer [30]. Gpr109A, activated by butyrate, suppresses intestinal inflammation by (1) induction of IL-18 secretion in colonic

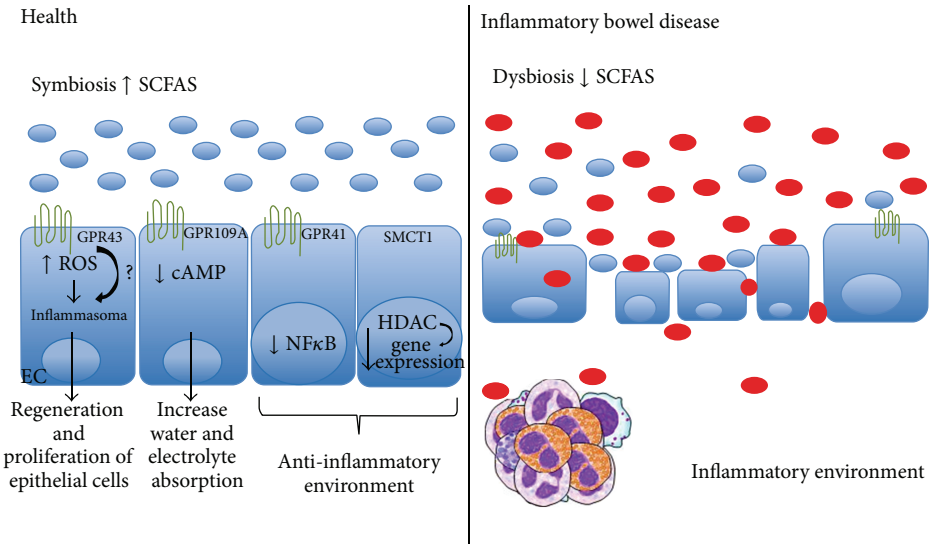

FIGURE 2: Schematic representation of a complex mucosal immune system composed of epithelial and hematopoietic cells that are able to react to pathogenic insults. Development of IBD occurs mainly when epithelial cells are damaged and/or the intestinal microbiota composition is not healthy.

epithelium, consequently inducing epithelium homeostasis, and (2) promoting an anti-inflammatory response in colonic macrophages and DCs that induce differentiation of Tregs. CD4+Foxp3+ T regulatory cells are indispensable for maintaining immune tolerance and are also an emerging therapeutic target for IBD. Recent studies have demonstrated that the metabolic products of certain bacterial strains in the intestines attenuated disease in animal models of colitis by inducing Treg proliferation [81–83]. These bacteria also promoted their peripherical generation by inducing T cell differentiation to Tregs through the generation of a TGF-β-rich environment [84].

The effects of SCFA may also result from its binding to GPR41 and GPR43. Indeed, GPR41-deficient mice have a higher susceptibility to experimental colitis, and this phenotype is associated with greater activation of NF-κB (Nuclear Factor kappa B). Activation of NF-κB induces expression of genes responsible for the production of proinflammatory cytokines such as TNF and IL-8 that contribute to the pathogenesis of IBD [85]. However, butyrate displays an anti-inflammatory effect by decreasing expression of proinflammatory cytokines via inhibition of NF-κB activation [86, 87]. A marked anti-inflammatory effect was observed by acetate as well. The effects of acetate have been demonstrated by Maslowski et al. to be due, in part, by the activation of GPR43 [22]. GPR43-deficient mice exhibit aggravated inflammation related to exacerbated production of inflammatory mediators and increased immune cell activation. Nevertheless, treatment with acetate promotes resolution of intestinal inflammation by GPR43 activation, thereby inducing apoptosis of inflammatory cells in colitis [22]. Acetate treatment has also been shown to reduce colonic inflammation in animal models by promoting Treg differentiation [88].

A recent study highlighted the important role of acetate production in preventing intestinal infection by its effect on the maintenance of gut epithelial barrier function [66]. Intriguingly, acetate may affect the production of reactive oxygen species (ROS) [22]. The production of ROS is involved in a wide spectrum of biochemical processes. The ability of ROS to activate an intracellular protein complex called the inflammasome is of crucial importance in IBD [54, 78, 79].

The role of the inflammasome in modulating the innate immune response in IBD is intimately related to the preservation of epithelial barrier integrity and the maintenance of gut homeostasis [50, 75]. Inflammasome complexes affect the innate immune response through activation by pathogen recognition NLRs [76]. NLRP6 and NLPR3 are key mediators of inflammasome complexes. NLRs activate caspase-1 and drive proteolytic processing of proinflammatory cytokines such as IL-1 and IL-18. These cytokines have evolved in intestinal epithelial cells to avoid overactive inflammatory responses against the host microbiota. Consequently, epithelial barrier integrity induces tissue repair following injury [65, 89, 90]. Several groups, using a common acute and chronic epithelial injury colitis mouse model based on the administration of DSS, reported an exacerbated disease severity in mice deficient in caspase-1, NLRP3, and NLPR6. These NLPRs are correlated with lower IL-1β and IL-18 production during colitis [89–92]. Interestingly, NLPR6-deficient mice have an altered gut microbiota (colitogenic bacteria), which together with the exacerbated colitis phenotype can be transferred to cohabitating WT mice. Therefore, NLRP6 participates in the steady-state regulation of the commensal microbiota and appears to be essential for preventing recurring colitis through the induction of basal secretion of IL-18 by epithelial cells [65]. Therefore, the inflammasome functions in the sensing of pathogens and the commensal microbiota by not only nonhematopoietic cells, such as the epithelial intestinal cells but also by hematopoietic cells [93]. Distinct inflammasome expression in different cell lineages

may orchestrate different functions during mucosal inflammation. They cooperate to maintain host tolerance towards commensal microbes and to initiate a potent immune response towards pathogens in the gut [94]. Nevertheless, the factors inducing the formation of inflammasomes and the precise effector mechanisms for regulation of the microbiota and inflammatory response remain elusive. We do not yet know whether SCFAs or GPCRs influence inflammasome activation. However, the induction of ROS by SCFAs could be a new mechanism by which microbial components trigger inflammasome formation. Nevertheless, the inflammasome regulates innate immune responses by sensing endogenous and exogenous stimuli. Considering that the inflammasome induces essential inflammatory responses in IBD, the sensing of the microbiota by the inflammasome through the action of SCFAs could be a new protective mechanism associated with microbiota metabolites. Furthermore, microbiota metabolites can be considered analogous to microbe-associated molecular patterns (MAMPs), which signal through GPCRs to convey information about the microbiota and the host. These receptors provide molecular mechanisms associated with innate immunity that are involved in the recognition of MAMPs as well as the classical innate immune receptors such as TLRs and NLRs.

5. Obesity

Obesity has reached epic proportions, with incidence rates above 20% in most western countries [95]. It is characterized by abnormal or extensive fat accumulation that negatively affects health. Such conditions lead to reduced life expectancy and/or increased health complications such as heart disease, type 2 diabetes, obstructive sleep apnea, certain types of cancer, and osteoarthritis [96]. The development of obesity is a complex process involving primarily a combination of excessive food energy intake, lack of physical activity, and genetic susceptibility. A few cases, however, are caused by genes, endocrine disorders, slow metabolism, medications, or psychiatric illness [97]. The rise in incidence rates of obesity can be attributed to the Western diet [98]. An imbalance in the human gut microbiota has been associated with metabolic diseases including obesity, diabetes, and atherosclerosis [99]. Studies in both animals and humans have found fewer Bacteroidetes and more Firmicutes colonizing the gut [99].

The first evidence of the role of the gut microbiota in adiposity came from GF animal studies. Mice raised in a conventional environment had more total body fat in comparison to those raised under GF conditions. When GF mice were conventionalized, they experienced a dramatic increase in total body fat, and this increase was not associated with differences in food consumption or decreased energy expenditure [100]. The relation between gut microbiota and obesity was also verified in knockout and diet-induced obese mice. In such animal models, obesity was associated with changes in the composition and metabolic function of the microbiome [98]. Further evidence of the influence of the gut microbiota on obesity is provided by brain-gut axis studies. An increased intake of dietary fiber, which is fermented in the colon, has been reported to decrease body weight and glucose control. De Vadder and colleagues [101] have shown that SCFAs activate intestinal gluconeogenesis via a cAMP-dependent mechanism and a gut-brain neural circuit involving the fatty acid receptor FFAR3. Frost and colleagues [102] have demonstrated that colonic acetate crosses the blood-brain barrier and is taken up by the brain. SCFAs is also associated with activation of acetyl-CoA carboxylase and changes in the expression profiles of regulatory neuropeptides that favor appetite suppression.

There are four main pathways that interfere with host energy storage. These pathways involve intestinal epithelial cells as sensors of microbial products and are believed to influence how the gut microbiome regulates host gene expression and affects energy expenditure and storage in the host [98, 103] (Figure 3). Colonization of GF mice with gut commensal bacteria alters the global intestinal transcriptional response and the cellular origins of selected responses by modulating the expression of genes involved in several important intestinal functions. These functions include nutrient absorption, mucosal barrier fortification, xenobiotic metabolism, angiogenesis, and postnatal intestinal maturation [103]. Studies using GF and conventionalized mice also revealed that the microbiota promotes the absorption of monosaccharides from the gut lumen, resulting in induction of *de novo* hepatic lipogenesis [104]. Fasting-induced adipocyte factor (FIAF), a circulating lipoprotein lipase inhibitor and member of the angiopoietin-like family of proteins, is selectively suppressed by conventionalism in the intestinal epithelium, liver, and adipose tissue of normal mice. Using GF, conventionalized, normal, and FIAF knockout mice, researchers established that FIAF suppression is essential for the microbiota-induced deposition of triglycerides in adipocytes. Their findings suggest that the gut microbiota is an important environmental factor that affects energy harvest from food and energy storage in the host.

A second pathway that affects host energy storage involves AMP-activated protein kinase (AMPK). AMPK is activated in response to metabolic stresses, and this activation results in an increased intracellular AMP to ATP ratio. Backhed and colleagues [105] reported that in contrast to mice with a gut microbiota, GF mice were protected against developing obesity after consuming a high-fat, sugar-rich Western diet. GF mice persistently remained lean despite a high caloric intake. This phenotype is associated with increased skeletal muscle and liver levels of phosphorylated AMPK, which stimulate fatty acid oxidation in peripheral tissues and lead to decreased glycogen content and increased insulin sensitivity in the liver [97]. These results suggest that the presence of a gut microbiota suppresses skeletal muscle fatty acid oxidation through a metabolic pathway that involves phosphorylation of AMPK. Moreover, GF knockout mice lacking FIAF were not protected from diet-induced obesity. GF FIAF−/−animals exhibited similar levels of phosphorylated AMPK compared to their wild-type littermates, but they had reduced expression of genes encoding for the peroxisomal proliferator-activated receptor coactivator Pgc-1α and enzymes involved in fatty acid oxidation. Based on these studies, GF mice are protected from diet-induced

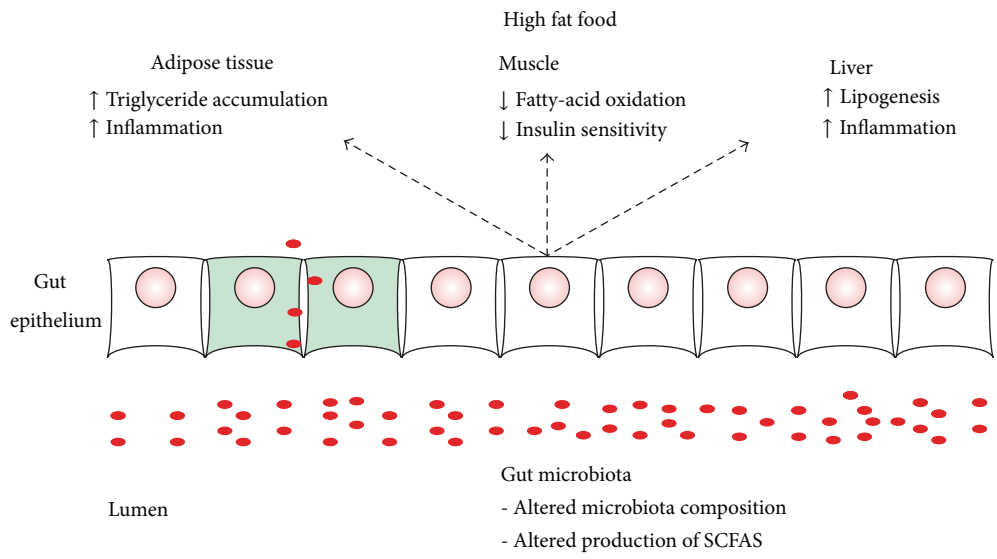

Figure 3: Effects of a high-fat diet. The altered microbial community of obese animals and humans promotes adiposity and decreased levels of short chain fatty acids and influences metabolic processes such as storage and metabolism of lipids in adipose tissue, muscle, and liver.

obesity by two independent but complementary mechanisms that result in increased fatty acid oxidation [98, 100, 105].

The host proteome has a limited number of glycoside hydrolases that are able to break down complex plant polysaccharides. The host microbiota synthesizes a large number of these enzymes, allowing them to break down complex carbohydrates into monosaccharides and SCFA. SCFAs diffuse passively and are recovered via monocarboxylic acid transporters, which also act as signaling molecules and ligands for GPR41, GPR43, and GPR109. SCFAs can be used as lipogenic substrates in host tissues but may promote fat storage via the activation of GPR41 and GPR43 receptors [26, 103, 106, 107]. Moreover, the activation of GPR43 by acetate and propionate contributes to the inhibition of lipolysis and adipocyte differentiation, thereby promoting the expansion of adipose tissue in animals fed a high-fat diet [108]. Because the capacity to ferment carbohydrates to SCFA varies among bacterial species (Bifidobacterium and *Bacteroides* species, e.g., are known to produce SCFAs), the actual composition of an individual's intestinal microbiota may play an important role in energy metabolism.

Finally, the low-grade inflammation and insulin resistance observed in obesity can be triggered by alteration of the gut barrier, leading to the higher plasma lipopolysaccharide (LPS) levels observed in obese individuals. Such conditions create a metabolic endotoxemia and drives obesity, insulin resistance, and systemic inflammation [108, 109].

6. Conclusions

Microbial signaling is required for immune development and homeostasis, whereas an intact immune system is necessary for maintenance of a healthy gut microbiota. Evidence presented herein suggests that some chronic inflammatory diseases are mediated or affected by the dysfunction of the gut microbiota and its metabolic products. Based on these observations, manipulation of intestinal microbiota may prevent or alleviate chronic inflammatory disease. The composition of the microbiota can be manipulated by antibiotics, probiotics, and dietary components. Probiotic consumption for the maintenance of a healthy gut has been practiced for over a century. In 1908, Elie Metchnikoff won the Nobel Prize for his discovery that ingestion of *Lactobacillus*-containing yogurt decreases the number of toxin-producing bacteria in the intestine. Several clinical and animal studies have suggested that probiotics and prebiotics can alleviate many inflammatory diseases such as asthma, obesity, and IBD. Clinical trials have indicated that feeding *L. rhamnosus* GG and *L. fermentum* to mothers during the prenatal and early postnatal periods may be effective in the treatment and prevention of early atopic disease in children. However, probiotics may not have the same positive effect on all subjects or on all chronic inflammatory diseases. One must also consider host dietary habits and probiotic actions such as production of SCFAs and direct DC activation. Additionally, many dietary components directly influence probiotic survival and activity. A high-fiber diet induces a healthy microbiota composition, leading to increased SCFA production, which has anti-inflammatory effects. Further studies are necessary to better understand the mechanisms by which probiotics improve chronic diseases. Additionally, probiotics could be genetically engineered to have desirable anti-inflammatory properties.

Conflict of Interests

The authors have declared that no conflict of interests exists.

Acknowledgments

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Research Article

Association of *Helicobacter pylori* and iNOS Production by Macrophages and Lymphocytes in the Gastric Mucosa in Chronic Gastritis

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Helicobacter pylori is one of the most common causes of chronic gastritis. With the development of the disease cellular inflammatory infiltrates composed of lymphocytes, plasma cells, and macrophages are formed in epithelium and lamina propria of the stomach. These cells are capable of secreting a number of active substances, including inducible nitric oxide synthase (iNOS). We examined the relationship between *H. pylori* and secretion of iNOS by cells of inflammatory infiltrates in chronic gastritis by light microscopy and immunohistochemistry. The data obtained indicate that stimulation of *H. pylori* immune system cells of the host organism during development of chronic gastritis causes increase in number of macrophages and lymphocytes in the inflammatory infiltrate of the gastric mucosa. This is accompanied with increased expression of inducible NO-synthase with excess free radicals in the tissues, which leads to secondary alterations and exacerbates the inflammation with impaired regeneration processes.

1. Introduction

Helicobacter pylori is one of the most common causes of chronic gastritis. The global human population gets infected by *H. pylori* as early as in childhood and adolescence. Chronic *H. pylori*-associated gastritis develops in more than 50% of infected people [1]. *H. pylori* has been proved to be the etiological factor of type B chronic gastritis, gastric and duodenal ulcer, and other gastrointestinal diseases associated with the morphological changes of gastric mucosa and such dysregenerative manifestations as atrophy, metaplasia, and dysplasia underlying neoplastic processes [2].

It is known that inflammatory cellular infiltrate, containing mainly lymphocytes, plasmocytes, and macrophages, is generated in epithelium and lamina propria of the stomach during the development of chronic gastritis, including chronic *H. pylori*-associated gastritis [3]. Lymphocytes, plasmocytes, and macrophages cause the cytokine damage of gastric mucosa with the inducible NO-synthase (iNOS) being a mixed factor [4].

The *H. pylori* antigens can induce iNOS expression by macrophages and lymphocytes of inflammatory cellular infiltrate in chronic gastrointestinal conditions. Urease, *H. pylori* pathogenicity factor, can directly inhibit the phagocytic activity of macrophages according to the literature data [5]. Urease can influence the level of iNOS expression by inflammatory infiltrate cells and the accumulation of nitrogen oxide and thereby regulate the inflammatory process [6–8]. The iNOS expression in chronic *H. pylori*-associated gastritis is also induced by bacterial outer membrane lipopolysaccharides that possess antigen properties and induce host antibacterial response and destructive changes in gastric mucosa [9].

However, contemporary literature lacks the data on the role of lymphocytes and macrophages in oxygen-dependent mechanisms of protection from *H. pylori* infection at the tissue and cellular levels, obtained by gastric mucosa biopsies study. Aforesaid the purpose of the current study was to investigate the *H. pylori*-induced iNOS expression by lymphocytes and macrophages of gastric mucosa in chronic gastritis.

2. Materials and Methods

For this investigation we used paraffin-embedded antrum biopsies from the archive of the clinic of Research Center of Clinical and Experimental Medicine (Novosibirsk, Russia). Tissue samples were obtained at endoscopy with biopsy gastric antral mucosa from patients with a first diagnosed chronic gastritis in 2009–2013. The urease test (Jatrox-H.p.-Test, Germany) was used to detect *H. pylori* in tissue samples indirectly.

Sections of 3-micron thickness were prepared on a rotary microtome HM355S ("Microm", Germany) and stained with hematoxylin and eosin by standard procedure to determine the severity and activity of chronic gastritis; light microscopy standard techniques were used. For *H. pylori* visualization Giemsa stain technique was used. Morphological assessment of biopsies was performed by visual analogue scale in accordance with the "Sydney system" and the classification of chronic gastritis described by Dixon et al. [10] and Aruin et al. [9] with a semiquantitative assessment of the degree of contamination of the gastric mucosa *H. pylori*.

After preliminary histological evaluation two study groups were formed. The first group (62 biopsy specimens) were patients with chronic moderate *H. pylori*-associated gastritis with moderate activity and low degree of bacterial contamination (*H. pylori* +). The average age of patients in this group was 56 years. The second group (56 biopsy specimens) consisted of patients with chronic moderate *H. pylori*-negative gastritis with moderate activity and an average age of 58 years.

Immunohistochemical (IHC) analysis was performed by using indirect streptavidin-peroxidase method with specific primary antibodies against inducible nitric oxide synthase (iNOS, "Spring BioScience") and macrophage marker CD68 ("DBS"). To visualize the antibodies "NovoLink" detection system ("Novocastra") was used. For IHC studies sections were dewaxed and rehydrated. After antigen unmasking in a microwave oven at 700 W power for 20–25 minutes and washing with distilled water, phosphate buffer, endogenous peroxidase was blocked within 5 minutes. Exposure time to the primary antibodies was 30–45 minutes at 37°C. Sections were incubated with streptavidin-peroxidase complex and DAB-substrate and were further counterstained with Mayer's hematoxylin.

Morphometric study of tissue structural elements was conducted using closed test system consisting of 100 points, square $3.6 \times 10^5\ \mu m^2$. There were registered volume density (Vv) of inflammatory infiltrates in the lamina propria and the numerical density (Nai) of lymphocytes, plasmocytes, and CD68+ macrophages and cells expressing iNOS [11]. Statistical analysis of the results was performed using the statistical analysis package Microsoft Office Excel 2007 and standard software package STATISTICA v.6. The arithmetic mean value (M) and standard error of the mean (m) were determined. To identify the probability of significance of differences of compared average values Student's t-test was used. Differences were considered statistically significant at the 5% significance level ($P < 0.05$).

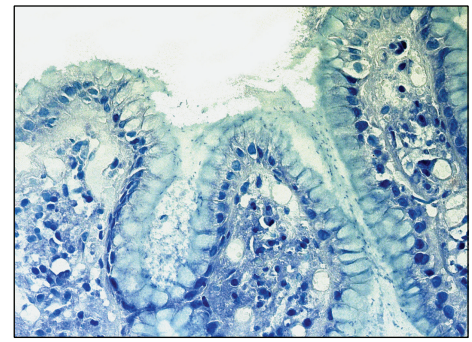

Figure 1: Antrum mucosa in *H. pylori*-associated gastritis: mucus masses with *H. pylori* agglomerations on mucosa surface, Giemsa staining, magnitude ×200.

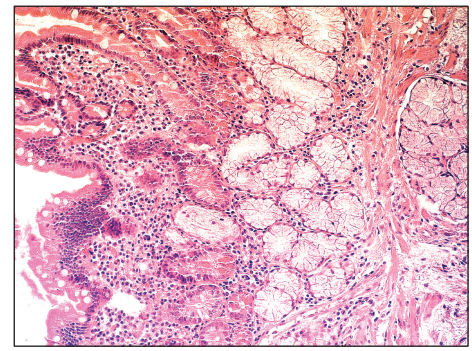

Figure 2: Antrum mucosa in *H. pylori*-associated gastritis: focal enteric metaplasia of epithelium, the lymphocytoplasmocytic infiltration of lamina propria with the admixture of neutrophils, focal fibrosis, hematoxylin and eosin staining, magnitude ×200.

3. Results

Signs of moderate chronic gastritis with moderate activity and low level of *H. pylori* contamination (+) were detected in the first study group using the general light microscopy of antrum biopsy material histological sections (Figure 1). Gastric mucosa represented a mature hypersecretory epithelium with erosions, sites of foveolar hyperplasia, and focal enteric metaplasia of foveolar epithelium. There were a mild edema, focal lymphocytoplasmocytic infiltration with more than 50% proportion of plasmocytes, and the admixture of neutrophils and a focal fibrosis in lamina propria (Figure 2).

Signs of moderate chronic gastritis with moderate activity and no signs of *H. pylori* contamination (–) were detected in the second study group. Gastric mucosa represented a mature epithelium with sites of enteric metaplasia of foveolar epithelium. Moderate lymphocytoplasmocytic infiltration with more than 60% proportion of plasmocytes and the admixture of neutrophils and small fibrosis foci were detected in lamina propria.

The morphometric study of histological sections in both groups has not revealed significant differences between the values of volume density of inflammatory infiltrates in lamina propria (Figure 3). The numerical density of lymphocytes in inflammatory infiltrate of gastric lamina propria

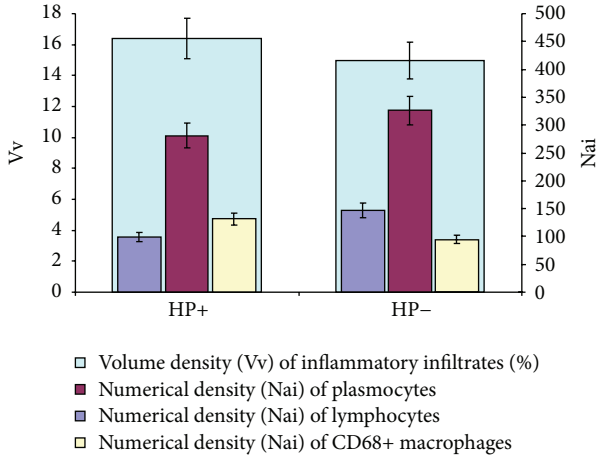

Figure 3: Volume density (Vv) of inflammatory infiltrates and numerical density (Nai) of lymphocytes, plasmocytes, and CD68+ macrophages study results.

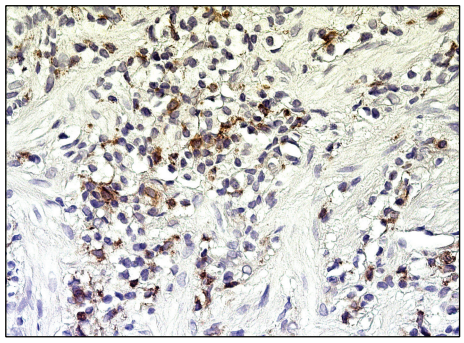

Figure 4: Antrum mucosa in *H. pylori*-associated gastritis: CD68+ macrophages in infiltrate of lamina propria, magnitude ×400.

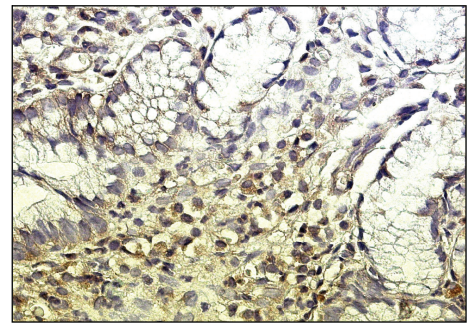

Figure 5: Antrum mucosa in *H. pylori*-associated gastritis: the iNOS expression by macrophages and lymphocytes of inflammatory infiltrate in lamina propria, magnitude ×400.

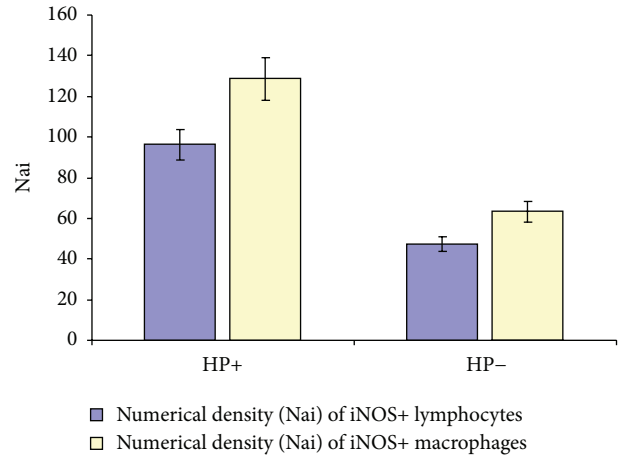

Figure 6: The numerical density (Nai) of lymphocytes and macrophages expressing iNOS.

in the second group was 1.5-fold higher than in the first group (Figure 3). Large number of CD68+ macrophages was detected in gastric mucosal biopsy material in the first study group. The numerical density in the first group was 1.4-fold higher than in the second group (Figures 3 and 4).

Numerical densities of iNOS+ lymphocytes and iNOS+ macrophages in the first study group (Figure 5) were 2-fold higher than those in the second study group (Figure 6). A 1.3-fold higher numerical density of iNOS+ macrophages in comparison with iNOS+ lymphocytes was noted in both groups. Thus the number of iNOS+ cells was significantly higher in antrum mucosa in case of chronic *H. pylori*-associated gastritis with low level of bacterial contamination than in case of chronic *H. pylori*-negative gastritis (Figure 6).

4. Discussion

Currently cytokines, *H. pylori* antigens, and its pathogenicity gene cluster are considered among the pathogenicity factors of *H. pylori*. Their activation launches a number of pathogenic mechanisms of gastric mucosal inflammation associated with destruction on molecular, cellular, and tissue level and with dysregenerative manifestations [12].

The results of this study suggest that the volume density of the inflammatory infiltrates in groups 1 and 2 did not have significant differences. However, the presence of *H. pylori* in the gastric mucosa had a significant effect on the cellular composition of infiltrates that exhibits a decrease in the number of lymphocytes and an increased number of macrophages in group 1 compared to group 2.

An activation of nuclear transcription factor NF-κB in epithelial cells and neutrophils of gastric mucosa during their interaction with CagA protein of *H. pylori* outer membrane is a key moment of inflammation initiation that results in the release of many proinflammatory cytokines [13]. Literature data suggest that during the chronization of inflammation these cytokines support the chemotaxis and chemokinesis of leucocytes and macrophages with an increase of their numbers in inflammation area [3, 14, 15].

It is known that the migration of leucocytes and macrophages to inflammation area is associated with generation of active oxygen forms and cell destruction with the release of cytotoxic enzymes determining the destructive changes in gastric mucosa [9]. Inflammatory process in

gastrointestinal tissues is also associated with an increase of secretory activity of lymphocytes and macrophages. Proinflammatory cytokines production can be accompanied with the iNOS expression by inflammatory infiltrate cells [16–18].

The number of lymphocytes and macrophages expressing iNOS in antrum mucosa was calculated to evaluate the inducible NO-synthase expression at the tissue and cellular levels. The data obtained showed that contamination of the gastric mucosa by *H. pylori* leads to activation of effector cells of the immune system of the host organism manifesting twofold increased number of macrophages and lymphocytes expressing an inducible form of NO-synthase.

The inducible NO-synthase is associated with the production of NO that is the factor of oxygen-dependent system of antiviral and antibacterial protection [19]. However, the overaccumulation of reactive oxygen metabolites in tissues causes the toxic effect on tissue cells, severe destructive changes, and dysregenerative disorders. This is consistent with more significant destructive changes of gastric mucosa, erosions, and such regeneration disorder as a focal enteric metaplasia detected in histological study of *H. pylori*-associated gastritis.

5. Conclusion

The data obtained as a result of histological examination of the gastric mucosa at the tissue and cellular levels indicate that *H. pylori* stimulation of immune cells of the host organism during development of chronic gastritis causes increase in number of macrophages and lymphocytes in the inflammatory infiltration of gastric mucosa with the activation of their functional activity, including oxygen-dependent mechanisms of immune response. It is associated with an increased macrophage and lymphocyte expression of inducible NO-synthase and the overaccumulation of free radicals in tissues leading to the secondary alteration, irrespective of etiological factor presence and intensity, and promotes persistence and aggravation of inflammatory process with dysregenerative manifestations.

Conflict of Interests

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

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Research Article

Appropriate Development of the Liver Treg Compartment Is Modulated by the Microbiota and Requires TGF-β and MyD88

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Neither the early postnatal development of the liver Treg compartment nor the factors that regulate its development has been characterized. We compared the early developmental patterns of Treg cell accumulation in murine liver, thymus, and spleen. A FoxP3^{EGFP} reporter mouse was employed to identify Treg cells. Mononuclear cells were isolated from organs postnatally, stained for CD4, and examined by flow cytometry to enumerate FoxP3^{+}CD4^{hi} cells. To assess roles for TGF-β1, MyD88, and TLR2, gene-specific knockout pups were generated from heterozygous breeders. To test the role of commensal bacteria, pregnant dams were administered antibiotics during gestation and after parturition. The pattern of appearance of Treg cells differed in liver, spleen, and thymus. Notably, at 1-2 weeks, the frequency of CD4^{hi} FoxP3^{+} T cells in liver exceeded that in spleen by 1.5- to 2-fold. The relative increase in liver Treg frequency was transient and was dependent upon TGF-β1 and MyD88, but not TLR2, and was abrogated by antibiotic treatment. A relative increase in liver Treg frequency occurs approximately 1-2 weeks after parturition that appears to be driven by colonization of the intestine with commensal bacteria and is mediated by a pathway that requires TGF-β1 and MyD88, but not TLR2.

1. Introduction

The immune system is tightly regulated, maintaining, in normal physiological conditions, a balance between immunity to challenge by pathogens and tolerance in order to suppress inappropriate immune responses. Regulatory T cells (Treg) have been recognized to play a major role in immune homeostasis by maintaining self-tolerance and preventing autoimmunity. Expression of the transcription factor forkhead box P3 (FoxP3) specifies the Treg lineage and confers suppressive function, through activation of a transcriptional program required for regulation [1–3]. The cytokine transforming growth factor beta1 (TGF-β1) plays a critical role in Treg development and function [4].

The liver can be considered an immune-privileged site [5] similar to other immune-privileged organs such as the eye and the gonads. The tolerogenic status of the liver is necessary because the liver receives blood not only from the systemic circulation via the hepatic artery, but also from the gastrointestinal (GI) tract via the portal vein. Portal vein flow results in a high concentration of non-pathogen-associated antigens reaching the liver, such as food antigens and bacterial breakdown products from commensal organisms residing in the gut. For example, lipopolysaccharide (LPS) is present in the portal venous blood at a concentration of about 1 ng/mL [6], a concentration which, if present in the systemic circulation, would lead to septic shock. To handle this immense load of bacteria-derived substances, the liver maintains a state of local immune tolerance, using a variety of mechanisms [5, 7]. The liver Treg compartment is one important component of the network of cells that mediates liver tolerance [8, 9].

Toll like receptors (TLRs) are pathogen recognition receptors that recognize conserved molecules found across diverse bacterial species; for example, TLR2 recognizes lipoteichoic acid present in most Gram-positive bacteria,

whereas TLR4 recognizes LPS, present in most Gram-negative bacteria [10, 11]. TLRs play critical roles in linking the innate and adaptive branches of the immune system. TLR ligands induce dendritic cell (DC) maturation from an immature phenotype and upregulate MHC class II and costimulatory molecules, necessary for proper activation of T cells by DCs [12]. Myeloid differentiation primary response 88 (MyD88) is a universal cytosolic adaptor protein that is downstream of all bacterial-responsive TLRs.

While published studies have looked at the development of Tregs in the neonatal thymus [13], and spleen [14], the development of Tregs in the neonatal liver has not been previously studied. Here, we assess the development of the Treg compartment in the postnatal liver and examine the contributions of TGF-β1 and MyD88 to liver Treg development.

2. Materials and Methods

2.1. Mice. FoxP3^{EGFP} mice on the BALB/c background and TLR2 knockout mice were obtained from the Jackson Laboratory (Bar Harbor, ME). FoxP3^{EGFP}.*Tgfb1*$^{-/-}$ knockout mice on the BALB/c background were generated by breeding FoxP3^{EGFP} with *Tgfb1*$^{+/-}$ heterozygous mice [15]. FoxP3^{EGFP} mice harbor a bicistronic *FoxP3* locus that coexpresses eGFP and FoxP3 [16]. The use of these reporter mice ensures that native FoxP3 protein remains unmodified, as it has been shown recently that a commonly used reporter mouse expressing a FoxP3^{gfp} fusion protein is a hypomorph with an abnormal Treg phenotype [17]. MyD88 knockout mice [18] were a kind gift from Dr. Brent Berwin at the Geisel School of Medicine. Timed pregnant C57BL/6 mice were obtained from the National Cancer Institute (NCI). Mice were bred at the Geisel School of Medicine according to Association for Assessment and Accreditation of Laboratory Animal care practices. FoxP3^{EGFP} and TLR2 knockout mice were genotyped by PCR as per the vendors' protocol. TGF-β1 knockout mice and MyD88 knockout mice were genotyped as previously described [19, 20], whereas Leadbetter et al. [20] indicate that PCR products expected from *Myd88*$^{+/+}$ mice and *Myd88*$^{-/-}$ mice are approximately 550 and 750 base pair (bp), respectively; in our hands these PCR products were approximately 600 and 300 bp, respectively. The mouse strain(s) (C57Bl/6 or BALB/c) used in each figure is indicated in the figure legends.

2.2. Isolation of Mononuclear Cells from Organs. Cardiac perfusion was carried out before removal and weighing of organs. Liver tissue was dissociated by chopping using a razor blade or by use of a tissue dissociator as per manufacturer's protocol (gentleMacs dissociator, Miltenyi Biotec) and subjected to treatment with 5 μg DNAse and 500 Units of Collagenase (Sigma). A cell suspension of liver nonparenchymal cells (NPC) was obtained by filtering and removal of hepatocytes, followed by red blood cell (RBC) lysis. For spleen and thymus, cell suspensions were prepared by grinding between frosted glass slides, followed by filtering and RBC lysis.

2.3. Flow Cytometry. Prior to staining with specific antibody, nonspecific binding was blocked using Fc block (anti-mouse CD16/CD32, clone 93, eBioscience). Antibodies used for staining were anti-CD4 (clone GK1.5, eBioscience), anti-CD3 (clone 145-2C11, BD Pharmingen), and anti-FoxP3 (clone FJK-16s, eBioscience). Intracellular staining for FoxP3 was carried out using a kit according to the manufacturer's protocol (eBioscience). Cell staining was acquired on either FACS Calibur or BD Accuri C6. Flow data analysis was carried out using either FlowJo version 7.6.5 (TreeStar) software or BD Accuri C6 software (Version 1.0.202.1).

2.4. Antibiotic Treatment. Timed pregnant mice were treated beginning at about two weeks of gestation with the following antibiotics (in the drinking water): ampicillin (1 g/L), neomycin sulfate (1 g/L), metronidazole (1 g/L), and vancomycin (0.5 g/L). Sucrose (10 g/L) was also added to the water. Antibiotic treatment in the drinking water was maintained until pups were 8-9 days old. Antibiotic water was changed twice weekly.

2.5. Statistics. Significance was determined using either the nonparametric Mann Whitney *U*-test for two-group comparisons or a two-way ANOVA for kinetic data. Statistical analyses were carried out using GraphPad Prism software (Version 6.0). Significance is denoted as follows: ns not significant ($P > 0.05$), $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, $^{****}P \leq 0.0001$, or as indicated in the figures. Error bars indicate mean ± standard deviation. Box and whisker plots show 5th to 95th percentile.

3. Results

3.1. Postnatal Development of FoxP3^{+}CD4^{hi} T Cells in Thymus, Spleen, and Liver. We employed a reporter mouse that provides convenience in the detection of FoxP3 expressing cells [16]. In FoxP3^{EGFP} transgenic reporter mice, FoxP3 expressing cells are identified as eGFP^{+} and are readily detected on flow cytometry without needing additional manipulations, such as intracellular staining. It has been shown recently that transgenic mice in which the eGFP reporter is expressed as a fusion protein with FoxP3 have subtle immunologic abnormalities, owing to unexpected effects of the eGFP fusion partner on FoxP3 functionality [17]. For this reason, we used reporter mice in which the FoxP3 protein is expressed from a bicistronic reporter construct that coexpresses FoxP3 and eGFP as separate proteins [16]. We obtained evidence that the expression of GFP is a reliable marker for FoxP3 expression. Using cell sorting to isolate GFP^{+} and GFP^{-} populations, we analyzed sorted populations by intracellular staining for the nuclear protein FoxP3 (Figure 1). The GFP^{-} population did not show expression of FoxP3, whereas greater than 95% of the GFP^{+} population was positive for the expression of FoxP3.

Next, we assessed the development of FoxP3^{+}CD4^{hi} T cells (regulatory T cells (Tregs)) in early postnatal thymus, liver, and spleen of FoxP3^{EGFP} reporter mice. We assessed both the frequency of Tregs, defined as the percentage of Tregs (GFP^{+} or FoxP3^{+}) among CD4^{hi} T cells, and the density of Tregs in each organ, defined as Treg cells/mg.

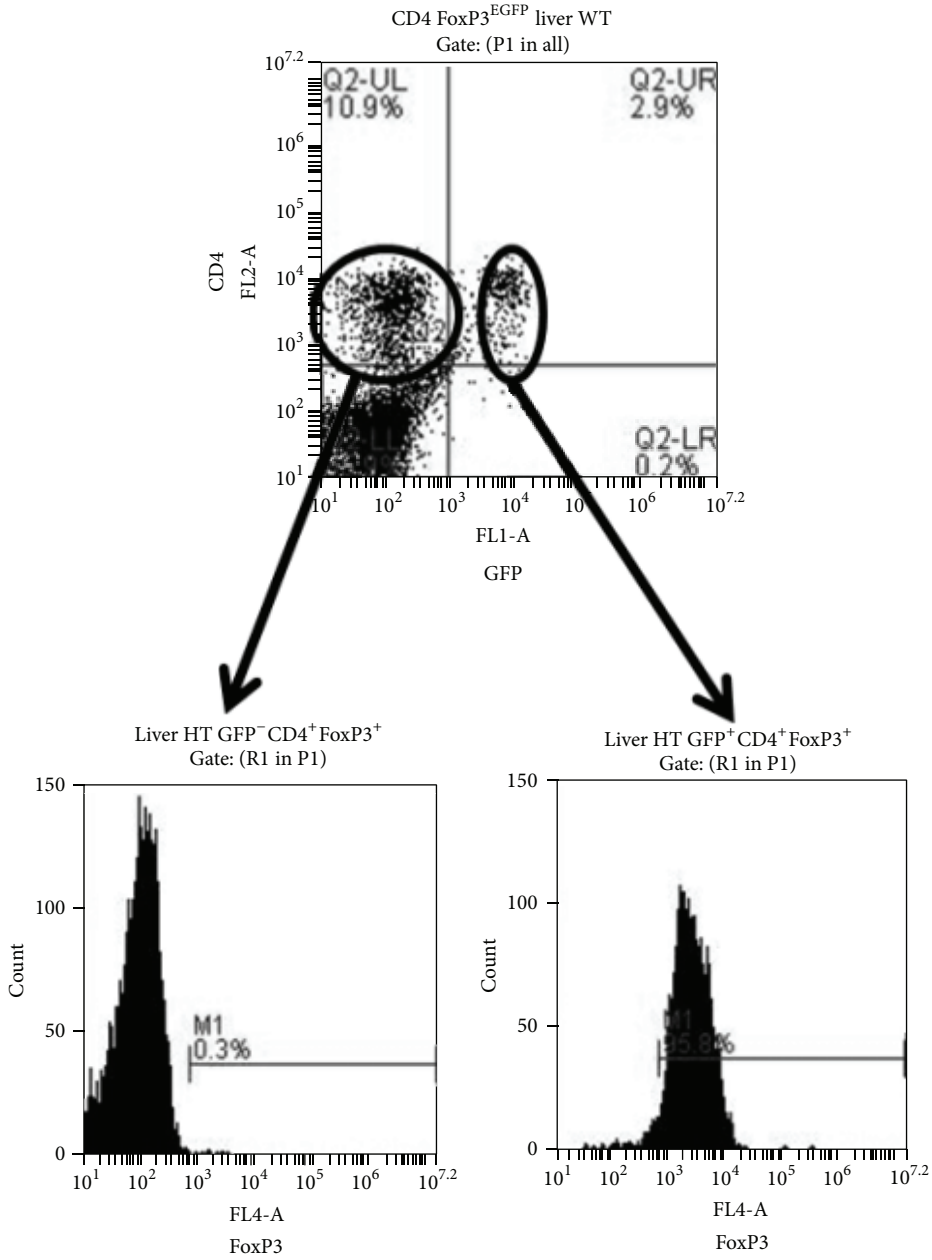

Figure 1: GFP expression is a reliable marker of FoxP3 expression in liver $CD4^+$ cells from $FoxP3^{EGFP}$ transgenic reporter mice. NPC were isolated from liver of adult BALB/c-background $FoxP3^{EGFP}$ mice and $CD4^+$ cells were sorted by GFP expression. Sorted cell populations were then analyzed by intracellular staining for FoxP3 expression.

Previous studies show that Tregs are detectable in thymus at postnatal days 3-4 [13, 21]. Consistent with this, about 3% of $CD4^{hi}$ cells in $FoxP3^{EGFP}$ mouse thymus were GFP^+ at postnatal days 3-4 (Figures 2(a) and 2(b)). Over the next two to three weeks, the frequency of GFP^+ cells among $CD4^{hi}$ cells progressively decreased to ~1% by weaning age (day 20-21). The density of Tregs in thymus remained relatively constant throughout the neonatal period (range 1,200 to 4,000 cells/mg; Figure 2(c)). In this analysis, we did not specifically stain for CD8 and therefore cannot formally determine whether some of these cells represent CD4CD8 double positive (DP) cells. However, this percentage is likely to be quite small, as it has been shown in several previous studies that, in the postnatal period through adulthood, greater than 95% of $CD4^+FoxP3^+$ thymocytes are $CD4^{hi}CD8^{neg}$ single positive cells and less than 5% of $CD4^+FoxP3^+$ thymocytes are DP cells [13, 22].

In spleen, the frequency of Tregs was ~6% at days 3-4 and quickly increased, reaching a steady state of ~10–12% at days 6–8, a frequency maintained at 11-12 days and 20-21 days (Figures 2(a) and 2(b)). The density of Tregs in the spleen was 500 cells/mg at days 3-4 and progressively increased over

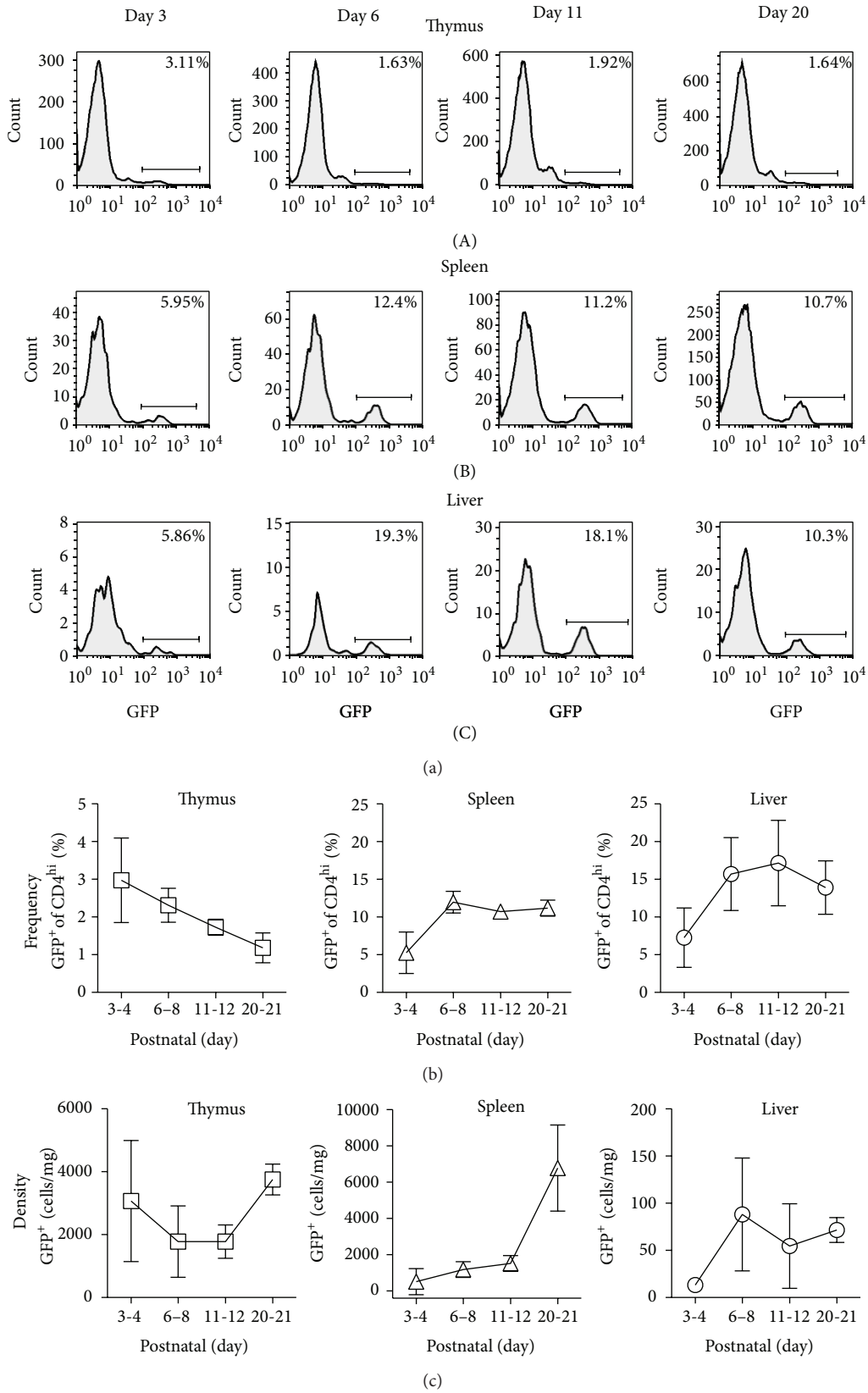

Figure 2: Postnatal development of FoxP3$^+$CD4$^+$ cells in thymus, spleen, and liver. NPC were isolated from thymus, spleen, and liver of BALB/c-background FoxP3^{EGFP} transgenic reporter mice of the indicated ages. (a) Individual GFP expression profiles of CD4$^+$ T cells are shown. (b) Composite data from several mice are shown. Frequency indicates the percentage of CD4$^+$ T cells that coexpress eGFP as a reporter of FoxP3 expression. $N = 3$ to 8 mice per time point. (c) Composite data from several mice are shown. Density indicates the number of CD4$^+$eGFP$^+$ cells per wet weight of the organ. $N = 3$ to 8 mice per time point.

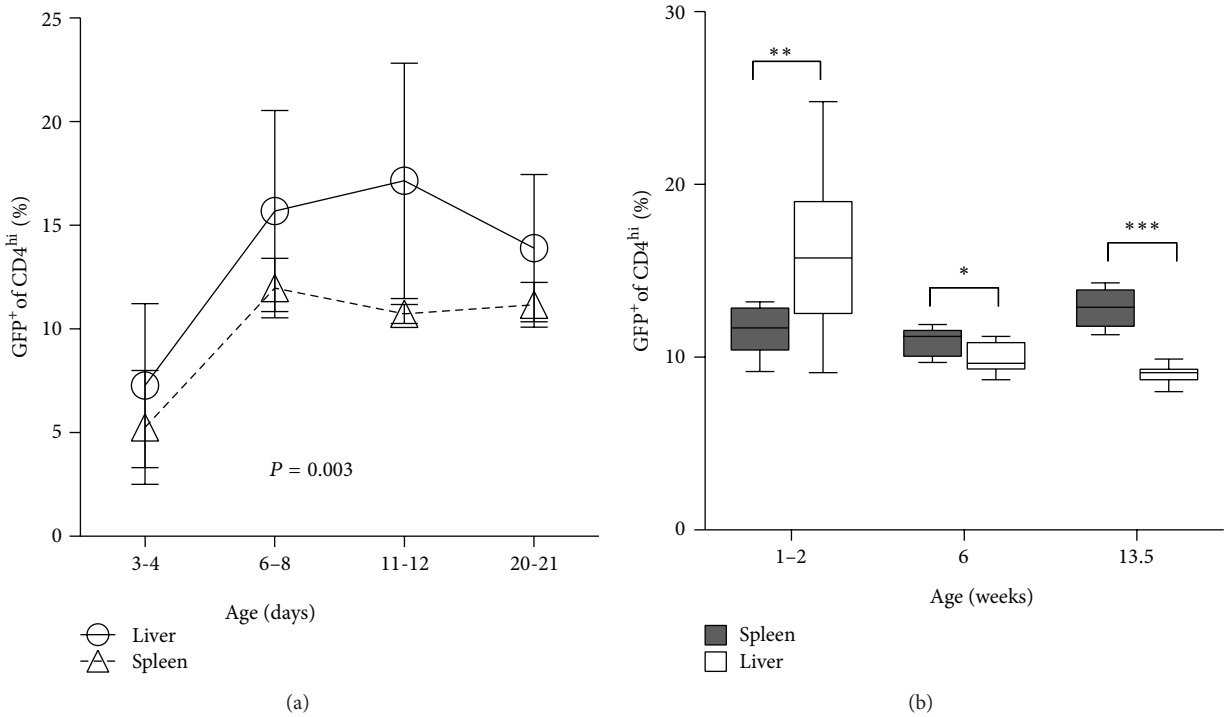

FIGURE 3: The increased frequency of FoxP3$^+$CD4$^+$ cells in postnatal liver reverses in the adult. (a) Liver and spleen FoxP3$^+$CD4$^+$ frequency data from BALB/c-background FoxP3^{EGFP} mice prior to weaning are displayed. Statistical analysis was by 2-way ANOVA. (b) FoxP3$^+$CD4$^+$ frequency data from preweaned mice (age 1-2 weeks) and adult mice (age 6 weeks; age 13.5 weeks) are shown. For 1-2 weeks, data for 6- to 8-day-old mice were combined with data from 11- to 12-day-old mice. $N = 7$ to 12 mice per time point. Statistical analyses used the nonparametric Mann-Whitney test.

the next several weeks. By weaning age, splenic Treg density increased significantly, to ~6,500 cells/mg (Figure 2(c)).

In liver, Tregs were detectable as early as days 3-4, with a frequency (~6%) comparable to that of spleen at similar age. At days 6–8 and 11-12, approximately 15–17% of CD4^{hi} T cells were Tregs. At days 20-21, the frequency was lower (13%; Figures 2(a) and 2(b)). At all ages tested, the density of Tregs in liver was lower than in either spleen or thymus (Figure 2(c)), consistent with the lower numbers of immune cells in this nonlymphoid organ.

A direct comparison of Treg frequency in the two organs shows that the spleen and liver were comparable at days 3-4 and 20-21. However, in between these two time points, the liver exhibited a significantly greater (~1.5-fold) Treg frequency (Figure 3(a)). Statistical analysis using ANOVA revealed that these curves are significantly different ($P = 0.003$). Assessing later time points, at 6 weeks, the relative frequency of Tregs in liver was lower than in spleen, a difference that was even more pronounced at 13.5 weeks (Figure 3(b)). Thus, at 1-2 weeks of age, the liver exhibits a relatively greater Treg frequency as compared with spleen; in the mature adult, the relative frequency is reversed, with liver showing lower Treg frequency among CD4^{hi} T cells.

3.2. TGF-β1 Is Required for the Normal Pattern of Development of FoxP3$^+$CD4^{hi} T Cells in Thymus, Spleen, and Liver. Because TGF-β1 plays an important role in the ontogeny and function of Tregs, we analyzed its contribution to the early development of Tregs in thymus, spleen, and liver. To facilitate this analysis, we crossed BALB/c background FoxP3^{EGFP} mice with BALB/c background TGF-β1 knockout (*Tgfb1*$^{-/-}$) mice [15]. BALB/c *Tgfb1*$^{-/-}$ mice develop histologically and biochemically detectable necroinflammatory disease in liver and other organs beginning at around 10 days of age, caused by an influx of CD4$^+$ T cells [23], and die at 15–17 days of age. Owing to this lethality, we could not measure Tregs at 20-21 days, so our analyses here are restricted to the 3-4, 6–8, and 11-12 day time points only. FoxP3^{EGFP} littermates with one intact *Tgfb1* allele (heterozygous *Tgfb1*$^{+/-}$ mice) were used as controls; *Tgfb1*$^{+/-}$ mice are healthy and phenotypically indistinguishable from wild type (*Tgfb1*$^{+/+}$) mice [19].

In thymus, Treg frequency in control *Tgfb1*$^{+/-}$ mice was ~4% at days 3-4 and diminished moderately over the next week (Figure 4(a)), indicating that the control mice exhibited the expected WT FoxP3^{EGFP} mouse pattern (Figure 2). In *Tgfb1*$^{-/-}$ mice at days 3-4, thymic Treg frequency was lower than in littermate *Tgfb1*$^{+/-}$ mice. The Treg frequency in *Tgfb1*$^{-/-}$ thymus increased dramatically over the next week, reaching 10% at days 11-12 (Figure 4(a)). The density of Tregs also increased in *Tgfb1*$^{-/-}$ thymus over this time period (Figure 4(b)). The dramatic increase in thymic Treg production at days 6–8 and 11-12 is consistent with previous reports showing a similar pattern in mice in which T cells

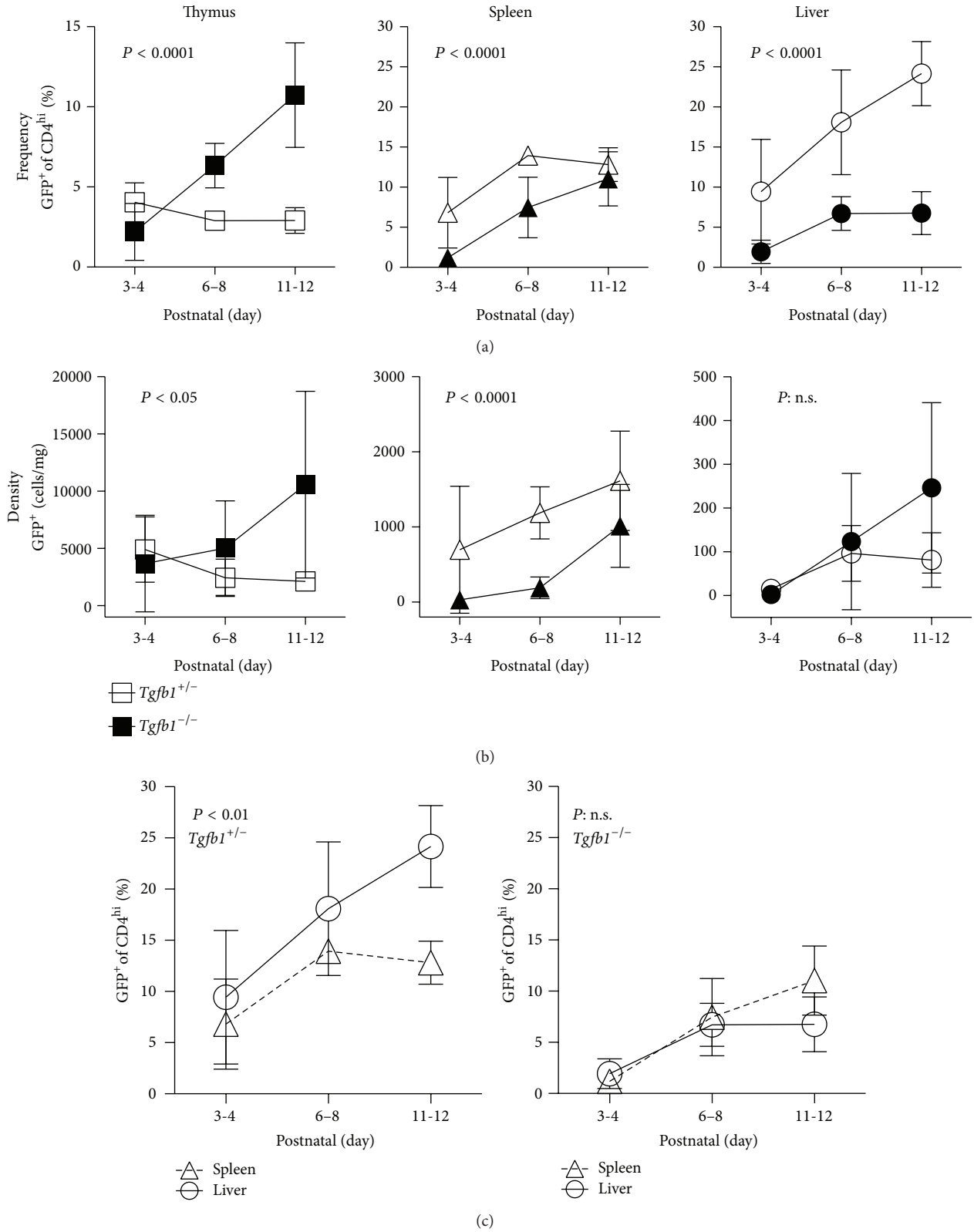

Figure 4: The increased frequency of FoxP3$^+$CD4$^+$ cells in postnatal liver depends on TGF-β1. NPC were isolated from thymus, spleen, and liver of BALB/c-background FoxP3^{EGFP}.*Tgfb1*$^{+/-}$ mice and littermate FoxP3^{EGFP}.*Tgfb1*$^{-/-}$ mice at the indicated ages. (a) Frequency data for FoxP3$^+$CD4$^+$ cells are shown. (b) Density data for FoxP3$^+$CD4$^+$ cells are shown. (c) Data for spleen and liver are shown for *Tgfb1*$^{+/-}$ mice and *Tgfb1*$^{-/-}$ mice. $N = 4$ to 9 mice per group at each time point. Statistical analyses were by 2-way ANOVA.

have been rendered conditionally deficient in one of the key components of the TGF-β receptor [21, 24].

In spleen, Treg frequency in control *Tgfb1*$^{+/-}$ mice exhibited the expected WT FoxP3^{EGFP} mouse pattern; that is, Treg frequency was ~7% at days 3-4 and increased to a steady state of ~12 to 13% over the next week. *Tgfb1*$^{-/-}$ mice exhibited a significant delay in the development of Tregs in the neonatal spleen; at days 3-4 and 6–8, both frequency and density of Tregs in *Tgfb1*$^{-/-}$ spleen were significantly lower compared to *Tgfb1*$^{+/-}$ littermates. By days 11-12, there was no difference in either frequency or density between *Tgfb1*$^{-/-}$ spleens and *Tgfb1*$^{+/-}$ spleens (Figures 4(a) and 4(b)).

In liver, Treg frequency in control *Tgfb1*$^{+/-}$ mice was ~ 10% at days 3-4 and increased to over 20% over the next week, an increase similar to that observed in WT FoxP3^{EGFP} liver. As in spleen, *Tgfb1*$^{-/-}$ liver exhibited a significant delay in the development of Tregs; at days 3-4, the frequency of Tregs in *Tgfb1*$^{-/-}$ liver was significantly lower compared to littermate control *Tgfb1*$^{+/-}$ livers. At days 11-12, the frequency of Tregs among CD4$^+$ T cells in *Tgfb1*$^{-/-}$ liver remained low (Figure 4(a)). This is a different pattern from that observed in *Tgfb1*$^{-/-}$ spleen, where the frequency of Tregs normalized by days 11-12. The low Treg frequency at day 11-12 is likely a function of the massive influx of (non-Treg) effector CD4$^+$ T cells into liver (but not spleen) that occurs just prior to 11 days of age in *Tgfb1*$^{-/-}$ mice [23]. Indeed, at days 11-12, the absolute density of *Tgfb1*$^{-/-}$ liver Tregs surpassed that of littermate control *Tgfb1*$^{+/-}$ liver Tregs, consistent with a large overall T cell influx (Figure 4(b)).

Next, we examined these data to test the hypothesis that TGF-β1 is required for the transient increase in liver Tregs observed at 1-2 weeks of age. We compared the frequency of Tregs in liver with the frequency of Tregs in spleen in *Tgfb1*$^{-/-}$ mice, as well as in littermate control *Tgfb1*$^{+/-}$ mice. At days 11-12, littermate control *Tgfb1*$^{+/-}$ mice had a significantly higher frequency of Tregs in liver as compared with spleen ($P < 0.01$ by ANOVA; Figure 4(c)), similar to what had been observed in WT FoxP3^{EGFP} mice. Importantly, in *Tgfb1*$^{-/-}$ mice at any age, Treg frequency was no different in spleen versus liver (*P*: n.s.). Therefore, the transient increase in liver Treg frequency observed in the postnatal period requires TGF-β1.

3.3. Commensal Bacteria and MyD88 Are Required for the Increase in Liver Treg Frequency in the Early Postnatal Period. We sought to further understand the factors that contribute to the transient increase in frequency of liver Tregs that occurs one week after parturition. We considered that the transient increase might represent a response to postnatal colonization of the murine intestine. Commensal organisms begin to colonize the murine intestinal tract as early as day 1 after birth [25]. The intestinal microbiota is known to play an important role in shaping the mature immune system in the intestine as well as extraintestinally [26]. Based on the anatomic relationship between the intestine and the liver, it is reasonable to conjecture that the gut-liver axis is important for the establishment of the immune system in the liver. We attempted to experimentally manipulate commensal colonization by treating pregnant dams with a cocktail of antibiotics before and after delivery. We obtained data from two litters in which the mothers had been treated successfully with oral antibiotics. Indeed, treatment with oral antibiotics abrogated the transient increase in liver Tregs (Figure 5(a)). In general, however, treatment with antibiotics resulted in unexpected and unacceptable morbidity and poor maternal behavior in mothers (not shown), so we took an orthogonal approach to test our hypothesis.

We hypothesized that the transient postnatal increase in liver Treg frequency is dependent on signals emanating from TLR responses to bacterial products. Since MyD88 is a common adaptor molecule that mediates downstream signaling from all bacterial responsive TLRs, we hypothesized that MyD88 is required for the transient postnatal increase in liver Treg frequency. We tested this hypothesis by examining Treg frequency in mice deficient in MyD88. We interbred heterozygous *Myd88*$^{+/-}$ mice to produce littermate *Myd88*$^{+/+}$ pups, *Myd88*$^{+/-}$ pups, and *Myd88*$^{-/-}$ pups. Importantly, a recent study shows that Myd88 gene status does not affect colonization of the intestine by commensal organisms [27]; therefore all mice from the same litter should become colonized with similar microbiota at similar concentrations, removing a potential artifact in interpretation of data. We analyzed mice at a time point (days 8-9) at which the difference in frequency between spleen Treg and liver Treg is expected to be maximal. Because these mice do not harbor the FoxP3^{EGFP} reporter construct, we directly analyzed intracellular FoxP3 expression on flow cytometry. As expected, wild type *Myd88*$^{+/+}$ pups had a higher frequency of Treg cells in liver than in spleen. Notably, the increase in liver Treg frequency at this age was completely abrogated in *Myd88*$^{-/-}$ pups, which had the same frequency of Treg cells in liver as in spleen (Figure 5(b)). *Myd88*$^{+/-}$ livers also showed a relative increase in liver Treg frequency, but this was more modest than in their wild type *Myd88*$^{+/+}$ littermates, suggesting there is a gene dosage effect in this pathway. Thus, MyD88 is required for the transient increase of Treg frequency seen in murine liver at 1-2 weeks of age, strongly supporting the hypothesis that the increase in Treg frequency in liver is a response to colonization of the gut by commensal organisms.

3.4. TLR2 Is Not Required for the Increase of Postnatal Liver Treg. As MyD88 is required for appropriate neonatal Treg development in liver, we next sought to identify upstream signaling molecule(s) that may be required for detection of gut colonization. TLR2 has been shown to be required for induction and upregulation of Tregs in mice [28, 29]. Moreover, TLR2 deficient mice exhibit a 50% decrease in the frequency of circulating Treg cells, whereas TLR4 deficient mice have normal Treg frequency [30, 31]. We therefore hypothesized that TLR2 is required for the transient increase of Treg frequency in murine liver during early development. We tested this hypothesis by examining Treg frequency in the liver and spleen of TLR2 deficient pups. As expected, *Tlr2*$^{+/+}$ mice had a higher frequency of Treg cells in liver than in spleen (analyzed at days 8-9). *Tlr2*$^{-/-}$ mice also had a

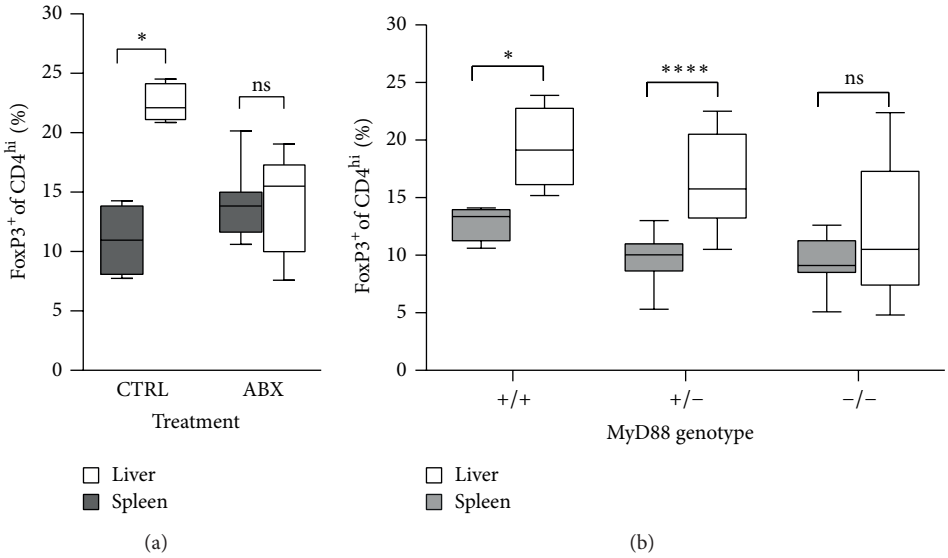

FIGURE 5: The increased frequency of FoxP3$^+$CD4$^+$ cells in postnatal liver is blocked by treatment with antibiotics and depends on MyD88. (a) Pregnant C57Bl/6 females were treated continuously with a cocktail of oral antibiotics from two weeks of gestation until pups were 8-9 days of age, at which time spleen and liver NPC were isolated and FoxP3$^+$CD4$^+$ cells were measured (n = 13 mice). Control mice are untreated C57Bl/6 background pups of the same age (n = 4 mice). Statistical analyses used the nonparametric Mann-Whitney test. (b) Liver and spleen FoxP3$^+$CD4$^+$ cell frequency data are shown for 8- to 9-day-old littermate *Myd88*$^{+/+}$ mice, *Myd88*$^{+/-}$ mice, *and Myd88*$^{-/-}$ mice. N = 8 to 13 mice per time point. *Myd88* mice were on a mixed BALB/c × C57Bl/6 background, but littermates were used, minimizing background effects. Statistical analyses used the nonparametric Mann-Whitney test.

higher frequency of Treg cells in liver than in spleen and were indistinguishable in this regard from their *Tlr2*$^{+/+}$ littermates (Figure 6). Thus, TLR2 is not required for the transient increase in Treg frequency. The involvement of MyD88 in this response may be downstream of a different TLR, or perhaps a combination of TLRs.

4. Discussion

While previous reports have studied the development of Tregs in the neonatal thymus [13], nothing is known about the development of this important T cell compartment in the neonatal liver. This study reveals that the development of the Treg compartment in the liver starts as early as day 3 after birth and that there is a pattern of a transient increase in the percentage of Tregs in the liver between days 6 and days 12. This pattern of development of postnatal liver Tregs requires TGF-β as well as MyD88. Colonization of the gut by commensal bacteria is seen as early as day 1 after birth [25]. It has been shown previously that the intestinal colonization of germ-free mice leads to the induction, activation, and expansion of mucosal Tregs [32] and the maintenance of immune homeostasis. Since the liver receives approximately 70% of its blood flow via the portal vein from the gut, the gut-liver axis plays a very important role in modulating the immune microenvironment of the liver.

Based on these previous observations and our findings here, we propose a model for liver Treg development. Microbial colonization of the intestine may be detected by

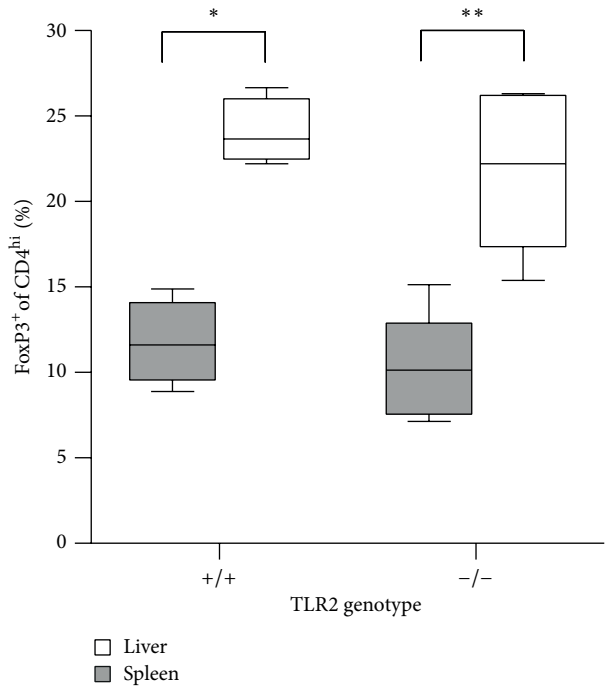

FIGURE 6: The increased frequency of FoxP3$^+$CD4$^+$ cells in postnatal liver is independent of TLR2. Liver and spleen FoxP3$^+$CD4$^+$ cell frequency data are shown for 8- to 9-day-old littermate C57Bl/6-background *Tlr2*$^{+/+}$ mice and *Tlr2*$^{-/-}$ mice. N = 4 to 5 mice per genotype. Statistical analyses used the nonparametric Mann-Whitney test.

intestinal cells by one or more TLR signaling pathways, mediated by the downstream adaptor molecule MyD88. The colonization of the gut also leads to the production of TGF-β1. Expression of TGF-β isoforms and receptors is detectable in rat gastric epithelium both during fetal development and in the neonatal stage [33]. In a model of induction of colonic Tregs with *Clostridium* species, it has been shown that, upon TLR ligation, intestinal epithelial cells increase TGF-β1 production and upregulate expression of matrix metalloproteinases (MMP2, MMP9, and MMP13) that hydrolyze latent TGF-β, converting it to its active form [34]. We propose that TLR/MyD88-mediated response to microbial colonization in the intestine enhances production of TGF-β1, resulting in an increase in Treg percentage in the liver. The observed transient increase in neonatal liver Tregs may result from expansion of Tregs within liver itself, or from an increased influx of Tregs from the intestine; the data presented here neither support nor exclude either possibility. TGF-β1 might act in the intestine to recruit naïve intestinal T cells along the Treg developmental pathway or cause expansion of an existing Treg population, which then traffic to the liver. Alternatively, microbial products resulting from gut colonization reaching the liver via the portal vein might only then be detected by TLRs expressed by resident liver cells. TLR expression in the liver is observed in several different cell populations, including Kupffer cells, hepatocytes, hepatic stellate cells, LSECs, hepatic dendritic cells, and liver NK cells [35, 36]. Interestingly, TLR4 ligation of quiescent HSCs results in increased expression of Bambi, the pseudoreceptor for TGF-β, increasing the sensitivity of HSCs to TGF-β [37].

TGF-β1 is critical for the development and maintenance of Treg cells in the thymus as well as in the periphery [4]. TGF-β1 is critical for the complete development of liver Tregs, since the increase in liver Tregs observed at one week postnatally is abrogated in mice deficient in the gene encoding TGF-β1. It has been shown that TGF-β1 may be acquired from the mother through breast-feeding [38] and pups here are indeed born from TGF-β1-replete mothers (*Tgfb1* heterozygous); moreover, there are two additional TGF-β isoforms expressed in mouse. Clearly, however, it is endogenously produced TGF-β1 that is critical for the Treg increase at one week after birth, and neither maternal TGF-β1 nor endogenous TGF-β2 or TGF-β3 is sufficient to rescue the liver Treg development phenotype.

In addition to TGF-β1, several additional secreted or membrane-bound factors are known to affect Treg development, including IL-2, retinoic acid and B-7 family molecules that signal through CD28 and CTLA-4 [39, 40]. It will be interesting to determine which, if any, of these factors participates in the regulation of liver Treg development and how they may interact with TGF-β1 and TLR/MyD88 to influence the liver Treg compartment.

Commensal bacteria play important roles in shaping the immune system [26, 41]. As previously noted, the presence or absence of an intact TLR/MyD88 response axis does not affect microbiota composition in steady state, and in fact maternal origin and vertical transmission are the important factors that define the structure of the microbiota in colonies of TLR knockout mice and MyD88 knockout mice [27]. In humans, it has been shown that maternal exposure to agriculture increases Tregs in cord blood, which might later affect allergic responses [42]. Our data using antibiotic treatment in wild-type mice, and the use of MyD88 deficient mice, suggest that commensals also contribute to shaping the composition of liver resident immune cells, specifically the Treg compartment. While MyD88 is a common adaptor molecule for all the bacterial responsive TLRs, it is also important in downstream signaling from IL-1R [43], and this pathway cannot be ruled out; however, the results of the antibiotics experiments argue that the relevant role of MyD88 is downstream of one or more TLRs. In our attempts to identify which TLR may be involved, we focused on TLR2 because of its defined role in Treg development [28, 29]. We found that TLR2 alone was dispensable for the Treg development pattern in the liver. The roles of other TLRs remain to be investigated.

Differential composition of the microbiota within the same genetic mouse strain, but obtained from different vendors, has been shown to result in differential development of the immune system [44, 45]. In this study we used mice bred in our animal facility, so we are not able to comment on whether differences in microbiota might differentially affect Treg development in the liver. We also do not know if the pattern of Treg development observed in liver is in response to one or more specific bacterial taxa, or if it represents a response to polymicrobial gut colonization.

It will be important to determine whether the transient increase in neonatal liver Treg frequency contributes to establishing proper liver immune cell function and if this increase in Treg frequency is important for the development of liver tolerance. A recent study using gene expression profiling has shown that exposure to microbiota in the neonatal period is essential for appropriate TLR responses, whose later exposure does not restore appropriately [41]. In this context, the timing of the increased liver Treg frequency may be important for establishing immune tolerance.

It is notable that the frequency of liver Tregs is very high one week after birth but then declines markedly; in the adult, the frequency of liver Tregs is much lower than the frequency of splenic Tregs. Presumably, as the frequency of liver Tregs declines, other tolerogenic pathways begin to have greater effect in maintaining the generally tolerogenic state of the liver. Such pathways likely include, as detailed in a comprehensive review by Crispe [5], expression of adhesion molecules to trap effector T cells in liver sinusoids, an abundance of immunosuppressive cytokines such as IL-10 and TGF-β, the expression of inhibitory T cell checkpoint molecules such as PD-L1, and the induction of apoptosis on T cells mediated by death ligands such as FasL that are expressed on various liver nonparenchymal cells, such as Kupffer cells.

5. Conclusion

In conclusion, our data suggest an important role for intestinal microbial colonization in the development of the liver Treg compartment. This might be important in the establishment of liver tolerance, and interruption or alteration

of this physiologic event might contribute to inflammatory liver disease. In light of these studies, it is worthwhile to consider whether there may be a relationship between the use of antibiotics in the neonatal period and the subsequent development of inflammatory liver disease later in life.

Conflict of Interests

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

Acknowledgments

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