IMMUNOMODULATORY FUNCTIONS OF NUTRITIONAL INGREDIENTS IN HEALTH AND DISEASE

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IMMUNOMODULATORY FUNCTIONS OF NUTRITIONAL INGREDIENTS IN HEALTH AND DISEASE

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Nutritional immunology is a rapidly developing field. An expanding body of evidence demonstrates the impact of foods and nutritional components on gut and systemic immunity of consumers. During recent years, the implications of nutrition and nutritional intervention on prevention of disease have become accepted and has become an important tool in management of several diseases.

Nutritional immunology might become even more important in the prevention of disease when the interplay between nutritional processes and immune system is better understood. Particularly, specific cellular and molecular immune responses provoked by nutrition and the role of the gut barrier and microbiota in the interplay needs more study.

This content of eBook was designed to provide a timely collection on mechanistic, translational and clinical research on the interplay between foods, nutritional components and immunity in physiological and pathophysiological conditions.

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EDITORIAL
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Editorial: Immunomodulatory Functions of Nutritional Ingredients in Health and Disease

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Keywords: nutritional intervention, gut microbiota, probiotics, dietary fibers, immune-related diseases

Editorial on the Research Topic

Immunomodulatory Functions of Nutritional Ingredients in Health and Disease

Nutritional immunology is a rapidly developing field. An expanding body of evidence demonstrate the impact of foods and nutritional components on gut and systemic immunity of consumers. During recent years, the implications of nutrition and nutritional intervention on prevention of disease have become accepted and has become an important tool in management of several diseases.

Nutritional immunology might become even more important in prevention of disease when the interplay between nutritional processes and immune system is better understood. Particularly, specific cellular and molecular immune responses provoked by nutrition and the role of the gut barrier and microbiota in the interplay needs more study.

This content of this Research Topic was designed to provide a timely collection on mechanistic, translational, and clinical research on the interplay between foods, nutritional components, and immunity in physiological and pathophysiological conditions.

We have a series of original or review articles featuring the role of gut microbiota impacted by various factors, specific probiotics in shaping immunity and implications in immune inflammatory diseases.

Fransen et al. focused on aged gut microbiota and health. Advanced age is associated with chronic low-grade inflammation, referred to as inflammaging. The elderly are also known to harbor an altered gut microbiota composition. It was unknown whether this altered gut microbiota was cause or consequence of inflammaging. To this end, Fransen et al. performed microbiota transfer from old mice to germ free young mice and demonstrated that the gut microbiota from old mice contributes to inflammaging after transfer to young GF mice. This knowledge might lead to targeted strategies to juvenile the microbiota in elderly to reduce inflammaging.

Besides aging, gender affects the immune system and gut microbiota composition. Also here cause or consequence was unknown. In this context, the same group demonstrate that gender differences in immunity are already present in GF mice, independent of gut microbiota and that microbiota-independent gender differences in the immune system select a gender-specific gut microbiota composition, which in turn further contributes to gender differences in the immune system (Fransen et al.). This research suggests that modulation of immunity by nutrition might need a gender specific approach.

Accumulating evidence supports an important role of diet and gut microbiota in immune-mediated diseases. Multiple sclerosis (MS) is an autoimmune neurological disease characterized by chronic inflammation of the central nervous system (CNS), leading to demyelination, axonal damage, and symptoms such as fatigue and disability. The infiltration of peripherally activated

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immune cells into the CNS has a key pathogenic role. Preclinical as well as clinical studies suggest a role for gut microbiota and dietary components in MS. The review from van Den Hoogan et al. focused on recent studies on gut microbiota and dietary interventions in MS and prospective animal models in which efficacy of dietary intervention can be tested.

In this Research Topic, we have also several studies focusing on different specific probiotic strains to modulate immune-related conditions. An imbalance in gut microbiota composition can lead to impaired intestinal homeostasis, chronic gut inflammation and a predisposition to developing, for example, colorectal cancer (CRC). The use of probiotic bacteria has emerged as an additive strategy to treat and prevent cancer. Moreover, consumption of beneficial bacteria may favorably modulate the composition of gut microbiota, which has been described in several studies to play an important role in preventing CRC carcinogenesis. In this regard, Jacouton et al. assessed the protective effect of oral treatment with *Lactobacillus casei* BL23, a probiotic strain well-known for its anti-inflammatory and anticancer properties in CRC. Their results demonstrate high potential of *L. casei* BL23 for the development of new, probiotic-based strategies to fight CRC.

Oral bacteria interact with intestinal mucosa and impact immunity. However, mechanistic insights are limited. de Vos et al. conducted a randomized placebo-controlled cross-over trial, to evaluate *Lactobacillus plantarum* supplementation (strain TIFN101, CIP104448, or WCFS1) or placebo in healthy human subjects for 7 days. Their data show that specific bacterial strains can prevent immune stress induced by commonly consumed painkillers such as non-steroidal anti-inflammatory drug (NSAID) and can have enhancing beneficial effects on immunity of consumers by stimulating antigen presentation and memory responses. This study demonstrates that probiotic species can serve as a mean to prevent side-effects of medication.

Indigenous *Clostridium* species have been recently demonstrated to induce colonic regulatory T cells (Tregs), and gut lymphocytes are able to migrate to pancreatic islets in an inflammatory environment. Jia et al. investigated whether supplementation with the well-characterized probiotics *Clostridium butyricum* CGMCC0313.1 (CB0313.1) may induce pancreatic Tregs and consequently dampen the diabetes incidence in non-obese diabetic (NOD) mice. This study provide the basis for future clinical investigations in preventing type 1 diabetes (T1D) by oral CB0313.1 administration.

In another animal model for T1D, in biobreeding diabetes-prone rats, it has been demonstrated that *Lactobacillus johnsonii* N6.2 mitigated the onset of diabetes, in part, through changes in kynurenine:tryptophan (K:T) ratios. As a step toward human application, Marcial et al. performed a pilot double-blind, randomized clinical trial to determine the safety, tolerance, and general immunological response of *L. johnsonii* N6.2 in healthy subjects. Forty-two healthy individuals with no known risk factors for T1D were involved to evaluate subject responses to the consumption of *L. johnsonii* N6.2. The data provide support for the safety and feasibility of using *L. johnsonii* N6.2 in prevention trials in subjects at risk for T1D.

Acute pancreatitis (AP) is a common abdominal inflammatory disorder and one of the leading causes of hospital admission for gastrointestinal disorders. No specific pharmacological or nutritional therapy is available but highly needed. In this Research Topic, Pan et al. provided a comprehensive review on recent advances on nutritional treatment of acute pancreatitis, focusing on enteral vs. parenteral nutrition strategies, and nutritional supplements such as probiotics, glutamine, omega-3 fatty acids, and vitamins in clinical AP. The review gives several leads to successful nutrition and nutritional supplements for clinical management of AP.

In addition, the same group have focused on dietary fibers in preventing or treating AP. Inulin-type fructans (ITFs) are capable of modifying gut immune and barrier homeostasis in a chemistry-dependent manner and hence potentially applicable for managing AP, but their efficacy in AP has not been demonstrated yet. He et al. examined and compared modulatory effects of ITFs with different degrees of fermentability on pancreatic-gut immunity and barrier function during experimentally induced AP in mice. The results demonstrate a clear chain length-dependent effect of inulin to alleviate AP.

Obesity and metabolic syndrome are currently recognized as worldwide epidemics that pose a profound socioeconomic impact and represent a concern to public health. Cells of the immune system contribute to both the maintenance of "lean homeostasis" and metabolic dysregulation observed in obese individuals. Mounting evidence suggest that food additives may also be important contributors to metabolic derangement. The latest review from Paula Neto et al. summarizes latest literature evidence that food additives have relevant effects on cells of the immune system that could contribute to immune-mediated metabolic dysregulation. The reviewed data suggest that some food additives should be avoided considering the adverse effects of these additives to predispose individuals to develop obesity and metabolic syndrome.

In another aspect, sexual dimorphism in immune response is widely recognized, but few human studies have observed this distinction. Food with endo-immunomodulatory potential may reveal novel sex-biased *in vivo* interactions. To this end, Jumat et al. compared immunomodulatory effects of *Carica papaya* compared between healthy male and female individuals. Their data show clear dissimilar immune profiles are elicited in the sexes after papaya consumption and may have sex hormone influence.

Lastly, we have two articles focusing on nutrient effects on health and diseases. Excessive sodium intake is often associated with high risk for cardiovascular disease. More recently, high-salt diets (HSDs) have been demonstrated to activate Th17 cells and increase severity of autoimmune diseases. Aguiar et al. evaluated the effects of a diet supplemented with NaCl in the colonic mucosa at steady state and during inflammation. They found that consumption of HSD *per se* triggered a histologically detectable inflammation in the colon and also exacerbated chemically induced models of colitis in mice by a mechanism dependent on IL-17 production most likely by both ILC3 and Th17 cells.

L-arginine deficiency is shown to be associated with a growing number of diseases in humans, including trauma, certain cancers, and infection. L-arginine supplementation is essential during pregnancy to support fetal development. In conditions of l-arginine depletion, T cell proliferation is impaired. Previously, it has been shown that neonatal blood has lower l-arginine levels than adult blood, which is associated with poor neonatal lymphocyte proliferation, and that l-arginine enhances neonatal lymphocyte proliferation through an interleukin (IL)-2-independent pathway. Yu et al. have further investigated how exogenous l-arginine enhances neonatal Treg function in relation to IL-10 production under epigenetic regulation. Their results suggest that l-arginine modulates neonatal Tregs through the regulation of IL-10 promoter DNA methylation. L-arginine supplementation may correct the Treg function in newborns with l-arginine deficiency.

The scientific contributions collectively show the important role of dietary components in immune homeostasis and the potential of specific food ingredients to prevent disease or manage disease symptoms. Insights in how specific food components might impact gut microbiota, barrier function or immunity-receptors might lead to targeted and rationally designed strategies to avoid immune related diseases. At the same time, the Research Topic shows that some ingredients may have adverse effects and should be avoided in sensitive subjects and that targeted groups such as different genders and age-classes have to be distinguished for optimal efficacy of nutritional interventions.

AUTHOR CONTRIBUTIONS

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

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REVIEW
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Modulation of Multiple Sclerosis and Its Animal Model Experimental Autoimmune Encephalomyelitis by Food and Gut Microbiota

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Multiple sclerosis (MS) is an autoimmune neurological disease characterized by chronic inflammation of the central nervous system (CNS), leading to demyelination, axonal damage, and symptoms such as fatigue and disability. Although the cause of MS is not known, the infiltration of peripherally activated immune cells into the CNS has a key pathogenic role. Accumulating evidence supports an important role of diet and gut microbiota in immune-mediated diseases. Preclinical as well as clinical studies suggest a role for gut microbiota and dietary components in MS. Here, we review these recent studies on gut microbiota and dietary interventions in MS and its animal model experimental autoimmune encephalomyelitis. We also propose directions for future research.

Keywords: prebiotic, probiotic, autoimmunity, immunomodulation, fecal transplant, Food, gut microbiome

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APPROACH

PubMed was used as search engine for this review. Combinations of the following keywords were used: microbiota, microbiome, experimental autoimmune encephalomyelitis, multiple sclerosis, probiotic, prebiotic, synbiotic, fecal microbiota transplantation, and diet (see **Box 1**). In addition, preliminary reports acquired from conference abstracts have been used in the case of multiple sclerosis microbiota studies, to increase the amount of studies that our findings are based on. The studies of which only abstracts were available are noted in **Table 3** as author, abstract. Since this article is not

Abbreviations: ADCC, antibody-dependent cell-mediated cytotoxicity; AHR, aryl hydrocarbon receptor; APC, antigen presenting cells; ATP, adenosine 5′-triphosphate; CDC, complement-dependent cytotoxicity; CFA, complete Freund's adjuvant; CNS, central nervous system; CRISPR, clustered regularly interspaced short palindromic repeats; DHA, docosahexaenoic acid; EAE, experimental autoimmune encephalomyelitis; EDSS, expanded disability status scale; EPA, eicosapentaenoic acid; FDC, follicular dendritic cells; FICZ, 6-formylindolo[3-2b]carbazole; GALT, gut-associated lymphoid tissue; GF, germfree; IBD, inflammatory bowel disease; IEL, intraepithelial lymphocytes; ILC, innate lymphoid cells; iNKT, invariant natural killer T cells; ITE 2-(1′H-indole-3′carbonyl)-thiazole-4-carboxylic acid methyl ester; KCV, kanamycin, colistin, and vancomycin; LCFA, long-chain fatty acids; MAIT, mucosa-associated invariant T; MAM, microbial anti-inflammatory molecule; MBP, myelin basic protein; MOG, myelin oligodendrocyte glycoprotein; MRI, magnetic resonance imaging; MS, multiple sclerosis; ODC, oligodendrocyte; PGN, peptidoglycan; PLP, proteolipid protein; PP, primary progressive; PSA, polysaccharide A; RR, relapsing remitting; SCFA, short-chain fatty acids; SFB, segmented filamentous bacteria; SGK-1, serum/glucocorticoid kinase 1; SP, secondary progressive; SPF, specific pathogen-free; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; TNF, tumor necrosis factor.

BOX 1 | List of definitions used in this article.

Microbiota: "'The population of microbes in a given anatomical niche in the human body (11)."

Microbiome: The collective genome of the microbiota (11).

Probiotic: "A live microbial feed supplement which beneficially affects the host animal by improving its microbial balance (154)."

Prebiotic: "Non-digestable food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already resident in the colon, and thus attempt to improve host health (155)."

Synbiotic: "A mixture of probiotics and prebiotics that beneficially affects the host by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract, by selectively stimulating the growth and/or by activating the metabolism of one or a limited number of health-promoting bacteria, and thus improving host welfare (155)."

a systematic review, the 155 cited articles are a balanced selection out of approximately 200 papers. PubMed was last checked for new articles on July 10, 2017.

INTRODUCTION

Roughly 2.5 million people worldwide are affected by multiple sclerosis (MS), an autoimmune neurological disease of the central nervous system (CNS). Frequently observed symptoms are fatigue, numbness, loss of coordination, vision loss, dizziness, pain, cognitive defects, depression, and bladder and bowel dysfunction (1). MS can lead to serious motoric disability, as approximately 50% of patients require permanent use of a wheelchair 25 years after diagnosis (2). Although the cause of MS is not known, several lines of evidence point to a crucial pathogenic role of the immune system. Genome-wide association studies, neuropathological analyses, and successful therapy trials support the concept that peripheral interactions of environmental risk factors with MS-predisposing genetic factors elicit an autoimmune attack on the CNS causing the formation of lesions. Classically, lesions are defined as usually sharply edged demyelinated areas within the white matter with a variable degree of inflammation, axonal damage, and gliosis. The presence of immune cells (T and B cells, macrophages) and immune molecules (antibodies, complement) supports the characterization of MS as an autoimmune-mediated inflammatory disease.

A growing body of evidence indicates that gut microbiota can modify the incidence and/or course of immune-mediated, extraintestinal diseases (3). Since the gut microbiota are greatly impacted by diet, studies have also started to assess the contribution of gut microbiota and diet in MS and animal models of autoimmune demyelinating diseases such as experimental autoimmune encephalomyelitis (EAE). The aim of this review is to determine whether MS can be treated *via* modification of gut microbiota and dietary components. We describe key players of the immune system that are involved in the pathogenesis of MS, and we discuss how the cross talk of gut microbiota with the immune system can affect the expression of MS/EAE. We then review recent studies on gut emicrobiota and dietary components in the animal model of EAE and MS patients. We also review how gut microbiota can be modulated and propose future research topics.

KEY PLAYERS IN MS IMMUNOPATHOLOGY

Multiple sclerosis presents itself in several forms of symptoms and disease courses. 85% of patients are diagnosed with relapsing remitting (RR) MS, which is characterized by alternating episodes of neurological symptoms (relapses) and recovery (remissions). During relapses, inflammatory active lesions can be detected in the CNS with imaging techniques, such as magnetic resonance imaging (MRI). As the disease progresses, recovery declines and 80% of RR MS patients develop secondary progressive (SP) MS within 10–20 years after initial diagnosis. SP MS does not come with periods of relapses and recovery, but increased disability gradually occurs along with axonal loss and decreased brain volume (atrophy). 10% of MS patients are diagnosed with primary progressive MS, which presents itself as SP MS directly from the start of the disease (2). The cause of MS is unknown, but various concepts have been proposed to explain disease etiology. An inside-out concept suggests that MS starts as a primary lesion in the CNS, for instance caused by an infection or by primary neurodegeneration inducing the release of self-antigens against which autoreactive T and B cells react. An outside-in concept, supported by animal research in EAE models, postulates that autoreactive T cells that have escaped thymic selection are activated by a peripheral infection. This activation can occur through antigens that closely resemble CNS antigens, bystander activation, novel autoantigen presentation, or recognition of sequestered CNS antigens. In both concepts, activated $CD4^+$ T_{h1} and T_{h17} cells infiltrate into the CNS where they can be reactivated by resident antigen-presenting cells (APC), microglia for example. $CD4^+$ T_{h1} and T_{h17} produce IFNγ and IL-17A, and the inflammatory reaction that follows increases the permeability of the blood–brain barrier and recruits other immune cells such as B cells and monocytes to the CNS. The inflammatory milieu also activates microglia, which in turn produce pro-inflammatory mediators which elicit demyelination and axonal loss (4). Naïve $CD4^+$ T cells infiltrating the CNS can broaden the pattern of autoimmune reactions by epitope spreading, adding to the inflammatory milieu (2). $CD8^+$ T cells likely also contribute to MS pathogenesis. They are found in high frequency in demyelinating lesions and correlate with axonal damage. Myelin-specific $CD8^+$ T cells can be activated by epitope spreading, and up to a quarter of $CD8^+$ T cells in active lesions are thought to be mucosa-associated invariant T (MAIT) cells. MAIT cells are able to produce IL-17 and are associated with the gut and liver (5). MS patients have increased serum levels of IL-18 (6), which activates MAIT cells and is inversely correlated with MAIT cell blood frequency (7). When costimulated by IL-18 and T-cell receptor stimulation, MAIT cells upregulate integrin very late antigen-4, which is involved in cell migration into the CNS (7). In addition, MAIT cells are depleted after efficacious autologous hematopoietic stem cell transplantation, a treatment which can be beneficial in MS patients (8). Therefore, MAIT cells are likely

involved in the immunopathogenesis of MS. The relative significance of the different T cell subsets in human MS is not yet completely understood, as EAE is usually induced *via* complete Freund's adjuvant (CFA), and interspecies immunological differences exist. Additionally, a more predominant $CD4^+$ driven disease course is seen in EAE, while a more $CD8^+$ T cell-driven immune response is seen in MS (2).

In addition to T cells, autoreactive B cells may be activated in the periphery. B cells infiltrating the CNS locally produce autoantibodies, which bind myelin and cause damage to myelin *via* complement- and/or macrophage-mediated cytopathic reactions (CDC and ADCC). These B cells are also able to migrate out of the CNS and mature in the lymph nodes before migrating back to the CNS (9). Protective T_{reg} cells and their anti-inflammatory effects could also be defective. Protective cells include $CD4^+$ Foxp3 expressing T_{reg}, IL-10 producing T_{r1}, and $CD39^+$ T_{reg} cells. In MS patients, these cells are found in reduced frequency in the periphery and they have reduced immunosuppressive capacity compared to healthy individuals. Other regulatory cell types could also play a role, as they may increase after treatments. These include the $CD8^+$ T_{reg} and IL-10 producing B_{reg} cells. In addition to defective regulatory cells, effector cells may escape their regulation when they are less sensitive to the suppression by T_{reg} cells (2). A clear imbalance of effector cells and regulatory cells is seen in early MS, which leads to a pro-inflammatory milieu in the CNS and promotes demyelination and axonal damage (see **Figure 1**) (2, 4, 10). In later stages of the disease immune cell migration from the periphery into the CNS subsides, but chronic CNS inflammation and neurodegeneration may continue to take place. This is associated with the formation of tertiary lymphoid-like structures within the CNS and associated meninges and with dysfunction of astrocytes and microglia. Microglial activation can promote dysfunction of astrocytes, after which astrocytes inhibit the maturation of myelin producing oligodendrocyte progenitors, resulting in reduced remyelination. Astrocytes can also produce CCL2 and GM-CSF, which further recruits and activates microglia and creates a self-sustaining feedback loop. Pro-inflammatory mediators such as reactive oxygen species (ROS) produced by astrocytes and microglia are neurotoxic, and the continuous CNS inflammation promotes gradual neurodegeneration (2) (see **Figure 2**).

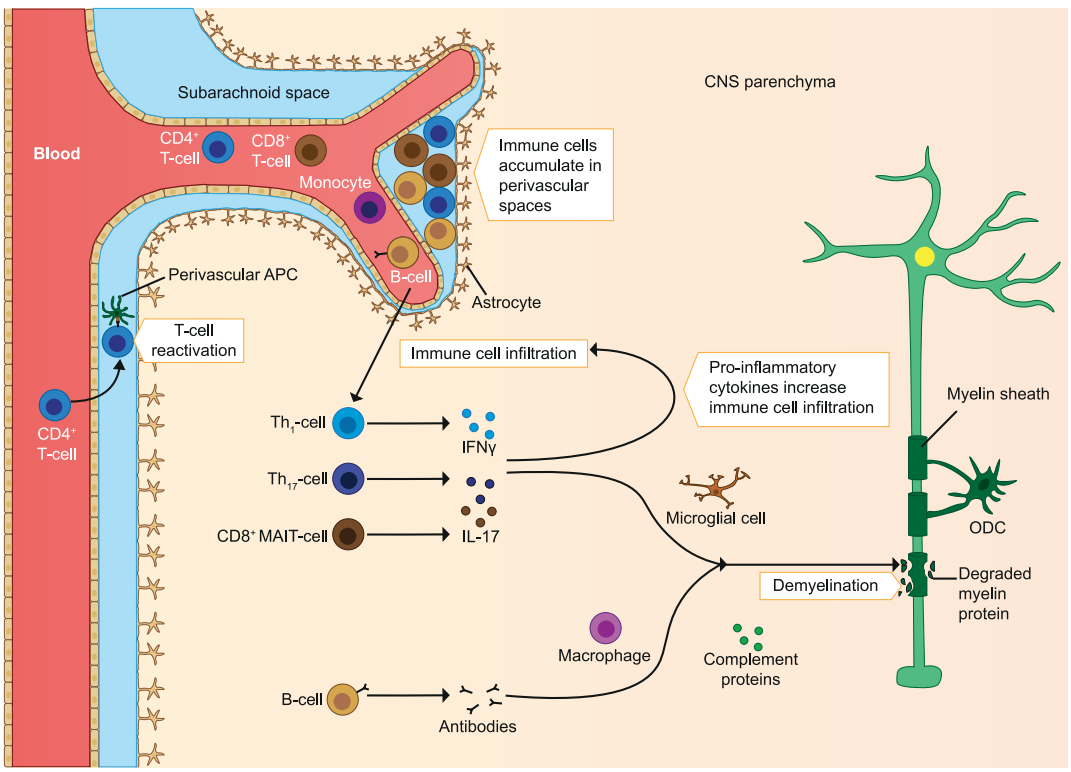

FIGURE 1 | Immune cells involved in the pathology of early MS. Immune cells infiltrate the CNS and are reactivated by APC. The infiltrating T cells produce pro-inflammatory cytokines, which increases immune cell infiltration. The inflammatory milieu also activates microglia, which produce pro-inflammatory mediators and elicit demyelination and axonal loss. Autoantibodies produced by B cells cause damage to myelin through complement-mediated cytotoxicity and macrophage-mediated cytopathic reactions. As the disease progresses, immune cells accumulate in perivascular spaces. ODC, oligodendrocyte; MAIT, mucosa-associated invariant T cells; APC, antigen-presenting cells; CNS, central nervous system; MS, multiple sclerosis. The figure has been inspired by: Fugger et al., Grigoriadis et al., and Goverman (2, 4, 10).

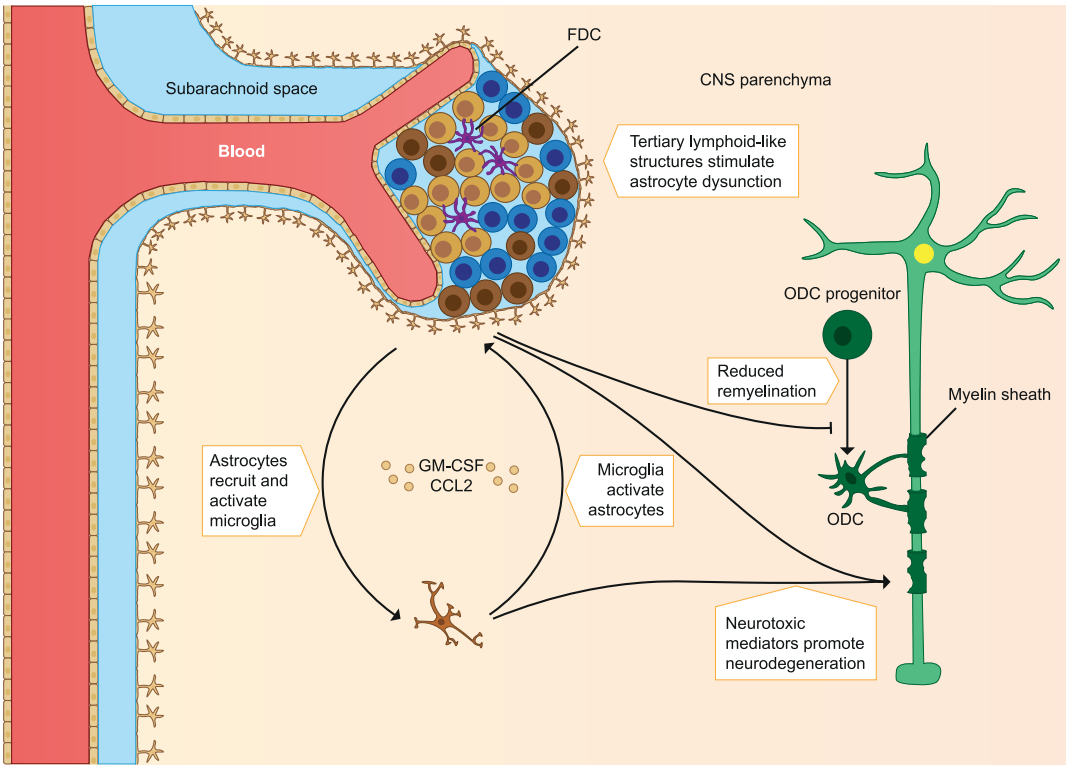

FIGURE 2 | Immune cells involved in the pathology of late MS. Immune cell migration from the periphery into the CNS subsides, but chronic inflammation of the CNS still takes place. Chronic CNS inflammation is associated with tertiary lymphoid-like structures in perivascular spaces and dysfunctional astrocytes and microglia. Microglia activation promotes astrocyte production of CCL2 and GM-CSF, which recruits and activates more microglia. Astrocytes inhibit remyelination, and both microglia and astrocytes produce pro-inflammatory mediators that are neurotoxic and contribute to gradual neurodegeneration. FDC, follicular dendritic cells; ODC, oligodendrocyte; CNS, central nervous system; MS, multiple sclerosis. The figure has been inspired by: Fugger et al. and Goverman (2, 10).

KEY PLAYERS OF THE HUMAN GUT MICROBIOTA

The human gut serves as a host to many microbes (bacteria, archaea, viruses, and fungi). Newborn humans have a sterile gut, and colonization occurs through exposure to new flora depending on the mode of delivery, diet, and hygiene (11). Over the years, the gut microbiota increase in diversity and reach a maximum at adolescence, after which they remain fairly stable (11). The diversity of gut microbiota can be expressed as α-diversity, which shows the richness and distribution of taxa within one population. β-Diversity is used to measure differences between multiple populations, and it measures how many taxa are shared between populations (12). Gut microbiota display a low diversity at the phylum level, as bacteria from only 8 out of 55 phyla have been detected in the human gut (13). Yet, large interindividual variation exists in the relative abundance of microbiota members. This variation is not only greatly impacted by short-term dietary alteration but also influenced by long-term dietary habits, host genotype, and stochastic processes such as history of colonization and ecological processes, such as selection and evolution (14) (see **Figure 3**). Additionally, the use of antibiotics can reduce the number and diversity of gut microbiota, but these are largely restored to the pretreatment composition after a recovery period (15).

Studies investigating the human gut microbiota often use the sequencing of specific genes such as the 16s ribosomal RNA gene in stool samples to determine which species are present. This has to be interpreted with care, as the microbiota composition differs from the small intestine to the large intestine. The abundance of microbiota is increased along the gastrointestinal tract and while the jejunum is host to mainly aerobic species, the colon is dominated by anaerobic species. Sequencing of stool samples is therefore most informative of microbiota in the large intestine (16).

Bacteria

The gut bacterial flora of healthy adults comprises mainly bacteria from the phyla Firmicutes and Bacteroidetes (16). These bacteria serve many functions. They aid in metabolism and nutrient availability by fermenting complex carbohydrates into short-chain fatty acids (SCFA), which can be used as an energy source by mucosa. Bacteria can also produce vitamins, such as vitamin K and components of certain vitamin B species (17), and play a role in the metabolism of medications. Gut microbiota also aid in the protection against pathogens by competitive exclusion (16, 17). The tremendous contribution of bacteria to

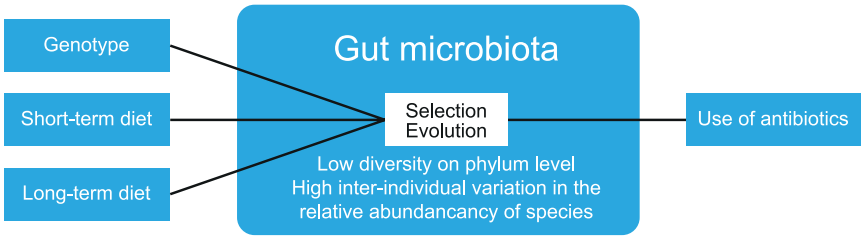

FIGURE 3 | Factors that determine gut microbiota composition. The composition of gut microbiota is influenced by multiple factors, such as diet and host genotype. Within the gut, ecological processes such as selection and evolution take place. The use of antibiotics reduces the numbers and diversity of gut microbiota. The figure has been modified after: Walter and Donaldson et al. (14, 15).

host physiology is best seen in germfree (GF) mice, which have impaired development of the epithelium, musculature, and vasculature of the intestines as well as systemic defects, such as in immune functions and brain development. The gut-associated lymphoid tissue (GALT) is strongly underdeveloped in GF mice, with a reduced number of immune cells and lymph node size. These defects can be restored by colonization with bacteria, indicating the strong influence of bacteria on the host immune system. Conversely, the immune system promotes optimal growth and nutritional benefit and influences the species composition in the gut by the production of secretory IgA antibodies and bactericidal products, such as antimicrobial peptides (defensins and cathelicidins) (18). In addition, epithelial cells are covered by a mucus layer that is continuously consumed by bacteria and renewed by goblet cells (19). This mucus layer also contains peptides which aggregate bacteria but are not bactericidal and functions to keep bacteria at a safe distance from epithelial cells (20). A fiber-deprived diet increases the number and activity of mucus-degrading bacteria, thus reducing the mucus layer and increasing susceptibility to pathogens (21), indicating the importance and interplay of dietary components with microbiota and the immune system.

Archaea

The majority of archaea found thus far in the human body are methanoarchaea, which all produce methane in the absence of oxygen. Because methods to efficiently detect archaea have only recently been developed, insight into the functional role of archaea in the gut is limited. Archaea seem to have a nutritional role as they form syntrophic interactions with bacteria and can favor the growth of fermenting bacteria (22). Archaea may also have immunogenic roles as lipids from *Methanobrevibacter* have powerful adjuvant properties (23) and exposure of monocyte-derived dendritic cells (DC) to archaea strains activates these cells (24).

Viruses

Most studies on viruses in the human gut microbiota focus on DNA viruses, as over 95% of RNA viruses in the human gut are of plant origin and may have little influence on the gut microbiota of human hosts. Among DNA viruses, double-stranded DNA viruses from the order Caudovirales (Podoviridae, Siphoviridae, and Myoviridae) or single-stranded DNA viruses from the family Microviridae (25) have been identified in the human gut. Viruses can prevent infection of the host epithelium by binding to mucin glycoproteins, thereby limiting bacterial–epithelium adhesion. Additionally, viruses (phages) infect bacteria and exert effects on the host through the modulation of bacteria in the gut. Phages use distinct surface molecules for infecting bacteria, and therefore, have a tropism for specific strains of bacteria. After infection, phages can display a lysogenic phase, in which the phage integrates into the viral genome and stays there in a latent phase, the phage is then called a called prophage. Alternatively, phages can kill bacteria after viral replication which is called the lytic phase. Most gut bacteria are believed to have at least one prophage latently incorporated in their genome, and prophages can enter the lytic phase from a lysogenic phase after exposure to a range of stimuli (13, 25).

Bacteria protect themselves from virus infections by clustered regularly interspaced short palindromic repeats (CRISPR) in their genome. These are short DNA regions that contain foreign virus DNA in between them. When phages infect bacteria, bacteria transcribe the foreign DNA in the CRISPR region, which guides the bacteria to cleave the intruding phage and fight off the infection. During the battle between bacteria and phages, phages may incorporate whole gene segments from previously infected bacteria into bacterial chromosomes. This may disrupt host bacteria genes. Alternatively, the incorporated gene segment may increase bacterial fitness, thereby promoting colonization. Viruses can also introduce toxin-encoding genes into bacteria, which may promote dysbiosis of gut microbiota. Bacteria may use their prophages to their advantage, by producing them for lysis of competitor bacterial species. Finally, viruses can protect the host from overgrowth of dominant bacterial species, by killing bacteria when a high density is reached (13).

THE INFLUENCE OF GUT MICROBIOTA ON EXTRAINTESTINAL TISSUES

In addition, gut microbiota may influence distant host tissues such as the CNS. Accumulating evidence indicates that gut microbiota affect various behaviors such as social interaction (26), nociceptive responses (27), depression (28), stress responsiveness, and anxiety (29). Gut microbiota also influence hippocampal neurogenesis (30), blood–brain barrier integrity (31), and microglia maturation (32).

Gut microbiota affect the CNS through multiple mechanisms. The gut enteric nervous system controls the motility and homeostasis of the gut, which influences the gut microbiota composition (33). Conversely, gut microbiota influence the enteric nervous system, and the vagus nerve provides a direct communicatory link between the gut and the CNS. Gut microbiota can produce neurotransmitters or precursors such as tryptophan, and microbiota metabolites can also directly influence the brain. For instance, fermenting bacteria produce SCFA, which can translocate into the brain and inhibit deacetylases, resulting in epigenetic changes (32). In addition, bacterial RNA, DNA, and proteins are detected in the human brain (34). Fragments of bacterial cell walls, such as peptidoglycan (PGN) may translocate to the brain and possibly influence brain development and social interaction (35). Bacteria also produce microbial-associated molecular patterns, which can be recognized by the host immune system with pattern recognition receptors, such as Toll- or Nod-like receptors and influence gut physiology (32, 35). The GALT is located along the small and large intestines and functions as immune surveillance of the gut. Gut microbiota can elicit an immune response in the GALT (36), which can be pro-inflammatory or anti-inflammatory, depending on the involved microbiota (37). The pro-inflammatory response of segmented filamentous bacteria (SFB) is best characterized. Studies in mice show that SFB enhance antigen presentation by DC resulting in greatly increased numbers of pro-inflammatory intraepithelial lymphocytes (IEL), such as γδ T cells and $CD8^+$ T cells. SFB also increase IgA^+ B cells and T_{h17} cells (13, 36). T_{h17} cells produce the cytokines IL-17A, IL-17F, IL-21, and IL-22. IL-17A and IL-17F control bacterial and fungal infections through the recruitment of neutrophils and more T_{h17} cells and increased production of β-defensin by epithelial cells. IL-22 induces epithelial cell proliferation, survival, tissue repair, and increased expression of antimicrobial molecules (13). In addition, gut bacteria such as *Helicobacter hepaticus* elicit a pro-inflammatory response, marked by increased numbers of T_{h17} and T_{h1} cells and pro-inflammatory cytokines excretion (38). T_{h1} cells are instrumental in fighting intracellular bacteria and viruses, *via* the production of IFNγ (13).

Gut microbiota are potent activators of innate lymphoid cells (ILC). ILC arise from common lymphoid precursors and respond rapidly to cytokines produced by the epithelium. ILC can be divided into three groups based on molecular markers: type 1 which expresses T box transcription factor (TBX21 or T-bet) in T cells, type 2 which expresses GATA binding protein and type 3 which expresses retinoid-related orphan receptor (ROR)γt. Type 3 ILC produce IL-17 and IL-22, and IL-22 production can be induced by bacterial metabolites acting on the aryl hydrocarbon receptor (AHR) (17). The pro-inflammatory reactions caused by gut microbiota may contribute to MS pathogenesis *via* the activation of autoreactive T-cells (36). Conversely, gut microbiota may also elicit an anti-inflammatory response, characterized by the induction of $CD4^+$ T_{reg} cells. T_{reg} cells produce TGF-β and IL-10, which decrease pro-inflammatory cytokine production and cell proliferation. Clostridia species promote production of TGF-β, which helps maintain an anti-inflammatory milieu and is involved in the induction of T_{reg} cells (13, 36). Additionally, Bacteroides species increase the production of anti-inflammatory cytokines and suppress IL-17 production (36).

Gut microbiota also affect invariant natural killer T cells (iNKT cells); GF mice have increased iNKT cell numbers in their colon. These are a pro-inflammatory subset of T cells that express the invariant T-cell receptor α, with which they detect lipid antigens presented by CD1d molecules. They can promote T_{h1} and T_{h17} responses by producing IFNγ, IL-2, IL17A and tumor necrosis factor (TNF) (13).

THE INFLUENCE OF PHAGES ON THE IMMUNE SYSTEM

The influence of phages on the immune system is likely mostly indirect, through modulation of bacteria by mechanisms mentioned earlier. Phages may also directly communicate with the immune system. Humans are constantly sensitized with phage antigens present in food. This induces a constant low level of phage-neutralizing antibodies in human serum. After systemic phage administration in animals, these neutralizing antibodies are produced at higher titers. In humans, administration of enterobacteria PhiX174 phages induced IgM antibodies, followed by IgG antibodies after a second exposure (39). The function of antibodies is clearance of phages as illustrated by the lower clearance in B cell-deficient mice. A reduction of macrophages, natural killer, or T cells does not influence phage clearance (40). Conversely, phages can influence T cell functions *in vitro*. Mycobacteriophages were found to suppress phytohemagglutinin-induced activation of T lymphocytes from guinea pigs in a dose-dependent matter (41). Moreover, purified T4 phages can inhibit human T cell proliferation *via* the CD3 T-cell receptor complex, while phage lysates of *Staphylococcus aureus* can induce T-cell proliferation (41). In addition, phages can affect host phagocytes. Phagocytosis and ROS production may limit bacterial densities in the gut. Excessive phagocyte activity may also lead to pathology. Preincubation of monocytes and neutrophils with T4 and F8 phages from *Pseudomonas aeruginosa* reduced *in vivo* phagocytosis of *Escherichia coli*. T4 phages also reduced the ROS production by phagocytes exposed to LPS and bacterial cells, although this effect may be phage strain-dependent, because staphylococcal A3/R-purified phage (lysates) did not affect ROS production (41). The effect of phages depends also on the type of preparation, as T4 lysates promote IL-6 production by monocytes but purified T4 phages do not (41). Most effects of phages are anti-inflammatory, as reflected by mitigated phagocytosis, ROS production, and lymphocyte proliferation. However, phage lysates of *S. aureus* are likely more pro-inflammatory. Overall, effects of phages on the host immune system are complex and depend on phage identity, phage tropism, and preparation.

Collectively, variable exposure to the environment and food greatly influences gut microbiota composition. Elements of the gut microbiota constantly interact with each other and with the host and are essential for the normal development of the immune system and CNS. Pro- and anti-inflammatory effects have been attributed to different microbial species and an imbalance may contribute to autoimmune disease may contribute to autoimmune disease (see **Figure 4**).

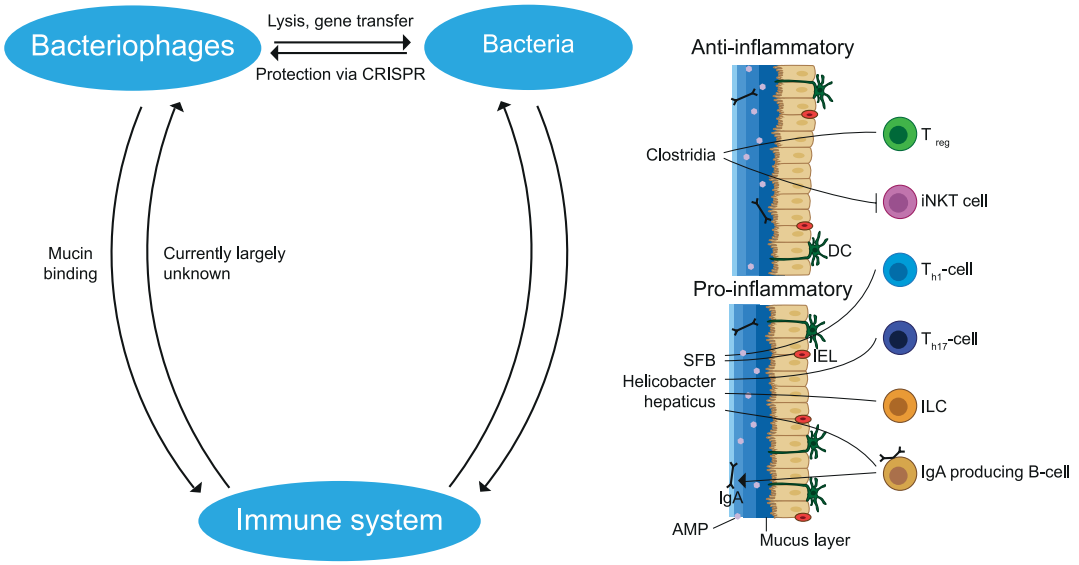

FIGURE 4 | Interactions between members of the gut microbiota and the immune system. Bacteriophages can infect and lyse bacteria or undergo a lysogenic cycle in which they stay dormant inside bacteria. During this process, gene segments may be transmitted which influences the fitness of the bacteria. Bacteria protect themselves from phage infection by CRISPR. Bacteria may cause a pro- and anti-inflammatory effect dependent on the bacterial species. Anti-inflammatory effects include the induction of T_{reg} cells and the reduction of iNKT cells. Pro-inflammatory effects include induction of T_{h1}, T_{h17}, IgA producing B cells and stimulation of IL-22 production by ILC, which increases AMP production. These immune cells and the mucus layer protect the epithelial cells from being infected by bacteria. In addition, phages limit bacteria–epithelial adhesion by binding to the mucus layer. The effects of the gut immune system on phages remain largely unknown. SFB, segmented filamentous bacteria; AMP, antimicrobial peptides; iNKT, invariant natural killer T; ILC, innate lymphoid cells; IEL, intraepithelial lymphocytes; DC, dendritic cell; CRISPR, clustered regularly interspaced short palindromic repeats. The figure has been inspired by: Glenn and Mowry (13).

EAE MODELS

Due to the inaccessibility of the CNS of MS patients, animal models are used for translational research into the pathogenesis of MS and for therapy development. As in none of the available models the immunological and pathological complexity of MS is fully replicated, multiple models are being used. Depending on the research question models of MS-like demyelination are induced chemically (cuprizone, lysolecithin), with neurotropic viruses (Theiler's murine encephalitis virus, Semliki Forest virus), through specific cytokine overexpression in the CNS, diphtheria toxin-based depletion of oligodendrocytes, and active immunization with myelin components in suitable adjuvants (42, 43). The active immunization model, known as EAE, is the most widely used preclinical MS model. EAE can be induced in a wide variety of laboratory animal species including mice, rats, rabbits, guinea pigs, and non-human primates by inoculation of myelin antigens formulated with a strong adjuvant, such as CFA. For synchronous EAE induction at high incidence in mice, the immunization is usually supplemented with injection of *Bordetella pertussis* toxin. Different myelin antigens and dosages are used to model the heterogeneity of MS. For example, SJL mice develop a RR type of EAE after sensitization against residues 79–87 of myelin basic protein (MBP_{79-87}) or residues 131–151 of proteolipid protein ($PLP_{131-151}$). C57Bl/6 mice can develop RR EAE after immunization with a low dose of myelin oligodendrocyte glycoprotein residues 35–55 (MOG_{35-55}) and develop chronic EAE without remission upon immunization with a high dose of MOG_{35-55} (44). It is increasingly felt that the usage of strong adjuvants precludes a role of subtle regulatory mechanisms. For such studies, passive EAE models induced by the transfer of activated T cells from a donor with actively induced EAE or spontaneous EAE models established in mice expressing transgenic T and/or B cell receptors specific for myelin antigens may be more useful (43).

Although EAE models have proven their relevance as a preclinical test system for new therapeutics they also have their limitations. The highly artificial way by which the autoimmune process is activated in CFA-based EAE models does not necessarily reflect the natural immune response toward self-antigens. As an illustration, the EAE model is dominated by $CD4^+$ T cells, whereas $CD8^+$ T cells are likely more immunodominant in MS patients (44). Another difference between EAE and MS is that mice with EAE mostly show lesions in the spinal cord, whereas MS patients mostly show lesions in the brain (43). A noticeable exception is formed by the atypical EAE models in marmosets, a small-bodied Neotropical primate, which more closely approximate MS with respect to clinical, pathological, and immunological presentation (45).

GUT MICROBIOTA-BASED INTERVENTIONS IN EAE MODELS

The first evidence that gut microbiota are involved in the pathogenesis of EAE stems from decades ago. Already in 1993, it

TABLE 1 | The role of gut microbiota in EAE.

Reference	Animal model	Intervention	Clinical score	Immune response
Berer et al. (47)	SJL anti-$MOG_{92–106}$ TCR^{tg}	Germfree housing	Protected	Reduced T_{h17}, impaired B-cell recruitment to brain-draining lymph nodes Reduced anti-MOG B cell response
Lee et al. (48)	C57Bl/6 $MOG_{35–55}$	Germfree housing	Decreased	Reduced T_{h1} and T_{h17}, increased T_{reg} Reduced DC capacity to induce T_{h1} and T_{h17} responses
Yokote et al. (49)	C57Bl/6 $MOG_{35–55}$	Broad spectrum antibiotics	Decreased	Decreased pro-inflammatory cytokines, decreased T_{h17}
Ochoa-Repáraz et al. (50)	SJL $PLP_{139–151}$; C57Bl/6 $MOG_{35–55}$	Broad spectrum antibiotics	Decreased	Reduced pro-inflammatory cytokines, increased T_{reg} Increased $CD11c^{high}CD103^{+}$ cells
Ochoa-Repáraz et al. (51)	C57Bl/6 $MOG_{35–55}$	Broad spectrum antibiotics	Decreased	Increased IL-10 producing $CD5^{+}$ B-cells Shift from T_{h1} and T_{h17} toward T_{h2} response
Ochoa-Repáraz et al. (52)	SJL $PLP_{139–151}$	Oral administration of *Bacteroides fragilis*	Decreased	Increased T_{reg}, reduced T_{h17}
Ochoa-Repáraz et al. (52)	SJL $PLP_{139–151}$	Oral administration of $PSA^{-/-}$ *B. fragilis*	Normal	Normal
Ezendam et al. (53)	Lewis rats MBP	Oral administration of *Bifidobacterium animalis*	Decreased duration	Not investigated
Lavasani et al. (54)	C57Bl/6 $MOG_{35–55}$	Oral administration of three *Lactobacilli* strains	Decreased	Reduced T_{h1} and T_{h17}, increased T_{reg}, IL-10 dependent
Takata et al. (55)	C57Bl/6 $MOG_{35–55}$; SJL $PLP_{139–151}$	Oral treatment with heat-killed *Pediococcus acidilactici*	Decreased	Reduced T_{h1} and T_{h17}, increased T_{reg}
Maassen and Claassen (56)	Lewis rats MBP; SJL $PLP_{139–151}$	Oral treatment with commercially available probiotic drinks containing *Lactobacillus casei*	Decreased in Lewis rats, no effect in SJL model	Not investigated
Kwon et al. (57)	C57Bl/6 $MOG_{35–55}$	Oral administration of *Bifidobacterium bifidum, Streptococcus thermophilus* and three *Lactobacillus* strains	Decreased	Reduced T_{h1} and T_{h17} response, increased T_{reg}
Rezende et al. (58)	C57Bl/6 $MOG_{35–55}$	Oral administration of recombinant HSP65-producing *Lactococcus lactis*^{tg}	Decreased	Decreased IL-17, increased IL-10, dependent on increased $CD4^{+}LAP^{+}$ T_{reg}
Wang et al.; Ochoa-Repáraz et al. (59–61)	SJL $PLP_{139–151}$; C57Bl/6 $MOG_{35–55}$	Oral treatment with *B. fragilis*-produced PSA	Decreased	Reduced T_{h1} and T_{h17}, increased T_{reg} and $CD103^{+}$ DC, increased $CD39^{+}$ T_{reg}
Kadowaki et al. (62)	2D2 anti-MOG TCR^{tg}; C57Bl/6 $MOG_{35–55}$	Adoptive transfer of $CD4^{+}$ induced IEL	Decreased	Reduced T_{h17} $CD4^{+}$ induced IEL are dependent on gut microbiota and diet
Maassen et al. (63)	Lewis rats, $MBP_{72–85}$	Oral administration of live/intranasal administration of soluble cell extracts from myelin proteins producing *L. casei*	Decreased, extracts from guinea pig MBP producing bacteria increased	Not investigated

Germfree housing, antibiotics, probiotics, and bacterial products affect the EAE clinical score.
DC, dendritic cells; EAE, experimental autoimmune encephalomyelitis; IEL, intraepithelial lymphocytes; MBP, myelin basic protein; MOG, myelin oligodendrocyte glycoprotein; PLP, proteolipid protein; PSA, polysaccharide A.

was discovered that transgenic mice expressing T-cell receptors specific for MBP fail to develop EAE when they were housed in a sterile, specific pathogen-free (SPF) environment, while mice housed in a non-sterile environment did develop EAE (46). To determine the role of gut microbiota in EAE, GF models, antibiotic treatments, probiotic mixtures, bacterial products, and diet-based interventions have been used. These studies are summarized in **Tables 1** and **2**.

GF Models

Germfree mice, which are bred and raised in a sterile environment, display significantly attenuated disease in both spontaneous and actively induced EAE models (47, 48). In the actively induced EAE model, GF mice develop EAE at a reduced incidence, while in mice developing overt disease, symptoms are milder and of shorter duration compared to conventionally colonized mice. This has been attributed to reduced mesenteric lymph node DC capacity to induce T_{h1} and T_{h17} responses. GF mice have reduced IL-17 and IFNγ producing $CD4^{+}$ T cells in their spinal cord. This is accompanied by increased $CD4^{+}CD25^{+}Foxp3^{+}$ T_{reg} cells in draining lymph nodes and spleen. Compared to GF mice, monocolonization with SFB significantly increases EAE clinical scores, with increased IL-17 and IFNγ production in the small intestines and spinal cord

TABLE 2 | Dietary interventions in EAE.

Reference	Animal model	Intervention	Clinical score	Immune response
Haghikia et al. (64)	C57Bl/6 $MOG_{35–55}$	Oral administration of propionic acid (short-chain fatty acid) and lauric acid (long-chain fatty acid)	Decreased	Increased T_{reg}, reduced T_{h17} on PA treatment Increased T_{h1} and T_{h17}, decreased *Prevotellaceae* and *Bacteroidetes* on lauric acid treatment
Lemire and Archer (65)	SJL spinal cord homogenate	Intraperitoneal vitamin D administration	Decreased	Reduced antibodies against MBP
Cantorna et al. (66)	B10.PL $MBP_{79–87}$	Dietary vitamin D supplementation	Decreased	Not investigated
Spach et al. (67)	C57Bl/6 $MOG_{35–55}$	Dietary vitamin D supplementation	Decreased	Reduced inflammatory cells, IFNγ in the spinal cord, IL-10 dependent
Piccio et al. (68)	SJL $PLP_{139–151}$; C57Bl/6 $MOG_{35–55}$	40% caloric restriction	Decreased	Increased plasma levels of corticosterone, adiponectin, reduced plasma levels of IL-6 and leptin
Esquifino et al. (69)	Lewis rats spinal cord homogenate	33 and 66% caloric restriction	66% caloric restriction protected from EAE signs	Reduced splenic $CD8^+$ T cells and B cells, reduced lymphoid and thymic $CD4^+$ T cells and B cells and IFNγ production
Kafami et al. (70)	C57Bl/6 $MOG_{35–55}$	Intermittent feeding	Decreased	Not investigated
Harbige et al. (71)	SJL $MOG_{92–106}$	Oral γ-linolenic acid treatment	Decreased	Increased TGF-β, prostaglandin E_2 production by spleen mononuclear cells
Harbige et al. (72)	Lewis rats, guinea pig spinal cord homogenate	Oral γ-linolenic acid treatment	Decreased	Not investigated
Kong et al. (73)	C57Bl/6 $MOG_{35–55}$	DHA-rich diet	Decreased	Reduced T_{h1} and T_{h17} cell differentiation, reduced amounts of T_{h1}, T_{h17} found in the spleen and spinal cord of mice on a DHA-rich diet. *In vitro*, DHA reduced the expression of costimulatory molecules on DC and reduced their production of pro-inflammatory cytokines
Unoda et al. (74)	C57Bl/6 $MOG_{35–55}$	EPA supplementation	Decreased	Increased expression of PPAR α, β, and γ on $CD4^+$ T cells in the spinal cord, reduced IFNγ and IL-17 cytokine production. $CD4^+$ T cells from the spleen of EPA-treated mice expressed increased mRNA levels of Foxp3, but also of IL-17 and RORγt
Salvati et al. (75)	Dark agouti rats, guinea pig spinal cord homogenate	EPA supplementation	Delayed time before EAE symptoms appeared	Increased myelination of axons in the spinal cord
Kim et al. (76)	C57Bl/6 $MOG_{35–55}$	Ketogenic diet	Decreased	Reduced T_{h1}, T_{h17}, and pro-inflammatory cytokines
Choi et al. (77)	C57Bl/6 $MOG_{35–55}$	Cycles of fasting Ketogenic diet	Decreased	Fasting increased T_{reg}, corticosterone, reduced $CD11^+$ DC, T_{h1}, T_{h17}, pro-inflammatory cytokines Ketogenic diet: not investigated
Jörg et al. (78)	C57Bl/6 $MOG_{35–55}$	High-salt diet	Increased	Increased T_{h17}
Krementsov et al. (79)	C57Bl/6 $MOG_{35–55}$, SJL $PLP_{135–151}$	High-salt diet	Increased in C57Bl/6 mice, in SJL only increased in females	No difference in T_{reg}, T_{h1}, and T_{h17} cells
Wu et al. (80)	C57Bl/6 $MOG_{35–55}$	High-salt diet	Increased	Increased T_{h17} in CNS and mesenteric lymph nodes, SGK-1 signaling dependent
Kleinewietfeld et al. (81)	C57Bl/6 $MOG_{35–55}$	High-salt diet	Increased	Increased inflammatory cell infiltration into the CNS, increased T_{h17}
Veldhoen et al. (82)	C57Bl/6 $MOG_{35–55}$	FICZ administration	Increased	Increased IL-17- and IL-22-producing $CD4^+$ T cells in the spinal cords
Quintana et al. (83)	C57Bl/6 $MOG_{35–55}$	FICZ, ITE, TCDD administration	FICZ increased, ITE and TCDD reduced	FICZ: increased $IL\text{-}17^+CD4^+$ and $IFNγ^+CD4^+$ T cells in the spleen TCDD: increased $Foxp3^+$ T_{reg}
Rothhammer et al. (84)	C57Bl/6 $MOG_{35–55}$	Tryptophan-deficient diet, supplementation with tryptophan metabolites and tryptophanase	Increased, supplementation reduced EAE scores	IFN-1 signaling induces AHR expression in astrocytes, supplementation does not reduce EAE scores in astrocyte-specific AHR knockout mice

(*Continued*)

TABLE 2 | Continued

Reference	Animal model	Intervention	Clinical score	Immune response
Stoye et al. (85)	SJL $PLP_{139–151}$	Intraperitoneal injection of ZnAsp	Decreased, but increased on high doses	Reduced proliferation of stimulated human T-cells, reduced pro-inflammatory cytokine production
Schubert et al. (86)	SJL $PLP_{139–151}$	Oral ZnAsp supplementation	Decreased, but increased on high doses	Reduced proliferation of stimulated human T-cells, reduced pro-inflammatory cytokine production
Kitabayashi et al. (87)	C57Bl/6 $MOG_{35–55}$	Oral Zink supplementation	Decreased	Not investigated
Rosenkranz et al. (88)	C57Bl/6 $MOG_{35–55}$	Intraperitoneal injection of ZnAsp	Decreased	Reduced systemic T_{h17} cells and increased $Foxp3^+$ T-cells in the spinal cord
Scelsi et al. (89)	Guinea pigs, spinal cord homogenate	Oral selenium supplementation	Increased on high doses, normal on normal doses	Not investigated
Chanaday et al. (90)	Wistar rats, whole MBP	Intraperitoneal and oral diphenyl diselenide	Diphenyl diselenide was toxic when intraperitoneally administered, reduced when orally administered	Reduced number of macrophages in the CNS, reduced MBP-specific T-cell proliferation
Xue et al. (91)	C57Bl/6 $MOG_{35–55}$	Intraperitoneal tocopherol administration	Decreased	Reduced MOG-specific splenocyte proliferation. Splenocytes incubated with tocopherol produced less IFNγ
Blanchard et al. (92)	C57Bl/6 $MOG_{35–55}$	Intraperitoneal TFA-12 administration	Decreased	Reduced inflammation of the CNS, astrogliosis and demyelination. Induces oligodendrocyte maturation
Racke et al. (93)	SJL, MOG-incubated lymph node cells	Dietary 13-cis-retinoic acid and 4-HPR	Decreased	Not investigated
Zhan et al. (94)	C57Bl/6 $MOG_{35–55}$	Intraperitoneal all-trans retinoic acid administration	Decreased	Reduced DC maturation, reduced pro-inflammatory monocytes in the subarachnoid space. Reduced numbers of T_{h1} and T_{h17} cells in the draining lymph nodes
Xiao et al. (95)	C57Bl/6 $MOG_{35–55}$	Intraperitoneal all-trans retinoic acid administration	Decreased	Inhibits T_{h17} differentiation by reducing expression of the IL-6 and IL-23 receptor

Dietary interventions affect the microbiota and EAE clinical scores.
AHR, aryl hydrocarbon receptor; CNS, central nervous system; DC, dendritic cells; DHA, docosahexaenoic acid; EAE, experimental autoimmune encephalomyelitis; EPA, eicosapentaenoic acid; FICZ, 6-formylindolo[3-2b]carbazole; ITE, 2-(1′H-indole-3′carbonyl)-thiazole-4-carboxylic acid methyl ester; MBP, myelin basic protein; MOG, myelin oligodendrocyte glycoprotein; PLP, proteolipid protein; SGK-1, serum/glucocorticoid kinase 1; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin.

(48). SPF-bred MOG TCR transgenic mice develop spontaneous EAE at high incidence, but appear completely EAE resistant under GF conditions (47). Upon colonization with conventional microbiota, EAE quickly developed (47).

Compared to conventionally colonized mice, GF mice have reduced IL-17 producing $CD4^+$ cells in Peyer's patches and lamina propria, but no difference is seen in mesenteric lymph nodes or other remote organs. Additionally, MOG-immunized GF mice had reduced autoreactive anti-MOG antibodies which could be increased by colonization. It is thought that MOG-specific B cells are activated and recruited into deep cervical lymph nodes by helper T cells. Once there, B cells encounter MOG imported from the lymphatic vessels and undergo proliferation, immunoglobulin class switching, and somatic hypermutation (47).

Germfree mice also have reduced gut luminal extracellular adenosine 5′-triphosphate (ATP). ATP can be derived from bacteria, and ATP activates lamina propria $CD70^{high}CD11c^{low}$ DC, leading to the production of IL-6 and IL-23 which are important for the differentiation of T_{h17} cells. Systemic and rectal administration of ATP increases the number of T_{h17} cells in GF mice. Although investigated in a colitis model, administration of ATP increases T_{h17} response (96). This may be relevant for EAE due to the role of T_{h17} cells in EAE. Administration of alkaline phosphatase, which can neutralize ATP and LPS, reduced the clinical signs of EAE when given presymptomatically but not in the acute or chronic phase (97). Besides reduced gut luminal ATP, GF animals have reduced tight junctions and increased permeability at the blood–brain barrier, potentially affecting EAE (32).

A general caveat in the usage of GF mice for the modeling of human autoimmune disease is that the immunocompetence of such mice is seriously disturbed. Even the frequently used SPF-bred mice are immunologically incomparable to adult humans. SPF-bred mice have reduced cervix mucosal memory T cells, and a relatively lower number of differentiated effector memory $CD8^+$ T-cells in their blood, a signature which is comparable to neonatal humans. Feral and pet store mice are immunologically more comparable to adult humans, and SPF-bred mice could be immunologically normalized by cohousing them with pet store mice (98). This indicates that exposure to pathogens affects the immune system and that studies using GF and SPF animals may not always translate well into the clinic.

Antibiotics

Antibiotic treatments modulate gut microbiota and this also affects EAE. An oral cocktail of non-absorbing antibiotics [kanamycin, colistin, and vancomycin (KCV)] administered 1 week before active EAE induction reduces disease scores. The impairment of normal EAE development is accompanied by a reduction of IFNγ, TNFα, IL-6, and IL-17 production by MOG-reactive T cells from draining lymph nodes. KCV also reduced the number of T_{h17} cells in mesenteric lymph nodes while this effect was not seen in iNKT cell-deficient mice, indicating that iNKT cells are mechanistically important in KCV treatment. Since no differences were found in mesenteric lymph node iNKT cells, Foxp3$^+$ T_{reg} cells and T_{h17} promoting cytokines, KCV treatment likely exerts its effect upstream of the mesenteric lymph nodes. In the KCV-treated group, reduced expression of IL-21 and IL-23 cytokines were found in lamina propria lymphocytes, and it is therefore speculated that KCV alters iNKT cells in the lamina propria, ultimately leading to T_{h17} cells in the mesenteric lymph nodes (49).

With a different antibiotics mixture (ampicillin, vancomycin, neomycin sulfate, and metronidazole) orally administered before EAE induction, normal EAE development was also impaired. This has been attributed to an increase in Foxp3$^+$ Treg cells in the mesenteric and cervical lymph nodes (deep or superficial lymph nodes not specified) of antibiotic-treated mice. The increase in Foxp3$^+$ Treg cells is likely a result of increased CD11c^{high}CD103$^+$ DC in Peyer's patches and mesenteric lymph nodes. CD11c^{high}CD103$^+$ DC enhance the conversion of naive CD4$^+$ T cells into Foxp3$^+$ Treg cells (50). In addition, this antibiotics mixture increases IL-10 producing CD5$^+$ B cells in cervical lymph nodes (deep/superficial lymph nodes not specified). Adoptive transfer of splenic CD5$^+$ B cells obtained from mice treated with antibiotics into naïve recipient mice which were $MOG_{35–55}$-immunized 1 day post transfer reduced EAE disease score. The reduced disease score was associated with a shift from a T_{h1}/T_{h17} cytokine profile toward a Th2 cytokine profile (51). Thus, this antibiotic treatment induced both regulatory T and B cells which protected against EAE.

Probiotics

The finding that gut microbiota can elicit pro- and anti-inflammatory reactions, has raised the interest for treatment of EAE with bacteria. Prophylactic treatment with *Bifidobacterium animalis* decreases the duration of EAE symptoms (53) and three commercially available probiotic drinks containing strains of *Lactobacillus casei* could reduce the EAE disease score in Lewis rats. However, no significant effects of these drinks have been observed in an SJL mouse model (56).

Prophylactic use of *Lactobacilli* monostrains reduces autoreactive T cells and prevents EAE. However, using these monostrains, established EAE could not be reversed. When three *Lactobacilli* strains are combined in a mixture, *Lactobacilli* therapeutically suppress disease progression and reduce clinical signs in $MOG_{35–55}$-immunized mice. The beneficial effect was IL-10 dependent, and treatment induced CD4$^+$CD25$^+$ T_{reg} cells in the mesenteric lymph nodes. The treatment also reduced T_{h1} and T_{h17} cytokines and increased IL-10 production in cultures of splenocytes cultured with autoantigen. In addition, reduced IL-17 and increased IL-10 levels were found in the CNS of treated animals (54).

Prophylactic treatment with heat-killed *Pediococcus acidilactici* resulted in reduced EAE scores and decreased $MOG_{35–55}$-induced IL-17 and IFNγ production from draining lymph node cells and splenocytes. Treatment also increased CD4$^+$ IL-10$^+$ cells in mesenteric lymph nodes and the spleen but not in the lamina propria. It is therefore thought that the treatment with heat-killed *P. acidilactici* activates inhibitory DC, which then migrate to the mesenteric lymph nodes to locally induce IL-10$^+$ T_{reg} cells. Treatment with heat-killed *P. acidilactici* also reduced the disease score in established EAE (55).

Prophylactic treatment with a cocktail of *Bifidobacterium bifidum, Streptococcus thermophilus*, and three *Lactobacillus* subspecies suppressed the EAE incidence and severity in $MOG_{35–55}$-immunized C57Bl/6 mice. The beneficial effect was associated with reduced T_{h1} and T_{h17} cell frequency and concomitant cytokine production along with increased IL-10 production in lymph nodes and spinal cord. IL-10 production by CD4$^+$ T cells and CD11c$^+$ DC is also increased in the spinal cord. When given after EAE immunization, this cocktail delays disease onset but cannot halt disease progression (57).

Intraepithelial lymphocytes are located in the epithelial layers of mucosal linings, e.g., of gastrointestinal and reproductive tracts. They comprise CD2$^-$CD5$^-$ natural, and CD2$^+$CD5$^+$ induced T cells, which release cytokines upon antigenic stimulation without the need of antecedent priming. Natural IEL acquire their activated phenotype in the presence of self-antigens in the thymus, while induced IEL acquire their phenotype in post-thymic cognate interaction with antigen. Adoptive transfer of CD4$^+$ IEL prior to EAE induction results in reduced disease severity. IEL cells transferred to the CNS upregulate LAG-3, CTLA-4, and TGF-β, but it is still unclear where and how they acquire their phenotype. It is evident, however, that IEL are affected by gut microbiota and can be induced by dietary components, such as aryl hydrocarbon ligands. It has not been tested whether CD4$^+$ IEL can reduce established EAE (62).

A series of observations shows the therapeutic value of polysaccharide A (PSA), produced by the bacterium *Bacteroides fragilis*. First, mice treated with antibiotics and recolonized with *B. fragilis* have reduced EAE disease scores compared to those not recolonized. Second, mice recolonized with PSA-deficient *B. fragilis* develop a normal EAE disease course (52), while mice colonized with wild-type *B. fragilis* have a milder disease course. The clinical effect is mirrored by reduced T_{h17} cells in the periphery and reduced IL-17 but increased IL-10 levels in the brains. Wild-type *B. fragilis* recolonized mice also have increased CD103$^+$ DC in their cervical lymph nodes (deep/superficial lymph nodes not specified). CD103$^+$ DC are known to convert naïve CD4$^+$ T cells into IL-10 producing Foxp3$^+$ T cells (52).

Bacterial Products

Oral treatment with purified PSA protects against EAE both in a prophylactic and therapeutic mode. PSA treatment led to increased CD103$^+$ DC in the cervical lymph nodes (deep/

superficial lymph nodes not specified) (59). Additionally, PSA is also recognized by DC near mesenteric lymph nodes in a toll-like receptor 2-dependent mechanism. PSA-exposed DC migrate to the mesenteric lymph nodes where they induce IL-10 producing T_{reg} cells (60, 99). A type of T_{reg} cell specifically induced by PSA has surface expression of ectonucleoside triphosphate diphosphohydrolase-1 (NTPDase-1; CD39). The NTPDase-1 converts extracellular pro-inflammatory ATP into 5′AMP, which can be further degraded to adenosine which, in contrast to ATP, has anti-inflammatory properties exerted *via* adenosine receptors (60, 99). $CD39^+$ T_{reg} cells have increased migratory capacity and are more abundant in the CNS of PSA-treated mice. Upon adoptive transfer, $CD39^+$ T_{reg} cells are protective against EAE in MOG-induced C57Bl/6 mice. PSA does not protect against EAE in CD39-deficient mice (61). Multiple bacteria species present in the large intestine are able to produce SCFA (acetate, propionate, and butyrate) by fermentation of dietary fibers. Through its effect on histone deacetylases butyrate can induce epigenetic modifications, such as acetylation of the Foxp3 locus. Butyrate can also stimulate DC and macrophages to secrete IL-10 and retinoic acid through G-protein-coupled receptors, such as Gpr41, 43, and 109a. SCFA may also act on epithelial cells, e.g., by stimulating TGF-β production. The acetylation of the Foxp3 locus as well as production of IL-10, retinoic acid, and TGF-β facilitates differentiation of naïve $CD4^+$ T cells into anti-inflammatory $Foxp3^+$ T cells (100).

Modified Probiotics

Bacteria can also be used as vector to deliver proteins into the gut and induce tolerance against these proteins. Oral pretreatment with recombinant *L. casei* which produce myelin antigens can reduce EAE scores in Lewis rats. In addition, intranasal pretreatment with soluble cell extracts of bacteria producing $MBP_{72–85}$ could also reduce the EAE disease score, while extracts of bacteria producing guinea pig MBP exacerbated the EAE disease score (63). Effects of *Lactobacilli* on the immune system are strain dependent, as different *Lactobacilli* induce distinct cytokine profiles in the mucosa (101). In addition, the growth phase (log vs stationary) of the bacterial culture is of importance, since this influences the IgG1/IgG2a antibody subclass ratio, which is indicative of the T_{h2}/T_{h1} pathway ratio (102). The differential effect of *Lactobacilli* strains and the influence of the phase of the bacterial culture should be taken into account when designing probiotic preparations.

Pretreatment with *Lactococcus lactis*, expressing heat shock protein 65 as a transgene, suppressed EAE development in $MOG_{35–55}$-immunized C57Bl/6 mice. The clinical effect was associated with reduced MOG-induced IL-17 production by splenocytes and increased IL-10 production by MOG-stimulated mesenteric lymph node cells. The treatment also caused increased T_{reg} cells in the spleen, inguinal and mesenteric lymph nodes, and spinal cord. *In vivo* depletion of $CD4^+LAP^+$ T_{reg} cells abrogated the protective effect of transgenic *L. lactis*, indicating that the increased T_{reg} cells are mechanistically important. The efficacy of this treatment during established EAE has not yet been investigated (58).

DIET-BASED INTERVENTIONS IN EAE MODELS

The notions that SCFA (<6 carbons) produced by gut microbiota modify immune functions and that SCFA are consumed through the diet underlie the hypothesis that the diet influences microbiota, the immune system, and ultimately EAE. Prophylactic oral treatment with propionic acid, a SCFA that is ingested with food, increases T_{reg} cells and reduces the EAE disease score. In contrast, long-chain fatty acids (LCFA; 13–21 carbons) increase EAE scores, which is associated with increased T_{h1} and T_{h17} cells (64). Of note, SCFA can migrate into the CNS and serve as fuel for CNS neurons and glial cells. The microbiota composition of these mice also differs, as those fed with lauric acid, a saturated LCFA, have reduced *Prevotellaceae* and *Bacteroidetes* in their gut microbiota. This illustrates the impact that dietary components can have on the immune system and EAE expression, possibly through the modification of gut microbiota. Fatty acids can also have beneficial effects on EAE, regardless of their length, when they are unsaturated. These lipids have one or more double bonds between the carbon atoms of their hydrocarbon chain. Fatty acids with a double bond at the third carbon atom counted from the methyl (–CH3) tail of the chain are Ω-3 fatty acids and with a double bond at the sixth carbon atom are Ω-6 fatty acids. Oral treatment with the Ω-6 fatty acid γ-linolenic acid reduced the EAE clinical scores in $MOG_{92–106}$-immunized SJL mice. This was associated with increased TGF-β, prostaglandin E_2 production by spleen mononuclear cells (71). Oral treatment with oils containing γ-linolenic acid also reduced the EAE clinical scores in Lewis rats, where EAE was induced with guinea pig CNS matter homogenate (72). Ω-3 fatty acids can also reduce the EAE clinical scores. A diet rich in docosahexaenoic acid (DHA) starting 5 weeks before induction of EAE with $MOG_{35–55}$ reduced the EAE clinical scores in C57Bl/6 mice. This was found associated with reduced T_{h1} and T_{h17} cell differentiation, and reduced amounts of these cells were found in the spleen and spinal cord of mice on a DHA-rich diet. *In vitro*, DHA reduced the expression of costimulatory molecules on DC and reduced their production of pro-inflammatory cytokines (73). A diet with the triglyceride form of DHA, starting before EAE induction also reduced the EAE clinical scores in the same mouse model. *In vitro*, pretreatment of microglia cells with the triglyceride form of DHA reduced microglial oxidative stress and production of nitric oxide and inflammatory cytokines (103). Eicosapentaenoic acid (EPA) supplementation starting 7 days after EAE induction reduced the EAE clinical scores in $MOG_{35–55}$-immunized C57Bl/6 mice. EPA is a ligand for PPAR α, β, and γ and increased their expression on $CD4^+$ T cells in the spinal cord, while reducing IFNγ and IL-17 cytokine production. $CD4^+$ T cells from the spleen of EPA-treated mice expressed increased mRNA levels of Foxp3, but also of IL-17 and RORγt (74). When an EPA rich diet was given at the time point of EAE induction, it delayed the time before EAE symptoms appeared in guinea pig spinal cord homogenate-immunized dark agouti rats. This was associated with increased myelination of axons in the spinal cord (75). A ketogenic diet, characterized by a high fat to protein and carbohydrate diet, also reduced the EAE clinical score when given

before EAE induction. This was associated with reduced T_{h1} and T_{h17} cells in the CNS and lymph nodes (the authors did not specify which lymph nodes). Additionally, mice on a ketogenic diet had reduced levels of pro-inflammatory cytokines in their lymph nodes and CNS (76). Another study confirmed the beneficial effect of the same ketogenic diet but did not investigate effects on the immune system (77). The proportion and types of lipids in the diet influence the gut microbiota composition. In healthy C57Bl/6 mice, mice were subjected to a high fat diet containing palm oil (high in saturated fatty acids), olive oil (high in monounsaturated fatty acids), safflower oil (high in Ω-6 polyunsaturated acids), or flaxseed/fish oil (high in Ω-3 polyunsaturated fatty acids). These diets were also compared to two low-fat diets, of which one contained a high percentage of calories from maize and one diet in which most calories came from sucrose. The cecum contents were tested for microbiota composition. In the palm oil group, the relative abundance of Bacteroidetes and *Bacteroidaceae* was reduced, while *Lachnospiraceae* was increased. Mice that received an olive oil-based diet had an increased proportion of *Bacteroidaceae*. In the flaxseed/fish oil group, the proportion of *Bifidobacteriaceae* and *Bifidobacterium* was increased. Within the low-fat diets, the relative abundance of *Ruminococcaceae* was increased and *Erysipelotrichaceae* was reduced in the high sucrose diet. Interestingly, cecal concentrations of SCFA were increased in the palm oil supplemented group (104). This indicates that dietary fats greatly influence the gut microbiota composition, which influences SCFA production or absorption. In this way, shifts in gut microbiota composition as a result from dietary fats may influence EAE/MS.

Other dietary components can also modify EAE disease scores. Potentially relevant for MS is the beneficial effect of vitamin D3 on EAE, as vitamin D3 deficiency is an established MS risk factor. Intraperitoneal administration of vitamin D prevents EAE development in SJL/J mice immunized with rat spinal cord homogenate when given prophylactically (65). Additionally, dietary vitamin D supplementation reduces EAE scores when given to $MBP_{79–87}$-immunized B10.PL mice (66) and $MOG_{35–55}$-immunized C57Bl/6 mice with established EAE, in which the effect was IL-10 dependent (67). DC isolated from $MOG_{35–55}$-immunized C57Bl/6 mice were also able to reduce EAE severity when incubated with 1,25-dihydroxyvitamin D3 prior to adoptive transfer into mice with EAE. Even though the proportions of T_{h1} and T_{h17} cells in lymph nodes and the spleen were increased during this treatment, their proportions in the spinal cord were reduced. Therefore, DC that were exposed to 1,25-dihydroxyvitamin D3 may reduce migration of pathogenic T cells from the periphery into the CNS (105). *In vitro* culture with high concentrations of vitamin D inhibits $CD4^+$ T cell proliferation, reduces IL-6, IL-17 producing T-cells while enhancing IL-10 producing and $CD4^+CD25^+Foxp3^+$ T cells (106). In other models, vitamin D reduced demyelination and increased remyelination (107) and exerted additional anti-inflammatory effects, such as inhibition of the T_{h1} and B-cell response and modulation of DC (108). Vitamin D may also inhibit the bacteria-induced pro-inflammatory NF-κB pathway and affect tight junction expression, contributing to proper intestinal barrier function (108).

Excess calorie intake and/or fasting may have a negative effect on MS/EAE. A prophylactic diet of 66% caloric restriction protected Lewis rats from developing EAE (69), while a prophylactic caloric restriction of 40% reduced the EAE score in several mouse models, which was associated with reduced spinal cord inflammation, demyelination, and axonal injury (68). Additionally, increased plasma levels of corticosterone, adiponectin and reduced plasma levels of IL-6 and leptin were found in animals with reduced food intake (68). These altered plasma concentrations are interesting, as corticosterone has broad inhibitory effects on the immune system, and adiponectin reduces IL-6 and TNFα production as well as induces production of IL-10R and IL-1R antagonists. Leptin induces T cell proliferation, T_{h1} differentiation, and pro-inflammatory cytokine production (68). Intermittent feeding (*ad libitum* access to food on alternating days) also had a positive effect on the EAE clinical score, but its effect on the immune system was not investigated (70). The beneficial effect of fasting on EAE disease score was confirmed in another study, in which mice were fed in cycles of 4 days *ad libitum*, followed by 3 days of fasting (first day of fasting 50%, then 2 days of 10% of normal caloric intake). This intervention was able to reduce the clinical EAE score, even in mice with established EAE. The suppression of EAE was associated with reduced immune cell infiltration into the spinal cord, reduced splenic $CD11^+$ DC, reduced pro-inflammatory cytokines, increased corticosterone in serum, increased T_{reg}, and reduced T_{h1} and T_{h17} cells in the lymph nodes and spleen. In addition, fasting protects oligodendrocytes from apoptosis and stimulates maturation of oligodendrocyte precursors (77). The effect on oligodendrocytes is seen in EAE and also in a cuprizone model, which demonstrates that fasting affects oligodendrocytes also in an autoimmunity-independent way (77).

Increased salt intake increased the EAE disease score in $MOG_{35–55}$-immunized C57Bl/6 mice (78–81). This effect was associated with increased activity of T_{h17} cells in the spinal cord and spleen. It is thought that the effect of high sodium is DC independent, as DC function is not altered by exposure to salt (78). Instead, sodium directly influences T_{h17} differentiation. Two studies found that high sodium induces the expression of serum/glucocorticoid kinase 1 (SGK-1) in naïve T cells (80, 81). SGK-1 promotes IL-23R expression and induces T_{h17} differentiation (80). The importance of SGK-1 signaling is shown by the observation that a high-salt diet increases the severity of EAE in mice, while SGK-1-deficient mice develop less severe EAE. This effect was associated with reduced T_{h17} cells in the CNS and mesenteric lymph nodes in SGK-1-deficient mice (80). Dietary tryptophan can be metabolized into a variety of AHR ligands. AHR ligands have shown different effects on EAE when given prophylactically. The tryptophan-derived AHR ligand 6-formylindolo[3-2b]carbazole (FICZ) accelerates EAE onset and increases pathology in $MOG_{35–55}$-immunized C57Bl/6 mice. This is associated with increased IL-17 and IL-22 producing $CD4^+$ T cells in the spinal cords (82). Another study verified that FICZ increases EAE severity and shows that FICZ-treated mice have increased $IL\text{-}17^+CD4^+$ and $IFN\gamma^+CD4^+$ T cells in the spleen (83). In addition, they show that tryptophan-derived

2-(1′H-indole-3′carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE) reduces EAE severity. Stimulation with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), a less natural AHR ligand, induces T_{reg} cells, which are protective of EAE also after adoptive transfer (83). Even though in these studies the AHR ligands were injected rather than supplemented through diet, it shows the immunomodulatory effects of different AHR ligands which may also be taken through the diet. AHR signaling may also have protective effects through astrocytes. Mice fed a tryptophan-deficient diet develop increased EAE severity. This effect could be reversed by tryptophan supplementation in control mice, but not in astrocyte-specific AHR knockout mice. Gut microbiota mediate the conversion of tryptophan into AHR ligands. Depletion of these microbiota by ampicillin treatment increased EAE disease scores, which can be reduced by supplementation with multiple tryptophan metabolites and the bacterial enzyme tryptophanase. This shows a protective effect of the interplay between dietary tryptophan, gut microbiota, AHR ligands, and astrocytes (84).

Zinc can also influence EAE. *In vitro*, salt composed of zinc with the amino acid aspartate (zinc aspartate = ZnAsp) reduces the proliferation of stimulated human T-cells (85, 86) and also reduces their production of IL-2, IL-10, IL-17 (85), IFNγ, TNFα, GM-CSF, and IL-5 (86). The same effects of ZnAsp were seen in mouse splenocyte cultures (85, 86). In $PLP_{139–151}$-immunized SJL mice, intraperitoneal injection of 30 μg ZnAsp per day reduced the EAE clinical score prophylactically as well as therapeutically, while 120 μg increased the EAE severity (85). An oral dose of 6 or 12 μg ZnAsp per day reduced the clinical disease score in the same EAE model, while an oral dose of 30 μg increased the disease score (86). Zinc supplementation also affected EAE in the $MOG_{35–55}$-immunized C57Bl/6 model. Zinc supplementation in drinking water (87) and daily intraperitoneal injections of 6 and 30 μg ZnAsp both reduced the EAE clinical score when given before EAE induction (88). The mechanisms underlying the effect of ZnAsp injections include reduced systemic T_{h17} cells and increased $Foxp3^+$ T-cells in the spinal cord (88). Lower plasma or serum concentrations of zinc have been found in MS patients compared to healthy individuals, while others have found no differences. Mice with EAE had reduced zinc plasma levels on day 21 after EAE induction compared to naïve mice (86). Given the beneficial effects of zinc supplementation in EAE, normalizing zinc levels in MS patients with zinc deficiency may be tested in clinical trials, but a high dosage or long-term supplementation may also have detrimental effects, thus patient serum concentrations in these trials must be tightly monitored (86). A zinc-deficient diet of 10 days did not alter the microbiota composition at phylum level and total bacterial abundance in healthy C57Bl/6 mice compared to those that were fed a normal diet. The relative abundance of several bacterial genera did differ between these diet groups, which included the genera *Enterococcus, Enterobacteriaceae, Paenibacillus, Granulicatella, Clostridium, Akkermansia*, and *Burkholderia* (109).

High dietary selenium (10× normal intake) increased EAE incidence and severity in guinea pigs, while those on normal and half amounts of selenium developed EAE in a normal fashion (89).

Intraperitoneal injection of diphenyl diselenide was toxic for whole MBP-immunized Wistar rats, while oral administration 1 week after immunization resulted in reduced EAE incidence and symptoms. This was associated with reduced macrophage numbers in the CNS and reduced MBP-specific T-cell proliferation. The EAE-reducing mechanism of diphenyl diselenide is thought to be based on reduced NF-κB signaling in macrophages and T-cells. In addition, diphenyl diselenide may increase ROS clearance as it mimics glutathione peroxidase activity, which protects against oxidative damage (90). The different effects of dietary selenium on EAE may be explained by differences in formulation.

Vitamin E has potent antioxidant properties. Multiple compounds are considered part of the vitamin E group, of which tocopherol is best studied. Intraperitoneal administration of tocopherol reduced the EAE clinical scores in $MOG_{35–55}$-immunized C57Bl/6 mice, which was associated with reduced MOG-specific splenocyte proliferation. In addition, splenocytes incubated with tocopherol produced less IFNγ (91). Vitamin E also increases remyelination and reduces demyelination in an animal model where demyelination is chemically induced (107). TFA-12, a synthetic tocopherol derivative, reduced the EAE clinical scores in $MOG_{35–55}$-immunized C57Bl/6 mice when injected intraperitoneally at the onset of EAE symptoms. This was associated with reduced inflammation of the CNS, astrogliosis, and demyelination. TFA-12 also accelerated remyelination in a chemically induced demyelination model. The mechanism behind this is believed to be due to the induction of oligodendrocyte maturation (92).

Vitamin A and its metabolites all-trans retinoic acid and 9-cis-retinoic acid can also reduce EAE severity. Prophylactic dietary 13-cis-retinoic acid and 4-HPR (a synthetic retinoid derivative) reduced EAE incidence in SJL mice which received MOG-incubated lymph node cells to induce EAE. Dietary 4-HPR can also reduce EAE severity in a therapeutic manner (93). In $MOG_{35–55}$-immunized C57Bl/6 mice, intraperitoneal injection with all-trans retinoic acid reduced the EAE clinical score when given prophylactically (94) as well as therapeutically (95). This was associated with reduced DC maturation and reduced pro-inflammatory monocytes in the subarachnoid space. Reduced T_{h1} and T_{h17} cells were also found in the draining lymph nodes. In addition, bone mesenchymal DC pretreated with all-trans retinoic acid were able to reduce T_{h1} and T_{h17} differentiation and lymphocyte proliferation *in vitro* (94). All-trans retinoic acid also directly affected naïve $CD4^+$ T cells, as it inhibited T_{h17} differentiation by reducing expression of the IL-6 and IL-23 receptor (95). Even though all-trans retinoic acid can induce $Foxp3^+$ T cells *in vitro*, the frequency of these cells was not increased in EAE-affected mice treated with the metabolite (95). *In vitro*, 9-cis-retinoic acid reduced pro-inflammatory cytokine production in LPS-stimulated microglia and reduced TNF-α and nitric oxide production in astrocytes (110). Mechanisms underlying gut microbiota and dietary interventions are summarized in **Box 2**.

BOX 2 | Mechanisms underlying microbiota and dietary interventions in experimental autoimmune encephalomyelitis (EAE).

Most interventions are mediated by the induction of anti-inflammatory IL-10 producing T_{regs}. These cells suppress pro-inflammatory cytokine production and T cell proliferation. In addition, multiple other mechanisms have been unraveled.

Germfree mice are resistant against EAE. The EAE resistance is attributed to reduced recruitment and activation of autoantibody producing B cells (47) as well as dendritic cells (DC) and the reduced capacity of these professional antigen-presenting cells (APC) to stimulate pro-inflammatory T cell responses (48).

Antibiotic treatments reduce EAE *via* invariant natural killer T (iNKT) cells and $CD1^{high}CD5^{+}$ B cells. Antibiotic treatments likely suppress pro-inflammatory cytokine production by iNKT cells located in the lamina propria, which reduces T_{h17} development (49). Antibiotic treatment also induces IL-10 producing $CD1^{high}CD5^{+}$ B cells in distant lymph nodes, and adoptive transfer of $CD1^{high}CD5^{+}$ B cells protect against EAE (51). Antibiotic treatments increase $CD11c^{high}CD103^{+}$ DC in mesenteric lymph nodes, which are able to induce $Foxp3^{+}$ T cells (50).

Polysaccharide A (PSA) is captured by DC in the gut *via* a TLR-2 dependent mechanism. DC then migrate to the mesenteric lymph nodes and induce IL-10 producing $CD4^{+}$ T_{regs}. A type of IL-10 producing T_{reg} specifically induced by PSA expresses the ectonucleosidase CD39 on its surface. $CD39^{+}$ T_{reg} cells have increased migratory capacity, are more abundant in the central nervous system (CNS) of PSA-treated mice, and are protective against EAE. The EAE modulatory capacity relies on the conversion of ATP into adenosine, which increases the anti-inflammatory effects of T_{regs} and suppresses effector cells, thus protecting against CNS inflammatory tissue damage (61, 99). Additionally, PSA treatment causes accumulation of $CD103^{+}$ DC in the cervical lymph nodes, which converses naïve $CD4^{+}$ T cells into protective IL-10 producing $Foxp3^{+}$ T cells. The mechanism causing the accumulation of $CD103^{+}$ DC in the cervical lymph nodes is unknown (59).

Short-chain fatty acids (SCFA), such as butyrate and propionic acid, are produced by bacterial fermentation of dietary fibers in the colon or consumed with the diet. SCFA induce T_{regs} through multiple known mechanisms. Oral administration of propionic acid can induce $CD4^{+}CD25^{+}Foxp3^{+}$ T_{reg} cells, while simultaneously reducing T_{h17} responses (64). Butyrate can stimulate DC, macrophages, and epithelial cells to produce cytokines that facilitate the differentiation of naïve $CD4^{+}$ T cells into anti-inflammatory $Foxp3^{+}$ T cells (100). *Dietary components* influencing EAE also include: (1) Different types and ratios of lipids, such as **Ω**-6 fatty acids, **Ω**-3 fatty acids and ketogenic diets reduce EAE by affecting splenic mononuclear cells (71) or T cells (73). (2) *Vitamin D3* has a broad effect on the immune system (108). In EAE, its effect is dependent on IL-10 (67). (3) *Vitamin E* has antioxidant properties (91), accelerates remyelination (107), and also affects splenocytes (91). (4) *Vitamin A* affects DC (94) and reduces T_{h1} and T_{h17} differentiation (95). (5) *Zinc* increases $Foxp3^{+}$ T cells (88), reduces T cell proliferation, and reduces their pro-inflammatory cytokine production (85, 86). (6) *Selenium* protects against oxidative damage and also affects T cells (90). (7) *Caloric restriction/fasting* reduces EAE, which is associated with changes in plasma levels of corticosterone, adiponectin, IL-6, and leptin (68). In addition, fasting reduces DC, T_{h1}, T_{h17} cells and increases T_{reg} cells as well as stimulates remyelination by oligodendrocytes (77). (8) A *high-salt* diet increases the T_{h17} response, which is likely DC independent. Instead, salt induces SGK-1 signaling, leading to increased T_{h17} differentiation. (9) *Aryl hydrocarbon receptor ligands* can have multiple effects on EAE. Cruciferous vegetables such as broccoli are rich in indole-3-carbinol, which upon contact with stomach acid, is converted into aryl hydrocarbon receptor ligands and can induce $CD4^{+}$ IEL. These cells can migrate into the CNS and are protective against EAE (62). 6-Formylindolo[3-2b]carbazole, a different aryl hydrocarbon receptor (AHR) ligand, increases the EAE disease score, while the tryptophan-derived AHR ligand ITE reduces EAE clinical signs. Tryptophan-derived AHR ligands limit astrocyte-mediated inflammation in the CNS (84). Aryl hydrocarbon receptor ligands may also act on type 3 ILC (17).

GUT MICROBIOTA IN MS PATIENTS

Comparing the Gut Microbiota of MS Patients with Healthy Controls

Colonization with bacteria and oral treatment with bacterial products and diet are all exogenous factors *via* which the clinical and/or pathological expression of EAE can be modified. It is therefore of considerable interest whether differences exist between the gut microbiota composition of MS patients compared to healthy individuals and to test whether treatment concepts developed in EAE can be translated to humans. Studies analyzing gut microbiota of MS patients are summarized in **Table 3**. Of these studies, eight have investigated whether differences exist in the microbiota composition of MS patients compared to healthy individuals, for which collectively almost 250 MS samples were tested. A reduced abundance of SCFA producers such as *Lachnospiraceae*, *Prevotella, Faecalibacterium prausnitzii*, and *Butyricimonas* in the gut of MS patients has been repeatedly reported. As SCFA reduced the disease score in several EAE models, reduced SCFA production in the gut of MS patients may have an impact on MS pathology. Of note, *F. prausnitzii* produces a microbial anti-inflammatory molecule (MAM), which has anti-inflammatory effects in a mouse model of colitis. MAM displays an inhibitory effect on nuclear factor-κB activity in human epithelial cells. Moreover, administration of *L. lactis* delivering a MAM-encoding plasmid reduced morbidity in an animal colitis model, whereas wild-type *L. lactis* had no effect (111). By producing MAM, *F. prausnitzii* adds to an anti-inflammatory milieu in the gut. Additionally, the gut of MS patients harbors higher numbers of the archaea *Methanobrevibacter* compared to healthy individuals. As mentioned earlier, lipids from *Methanobrevibacter* are thought to elicit inflammatory reactions (112) and therefore possibly add to a pro-inflammatory milieu in the gut of MS patients. Two studies found that *Enterobacteriaceae* are increased in MS patients compared to healthy individuals. The *Enterobacteriaceae* family of gut bacteria includes pathogenic as well as non-pathogenic/opportunistic species. Increased *Enterobacteriaceae* abundance has been found in patients with inflammatory bowel disease (IBD). However, it is unclear if this dysbiosis is a cause or consequence of the IBD (3). Several *Enterobacteriaceae* members have a competitive growth advantage under inflammatory conditions in the gut of mice (113), and therefore, it is conceivable that *Enterobacteriaceae* abundance increases after IBD develops. Studies investigating the role of *Enterobacteriaceae* in EAE models were not found. Of note, children with autism and patients with Parkinson's disease have increased *Enterobacteriaceae* abundance in their gut microbiota, indicating that *Enterobacteriaceae* could possibly play a role in CNS disorders (114). Only one study found that *B. fragilis* was reduced in a cohort of 18 pediatric MS patients compared to healthy individuals (115). *In vitro,* PSA induced expression of CD39 and Foxp3 on naïve human $CD4^{+}$ T cells and increased IL-10 production (116). Even though only one study found a difference in the abundance of *B. fragilis* (115), the beneficial effect of PSA on EAE may be translatable to corresponding human diseases, such as MS.

TABLE 3 | Gut microbiota studies in MS patients.

Reference	Number of subjects, type of MS	Main findings
Tremlett et al. (115)	18 RR pediatric, 17 HC	No difference in species richness. Increased *Desulfovibrionaceae, Methanobrevibacter*[b], *Enterobacteriaceae*[b]. Reduced *Lachnospiraceae*[a], *Ruminococcaceae*[a], *Faecalibacterium prausnitzii*[d], and *Butyricimonas*[a] *Bacteroides fragilis*[c]. Functional prediction of microbial genes indicated increased glutathione metabolism in patients
Tremlett et al. (117)	17 RR pediatric	Depletion of Fusobacteria is associated with increased risk on earlier relapses. Higher abundance of Firmicutes and Euryarchaeota trended to be associated with increased risk on earlier relapses
Tremlett et al. (118)	15 RR pediatric, 9 HC	No difference in blood Foxp3$^+$ T_{reg} frequency and intracellular production of IFNγ, IL-17, IL-4, and IL-10 by CD4$^+$ T cells. IL-17$^+$ T cells correlated with gut microbiota richness in MS patients. IL-17$^+$ T cells inversely correlated with Bacteroidetes abundance in patients. T_{reg} frequency correlated with Fusobacteria abundance in healthy controls
Miyake et al. (119)	20 RR, 40 HC, 18 HC	No difference in species richness. Reduced *Faecalibacterium*[d], *Prevotella*[a], *Anaerostipes*, butyrate-producing bacterium A2-175[a], and SL7/1[a]. Trends in increased *Bifidobacterium, Streptococcus*
Chen et al. (120)	31 RR, 36 HC	No difference in species richness. Increased *Pedobacter, Flavobacterium, Pseudomonas, Mycoplana, Blautia, Dorea,* and *Haemophilus*. Reduced *Parabacteroides, Adlercreutzia, Collinsella, Lactobacillus,* and *Prevotella*[a]. Reduced fatty acids metabolism and increased phytoestrogen metabolism were predicted by functional analysis
Jangi et al. (112)	60 RR, 43 HC	Increased *Methanobrevibacter*[b], *Akkermansia.* Decreased *Butyricimonas*[a]. Untreated patients had reduced *Prevotella*[a], *Sutterella,* increased *Sarcina*. Positive correlations between *Methanobrevibacter*[b], *Akkermansia*, and T-cell/monocyte gene expression implicated in MS pathology. Negative correlations for *Butyricimonas*[a] with gene expressions implicated in MS pathology
Jangi, et al., abstract (121)	22 untreated, 13 GA, 18 IFN-β, 44 HC	Increased *Methanobrevibacteriaceae*[b] in patients compared to HC. Reduced *Butyricimonas*[a] in untreated patients compared to HC. Both treatment groups had increased *Lachnospiraceae*[a] abundance compared to untreated MS patients
Baum et al.,abstract (122)	54 MS patients vs healthy controls (the amount of controls is not stated in the abstract)	Increased *Atopobium*, *Bifidobacteriae*. Reduced *Bacteroidaceae* in MS patients
Cantarel et al. (123)	7 RR vitamin D-deficient, before and after vitamin D supplementation, 8 HC	Reduced *Bacteroidaceae, Faecalibacterium*[d], increased *Ruminococcus*[a] in patients. Vitamin D supplementation increased *Faecalibacterium*[d], *Akkermansia*, and *Coprococcus*[a]. GA treatment affected gut microbiota
Sand et al., abstract (124)	Not specified in abstract	Increased *Enterobacteriaceae*[b] in female patients compared to HC. GA treatment affects gut microbiota
Tankou et al., abstract (125)	43 untreated MS patients, disease subtype not specified	Patients with <40 ng/mL serum vitamin D concentration had lower *Ruminococcaceae*[a] compared to patients with higher vitamin D levels
Telesford et al. (116)	*In vitro* cultures of patients and HC	PSA from *B. fragilis*[c] induced CD39$^+$ T_{reg} cells *in vitro*

[a]*SCFA producer.*
[b]*Likely pro-inflammatory.*
[c]*PSA producer.*
[d]*MAM producer.*
MAM, microbial anti-inflammatory molecule; MS, multiple sclerosis; PSA, polysaccharide A; RR, relapsing remitting; SCFA, short-chain fatty acids.

Taken together, the gut microbiota composition of MS patients and healthy individuals likely differs, as reflected by a different abundance of SCFA producing bacteria, *F. prausnitzii, Methanobrevibacter,* and *Enterobacteriaceae* (see **Figure 5**). Not all studies reported the same differences between MS patients and healthy individuals. This may be explained by differences in sampling methods, molecular techniques, and differences in study populations such as their diet, received treatments, type of MS, or ethnicity. In addition, the populations of tested MS patients varied among studies. Differences in the gut microbiota of pediatric MS patients compared to adult MS patients may exist. Moreover, some studies divided patients in groups based on treatments, while others only separated patients from healthy individuals. Therefore, there is a great need of large controlled studies to determine which results from multiple small studies can be replicated in a bigger cohort. It is also not clear whether the dysbiosis in SCFA-producing bacteria, *F. prausnitzii, Methanobrevibacter*, and *Enterobacteriaceae* adds to the pathology of MS, or if it is a result of MS pathology or treatments.

Prospective Microbiota Studies in MS Patients

Two relatively small studies have tried to link gut microbiota to clinical outcomes or changes in the immune system of MS patients.

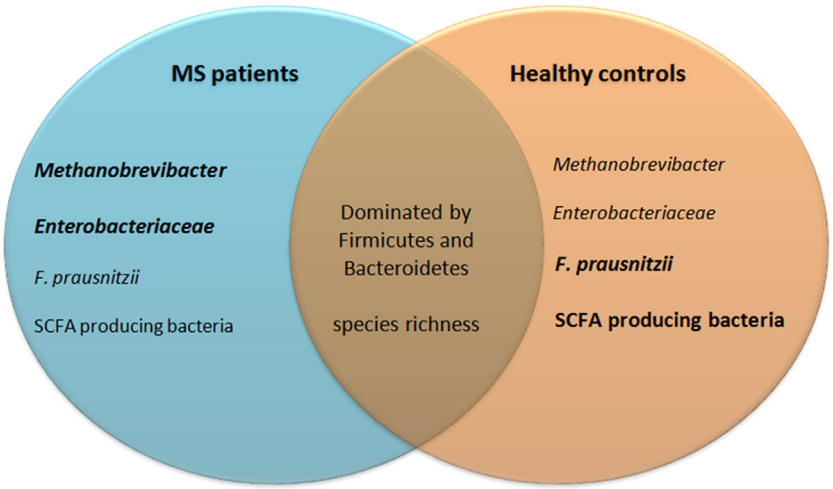

FIGURE 5 | Similarities and differences in gut microbiota of MS patients and healthy controls. Eight studies investigating almost 250 patient fecal samples for differences in microbiota composition were analyzed. Many species were shown differently present. Only differences that have been reproduced by at least one other study are included in this figure. The gut microbiota of both MS patients and healthy controls are dominated by bacteria from the phyla *Firmicutes* and *Bacteroidetes* and their species richness does not differ. MS patients may have increased *Methanobrevibacter* and *Enterobacteriaceae*, but reduced *Faecalibacterium prausnitzii* and SCFA producing bacteria. SCFA, short-chain fatty acids; MS, multiple sclerosis.

In a cohort of 17 pediatric MS patients, the absence of *Fusobacteria* was associated with increased risk on earlier relapses. Additionally, trends in increased abundance of *Firmicutes* and *Euryarchaeota* were observed which may be associated with increased risk of earlier relapses (117). In 15 pediatric MS patients, no difference was found in blood $CD4^+$ T_{reg} cells compared to healthy individuals, but IL-17^+ T cells correlated with species richness and inversely correlated with *Bacteroidetes* in patient stools (118). These studies are an interesting indication that it may be possible to modulate the gut microbiota as a treatment of MS. However, studies performed thus far included far too few patients to be able to determine a causal relationship between the abundance of certain microbial species and symptoms or immune responses. The effect of MS treatments on gut microbiota has also only been investigated in relatively small studies thus far. Preliminary data suggest that both glatiramer acetate treatment and vitamin D supplementation affect gut microbiota (see **Table 3**).

The aforementioned microbiota studies in MS patients are possibly biased as fecal/stool samples were used for characterization of gut microbiota. The composition of microbiota varies from the mucosal surface to the lumen, and fecal samples mostly detect luminal microbiota. Hence, differences in mucosa-associated species may be underappreciated (17). *B. fragilis* is one of the few species that can penetrate the tight inner mucus layer of the colon and can colonize the colonic crypts (15). It is, therefore, interesting to investigate whether mucosa-associated *B. fragilis* is differently present in patients. Moreover, as fecal samples more closely reflect microbiota composition of the colon than of the small intestines, differences in microbiota composition of the small intestines are undetected (16).

Potential Role of PGN in MS

Another way in which gut microbiota may influence MS is through PGN, a bacterial cell wall component. PGN is ubiquitously present in the gut and can be phagocytosed into APC (142). PGN is sensed through NOD-like and toll-like receptors, which induces pro-inflammatory mitogen-activated protein kinase and NF-κB (143). Microbiota-dependent NOD1 signaling increases the lifespan of circulating neutrophils and monocytes (144), and PGN has strong adjuvant properties, as mice injected with $MOG_{35–55}$ and incomplete Freund's adjuvant did not develop EAE, while when PGN was added to the mixture mice did develop EAE (145). Mechanistically, PGN modulates DC, which leads to T_{h1} cell expansion (145). In the rhesus monkey and marmoset EAE model (146) as well as human MS (147), PGN is found in APC located in the brain. Another study in autopsied brain samples revealed that the expression of bacterial components in MS samples differed from non-MS samples. In both sample groups, the dominant phylum was Proteobacteria, but less diversity was observed in progressive MS samples. In addition, Actinobacteria were enriched in RRMS samples. The total amount of PGN in the brain did not distinguish MS samples from non-MS samples. But within MS lesions, PGN inversely correlated with myelin density and was associated with several genes of the immune system, including NF-κB (34). The presence of a TLR/NLR ligand in the brain, possibly originating from gut microbiota, may contribute to MS pathology by influencing inflammation, demyelination or remyelination, and therefore, more research is needed to elucidate the role of microbial compounds in MS brains.

DIETARY STUDIES IN MS PATIENTS

How different food components may affect MS has been reviewed comprehensively by Schmitz et al. (141) of which the most important ones are summarized in **Table 4**. Multiple dietary intervention studies have been done in MS patients, but most were unsuccessful in reducing MS severity and have not

examined the effects on microbiota. Vitamin D supplementation did show positive outcomes as it led to fewer relapses and reduced pro-inflammatory cytokines such as IFNγ and IL-4 in T-cells. Additionally, vitamin D supplementation increased peripheral IL-10^{+}CD4^{+} T cells, suppressed T-cell proliferation, and reduced the number of gadolinium-enhancing regions per patient (13, 141). However, these results came from small studies and more randomized controlled trials investigating the effect of vitamin D for MS treatment are now taking place. The effect of vitamin D might in part be exerted *via* the gut microbiota, as in healthy individuals high vitamin D intake was associated with increased proportions of *Prevotella* and reduced proportions of *Haemophilus* and *Veillonella* (148). Vitamin D supplementation in vitamin D-deficient MS patients altered the relative proportions of different genera, but this study included very few subjects (123). In a study of 70 and a replication study of 59 RR MS patients, the influence of salt on MS was studied. As 80–90% of salt intake is excreted in urine, salt excretion rate in urine was measured as a proxy for salt intake. High salt excretion was associated with increased relapse rates, increased risk on developing a new lesion detected by MRI scans, and increased T2 lesion load. However, causality was not established. Patients with relapses are

TABLE 4 | Dietary studies in MS patients.

Reference	Number of subjects, type of MS	Diet-groups	Main findings
Bates et al. (126)	292 RRMS	Ω-3 EPA and DHA vs oleic acid supplementation. Both groups also had vitamin E and antioxidant supplementation	No difference in relapse rate or EDSS
Weinstock-Guttman et al. (127)	27 RRMS	Low-fat diet (<15% calories) with Ω-3 EPA and DHA supplements vs low fat (<30% calories) with oleic acid supplements. Both groups received vitamin E, multivitamin, and calcium supplementation	No difference in relapse rate or EDSS between groups. Relapse rates, reduced compared to 1 year before the start of this study. EPA/DHA group had increased physical and mental parameters. Reduced fatigue score in the oleic acid group
Torkildsen et al. (128)	99 RRMS	Ω-3 EPA and DHA vs corn oil supplementation	No difference in relapse rate, EDSS, quality of life, and fatigue scores
Bates et al. (129)	134 SPMS	Ω-6 Linoleic acid and γ-linolenic acid vs linoleic acid vs oleic acid supplementation	No difference in relapse rate or EDSS
Bates et al. (130)	104 PPMS	Ω-6 Linoleic acid and γ-linolenic acid vs linoleic acid vs oleic acid supplementation	No difference in relapse rate or EDSS. High-dose linoleic acid group had less severe relapses
Harbige and Sharief (131)	28 RRMS	Ω-6 Linoleic acid supplementation vs placebo	Reduced relapse rate, improved EDSS
Jafarirad et al. (132)	35 RRMS	Vitamin A (retinyl palmitate) or placebo supplementation	Reduced T cell proliferation when incubated with MOG
Wingerchuk et al. (133)	15 RRMS	Vitamin D supplementation, uncontrolled	Reduced EDSS compared to baseline
Mahon et al. (134)	39 MS patients, subtype not specified	Vitamin D3 supplementation vs placebo. Both groups received calcium supplementation	Increased TGF-β concentration in serum of vitamin D3 supplemented group. No differences in TNFα, IFNγ, and IL-13 concentrations
Goldberg et al. (135)	10 MS patients, subtype not specified	Vitamin D3, calcium, and magnesium supplementation, uncontrolled	Fewer relapses than expected
Choi et al. (77)	48 RRMS	Cycles of fasting vs ketogenic diet vs control	Fasting and diet group had improved health related quality of life, reduced disability scores. Fasting and ketogenic diet were well tolerated
Haghikia et al. (136)	Not specified in abstract	Propionic acid treatment in patients and HC	No side effects of PA. 25–30% increase of T_{reg} and reduced T_{h17} cells in both groups
Farez et al. (137)	70 RRMS, replicated by a separate group of 52 patients		High salt excretion is associated with increased disease activity
Hadgkiss et al. (138)	2,087 MS patients, subtype not specified		A general healthy diet, based on fruit, vegetable, fat, meat and dairy consumption was associated with better clinical scores
Rezapour-Firouzi et al. (139)	65 RRMS	Three groups: 1. hemp seed/evening primrose oil; 2. olive oil; and 3. cosupplemented oil vs baseline measurements. Subjects were advised to have a general healthy diet	Reduced relapse rates, EDSS in groups 1 and 3 compared to baseline
Nordvik et al. (140)	16 RRMS	General health lifestyle and A, B, D, E, Ω-3 fatty acid supplementation	Reduced EDSS compared to baseline

Dietary studies in MS patients have been extensively reviewed by Schmitz et al. (141).

AHR, aryl hydrocarbon receptor; CNS, central nervous system; DC, dendritic cells; DHA, docosahexaenoic acid; EDSS, expanded disability status scale; EAE, experimental autoimmune encephalomyelitis; EPA, eicosapentaenoic acid; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; SGK-1, serum/glucocorticoid kinase 1.

often treated with steroids, which influences salt excretion (137). Clinical trials with controlled salt intake are needed to assess the impact of increased salt intake on MS severity.

In a cohort of 2,087 MS patients, a general healthy diet (based on fruit/vegetable, fat, meat, and dairy consumption) was associated with better clinical scores (138). Therapeutically, two studies have shown effect of a general healthy diet (low intake of saturated fatty acid, sugar, coffee, alcohol, and high intake of vegetables, fruits, fish and whole grain products) on MS clinical scores when combined with supplementation of oils rich in Ω-6 or Ω-3 fatty acids (139). Of note, one of these studies also supplemented multiple vitamins including vitamin A, B, D, and E (140). Since these studies used multiple dietary components as intervention, it is hard to determine which part of the intervention exerted the beneficial effects. A difference in fat consumption may be responsible, since a third study found that reduced fat intake supplemented with Ω-3 fatty acids reduces MS clinical scores (127). It is noteworthy that the abovementioned studies related results to baseline measurements instead of a control group. Because dietary studies in MS can hardly be blinded and patients in the control groups have little incentive to complete a study, control groups have high dropout rates. Efforts are now being made to develop study designs that increase the participation of patients in control groups and to test if an observation period prior to a test diet can serve as a control group (149). Multiple studies are currently listed on www.clinicaltrials.gov as recruiting or ongoing, including studies testing diets such as ketogenic, low sodium, low saturated fat, caloric restriction, and a variation of Paleolithic diet (low grain, high vegetables). These studies should provide additional insight into which compounds may be responsible for the positive effects of a generally healthy diet. Preliminary results of oral treatment with propionic acid are promising. Healthy individuals and patients were treated with propionic acid, which increased T_{reg} frequency and reduced T_{h17} frequency in both groups without any side effects; only an abstract of this study is available (136).

METHODS TO MANIPULATE GUT MICROBIOTA FOR THERAPEUTIC PURPOSES

Current evidence suggests that MS patients may have a different gut microbiota composition compared to healthy individuals and that gut microbiota may affect the relapse rate (see **Table 3**). If these results will be verified in larger studies, it would be interesting to test whether manipulation of the gut microbiota of MS patients induces immunosuppression. Multiple approaches can be used to manipulate the gut microbiota (see **Box 3**). Probiotics involve the treatment with live microorganisms that are absent or present in too low abundance in the host. In this way, bacteria that induce immunosuppression can be introduced in patients. Probiotics are already being used in intestinal diseases such as IBD or antibiotic-associated diarrhea (17). Prebiotics are food ingredients with which gut microbiota may be modified. Resistant starches, which can reach the colon before being metabolized, alter the gut microbiota and have been associated with increased butyrate production. Additionally, fibers can be fermented by gut microbiota that produce SCFA. However, the effect of prebiotics depends on microbial strains that are already present in the host. Therefore, high individual variation and a large number of non-responders are seen (150). Probiotics and prebiotics can also be used in conjunction, i.e., as synbiotic, to augment their individual effectiveness. The probiotics part introduces new strains, while the prebiotic part synergistically increases the efficacy of the introduced species due to increased survival, competition, or metabolic activity (150). Antibiotics can be used to remove or suppress unwanted, immunogenic strains from the gut microbiota. A downside of antibiotic treatment is that multiple strains are targeted and beneficial bacteria may also be depleted. This increases the risk of opportunistic infections. Moreover, in mice, vancomycin treatment disrupts complex carbohydrate fermentation and leads to reduced concentrations of SCFA, including butyrate and propionic acid (151). Phages may be employed for more specific targeting of immunogenic parts of the gut microbiota. In this way, beneficial bacteria remain untouched and immunosuppression may still occur. Phages may also be used to increase the competitiveness of strains that need to be introduced. However, phage therapy still requires more research before it can be implemented, as there is limited evidence of efficacy in human clinical trials (152). Fecal microbiota transplantation involves the administration of a liquid filtrate of a healthy person's stool to a diseased recipient. In this way, the recipient gut microbiota can be modulated to resemble the gut

BOX 3 | Methods to alter or use microbiota as a therapeutic intervention.

Probiotics can be used to introduce strains that are missing in hosts. Since multiple sclerosis (MS) patients have reduced butyrate producing strains in their gut flora, these strains may be restored using probiotics.

Prebiotics can be used to enrich strains that are deficiently present in the hosts. Resistant starches have been associated with increased butyrate production. Additionally, fibers can be fermented by gut microbiota which produce SCFA. The effect of prebiotics is dependent on microbial strains that are already present in the host. Therefore, high individual variation and a large number of non-responders are seen (150).

Synbiotics contain a mixture of probiotics and prebiotics. This mixture introduces new strains while synergistically increasing their effectiveness due to increased survival, competition, or metabolic activity of the introduced strain (150).

Antibiotics can be used to reduce unwanted strains in the gut microbiota. However, the effect of antibiotics is often very broad and targeting many strains at once also depletes beneficial bacteria, including short-chain fatty acids (SCFA)-producing bacteria (151).

Phage therapy has the key advantage that it can be used to selectively reduce strains of bacteria, but is not yet in clinical use (152). In the future, phage therapy may specifically target pro-inflammatory bacteria that are increased in MS patients, while beneficial strains remain untouched.

Fecal microbiota transplantation can be used in patients who have a dysbiosis to make their gut microbiota composition more closely resemble the composition of a healthy donor. Strains that are deficient in patients with a dysbiosis can be induced in the recipient using fecal microbiota transplantation, while excessive strains may be reduced through competition and possibly cross talk between gut flora and the immune system (153).

Microbial products such as polysaccharide A (PSA) or SCFA may be administered to induce an anti-inflammatory response in the gut. Chemical alterations may be needed to promote therapeutic efficacy and dosage/kinetic studies will need to elucidate how to best use these products (100).

Dietary interventions such as SCFA, Ω-3/6 fatty acid, vitamin, or trace element supplementation, reduced salt intake or caloric restriction may also be used to reduce MS.

microbiota of the donor. New strains may be introduced by the transplantation, and through competition and possibly cross talk between gut flora and the immune system, excessive strains may be reduced and dysbiosis could be normalized. Fecal microbiota transplantation has been effective in small randomized controlled trials for the treatment of recurrent *Clostridium difficile* infection. Based on these successes, clinical trials are now being done to investigate the efficacy of fecal microbiota transplantation in IBDs (153). Fecal microbiota transplantation may be a relatively safe option for restoration of a healthy microbiome in MS patients. Instead of manipulating microbiota, microbial products that induce immunosuppression may also be used for therapeutic purposes. Chemical alterations may have to be made to make sure that the compounds reach their site of action and act through their intended mechanism. SCFA are produced by bacteria in the colon, but when orally administered, a large proportion of SCFA are thought to be absorbed in the small intestine (100). Therefore, dosage and kinetic studies will have to point out how optimal use can be made of these products. The advantage of bacterial compounds over live bacterial species is that bacterial compounds can be dosed like any other drug, while live bacterial species are affected by colonization and may therefore lead to variable interindividual effects (37).

CONCLUSION AND FUTURE DIRECTIONS

This review shows that many different microbial species and dietary interventions affect EAE disease expression and differences in gut microbiota between MS patients and healthy individuals may exist. Therefore, altering the gut microbiota of MS patients, possibly through dietary interventions is a potential therapeutic aim. Thus far, multiple small studies found that SCFA producing bacteria are reduced in patients with MS, and the PSA producing bacterium *B. fragilis* may also be reduced although *B. fragilis* is only found differently present in the gut by one study. Potentially pro-inflammatory *Methanobrevibacter* and *Enterobacteriaceae* may be increased in patients with MS. In addition, small studies have tried to determine if a causal relationship exists between gut microbiota and MS severity or immune responses. To determine if the results found in small studies are true and to determine if a causal relationship exists, controlled, prospective studies are eagerly awaited. Because it is relatively easy to frequently collect stool samples, it is feasible to analyze a few 100 samples of MS patients. Of these patients, clinical data need to be gathered, such as which treatments they are on, when they have relapses, as well as immunological data such as the frequency of T cell subsets and their cytokine production. In this way, the effects of vitamin D, glatiramer acetate, and other treatments on gut microbiota can be assessed, and it will be possible to determine whether a causal relationship exists between microbiota of MS patients and their immune response in relation to disease progression.

We also propose to collect mucosa biopsies to investigate the contribution of mucosa-associated bacterial species in MS. Thus far, all studies on gut microbiota in MS patients used fecal samples, which focus on luminal microbiota. Therefore, differences in mucosa-associated species may have been left undetected. Comparing intestinal biopsies of MS patients with healthy individuals may show differences, which could be additional targets for treatment. Geva-Zatorsky et al. have recently monocolonized gnotobiotic mice with one of 53 different bacterial species and assessed their immunomodulatory effects. Even though the effects of bacteria may be different in SPF or conventionally housed mice, this does provide additional immunosuppressive species which may be combined to rationally develop probiotic mixtures (37).

In the case that species that worsen or improve MS will be identified, the gut microbiota of MS patients may be modified for therapeutic benefit. This may be achieved by using probiotics, prebiotics, or a mixture of these two: synbiotics. The role of viruses in this process needs deeper investigation. As viruses can greatly influence the bacterial diversity, phage therapy may be used in conjunction with pre-, pro-, or symbiotic mixtures to reduce competitiveness of microbiota that are already present and increase colonization of introduced microbiota. These may also be used to reduce the abundance of unwanted microbial species. An already used and relatively safe option for altering the gut microbiota is fecal microbiota transplantation. The gut microbiota of MS patients may be restored with fecal microbiota transplantation to resemble the gut microbiota of healthy individuals.

An alternative for restoration of the gut microbiota of patients is treatment with bacterial products or dietary components that may dampen immune responses, such as purified PSA or SCFA. PSA is effective in EAE models through well-known mechanisms (99), and *in vitro* studies indicate that these results may translate well into humans (116). Therefore, it would be interesting to investigate if PSA would be equally effective in MS patients. Preliminary results of treatment with the dietary component propionic acid in MS patients are promising, as it shows immunological efficacy without safety concerns (136). This makes it a very promising therapeutic that needs to be tested in controlled clinical trials. The efficacy of vitamin D supplementation and reduced salt intake will also need to be tested in large randomized controlled trials.

In conclusion, gut microbiota and dietary interventions are promising treatments for MS, but there are still many questions that need to be investigated before we can conclude which bacterial species and which dietary components play a role in MS pathogenesis and before it will be possible to selectively target detrimental species as a treatment in MS patients.

AUTHOR CONTRIBUTIONS

All the authors developed tables, boxes, and figures. WH prepared and revised the manuscript. BH wrote part of the EAE/MS paragraphs. JL and BH critically reviewed the different versions of the manuscript and provided comments and additional research articles.

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Clostridium butyricum CGMCC0313.1 Protects against Autoimmune Diabetes by Modulating Intestinal Immune Homeostasis and Inducing Pancreatic Regulatory T Cells

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Recent evidence indicates that indigenous *Clostridium* species induce colonic regulatory T cells (Tregs), and gut lymphocytes are able to migrate to pancreatic islets in an inflammatory environment. Thus, we speculate that supplementation with the well-characterized probiotics *Clostridium butyricum* CGMCC0313.1 (CB0313.1) may induce pancreatic Tregs and consequently inhibit the diabetes incidence in non-obese diabetic (NOD) mice. CB0313.1 was administered daily to female NOD mice from 3 to 45 weeks of age. The control group received an equal volume of sterile water. Fasting glucose was measured twice a week. Pyrosequencing of the gut microbiota and flow cytometry of mesenteric lymph node (MLN), pancreatic lymph node (PLN), pancreatic and splenic immune cells were performed to investigate the effect of CB0313.1 treatment. Early oral administration of CB0313.1 mitigated insulitis, delayed the onset of diabetes, and improved energy metabolic dysfunction. Protection may involve increased Tregs, rebalanced Th1/Th2/Th17 cells and changes to a less proinflammatory immunological milieu in the gut, PLN, and pancreas. An increase of $\alpha4\beta7^+$ (the gut homing receptor) Tregs in the PLN suggests that the mechanism may involve increased migration of gut-primed Tregs to the pancreas. Furthermore, 16S rRNA gene sequencing revealed that CB0313.1 enhanced the Firmicutes/Bacteroidetes ratio, enriched *Clostridium*-subgroups and butyrate-producing bacteria subgroups. Our results provide the basis for future clinical investigations in preventing type 1 diabetes by oral CB0313.1 administration.

Keywords: type 1 diabetes, butyrate-producing bacteria, regulatory T cells, gut microbiota, regulatory T cells migration

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INTRODUCTION

Type 1 diabetes (T1D) is a condition in which pancreatic beta cell destruction leads to absolute insulin deficiency (1), which is caused by multiple-factors. The genetic background is essential, but not sufficient for causing the disease. T1D incidence has been rising more rapidly than can be accounted for by genetic changes, and evidence suggests that intestinal-related environmental factors, in particular the gut microbiota, are critical to the development of T1D (2, 3). The increasing incidence of T1D is most pronounced in children aged 1–5 years (4), suggesting that early life exposure is critical in shaping the autoimmune response. Moreover, young T1D patients have increased IL-4-producing cells in the small intestinal lamina propria, and interferon-γ (INF-γ)-producing cells were positively correlated with the degree of celiac disease, reflecting that gut immune reactivity is skewed in young T1D patients (5, 6). These findings raise the possibility that early life exposure to intestinal microbiota or its metabolites may be involved in immune responses associated with T1D.

Healthy children have a higher proportion of butyrate-producing bacteria in their microbiomes compared with children expressing at least one beta islet cell autoantibody (7, 8). Based on this, we speculate that the proportion of butyrate-producing bacteria may be a key regulator in determining the gut health of children at high risk of T1D. However, there is no evidence to date that any butyrate-producing bacteria protect against T1D in either animals or humans.

Additionally, several studies indicated that oral administration of CB0313.1 alleviated ovalbumin-induced allergic airway inflammation and food allergy in mice (9, 10). However, the impact of CB0313.1 on the development of T1D during different periods of life remains to be elucidated.

Recent reports have suggested that taxonomic groups Clostridia and Clostridiales prevent inflammation by a mechanism involving type 2 immunity (11), which may offer a feasible strategy to counteract T1D. Furthermore, previous studies have shown an induction of colonic regulatory T cells (Tregs) by indigenous *Clostridium* species such as *Clostridium butyricum* MIYAIRI 588 (12, 13). Thus, we speculated that supplementation with *Clostridium butyricum* CB0313.1 might induce pancreatic Tregs, thereby exerting beneficial effects on T1D.

Selective destruction of pancreatic beta cells induced by autoreactive T cells would be the primary cause of T1D. Autoimmunity is thought to escalate silently over a prolonged period of time before T1D is diagnosed; therefore, the failure to develop proper tolerogenic immune causes T1D. Immune regulation mediated by dedicated subsets of T lymphocytes, Tregs plays a critical role in immune homeostasis and self-tolerance. The major goal of immune therapies in T1D is to re-establish the balance of self-tolerance, particularly in the pancreas (14). Tregs suppress autoreactive T cells and induce immune tolerance *via* four main regulatory mechanisms: cell-to-cell contact, secretion of immuno-suppressive cytokines (e.g., TGF-β and IL-10), killing or modification of antigen-presenting cells (APCs), and competition for growth factors (15). Tregs exert their anti-diabetes effects within the pancreas as well as pancreatic lymph node (PLN) (16). Tregs in the PLN of non-obese diabetic (NOD) mice suppress the initial T cell differentiation and suppress the activation and secretion of IFN-γ, reverse the Th1/Th2/Th17 skewing to shape proper immune tolerance and prevent tissue inflammation (15–18).

Even more impressively, Tregs are easily attracted to sites of inflammation, then colocalize with and dampen the activity of Teffs. In addition, infiltration of both Teffs and Tregs was observed in the pancreas, suggesting that Tregs can be active at the inflammatory site (15).

The link between the gut and pancreas has also been emphasized in studies. Previous reports demonstrated that pancreatic islet T cells express α4β7 integrin, a gut homing receptor (6). Integrin α4β7 binds its ligands, VCAM-1 (CD106), MAdCAM-1, and fibronectin, and plays an important role in directing the migration of blood lymphocytes to the intestine and associated lymphoid tissues (19). Therefore, α4β7 can be used as a marker of gut homing cells.

Non-obese diabetic (NOD) mice share many similarities to T1D in human subjects. The incidence of spontaneous diabetes is 60–80% in the female NOD mice, whereas 20–30% in males. Therefore, we used female NOD mice in experiment (20).

Our study aimed to elucidate the effects of CB0313.1 on the progression of autoimmune diabetes and to investigate the influence of CB0313.1 on the gut microbiota, Tregs, and Th1/Th2/Th17 cell balance. This study will facilitate the development of probiotic-based therapies of T1D.

MATERIALS AND METHODS

Animal Experiment Design

Three-week old female NOD mice (Su Pu Si Biotechnology, Co., Ltd., Suzhou, Jiangsu, China) were housed under specific pathogen-free conditions in the animal facility of Jiangnan University (Jiangsu, China). Animals had free access to water and food. All mice were housed in individual ventilated caging systems (TECNIPLAST, Italy) under SPF conditions. CB0313.1 powder and/or sodium butyrate (NaB) was suspended in sterile water, then given to the corresponding mice by gavage. Since CB0313.1 is a spore-producing probiotics, it can avoid destruction by intestinal acidity and efficiently colonize the colon (21). The animals were anesthetized, then euthanized by cervical spine dislocation. All studies were approved by the Institutional Animal Ethics Committee of Jiangnan University (JN No. 20131205) and carried out in compliance with national and international guidelines for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering.

The study was designed as summarized in **Figure 1A**. To evaluate the effect of CB0313.1 administration on the onset of diabetes, the NOD mice were randomly assigned to two groups to receive CB0313.1 suspended in sterile water (2.5×10^8 CFU/kg/day, 400-fold of the dosage that used in colitis patients in clinics, Qingdao East Sea Pharmaceutical Co. Ltd., Shandong, China) or equal volume of sterile water by gavage from 3 to 45 weeks of age ($n = 25–28$/group). The mice were euthanized when a diagnosis of diabetes was made or at 45 weeks of age when the study ended. At that time, all remaining mice from all groups were euthanized for immune cells detection by flow cytometry (**Figure 1A**).

In parallel studies, NOD-CB or NOD control mice were euthanized at 6 (n = 10), 9 (n = 9), and 13 (n = 23, on two independent repeats) weeks of age (after 3, 6, and 10 weeks of treatment, respectively). The MLN, PLN, pancreas, and spleen were collected for T cell detection by flow cytometry.

To block egress of lymphocytes, 9-week-old female NOD mice were either given 1.5 mg/kg FTY720 (MCE, cat. NO. HY-12005) in sterile water or equal volume of sterile water by gavage daily for a week. Next the mice were sacrificed and immune cell populations were detected in MLN, PLN, and spleen by flow cytometry.

Blood Glucose Measurement

A glucometer (Roche, NSW, USA) was used to measure the glucose from tail vein samples, expressed in mmol/dl glucose. T1D is typically diagnosed using the criteria of the American Diabetes Association, which include acute onset of symptoms, glycosuria, plasma glucose of >11.1 mmol/l (22, 23). Polydipsia, polyphagia, and polyuria (the classic trio of symptoms associated with disease onset) along with overt hyperglycemia remain diagnostic hallmarks in children and adolescents, and to a lesser extent in adults (24, 25). In addition, NOD mice with hyperglycemia (>11.1 mmol/l) will probably require urgent implementation of insulin therapy (26, 27). Hence, NOD mice with hyperglycemia (>11.1 mmol/l) on two consecutive daily readings along with polyuria were considered to be diabetic.

Comprehensive Laboratory Animal Monitoring System (CLAMS) Metabolic Chamber

Respiratory exchange rate (RER), spontaneous physical activity, and heat production were monitored with CLAMS (Oxymax/

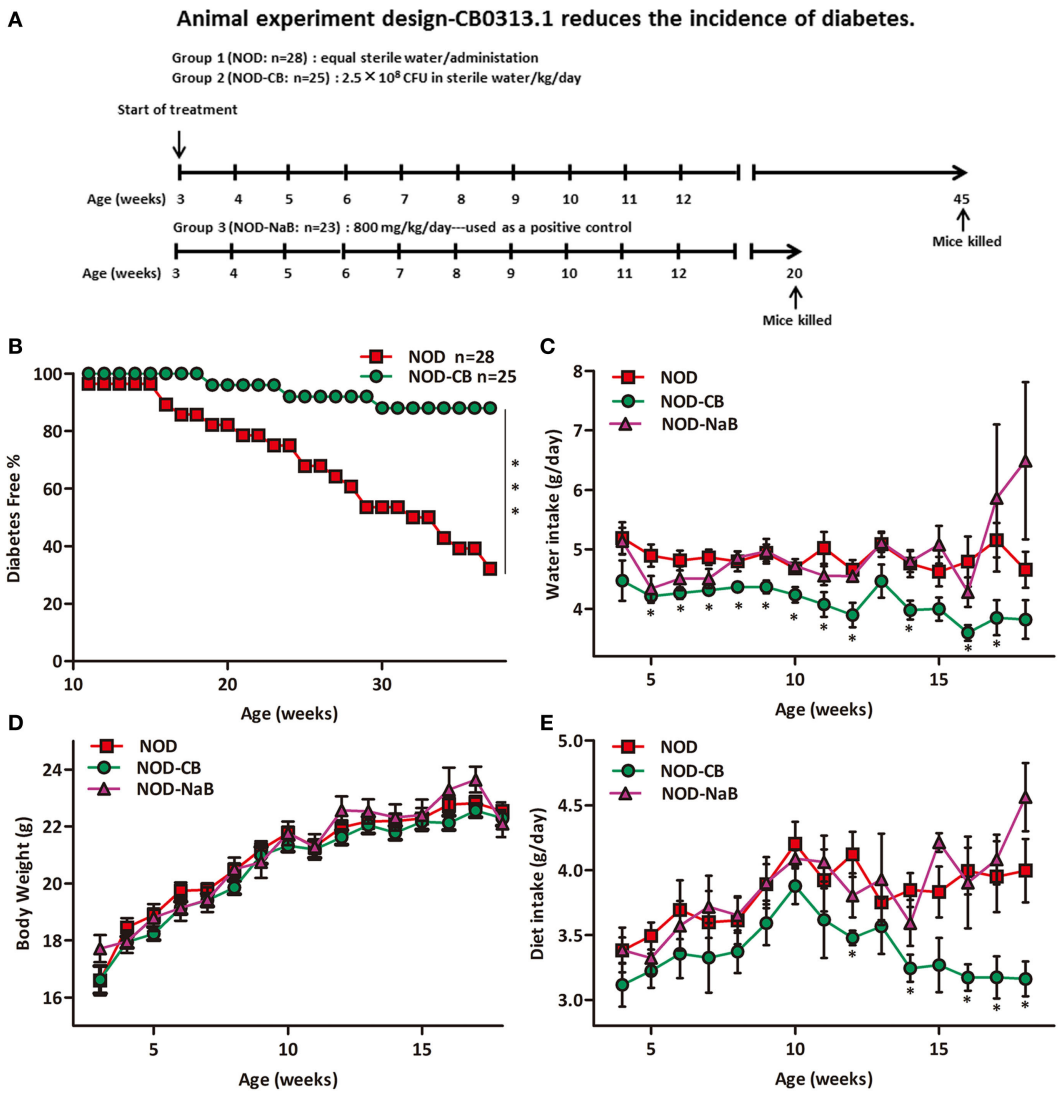


FIGURE 1 | Continued

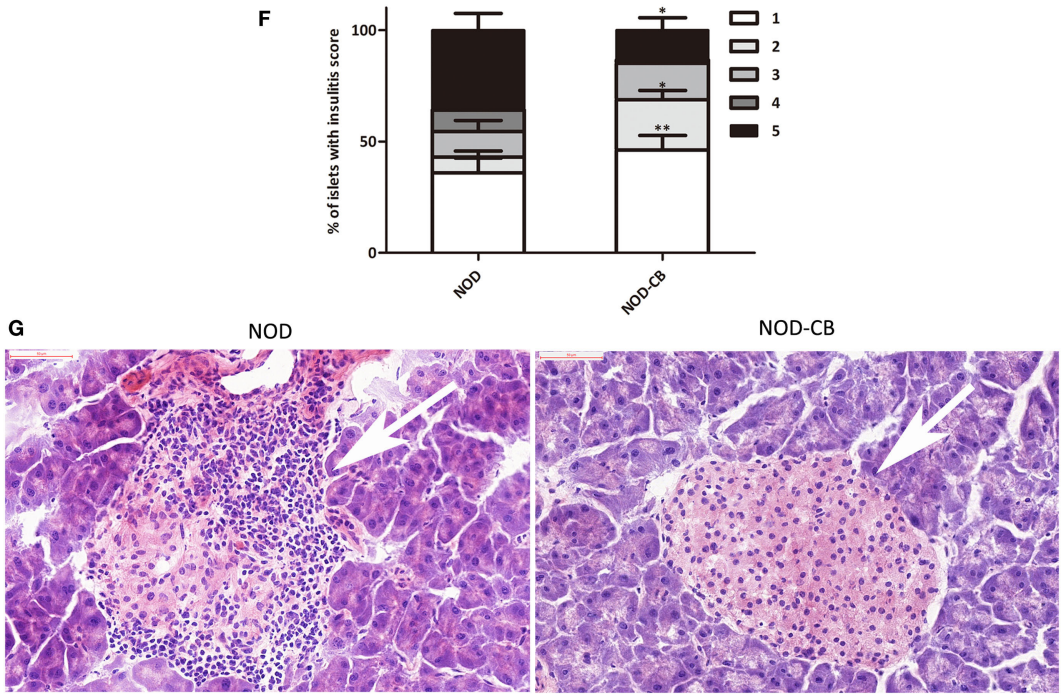


FIGURE 1 | CB0313.1 delays the onset and reduces the incidence of diabetes. **(A)** To evaluate the effect of CB0313.1 on the onset of type 1 diabetes, NOD mice were randomly assigned to two groups to receive CB0313.1 or equal volumes of sterile water by gavage from 3 to 45 weeks of age. NaB was used as positive control. Mice were monitored for the appearance of clinical signs of diabetes and euthanized at disease occurrence for immune cell detection by flow cytometry. Experiment was carried up to 45 weeks of age and all remaining non-diabetic mice from all groups were euthanized for the detection of immune cells by flow cytometry. **(B)** Delayed onset and reduced incidence of diabetes in NOD-CB mice. Mice received CB0313.1, NaB, or sterile water treatment from 3 weeks of age with fasting glucose monitoring. Mice were diagnosed as diabetic and euthanized when blood glucose levels exceeded 11.1 mmol/L on 2 consecutive days along with polyuria. **(C)** Water intake. **(D)** Body weight. **(E)** Diet intake. **(F)** Insulitis score for NOD-CB and NOD control mice at 13 weeks of age. Percentage of islets with a given score at 13 weeks of age in NOD-CB (n = 23) and NOD control (n = 23) mice. 1 = white, no infiltration; 2 = light gray, few mononuclear cells infiltrated; 3 = gray, peri-insulitis; 4 = dark gray, <50% islet infiltration; 5 = black, >50% islet infiltration. **(G)** Histological examination of pancreatic islet infiltration by immune cells in female NOD mice at 13 weeks of age. Analysis of variance followed by the indicated *post hoc* test was performed to determine the significance among the three groups. t-test was used for two independent groups. *, **, *** $p < 0.05$, $p < 0.01$, $p < 0.001$ *vs* NOD control mice.

CLAMS system, Columbus, OH, USA). The mice (12 weeks old) were housed individually in the metabolic chambers. After 12 h of adaptation, the data for all parameters were recorded and analyzed.

Preparation of Single Cell Suspensions

At 6, 9, 13, and 45 weeks of age, mice were sacrificed and immune cell populations were detected in mesenteric lymph nodes (MLNs), PLN, pancreas, and spleen. After the mice were euthanized, the indicated organs were placed in cold PBS immediately.

MLN and PLN were harvested and ground with frosted glass plates along with PBS scouring until there were no visible flakes, and the turbid solution was passed through a 70-μm polypropylene mesh (28).

Fresh pancreas was harvested and cut into small pieces. After being digested in PBS at 37°C for 15 min with rotation, the digested pancreatic pieces were passed through 70-μm polypropylene mesh along with grinding using a 2.5 ml syringe plunger and PBS scouring (29, 30).

Spleen was harvested and immediately ground with a 2.5 ml syringe plunger, accompanied by PBS scouring. The single spleen cell suspensions were centrifuged at 300 *g* for 5 min; then, red blood cell lysis buffer was added to lyse the red blood cells. After resting for 15 min at room temperature, the suspensions were centrifuged and resuspended in PBS (30).

Flow Cytometry

Single cell suspensions prepared from the indicated tissues were stained for 30 min at 4°C after FcγRII/III blocking with anti-CD16/CD32 monoclonal antibody. Flow staining buffer was purchased from eBiosciences. Antibodies were purchased from eBiosciences (San Diego, CA, USA), Miltenyi (Bergisch Gladbach, Germany), and BioLegend (San Diego, CA, USA); detailed information is listed in Tables S1, S3, and S4 in Supplementary Material. For Tregs staining, cells were first surface stained, then fixed, and stained for intracellular (nuclear) forkhead box P3 (Foxp3) according to the manufacturer's protocol. For the detection of intracellular (cytoplasmic) cytokine expression, cell suspensions were incubated at 37°C for 5 h with Cell Stimulation Cocktail (eBioscience, San Diego, CA, USA), then cells were stained and fixed according to the manufacturer's protocol. Isotype-matched controls were included in all experiments. Stained cells were

analyzed on an Attune NxT flow cytometer (Thermo Fisher Scientific, MA, USA).

Histological Evaluation

Fresh tissues were collected at 13 weeks of age after 10 weeks of CB0313.1 administration. Tissues were fixed in NEG-50 (Thermo Scientific, MA, USA) and immediately stored at −80°C until used for frozen sections. 8-μm sections were stained with Hematoxylin and Eosin (H&E) following the standard procedure.

Stool Sampling, DNA Extraction, and Sequencing

Stool samples were collected and immediately stored at −80°C until used for DNA extraction. Microbial genomic DNA was extracted from fecal samples using Fast DNA Spin Kit for Soil (MP Biomedicals, cat. # 6560-200, CA, USA) following the manufacturer's instructions. The V3, V4 region of 16S rRNA was PCR-amplified using specific primers (sense: 5′-AYTGGGYDTAAAGNG-3′; antisense: 5′-TACNVGGGTATC TAATCC-3′). Reaction conditions were 95°C for 5 min; 95°C for 30 s, 64°C for 30 s, and 72°C for 30 s, then repeated for 40 cycles, with a final incubation at 72°C for 10 min. The PCR products were excised from a 1.5% agarose gel, purified by Gene Clean Turbo (MP Biomedicals, cat. #: 111102400), and quantified by Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies, cat. # P7589, Carlsbad, CA, USA) following the manufacturer's instructions. Libraries were prepared using TruSeq DNA LT Sample Preparation Kit (Illumina, cat. # FC-121-2001, San Diego, CA, USA) and sequenced for 500 + 7 cycles on Illumina MiSeq using the MiSeq Reagent Kit (500 cycles-PE, cat. # MS-102-2003). The sequences reported in this paper have been deposited in the BioProject of NCBI under the accession NO. PRJNA412689.

Real-time PCR for Detecting Butyrate-Producing Bacteria

The final step for conversion of butyryl-CoA to butyrate is either catalyzed by butyrate kinase (*buk*) or acetate CoA-transferase (*butyryl-CoA*) kinase. Typically, these two genes are used as biomarkers for the identification/detection of butyrate-producing communities (31). Targeting the whole pathway for functional predictions is a robust way to circumvent difficulties associated with the analysis based on specific genes only (32, 33). The levels of *buk* and *butyryl-CoA* gene expression were normalized by total bacterial DNA and compared with NOD control mice. Primer sequences are given in Table S2 in Supplementary Material.

SCFAs Analysis

Acetate, propionate, and butyrate in stool samples were analyzed by gas chromatography coupled mass spectrometry (GC-MS) (34).

ELISA

Colonic and pancreatic TGF-β1, IL-10, IL-4, and IL-17A were measured by ELISA kit (Fcmacs, Nanjing, China) according to the manufacturer's instructions. The tissue was homogenized with physiological saline (1:19), then the homogenate was centrifuged at 4°C for 10 min at 12,000 *g*, and the supernatant was used for ELISA analysis.

Statistics

All data were analyzed using GraphPad Prism 5 software (San Diego, CA, USA). Cumulative diabetes incidence was calculated using the Kaplan–Meier estimation while statistical significance was evaluated by the log rank test. All data are presented as mean ± SEM ($n = 3$–28). One-way analysis of variance (ANOVA) was performed to determine the significance among three groups followed by the indicated *post hoc* test. *t*-test was used for two independent groups. $p < 0.05$ was considered statistically significant. Linear regression with a Pearson correlation analysis was performed for determining the correlation between specific bacterial clusters and fasting glucose. Optimization of 1% was performed using the unweighted pair group method with arithmetic averages clustering algorithm (UPGMA) and by principal component analysis (PCA) using Past v2.16.

RESULTS

CB0313.1 Delays the Onset and Reduces the Incidence of Diabetes

The effect of CB0313.1 on the development of autoimmune diabetes in NOD mice was studied by evaluating the time of onset and the incidence of diabetes. As shown in **Figure 1B**, treatment at weaning (3 weeks of age) resulted in a delay of diabetes onset in NOD-CB mice (19 weeks of age) compared with the NOD control mice (11 weeks of age). In addition, at 37 weeks of age, 32.14% (9/28) of NOD control mice were diabetes free, in comparison with 88% (22/25) of mice in the NOD-CB mice (**Figure 1B**, $p < 0.001$ by log rank test). Moreover, CB0313.1 treatment attenuated water intake and food intake significantly, and this effect was not associated with body weight (**Figures 1C–E**).

Furthermore, in the group of mice treated from 3 to 13 weeks of age, we evaluated the degree of insulitis. The average insulitis score of NOD-CB mice was significantly lower than in NOD controls (**Figures 1F,G**; Figure S1 in Supplementary Material).

CB0313.1 but Not Butyrate Improves Energy Metabolic Dysfunction

Next, we investigated the therapeutic effect of CB0313.1 on energy metabolic dysfunction at 12 weeks of age. NOD-CB mice showed a significantly lower respiratory exchange ratio (RER) compared to NOD controls, indicating decreased glucose oxidation, and increased fat and protein oxidation in NOD-CB mice (**Figure 2A**, $p < 0.001$). Meanwhile, we observed significantly increased physical activity (**Figure 2B**, $p < 0.01$, in the nighttime) and decreased heat production (**Figure 2C**, $p < 0.001$ for day and $p < 0.05$ for night) in NOD-CB mice *vs* NOD controls. These findings demonstrate that CB0313.1 prevents diabetes-induced energy metabolic dysfunction, and this effect may be associated with changes in the ratio of energy expenditure, increased physical activity and decreased heat production.

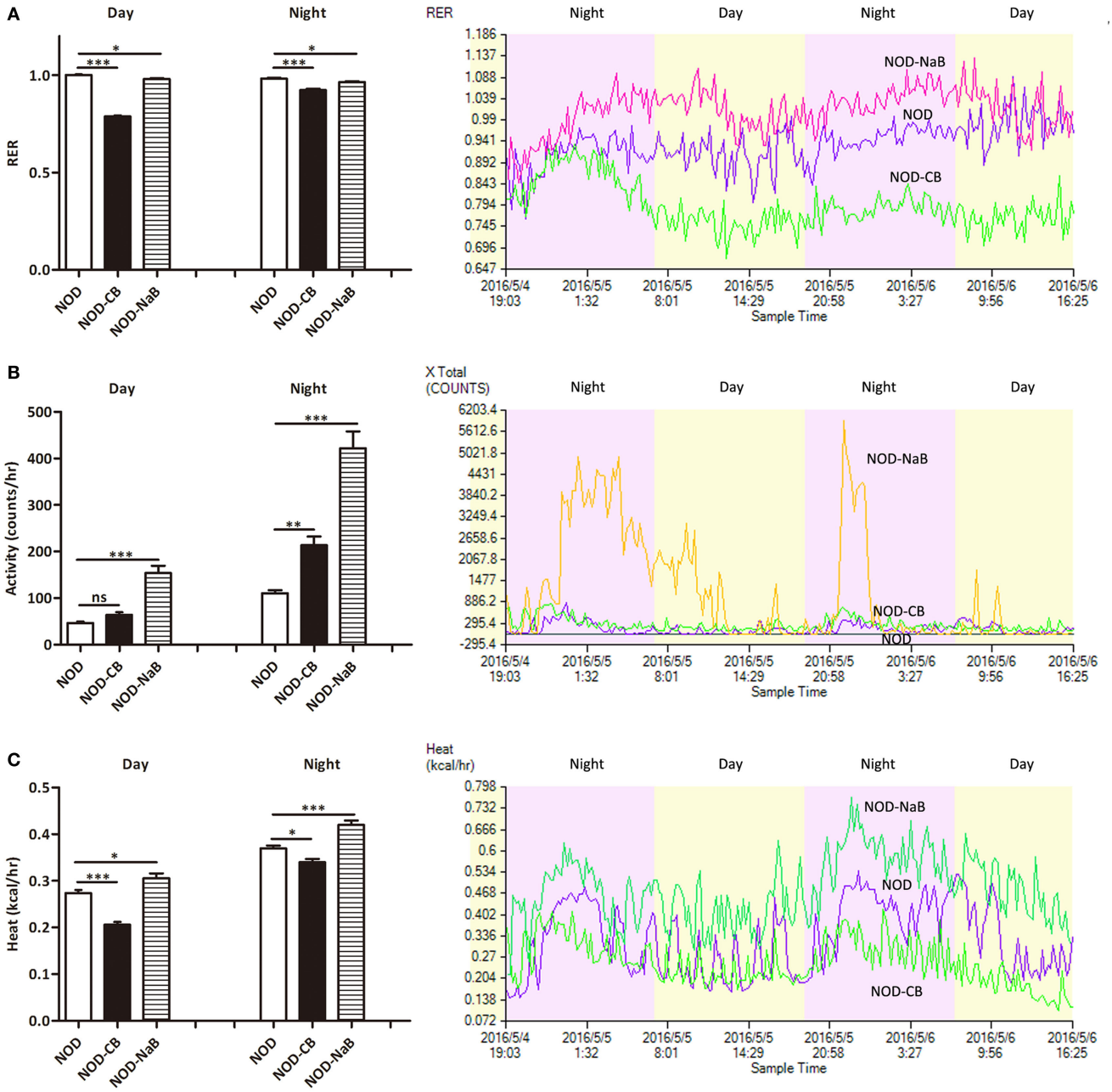


FIGURE 2 | CB0313.1 but not butyrate improves energy metabolic dysfunction. Energy metabolism was examined using a metabolic chamber at 12 weeks of age. **(A)** Substrate utilization is expressed as the respiratory exchange ratio (RER), the ratio of O_2 consumption to CO_2 exhalation volume; **(B)** spontaneous physical activity; **(C)** heat production. Data are mean ± SEM ($n = 4$ mice per group). Analysis of variance followed by the indicated *post hoc* test was performed to determine the significance among the three groups. *t*-test was used for two independent groups. *, **, *** $p < 0.05$, $p < 0.01$, $p < 0.001$ *vs* NOD control mice.

To investigate how CB0313.1 affected energy metabolism, we administered NOD mice with metabolite NaB by gavage at 800 mg/kg/day from 3 weeks of age according to the recommended dosage of a previous study (35), and observed an earlier onset of T1D at 9 weeks of age in NOD-NaB mice *vs* 11 weeks of age in NOD controls (data not shown). CLAMS metabolic chambers were used to monitor metabolism of NOD-NaB mice. Interestingly, the physical activity of NOD-NaB mice was dramatically increased to 3.32-fold in the daytime, and 3.82-fold in the evening *vs* NOD controls (**Figure 2B**). In addition, significantly increased heat production (**Figure 2C**) and decreased RER (**Figure 2A**) were observed in NOD-NaB mice (**Figure 2C**). These findings demonstrate that NOD-CB mice exhibited different metabolic behaviors compared with NOD-NaB mice, indicating that the beneficial effects of CB0313.1 on T1D are associated with improvement of the energy metabolic dysfunction, but not only *via* butyrate.

CB0313.1 Restores the Diabetes-Induced Gut Microbial Dysbiosis

16S rRNA gene sequencing data showed that NOD-CB mice and NOD controls harbored distinct microbial communities. The vast majority (> 95%) of the annotated reads in NOD mice at 40 weeks of age were distributed among three bacterial phyla: Firmicutes, Bacteroidetes, and Proteobacteria (**Figure 3A**). CB0313.1 administration was associated with a significant decrease in Bacteroidetes (by 26.39%, $p < 0.05$) and a significant increase in Firmicutes (by 30.59%, $p < 0.05$) compared to NOD controls (**Figures 3A,B**). **Figures 3C,D** illustrate the microbial alterations at the class and order levels, respectively. Notably, taxonomic groups Clostridia and Clostridiales increased consistently, indicating that CB0313.1 promoted the establishment of a protective microbiome enriched in Clostridiales (38.01% in NOD-CB mice *vs* 30.56% in NOD controls) (**Figures 3C,D**) (11).

At the family level, 8 out of 48 families identified were markedly changed in response to CB0313.1. Major differences were observed at the level of dominant families: Bacteroidaceae, Prevotellaceae, Rikenellaceae, Streptococcaceae, and F16 decreased by 46.27%, 66.67% ($p < 0.05$), 27.56% ($p < 0.05$), 92.09% ($p < 0.01$), and 55.63% ($p < 0.05$), respectively, in NOD-CB mice *vs* NOD controls. Lactobacillaceae, Clostridiaceae, Desulfovibrionaceae increased by 49.97%, 161.26% ($p < 0.05$), 200.07% ($p < 0.01$), respectively, in NOD-CB mice *vs* NOD controls (**Figure 3E**).

Additionally, the following genera were increased significantly in NOD-CB mice *vs* NOD controls: *Clostridium* (by 3.54-fold, $p < 0.05$), Desulfovibrionaceae;g (by 2.76-fold, $p < 0.05$), Helicobacteraceae;g (by 2.52-fold, $p < 0.05$), *Lactobacillus* (by 1.72-fold, $p < 0.05$), and *Allobaculum* (by 111-fold), whereas the following genera decreased significantly in NOD-CB mice *vs* NOD controls: Bacteroides (by 41.5%, $p < 0.05$), *Prevotella* (by 65.43%, $p < 0.05$), *Odoribacter* (by 44.39%, $p < 0.05$), *Lactococcus* (by 93.18%, $p < 0.01$), *Candidatus Arthromitus* (by 98.94%, $p < 0.05$), *Helicobacter* (by 95.06%, $p < 0.05$), and F16;g (by 55.63%, $p < 0.05$) (**Figure 3F**). Most of them are still poorly characterized and could be further studied in the context of T1D.

Principal coordinate analysis (PCoA) showed that CB0313.1 significantly modified the overall structure of the gut microbiota along the first principal component (PC1) (**Figure 3G**, $p < 0.01$).

We found that supplementation with CB0313.1 effectively increased intestinal CB0313.1 by 12,370-fold, demonstrating its ability to efficiently colonize the gut (**Figure 3H**).

Typically, two genes, *buk* and *butyryl-CoA*, are used as biomarkers for the detection of butyrate-producing communities. We found that CB0313.1 only carried the *buk* gene but no *butyryl-CoA* gene, suggesting that CB0313.1 produces butyrate *via* the *buk* pathway. However, CB0313.1 administration significantly increased not only bacteria carrying the *buk* gene (by 7-fold, $p < 0.01$) but also those carrying the *butyryl-CoA* gene (by 4-fold, $p < 0.05$) (**Figures 3I,J**), demonstrating that probiotic CB0313.1 also promoted the growth of other butyrate-producing bacteria. These data suggest that CB0313.1 promotes the growth of intestinal butyrate-producing bacteria.

However, we observed an increase trend of butyric acid in NOD-CB mice but not in NOD-NaB mice (**Figure 3K**), suggesting NaB by oral gavage was not efficiently delivered to the lower digestive tract in our experiment.

Overall, the NOD-CB mice harbored more microbial clusters previously described as beneficial to T1D, whereas the NOD control mice harbored more microbial clusters previously described as pathogenic to T1D.

Specific Bacterial Groups in the Gut Correlate with Fasting Glucose

Since the gut microbes might be involved in the gut-pancreas axis, we next investigated whether bacterial clusters from the gut microbiota were associated with fasting glucose. We performed linear regression with a Pearson correlation analysis to determine the correlation between specific bacterial clusters and fasting glucose.

As presented in **Figure 4**, the relative abundance of Bacteroidetes (**Figure 4A**, $p < 0.01$), Bacteroidia (**Figure 4C**, $p < 0.01$), Bacteroidales (**Figure 4D**, $p < 0.05$), Bacteroidaceae (**Figure 4G**, $p < 0.05$), Rikenellaceae (**Figure 4H**, $p < 0.05$), Prevotellaceae (**Figure 4I**, $p < 0.001$), Bacteroides (**Figure 4J**, $p < 0.05$), *Lactococcus* (**Figure 4M**, $p < 0.001$), and *Prevotella* (**Figure 4N**, $p < 0.001$) were positively associated with fasting glucose, whereas the relative abundance of Firmicutes (**Figure 4B**, $p < 0.01$), Lactobacilales (**Figure 4E**, $p < 0.05$), Desulfovibrionales (**Figure 4F**, $p < 0.01$), *Clostridium* (**Figure 4K**, $p < 0.05$), *Lactobacillus* (**Figure 4L**, $p < 0.0767$), and *Desulfovibrio* (**Figure 4O**, $p < 0.01$) were negatively associated with fasting glucose. We challenged these associations by performing single linear regression analyses and found that the correlations between these relative abundance and fasting glucose were significant.

Among those that were significantly correlated with fasting glucose, Prevotellaceae (belonging to the Bacteroidetes phylum) showed the most robust positive correlation (**Figure 4I**, $p < 0.001$). Consequently, we propose a new link between gut microbiota and fasting glucose.

CB0313.1 Changes Cytokine Profiles and Induces Tregs in the MLN

Since the NOD-CB mice harbored a microbiome which protected against T1D onset, we hypothesized that these protective gut microbial communities might exert their beneficial effects on host intestinal immunity, which subsequently affected systemic and pancreatic immunity. To test our hypothesis, we investigated the cytokine profile of T cells from MLN.

Intracellular staining for IFN-γ, IL-4, and IL-17A demonstrated lower percentages of IFN-γ^+, IL-4^+ $CD4^+$ T cells and similar percentages of IL-$17A^+$ $CD4^+$ T cells from MLN of NOD-CB mice *vs* NOD controls at 13 weeks of age [**Figure 5A** ($p < 0.05$), **Figure 5B** ($p < 0.01$), and **Figure 5C**, respectively].

Intranuclear staining from MLN cells showed an increasing trend of $Foxp3^+$ abundance (11.60% $Foxp3^+$ in $CD4^+$ T cells in NOD-CB mice *vs* 8.99% in NOD controls) at 6 weeks (**Figure 5D**). Among the $\alpha4\beta7^+$ T cells in MLN, a similar proportion of $CD4^+Foxp3^+$ cells were observed in the two groups. Moreover, at 9 weeks, a similar proportion of $CD4^+Foxp3^+$ cells were also observed in the two groups (**Figure 5E**).

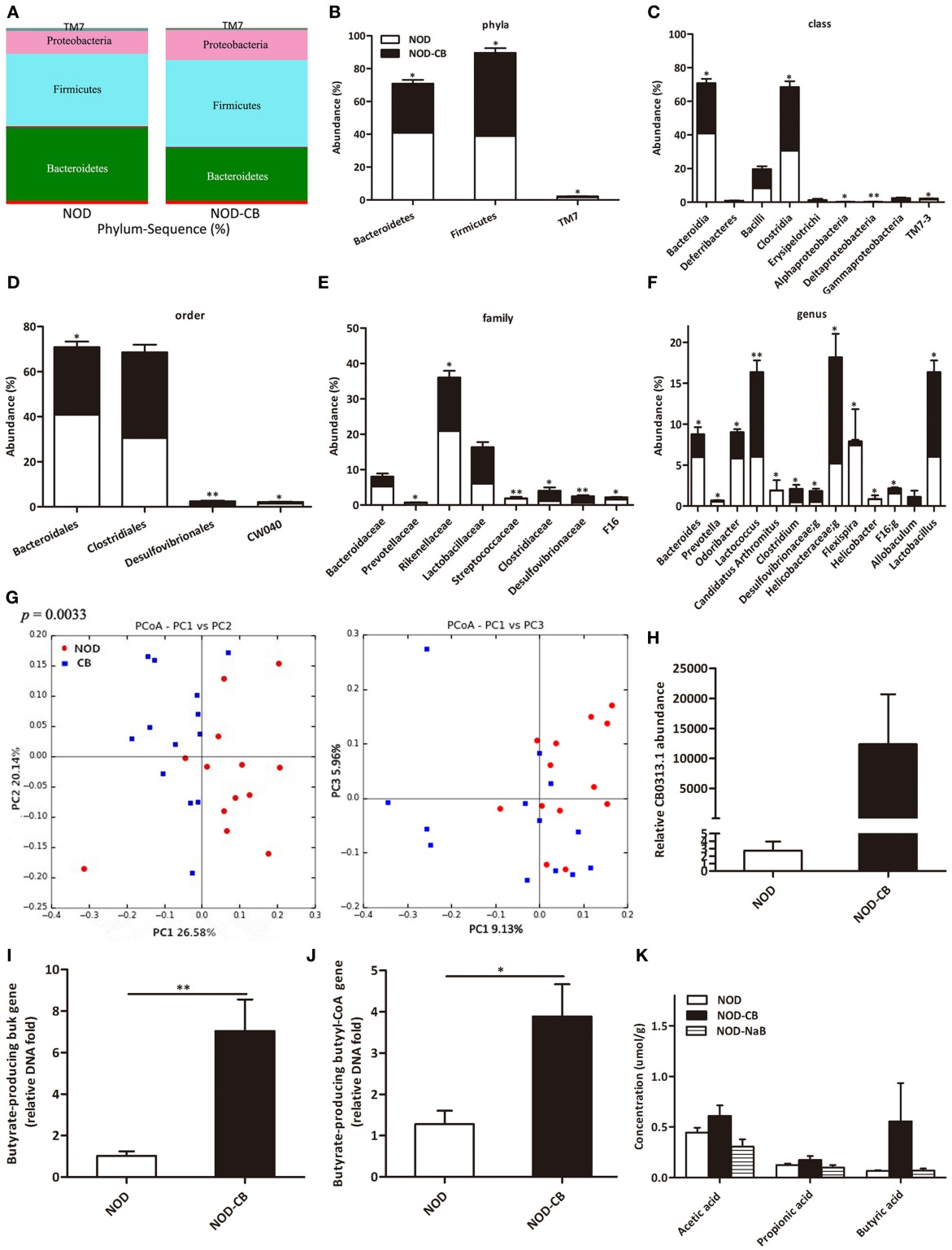


FIGURE 3 | CB0313.1 restores the diabetes-induced gut microbial dysbiosis. **(A,B)** Abundance of the most important phyla in each group. Abundance of the main altered classes **(C)**, orders **(D)**, families **(E)**, and genera **(F)** in each group. **(G)** Principal coordinate analysis (PCoA) plot of weighted UniFrac distances, each dot representing a feces community; the percentage of variation explained by each principal coordinate is shown in parentheses. **(H)** Relative abundance of CB0313.1. Predominant butyrate producing genes: relative abundance of butyrate kinase (*buk*) and *butyryl-CoA* DNA in feces **(I,J)**. **(K)** SCFA concentration in feces measured by GC-MS. Analysis of variance followed by the indicated *post hoc* test was performed to determine the significance among the three groups. Data are mean ± SEM ($n = 8$ mice per group). *, **, *** $p < 0.05$, $p < 0.01$, $p < 0.001$ *vs* NOD control mice by *t*-test.

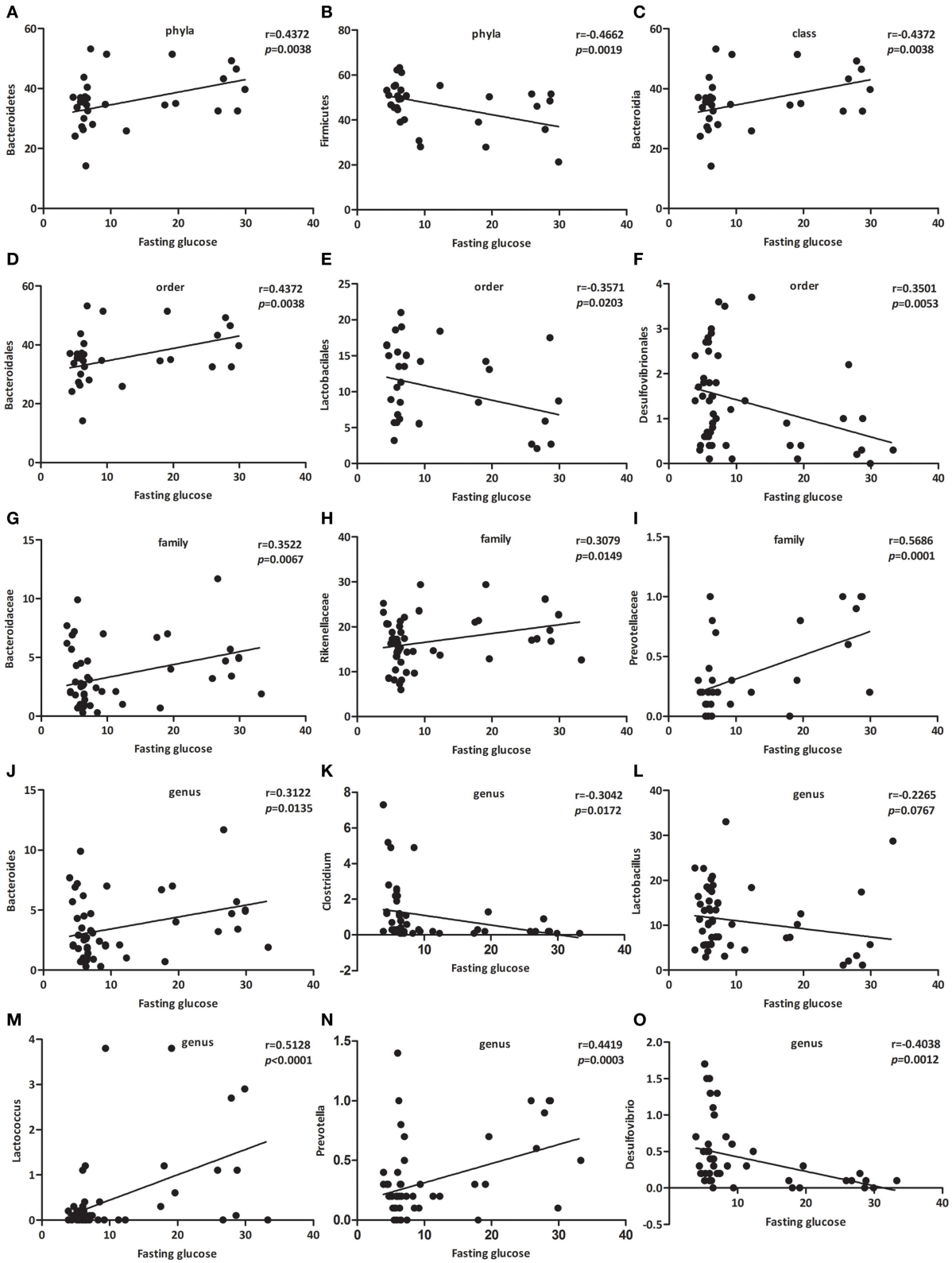
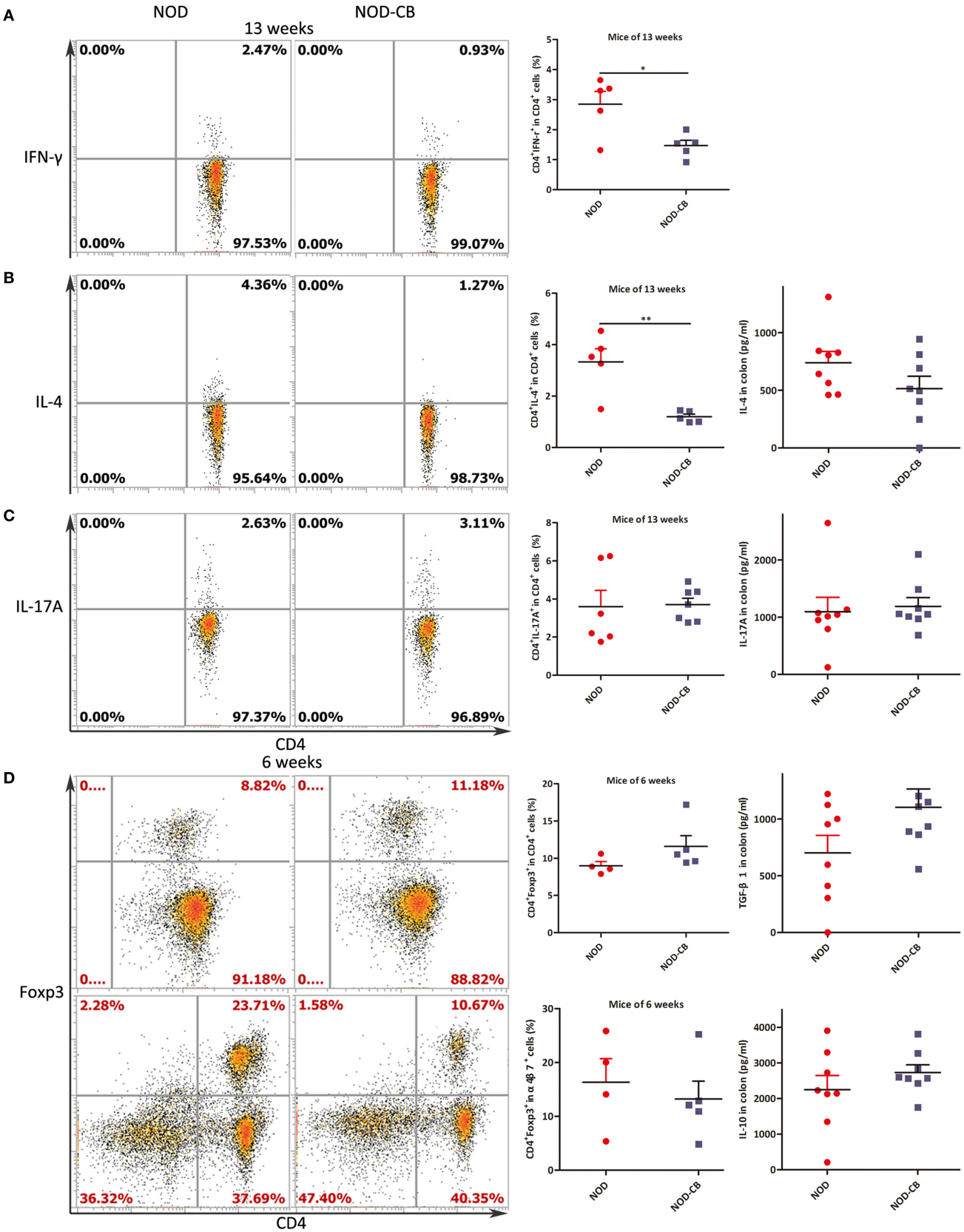


FIGURE 4 | Specific bacterial groups in the gut correlate with fasting glucose. Correlation analyses between fasting glucose and the relative abundance (%) of **(A)** Bacteroidetes, **(B)** Firmicutes, **(C)** Bacteroidia, **(D)** Bacteroidales, **(E)** Lactobacilales, **(F)** Desulfovibrionales, **(G)** Bacteroidaceae, **(H)** Rikenellaceae, **(I)** Prevotellaceae, **(J)** Bacteroides, **(K)** *Clostridium*, **(L)** *Lactobacillus*, **(M)** *Lactococcus*, **(N)** *Prevotella*, **(O)** *Desulfovibrio*. $n = 42–62$. *, **, *** $p < 0.05$, $p < 0.01$, $p < 0.001$ *vs* NOD control mice by *t*-test.



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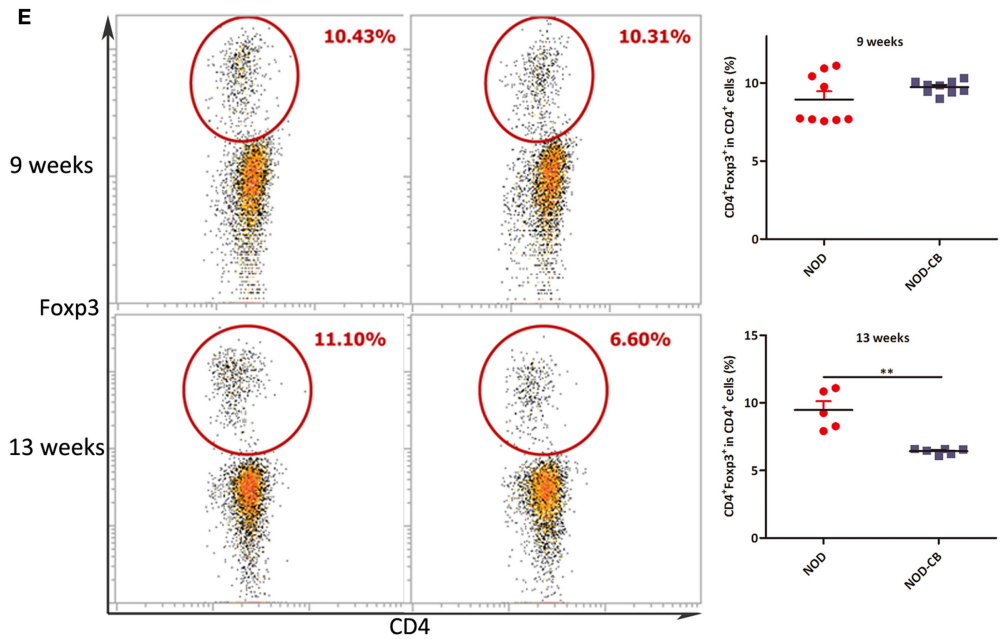


FIGURE 5 | CB0313.1 changes cytokine profiles and induces Tregs in the MLN. **(A)** Percent IFN-γ+ in CD4+ cells in MLN as indicated at 13 weeks of age. **(B)** Percent IL-4+ in CD4+ cells in MLN as indicated at 13 weeks of age, and protein level of IL-4 determined by ELISA at 13 weeks of age. **(C)** Percent IL-17A+ in CD4+ cells in MLN as indicated at 13 weeks of age, and protein level of IL-17A determined by ELISA at 13 weeks of age. **(D)** Percent Foxp3+ in CD4+ cells, percent CD4+Foxp3+ in α4β7+ cells in MLN as indicated at 6 weeks of age, and protein levels of TGF-β, IL-10 determined by ELISA at 13 weeks of age. **(E)** Percent Foxp3+ in CD4+ cells in MLN as indicated at 9, 13 weeks of age. Data are mean ± SEM (n = 5–9 mice per group). *, **, *** $p < 0.05$, $p < 0.01$, $p < 0.001$ *vs* NOD control mice by t-test. For **(A–C,E)**, the number of cells is 5,000; For **(D)**, the number of cells is 10,000.

Of note, at 13 weeks of age (when the NOD mice start to develop into T1D form insulitis phase gradually), the percentage of Foxp3+ in CD4+ T cells of NOD-CB mice markedly decrease to 6.43% ($p < 0.001$), while the percentage of NOD controls still held at 9.48% (**Figure 5E**). These data indicate that the Tregs in MLN of NOD-CB mice started to be reduced or migrate to other organs at 13 weeks of age.

CB0313.1 Promotes the Migration of Gut-Primed Tregs to the PLN

We next examined the percentage of Foxp3+ in CD4+ T cells from PLN, and found a marked increase of Foxp3+ in CD4+ T cells in PLN (12.47% in NOD-CB mice *vs* 7.72% in NOD control mice, $p < 0.01$), as early as 6 weeks of age (**Figures 6A,B**). Even more impressively, the percentage of CD4+Foxp3+ in α4β7+ cells increased to 34.47 *vs* 11.92% in NOD controls ($p < 0.01$), suggesting that CB0313.1 promotes the migration of gut and associated lymphoid tissues-primed Tregs to the PLN (**Figures 6C,D**). It has been speculated that Tregs may circulate between gut and pancreas in NOD mice.

FTY720 Suppresses the Accumulation of α4β7+ Tregs but Not the Total Tregs in the PLN

To confirm if CB0313.1 promoted the migration of gut-primed Tregs to PLN, we administrated NOD mice with FTY720 by gavage, which inhibits T cells circulation and traps them in the lymph node (36). Indeed, FTY720 resulted in a decreased proportion of splenic Tregs (**Figure 7A**), whereas an increased fraction of Tregs in the MLN compared to the NOD-CB mice (**Figure 7B**). FTY720 inhibited the migration of α4β7+ Tregs away from MLN (**Figure 7C**). In the PLN, FTY720 suppressed the accumulation of α4β7+ Tregs (**Figure 7E**), however, did not significantly change the total proportion of Tregs (**Figure 7D**), suggesting that the majority of Tregs in the PLN might be induced locally and migrated α4β7+ Tregs represented only a small fraction (Figure S6 in Supplementary Material).

CB0313.1 Induces Pancreatic Tregs and Reduces Pancreas Inflammation

To elucidate the influence of CB0313.1 on pancreatic immunity, we used flow cytometry to examine the CD4+Foxp3+ T cells in the pancreas. At 6 and 9 weeks of age, we found similar proportions of pancreatic Tregs. However, at 13 weeks of age, we observed a significantly increased proportion of pancreatic Tregs in NOD-CB mice *vs* NOD controls as well as increased TGF-β and IL-10, supporting the hypothesis that oral CB0313.1 may exert its protective effects *via* Tregs and associated cytokines (**Figure 8A**, $p < 0.01$). Increased TGF-β in pancreas also suggests that CB0313.1 may induce Tregs *via* TGF-β related mechanism.

Although we showed that CB0313.1 could induce pancreatic Tregs at 13 weeks of age, whether this induction is a long-standing effect remained to be determined. An increased trend of pancreatic

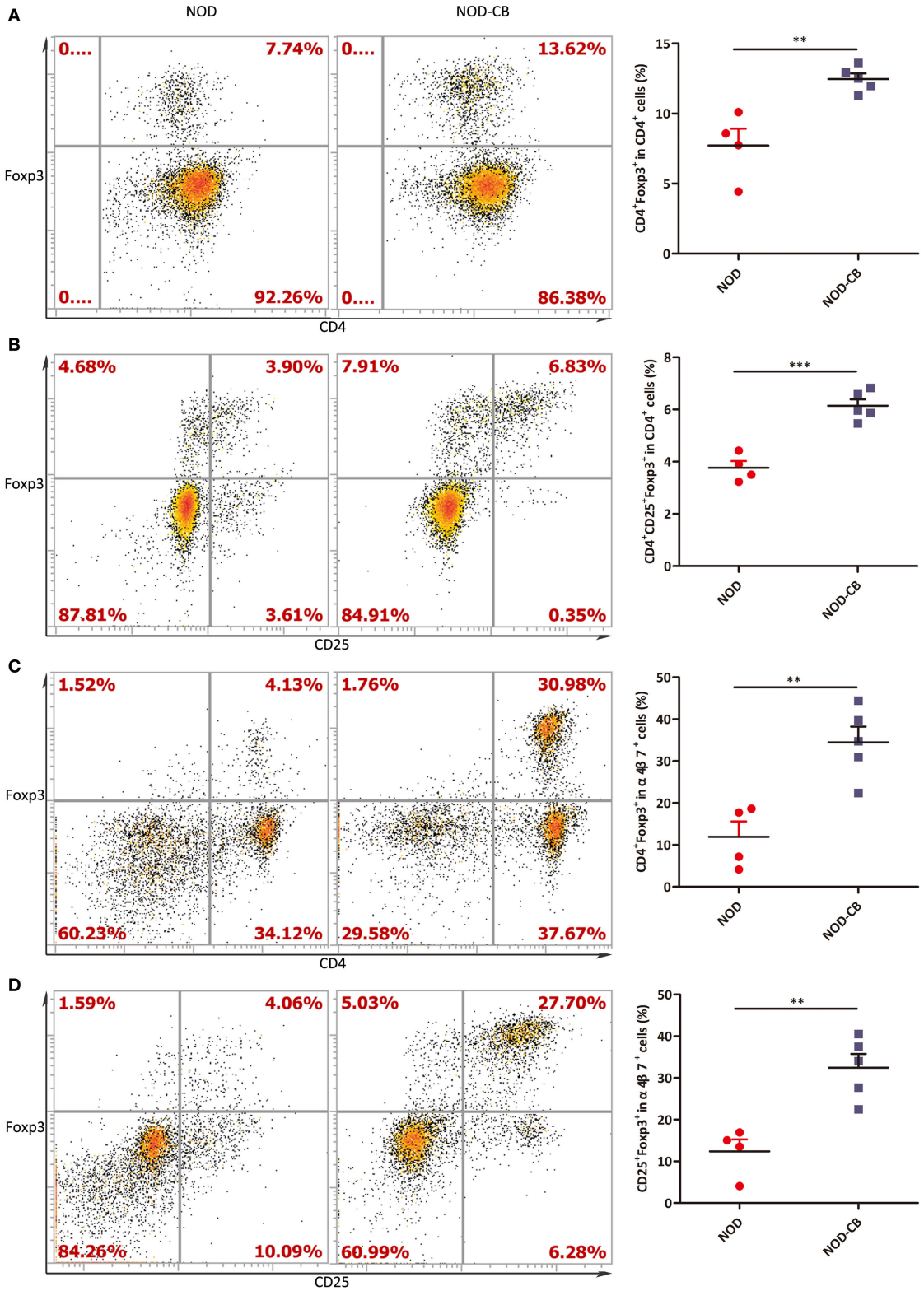


FIGURE 6 | CB0313.1 promotes the migration of gut-primed Tregs to the PLN. **(A)** Percent CD4$^+$ Foxp3$^+$ cells in PLN. **(B)** Percent CD4$^+$CD25$^+$Foxp3$^+$ cells in PLN. **(C)** Percent CD4$^+$ Foxp3$^+$ cells in α4β7$^+$ cells in PLN. **(D)** Percent CD25$^+$Foxp3$^+$ cells in PLN. ***$p < 0.05$, $p < 0.01$, $p < 0.001$ *vs* NOD controls mice by *t*-test. The number of cells is 10,000.

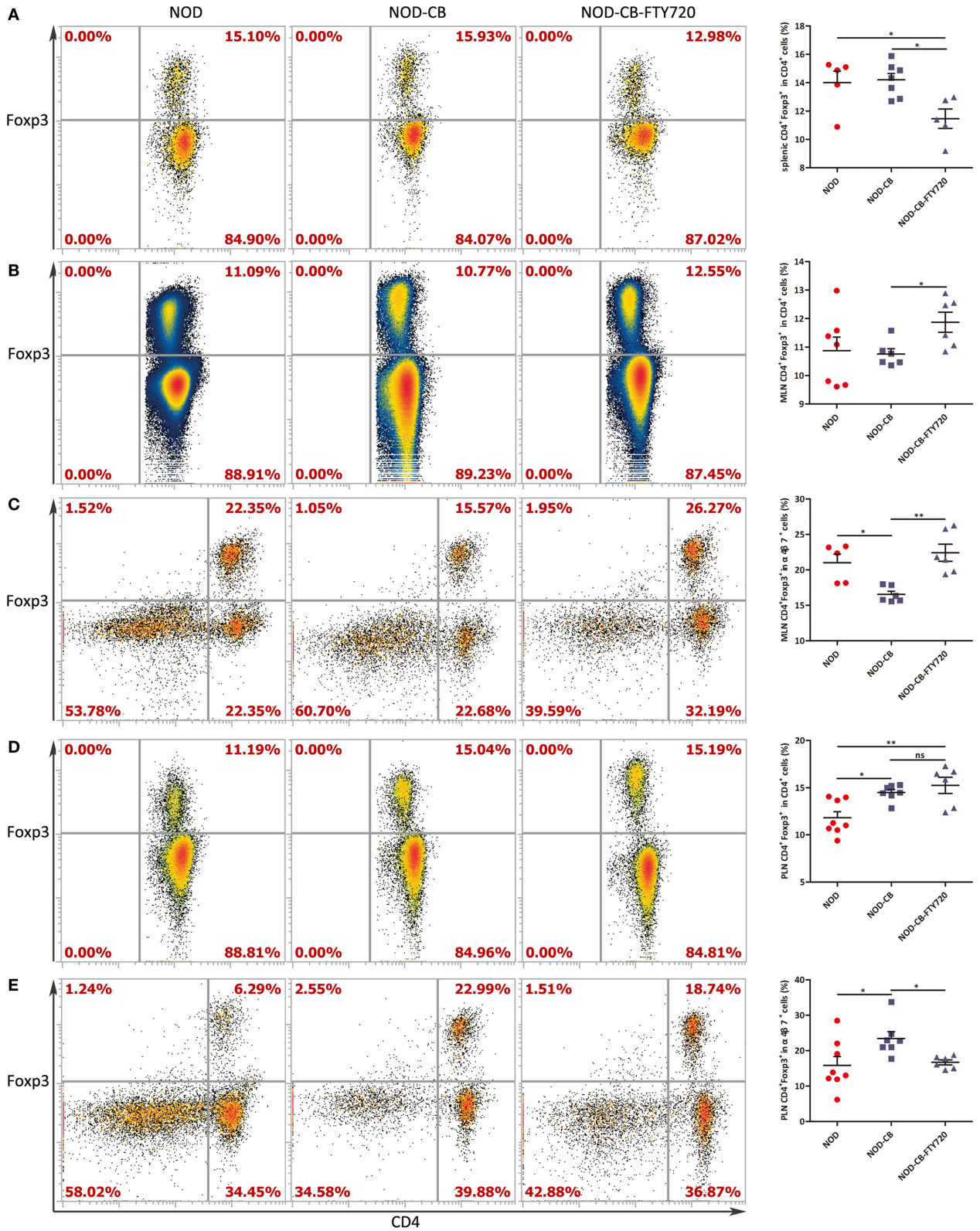


FIGURE 7 | FTY720 suppresses the accumulation of α4β7+ Tregs but not the total Tregs in the PLN. **(A)** Percent CD4+Foxp3+ in CD4+ spleen cells. **(B)** Percent CD4 +Foxp3+ in CD4+ MLN cells. **(C)** Percent CD4+Foxp3+ in α4β7+ MLN cells. **(D)** Percent CD4+Foxp3+ in CD4+ PLN cells. **(E)** Percent CD4+Foxp3+ in α4β7+ PLN cells. Data are mean ± SEM (n = 5–8 mice per group). *, **, *** $p < 0.05$, $p < 0.01$, $p < 0.001$ *vs* NOD control mice by t-test. The number of cells is 10,000–300,000.

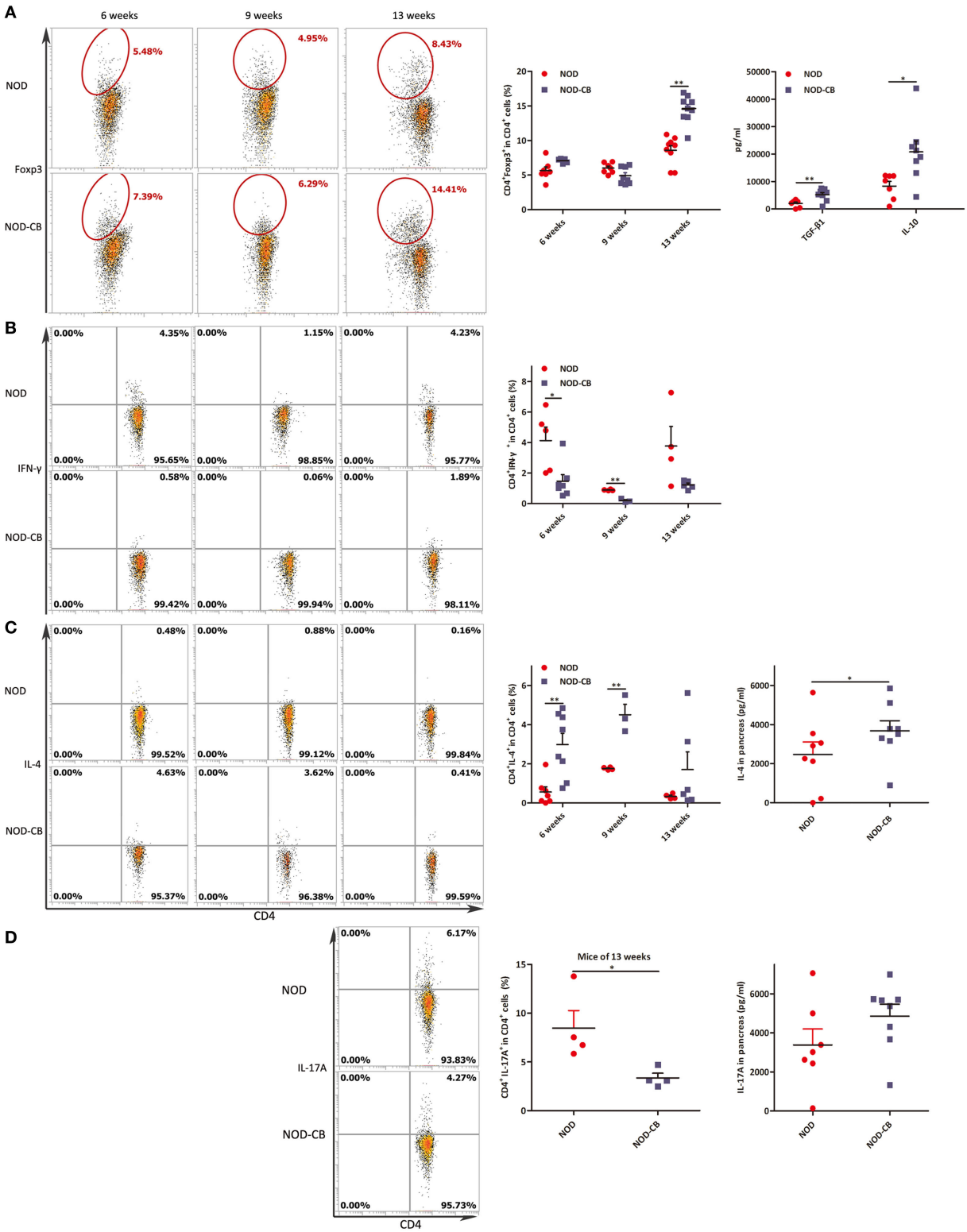


FIGURE 8 | CB0313.1 induces pancreatic Tregs and reduces pancreas inflammation. **(A)** Percent $CD4^+Foxp3^+$ cells in pancreas as indicated at 6, 9, and 13 weeks of age, and protein levels of TGF-β, IL-10 determined by ELISA at 13 weeks of age. **(B)** Percent $CD4^+IFN\text{-}\gamma^+$ cells in pancreas as indicated at 6, 9, and 13 weeks of age. **(C)** $CD4^+IL\text{-}4^+$ cells in pancreas as indicated at 6, 9, and 13 weeks of age, and protein level of IL-4 determined by ELISA at 13 weeks of age. **(D)** $CD4^+IL\text{-}17A^+$ in pancreas as indicated at 13 weeks of age, and protein level of IL-17A determined by ELISA at 13 weeks of age. Data are mean ± SEM (n = 4–15 mice per group). *, **, *** $p < 0.05$, $p < 0.01$, $p < 0.001$ *vs* NOD control mice by t-test. For **(A)**, the number of cells is 5,000. For **(B–D)**, the number of cells is 3,000–5,000.

Tregs was observed in the NOD-CB mice *vs* NOD controls at the end of the experiment, indicating a higher immune tolerance locally in the pancreas due to CB0313.1 treatment (Figure S4 in Supplementary Material).

Despite the importance of Tregs in diabetes prevention, the participation of other key players cannot be ruled out. Consequently, we examined the Th1/Th2/Th17 balance in pancreas. Intracellular staining for cytokine profile of T cells from the pancreas demonstrated a lower percentage of IFN-γ^+, IL-17A$^+$ and a higher percentage of IL-4$^+$ CD4$^+$ T cells in NOD-CB mice *vs* NOD controls, as well as increased protein level of IL-4 (**Figures 8B–D**).

These findings demonstrate that CB0313.1 reduces pancreas inflammation and reversed the imbalance in Th1/Th2/Th17/Tregs.

CB0313.1 Changes the Splenic Cytokine Profiles

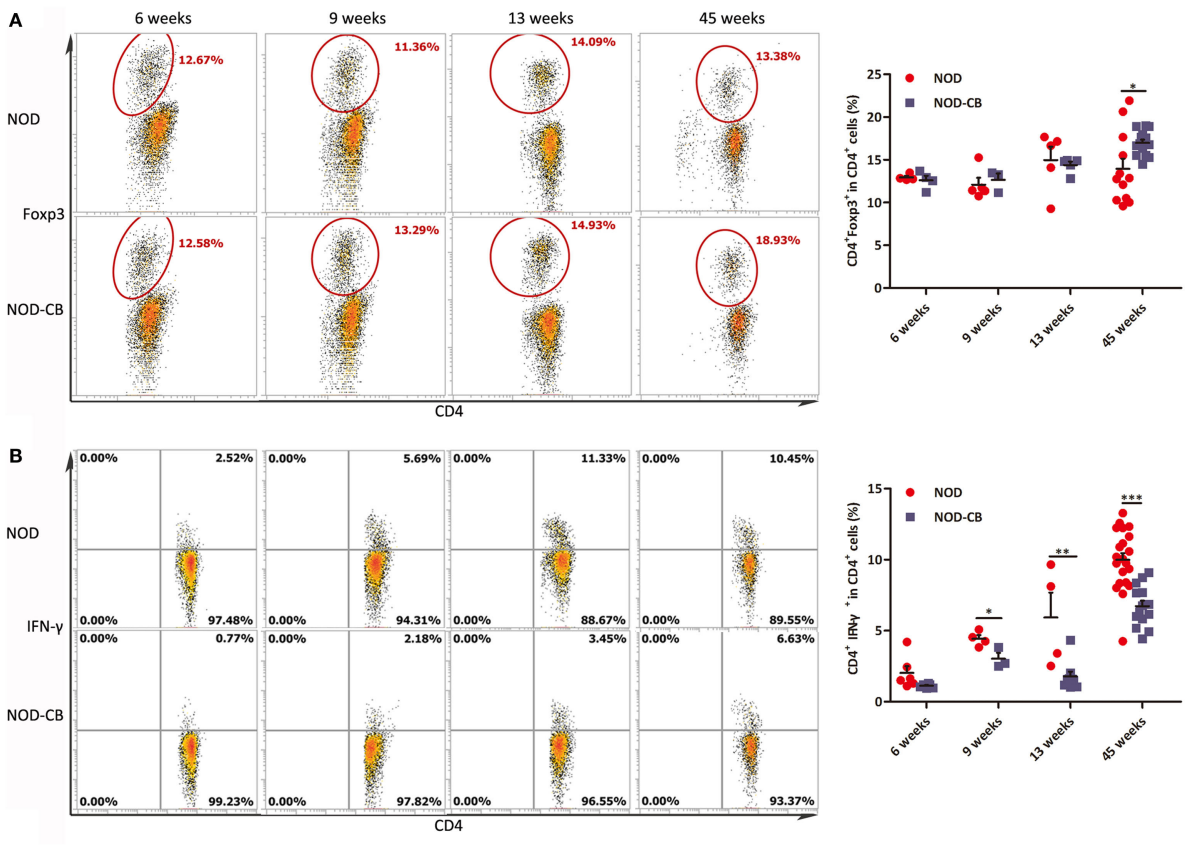
We analyzed splenic Tregs and found no differences in Treg levels among NOD-CB mice and NOD controls until 45 weeks (**Figure 9A**).

Furthermore, we analyzed cytokine profiles in the spleen and found a significant decrease in IFN-γ-producing Th1 cells and an increase in IL-4-producing Th2 cells (**Figures 9B,C**). Unexpectedly, the frequencies of Th17 cells were increased significantly in the spleen of NOD-CB mice *vs* NOD controls at 45 weeks of age (**Figure 9D**). These findings demonstrate that CB0313.1 decreases systemic inflammation primarily by reducing IFNγ$^+$CD4$^+$ T cells.

DISCUSSION

This study demonstrated that CB0313.1 limits the development of T1D primarily by modulation of intestinal immune homeostasis and induction of pancreatic Tregs in the early life of NOD mice. To the best of our knowledge, this is the first study that has shown a positive effect of CB0313.1 on T1D.

The development of anti-islet cell autoimmunity precedes the onset of clinical T1D and is already initiated at the age of 3–5 weeks in NOD mice (20). During the same period, dietary factors have a strong impact on the gut microbiome, which plays a central role in the development of the infant immune system (20). Therefore, weaning period (at 3 weeks of age) is an ideal time for probiotic intervention. The gut microbiome and the immune system develop synchronously. Literature shows varying effects of probiotics and antibiotics on T1D, and specific microbiome compositions may affect the risk of developing T1D (in either direction) (37). However, to date, the definitive demonstration



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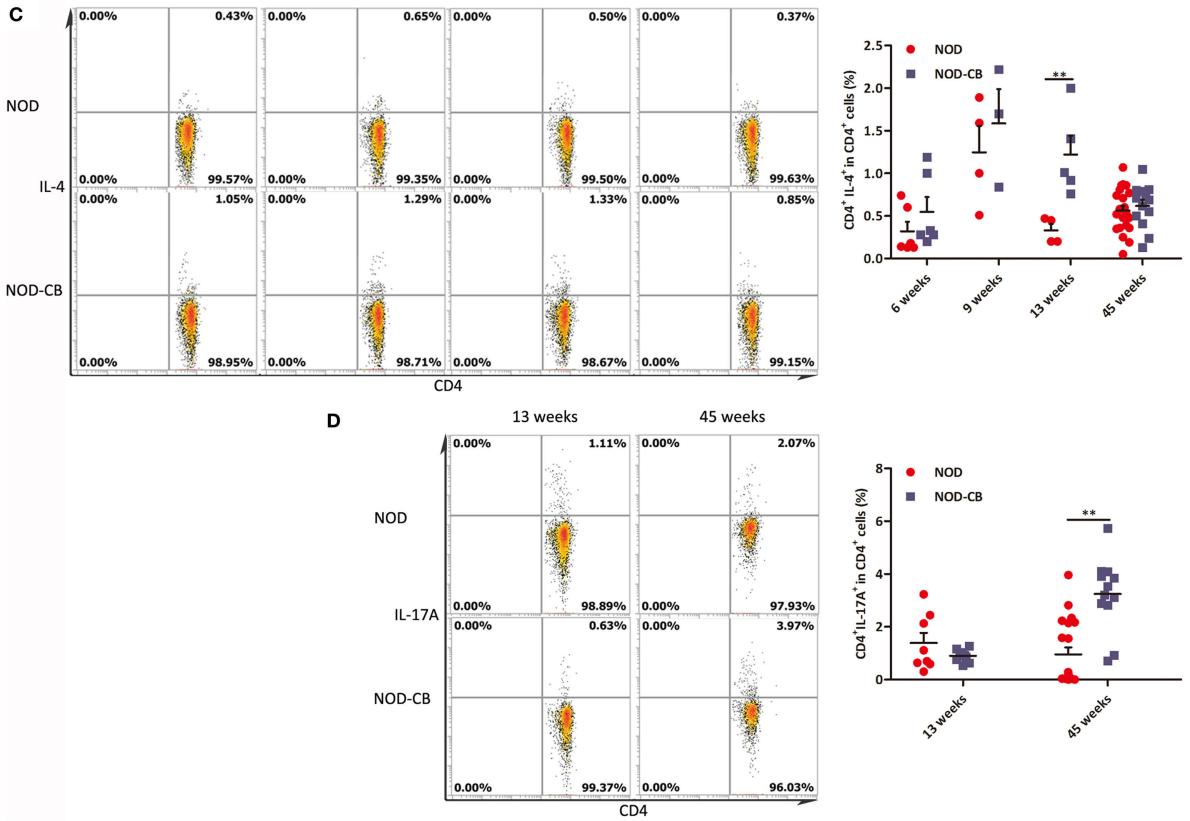

FIGURE 9 | CB0313.1 changes the splenic cytokine profiles. **(A)** Percent CD4+Foxp3+ cells in spleen as indicated at 6, 9, 13, and 45 weeks of age. Percent CD4+IFN-γ+ **(B)** CD4+IL-4+ **(C)** and CD4+IL-17A+ **(D)** cells in spleen as indicated. Data are mean ± SEM (n = 3–21 mice per group). *, **, *** $p < 0.05$, $p < 0.01$, $p < 0.001$ *vs* NOD control mice by t-test. The number of cells is 10,000.

of causal relationship regarding the specific microbiome composition and the development of T1D is still lacking (37). The main reason is that interactions between environment, gut microbiome, and the host organism were complex and numerous (38). Studies of the relationships between microbiome, immune system, and T1D may discover several microbial biomarkers of T1D, which may be used to better predict the risk for T1D. Moreover, specific bacterial or microbial metabolites can also serve as future interventions to combat autoimmune destruction of β-cells (39, 40). Our work is aimed to find out how the host immune system responds to probiotics in diabetic subjects, providing a therapeutic possibility by targeting the microbiome.

Based on the beneficial effects of CB0313.1 on preventing T1D and insulitis (**Figure 1**) and on improving metabolic dysfunction (**Figure 2**), we continued to investigate the impacts of butyrate (a major metabolite of CB0313.1) on NOD mice. However, we observed an early onset of T1D in NaB-treated mice. The dosage of NaB used in this study appeared to affect the nervous system, because the physical activity and heat production of NaB-treated mice were significantly increased *vs* NOD controls (**Figure 2**). As T1D patients are often accompanied by insufficient insulin, strenuous exercise may also lead to hyperglycemia and ketoacidosis (41).

Establishing causal relationship between the microbiome and physiology is critical to the ultimate goal of modifying the microbiome to prevent or treat diabetes. A key for deriving a mechanistic explanation of the described hypotheses may lie in the metabonomics analysis of intestinal contents from CB0313.1-treated mice.

We found that CB0313.1 treatment could increase the levels of acetic acid, propionic acid, and butyric acid in high fat diet mice model in our previous study; however, in this study, we only observed an increased trend of butyric acid in NOD-CB mice (**Figure 3K**). We did not treat our mice with acetate or propionate alone. There are several reports about propionic acid improving glycometabolism *via* several mechanisms, such as GLP-1, FFAR2, FFAR3, and intestinal gluconeogenesis, etc. (42). Therefore, it is possible that propionic acid can protect against T1D; however, there is no detailed investigation on the effect of propionic acid on T1D to date.

We found that Bacteroidetes abundance was significantly decreased in NOD-CB mice *vs* NOD controls (**Figures 3A,B**). The relative abundance of Bacteroidetes (**Figure 4A**), Bacteroidia (**Figure 4C**), Bacteroidales (**Figure 4D**), Bacteroidaceae (**Figure 4G**), and Bacteroides (**Figure 4J**) were positively associated with fasting glucose. Furthermore, the relative abundance

of Rikenellaceae, Prevotellaceae, Prevotella (belonging to the Bacteroidetes phylum) also showed positive correlation with fasting glucose in our study (**Figures 4H,I,N**). This is consistent with previous studies showing that the Bacteroides subgroups positively correlated with increased risk of early autoantibody development and decreased butyrate-producing bacteria (43, 44).

Clostridia is one of the most prominent Gram-positive and spore-forming bacterium indigenous to the murine gastrointestinal tract; and it becomes prominent after weaning and persists in the adult animals (45). Moreover, some *Clostridium* clusters were shown to induce colonic Tregs (13) and were involved in the maintenance of mucosal homeostasis (11) as well as the prevention of inflammatory bowel disease (46, 47). In our study, taxonomic groups Clostridia, Clostridiales, Clostridiaceae, and *Clostridium* were increased consistently in NOD-CB mice *vs* NOD controls (**Figures 3B–F**), and we found that the relative abundance of *Clostridium* was negatively associated with fasting glucose (**Figure 4K**), indicating that CB0313.1 formed an environment with enriched *Clostridium*-clusters. Thus, we speculate that CB0313.1 might be involved in type 2 immunity, inducing Tregs, thereby retarding and/or suppressing the onset of T1D (48, 49).

Although CB0313.1 only carries the *buk* gene, oral administration of CB0313.1 also causes a significant increase in butyrate-producing bacteria carrying the *butyryl-CoA* gene (**Figure 3J**), which might be explained by a modification of the other butyrate-producing bacteria by CB0313.1 *via* an unknown mechanism. However, this hypothesis merits further investigation.

Thus, the selective modulation of gut microbial phenotypes, particularly the enrichment of *Clostridium* subgroups and of the butyrate-producing bacteria, may contribute to the improvement of T1D and associated immune imbalance. Therefore, we speculate that CB0313.1 may exert its beneficial effect via itself and/or through its modification of the gut microbiota composition.

Previous reports have demonstrated that CB0313.1 alleviated intestinal inflammation (50), but the exact mechanism remains elusive. Intracellular staining for cytokine profiles of T cells from MLN demonstrated lower percentages of IFN-γ^+, IL-4^+ and similar percentages of IL-$17A^+CD4^+$ T cells in NOD-CB mice *vs* NOD controls (**Figure 5**). These findings are consistent with previous observations of young T1D patients (6).

Two weeks after birth, the peripheral lymph nodes of mice start to separate into T zone and B zones, whereas in germ-free mice, the development is repressed, suggesting that the gut

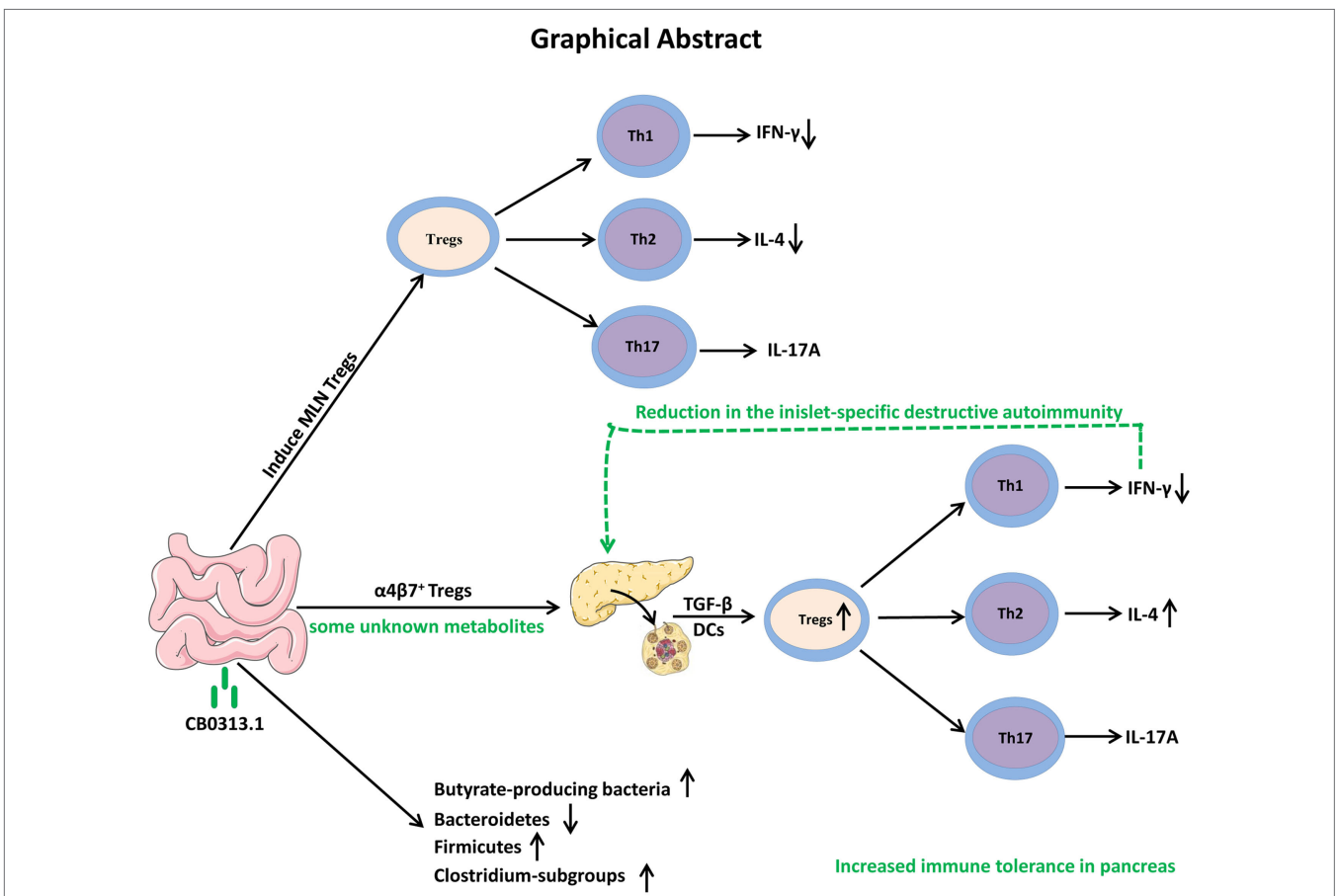

FIGURE 10 | Graphical abstract. Early oral CB0313.1 administration (starting at weaning in diabetes-prone NOD mice) induced Tregs in PLN and pancreas, balanced pancreatic Th1/Th2/Th17 cells, enhanced Firmicutes/Bacteroidetes, enriched *Clostridium*-subgroups and butyrate-producing bacteria subgroups, eventually preventing the onset and progression of type 1 diabetes.

microbiota affects the development of peripheral lymph nodes (51). In addition, recent reports indicated that the frequency of Foxp3$^+$ Tregs in colonic and small intestinal lamina propria increased after weaning (13), indicating that dietary factors influence the accumulation of intestinal Tregs, particularly after weaning. In our study, at 6 weeks of age, the percentages of CD4$^+$ T cells in MLN and PLN were significantly greater in NOD-CB mice *vs* NOD controls (Figure S5 in Supplementary Material), indicating that CB0313.1 promotes the development of MLN and PLN after weaning, but the molecular mechanism is unknown.

Recent reports have suggested that gut-associated lymphoid tissues might play a critical role in islet-specific autoimmunity in diabetes-prone individuals, even in humans (52–55). Consistent with these studies, we observed a significantly decreased MLN Tregs at 13 weeks of age accompanied by an increased PLN and pancreatic Tregs at in NOD-CB mice (**Figures 5E** and **6A**). Consequently, we speculated that the MLN Tregs might migrate to PLN *via* blood circulation or lymphocinesia in response to CB0313.1. Therefore, we used α4β7 as a gut homing marker to detect the trend of MLN Tregs. At 6 weeks of age, in the PLN, the percentage of CD4$^+$Foxp3$^+$ among α4β7$^+$ cells increased markedly, suggesting that CB0313.1 promotes the migration of gut-primed Tregs to the PLN. However, FTY720 experiment indicated that the majority of Tregs in the PLN might be induced locally and migrated α4β7$^+$ Tregs represented only a small fraction (**Figure 7**).

In addition, we found CB0313.1 treatment can effectively elevate the frequency of splenic CD11c$^+$ in CD45$^+$ cells (Figure S7 in Supplementary Material). Furthermore, an increased pancreas TGF-β was observed. Taken together, we speculate that in the pancreas and PLN, the Tregs may be induced by the DCs, TGF-β, or some other way.

It seems that CB0313.1 contributes to the reduced onset of diabetes by the gut-pancreas axis. However, the exact mechanism of immune cell circulation between gut and pancreas needs to be further investigated.

Moreover, in the PLN and pancreas, a higher proportion of CD4$^+$ T cells was also marked with Foxp3$^+$ in NOD-CB mice (**Figures 6A**, **7D** and **8A**), indicating a higher immune tolerance environment.

IL-17A is generally considered to be a proinflammatory cytokine in T1D (56). However, we analyzed splenic cytokine profiles and found the splenic IL-17A level significantly increased at the end of study in response to CB0313.1 (**Figure 9D**). It is possible that promoting insulitis, pancreatic inflammation (57), and progression to T1D (58, 59) only occurs after the conversion of Th17 cells to Th1, while in our study the IFNγ-producing Th1 cells did not increase.

In conclusion, our study demonstrates that oral CB0313.1 administration (starting at weaning) in diabetes-prone NOD mice induces the accumulation of PLN and pancreatic Tregs, which is associated with a reduction in the islet infiltrates and islet-specific destructive autoimmunity, eventually preventing the onset and progression of diabetes (**Figure 10**). Given the absence of side effects of CB0313.1 treatment for 45 weeks in NOD mice and the demonstrated safety of CB0313.1 in patients with gut related diseases (21, 50), CB0313.1 is likely to be a relative safe agent. Our results provide a rationale for future clinical trials on primary prevention of T1D by oral CB0313.1 administration.

ETHICS STATEMENT

All studies were approved by the Institutional Animal Ethics Committee of Jiangnan University (JN. No 20131205) and carried out in compliance with national and international guidelines for the Care and Use of Laboratory Animals.

AUTHOR CONTRIBUTIONS

YC, JS conceived the idea and reviewed the final manuscript. YC, JS, LJ designed the experiments. LJ wrote the manuscript with the assistance of YC and JS. LJ, KS, NF, YS, and JL performed flow cytometry. LJ performed the other experiments. LJ, L-LP, and CW analyzed the data. L-LP, ZL, HZ, WC, and JD revised the manuscript, provided intellectual input, and contributed to data acquisition. All authors participated in the discussion and commented on the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/article/10.3389/fimmu.2017.01345/full#supplementary-material.

FIGURE S1 | Insulitis score for non-obese diabetic (NOD)-CB and NOD control mice. Percentage of islets with a given score at 13 weeks of age in NOD-CB (n = 23) and NOD control (n = 23) mice. **(A)** 1 = white, no infiltration; **(B)** 2 = light gray, few mononuclear cells infiltrated; **(C)** 3 = gray, peri-insulitis; **(D)** 4 = dark gray, <50% islet infiltration; **(E)** 5 = black, >50% islet infiltration. Data are mean ± SEM. $^*p < 0.05$, $^{**}p < 0.01$.

FIGURE S2 | **(A)** Relative clostridial cluster IV abundance; **(B)** Relative clostridial cluster XIVa abundance. Data are mean ± SEM, (n = 3–5 mice per group). *, **, *** $p < 0.05$, $p < 0.01$, $p < 0.001$ *vs* NOD control mice by t-test.

FIGURE S3 | Methods of gating for FACS.

FIGURE S4 | Percent $CD4^+Foxp3^+$ cells in pancreas as indicated at 45 weeks of age.

FIGURE S5 | Percent $CD4^+$ cells in MLN and PLN. **(A)** Percent $CD4^+$ cells in MLN as indicated at 6, 9, and 13 weeks of age in the previous experiments using the antibody of CD4 (eBioscience, FITC). **(B)** Percent $CD4^+$ cells in MLN e as indicated at 6 weeks of age in the additional experiments using the new antibody of CD4 (Miltenyi, PE-vio770). **(C)** Percent $CD4^+$ cells in PLN as indicated at 6 weeks of age in the additional experiments using the antibody of CD4 (Miltenyi, PE-vio770).

FIGURE S6 | Percent $\alpha4\beta7^+$ cells in PLN Tregs. Data are mean ± SEM (n = 5–8 mice per group). *, **, *** $p < 0.05$, $p < 0.01$, $p < 0.001$ *vs* NOD control mice by *t*-test.

FIGURE S7 | Percent $CD11c^+$ cells in splenic $CD45^+$ cells. *, **, *** $p < 0.05$, $p < 0.01$, $p < 0.001$ *vs* NOD control mice by *t*-test.

TABLE S1 | Information of antibody for FACS.

TABLE S2 | Primer sequences used in this study for microbial abundance.

TABLE S3 | Information of antibody for FACS for the experiment of regulatory T cells migration.

TABLE S4 | Information of antibody for FACS for the experiments of $CD45^+CD11c^+$.

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frontiers in Immunology

ORIGINAL RESEARCH
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Aged Gut Microbiota Contributes to Systemical Inflammaging after Transfer to Germ-Free Mice

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Advanced age is associated with chronic low-grade inflammation, which is usually referred to as inflammaging. Elderly are also known to have an altered gut microbiota composition. However, whether inflammaging is a cause or consequence of an altered gut microbiota composition is not clear. In this study, gut microbiota from young or old conventional mice was transferred to young germ-free (GF) mice. Four weeks after gut microbiota transfer immune cell populations in spleen, Peyer's patches, and mesenteric lymph nodes from conventionalized GF mice were analyzed by flow cytometry. In addition, whole-genome gene expression in the ileum was analyzed by microarray. Gut microbiota composition of donor and recipient mice was analyzed with 16S rDNA sequencing. Here, we show by transferring aged microbiota to young GF mice that certain bacterial species within the aged microbiota promote inflammaging. This effect was associated with lower levels of *Akkermansia* and higher levels of TM7 bacteria and *Proteobacteria* in the aged microbiota after transfer. The aged microbiota promoted inflammation in the small intestine in the GF mice and enhanced leakage of inflammatory bacterial components into the circulation was observed. Moreover, the aged microbiota promoted increased T cell activation in the systemic compartment. In conclusion, these data indicate that the gut microbiota from old mice contributes to inflammaging after transfer to young GF mice.

Keywords: gut microbiome, immune system, inflammaging, germ-free mice, aging

INTRODUCTION

The gut microbiota is a highly complex and diverse community of bacteria that closely interacts with the epithelium and underlying immune cells in the gut (1). The bacterial divisions that dominate the human gut microbiota are *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Proteobacteria* (2). The dominance of these bacterial divisions is evolutionary conserved and has been confirmed

in different mammalian species (3, 4). In recent years, it has become clear that the gut microbiota has a major impact on the immune system, metabolism, and even behavior of the host (5). Moreover, an imbalance in gut microbiota composition (dysbiosis) has been associated with several immunological, metabolic, and mental disorders (6). However, for the majority of these diseases it remains unclear whether dysbiosis is a cause or consequence of the disease.

In adults, the gut microbial community remains relatively stable (7). However, a number of studies have shown that gut microbiota composition is different in the elderly. For example, it has been demonstrated that *Firmicutes* was dominant in the gut microbiota of young individuals, whereas *Bacteroidetes* was more prevalent in the gut microbiota in the elderly (8, 9). Others found a decrease in anaerobes such as *Bifidobacteria*, but an increase in *Enterobacteria*, such as Escherichia *coli* in the elderly (10, 11). In people above 100 years old, an increase in pathobionts was observed (12). Also bacteria with anti-inflammatory properties such as *Faecalibacterium prauznitzii* were decreased in older individuals (13).

Concomitantly with microbiota changes, immunity becomes impaired in elderly (14). Elderly are known to be more susceptible to infections and mount less effective immune responses after vaccination. Moreover, homeostasis between pro-inflammatory and regulatory responses is lost, which results in a state of low-grade chronic systemic inflammation (14). The age-related chronic inflammation, which is called inflammaging, likely contributes to the pathology of several diseases typically associated with aging such as dementia, stroke, and cardiovascular diseases. In addition, advanced age has been reported to increase intestinal permeability in rodents and non-human primates and may subsequently enhance translocation of luminal bacterial products and induce inflammation (15, 16).

Whether age-induced microbiota changes are associated with inflammaging is not entirely clear, but there are some indications that intestinal microbes are involved in this process (17). To address the influence of the aged gut microbiota on the immune system, we transferred the gut microbiota from young or old conventional mice to germ-free (GF) mice. We demonstrate that the aged microbiota induced higher frequencies of several T helper (Th) cell subsets, in particular in the spleen. Moreover, expression of several inflammatory markers was elevated in the ileum after transferring microbiota of aged mice. Presumably translocation of bacterial components occurred, since the serum after transfer of aged microbiota contained higher levels of immunostimulatory bacterial components. Finally, gut microbiota composition analysis revealed differences in abundance of bacterial species such as *Akkermansia*, TM7, and *Proteobacteria*, which are potentially involved in the increased inflammatory potential of the microbiota of aged mice.

MATERIALS AND METHODS

Study Design

All animal experiments were approved by the local ethical committee of the University of Groningen (project number 6543) and adhered to FELASA guidelines. The objective of this study was to determine whether the gut microbiota from aged mice contributes to the aging of the immune system. To this end, gut microbiota from young or old conventional mice was transferred to young GF mice. Effects on the immune system were compared between young or old conventional mice, GF recipients of young or old microbiota, and GF mice that remained GF throughout the experiment. Each experimental group consisted of 10 mice per group, except the control group of GF mice, which consisted of 5 mice per group. After an acclimatization period of at least 4 weeks, feces were freshly collected from the conventional mice. Feces from the same group was pooled and mixed in PBS. Next, 200 µl of 100 mg/ml of this mixture were given by oral gavage to GF mice of 12–14 weeks old. After transfer, recipient mice were individually housed in IVC cages for another 4 weeks. Mice were sacrificed on 10 different days, each day one mouse per group.

Mice

Young (7–10 weeks) or old (17 months) C57BL/6JRccHsd conventional female mice were purchased from a commercial supplier (Envigo, Horst, the Netherlands). Female GF mice of 12–14 weeks were obtained from a breeding colony at the animal facility of Radboud University Nijmegen Medical Centre (Nijmegen, the Netherlands). All animals were put on an autoclaved Rat/mouse maintenance V153X R/M-H diet (Ssniff, Soest, Germany) directly after weaning in the case of GF mice, or directly after arrival in the case of conventional mice. The mice were kept on this diet throughout the experiment. Conventional mice were housed in IVC cages and GF mice were housed in GF isolators.

Organ and Tissue Collection

Mice were sacrificed at the following ages: young conventional mice 16–19 weeks, old conventional mice 19–20 months, GF recipient mice 16–18 weeks, GF mice 13–15 weeks. Mice were anesthetized with isoflurane, bled, and sacrificed by cervical dislocation. Serum was collected and stored at −80°C. Colon content and a piece of terminal ileum were snap frozen in liquid nitrogen and stored at −80°C. In addition, spleen, Peyer's patches (PPs), and mesenteric lymph nodes (MLNs) were collected for FACS analysis.

Flow Cytometry

Single cell suspensions were obtained from spleen, PPs, and MLNs. Cells were stained with Fixable Viability Dye eFluor 506 (eBioscience, Vienna, Austria) for exclusion of dead cells. A-specific binding to Fc receptors was blocked by incubating the cells with anti-CD16/32 (clone 93, Biolegend, Uithoorn, the Netherlands) for 15 min on ice. For extracellular staining, cells were incubated with the desired mixture of antibodies for 30 min on ice. After washing, cells were fixed with FACS lysing solution (BD Biosciences, Breda, the Netherlands). For intracellular staining, fixed cells were permeabilized with PERM (eBioscience, Vienna, Austria) and subsequently stained with the desired antibodies for 30 min on ice. For identification of the different Th cell subsets, cells were stained with antibodies against: CD3e

(clone 17A2), CD4 (clone GK1.5), T-bet (clone 4B10), RORγt (clone B2D), Gata-3 (clone TWAJ), CD25 (clone PC61), and Foxp3 (clone FJK-16S). Appropriate isotype controls were used to determine specificity of the staining. Samples were acquired with the FACSVerse (BD Biosciences, Breda) and analyzed with FlowJo software (FlowJo, LLC, Oregon, USA).

Transcriptome Microarray

A piece of terminal ileum from each mouse was snap frozen in liquid nitrogen and stored afterward at −80°C. From these samples, RNA was isolated with the RNeasy kit (Qiagen, Valencia, CA, USA) and whole-genome gene expression was analyzed with Affymetrix GeneChip Mouse Gene 1.1 ST arrays as described previously (18). The gene expression datasets were deposited in NCBI's Gene Expression Omnibus (GEO) and are accessible through GEO Series accession number: GSE104063.

HEK293 toll-like receptor (TLR)2/TLR4 Assay

Human Embryonic Kidney 293 cells stably transfected with mouse TLR2/CD14 or TLR4/MD-2/CD14 and the secreted embryonic alkaline phosphatase reporter coupled to the NF-kB/AP-1 promoter were purchased from Invivogen (San Diego, CA, USA). Every cell line was grown at 37°C, 5% CO_2 in DMEM medium (Lonza B.V., Basel, Switzerland), supplemented with 4.5 g/l glucose, 10% heat-inactivated FBS, 2 mM L-glutamine, 50 U/ml penicillin, 50 mg/ml streptomycin, and 100 mg/ml Normocin. After two passages, the cells were cultured in the presence of HEK-Blue selection medium (Invivogen, San Diego, CA, USA) in order to maintain the transfected constructs. Cells were stimulated with 2.5% mouse serum for 20 h at 37°C, 5% CO_2. As a control, cells were stimulated in triplo with medium only (negative control), 100 ng/ml lipopolysaccharide (LPS)-EK (InvivoGen; positive control for TLR4), or 10^7 bacteria/well of heat-killed Listeria monocytogenes (InvivoGen; positive control for TLR2). Next, 20 µl medium from each well was aliquoted and mixed with 180 µl QUANTI-Blue reagent (Invivogen, San Diego, CA, USA). After incubation at 37°C for 2 h, OD at 650 nm was measured with a microplate absorbance spectrophotometer (Bio-Rad Laboratories, Veenendaal, the Netherlands).

Microbiota Analysis

Fresh feces samples obtained just after defecation were collected from all mice at different time points during the experiment. In addition, colonic content samples from these mice were collected at the end of the experiment. All samples were snap frozen in liquid nitrogen and stored at −80°C. These samples were used for 16S rRNA gene analysis for microbiota profiling with barcoded amplicons from the V1–V2 region of 16S rRNA genes as described previously (18). Briefly, amplicon pools were 250 bp paired-end sequenced using Illumina Miseq (GATC-Biotech, Konstanz, Germany). The Illumina Miseq data analysis was carried out with a workflow employing the Quantitative Insights Into Microbial Ecology pipeline (19) and a set of in-house scripts as described before for Illumina Hiseq 16S rRNA gene sequences (Hermes et al., manuscript in preparation).

Statistics

Flow cytometry data and HEK293 TLR assay data are expressed as means, error bars represent SEM. To verify whether data were normally distributed, the Kolmogorov–Smirnov test was performed. In cases where data were not normally distributed, data were log transformed before analysis. For comparing two groups, the unpaired two-tailed Student's *t* test was used. For comparing more than two groups with each other, one way ANOVA was performed followed by the Bonferroni test to compare specific groups. *P*-values below 0.05 were considered significant. All tests were performed with Graphpad software (Prism, La Jolla, CA, USA).

RESULTS

Microbiota of Old Mice Enhances CD4$^+$ T Cell Differentiation in the Spleen

In order to investigate how aging influences the interplay between the gut microbiota and the immune system of the host, we transferred gut microbiota from young (11–14 weeks) or old (18 months) conventional mice to young GF mice (12–14 weeks). Four weeks later, the mice were sacrificed and the frequency of the different CD4$^+$ Th subsets were identified in the PPs, MLNs, and the spleen by flow cytometry (see Figure S1 in Supplementary Material for gating strategy). Conventional mice, aged 11–14 weeks, or 18 months served as control.

In conventional mice, a higher frequency of Th2 cells was found in the spleen of old mice compared with young mice (**Figure 1A**). This enhanced Th2 frequency could be induced by transfer of the old microbiota to young GF mice and was not observed when young microbiota was transferred. Also the high T_{reg} (**Figure 1B**) and Th1 (**Figure 1C**) numbers in spleens of old conventional mice could be induced by transfer of old microbiota. GF mice, which received the old microbiota had a higher frequency of splenic T_{regs} (**Figure 1B**) and Th1 cells (**Figure 1C**) than GF mice which received the young microbiota. No differences were observed in Th17 cells (data not shown). Furthermore, in the PPs and MLNs no differences were observed in Th frequencies (data not shown), except for Th1 cells in PPs, which were significantly higher in GF mice after transfer of the old microbiota (**Figure 1D**) when compared with GF mice after transfer of microbiota of young mice. In conclusion, the old microbiota enhanced CD4$^+$ T cell differentiation or distribution of several Th subsets, in particular in the systemic compartment.

Microbiota of Old Mice Upregulates Inflammation-Associated Immune Pathways in the Ileum

To study the effect of the microbiota on the host in an unbiased manner, we performed genome-wide gene expression analysis of

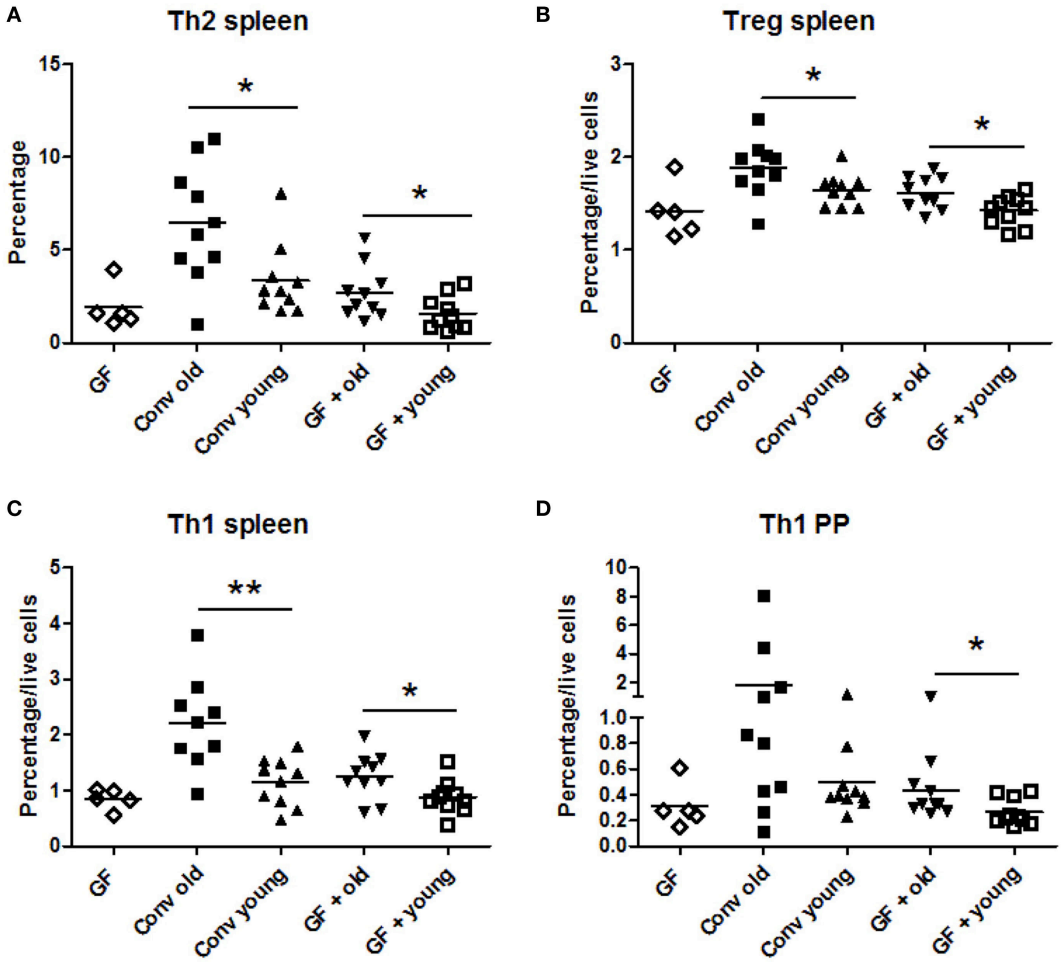

FIGURE 1 | Old microbiota induces higher frequencies of T helper (Th) subsets in the spleen. Spleen, mesenteric lymph node, and Peyer's patch (PP) CD4$^+$ T cell populations were analyzed with flow cytometry after isolation from young or old conventional (conv) mice ($n = 10$), germ-free (GF) recipient mice of young or old microbiota ($n = 10$), and GF control mice ($n = 5$). **(A)** Percentage of splenic CD4$^+$ T cells expressing GATA-3 (Th2). **(B)** Percentage among total live cells of splenic CD4$^+$ T cells expressing CD25 and Foxp3 (T_{reg}). **(C)** Percentage among total live cells of splenic CD4$^+$ T cells expressing T-bet (Th1). **(D)** Percentage among total live PP cells of CD4$^+$ T cells expressing T-bet (Th1). All data are expressed as means. $^*P < 0.05$, $^{**}P < 0.01$.

the ileum with microarray. Genes that were significantly higher expressed in the ileum of old conventional mice compared with young conventional mice were analyzed with the STRING database (20). We identified a large cluster of genes involved in the immune response that were upregulated in the ileum of old conventional mice (**Figure 2A**). The function of these genes included antigen processing and presentation, activation of the complement pathway, recognition of microbe-associated molecular patterns, and migration of B cells. TNF-α was in the center of this network, which might suggest that TNF-α plays an important role in these processes.

Also genes that were significantly higher expressed in GF mice that received the old microbiota compared with recipients of young microbiota were analyzed with the STRING database. Also here we identified a cluster of genes with TNF-α in the center of the network (**Figure 2B**). These results might suggest that TNF-α production is specifically enhanced by the old microbiota.

Identification of Immune Pathways Specifically Affected by Old and Young Microbiota

Microarray data were further analyzed with Ingenuity Pathway Analysis (IPA), only focusing on genes that were significantly differentially expressed ($P < 0.05$, fold-change >1.2 or <-1.2) when comparing old versus young conventional mice, or GF recipient mice that received young versus old microbiota. Interestingly, we observed three canonical pathways that were significantly affected both in the conventional mice and in the GF recipient mice (**Figure 3A**). The canonical pathways "role of PRRs in recognition of bacteria and viruses," "Th cell differentiation," and "B-cell development" were upregulated in old conventional mice compared with young conventional mice and also in GF recipients of old microbiota compared with recipients of young microbiota. Therefore, these pathways might be in particular influenced by the microbiota during aging.

Also predicted upstream regulators were identified with IPA. Upstream regulators are the upstream transcriptional regulators that potentially explain the observed gene expression differences in the dataset. The most significantly predicted upstream regulator that could cause the gene expression profile in old conventional mice in comparison with young conventional mice was LPS (**Figure 3B**). Importantly, LPS from *Salmonella enterica* was among the most significantly predicted upstream regulators of old microbiota after transfer to GF mice (**Figure 3B**). Thus, LPS is a component of the old microbiota that is possibly involved in mediating its effects on the immune system of the host.

To further identify the genes that were specifically influenced by the old microbiota, we compared the genes that were differentially expressed between young versus old conventional mice and GF recipients of young versus old microbiota (**Figure 3C**). We identified 27 genes that were differentially expressed in both datasets. This list of genes was further narrowed down to genes that were up- or down-regulated in both datasets and are known to play a role in the immune response. As mentioned above, TNF-α was upregulated both in old conventional mice and in GF recipients of old microbiota. Also, TNFSF8, which is the ligand for CD30, was more highly expressed in these groups of mice. On the other hand, several genes encoding for the lambda immunoglobulin light chain were more highly expressed both in young conventional mice and in GF recipients of young microbiota.

Higher Amounts of Bacterial Components in Systemic Circulation after Old Microbiota Transfer

As LPS was a prominent predicted upstream regulator in the ileal IPA analysis, we investigated whether there were innate immune activating components in sera of animals receiving old microbiota. These components can possibly be transferred from the intestine by translocation of bacterial components due to a compromised intestinal barrier (21, 22). To this end, we incubated the sera of these mice with HEK293 cells transfected with TLR 2 or TLR4. Activation of NFκB was measured with a reporter gene. No differences were observed between old conventional mice and young conventional mice. However, the sera from GF mice, which had received old microbiota showed significantly higher activation of TLR2 compared with sera from recipients

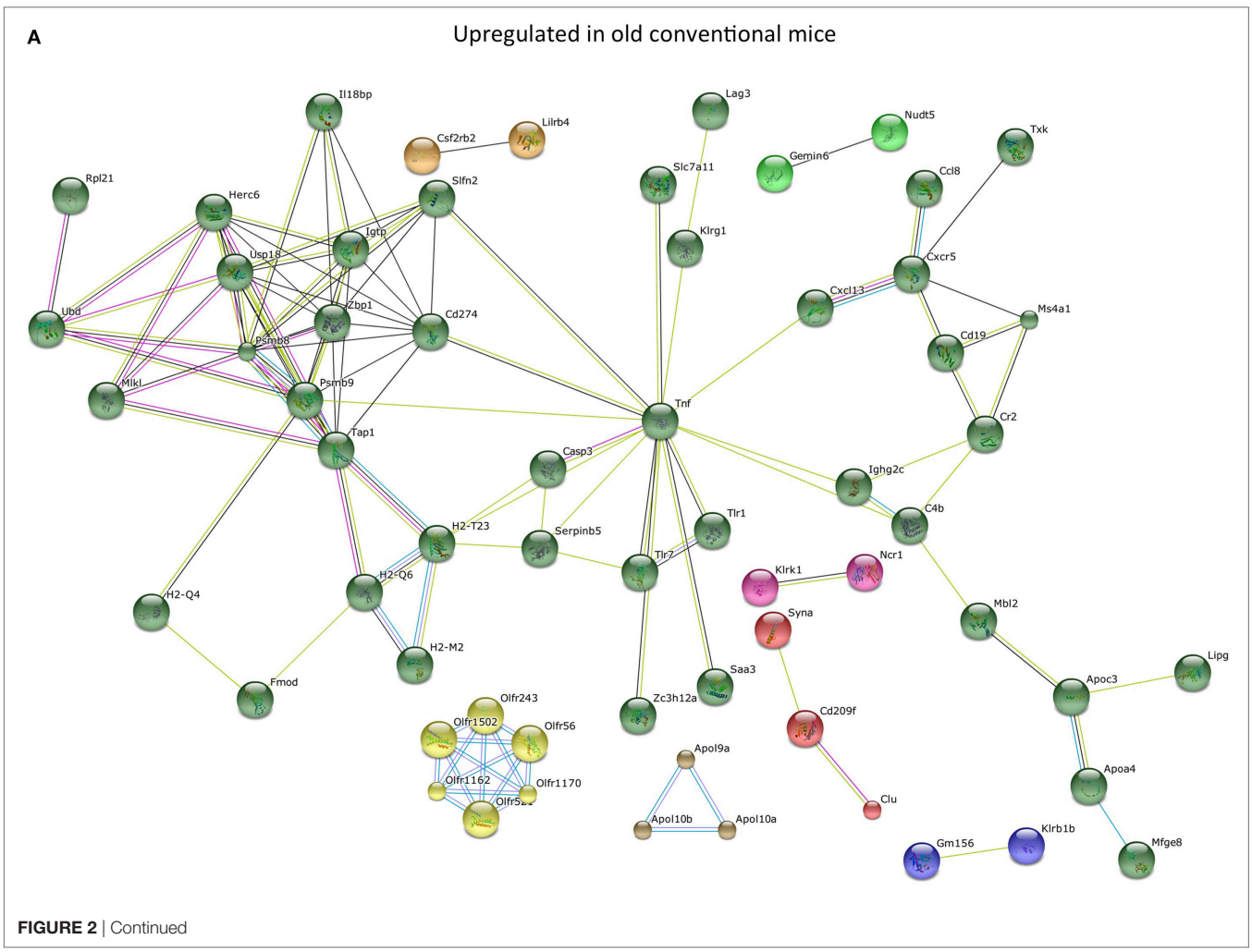

FIGURE 2 | Continued

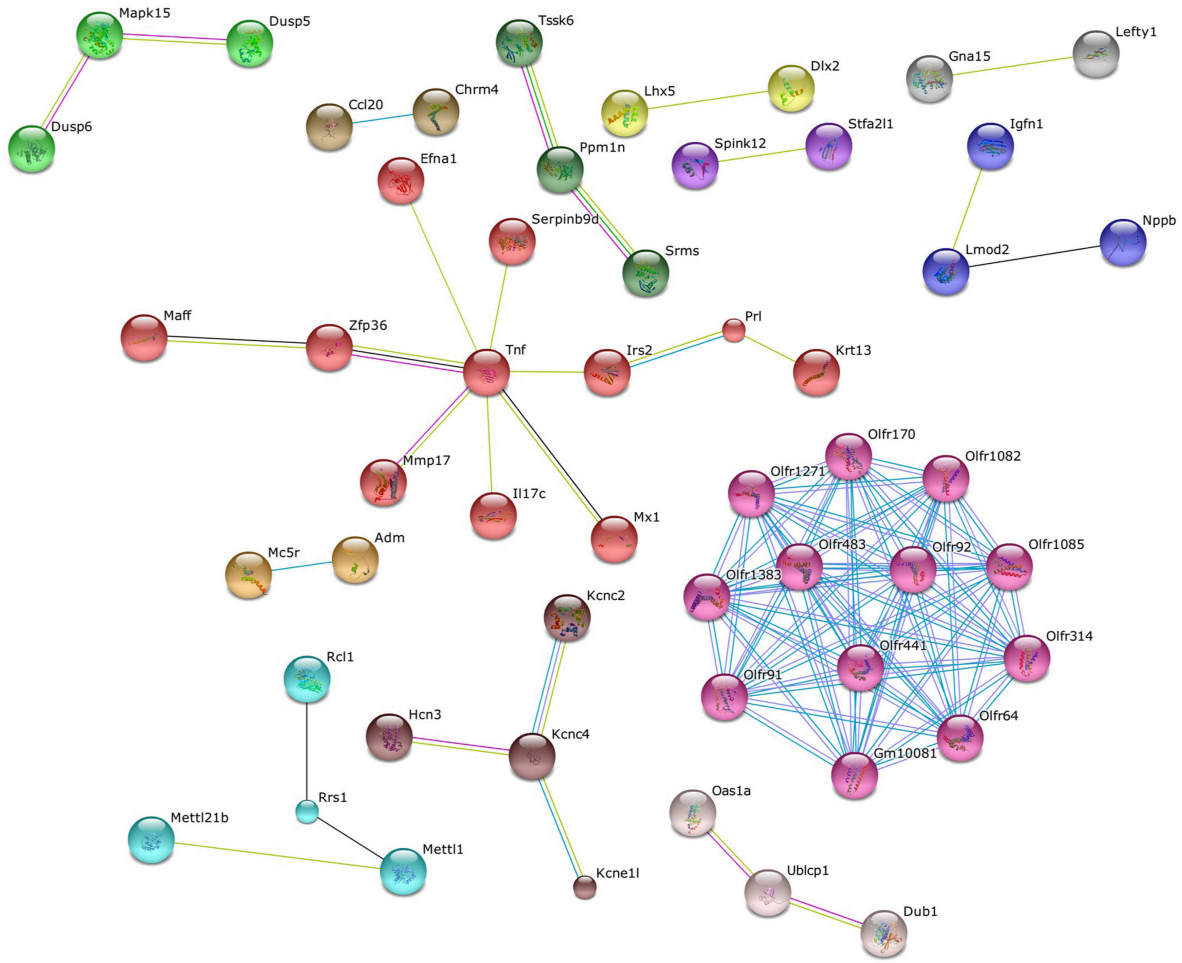

FIGURE 2 | Old microbiota upregulates several immune pathways in the ileum. Whole-genome gene expression in the ileum of mice ($n = 5$ per group) was assessed with Affymetrix GeneChip Mouse Gene 1.1 ST arrays. The most highly upregulated genes were analyzed with the STRING database. Only genes with at least one interaction are shown. The following interactions are indicated: from curated databases (blue), experimentally determined (pink), textmining (yellow), co-expression (black), and protein homology (purple). **(A)** Genes that were upregulated ($P < 0.05$, fold-change >1.3) in the ileum of old conventional mice compared with young conventional mice. **(B)** Genes that were upregulated ($P < 0.05$, fold-change >1.2) in the ileum of germ-free (GF) mice that received old microbiota compared with GF mice that received the young microbiota.

of young microbiota (**Figure 4A**). A similar trend was observed for TLR4 activation, although this difference did not reach statistical significance (**Figure 4B**). In summary, these data indicate that old microbiota transfer leads to increased translocation of inflammatory bacterial products into the circulation.

Bacterial Groups Associated with Increased Inflammatory Potential of Old Microbiota

Next, we investigated how the gut microbiota composition changes over time in the recipient mice. To this end, the composition of the gut microbiota of the different experimental groups was analyzed with 16S rDNA sequencing. From the GF recipient mice that received old or young microbiota, we analyzed feces 1 week after transfer or 4 weeks after transfer (**Figure 5**). We were particularly interested to see whether the gut microbiota evolves into a community similar to the donor, or whether it adapts to its host. Redundancy analysis at the genus level confirmed that gut microbiota composition was different between old conventional mice and young conventional mice, since the samples separated into two distinct clusters (**Figure 6A**). The samples collected 1 week after transfer also separated into two different clusters, suggesting that the transfer of different gut microbiota communities also led to the establishment of different microbiota communities in the recipients. However, after 4 weeks gut

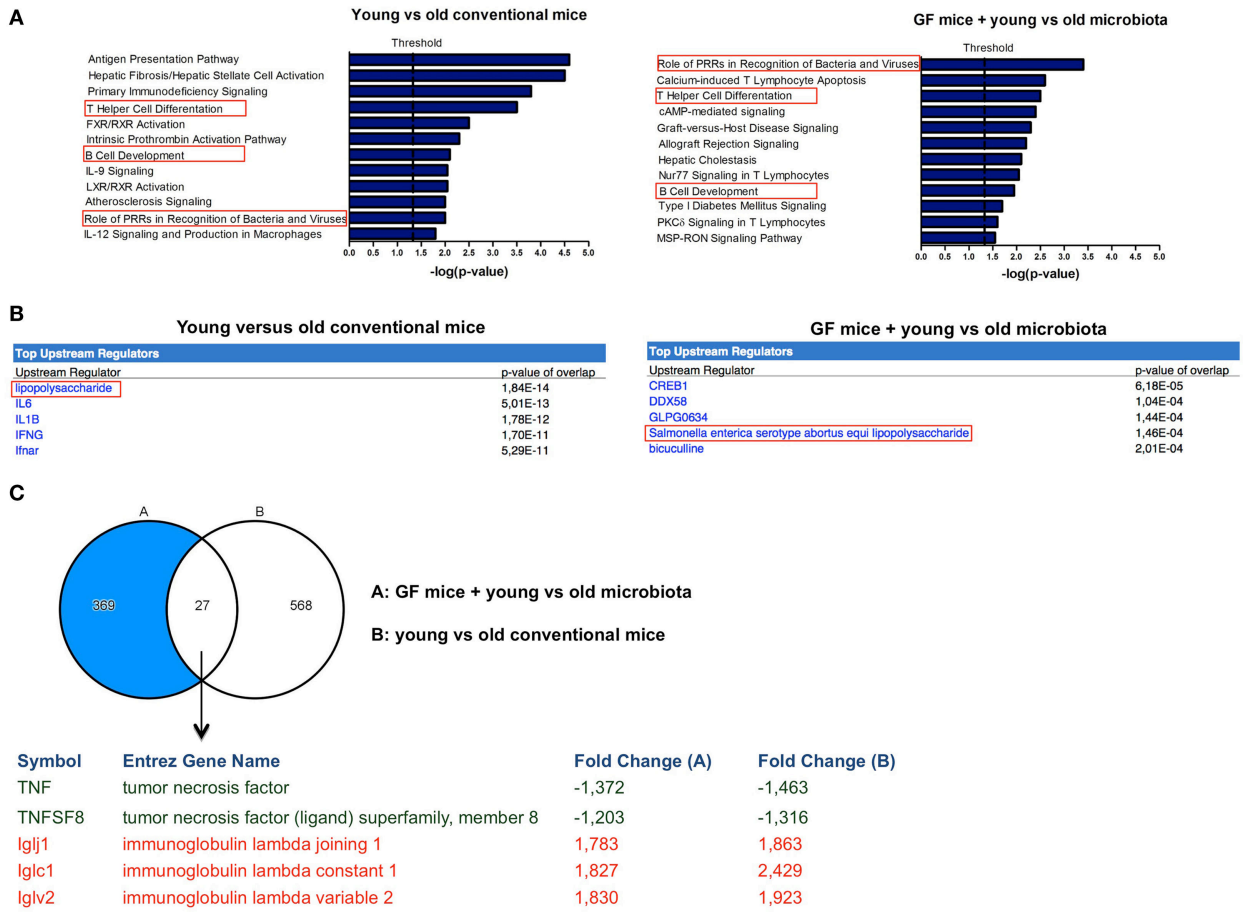

FIGURE 3 | Markers of inflammation are upregulated in the presence of old microbiota. Whole-genome gene expression in the ileum of mice ($n = 5$ per group) was assessed with Affymetrix GeneChip Mouse Gene 1.1 ST arrays. Genes that were significantly differentially expressed ($P < 0.05$ and fold-change >1.2) between young and old conventional mice, or germ-free (GF) recipients of young or old microbiota were analyzed with Ingenuity Pathway Analysis. **(A)** Canonical pathways that were most significantly affected by age or after transfer of aged microbiota. **(B)** Most significantly predicted upstream regulators by comparing young and old conventional mice, or GF recipients of young or old microbiota. **(C)** Venn diagram of differentially expressed genes between young and old conventional mice, and GF recipients of young or old microbiota.

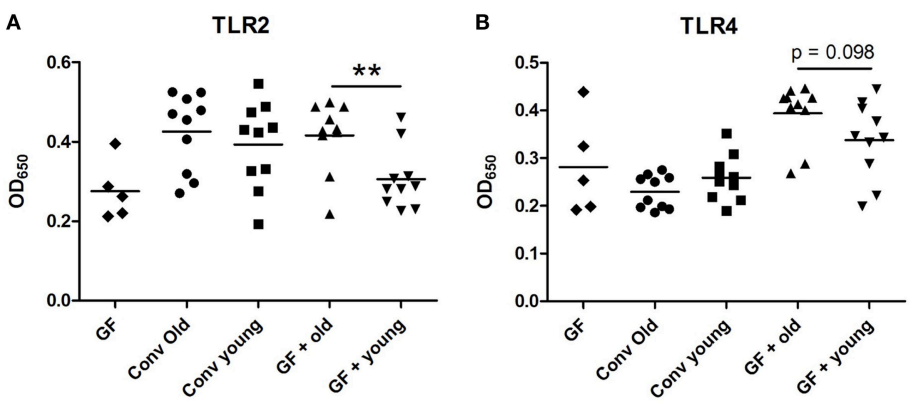

FIGURE 4 | Transfer of old microbiota enhances inflammatory bacterial components in serum. Human Embryonic Kidney 293 cells transfected with mouse toll-like receptor (TLR)2/CD14 **(A)** or mouse TLR4/MD-2/CD14 **(B)** were stimulated with 2.5% serum from young or old conventional (conv) mice ($n = 10$), germ-free (GF) recipient mice of young or old microbiota ($n = 10$), and GF control mice ($n = 5$). Activation of these receptors was measured with a secreted embryonic alkaline phosphatase reporter coupled to the NF-kB/AP-1 promoter. All data are expressed as means. $^{**}P < 0.01$.

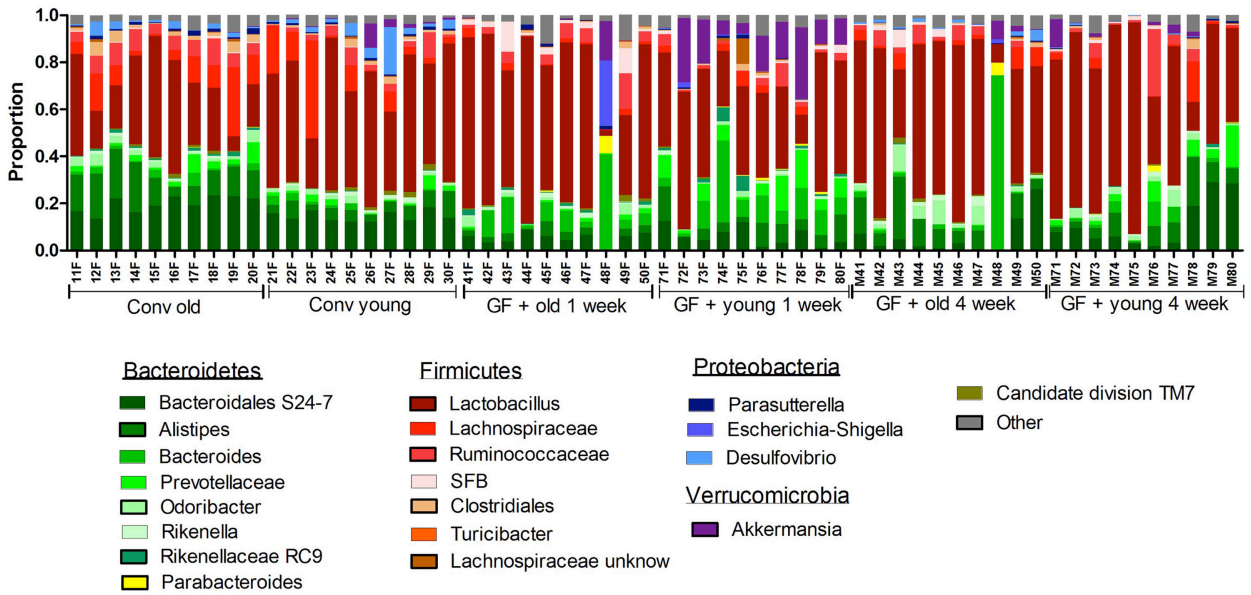

FIGURE 5 | Gut microbiota composition in conventional and conventionalized mice. Fecal samples were collected from conventional (conv) mice ($n = 10$) at the time of transfer to the germ-free (GF) recipient mice, or from the GF recipient mice ($n = 10$) 1 and 4 weeks after the transfer. Gut microbiota composition was analyzed with 16S rDNA sequencing and data are presented as the relative abundance of the different bacterial groups for each individual mouse. The most highly abundant bacterial groups are indicated.

microbiota composition in the recipient mice was most similar to the gut microbiota composition in young conventional mice. Moreover, at this time point the clusters of samples derived from the recipient mice were showing more overlap, which suggests gut microbiota composition of the two groups became more similar to each other compared with the first week time point. Together these results indicate that at 1 week the gut microbiota composition of the donor dictates the gut microbiota composition in the recipient, but at later time points the gut microbiota composition adapts to the host.

To look more specifically at the bacterial groups that were responsible for the observed differences in immune responses, we investigated which bacterial phyla had a significant difference in abundance (**Figure 6B**). Compared with young conventional mice, old conventional mice had higher abundance of *Tenericutes*, but lower abundance of *Verrucomicrobia*. *Akkermansia* is the only genus known to belong to the *Verrocumicrobia* phylum. Indeed we observed a similar difference in abundance for *Akkermansia* (data not shown). In addition, age influenced the *Firmicutes/Bacteroidetes* ratio. Old conventional mice had more *Bacteroidetes*, but less *Firmicutes* compared with young conventional mice. Interestingly, 1 week after transfer of old microbiota, recipient mice had significant less *Verrucomicrobia* than GF mice that received young microbiota (**Figure 6B**). There was also a difference in the *Firmicutes/Bacteroidetes* ratio, but surprisingly recipients of old microbiota had significantly less *Bacteroidetes* and more *Firmicutes* compared with recipients of young microbiota. Four weeks after transfer, the differences at 1 week were no longer present. However, at this time point recipients of old microbiota had a higher abundance of TM7 and *Proteobacteria* (**Figure 6B**). The difference in *Proteobacteria* was likely due to a difference in abundance of *Desulfovibrio*, since this was the only *Proteobacterium* that was significantly more abundant at the genus level after transfer of old microbiota. In summary, a number of bacterial groups were identified that were affected by age, which included *Akkermansia*, TM7, and *Proteobacteria*. These bacterial groups are possibly involved in the increased inflammatory potential of the old microbiota.

DISCUSSION

Several studies have demonstrated that aging is associated with an altered gut microbiota composition, inflammaging, and increased gut permeability (17). However, whether the aged microbiota is a cause or consequence of inflammaging is not known. To the best of our knowledge, we are the first to show that some characteristics of this typical immunosenescence can be induced by microbiota of aged mice after transfer into young GF mice. Microorganisms associated with this effect were found to be *Akkermansia*, TM7 bacteria, and *Proteobacteria*. Our results indicate that increased amounts of TLR2-stimulating components were found in the circulation of recipients of old microbiota, which may have promoted increased inflammation and enhanced T cell differentiation. Interestingly, dysbiosis and a comprised intestinal barrier are also observed in several other disorders such as IBD and metabolic syndrome (23, 24).

Certain bacterial species colonizing the gut have been shown to induce a specific subset of Th cell. For example, segmented filamentous bacterium was found to specifically induce Th17 cells in the gut (25). On the other hand, polysaccharide

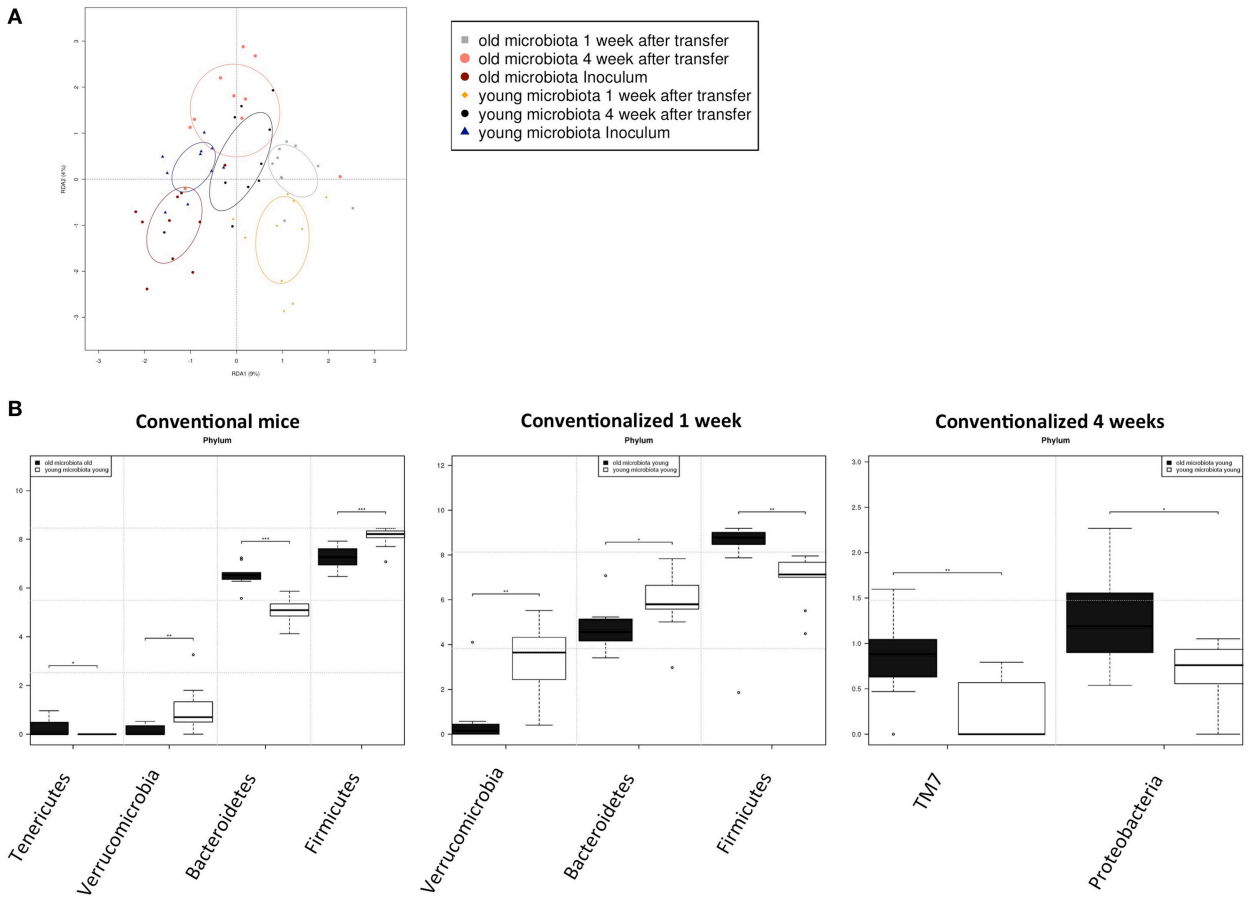

FIGURE 6 | Transfer of aged microbiota to germ-free (GF) mice leads to altered gut microbiota composition. Fecal samples were collected from conventional (conv) mice ($n = 10$) at the time of transfer (inoculum) to the GF recipient mice, or from the GF recipient mice ($n = 10$) 1 and 4 weeks after the transfer. Gut microbiota composition was analyzed with 16S rDNA sequencing. **(A)** Redundancy analysis of gut microbiota composition of the different experimental groups at the genus level. **(B)** Bacterial phyla that were significantly different in abundance in young or old conventional mice, or in GF mice conventionalized with young or old microbiota after 1 or 4 weeks $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.01$.

A produced by the human symbiont *Bacteroides fragilis* was shown to promote expansion of IL-10-producing T_{reg} cells in a TLR2-dependent manner (26–28). It has also been demonstrated that certain *Clostridium* species induce T_{reg} cells in the colon (29, 30). In our study, the aged microbiota did not promote differentiation of a specific Th cell subset. However, after transfer of aged microbiota to GF mice, we rather observed increased levels of several Th cell subsets. This effect was almost exclusively observed in the spleen, but not in the PPs or MLNs. These results do not suggest an association with any of the bacterial species mentioned above. An increased exposure of naïve T cells in the systemic compartment to bacterial compounds in general as a result of a reduced intestinal barrier seems a more likely explanation.

The transfer of old microbiota into young GF mice induced differential regulation of pathways including T cell differentiation, B-cell development, and recognition of microbes by pattern recognition receptors. A central regulatory cytokine was TNF-α, which was consistently upregulated by the old microbiota both in the conventional mice and GF recipient mice. TNF-α is well known for its role in the pro-inflammatory response (31). TNF-α also plays a central role in the pathogenesis of IBD and anti-TNF-α agents are used in the clinic to treat the disease (32). TNF-α was also shown to increase intestinal epithelial permeability (33). Interestingly, in agreement with our data it was recently demonstrated that age-associated inflammation depends on the microbiota and TNF- α (34).

Young microbiota had a different effect and increased expression of lambda immunoglobulin light chain genes both in conventional and GF recipient mice. B cells express only one class of light chain, lambda (λ), or kappa (κ). It has been observed previously that the gut microbiota can influence the ratio of these two light chains. Microbial colonization of GF mice was shown to increase the ratio of $Ig\lambda^{+}$ to $Ig\kappa^{+}$ B cells in the lamina propria (35). Since increased Igλ usage by B cells is considered a marker for B-cell receptor editing (36–38), these results might suggest that the young microbiota promote a more diverse B-cell repertoire. Another possibility is that the young microbiota contains more antigens that are recognized by B-cell clones that express Igλ.

The increased level of differentiated $CD4^+$ T cells in the spleen, the elevated inflammation in the ileum, and the prediction of LPS as an upstream regulator in the presence of aged microbiota led us to hypothesize that more bacterial components had translocated into the circulation in animals containing old microbiota. Indeed we observed that serum of GF recipients of old microbiota had an increased ability to activate TLR2 and trend toward increased TLR4 stimulation. Similar mechanisms seem to contribute to other disorders such as type 2 diabetes and metabolic syndrome. High-fat diet was shown to alter gut microbiota composition, which increased the permeability of the small intestine (22). The increased permeability allowed bacterial components to reach distal sites, which induced low-grade inflammation and subsequent insulin resistance (21). Importantly, the mucin-degrading bacterium *A. muciniphila* was shown to reverse these metabolic disorders by strengthening the intestinal barrier (39). In our study, old conventional mice had lower abundance of *Akkermansia*, which has also been reported previously both in humans and mice (40, 41). *Akkermansia* was also less abundant after transfer of old microbiota to GF mice at early time points. Therefore, it is tempting to speculate that the absence of *Akkermansia* in recipients of old microbiota might be associated with translocation of inflammatory bacterial components into the circulation.

As mentioned previously, certain members of the gut microbiota modulate the immune system (42). However, components of the immune system such as IgA antibodies also shape gut microbiota composition (43–45). Therefore, we investigated whether after transfer to GF mice the aged microbiota remained similar in composition to the donor or would quickly adapt to the young host. One week after transfer, the composition of old and young microbiota was clearly different, but after 4 weeks the difference was less pronounced, and both the microbiota from the old and young mice were more similar to the microbiota of the young mice. This suggests that the aged microbiota had partially adapted to the young host. As described for aged humans (8, 9), old conventional mice had a lower *Firmicutes*/*Bacteroidetes* ratio. However, this trait was not transferable to GF mice. Four weeks after transfer, GF recipients of old microbiota had more TM7 bacteria and *Proteobacteria*. The difference in *Proteobacteria* was at least partially due to a significant lower abundance of *Desulfovibrio* after transfer of old microbiota. Interestingly, *Desulfovibrio* and TM7 bacteria have recently been associated with a compromised intestinal barrier due to an altered mucus structure that was more penetrable by bacteria leading to increased intestinal immune infiltration (46). Further indications that TM7 phyla and *Proteobacteria* such as *Desulfovibrio* can contribute to intestinal inflammation comes from observations that these bacteria are associated with the pathogenesis of IBD (47–49). In conclusion, our data seem to support the hypothesis that the altered gut microbiota composition in aged individuals contributes at least partially to the chronic low-grade inflammatory state observed during aging. Therefore, strategies to modify gut microbiota composition of the elderly with, for example, probiotics or prebiotics (50) might help to reduce inflammation and thereby promote healthy aging.

ETHICS STATEMENT

All animal experiments were approved by the local ethical committee (DEC) of the University of Groningen (project number 6543) and adhered to FELASA guidelines.

AUTHOR CONTRIBUTIONS

FF designed the experiments and wrote the manuscript. FF, AB, and TB performed the experiments. SA, FH, and HFS generated and analyzed the microbiota data. CJ and MJ provided material and resources. MB generated and analyzed microarray data. HS, MF, and PV supervised the study.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/article/10.3389/fimmu.2017.01385/full#supplementary-material.

FIGURE S1 | Gating strategy to identify T helper subsets. Gating strategy and representative FACS plots (spleen sample) for identifying T cell subsets. Among $CD3^+CD4^+$ T cells, the percentage of Th1 cells (T-bet+), Th2 cells (GATA-3+), Th17 cells (RORgT+), and T_{regs} (Foxp3+CD25+) was identified. Percentage of cells stained with isotype control was subtracted to determine the percentage of true positive cells.

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The Impact of Gut Microbiota on Gender-Specific Differences in Immunity

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Males and females are known to have gender-specific differences in their immune system and gut microbiota composition. Whether these differences in gut microbiota composition are a cause or consequence of differences in the immune system is not known. To investigate this issue, gut microbiota from conventional males or females was transferred to germ-free (GF) animals of the same or opposing gender. We demonstrate that microbiota-independent gender differences in immunity are already present in GF mice. In particular, type I interferon signaling was enhanced in the intestine of GF females. Presumably, due to these immune differences bacterial groups, such as *Alistipes*, *Rikenella*, and Porphyromonadaceae, known to expand in the absence of innate immune defense mechanism were overrepresented in the male microbiota. The presence of these bacterial groups was associated with induction of weight loss, inflammation, and DNA damage upon transfer of the male microbiota to female GF recipients. In summary, our data suggest that microbiota-independent gender differences in the immune system select a gender-specific gut microbiota composition, which in turn further contributes to gender differences in the immune system.

Keywords: gender, gut microbiota, germ-free mice, immunity, inflammation

INTRODUCTION

It is widely accepted that there are differences in the immune system between males and females. Males are generally more susceptible to infections (1), whereas prevalence of various autoimmune disorders is much higher in females (2, 3). Different concentrations of sex steroids, such as testosterone, estrogens, and progesterone, could contribute to these immune differences, since sex steroids can influence the function of immune cells by binding to specific receptors expressed on these cells (1). Genetic differences between males and females are also considered to contribute, since the X chromosome is known to contain the largest number of genes involved in immunity of the whole genome (4, 5).

A third factor that could contribute to gender differences in the immune system is the gut microbiota. Our intestine harbors a highly complex community of bacteria, which is separated from a large pool of immune cells by only a single layer of epithelial cells (6). Consequently, gut microbiota composition shapes the immune system and *vice versa* (7). Several studies have shown that gut microbiota composition differs between males and females (8–11), which could potentially contribute to the observed gender-specific differences in immunity. The importance of these differences for gender-specific disease development has been shown in type 1 diabetes in which a higher prevalence of the disease in females was shown to be critically dependent on the gut microbiota (10, 11), demonstrating a link between gut microbes, gender, and immunity. However, whether gender-dependent immunity differences are a cause or consequence of an altered gut microbiota composition is not clear.

To determine the contribution of the gut microbiota to gender-specific differences in the immune system, in this study, we performed gut microbiota transfer experiments from conventional male or female mice to germ-free (GF) recipient mice of the same or opposing gender. Effects on the immune system were assessed by measuring genome-wide gene expression levels in the ileum and by analyzing different immune cell populations in the thymus, spleen, mesenteric lymph nodes (MLNs), and Peyer's patches (PPs).

MATERIALS AND METHODS

Mice

Female and male C57BL/6JRccHsd conventional 7- to 10-week-old mice were purchased from a commercial supplier (Envigo, Horst, the Netherlands). Female and male GF mice, 12- to 14-weeks old, were obtained from a breeding colony at the animal facility of Radboud University Nijmegen Medical Centre (Nijmegen, the Netherlands). All animals were put on an autoclaved rat/mouse maintenance V153X R/M-H diet (Ssniff, Soest, Germany) directly after weaning in the case of GF mice, or directly after arrival in the case of conventional mice. The mice were kept on this diet throughout the experiment. Conventional mice were housed in IVC cages and GF mice were housed in GF isolators. All experiments were approved by the local ethical committee of the University of Groningen.

Experimental Design

After an acclimatization period of at least 4 weeks, feces were freshly collected from the conventional males ($n = 8$) or conventional females ($n = 10$). Feces from the same group was pooled and mixed in PBS. Next, 200 µl of 100 mg/ml of this mixture was given by oral gavage to age-matched GF mice of the same or opposing gender ($n = 10$ per experimental group). After transfer, recipient mice were individually housed in IVC cages for another 4 weeks. As a control, male and female GF mice ($n = 5$) were kept germ free throughout the experiment. In summary, there were the following experimental groups:

1. Conventional males ($n = 8$)
2. Conventional females ($n = 10$)
3. GF males that received microbiota from conventional males ($n = 10$)
4. GF females that received microbiota from conventional females ($n = 10$)
5. GF males that received microbiota from conventional females ($n = 10$)
6. GF females that received microbiota from conventional females ($n = 10$)
7. GF males as a control ($n = 5$)
8. GF females as a control ($n = 5$)

Organ and Tissue Collection

Mice were sacrificed at the following ages: conventional mice 16–19 weeks, GF recipient mice 16–18 weeks, and GF mice 13–15 weeks. The GF control mice were slightly younger than the other groups of mice since it was logistically not feasible to obtain enough GF mice of the same age of both sexes from our breeding colony at the same time. Mice were anesthetized with isoflurane, bled, and sacrificed by cervical dislocation. Serum was collected and stored at −80°C. Colon content and a piece of terminal ileum were snap frozen in liquid nitrogen and stored at −80°C. In addition, spleen, thymus, PPs, and MLNs were collected for FACS analysis.

Flow Cytometry

Single cell suspensions were obtained from spleen, thymus, PPs, and MLNs. Cells were stained with Fixable Viability Dye eFluor 506 (eBioscience, Vienna, Austria) for exclusion of dead cells. A-specific binding to Fc receptors was blocked by incubating the cells with anti-CD16/32 for 15 min on ice. For extracellular staining, cells were incubated with the desired mixture of antibodies for 30 min on ice. After washing, cells were fixed with FACS lysing solution (BD Biosciences, Breda, the Netherlands). For intracellular staining, fixed cells were permeabilized with PERM (eBioscience, Vienna, Austria) and subsequently stained with the desired antibodies for 30 min on ice. For identification of the different T helper (Th) cell subsets, cells were stained with antibodies against CD3e (clone 17 A2), CD4 (clone GK1.5), T-bet (clone 4B10), RORyt (clone B2D), Gata-3 (clone TWAJ), CD25 (clone PC61), and Foxp3 (clone FJK-16S). Appropriate isotype controls were used to determine specificity of the staining. T cell precursors in thymus were identified by excluding other lineages: CD11b (clone M1/70), CD11c (clone HL3), CD19 (clone 1D3), CD45R/B220 (clone RA3-6B2), NK1.1 (clone PK136), and TER119 (clone TER-119). Next, cells were gated on populations that were double negative (DN), single positive, or double positive for CD4 (clone GK1.5) and CD8a (clone 53-6.7). To identify earliest T cell precursors (triple negative), cells positive for CD3e (clone 145-2C11) were excluded. Samples were acquired with the FACSVerse or FACSCanto II (BD Biosciences, Breda, the Netherlands) and analyzed with FlowJo software (FlowJo, LLC, OR, USA).

Transcriptome Microarray

A piece of terminal ileum from each mouse was snap frozen in liquid nitrogen and stored afterward at −80°C. RNA was isolated with the RNeasy kit (Qiagen, Valencia, CA, USA). Quantity of RNA was measured with the ND-1000 (NanoDrop Technologies,

Thermo Fisher Scientific, Breda, the Netherlands) and quality of RNA was assessed with the Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA). Total RNA (100 ng) was labeled utilizing the Ambion WT Expression kit (Life Technologies Ltd., Bleiswijk, the Netherlands) and the Affymetrix GeneChip WT Terminal Labeling kit (Affymetrix, Santa Clara, CA, USA). After labeling, samples were hybridized to Affymetrix GeneChip Mouse Gene 1.1 ST arrays. An Affymetrix GeneTitan Instrument was used for hybridization, washing, and scanning of the array plates. Bioconductor packages integrated in an online pipeline were used for quality control of the data (12, 13). Probe sets were redefined using current genome information (14). Probes were reorganized based on the Entrez Gene database (remapped CDF v14.1.1). Robust Multi-array Analysis preprocessing algorithm available in the Bioconductor library affyPLM (15) was used to obtain normalized expression estimates from the raw intensity values.

Serum Antibody Isotypes

Mouse serum samples were assayed for mouse IgG1, IgG2a, IgG2b, IgG3, IgA, IgE, and IgM using ProcartaPlex Mouse Antibody Isotyping Panel on the Luminex platform (Affymetrix, Santa Clara, CA, USA), according to manufacturer's instructions. In brief, samples were thawed on ice. Beads were mixed and washed and subsequently incubated overnight at 4°C with standards or with 1:500 or 1:50,000 diluted samples. After washing, the beads were incubated with detection antibody mix for 30 min at room temperature. The beads were washed and incubated for 30 min at room temperature with streptavidin-PE. After washing the beads were measured with a Luminex instrument (Bio-Plex 200, Bio-Rad), which was calibrated using Bio-Rad calibration beads. Standard curves were calculated using 5-parameter logistic regression in Bio-plex 5.0 software.

Microbiota Analysis

Fresh feces samples obtained just after defecation were collected from all mice at different time points during the experiment. In addition, colonic content samples from these mice were collected at the end of the experiment. All samples were snap frozen in liquid nitrogen and stored at −80°C. These samples were used for 16S rRNA gene analysis for microbiota profiling with barcoded amplicons from the V1–V2 region of 16S rRNA genes generated using a 2-step PCR strategy that reduces the impact of barcoded primers on the outcome of microbial profiling (16). DNA extraction was performed using a combination of the bead-beating-plus column method and the Maxwell 16 Tissue LEV Total RNA purification kit (Promega, Leiden, the Netherlands). Beating of the fecal pellets took place as described before (17), but with STAR (Stool transport and recovery) buffer (Roche, Basel Switzerland). 250 µl supernatant after centrifugation was taken for the Maxwell 16 Tissue LEV Total RNA Purification Kit, and the DNA was eluted in 50 µl DNAse-free water. Twenty nanograms of DNA were used for the amplification of the 16S rRNA gene with primers 27F-DegS and 338R I + 338R II for 25 cycles as described before (18), only primers had a Universal Tag (UniTag) linkers attached; UniTag I (forward) and II (reverses) (I—GAGCCGTAGCCAGTCTGC; II—GCCGTGACCGTGACATCG). The first PCR was performed in a total volume of 50 µl containing 1× HF buffer (Finnzymes, Vantaa, Finland), 1 µl dNTP Mix (10 mM; Promega, Leiden, the Netherlands), 1 U of Phusion® Hot Start II High-Fidelity DNA polymerase (Finnzymes Vantaa, Finland), 500 nM of the 27F-DegS primer (18, 19) that was appended with UniTag 1 at the 5′ end, 500 nM of an equimolar mix of two reverse primers, 338R I and II (19) based on three previously published probes EUB 338 I, II, and III (18), that were 5′-extended with UniTag 2, and 0.2–0.4 ng/µl of template DNA. The sequence of the UniTags were selected to have a GC content of ~66% and a minimal tendency to form secondary structures, including hairpin loops, heterodimers, and homodimers as assessed by the IDTDNA Oligoanalyzer 3.1 (Integrated DNA Technologies). Moreover, sequences were selected that had no matches in 16S rRNA gene databases (based on results of the "TestProbe" tool offered by the SILVA rRNA database project (20) using the SSU r117 database), and no prefect matches in genome databases with the Primer-BLAST tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The size of the PCR products (~375 bp) was confirmed by gel electrophoresis using 5 µl of the amplification reaction mixture on a 1% (w/v) agarose gel containing 1× SYBR® Safe (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). Five microliters of these PCR products were taken to add adaptors and a 8-nt sample-specific barcode in an additional 5 cycle PCR amplification. This second PCR was performed in a total volume of 100 µl containing 1× HF buffer, dNTP Mix, 2 U of Phusion® Hot Start II High-Fidelity DNA polymerase, 500 nM of a forward and reverse primer equivalent to the Unitag1 and UniTag2 sequences, respectively, that were each appended with an 8 nt sample-specific barcode (G. Hermes and J. Ramiro-Garcia, et al., in preparation) at the 5′ end. PCR products were purified with the magnetic beads (MagBio, London, UK) according to the HighPrepTM protocol of the manufactures instructions using 20 µl nuclease-free water (Promega Leiden, the Netherlands) and quantified using the Qubit (Life Technologies, Bleiswijk, the Netherlands). Purified PCR products were mixed in approximately equimolar amounts and concentrated by the magnetic beads as the purification before. Purified amplicon pools were 250 bp paired-end sequenced using Illumina Miseq (GATC-Biotech, Konstanz, Germany).

The Illumina Miseq data analysis was carried out with a workflow employing the Quantitative Insights Into Microbial Ecology (QIIME) pipeline (21) and a set of in-house scripts as described before for Illumina Hiseq 16S rRNA gene sequences (G. Hermes and J. Ramiro-Garcia, et al., in preparation). The set of in-house scripts processed the reads as follows: reads were filtered for not matching barcodes; OTU picking and chimera removal was done *via* matching the sequences to the Silva 111 database, with only one mismatch allowed, and a biom and with ClustalW a multiple alignment and phylogenetic tree file was generated. Further outputs were generated *via* QIIME, such as filtered reads per sample, PD whole tree diversity measurements and the level 1 to 6 taxonomic distributions with relative abundances.

Statistics

Flow cytometry data and serum antibody isotype data are expressed as means. To verify whether data were normally distributed the Kolmogorov–Smirnov test was performed. In cases where data were not normally distributed, data were log

transformed before analysis. For comparing two groups, the unpaired two-tailed Student's *t*-test was used. For comparing more than two groups with each other, one way ANOVA was performed followed by the Bonferroni test to compare specific groups. *p*-Values below 0.05 were considered significant, and *p*-values below 0.1 were considered a trend. All tests were performed with GraphPad software (Prism, La Jolla, CA, USA).

Differentially expressed probe sets were identified using linear models, applying moderated T-statistics that implemented empirical Bayes regularization of SEs (22). A Bayesian hierarchical model was used to define an intensity-based moderated T-statistic, which takes into account the degree of independence of variances relative to the degree of identity and the relationship between variance and signal intensity (23).

Statistical tests for gut microbiota composition were performed using R and Calypso (24). Where the count data were not normally distributed and variances between groups were not equal, the Mann–Whitney *U* test was used.

RESULTS

Male Microbiota Induces Lower Weight in Female Mice

In order to investigate how gender influences the interplay between the gut microbiota and the immune system of the host, we transferred gut microbiota from male or female conventional mice to GF mice of the same or opposing gender. After microbiota transfer, the weight of the GF recipient mice was followed for 4 weeks. All GF recipient mice lost some weight in the first week after the microbiota transfer (**Figure 1**). However, GF females that received male microbiota had lost significantly more weight ($p < 0.05$) compared to GF females with female microbiota. The weight of the female recipients of a male microbiota developed in a similar fashion as the male recipients of male microbiota. This weight difference remained until the end of the experiment ($p < 0.01$). A gender-dependent effect of the microbiota on weight was not observed in male GF recipients.

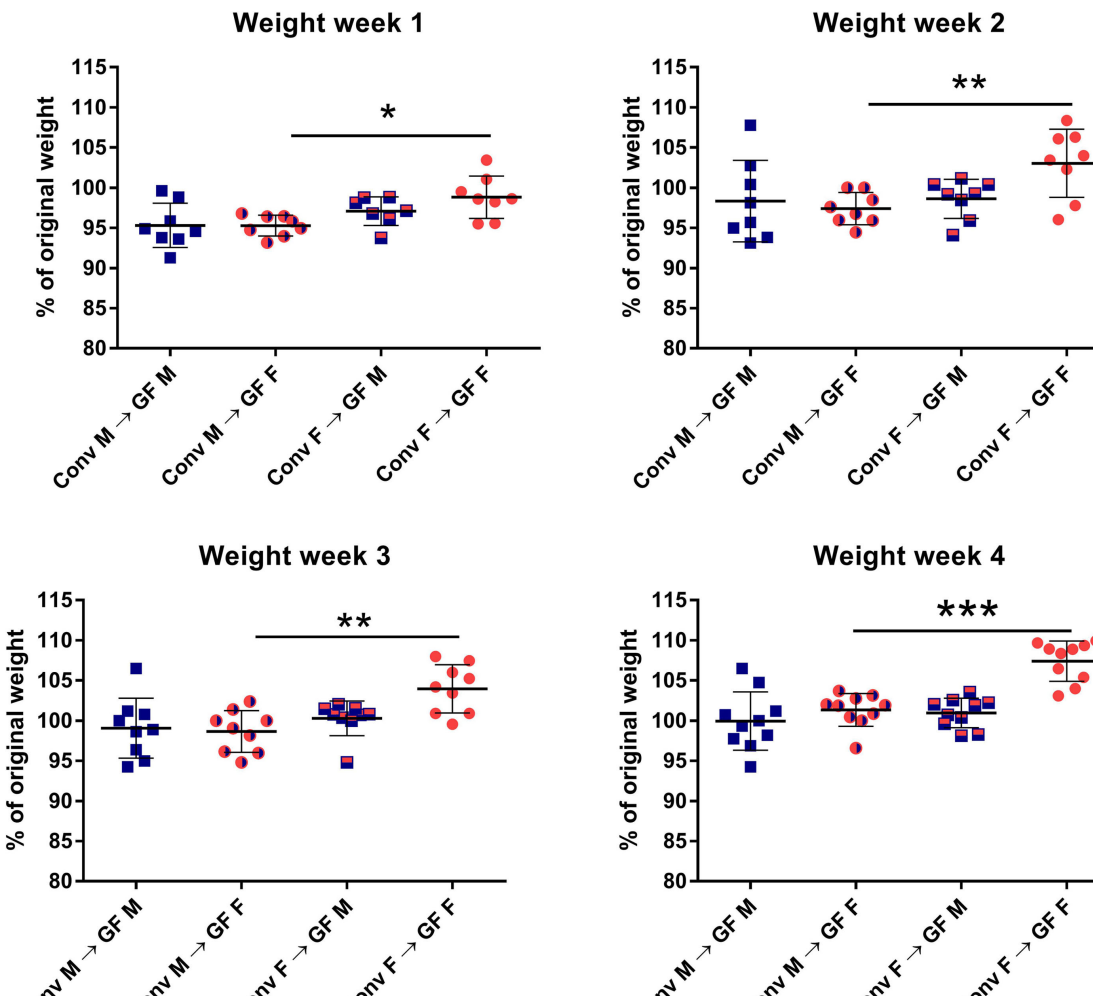


FIGURE 1 | Feces of conventional (conv) males (M) or females (F) was transferred to germ-free (GF) male or female recipient mice ($n = 10$) by oral gavage. Weight was measured once a week for 4 weeks and is presented as the percentage of the original weight just before gut microbiota transfer. * indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.001$.

Female Microbiota Enhances T Cell Precursors in Thymus but Lowers RORγt$^+$ Foxp3$^+$ T Cells in Male Recipients

Four weeks after microbiota transfer the mice were sacrificed and cells were isolated from the thymus, spleen, PPs, and MLNs to study the effect of the gut microbiota transfer on T cell development and differentiation. Conventional and GF mice of both genders were included as controls. Male conventional mice had a higher number of thymic cells than germ-free males ($p < 0.05$). In addition, both conventional and GF females had a higher number of thymic cells ($p < 0.01$) compared to males (**Figure 2A**). Intriguingly, female recipients of female microbiota also had significantly more thymic cells ($p < 0.05$) than female recipients of male microbiota (**Figure 2A**). There was no gender-dependent effect of the microbiota on thymic cells in male recipients.

Early T cell precursors in the thymus are DN for CD4 and CD8, after which they become double positive for CD4 and CD8, and finally develop into cells single positive for one of these molecules (25). Frequencies of DN T cell precursors tended to be higher in females (**Figure 2B**). Moreover, absolute numbers of DN T cell precursors were significantly higher ($p < 0.001$) in conventional and GF females compared to males (**Figure 2C**). This higher number of DN T-cells was present in female microbiota recipients (both male and female) compared to recipients of male microbiota ($p < 0.05$). Taken together these results suggest that the gut microbiota influences T cell development in a gender-dependent manner.

To study the impact of the gut microbiota on T cell differentiation, the composition of the different CD4$^+$ Th subsets were studied in the PPs, MLN, and the spleen. The frequency of the recently described RORγt$^+$ Foxp3$^+$ population (26) was much lower in the PPs (**Figure 2D**) and MLN (**Figure 2E**) of GF mice. Moreover, transfer of male microbiota led to a significantly higher percentage of RORγt$^+$ Foxp3$^+$ cells in PPs and MLN ($p < 0.05$) compared to male recipients of female microbiota (**Figures 2D–E**). Frequencies of conventional Tregs were also higher ($p < 0.001$) in male recipients compared to female recipients, but this was not dependent on the microbiota (**Figure 2F**). No differences between the experimental groups were observed for the Th1, Th2, or Th17 subsets in any of the organs tested (Figure S1).

Microbiota Influences Antibody Production in a Gender-Specific Manner

To study the effect of microbial colonization on antibody production, total levels of the different antibody isotypes were measured in the serum (**Figure 3**). GF females tended to have higher levels

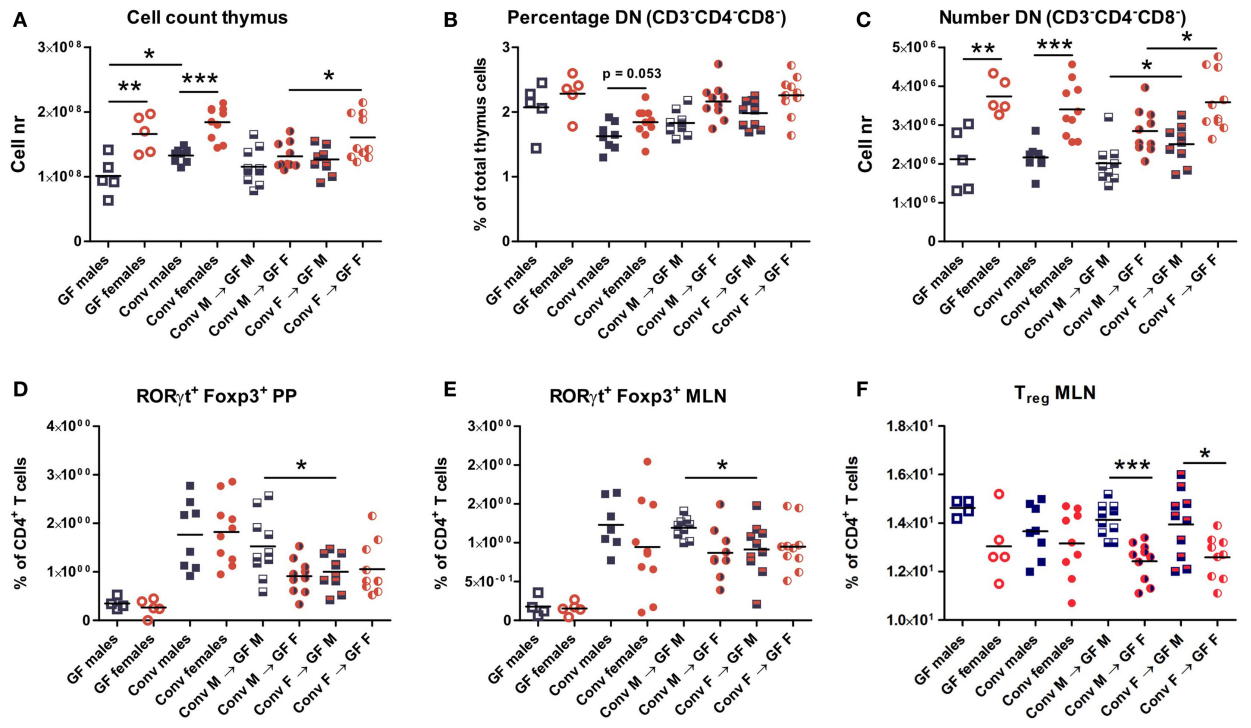


FIGURE 2 | Cells from thymus, spleen, Peyer's patches (PPs), and mesenteric lymph nodes (MLNs) were analyzed with flow cytometry. Germ-free (GF) males (M) ($n = 5$), GF females (F) ($n = 5$), conventional (conv) males ($n = 8$), and females ($n = 10$) were included as controls. Experimental groups of GF recipients of gut microbiota each contained 10 mice per group. **(A)** Total number of thymus cells. **(B)** Percentage among live thymus cells of lineage negative (CD11b, CD11c, CD19, CD45R/B220, NK1.1, and TER119) cells that were also double negative (DN) for CD4 and CD8. **(C)** Absolute numbers of thymus cells that were lineage negative (CD11b, CD11c, CD19, CD45R/B220, NK1.1, and TER119) and also DN for CD4 and CD8. **(D)** Percentage of RORγt$^+$Foxp3$^+$ cells among CD4$^+$ T cells in PPs. **(E)** Percentage of RORγt$^+$Foxp3$^+$ cells among CD4$^+$ T cells in MLN. **(F)** Percentage of CD25$^+$Foxp3$^+$ regulatory cells (Treg) among CD4$^+$ T cells in MLN. * indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.001$.

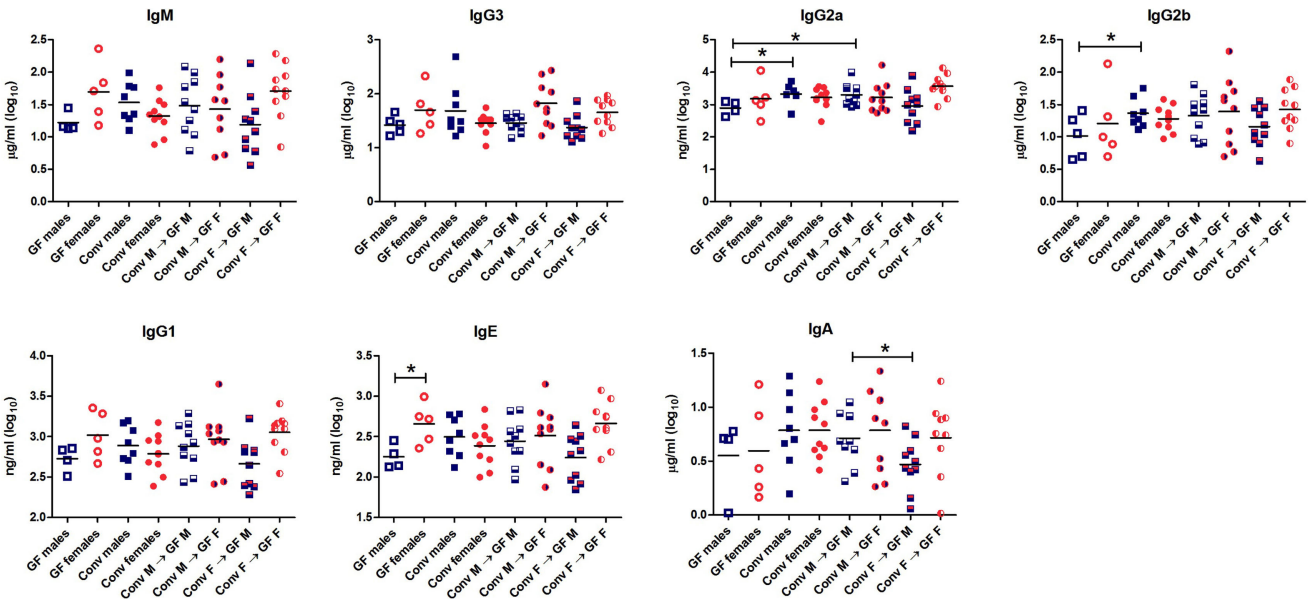


FIGURE 3 | Total levels of IgM, IgG3, IgG2b, IgG2a, IgG1, IgE, and IgA were measured with luminex in serum of germ-free (GF) males (M) ($n = 5$), GF females (F) ($n = 5$), conventional (conv) males ($n = 8$), conventional females ($n = 10$), and the GF recipient male or female mice that received the gut microbiota from the same or opposite gender ($n = 10$ per group). * indicates $p < 0.05$.

of IgM ($p < 0.1$) and significantly higher levels of IgE ($p < 0.05$) compared to GF males. These data suggest that females intrinsically produce higher levels of these antibody isotypes compared to males independent of the microbiota. Moreover, conventional females did not have different levels of any antibody isotype compared to GF females, which might suggest that in females gut microbiota did not influence antibody production in the systemic compartment. On the other hand, conventional males tended to have higher levels of IgM ($p < 0.1$) and significant higher levels of IgG2a and IgG2b ($p < 0.05$) compared to GF males (**Figure 3**). Thus gut microbiota seemed to influence antibody production in males but not in females. Interestingly, the female microbiota significantly ($p < 0.05$) lowered IgA levels compared to male microbiota in male GF recipients.

Microbiota-Independent and Dependent Differences in Gender-Specific Immunity

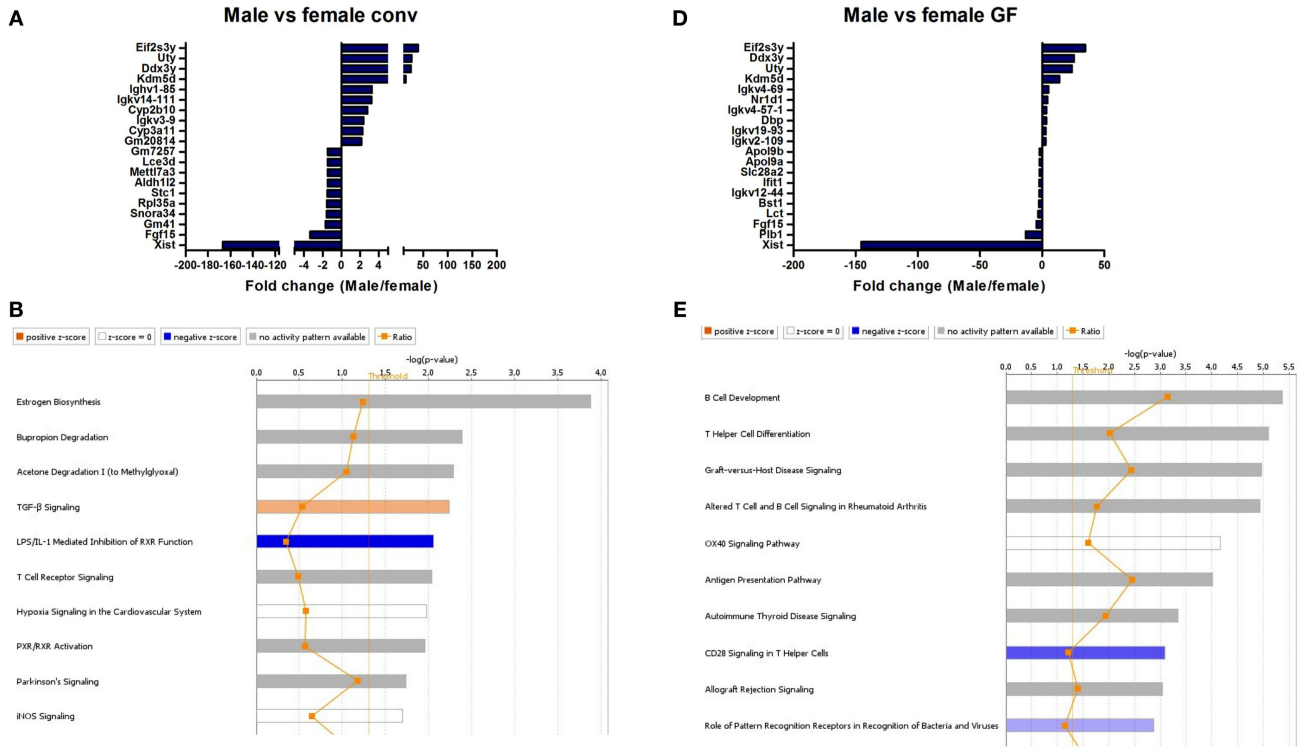
To determine microbiota-dependent and -independent differences in gender-specific immunity in the intestine, we performed genome-wide gene expression analysis of the ileum with microarray. Ileum was chosen because of its primary intestinal role in immune signaling (6). Data were analyzed with Ingenuity Pathway Analysis (IPA), only focusing on genes that were significantly differentially expressed ($p < 0.05$, fold change >1.2 or <−1.2).

The microbiota-independent differences were studied by comparing gender-specific differences in GF animals and conventional mice. A number of genes were identified that were exclusively expressed in females (Xist) or in males (Eif2s3y, Uty, Ddx3y, Kdm5d). This difference was similar between conventional (**Figure 4A**) and GF mice (**Figure 4D**). Canonical pathways that were differentially regulated in different genders in conventional mice included "estrogen biosynthesis," but also immune pathways such as "TGF-β signaling" and "T cell receptor signaling" (**Figure 4B**). The prediction by IPA of the pro-inflammatory cytokine IL-1 β as the most significant upstream regulator further confirmed the prominent differences in immunity between conventional males and females (**Figure 4C**).

Strikingly, the canonical pathways most significantly affected by gender in GF mice were all immune-related (**Figure 4E**). These pathways for example included "B cell development," "Th cell differentiation," and "antigen presentation pathway." This difference between GF males and females could be due to differences in the regulation of type I interferon (IFN) production, since the receptor for type I IFN (IFNAR) and a transcription factor involved in type I IFN production (IRF7) were predicted as most significant upstream regulators by IPA (**Figure 4F**). In conclusion, some of the gender-dependent differences in immunity are not dependent on the gut microbiota, but are also present in GF mice.

To determine gender-specific microbiota effects, we compared ileal microarray data of (i) GF female recipient mice that received male or female microbiota and (ii) GF male recipient mice that received male or female microbiota.

In female recipients, the male microbiota upregulated several genes involved in the immune response, including a number of immunoglobulin variants, Dennd1b and Lcn2 (27–29) (**Figure 5A**). Female microbiota in GF females resulted in different effects. Here, the most highly upregulated genes were proteases Mcpt1 and Mcpt2, which are expressed by intestinal mucosal mast cells (30). Most canonical pathways that were significantly affected by the gender of the microbiota in GF recipients mice were involved in DNA repair and the cell cycle (**Figure 5B**). Moreover, the most



C

Top Upstream Regulators	
Upstream Regulator	p-value of overlap
IL1B	2,91E-07
B2M	1,32E-06
dichlorodiphenyl dichloroethylene	1,91E-06
ethanol	1,13E-05
dieldrin	3,72E-05

F

Top Upstream Regulators	
Upstream Regulator	p-value of overlap
Ifnar	1,51E-14
IRF7	1,91E-14
PTGER4	1,94E-13
lipopolysaccharide	3,10E-13
IFNAR1	4,96E-13

FIGURE 4 | Microbiota-independent differences in immunity between males and females. Whole-genome gene expression in the ileum of male and female conventional (conv) mice ($n = 5$ per group) or male and female germ-free (GF) mice ($n = 4$) was assessed with Affymetrix GeneChip Mouse Gene 1.1 ST arrays. Genes that were significantly differentially expressed ($p < 0.05$ and fold change >1.2) between males and females, either conventional or germ free were analyzed with Ingenuity Pathway Analysis. **(A)** Top 10 of most upregulated genes and top 10 of most downregulated genes by comparing conventional males with conventional females. **(B)** Canonical pathways that were most significantly affected by gender in conventional mice. **(C)** Most significantly predicted upstream regulators of the pathways affected by gender in conventional mice. **(D)** Top 10 of most upregulated genes and top 10 of most downregulated genes by comparing GF males with GF females. **(E)** Canonical pathways that were most significantly affected by gender in GF mice. **(F)** Most significantly predicted upstream regulators of the pathways affected by gender in GF mice.

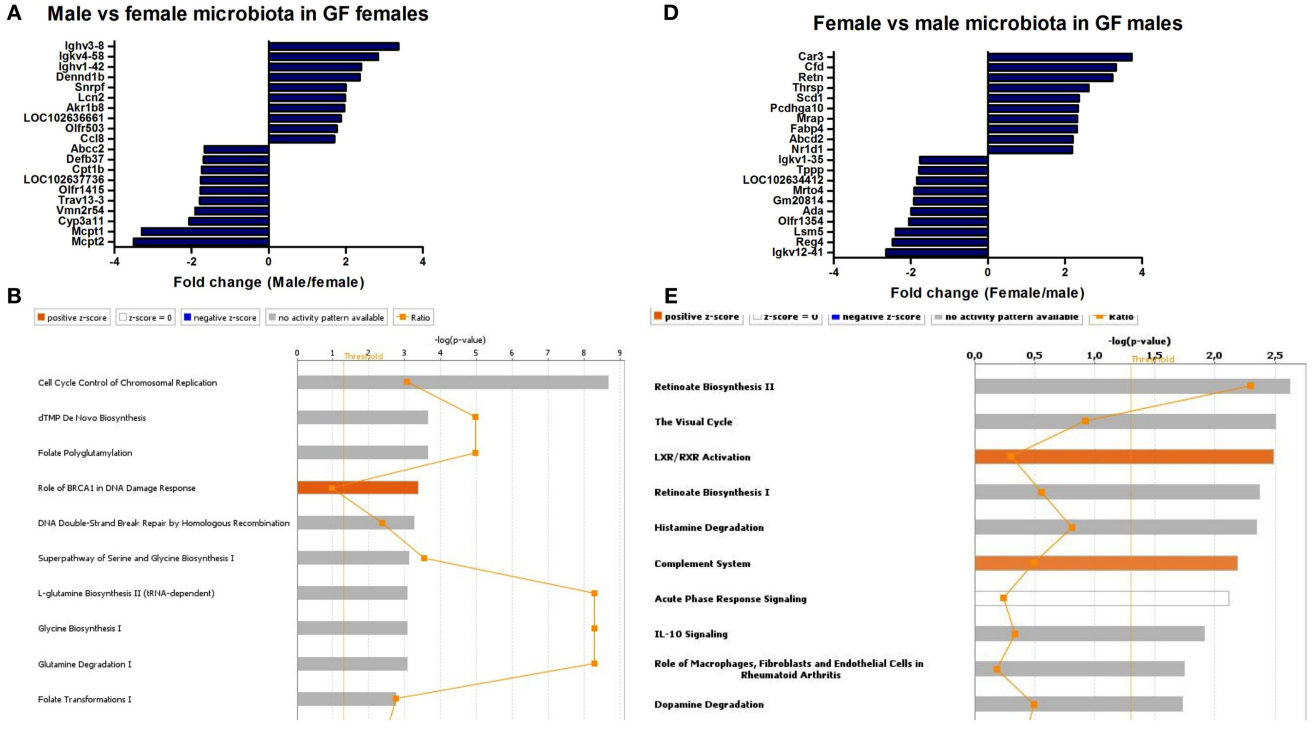
significant predicted upstream regulator of these pathways was dextran sulfate (**Figure 5C**), which is a well-known inducer of experimental colitis (31).

In male recipients of female microbiota, the most highly upregulated genes included genes with known roles in the immune response, but also metabolism, including Cfd and Retn (32, 33) (**Figure 5D**). The most highly upregulated genes by the male microbiota in GF male recipients included Reg4, an antimicrobial protein recognized for its role in host–microbiota interactions (34), and ADA, which is crucial for the development of the immune system (35). The most significantly affected canonical pathways by the gender of the microbiota in GF male recipients included "complement system," "acute phase response signaling," and "IL-10 signaling" (**Figure 5E**). The most significant predicted upstream regulators were rosiglitazone, which is used for the treatment of type 2 diabetes (36) and the pro-inflammatory cytokine TNF-α (**Figure 5F**).

Next, genes that were induced by the male microbiota both in male and female GF recipients, but not by the female microbiota in neither male nor female GF recipients, were analyzed with the STRING database (37). We identified a number of gene clusters specifically induced by the male microbiota, which are significantly predicted to be involved in the gene ontology biological processes DNA replication and the cell cycle (**Figure 6**). A similar analysis of the response induced by the female microbiota did not reveal any clusters or pathways that were significantly affected (data not shown).

Bacterial Groups Associated With Gender-Specific Microbiota Differences

To investigate how the gut microbiota composition changes over time in the recipient mice, composition of the gut microbiota of the different experimental groups was analyzed with 16S rRNA



C

Top Upstream Regulators	
Upstream Regulator	p-value of overlap
dextran sulfate	9,69E-11
calcitriol	2,92E-10
CCND1	3,04E-10
TBX2	1,51E-09
RB1	2,83E-09

F

Top Upstream Regulators	
Upstream Regulator	p-value of overlap
rosiglitazone	2,81E-11
TNF	1,58E-08
IFNAR1	1,96E-07
progesterone	8,55E-07
CEBPB	2,64E-06

FIGURE 5 | Microbiota-dependent differences in gender-specific immunity. Whole-genome gene expression in the ileum of male and female germ-free (GF) mice ($n = 5$) that received the gut microbiota from male or female conventional mice was assessed with Affymetrix GeneChip Mouse Gene 1.1 ST arrays. Genes that were significantly differentially expressed ($p < 0.05$ and fold change >1.2) between recipients of male and female microbiota were analyzed with Ingenuity Pathway Analysis. **(A)** Top 10 of most upregulated genes and top 10 of most downregulated genes by comparing male microbiota with female microbiota in GF female recipients. **(B)** Canonical pathways that were most significantly affected by gender of the microbiota in GF female recipient mice. **(C)** Most significantly predicted upstream regulators of the pathways affected by gender of the microbiota in GF female recipient mice. **(D)** Top 10 of most upregulated genes and top 10 of most downregulated genes by comparing female microbiota with male microbiota in GF male recipients. **(E)** Canonical pathways that were most significantly affected by gender of the microbiota in GF male recipient mice. **(F)** Most significantly predicted upstream regulators of the pathways affected by gender of the microbiota in GF male recipient mice.

gene sequencing. From the male and female conventional mice, we analyzed feces at the time of transfer or 4 weeks after transfer (**Figure 7**). From the GF recipient mice, we analyzed feces 1 week after transfer or 4 weeks after transfer. A fundamental question to answer was whether the gut microbiota evolves into a community similar to the donor or whether it adapts to its host. Redundancy analysis (RDA) at the genus level confirmed that gut microbiota composition was different between male conventional mice and female conventional mice, since the samples separated into two distinct clusters at the time of transfer and 4 weeks after transfer (**Figure 8A**). One or four weeks after transfer of male or female microbiota to GF mice of both genders, four separate clusters of each experimental group could be distinguished (**Figure 8A**). These results imply that both the donor and host shape the gut microbiota community, leading to a unique composition in each experimental group. To further investigate how the gut microbiota communities from the different groups were related to each other, we performed another RDA analysis including all the groups and time points (**Figure 8B**) Surprisingly, this analysis revealed that for both genders the microbiota first adapted to the gender of the recipient 1 week after the transfer, but 4 weeks after the transfer gut microbiota composition was similar to the gender of the donor, regardless the gender of the recipient.

To study more specifically the bacterial groups that were potentially responsible for the observed differences in immune responses, we investigated which bacterial groups had a significant difference in abundance at the family level (**Figure 9A**) or genus level (**Figure 9B**). Conventional females had higher abundance of Desulfovibrionaceae, Lactobacillaceae (*Lactobacillus* at the genus level), and Verrucomicrobiaceae (*Akkermansia* at the genus level), whereas conventional males had higher abundance of Ruminococcaceae and Rikenellaceae (*Alistipes* at the genus

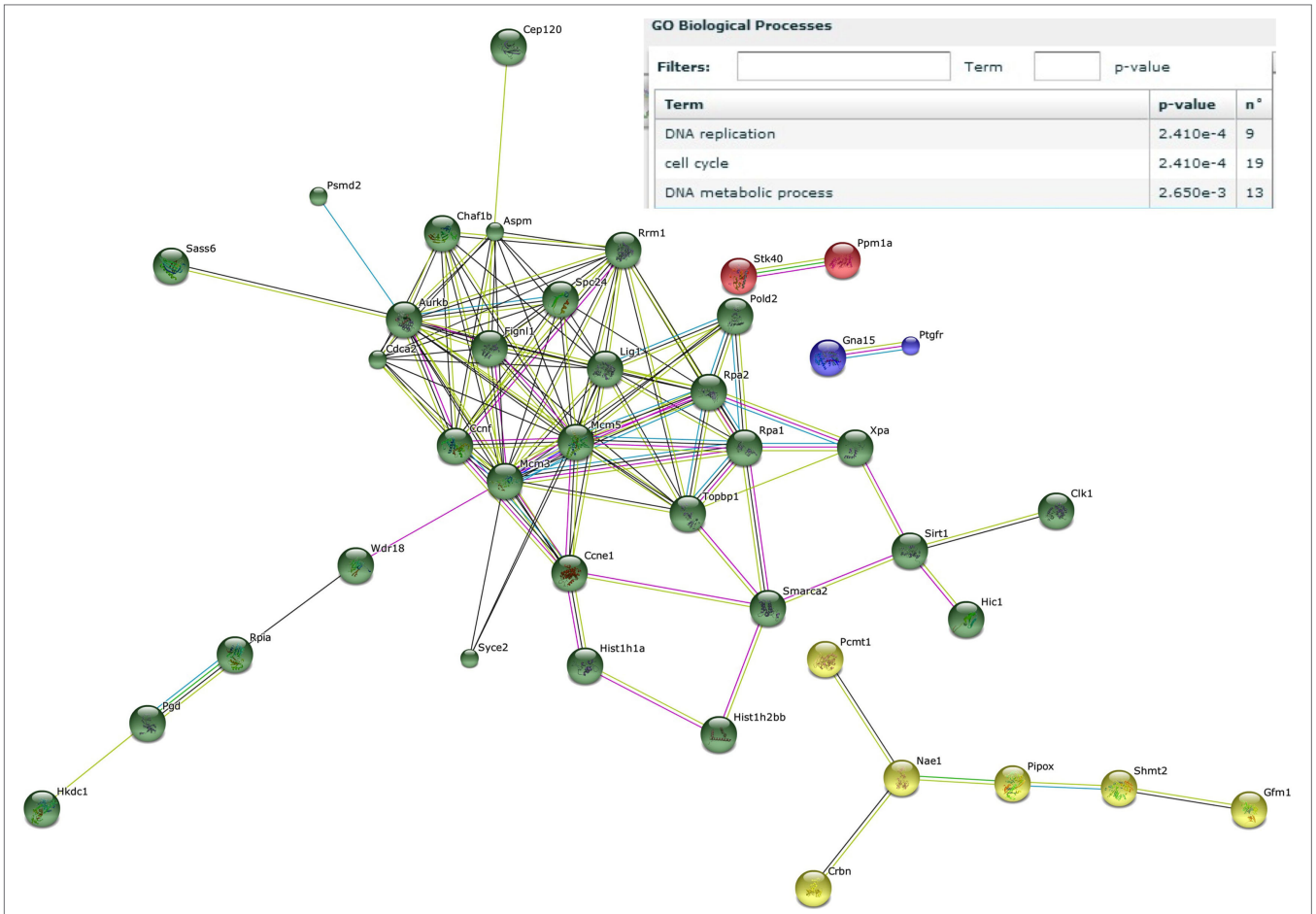


FIGURE 6 | Male microbiota influences DNA replication and the cell cycle. Whole-genome gene expression in the ileum of male and female germ-free (GF) mice ($n = 4$), or male and female GF recipient mice ($n = 5$) that received the gut microbiota from male or female conventional mice was assessed with Affymetrix GeneChip Mouse Gene 1.1 ST arrays. Only genes that were significantly differentially expressed ($p < 0.05$ and fold change >1.2) were included in the analysis. Genes that were specifically affected by the male microbiota were selected as follows: affected by male microbiota in male GF recipients compared to male GF controls AND affected by male microbiota in female GF recipients compared to female GF controls, but NOT affected by female microbiota in female GF recipients compared to female GF controls, OR female microbiota in male GF recipients compared to male GF controls. The resulting list of genes was analyzed with the STRING database. Only genes with at least one interaction are shown. The following interactions are indicated: from curated databases (blue), experimentally determined (pink), textmining (yellow), co-expression (black), and protein homology (purple).

level). One week after transfer to GF recipients, the strong influence of the gender of the recipient was very clear, since all significant differences were dependent on the gender of the host. Female recipients had higher abundance of Lactobacillaceae (*Lactobacillus* at the genus level) and male recipients had higher abundance of Desulfovibrionaceae, Ruminococcaceae, and Porphyromonadaceae (*Odoribacter* at the genus level). Four weeks after transfer, most differences were still dependent on the gender of the host, but there were also some donor-dependent differences. For example, lactobacilli were still more abundant in female recipients, whereas *Akkermansia* and *Prevotellaceae* were more abundant in male recipients. However, in particular in GF female recipients, there was also a clear influence of the gender of the donor. In these mice, *Rikenella*, Lachnospiraceae, and Desulfovibrionaceae were increased after transfer of male microbiota, but Prevotellaceae was increased after transfer of the female microbiota.

DISCUSSION

Several studies have demonstrated that gender influences gut microbiota composition (8–11) and the immune system (1–5). However, whether the differences in gut microbiota composition between males and females are a cause or consequence of gender-specific differences in the immune system is not known. Here, we demonstrate that some characteristics of gender-specific immune differences can be induced by the gut microbiota.

Some differences in the immune system between males and females were also present in GF mice, suggesting these differences were not dependent on the gut microbiota. In particular, the type I IFN pathway was enhanced in the intestine of GF females. This can explain several gender-effects reported in literature. It has been shown that plasmacytoid DCs of females produce higher levels of IFN-α after TLR7 activation compared to males (38, 39). Furthermore, type I IFN has been shown to regulate intestinal

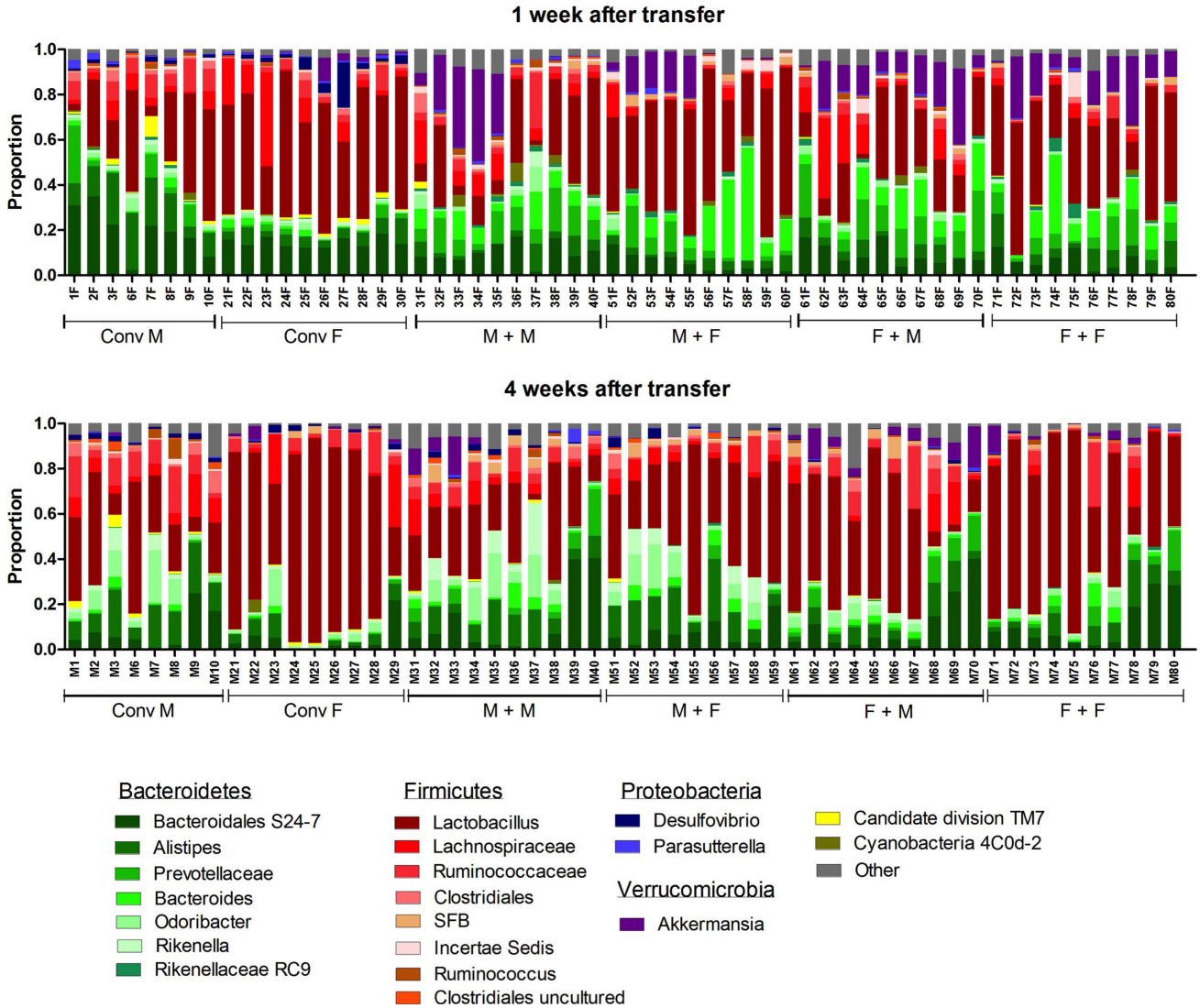


FIGURE 7 | Gut microbiota composition in conventional and conventionalized mice. Fecal samples were collected from conventional (conv) males (M) ($n = 8$) and conventional females (F) ($n = 10$) at the time of transfer or 4 weeks after transfer to the germ-free (GF) recipient mice. In addition, fecal samples 1 and 4 weeks after the transfer were collected from the following groups (all $n = 10$ per group): male GF recipient mice that received male microbiota (M + M), female GF recipient mice that received male microbiota (F + M), male GF recipient mice that received female microbiota (M + F), and female GF recipient mice that received female microbiota (F + F). Gut microbiota composition was analyzed with 16S rDNA sequencing and data are presented as the relative abundance of the different bacterial groups for each individual mouse. The most highly abundant bacterial groups are indicated.

homeostasis (40). For example, mice with conditional deletion of the type I IFN receptor (IFNAR1) in intestinal epithelial cells had an altered gut microbiota composition, which promoted epithelial hyperproliferation and experimental colitis-associated cancer (41). Therefore, it is conceivable that our observed enhanced type I IFN production in female intestines contributed to the selection of a gender-specific gut microbiota composition.

Interestingly, several bacterial groups, such as *Alistipes*, *Rikenella*, and Porphyromonadaceae, known to expand in the absence of innate immune defense mechanisms were over-represented in the male microbiota in our study before or after transfer to GF mice. This is corroborated by the observation that Rikenellaceae and Porphyromonadaceae were more abundant in the gut microbiota of MyD88-deficient mice also lacking adequate innate immunity (42). Moreover, NOD2-deficiency has been shown to cause dysbiosis in mice, including a higher abundance of *Rikenella*, which induced transmissible colitis and colorectal cancer (43). Finally, in mice deficient in IL-10 and the antimicrobial peptide Lipocalin-2, *Alistipes* was shown to flourish and was sufficient to induce colitis and tumorigenesis in IL-10 deficient mice (44). Notably, Lipocalin-2 was among the genes that were most highly upregulated in female recipients of male microbiota in our study. In summary, these results may indicate that a lower innate immune response in the gut of males promoted growth of specific bacteria with the potential to promote intestinal inflammation.

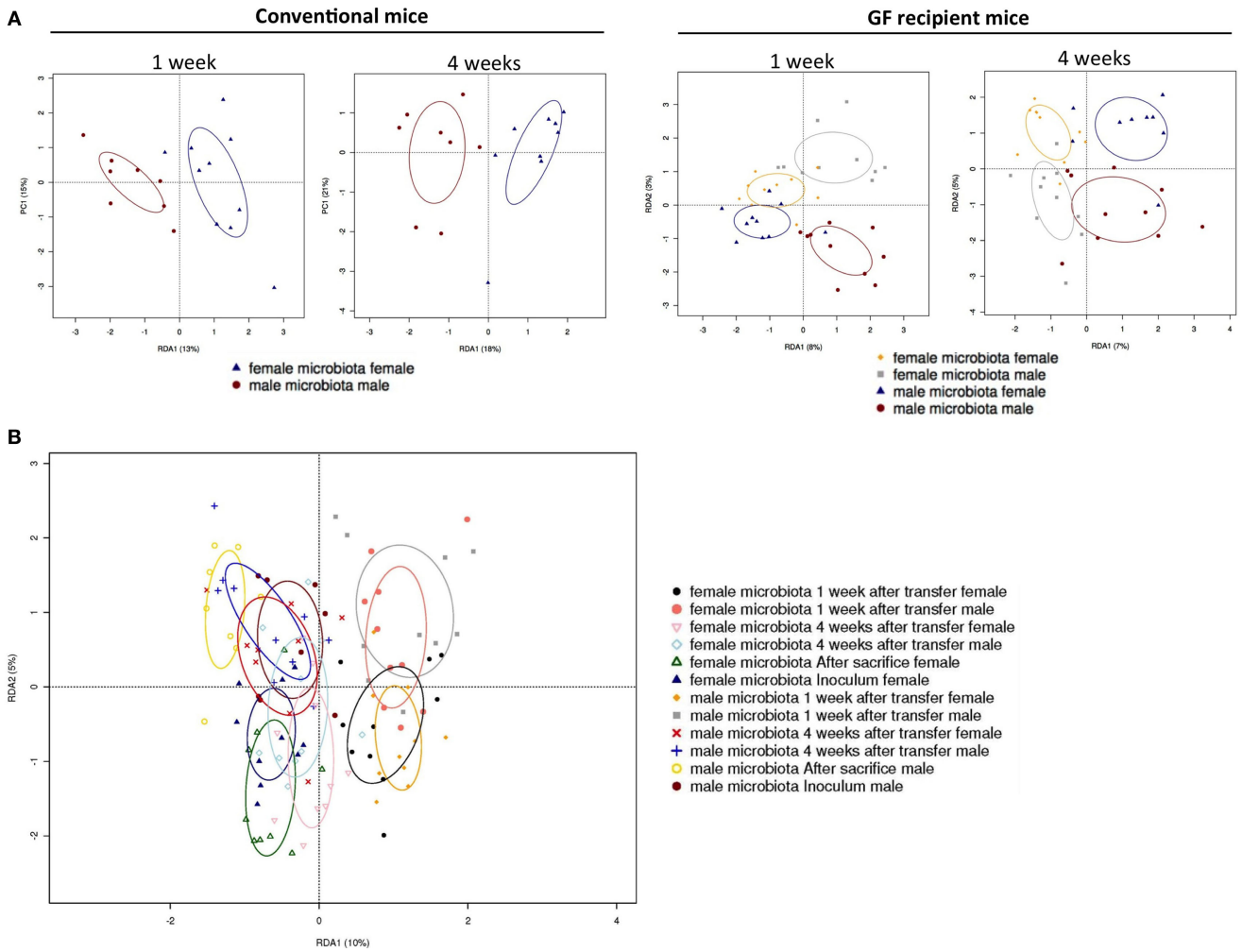


FIGURE 8 | Redundancy analysis (RDA) of gut microbiota composition. Fecal samples were collected from conventional (conv) males (M) ($n = 8$) and conventional females (F) ($n = 10$) at the time of transfer or 4 weeks after transfer to the germ-free (GF) recipient mice. In addition, fecal samples 1 and 4 weeks after the transfer were collected from the following groups (all $n = 10$ per group): male GF recipient mice that received male microbiota (male microbiota male), female GF recipient mice that received male microbiota (male microbiota female), male GF recipient mice that received female microbiota (female microbiota male), and female GF recipient mice that received female microbiota (female microbiota female). Gut microbiota composition was analyzed with 16S rRNA gene sequencing. **(A)** RDA of gut microbiota composition of conventional males and females at the time of transfer (first panel), or conventional males and females 4 weeks after transfer (second panel), or GF recipient mice 1 week after transfer (third panel), or GF recipient mice 4 weeks after transfer (fourth panel). **(B)** RDA of gut microbiota composition of all groups combined.

Several lines of evidence suggest that in our study the male microbiota induced more gut inflammation after transfer. Female recipients lost significantly more weight after receiving male microbiota compared to female microbiota. Weight loss is a sign of discomfort and a hallmark of dextran sulfate sodium (DSS)-induced colitis (31). Interestingly, DSS was also most significantly predicted as upstream regulator when comparing gene expression in the ileum of female recipients of male or female microbiota. We also observed that DNA repair and cell cycle pathways were specifically induced by the male microbiota. This response could be due to increased inflammation induced by the male microbiota, since inflammation was previously shown to promote DNA damage and subsequent carcinogenesis in the colon (45).

The gut microbiota also influenced T cell precursors in the thymus and T cell differentiation in PPs and MLN in a gender-specific manner. We found that the thymus of females contained more cells compared to males. This corroborates the observation that thymus size is influenced by sex hormones and is larger in females (46). However, to our knowledge, we are the first to show that thymus size is also influenced by the gut microbiota in a gender-specific manner. In addition, the female microbiota induced less RORγt$^+$Foxp3$^+$ T cells in PPs and MLN in male recipients compared to the male microbiota. This cell population has recently been shown to be induced by the microbiota and to inhibit Th2-associated pathology (26). Thus, the female microbiota might be less efficient in preventing allergies. Indeed

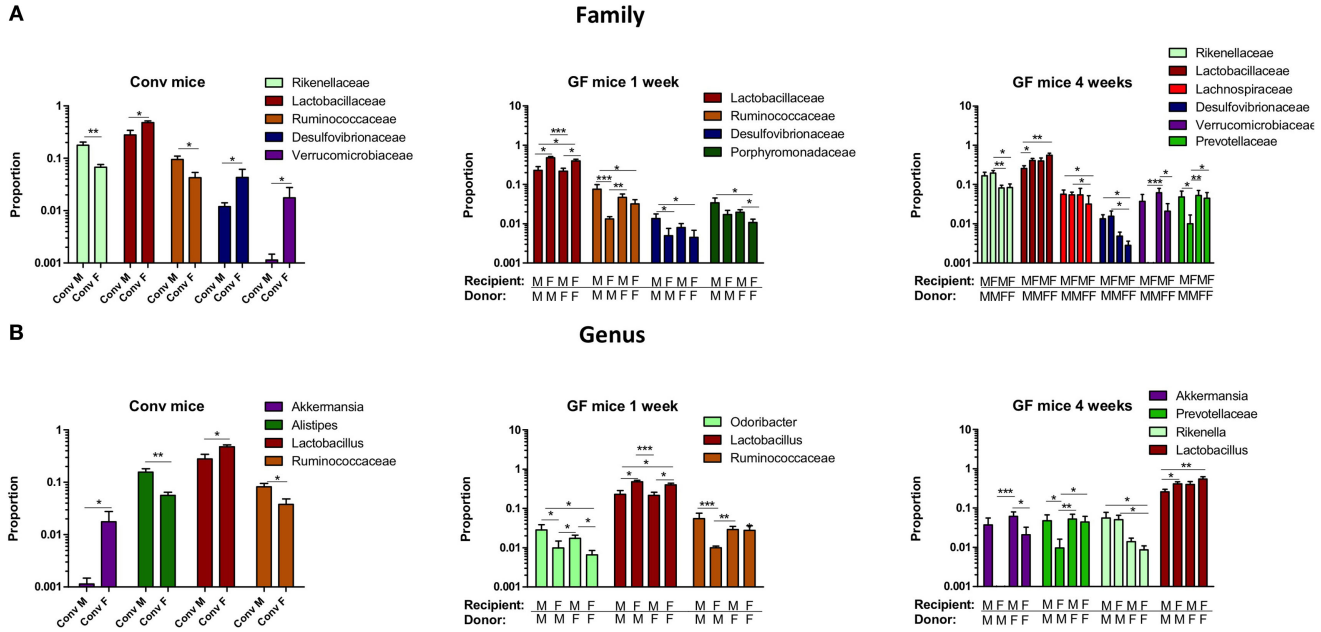


FIGURE 9 | Gender-specific differences in gut microbiota composition. Fecal samples were collected from conventional (conv) males (M) ($n = 8$) and conventional females (F) ($n = 10$) at the time of transfer or 4 weeks after transfer to the germ-free (GF) recipient mice. In addition, fecal samples 1 and 4 weeks after the transfer were collected from the following groups (all $n = 10$ per group): male GF recipient mice that received male microbiota (male microbiota male), female GF recipient mice that received male microbiota (male microbiota female), male GF recipient mice that received female microbiota (female microbiota male), and female GF recipient mice that received female microbiota (female microbiota female). Gut microbiota composition was analyzed with 16S rRNA gene sequencing. **(A)** Bacterial families that were significantly different in abundance in the different experimental groups. **(B)** Bacterial genera that were significantly different in abundance in the different experimental groups.

IgE-mediated food allergies are known to be more prevalent in adult females (47).

Together our results suggest that microbiota-independent gender immune differences contribute to the selection of a gender-specific gut microbiota composition, which in turn further drives gender immune differences. Therefore, gender should be considered in the development of strategies to target the gut microbiota in different disorders.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of FELESA guidelines and the ethical committee for animal experiments from the University of Groningen (DEC-RUG). The protocol was approved by the ethical committee for animal experiments from the University of Groningen (DEC-RUG).

AUTHOR CONTRIBUTIONS

FF designed the experiments and wrote the manuscript. FF, AB, TB, and BM performed the experiments. SA, FH, and HS generated and analyzed the microbiota data. CJ and MJ provided material and resources. MB generated and analyzed microarray data. HFS, MF, and PV supervised the study.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.00754/full#supplementary-material.

FIGURE S1 | Cells from spleen, Peyer's patches (PPs), and mesenteric lymph nodes (MLNs) were analyzed with flow cytometry. Germ-free (GF) males (M) ($n = 5$), GF females (F) ($n = 5$), conventional (conv) males ($n = 8$), and females ($n = 10$) were included as controls. Experimental groups of GF recipients of gut microbiota each contained 10 mice per group. **(A)** Percentage of RORγt$^+$Foxp3$^+$ cells among CD4$^+$ T cells in the spleen. **(B)** percentage of CD25$^+$Foxp3$^+$ regulatory cells (Treg) among CD4$^+$ T cells in the spleen and PPs. **(C)** Percentage of T-bet$^+$ Th1 cells among CD4$^+$ T cells in the spleen, MLNs, and PPs. **(D)** Percentage of Roryt$^+$ Th17 cells among CD4$^+$ T cells in the spleen, MLNs, and PPs. **(E)** Percentage of Gata-3$^+$ Th2 cells among CD4$^+$ T cells in the spleen, MLNs, and PPs.

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Lactobacillus johnsonii N6.2 Modulates the Host Immune Responses: A Double-Blind, Randomized Trial in Healthy Adults

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Lactobacillus johnsonii N6.2 mitigates the onset of type 1 diabetes (T1D) in biobreeding diabetes-prone rats, in part, through changes in kynurenine:tryptophan (K:T) ratios. The goal of this pilot study was to determine the safety, tolerance, and general immunological response of *L. johnsonii* N6.2 in healthy subjects. A double-blind, randomized clinical trial in 42 healthy individuals with no known risk factors for T1D was undertaken to evaluate subject responses to the consumption of *L. johnsonii* N6.2. Participants received 1 capsule/day containing 10^8 colony-forming units of *L. johnsonii* N6.2 or placebo for 8 weeks. Comprehensive metabolic panel (CMP), leukocyte subpopulations by complete blood count (CBC) and flow cytometry, serum cytokines, and relevant metabolites in the indoleamine-2,3-dioxygenase pathway were assessed. *L. johnsonii* N6.2 survival and intestinal microbiota was analyzed. Daily and weekly questionnaires were assessed for potential effects of probiotic treatment on general wellness. The administration of *L. johnsonii* N6.2 did not modify the CMP or CBC of participants suggesting general safety. In fact, *L. johnsonii* N6.2 administration significantly decreased the occurrence of abdominal pain, indigestion, and cephalic syndromes. As predicted, increased serum tryptophan levels increased resulting in a decreased K:T ratio was observed in the *L. johnsonii* N6.2 group. Interestingly, immunophenotyping assays revealed that monocytes and natural killer cell numbers were increased significantly after washout (12 weeks). Moreover, an increase of circulating effector Th1 cells ($CD45RO^+CD183^+CD196^-$) and cytotoxic $CD8^+$ T cells subset was observed in the *L. johnsonii* N6.2 group. Consumption of *L. johnsonii* N6.2 is well tolerated in adult control subjects, demonstrates systemic impacts on innate and adaptive immune populations, and results in a decreased K:T

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ratio. These data provide support for the safety and feasibility of using *L. johnsonii* N6.2 in prevention trials in subjects at risk for T1D.

Trial registration: This trial was registered at http://clinicaltrials.gov as NCT02349360.

Keywords: *Lactobacillus johnsonii*, diabetes type I, probiotic, indoleamine-2,3-dioxygenase, microbiome, gastrointestinal symptom, T cell, immunological response

INTRODUCTION

Commensal bacteria regulate a myriad of host processes and provide several nutrients to their host as well as their symbionts within the microbial community (1, 2). In healthy individuals, these relationships are thought to occur in equilibrium (3). However, disruption of this equilibrium may contribute to various conditions including inflammatory bowel disease and atopy [for a review, see Ref. (4)]. As a result of the multiple microbiome studies being performed, this connection has gained credibility as associations between gut microbiota and either the risk for or presence of a variety of specific human diseases have been shown [for a review, see Ref. (5–7)]. While genetics has been demonstrated to represent a major risk factor for the development of type 1 diabetes (T1D), numerous environmental factors have been suggested that could further elicit a break in immunological tolerance and initiate or perpetuate β-cell autoimmunity (8, 9). Recent studies have looked at the fluctuations in the microbiota and the rate of diabetes development in infant cohorts. These studies have shown a low abundance of lactate-producing and butyrate-producing species and an increase of the *Bacteroides* genus in children with autoimmunity when compared to controls (10, 11). Kostic et al. (12) further showed that the fluctuations in the microbiota composition occur prior to the onset of disease but after seroconversion.

Interactions between the intestinal environment, epithelial barrier function, and the immune system have all been shown to have a major impact on the rate of T1D development in rodent models (13–15). In order to understand the role of the resident microbiota in T1D, we performed a culture-independent analysis of the bacteria in fecal samples collected from biobreeding diabetes-resistant (BB-DR) and diabetes-prone (BB-DP) rats. These experiments demonstrated a significant difference in *Lactobacillus* and *Bifidobacterium* species in the intestinal microbiota of DR and the DP rats, which were correlated with health status (16). Members of these bacterial genera are widely used in dietary supplements as probiotics worldwide. However, the mechanisms by which these individual probiotics modulate host responses and immunity are diverse and are often strain specific, rather than shared among genera (17, 18).

Given the observation of *L. johnsonii* N6.2 in protected DR rats, we performed an intervention study using *L. johnsonii* N6.2 in BB-DP animals. It was found that the administration of *L. johnsonii* N6.2 to BB-DP rats reduced the incidence of T1D (14). The feeding of this microorganism postweaning was followed by changes in the native microbiota, host mucosal proteins, and oxidative stress response. In the ileum, lower levels of the pro-inflammatory cytokines IFN-γ and TNF-α were also observed in the *L. johnsonii* fed group. *L. johnsonii*-mediated diabetes prevention correlated with a Th17 cell bias and elevated IL-23 levels within the mesenteric lymph nodes. Further *in vitro* studies indicate that the modification of dendritic cells (DCs) by oral feeding of *L. johnsonii* N6.2 contributed to the Th17 bias (15).

One potential mechanism by which the host microbial composition may alter immune responses is through the metabolism of tryptophan. This essential amino acid acts as a substrate for the enzyme indoleamine-2,3-dioxygenase-1 (IDO), which converts tryptophan to kynurenine (19–21). T helper subset activation and differentiation has been demonstrated to depend on the bioavailability of local tryptophan (22), and seminal studies showed that murine IDO expression was necessary for T cell tolerance during pregnancy to the semi-allogeneic fetus (23, 24). In an *in vivo* feeding assay performed in BB-DP rats, *L. johnsonii* N6.2 lowered intestinal IDO gene transcription, which in turn correlated with decreased blood plasma kynurenine levels (25). During *in vitro* studies, *L. johnsonii* N6.2 produced H_2O_2 that strongly inhibited IDO activity. Mass spectrometry analysis of the IDO catalytic heme-center supported the presence of a molecule in the *L. johnsonii* culture cell-free supernatant that modifies this immunoregulatory enzyme's prosthetic group, and as a consequence, its activity. These data suggest that this bacterium alters host IDO activity, with the potential for downstream effects on T-cell development, intestinal physiology, and ultimately T1D development.

Translating this work toward a potential method for T1D prevention in humans required a pilot study in healthy individuals. Hence, the primary aim of this study was to assess the safety and tolerability of *L. johnsonii* N6.2. A secondary mechanistic aim was to characterize the host immune response to *L. johnsonii* N6.2 consumption, specifically the impact of this bacterium on circulating immunoglobulin, cytokines, leukocyte subpopulations, and relevant metabolites in the IDO pathway in healthy adults.

MATERIALS AND METHODS

Subjects

Forty-two healthy adults (female = 30, male = 12; mean age ± SD = 23.2 ± 5.5 years) participated in the study. Participants were recruited from the community and the University of Florida campus in Gainesville, FL, USA in accordance with an Institutional Review Board (IRB) approved study at the University of Florida. Exclusion criteria included gastrointestinal disease (gastric ulcers, Crohn's, ulcerative colitis, etc.), chronic disease such as diabetes, kidney disease, and heart disease; current or past treatment for immune-compromising diseases or conditions; currently working or living with an immunocompromised person; currently taking medications for constipation, diarrhea, or a psychological

disorder (depression, anxiety, insomnia, etc.); antibiotics within the past 4 weeks prior to randomization; currently taking a probiotic supplement and unwilling to discontinue a minimum of 2 weeks prior to the study start; current smoker; pregnant or lactating or a female who plans to become pregnant in the next 6 months; and a known allergy to milk. Inclusion criteria included men and women 18–50 years of age and approval to participate following screening blood work and physical examination by the advising physician.

Experimental Design

In a double-blinded study, healthy volunteers were randomly assigned to one of two treatments, *L. johnsonii* N6.2 at 5×10^8 colony-forming units (CFU) per capsule or placebo (skim milk) capsule for 8 weeks in a parallel design. Prior to treatment, there was a 1-week pre-baseline period, and treatment was followed by a 4-week washout period. One week prior to randomization, consented participants underwent a physical examination and were screened *via* a comprehensive metabolic panel (CMP), and females received a pregnancy test. During pre-baseline, intervention and washout periods, participants completed a daily online questionnaire, reporting on: study supplement intake, hours of sleep, bowel movement frequency, gastrointestinal symptoms, general wellness, and medication use. In addition, participants completed the gastrointestinal symptom rating scale (GSRS), and quality of life was assessed with the quality of life questionnaire, SF-36v2® on a weekly basis. At the randomization appointment, height, weight, vitals (blood pressure, heart rate), and demographic information were obtained. CMP and complete blood count were assessed at baseline, during weeks 2, 4, and 8 of the study intervention, and during washout. An additional pregnancy test was given during week 4 of the intervention phase. At a final appointment, participants returned any unconsumed supplements.

L. johnsonii N6.2 Culture and Capsules Elaboration

Lactobacillus johnsonii N6.2 was grown in modified MRS medium (FGM-LJ2). The media contained peptone 10 g, meat powder 10 g, yeast peptone 5 g, table sugar 20 g, K_2HPO_4 2 g, sodium acetate 5 g, ammonium citrate tribasic 2 g, $MgSO_4 \cdot 7H_2O$ 0.2 g, $MnSO_4 \cdot H_2O$ 0.05 g, tween 80 1 g; final volume of 1 L with DI water. *L. johnsonii* N6.2 was incubated at 37°C for 16 h under microaerophilic conditions. Cells were pelleted by centrifugation at 6,000 *g* for 20 min at 4°C and washed twice with BAM R61 0.02 M phosphate buffer pH 7.3 (Bacteriological Analytical Manual, 8th Edition, Revision A, 1998). The cell pellet was resuspended in sterile reconstituted food grade skim milk at 100 g/L (Real Food, IL, USA), transferred to sterile bags (Whirl-Pak, USA), and frozen at −80°C for at least 2 h. The frozen samples were freeze dried (LabConco FreeZone, LabConco Corp., MO, USA) for 48 h. The dried powder was saved at 4°C until capsule filling. Acid resistant capsules (AR Caps, Size #1, CapsCanada, Pompano Beach, FL, USA) were filled using a sterilized Profiller (Torpac®, NJ, USA) with the freeze-dried preparation of *L. johnsonii* N6.2 in skim milk (Real Food, IL, USA). Lyophilized skim milk in identical capsules was used as the placebo. The study capsules were provided in bottles labeled with treatment codes by a study collaborator who did not have contact with study participants.

Stool Sample Collection and Transit Survival of *L. johnsonii* N6.2

Single stools were collected using a commode specimen collection system (Fisher Scientific, Pittsburgh, PA, USA) during the last 2 days of pre-baseline, during weeks 2, 4, and 8 of the intervention, and during washout. Participants were instructed to place the stool containers on ice immediately after defecation and deliver samples to study personnel within 4 h of defecation. Samples were homogenized and fractionated on sterile vials (approximately 1.0 g/vial) and saved at −80°C until use. Fresh samples (approximately 1 g) were immediately diluted (1/10 w/v) in phosphate buffer solution (pH: 7.4), and serial dilutions were made and plated on MRS agar media (pH; 5.5 ± 0.1). Plates were incubated for 48 h under microanaerobic conditions. Values were referred as CFU per wet gram stool (CFU/g). The identity of *L. johnsonii* N6.2 was confirmed by PCR amplification of the strain-specific gene T285_00345 gene (26).

Blood Sample Collection and CMP

From fasting blood samples, serum (Red top Tube, BD, USA) and plasma (EDTA Purple top Tube, BD, USA) were collected. The CMP was obtained from serum samples evaluating glycemia (glucose level), kidney function (creatinine and urea level), and liver function (aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, and bilirubin level). The analysis was performed by Vista Clinical Diagnostics, Clermont, FL, USA. Additionally, plasma and serum samples were aliquoted, flash frozen in liquid nitrogen, and stored at −80°C for further assays. ELISA assays were used to quantify serum insulin levels (eBioscience, CA, USA) and C-reactive protein (CRP) (Cayman Chemical, MI, USA).

IDO Activity: Tryptophan Metabolites Pathway

Quantification of tryptophan catabolites and other metabolites in blood plasma samples (i.e., tryptophan, kynurenine, kynurenic acid, xanthurenic acid, serotonin, and anthranilic acid) were performed using global high-performance liquid chromatography and mass spectrometry (LC-HRMS/HRMS) at the Southeast Center for Integrated Metabolomics of the University of Florida.

Cytokine Determinations

The following ELISA kits were used: IL-2, IL-6, and TNF-α from eBioscience (CA, USA); IFN-γ and IFN-α from Abcam (MA, USA); IL-2SsRα from BD (NJ, USA) following the manufacturers' instructions.

Flow Cytometry

Direct immunofluorescence surface staining of whole blood samples with six antibody panels was performed to provide a detailed assessment of immune cell subsets. Five of the panels and gating strategies are emulated from Maecker (27) and

consist of a B cell subset panel (CD3, CD19, CD20, CD24, CD27, CD38, and IgD), innate cell panel (CD3, CD11c, CD14, CD16, CD19, CD20, CD56, CD123, and HLA-DR), T cell naïve and memory panel (CD3, CD4, CD8, CD38, CD45RA, CD197, and HLA-DR), T cell effector subset panel (CD3, CD4, CD38, CD45RO, CD183, CD196, and HLA-DR), and a Treg panel (CD3, CD4, CD25, CD45RO, CD127, CD194, and HLA-DR). A sixth follicular helper T (Tfh) panel (CD3, CD4, CD45RA, CD183, CD196, CD197, and CD279) was designed to assess precursor Tfh (28) and memory Tfh (29). Antibodies against the following antigens were used: CD3 (SK7), CD8 (SK1), CD19 (HIB19), CD20 (2H7), and CD45RO (UCHL1) from BD Biosciences, HLA-DR (LN243), IgD (IA6-2), CD3 (UCHT1), CD4 (RPA-T4), CD11c (Bu15), CD14 (M5E2), CD16 (3G8), CD24 (ML5), CD25 (BC96), CD27 (O323), CD38 (HB7), CD45RA (HI100), CD56 (HCD56), CD123 (6H6), CD127 (A019D5), CD183 (G025H7), CXCR3 (G025H7), CD185 (J252D4), CD194 (L291H4), CD196 (G034E3), and CD197 (G043H7) from BioLegend (USA), and CD279 (eBioJ105) from eBiosciences (USA). Whole blood (200 µL/stain) was incubated for 30 min at room temperature and protected from light. Afterward, 2 mL of Fix/lyse 1× (eBioscience, USA) was added and incubated at room temperature for 5 min. Successive washing/centrifugation (5 min, 450 g) steps were performed until hemolysis color completely faded. The samples were acquired on a BD Fortessa cytometer, and data were analyzed by FlowJo software.

Extraction of Fecal Microbiota

DNA was extracted from fecal samples and preserved at −80°C using the PowerFecal® DNA isolation kit (MoBio Lab, Inc., USA) with the following modification. 250 mg of fecal sample were homogenized in 750 µL of bead solution and 100 µL of Protease from *Streptomyces griseus* 20 mg/mL (Sigma-Aldrich, Steinheim, Germany) were added. The mixture was incubated at 37°C for 15 min and samples were processed according to the manufacturers' protocol. In the elution step, the DNA was collected in 70 µL of water and quantified. The DNA concentration was standardized to 1 ng/µL before the amplification of the V4 region using primers for paired-end sequencing on the Illumina MiSeq platform as described earlier (30).

Microbiota Analysis

Clustering of operational taxonomic units (OTUs) at 97% similarity was performed with the subsampled open-reference OTU picking method (31), with no removal of singletons. The Greengenes reference dataset version 13.8 (32) was used as the reference for OTU picking and for taxonomy assignment with uclust (33). OTUs identified as mitochondrial DNA or as chloroplasts were removed from further analyses. Parsed raw sequencing reads are publicly available through NCBI's Sequence Read Archive under the BioProject accession number PRJNA378749.

Statistics

Unless otherwise noted, statistical analysis was performed using JMP Pro software (SAS Institute, Cary, NC, USA). Multivariate analysis was performed by two-way analysis of variance (ANOVA) with a *post hoc* Tukey's honestly significant difference test. Bivariate analysis was performed using Student's *t*-tests. Numerical data are summarized as mean ± SE. Significance was defined as $p < 0.05$.

Analyses of Surveys

For each of the response variables, a repeated measures analysis was performed by fitting a linear mixed model that considered the repeated nature of the data. The fitted model had the following form: $y = \mu + \text{gender} + \text{supp} + \text{group(supp)} + e$ where µ is the overall mean, gender is the gender effect, supp is a diet supplementary effect, group (supp) corresponds to the combination of measurement week within a diet supplement effect, and *e* corresponds to an error term, where measurements from the same individual were correlated using an unstructured error with a different correlation for each pair of time points and a different error variance for each time point. The models were fitted using SAS v. 9.4 with the procedure MIXED and degrees of freedom were adjusted using the Kenward–Rogers correction. Comparisons of means for the diet supplement levels at a given week were obtaining constructing specific contrasts, and for all tests a significance level of 5% was considered.

Analyses of Immune Cells

For each of the response variables, a repeated measures analysis was performed by fitting a linear mixed model that considered the repeated nature of the data. The fitted model had the following form: $y = \mu + \beta^{*}x0 + \text{gender} + \text{supp} + \text{group} + \text{time} + \text{supp}^{*}\text{time} + \text{group}^{*}\text{time} + \text{supp}^{*}\text{group} + \text{supp}^{*}\text{group}^{*}\text{time} + e$ where µ is the overall mean, $\beta^{*}x0$ is the regression coefficient associated with the covariate for initial measurement $\beta^{*}x0$, gender is the gender effect, supp is a diet supplementary effect, group is the group effect, and time is the time of measurement. The other terms are the two- and three-way interactions. Also, *e* corresponds to an error term, where measurements from the same individual were correlated using an unstructured error with a different correlation for each pair of time points and a different error variance for each time point. The models were fitted using SAS v. 9.4 with the procedure MIXED and degrees of freedom were adjusted using the Kenward–Rogers correction. Comparisons of means for a given model term were obtained with the least significance difference, and for all tests a significance level of 5% was considered.

Analysis of Microbiota

Community structure was analyzed in R with phyloseq (34) and plotted with ggplot2 (35). Analysis of similarities (ANOSIM) and Permutational Multivariate Analysis of Variance (PERMANOVA) were performed in R using VEGAN v2.0-8 (36). Differences in taxonomic profiles were analyzed by Welch's *t*-test (for two groups) or by ANOVA (for multiple groups) with Tukey–Kramer *post hoc* tests with STAMP (37).

Study Approval

The study was approved by the IRB (# 201400370) at the University of Florida and conducted according to guidelines established by the Declaration of Helsinki. Participants were informed of the aims, requirements, and risk/benefits of the study, and written informed consent indicating their full knowledge of the study

protocol was received from participants prior to study enrollment. In addition, an Investigational New Drug (IND#016829) has been filed with the Food and Drug Administration of the United States of America.

RESULTS

The Administration of *L. johnsonii* N6.2 Decreases Indigestion, Abdominal Pain, and Cephalic Syndrome Scales

Of the 92 individuals initially screened and assessed for eligibility, 50 were consented, and 42 randomized to the treatment groups (**Figure 1**) in a double-blind parallel study design. **Table 1** summarizes the characteristics and compliance of the subjects that participated in the study.

The analysis of the hemogram and CMP data showed no statistically significant differences between the *L. johnsonii* N6.2 and placebo groups (Table S1 in Supplementary Material). The only parameter that showed statistical significance after 8 weeks of treatment was total bilirubin values (placebo $= 0.54 \pm 0.05$ versus *L. johnsonii* N6.2 $= 0.70 \pm 0.05$, $p < 0.05$); however, both groups were within the reference range of 0.2–1.9 mg/dL (38). These differences were not observed after the washout period (12 weeks). No statistically significant differences were observed in either the control or treatment cohorts with regards to kidney and liver function (Table S1 in Supplementary Material). As expected, no alterations in the circulating levels of insulin and CRP were observed in the *L. johnsonii* N6.2 group at 8 weeks or 12 weeks compared to placebo. Based on these results, the consumption of *L. johnsonii* N6.2 was well tolerated without apparent risks or deviations from the reference ranges for standard clinical assessments.

We next assessed general digestive health over the course of the trial. During pre-baseline, intervention, and washout periods, participants completed a daily online questionnaire reporting study supplement intake, hours of the sleep, bowel movement frequency, gastrointestinal symptoms, general wellness, and medication use. Of the five domains of the weekly GSRS questionnaire, indigestion ($p < 0.05$) and abdominal pain ($p < 0.05$) were significantly lower among *L. johnsonii* N6.2 treated subjects during treatment weeks 2 to 8, and during washout weeks 1–4 compared to placebo (**Table 2**). Syndrome scores from the daily questionnaire indicated that cephalic syndrome, including the symptoms of headache and dizziness, was significantly lower ($p < 0.05$) for *L. johnsonii* N6.2 versus placebo for treatment weeks 2 and 4–8. The gastrointestinal distress syndrome was lower ($p < 0.05$) for *L. johnsonii* N6.2 versus placebo during most treatment weeks and neared significance at baseline ($p = 0.05$). Interestingly, the probiotic group showed a significant decrease ($p < 0.05$) in the epidermal syndrome at the end of the washout period (**Table 3**). Stomach ache or pain as an individual symptom was significantly lower in the group receiving *L. johnsonii* N6.2 during treatment weeks 1–8 compared to the placebo group, with a similar trend at baseline ($p = 0.06$) (Table S2 in Supplementary Material). Bloating as an individual symptom was lower ($p < 0.01$) in *L. johnsonii* N6.2 versus placebo at baseline and during most treatment and washout weeks (Table S2 in Supplementary

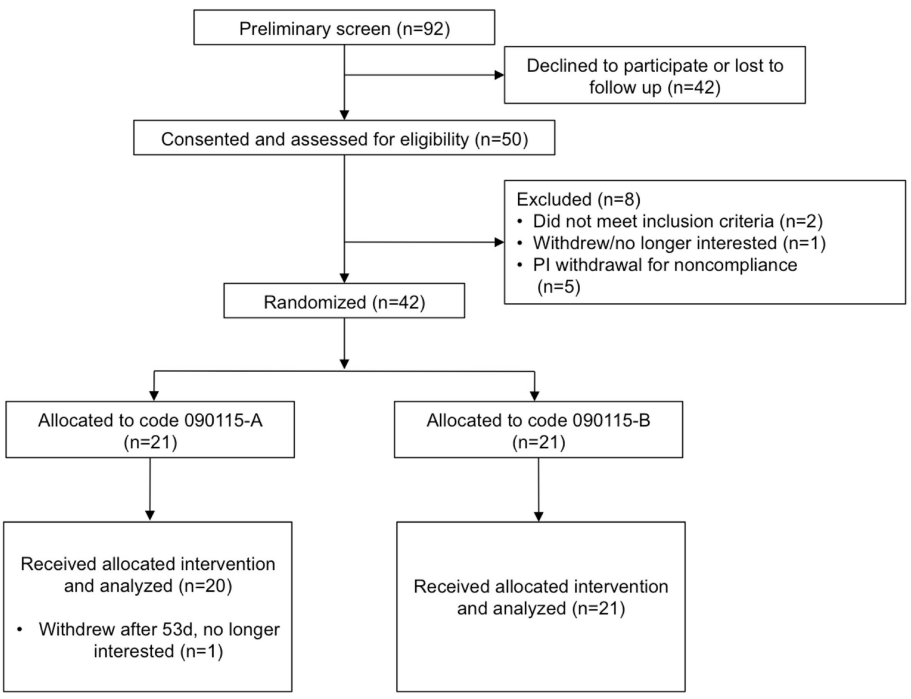

FIGURE 1 | Study flow diagram to illustrate the number of subjects that were screened, consented, and randomized. Code 090115-A was used for the placebo, whereas Code 090115-B was used for *Lactobacillus johnsonii* N6.2.

Material). Administration of *L. johnsonii* N6.2 also resulted in lower individual daily symptoms of cramping, abdominal noises, and headache for most treatment weeks. Furthermore, a significant decrease in the anxiety symptom after the washout period in the probiotic group was observed (Table S2 in Supplementary Material). The anxiety changes may have affected the psychology syndromes where a trend to decrease ($p < 0.1$) during the washout was observed (**Table 3**).

L. johnsonii N6.2 Survives Human Gastrointestinal Transit

Gastrointestinal transit survival of *L. johnsonii* N6.2 was evaluated by following the total CFU/g of stools counts of lactic acid bacteria (LAB) in fresh fecal samples. Additionally, the presence of *L. johnsonii* N6.2 was confirmed by RT-PCR of the T285_00345 gene.

Overall, a large variability in the amount of the total CFU of LABs was observed between subjects (from 10^2 to 10^8 CFU/g stools) at time 0, and no significant changes were observed over time either in the placebo or in the *L. johnsonii* N6.2 treatment group (**Figure 2A**). However, it was possible to observe three groups of subjects within each treatment: (a) subjects with high counts of LAB throughout the study (>10^5 CFU/g), (b) a group that at time 0 showed low concentrations of LAB that increased over time (from 10^4 to 10^8 CFU/g), and (c) subjects with low counts of LAB throughout the study (<10^5 CFU/g). To quantify the variation of the LAB population, the log CFU/g values for time 0 was subtracted at each time point and expressed as relative change (log CFU/$g_{\text{at each time point}}$/log CFU/$g_{\text{at time 0}}$). For subjects with consistently high or low LAB populations (groups a and c), the relative fold change in LAB was 1.0 ± 0.11 and 0.8 ± 0.02, respectively, in the probiotic group (**Figure 2B**). Similar results were obtained in the placebo group (**Figure 2C**). As expected, subjects in group b that received *L. johnsonii* N6.2 capsules displayed the highest relative change in LAB counts (**Figure 2B**). After the washout period, the LAB counts appeared to return to baseline levels (**Figures 2B,C**). These results suggest that *L. johnsonii* N6.2 survived the transit through the gastrointestinal system and potentially may not colonize the gut.

To verify this hypothesis, the presence of *L. johnsonii* N6.2 was confirmed using specific RT-PCR of the T285_00345 gene and expressed as genomic equivalents/100 ng of DNA (**Figure 2D**). This gene was found in the genome of *L. johnsonii* N6.2 but not in others in the NCBI database. It was found that the T285_00345

TABLE 1 | Characteristics of the participants and compliance.

Measure	Placebo (*n* = 21)	Ljo[a] (*n* = 21)
Gender (M/F), *n*	7/14	5/16
Age, years median (range)	21 (18–48)	23 (18–36)
Race/ethnicity, *n* (%)		
Asian	2 (10%)	4 (19%)
African-American	1 (5%)	1 (5%)
Hispanic	3 (14%)	4 (19%)
White	18 (86%)	12 (57%)
Other	0	3 (14%)[b]
BMI, mean (SD)	23.6 ± 4.7	23.6 ± 4.2
Blood pressure (mean mm Hg)	116/75	119/74
Compliance (%)		
Supplement protocol	88.7 ± 10.0	92.9 ± 8.3
Questionnaires protocol	86.6 ± 14.4	90.7 ± 9.7

[a]*Lactobacillus johnsonii N6.2.*

[b]*Participants who classified themselves as other included n = 2 Hawaiian and n = 1 Unknown.*

TABLE 2 | Gastrointestinal symptom rating scale scores.

Period	Abdominal pain[a]		Reflux[b]		Diarrhea[c]		Indigestion[d]		Constipation[e]	
	Placebo	Ljo	Placebo	Ljo	Placebo	Ljo	Placebo	Ljo	Placebo	Ljo
Baseline	1.6 ± 0.1	1.3 ± 01	1.2 ± 0.1	1.1 ± 0.1	1.4 ± 0.2	1.3 ± 0.1	1.9 ± 0.1	1.4 ± 0.1*	1.4 ± 0.1	1.3 ± 0.1
Week 1	1.7 ± 0.1	1.2 ± 0.1*	1.1 ± 0.1	1.0 ± 0.1	1.3 ± 0.1	1.4 ± 0.1	1.7 ± 0.1	1.4 ± 0.1*	1.3 ± 0.1	1.1 ± 0.1
Week 2	1.7 ± 0.1	1.1 ± 0.1***	1.2 ± 0.1	1.1 ± 0.1	1.5 ± 0.2	1.3 ± 0.2	1.8 ± 0.1	1.3 ± 0.1***	1.5 ± 0.2	1.2 ± 0.2
Week 3	1.4 ± 0.1	1.1 ± 0.1*	1.3 ± 0.1	1.1 ± 0.1	1.4 ± 0.1	1.3 ± 0.1	1.7 ± 0.1	1.3 ± 0.1*	1.4 ± 0.1	1.2 ± 0.1
Week 4	1.5 ± 0.1	1.1 ± 0.1*	1.2 ± 0.1	1.1 ± 0.1	1.5 ± 0.2	1.4 ± 0/1	2.0 ± 0.1	1.3 ± 0.1**	1.5 ± 0.2	1.2 ± 0.1
Week 5	1.5 ± 0.1	1.1 ± 0.1**	1.2 ± 0.1	1.0 ± 0.1	1.4 ± 0.1	1.2 ± 0.1	1.8 ± 0.2	1.3 ± 0.1*	1.3 ± 0.1	1.2 ± 0.1
Week 6	1.4 ± 0.1	1.1 ± 0.1*	1.3 ± 0.2	1.2 ± 0.2	1.5 ± 0.2	1.2 ± 0.2	1.6 ± 0.1	1.3 ± 0.1*	1.3 ± 0.1	1.2 ± 0.1
Week 7	1.6 ± 0.1	1.0 ± 0.1**	1.2 ± 0.1	1.0 ± 0.1*	1.2 ± 0.1	1.1 ± 0.1	1.7 ± 0.1	1.2 ± 0.1**	1.4 ± 0.1	1.1 ± 0.1*
Week 8	1.6 ± 0.1	1.1 ± 0.1**	1.2 ± 0.1	1.0 ± 0.1	1.4 ± 0.1	1.1 ± 0.1	1.7 ± 0.1	1.2 ± 0.1**	1.6 ± 0.1	1.1 ± 0.1*
Washout 1	1.5 ± 0.1	1.0 ± 0.1**	1.2 ± 0.1	1.0 ± 0.1*	1.5 ± 0.1	1.1 ± 0.1*	1.8 ± 0.1	1.2 ± 0.1**	1.4 ± 0.1	1.1 ± 0.1*
Washout 2	1.4 ± 0.1	1.0 ± 0.1***	1.1 ± 0.1	1.0 ± 0.1	1.3 ± 0.2	1.2 ± 0.2	1.7 ± 0.1	1.3 ± 0.1*	1.5 ± 0.1	1.1 ± 0.1*
Washout 3	1.7 ± 0.1	1.1 ± 0.1***	1.3 ± 0.1	1.1 ± 0.1	1.4 ± 0.1	1.2 ± 0.1	1.9 ± 0.1	1.3 ± 0.1***	1.4 ± 0.1	1.1 ± 0.1*
Washout 4	1.5 ± 0.1	1.2 ± 0.1	1.3 ± 0.1	1.2 ± 0.1	1.4 ± 0.1	1.1 ± 0.1*	1.8 ± 0.1	1.2 ± 0.1***	1.3 ± 0.1	1.1 ± 0.1

[a]*Abdominal pain syndrome includes abdominal pain, hunger pains, and nausea symptoms.*

[b]*Reflux syndrome includes heartburn and acid regurgitation symptoms.*

[c]*Indigestion syndrome includes stomach rumbling, bloating, burping, and increased flatus symptoms.*

[d]*Constipation syndrome includes constipation, hard stools, and feeling of incomplete evacuation symptoms.*

[e]*Diarrhea syndrome includes diarrhea, loose stools, and urgent need for defecation symptoms.*

Ljo correspond to Lactobacillus johnsonii N6.2.

Data presented as least squares mean ± SEM.

*$p < 0.05$.

**$p < 0.01$.

***$p < 0.001$.

TABLE 3 | Daily questionnaire syndrome scores.

Period	GI distress[a]		Epidermal[b]		Cephalic[c]		Ear–nose–throat[d]		Psychological[e]		Emetic[f]	
	Placebo	Ljo	Placebo	Ljo	Placebo	Ljo	Placebo	Ljo	Placebo	Ljo	Placebo	Ljo
Baseline	3.1 ± 0.5	1.6 ± 0.5	0.3 ± 0.2	0.1 ± 0.2	0.4 ± 0.1	0.5 ± 0.1	0.7 ± 0.2	0.6 ± 0.2	1.7 ± 0.6	2.6 ± 0.6	0.07 ± 0.06	0.13 ± 0.06
Week 1	2.2 ± 0.3	1.1 ± 0.3*	0.5 ± 0.2	0.01 ± 0.2	0.4 ± 0.1	0.4 ± 0.1	1.2 ± 0.4	0.7 ± 0.4	1.2 ± 0.5	2.0 ± 0.5	0.06 ± 0.03	0.02 ± 0.03
Week 2	1.7 ± 0.3	1.1 ± 0.3	0.6 ± 0.2	0.01 ± 0.2*	0.9 ± 0.2	0.2 ± 0.2**	1.3 ± 0.3	0.4 ± 0.3*	2.1 ± 0.6	1.4 ± 0.6	0.09 ± 0.06	0.01 ± 0.06
Week 3	2.0 ± 0.4	0.8 ± 0.4*	0.5 ± 0.2	0.07 ± 0.2	0.4 ± 0.1	0.3 ± 0.1	1.1 ± 0.4	0.8 ± 0.4	1.7 ± 0.4	0.9 ± 0.4	0.08 ± 0.07	0.13 ± 0.07
Week 4	2.2 ± 0.3	1.0 ± 0.3**	0.4 ± 0.2	0.07 ± 0.2	0.5 ± 0.1	0.05 ± 0.1**	1.1 ± 0.3	0.3 ± 0.3*	1.7 ± 0.6	1.6 ± 0.6	0.10 ± 0.05	0.01 ± 0.05
Week 5	1.9 ± 0.3	1.1 ± 0.3*	0.4 ± 0.2	0.03 ± 0.2	0.6 ± 0.1	0.2 ± 0.1*	1.2 ± 0.3	0.4 ± 0.3	1.9 ± 0.5	1.2 ± 0.5	0.10 ± 0.05	0.02 ± 0.05
Week 6	1.5 ± 0.2	0.9 ± 0.2	0.3 ± 0.2	0.02 ± 0.2	0.6 ± 0.1	0.02 ± 0.1**	1.0 ± 0.3	0.3 ± 0.3	1.8 ± 0.5	1.0 ± 0.5	0.08 ± 0.03	0.02 ± 0.03*
Week 7	2.0 ± 0.2	0.8 ± 0.2**	0.3 ± 0.1	0.01 ± 0.1	0.6 ± 0.1	0.04 ± 0.1***	0.8 ± 0.2	0.2 ± 0.2*	1.7 ± 0.5	1.0 ± 0.5	0.19 ± 0.07	0.01 ± 0.07
Week 8	1.6 ± 0.3	0.7 ± 0.3*	0.4 ± 0.2	0.02 ± 0.2	0.3 ± 0.1	0.04 ± 0.1*	0.5 ± 0.2	0.1 ± 0.2	1.8 ± 0.4	0.8 ± 0.4	0.04 ± 0.03	0.02 ± 0.03
Washout 1	2.1 ± 0.3	0.8 ± 0.3**	0.2 ± 0.1	0.01 ± 0.1	0.3 ± 0.1	0.03 ± 0.1	0.8 ± 0.3	0.2 ± 0.3	1.8 ± 0.4	0.8 ± 0.4	0.06 ± 0.04	0.02 ± 0.04
Washout 2	2.2 ± 0.4	0.7 ± 0.4**	0.2 ± 0.1	0.01 ± 0.1*	0.6 ± 0.1	0.04 ± 0.1***	0.6 ± 0.2	0.3 ± 0.2	1.6 ± 0.4	0.8 ± 0.4	0.11 ± 0.03	0.02 ± 0.03*
Washout 3	2.6 ± 0.5	1.0 ± 0.5*	0.3 ± 0.1	0.07 ± 0.1*	0.4 ± 0.1	0.1 ± 0.1	0.7 ± 0.2	0.4 ± 0.2	1.9 ± 0.4	1.1 ± 0.4	0.17 ± 0.06	0.06 ± 0.06
Washout 4	1.9 ± 0.4	0.7 ± 0.4*	0.3 ± 0.1	0.07 ± 0.1*	0.4 ± 0.1	0.1 ± 0.1	1.0 ± 0.3	0.4 ± 0.3	2.1 ± 0.5	1.0 ± 0.5	0.08 ± 0.05	0.08 ± 0.05

[a]*Gastrointestinal distress syndrome includes daily symptoms of bloating, flatulence, stomach noises, and abdominal cramps.*
[b]*Epidermal syndrome includes daily symptoms of itching, skin rash, and skin redness/flushing.*
[c]*Cephalic syndrome includes daily symptoms of headache and dizziness.*
[d]*Ear–nose–throat syndrome includes daily symptoms of sore throat, runny eyes, nasal congestion, and blocked ear canal.*
[e]*Psychological syndrome includes daily symptoms of anxiety, depression, and stress.*
[f]*Emetic syndrome includes daily symptoms of nausea and vomiting.*
Ljo correspond to Lactobacillus johnsonii N6.2.
Data presented as least squares mean ± SEM.
*$p < 0.05$.
**$p < 0.01$.
***$p < 0$.

gene gave background amplification on the placebo-treated group (**Figure 2F**), while a significant increase in genomic equivalents over time ($p < 0.05$) were observed in the *L. johnsonii* N6.2 group (**Figure 2E**). Interestingly, the presence of *L. johnsonii* N6.2 was confirmed in all the subgroups (a, b, and c) independently of the total LAB counts (see **Figures 2E,F**). However, after washout, the genomic equivalents in *L. johnsonii* N6.2 group were similar to time 0 or below the detection limit.

L. johnsonii N6.2 Modulates the Concentration of Metabolites in the IDO-Dependent Pathway in Healthy Subjects

We previously reported that the administration of *L. johnsonii* N6.2 to BB-DP rats resulted in decreased expression of IDO and, consequently, changes in the kynurenine:tryptophan (K:T) ratios in peripheral serum (25). Here, the impact of *L. johnsonii* N6.2 on IDO activity was evaluated by quantifying plasma levels of the following metabolic intermediates in the tryptophan pathway: tryptophan, kynurenine, serotonin, xanthurenic acid, anthranilic acid, and kynurenic acid. Samples taken at different time points (0, 8, and 12 weeks) were quantified using liquid chromatography-mass spectrometry (LC-HRMS/HRMS) (Table S3 in Supplementary Material). Based on our findings in rodent studies, it was expected that a decrease in IDO activity or expression would increase the concentration of tryptophan, while decreasing the concentrations of kynurenine, xanthurenic acid, anthranilic acid, and kynurenic acid associated also with a possible increase in serotonin levels (25). For each of the metabolites, we observed no significant differences between the treatment groups during the treatment period (Table S3 in Supplementary Material). Similarly, the K:T ratio was not affected, being similar for both groups during treatment with placebo or *L. johnsonii* N6.2 (Table S3 in Supplementary Material).

Similar statistical analyses of the metabolic intermediates were also conducted considering the LAB counts (groups a, b, or c as described earlier). After 8 and 12 weeks, the kynurenine values did not change significantly ($p > 0.1$) in the different subgroups (**Figure 3A**). After 8 weeks (last day of treatment), a slight increase in the tryptophan concentration was observed, which correlated with a decrease in the K:T ratio in the *L. johnsonii* N6.2 versus placebo-treated group b subjects (low to high LAB counts), although statistical significance was not reached ($p = 0.17$ and $p = 0.13$, respectively) (**Figures 3B,C**). Interestingly, at 12 weeks (after 4 weeks of washout), the changes in tryptophan and K:T ratio reached statistical significance with $p < 0.01$ and $p < 0.05$, respectively (**Figures 3B,C**). These results suggest that the effects of *L. johnsonii* N6.2 supplementation may take longer than 8 weeks to be quantified.

The fact that the expected modulation of the tryptophan pathway was only observed in one group of subjects (low to high LAB counts), suggests that the effects of *L. johnsonii* N6.2 supplementation may require an intestinal environment that is permissive to microbe colonization over time. These results indicate that the counts of LAB during baseline may also be used as biomarkers to predict responders from non-responders in a heterogeneous population, although this will require confirmation.

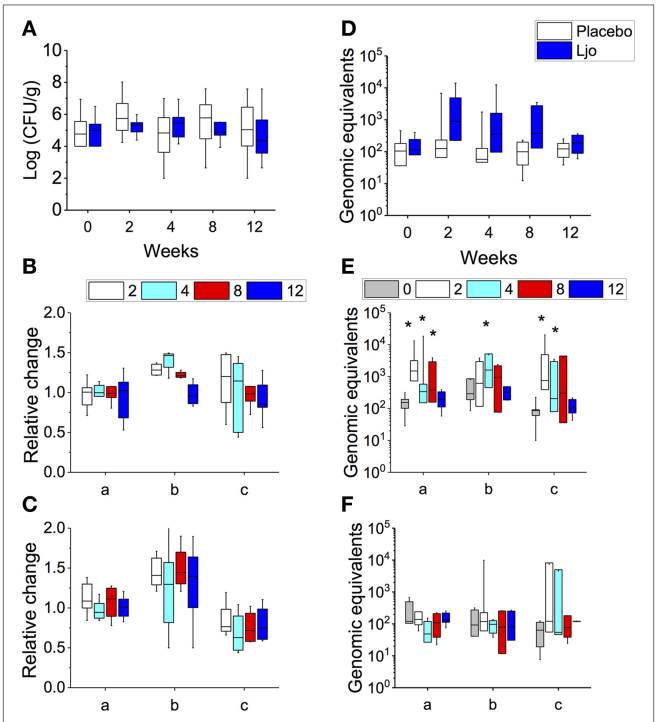

FIGURE 2 | Determination of total lactic acid bacteria (LAB) and *L. johnsonii* N6.2 (Ljo) in stool samples. In placebo and Ljo groups, it was determined: **(A)** total number of LAB (Log CFU/g). Based on the numbers of LAB obtained, three subgroups were defined: (a) high LAB, (b) low to high LAB, and (c) low LAB. **(B)** Relative change in LAB for Ljo. **(C)** Relative change in LAB for placebo. **(D)** The presence of Ljo was confirmed by performing qRT-PCR of the T285_00345 gene and expressed as genomic equivalents. These data were further stratified based on the determination of total LAB numbers for the Ljo **(E)** and placebo **(F)** treatment groups. * indicates statistical differences ($p < 0.05$) between the groups and time points shown in panels **(E,F)** using analysis of variance. Comparison of the treatment combinations was performed by least significance difference with a significance level of 5%.

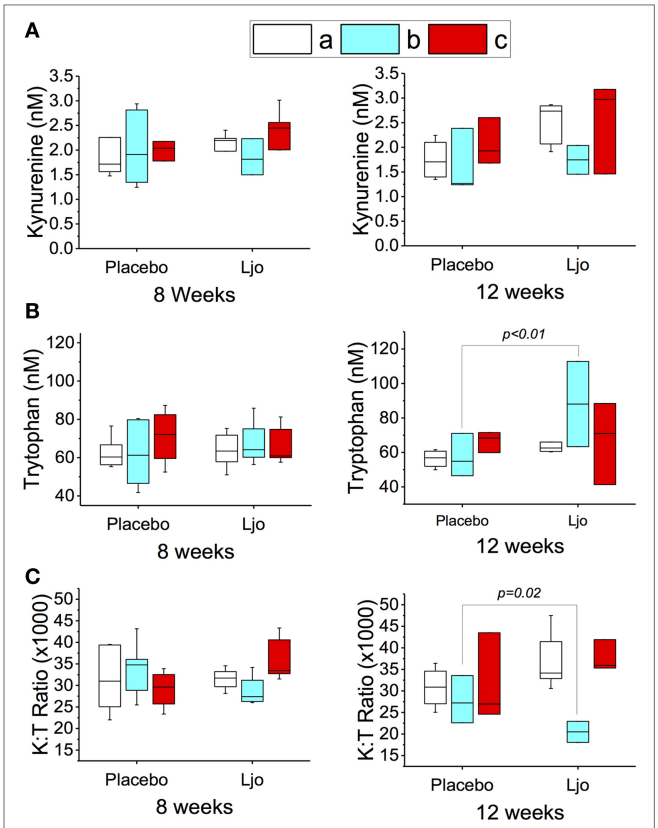

FIGURE 3 | Peripheral tryptophan and kynurenine concentration in plasma of healthy subjects. The concentrations of kynurenine **(A)** and tryptophan **(B)** were determined by LC-HRMS/HRMS after 8 or 12 weeks in the placebo or *L. johnsonii* N6.2 (Ljo) treatment groups. Panel **(C)** is shown the kynurenine:tryptophan (K:T) ratio. The concentration of the metabolites shown has been normalized to the concentration found at time 0 for each subject. The results obtained were further stratified based on the number of LAB present as described in the Section "Results." (a) High LAB; (b) low to high LAB, and (c) low LAB.

L. johnsonii N6.2 Supplementation Alters the Frequency of Immune Subsets in Peripheral Blood

The impact of *L. johnsonii* supplementation on the immune system was evaluated by flow cytometry of PBMCs as described by Maecker (27). The identification of immune cell subsets was performed by eight-color antibody staining at time 0, after 8 weeks of treatment or after the washout period (12 weeks). Six antibody staining panels were used to differentiate the following immune cell subsets of the innate and adaptive arms of the immune system: (i) B cells, (ii) natural killers (NKs), monocytes, and DCs, (iii) naïve and memory T cells, (iv) Tfh cells, (v) differentiated effector T cells (Teff), and (vi) regulatory T cells (Tregs).

B Cell Subsets

From the B cell population ($CD3^-CD19^+$), we analyzed the frequencies of transitional ($CD27^-IgD^+CD24^{hi}CD38^{hi}$), naïve ($CD27^-IgD^+CD24^{lo/-}CD38^{lo/-}$), non-class switched memory ($CD20^{hi}CD27^+IgD^+$), class switched memory ($CD20^{hi}CD27^+IgD^-$), or plasmablast ($CD20^{lo/-}CD38^+$) cells (Figure S1 in Supplementary Material). Analyses of these cell populations either after 8 weeks of treatment or following the washout period indicated that no significant changes were observed upon administration of *L. johnsonii* N6.2 (Table S4 in Supplementary Material).

NK, Monocytes, and DC Subsets

This staining panel facilitated the discrimination of B and T cell lineage negative cells ($CD3^-CD19^-CD20^-$) into NK cells ($CD56^+$), monocytes ($HLA\text{-}DR^+CD14^+$), myeloid DCs (mDCs) ($HLA\text{-}DR^+CD14^-CD16^-CD11c^+CD123^-$), and plasmacytoid DCs (pDCs) ($HLA\text{-}DR^+CD14^-CD16^-CD11c^-CD123^+$) (Figure S2 and Table S4 in Supplementary Material). It was found that the numbers of mDCs and pDCs neither changed over time nor as a result of the probiotic supplementation. By contrast, the frequencies of monocytes and NK cells were increased as a result of the probiotic treatment reaching statistical significance at 12 weeks (**Figure 4A**). Specifically, a subset of NK cells ($CD16^+CD56^{hi}$)

showed a trend toward increasing expression of HLA-DR after 8 weeks and after the washout period ($p < 0.1$, **Figure 4B**). The relative frequency of monocytes was not affected significantly after 8 weeks of treatment ($p > 0.1$); however, at 12 weeks, monocyte frequencies increased significantly among *L. johnsonii* N6.2 treated subjects ($p < 0.05$) (**Figure 4A**; Table S4 in Supplementary Material).

Naïve and Memory T Cell Subsets

$CD4^+$ and $CD8^+$ T cells were divided into naïve (Tn, $CD197^+CD45RA^+$), T effector memory (Tem, $CD197^-CD45RA^-$), T central memory (Tcm, $CD197^+CD45RA^-$), and T effector memory expressing CD45RA (Temra, $CD197^-CD45RA^+$) (Figure S3 and Table S5 in Supplementary Material). Antibodies against CD38 and HLA-DR antigens were also included in this panel to assess activation state. It was found that the administration of *L. johnsonii* N6.2 decreased the number of $CD4^+$ cells after 8 weeks of treatment ($p < 0.05$), while after 12 weeks, the numbers of $CD4^+$ cells were similar between the two treatment groups ($p > 0.1$) (**Figure 5A**; Table S5 in Supplementary Material). No changes were observed in the $CD4^+CD38^+HLA\text{-}DR^+$ subset (**Figure 5C**). However, the most notable changes were obtained in the activated ($CD38^+HLA\text{-}DR^+$) CD8 T cells after 8 weeks of treatment ($p < 0.05$) (**Figure 5B**), as well as in the activated Temra subset which increased significantly in subjects treated with probiotic compared to placebo ($p < 0.05$) (**Figure 5C**). The probiotic treatment decreased the relative amount of naïve $CD8^+$ T cells ($p < 0.05$) while increasing the frequency of $CD8^+$ Tem ($p < 0.05$). The concentrations of both cell types were similar after the washout period (**Figure 5B**; Table S5 in Supplementary Material). $CD4^+$ T cells showed strong trends toward decreased CD185 (CXCR5) and CD279 (PD-1) expression levels on naïve and Tem subsets as a result of the *L. johnsonii* N6.2 supplementation; however, these changes only reached statistical significance after the washout period (for CD279, $p = 0.05$ and $p = 0.07$; for CD185, $p < 0.01$ and $p = 0.05$) (**Figures 6A,B**). Furthermore, it was found that the administration of *L. johnsonii* N6.2 for 8 weeks significantly decreased the expression of CD279 on $CD8^+$ Tem and $CD8^+$ Tcm ($p < 0.05$ and $p = 0.05$, respectively) while a trend toward increased the expression of CD279 on $CD8^+$ Temra cells was also observed ($p < 0.1$). These changes in $CD8^+$ Tem and Tcm cells were sustained after the washout period ($p < 0.05$ and $p < 0.05$, respectively) (**Figure 6C**). A significant decrease of CD185 expression on $CD8^+$ naïve and Tem cells was also observed at 8 weeks ($p < 0.06$ and $p < 0.05$, respectively) and sustained even after the washout period in both cell populations ($p < 0.05$ and $p = 0.05$, respectively) (**Figure 6D**).

Differentiated Teff Subsets

$CD4^+CD45RO^+$ T cells were separated into Th1 ($CD183^+CD196^-$), Th2 ($CD183^-CD196^-$), Th17 ($CD183^-CD196^+$), and Th1/Th17 ($CD183^+CD196^+$). CD38 and HLA-DR were included to indicate activation (Figure S4 in Supplementary Material). While significant differences were not observed among the total numbers for each of the Teff subsets (**Figure 7A**), it was found that the number of activated Th1 ($HLA\text{-}DR^+$ and $HLA\text{-}DR^+CD38^+$) were significantly increased ($p < 0.05$) in the *L. johnsonii* N6.2 treatment group (**Figures 7B,C**). Interestingly, the numbers remained stable

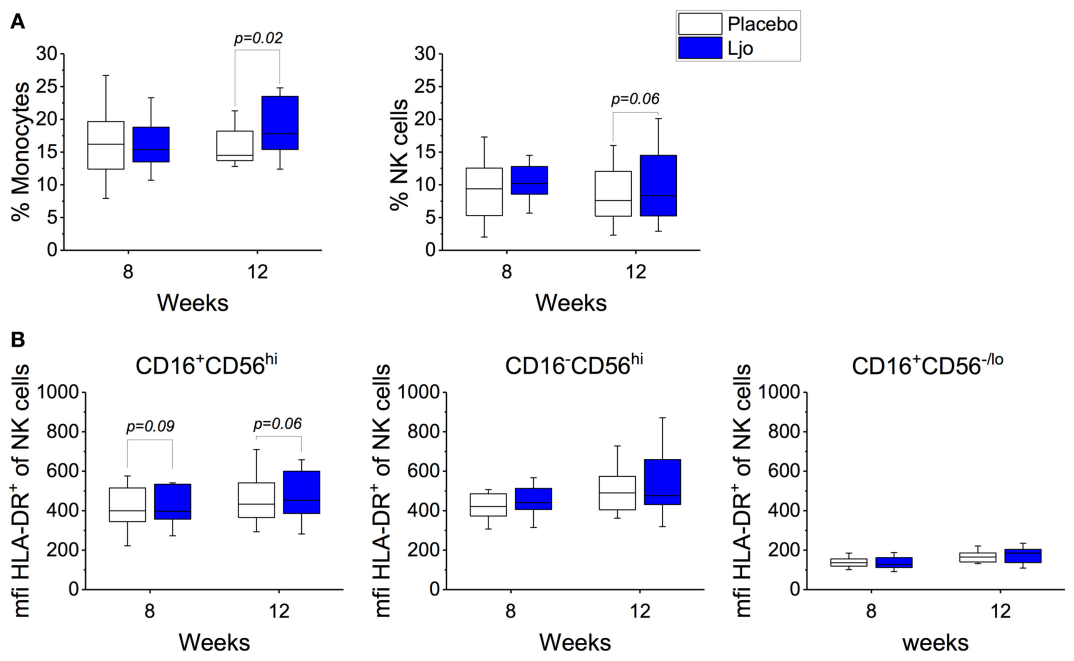

FIGURE 4 | Monocytes and natural killer (NK) cells in healthy subjects. **(A)** Mononuclear cells ($CD3^-CD19^-$) were stained with specific antibodies to define monocytes ($CD14^+$) and NK cells ($CD14^-$) in the placebo and in the *L. johnsonii* N6.2 (Ljo) groups. **(B)** Expression of HLA-DR (mfi) in different NK cells subset: $CD16^-CD56^{hi}$, $CD16^+CD56^{lo/-}$, and $CD16^+CD56^{hi}$ after 8 weeks of treatment or 12 weeks (4 weeks into the washout). The concentration of cells shown has been normalized to the concentration found at time 0 for each subject.

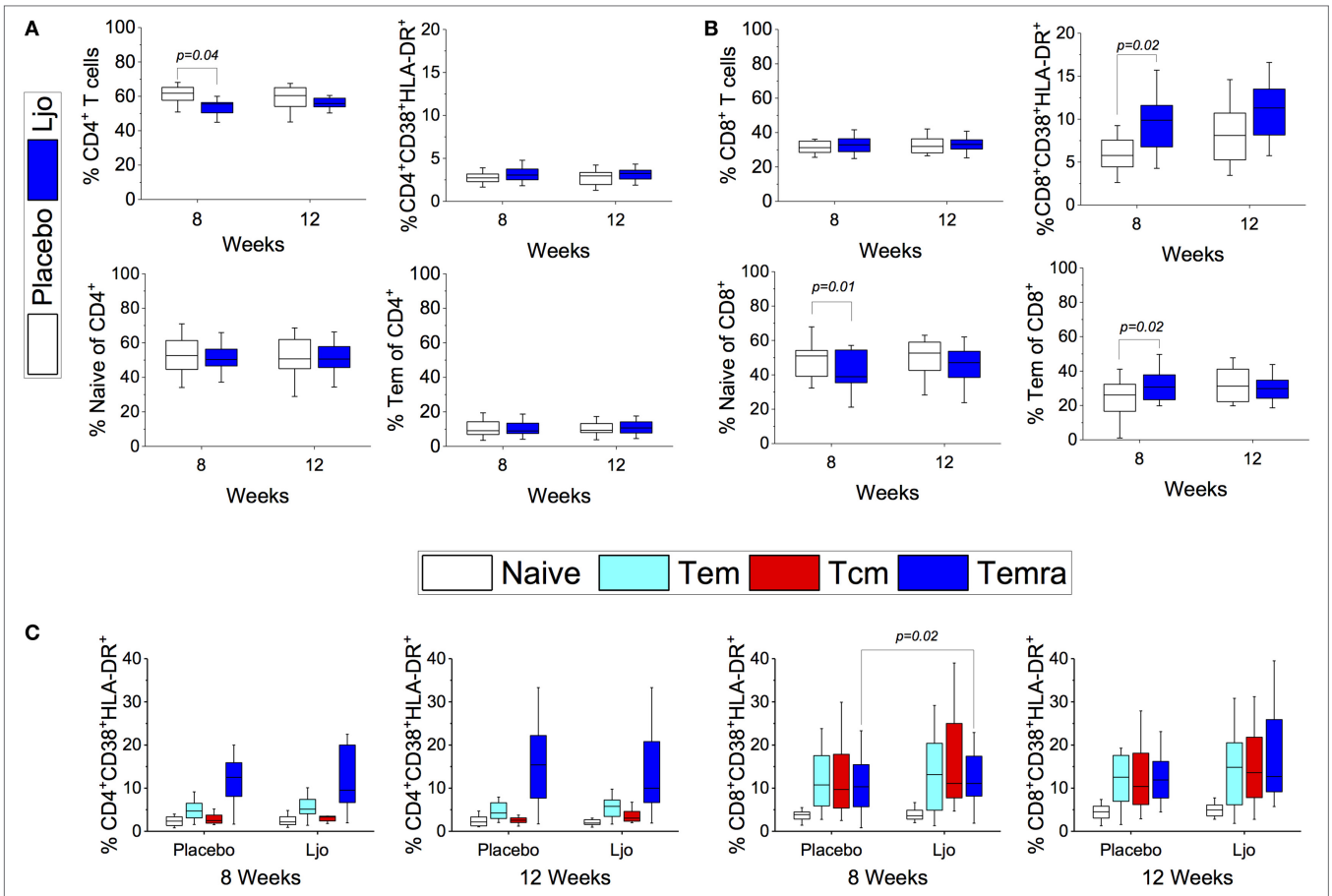

FIGURE 5 | T cell subset. CD4+ **(A)** or CD8+ **(B)** T cells populations subsets [Naïve, Tem, activated (CD38+HLA-DR+)] were quantified after 8 or 12 weeks of treatment in the placebo and *L. johnsonii* N6.2 (Ljo) groups. Naïve (CD197+CD45RA+), Tem (CD197−CD45RA−), Tcm (CD197+CD45RA−), and Temra (CD197−CD45RA+) by labeling with specific antibodies **(C)**. The concentration of cells shown has been normalized to the concentration found at time 0 for each subject.

after 4 weeks of washout ($p < 0.05$ and $p < 0.09$, respectively). No significant differences were observed in the activation state of the Th2, Th17, or Th1/Th17 subsets during the treatment period; however, a trend toward increased Th17 (HLA-DR+) and Th1/Th17 (HLA-DR+) cells was observed after the washout period ($p < 0.1$) (**Figures 7A,B**).

Tfh Subsets

CD4+CD45RA−CD185+ cells were separated into precursor (CD279+CD197−) and memory (CD279+CD183−) Tfh cells (Figure S4 in Supplementary Material). Precursor Tfh subset was significantly ($p < 0.05$) decreased in the *L. johnsonii* N6.2 group after the washout period (12 weeks). Memory Tfh was also decreased in the *L. johnsonii* N6.2 group but not statistically significant compared to placebo (Table S5 in Supplementary Material).

Treg Subsets

CD4+CD127−/loCD25+ Tregs were separated into naïve (CD45RO−) and memory (CD45RO+). HLA-DR and CD194 expression was evaluated as well (Figure S4 in Supplementary Material). No differences were observed among the groups after 8 weeks of treatment; however, memory Tregs showed a strong trend toward increased activation (HLA-DR+CD194+) in the *L. johnsonii* N6.2 treatment group after the washout period ($p = 0.07$) (Table S5 in Supplementary Material).

L. johnsonii N6.2 Increased Circulating Levels of IgA

Based on the results obtained by immunophenotyping, we determined the levels of the following serum-soluble cytokines and immune markers: IL-6, TNF-α, IFN-γ, IFN-α, IL-2, soluble CD25 (IL-2Rα), and IgA by ELISA. IL-2 and IFN-α were below the detection limit and were not further analyzed. No statistical differences were obtained between the treatment groups or the time points for IL-6, TNF-α, IFN-γ, TNFα, and soluble CD25 ($p > 0.1$). A significant ($p < 0.05$) increase in the concentration of IgA was observed during the washout period in the *L. johnsonii* treatment group, while no differences observed in the placebo group (**Figure 8**).

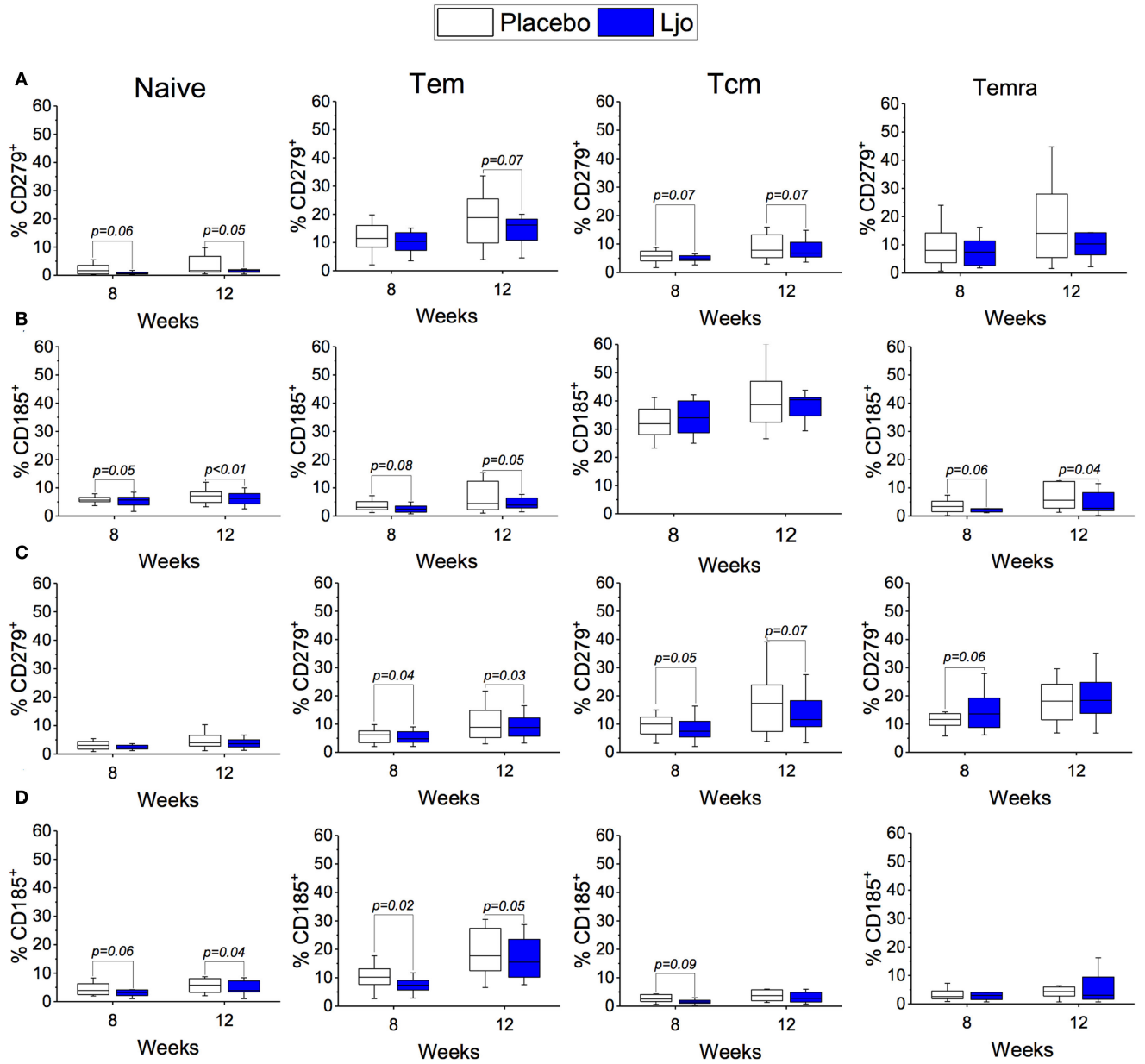

FIGURE 6 | Expression of CD185+ and CD279+ on T cells subset. Expression of CD279+ **(A)** or CD185+ **(B)** on Naïve, Tem, Tcm, and Temra CD4+ T cells. Expression of CD279+ **(C)** or CD185+ **(D)** on Naïve, Tem, Tcm, and Temra CD8+ T cells. The number of cells in each population was evaluated for the placebo (white bars) and the *L. johnsonii* N6.2 (Ljo, blue bars) group at 8 and 12 weeks. The concentration of cells shown has been normalized to the concentration found at time 0 for each subject.

L. johnsonii N6.2 Induces Minor Changes in the Microbiota of Healthy Subjects

Based on the observations that many significant changes in the immune cell populations and IgA levels were observed 4 weeks after finalizing the *L. johnsonii* N6.2 treatment (washout period), we investigated whether *L. johnsonii* N6.2 induced changes in the microbiota.

The microbiome was analyzed at time 0 and after 8 or 12 weeks of administration of the placebo or *L. johnsonii* N6.2. DNA was extracted from all stool samples, and the microbial communities were characterized by sequencing the V4 region of the 16S rDNA with Illumina MiSeq. An average of 54,743 ± 15,800 sequencing reads per sample were obtained. Approximately 380,933 OTUs were detected, representing 173 families. The 10 most abundant families detected were *Bacteroidaceae*, *Lachnospiraceae*, *Ruminococcaceae*, *Prevotellaceae*, *Paraprevotellaceae*, an unclassified Clostridiales family, *Bifidobacteriaceae*, *Desulfovibrionaceae*, *Porphyromonadaceae*, and *Veillonellaceae* (Figure S5 in Supplementary Material), which was consistent throughout the time course of the study, although variation between individuals was

observed. Bacterial communities clustered only by individual (ANOSIM $R = 0.921$, $p < 0.01$), and the community structure did not differ significantly by treatment (ANOSIM $R = 0.011$, $p = 0.05$) or over time (ANOSIM $R = -0.017$, $p > 0.99$). In addition, community structure was not correlated with the combined effects of treatment and time (PERMANOVA $R^2 = 0.002$, $p = 1.00$) (Figure S6 in Supplementary Material). The statistical analysis showed that the relative abundance of genera or families was not significantly different between treatment groups or time points.

Due to the high variability observed in the microbiome among the subjects, we tested if the microbiome of each subject could be used to determine changes in each individual over time. Using this normalization approach, it was found that of the 173 families in the dataset, only 34 changed in relative abundance between weeks 0 and 8. The change per family was compared between the two treatment groups by Welch's two-sample *t*-test. Although no significant differences at $p < 0.05$ were obtained, two families, *Prevotellaceae* and *Ruminococcaceae* showed trends with $p < 0.1$ (Table S6 in Supplementary Material) while families *Lactobacillaceae*, *Erysipelotrichaceae*, and *Odoribacteraceae* showed values of $p = 0.17$.

After the washout period, the observed microbial changes induced by *L. johnsonii* N6.2 supplementation (i.e., increase in *Ruminococcaceae, Lactobacillaceae*, and *Erysipelotrichaceae*; or decrease in *Prevotellaceae* and *Odoribacteraceae*) were reverted such that the families returned to their initial abundancies. Interestingly, one family, *Christensenellaceae* significantly increased in concentration in the *L. johnsonii* N6.2 treated group after 12 weeks (after the washout period) ($p < 0.05$), while the *Clostridiaceae* and *Bacteroidaceae* families showed trends to increase or decrease, respectively ($p = 0.06$ and $p = 0.09$, respectively). However, when this normalization method was tested at the genus level, no statistical differences were observed.

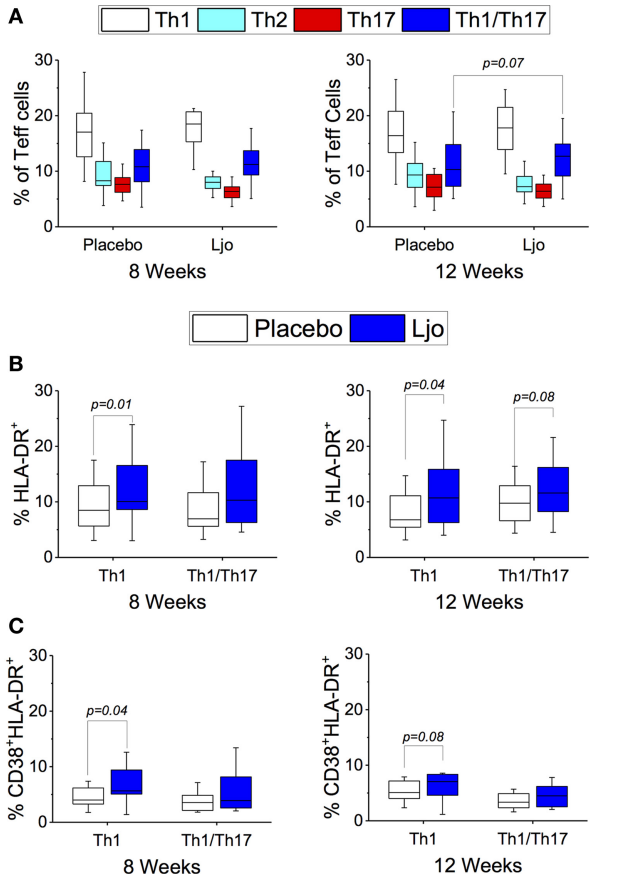

FIGURE 7 | T effector cells subset ($CD3^+CD4^+CD45RO^+$). **(A)** Th1 ($CD183^+CD196^-$), Th2 ($CD183^-CD196^-$), Th17 ($CD183^-CD196^+$), and Th1/Th17 ($CD183^+CD196^+$) were labeled with specific antibodies and quantified in the placebo (white bars) and *L. johnsonii* N6.2 (Ljo, blue bars) group at 8 and 12 weeks of treatment. **(B)** HLA-DR^+ and **(C)** HLA-DR^+CD38^+ are shown for the Th1 and Th1/Th17 effector T cells. The concentration of cells shown has been normalized to the concentration found at time 0 for each subject.

DISCUSSION

While the etiology of T1D is known to involve an autoimmune component, the contribution of environment to disease development remains poorly understood. However, the notion of modulating gut homeostasis with supplementation of tolerogenic "normal" commensal microbes offers a presumably safe method of intervention in the disease prevention setting. To date, a relatively limited number of studies have

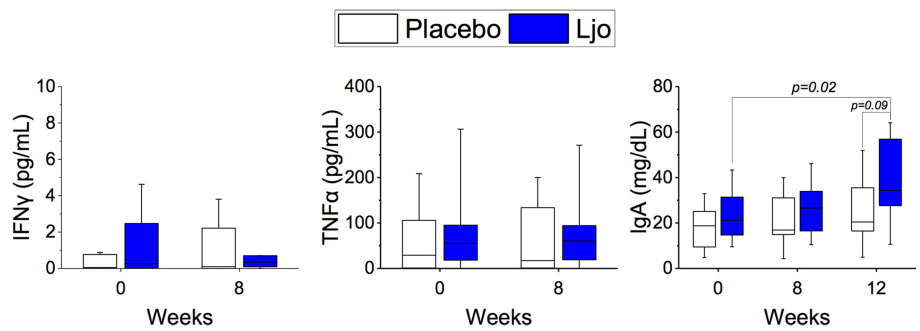

FIGURE 8 | Determination of soluble markers. The concentrations of IFNγ, IgA, and TNFα were quantified in the placebo (white bars) and *L. johnsonii* N6.2 (Ljo, blue bars) group at time 0 and after 8 weeks of treatment or after the washout (12 weeks).

been performed directed at the prevention of TID, with prior trials primarily focused on nutrition-related interventions. For example, compared to standard infant formula with 20% hydrolyzed casein, the administration of hydrolyzed casein showed no significant effects on progression to autoimmunity (defined as positivity for at least two diabetes-associated autoantibodies) after 7 years of follow-up in infants at risk for T1D (39). Similarly, docosahexaenoic acid provided to at-risk infants in the first 5 months of life had no effect on inflammatory cytokine production (40).

In a recent publication from The Environmental Determinants of Diabetes in the Young (TEDDY) study group, there was a reported association between decreased risk of islet autoimmunity and early supplementation of probiotics (between the age of 0 and 27 days) when compared to no supplementation (41). Probiotics are "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (42). A number of *Lactobacillus* and *Bifidobacterium* species are Generally Regarded as Safe microorganisms and are widely used in dietary supplements as probiotics worldwide. The mechanism of activity of probiotics is diverse and strain specific [reviewed in Ref. (43, 44)]. While the effect of different *Lactobacillus* strains on the immunological response was evaluated in several human trials (45–48), comprehensive analyses or immunophenotyping has not been performed.

Gastrointestinal microbe-based strategies for the prevention of T1D onset in humans have not yet been explored, although it has been hypothesized that the presence of certain *Lactobacillus* spp. strains may be involved in the pathogenesis of TID. *L. johnsonii* N6.2 is prevalent in BB-DR rats (16). This strain has been shown to have decreased autoimmunity onset compared to their BB-DP counterparts (14). Translating this work toward the prevention of T1D in humans first required a pilot study in healthy individuals. We performed a human trial to evaluate the safety, tolerability, and general response to consumption of this microorganism in healthy individuals. The primary outcome was the determination of safety and tolerability of oral *L. johnsonii* N6.2. Assessment of probiotic safety implicates several parameters such as immunological, microbiological, and metabolic shifts associated with the microbes' nature, method of administration, doses, and duration of consumption (49, 50). It was found that *L. johnsonii* N6.2 preparation was well tolerated with no risks for healthy subjects. The hemogram and CMP data showed no significant differences between the probiotic and placebo groups throughout the treatment and after washout periods. No adverse events related to *L. johnsonii* N6.2 preparation were observed. Because probiotics are non-pathogenic, few of them may be related to health risks; for *Lactobacillus*, infection (lactobacillemia) is estimated to occur only once per 100 million people and has therefore been considered "unequivocally negligible" (49). *L. johnsonii* N6.2 survived intestinal transit, although no significant differences in the total numbers of LAB were observed among treatments. Results showed that *L. johnsonii* N6.2 has the ability to survive and may colonize the intestinal tract without affecting the residing microbiota in healthy subjects. Remarkably, significant changes in the kynurenine pathway metabolites as well as immune responses were observed in serum and peripheral blood, while significant changes in certain GSRS syndromes were observed in the probiotic treatment group.

Although developed for patient populations, the GSRS has been used to evaluate gastrointestinal symptoms in healthy adults (51–53). This study provides supporting data that the tool is sufficiently sensitive to detect differences in healthy individuals. A lower rating of the abdominal pain scale of the GSRS was demonstrated for *L. johnsonii* N6.2 versus placebo. The individual symptom data suggest that *L. johnsonii* N6.2 may demonstrate a beneficial effect by reducing stomach ache or pain in the healthy adults studied, but the strong trend for significance suggests that the groups may have differed at baseline and thus, this may also be a carryover effect. However, the data suggest that the difference between the groups may have increased with *L. johnsonii* N6.2. Although this potential mitigating effect on abdominal pain has not been reported previously for *L. johnsonii*, other *Lactobacillus* spp. have been evaluated for efficacy in improving abdominal pain. For *L. rhamnosus* GG (LGG), the reported effects in human trials were inconsistent. Francavilla et al. (54) reported that LGG was effective at 3×10^9 to 1×10^{10} CFU/day on reducing abdominal pain severity/intensity or frequency in children with irritable bowel syndrome (IBS) and functional abdominal pain (FAP). In addition, Gawrońska et al. (55) reported that LGG may moderately increase treatment success without effect on pain severity or may result in no differences at all compared to placebo according to Bauserman and Michail (56). Conversely, *Lactobacillus reuteri* showed no improvement over placebo in FAP in children (57). In adults with IBS, provision of *Lactobacillus plantarum* 299v at a dose of 1×10^{10} CFU/day showed significantly lower abdominal pain severity and frequency (58). Similarly, *Lactobacillus casei rhamnosus* at 6×10^8 CFU/day demonstrated a clinically significant improvement in abdominal pain in a subgroup of IBS patients with a predominance of diarrhea, although small sample size precluded statistical analysis (59). By contrast, *L. reuteri* was ineffective in lessening abdominal pain in IBS patients (60). As mitigation of abdominal pain may be strain specific, the results of this study suggest that further research is needed to explore the potential efficacy of *L. johnsonii* N6.2 in individuals with abdominal pain such as those with IBS.

Of interest is the effectiveness of *L. johnsonii* N6.2 on lessening the daily symptom reporting of headache and cramping. Very little research has explored the effect of probiotic supplementation on headache. In a dose–response trial of healthy adults, the effect of *Bifidobacterium animalis* subspecies *lactis* (BB-12) and *Lactobacillus paracasei* subspecies *paracasei* (CRL431) on well-being including the symptoms of bloating, flatulence, and headache were evaluated, but no changes in the interventions were observed (61). In regards to cramping, a 4-week intervention of *Lactobacillus acidophilus* DDS-1 improved abdominal cramping with a lactose challenge in adults with lactose intolerance (62). Further research is needed to determine if *L. johnsonii* N6.2 is effective in mitigating headache and cramping in clinical populations.

In BB-DR rats, the mechanism of *L. johnsonii*–host interactions related to prevention of TID may involve downregulation of the production of kynurenine and increasing tryptophan flux toward the synthesis of serotonin (25). To evaluate whether or not these fluctuations in tryptophan metabolites observed in rats could be used as a marker of *L. johnsonii* activity in humans,

the levels of tryptophan metabolites were determined in serum throughout the study. A strong trend toward decreased serum levels of kynurenine along with increased amounts of tryptophan was observed in a subgroup of participants who consumed *L. johnsonii* N6.2 and exhibited an increase of LAB CFU/g stool over the treatment period. These fluctuations in the metabolites may be related to IDO activity, which has been associated with immune regulation and modulation of chronic inflammation, as well as allergic and autoimmune disorders. Many studies have focused on the inhibition of IDO to regulate effector metabolites as kynurenine derivate, serotonin, and tryptophan [for a review, see Ref. (21)]. Our group originally described the use of *L. johnsonii* N6.2 to modulate IDO activity *in vitro* and *in vivo* (25). In a recent study, the administration of commercial probiotics (Vivomixx in Europe or Visbiome in USA) to HIV^+ patients decreased the IDO mRNA expression levels in gut-associated lymphoid tissue after 6 months of administration. In these patients, IDO was overexpressed in the gut mucosa, and it was proposed that the downregulation of IDO would be necessary to decrease its harmful effects on the mucosal barrier (63). In this study, we determined the concentrations of several intermediates in the tryptophan degradation pathway. Although statistical significance was not achieved for any metabolite during the treatment period, a significant increase in the tryptophan concentrations and a decrease in the tryptophan/kynurenine ratio were observed after the washout period (12 weeks) in the *L. johnsonii* N6.2 group. An interesting finding of this study was that the symptom of anxiety was significantly lower in the *L. johnsonii* N6.2 group during the washout period. This symptom has been associated with decreased levels of serotonin (64, 65), and since we observed differences in the symptom score in the *L. johnsonii* N6.2 group, we speculate that the modulation of IDO activity by the probiotic may have channeled the tryptophan concentrations toward the production of serotonin. However, the serotonin levels were highly variable, and no statistical differences were observed.

Modification in the activity of IDO activity in antigen-presenting cells (APCs), such as DCs and macrophages, or NK cells has been reported to have a broad impact on the immune system directly affecting T cells (22, 23, 66). Previous reports have shown that several species of LAB may exert direct effects on APCs (DC cells and monocytes), macrophages, and to a lesser extent on B cells (67–71). To assess the global impact of *L. johnsonii* N6.2's modifications of IDO, we performed immunophenotyping and quantified the relative concentrations of B cells, Monocytes, NK and T cells (including Teff, Treg, and Tfh). The administration of *L. johnsonii* N6.2 resulted in a progressive increase in the frequencies of monocytes and NK cells (specifically the activated NK $CD16^+CD56^{hi}$ subset), reaching statistical significance after the washout period. However, B cells or DCs did not show differences between the groups during the treatment period or after washout. NK cell activation may result from cell-to-cell contact as result of NK/DC cross talk (72) or it may result from to the direct interaction of *L. johnsonii* N6.2 associated molecules, such as lipids or DNA, with NK cells. As we have previously observed *in vitro* that *L. johnsonii* N6.2 can stimulate the innate immune response through TLR9 signaling (73), we hypothesize that TLR9 activation is a likely mechanism.

Mailliard et al. (74) reported that the interaction of NK cells with DCs contributed to the maturation of Th1 cells and IFN-γ^+CD8^+ T cells. In this study, we observed a significant increase in the activated $HLA\text{-}DR^+CD38^+$ Th1 population after the 8 weeks of treatment and after the washout (12 weeks). These results are in agreement with previous reports seeking allergy treatments where it was observed that the administration of *L. paracasei* induced a Th1 type response in mice (75).

Notably, we observed significant changes in most $CD8^+$ T cells subsets: Tn, Tcm, Tem, and Temra. Among them, activated $CD38^+HLA\text{-}DR^+$ $CD8^+$ T cells increased significantly at 8 weeks and after washout (12 weeks). These results are in agreement with the activation of Th1 responses mediated by NK cells. The decrease in $CD8^+$ Tn and increase in $CD8^+$ Tem cells observed in this study has been reported previously after antigenic stimulation (Type 1 response) where Tn cells differentiated into two main subsets as Tem and Tcm cells (76). However, in the previous study, after antigen stimulation ceased, most of the $CD8^+$ Teff cells decreased by apoptosis, while a small percentage remained as mature memory $CD8^+$ T cells (77). Here, we observed that the populations remained either decreased or increased after the washout period. This observation coincided with a significantly decrease expression of the inhibitory coreceptor, $CD279^+$ (PD-1), and of the follicular homing chemokine receptor, $CD185^+$ (CXCR5), on $CD8^+$ Tem in the *L johnsonii* N6.2 group after 8 weeks of treatment. These results indicate that *L. johnsonii* N6.2 may reduce or delay apoptosis of memory $CD8^+$ T cells.

In summary, we identified systemic biomarkers [such as the increase of circulating effector Th1 cells ($CD45RO^+CD183^+CD196^-$) and cytotoxic $CD8^+$ T cells] that can be utilized to follow the effects of *L. johnsonii* N6.2 consumption in healthy subjects. The results of this pilot study provide a solid foundation for an investigation into prevention of T1D onset by *L. johnsonii* N6.2 in an at-risk human population.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Institutional Review Board (# 201400370) at the University of Florida with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Institutional Review Board at University of Florida (this trial was registered at http://clinicaltrials.gov as NCT02349360).

AUTHOR CONTRIBUTIONS

GM, AF, DC, and NH performed research. GM, AF, SG, DC, NH, and JM analyzed data. WD, DP, TG, TB, MA, MH, CG, and CW contributed to discussion and reviewed the manuscript. WD and GL conceived the study.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.00655/full#supplementary-material.

FIGURE S1 | Flow cytometry gating strategy for evaluation of the B cells subset in healthy subjects.

FIGURE S2 | Flow cytometry gating strategy for evaluation of the NK, monocytes and dendritic cells subsets in healthy subjects.

FIGURE S3 | Flow cytometry gating strategy for evaluation of naïve and memory T cells subsets in healthy subjects.

FIGURE S4 | Flow cytometry gating strategy for evaluation of differentiated effector T (Teff), T follicular helper, and regulatory T (Treg) cells subsets in healthy subjects.

FIGURE S5 | Relative abundance of bacterial families in gut microbiota. The 10 most abundant families in stool samples from subjects given a placebo **(A)** or *Lactobacillus johnsonii* N6.2 **(B)** treatment. The *Lactobacillaceae* family was also included for comparison.

FIGURE S6 | Non-metric multidimensional scaling plot of microbial community similarity based on Bray–Curtis beta diversity of Illumina MiSeq 16S rRNA gene libraries. Points shown belong to the sampling time points: T0, T1 = 2 weeks, T2 = 4 weeks, T3 = 8 weeks (end of treatment), and T4 = 12 weeks. Polygons connect all the samples for one subject, and the bounding lines are colored according to the treatment group.

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Lactobacillus plantarum Strains Can Enhance Human Mucosal and Systemic Immunity and Prevent Non-steroidal Anti-inflammatory Drug Induced Reduction in T Regulatory Cells

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Orally ingested bacteria interact with intestinal mucosa and may impact immunity. However, insights in mechanisms involved are limited. In this randomized placebo-controlled cross-over trial, healthy human subjects were given *Lactobacillus plantarum* supplementation (strain TIFN101, CIP104448, or WCFS1) or placebo for 7 days. To determine whether *L. plantarum* can enhance immune response, we compared the effects of three stains on systemic and gut mucosal immunity, by among others assessing memory responses against tetanus toxoid (TT)-antigen, and mucosal gene transcription, in human volunteers during induction of mild immune stressor in the intestine, by giving a commonly used enteropathic drug, indomethacin [non-steroidal anti-inflammatory drug (NSAID)]. Systemic effects of the interventions were studies in peripheral blood samples. NSAID was found to induce a reduction in serum $CD4^+$/Foxp3 regulatory cells, which was prevented by *L. plantarum* TIFN101. T-cell polarization experiments showed *L. plantarum* TIFN101 to enhance responses against TT-antigen, which indicates stimulation of memory responses by this strain. Cell extracts of the specific *L. plantarum* strains provoked responses after WCFS1 and TIFN101 consumption, indicating stimulation of immune responses against the specific bacteria. Mucosal immunomodulatory effects were studied in duodenal biopsies. In small intestinal mucosa, TIFN101 upregulated genes associated with maintenance of T- and B-cell function and antigen presentation. Furthermore, *L. plantarum* TIFN101 and WCFS1 downregulated immunological pathways involved in antigen presentation and shared downregulation of snoRNAs, which may suggest cellular destabilization, but may also be an indicator of tissue repair. Full sequencing of the *L. plantarum* strains revealed possible gene clusters that might

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be responsible for the differential biological effects of the bacteria on host immunity. In conclusion, the impact of oral consumption *L. plantarum* on host immunity is strain dependent and involves responses against bacterial cell components. Some strains may enhance specific responses against pathogens by enhancing antigen presentation and leukocyte maintenance in mucosa. In future studies and clinical settings, caution should be taken in selecting beneficial bacteria as closely related strains can have different effects. Our data show that specific bacterial strains can prevent immune stress induced by commonly consumed painkillers such as NSAID and can have enhancing beneficial effects on immunity of consumers by stimulating antigen presentation and memory responses.

Keywords: intestinal mucosal immunity, adaptive immunity, *Lactobacillus plantarum*, indomethacin, non-steroidal anti-inflammatory drug, probiotics

INTRODUCTION

Commensal Lactobacilli species may play an active role in intestinal immune homeostasis (1–9). Lactobacilli likely regulate immune cells *via* the interaction of bacterial cell-wall components or secreted bacterial products with immune or epithelial cells in human gut mucosa (8, 10, 11). These interactions seem to contribute to more than just tolerance to the beneficial microbes. As demonstrated in several vaccination studies Lactobacilli, such as *plantarum* strains derived from WCFS1, have a positive impact on immune responses (12–18). Furthermore, these bacteria can activate tolerogenic cellular pathways in human intestinal mucosal cells (3, 5, 10).

In previous studies, we demonstrated that *Lactobacillus plantarum*, a member of lactic acid bacteria with a "generally recognized as safe" status (3, 19), had different effects on human dendritic and peripheral blood mononuclear cells (10, 11). Using comparative genomic hybridization, we identified a number of bacteriocins and cell-wall components involved in the glycosylation of cell wall teichoic acids associated with these differential effects (8, 10). The differential expression of *L. plantarum* genes may contribute to the observed differences in activation of toll-like receptor (TLR) 2–4 and CD14 antigens in the host (10). As a consequence of differences in TLR-binding dendritic cells *L. plantarum* strains induce alterations in production of the pro-inflammatory cytokine IL-12 and the regulatory cytokine IL-10 (10). The immunomodulatory effects of *L. plantarum* may benefit human gut homeostasis, in particular in case of pathogenic or pharmacological induced mucosal stress. Non-steroidal anti-inflammatory drugs (NSAIDs), which are commonly used painkillers, are well known for their negative side effects on gut mucosal integrity and immunity, which are mediated through inhibition of cyclooxygenase and subsequent prostaglandin deficiency. But these effects have also been shown to be modulated by gut microbiota *via* among others TLR4 signaling (20).

The current clinical trial has been conducted to study the effects of three *L. plantarum* strains on immunity and intestinal barrier function. The findings regarding immune responses are presented in the current report, while the results on gut barrier function have been published previously (2). We have shown that small intestinal permeability of healthy volunteers increased, indicating gut barrier dysfunction, after administration of indomethacin (a NSAID) which could not be reversed by intake of *L. plantarum* strains. However, one of the strains, *i.e.*, *L. plantarum* TIFN101, did demonstrate profound effects on mucosal gene transcription related to mucosal structure, while the findings regarding the modulation of these processes by *L. plantarum* WCFS1 and CIP4448 were more moderate. This indicates strain-dependent modulation of gut barrier function by the tested *L. plantarum* strains (2).

To investigate mechanism by which *L. plantarum* may influence *in vivo* human mucosal and systemic immune activity under mild mucosal stress conditions such as intake of NSAIDs, we conducted a randomized double-blind placebo-controlled cross-over human trial. Three *L. plantarum* strains were selected for their different immunomodulating effect *in vitro* (3, 10, 11). The three strains have different effects on TLRs signaling and differently stimulate cytokine production *in vitro* in immune cells (3, 10, 11). Healthy volunteers received the NSAID indomethacin, which induces mild and reversible damage to the gastrointestinal lining (21–24). Study participants subsequently consumed one of the three *L. plantarum* strains or placebo for a week. Polarized T-cell responses in the peripheral circulation, as well as the responses after re-stimulation with cell extract of *L. plantarum* strains, superantigen, or tetanus were studied to determine whether these bacteria could enhance systemic immunity against one of the stimuli. Duodenal biopsies were taken and used to study the effect of the strains on mucosal gene regulation. Furthermore, the bacterial strains were sequenced and annotated to identify putative gene clusters associated with the differential responses induced by the probiotics.

MATERIALS AND METHODS

This study was approved by the University Hospital Maastricht Ethical Committee and has been registered in the US National Library of Medicine (*NCT01456767*).[1] A more detailed description of the trial design has been published previously (2).

[1] http://www.clinicaltrials.gov.

Study Design and Study Participants

Ten healthy volunteers, seven females and three males (26.3 ± 10.1 years, BMI of 21.8 ± 2.40 kg/m^2), without a history of gastrointestinal symptoms and free of medication, were tested on four separate occasions in a randomized cross-over design. Four intervention periods included the 7-day intake of *L. plantarum*

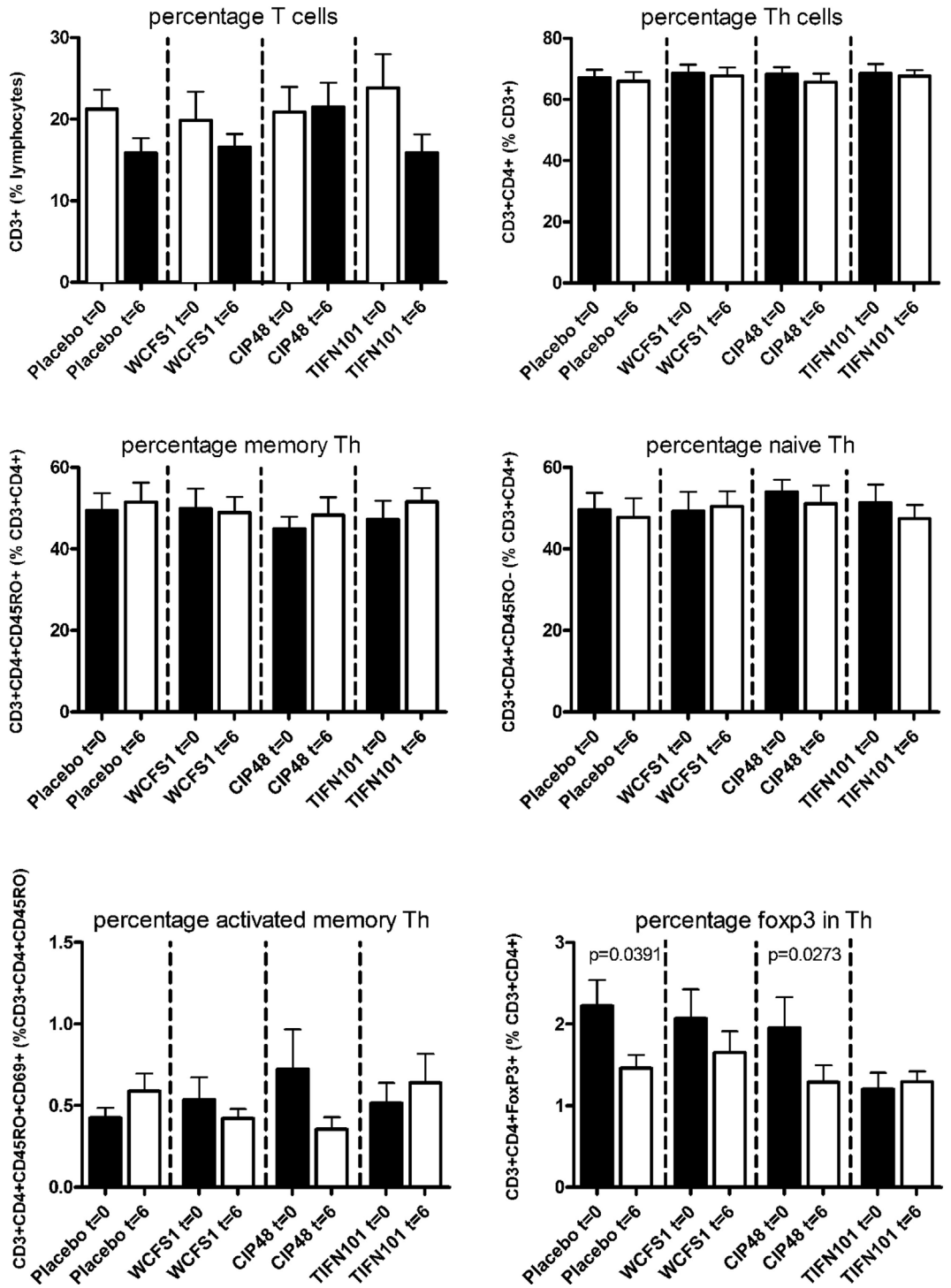

FIGURE 1 | Effects of three *Lactobacillus plantarum* strains on the frequency of CD4$^+$ T-cell populations in systemic circulation. Data are expressed as mean ± SEM. Statistical significant differences were determined by using two-sided Student's *t*-tests. A *p*-value <0.05 was considered statistically significant. A *p*-value <0.1 was considered a statistical trend.

strains WCFS1 (WCFS), CIP104448 (CIP48), or TIFN101, or placebo. Before and after the intervention, blood samples were taken to study the effect of *L. plantarum* consumption on T-cell polarization. Furthermore, at day 7, duodenal biopsies were taken for whole-genome expression microarrays. No side effects or complications were reported.

All included subjects gave their written informed consent. The healthy volunteers consumed their habitual diet and kept a gastrointestinal symptoms diary. The night before the start day, the volunteers ingested 75 mg of the NSAID indomethacin. On the start day, the volunteers ingested another dosage of 50 mg indomethacin to conform to a previously established protocol to establish mild gastrointestinal mucosal stress (25, 26). Subsequently, the volunteers consumed the bacterium or placebo supplements for a period of 7 days during breakfast and during dinner. The vials containing bacteria or placebo were non-transparent. On the seventh day, blood samples were taken again and tissue samples were obtained from the horizontal part of the duodenum by standard flexible gastroduodenoscopy at approximately 15 cm distal to the pylorus. The duodenal mucosa was chosen as the duodenum is readily accessible for sampling. The interventions were performed with an interval of 4 weeks to allow a wash-out period and also healing of the biopsy-sampling region.

Bacterial Strains and Growth Conditions

Three *L. plantarum* strains form the NIZO strain-collection were selected: WCFS1, CIP104448, and TIFN101. In previous studies TIFN101 has been referred to as CIP104450 (11, 27). The bacteria were cultured at 37°C in man, rogosa, and sharpe medium (Merck). Detailed protocols for culturing, harvesting, freeze drying, storing, and viability determination of *Lactobacillus* species have been published (1). Maltodextrin and glucose were added to a final concentration of 20 and 2% (wt/vol), respectively, to obtain bacterial preparations (WCFS1, 2.6×10^9 CFU; CIP104448, 2.4×10^9 CFU; and TIFN101, 5.6×10^9 CFU); placebo controls contained the two sugars.

Cell Staining

Blood of study participants was collected in EDTA-containing tubes and processed for fluorescence-activated cell sorting (FACS) analysis (Table S1 in Supplementary Material). Isotype controls were used at the same dilution as the antibody.

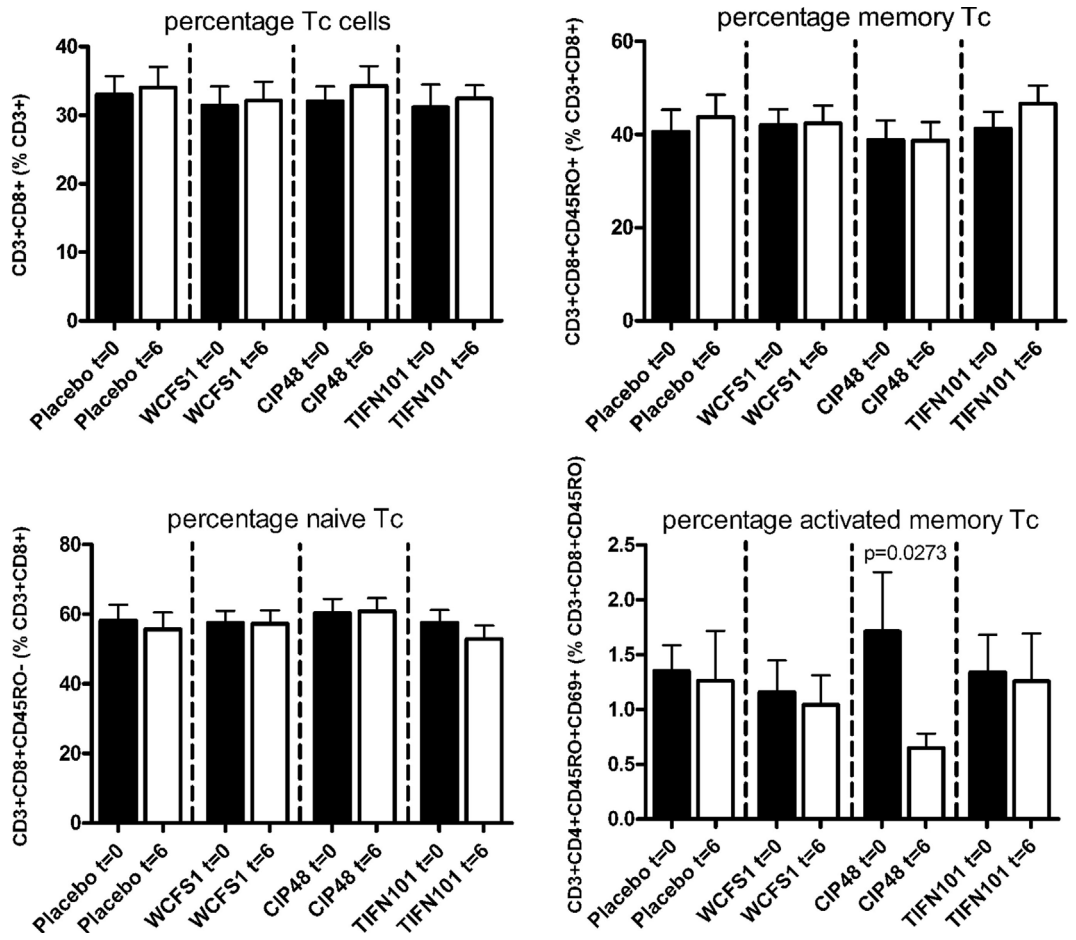

FIGURE 2 | Effects of three *Lactobacillus plantarum* strains on the frequency of CD8+ T-cell populations in systemic circulation. Data are expressed as mean ± SEM. Statistical significant differences were determined by using two-sided Student's *t*-tests. A *p*-value <0.05 was considered statistically significant. A *p*-value <0.1 was considered a statistical trend.

To study T-cell polarization, 200 µl of blood was diluted with 200 µl of RPMI1640 supplemented with heat-inactivated 10% fetal calf serum (FCS) and incubated with either phorbol-myristate-acetate (PMA; 80 nM Sigma-Aldrich, Steinheim, Germany) and 2 nM calcium ionophore (Ca-Io; Sigma-Aldrich) (4 h), *Staphylococcus aureus*-enterotoxin B (SEB) (5 µg/ml Sigma, Deisenhofen, Germany) (24 h), tetanus toxoid (TT; 1.5 Lf/ml, RIVM, Bilthoven, The Netherlands) (24 h), or bacterial lysates (30 µg/ml) (24 h). Stimulation with bacterial lysates was performed 1 week after treatment with one of the three strains. After stimulation, red blood cells were lysed with ammonium chloride, washed (PBS with 2% FCS), and incubated with different antibody cocktails.

To stain for T-cells and T-cell subsets, cells were incubated with an antibody cocktail consisting of anti-CD3, anti-CD8, and anti-CD45RO for 30 min in the dark on ice. After washing with buffer, cells were incubated with streptavidin-Pacific Orange (1:100 Invitrogen) for 15 min on ice. After washing and centrifugation, pelleted cells were resuspended in Fix/Perm solution (eBioscience) for 45 min on ice. After washing in Perm-solution cells were incubated in mouse-serum for 15 min, followed by incubation with the cytokine antibody mix (anti-IL-4, anti-IFNγ, anti-IL-17, and anti-IL-21) or an isotype control mix for 30 min on ice. Cells were then washed with Perm solution (three times), resuspended in wash-buffer and measured by flow-cytometry.

For staining NK-cells, cells were incubated with an antibody cocktail consisting of anti-CD3, anti-CD16, anti-CD56, anti-CD335, and anti-CD161 (NK-cell staining), or with an isotype control cocktail for NK-cells consisting of anti-CD3, anti-CD16, anti-CD56, and isotype controls for anti-CD335 and CD161 for 30 min in the dark on ice. After washing with washing buffer, cells were fixed in FACS-lysis solution (BD Biosciences) for 30 min on ice, washed and analyzed by flow-cytometry.

RNA Isolation and Microarray

Total RNA was isolated from duodenal biopsy samples of the study participants using Trizol reagent (1 ml) (Invitrogen, Breda, The Netherlands). RNA was purified using the Qiagen RNeasy Micro kit (Qiagen, The Netherlands) and quantified on a NanoDrop ND-1000 spectrophotometer (Isogen Life Science, The Netherlands). RNA quality was confirmed using an Agilent 2100 bioanalyzer (Agilent Technologies, The Netherlands). RNA

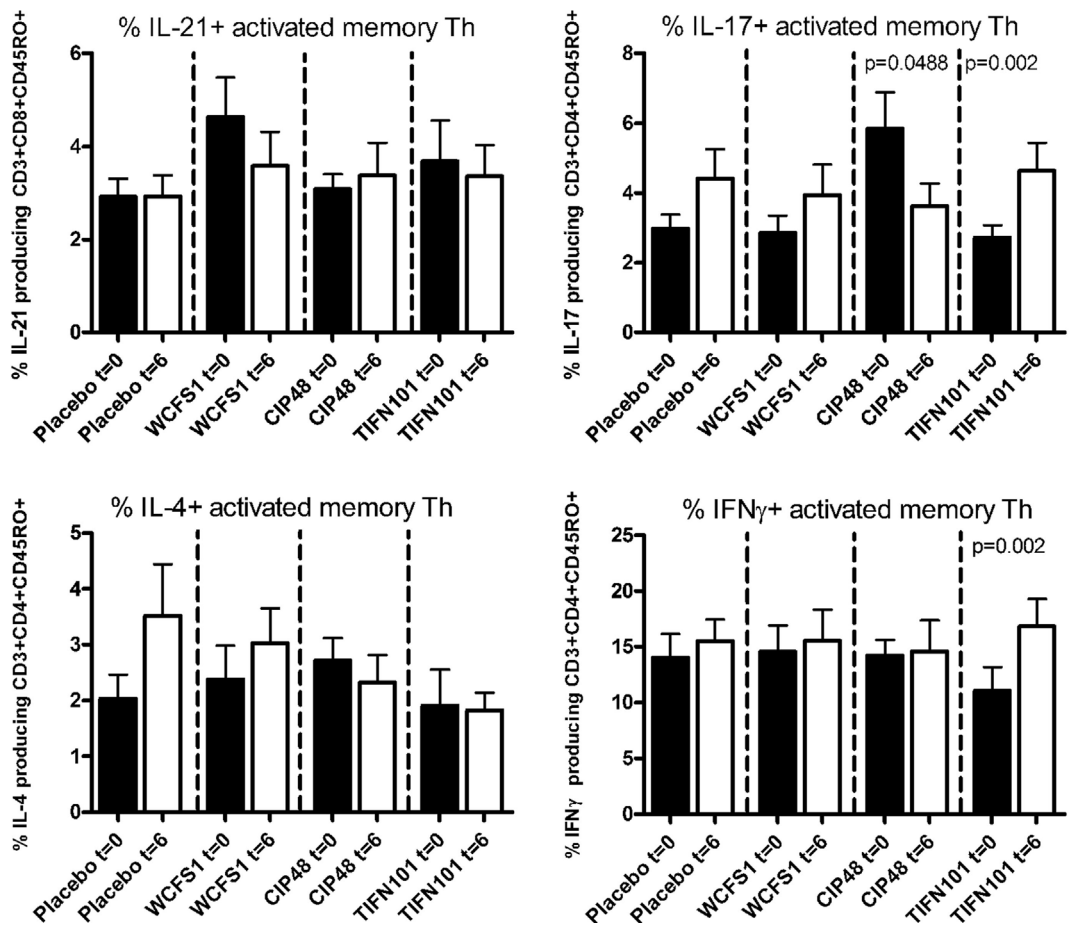

FIGURE 3 | Effects of three *Lactobacillus plantarum* strains on the frequency of IL-21, IL-17, IL-4, and IFN-γ-producing *Staphylococcus aureus* enterotoxin B superantigen (SEB)-stimulated memory-CD45RO+ Th cells. Data are expressed as mean ± SEM. Statistical significant differences were determined by using two-sided Student's *t*-tests. A *p*-value <0.05 was considered statistically significant. A *p*-value <0.1 was considered a statistical trend.

was judged suitable for array-hybridization only if samples exhibited intact bands corresponding to 18S and 28S ribosomal subunits and displayed no chromosomal peaks or RNA-degradation products.

Total RNA (100 ng) was used for whole transcript cDNA synthesis using the Ambion WT expression kit (Life Technologies, The Netherlands) and was subsequently labeled using the Affymetrix GeneChip WT Terminal Labeling Kit (Affymetrix, Santa Clara, CA, USA). Samples were hybridized to human whole genome Affymetrix GeneChip Human Gene 1.1 ST arrays, washed, stained, and scanned on an Affymetrix GeneTitan instrument. Details on array handling can be found in the Affymetrix GeneTitan Instrument User Guide for Expression Array Plates (P/N 702933 Rev.2).

Duodenal Mucosa Microarray Data Analysis

Microarray analysis was performed utilizing MADMAX for statistical analysis (28). All arrays met the quality criteria. The probes on the Human Gene 1.1 ST arrays were redefined according to Dai et al. (29) based on the NCBI Entrez database (CDF version 15.1). In this way, the Human Gene 1.1 ST array targets 19,682 unique genes. Normalized expression values were obtained from the raw intensity values by using the robust multi-array analysis preprocessing algorithm available in the AffyPLM library using default settings (30). Microarray data were filtered and probe sets with at least five probes and expression values higher than 20 on at least five arrays with an interquartile range (IQR) >0.2 (log2 scale) across all samples were selected for further statistical analysis. In addition, an IQR cut-off of 0.2 was used to filter out genes that showed no variation between the conditions. Differentially expressed genes were identified using linear models, applying moderated t-statistics that implemented empirical Bayes regularization of SEs in the library limma (31). The moderated *t*-statistic was extended by a Bayesian hierarchical model to define an intensity-based moderated t-statistic to adjust for independence of variances relative to the degree of identity and relation between variance and signal intensity (32). Genes were defined as significantly changed when the *p*-value was <0.05 for

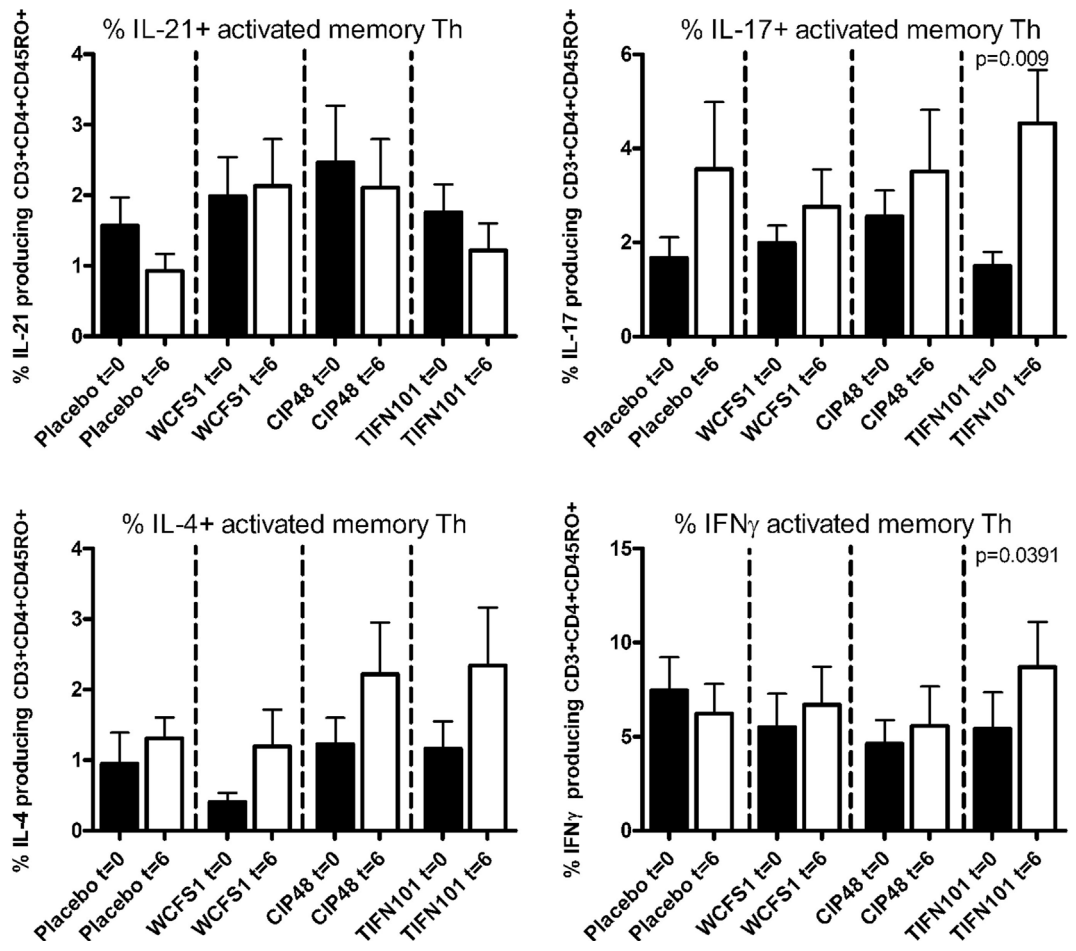

FIGURE 4 | Effects of three *Lactobacillus plantarum* strains on the frequency of IL-21, IL-17, IL-4, and IFN-γ-producing tetanus toxoid-stimulated memory-CD45RO+ Th cells. Data are expressed as mean ± SEM. Statistical significant differences were determined by using two-sided Student's *t*-tests. A *p*-value <0.05 was considered statistically significant. A *p*-value <0.1 was considered a statistical trend.

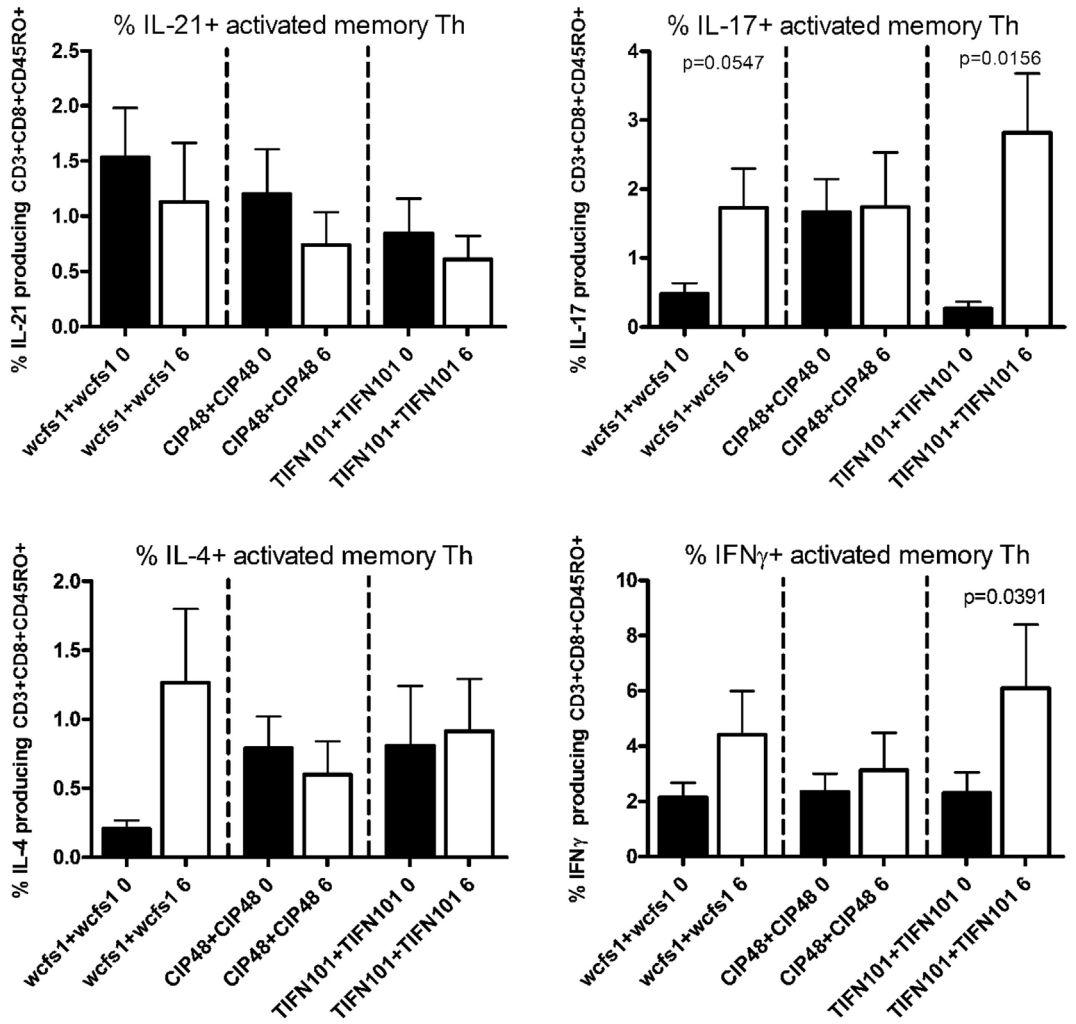

FIGURE 5 | Effects of three *Lactobacillus plantarum* strains on the frequency of IL-21, IL-17, IL-4, and IFN-γ-producing memory-CD45RO+ Th cells stimulated with bacterial cell extracts, matched to the strain consumed. Data are expressed as mean ± SEM. Statistical significant differences were determined by using two-sided Student's *t*-tests. A *p*-value <0.05 was considered statistically significant. A *p*-value <0.1 was considered a statistical trend.

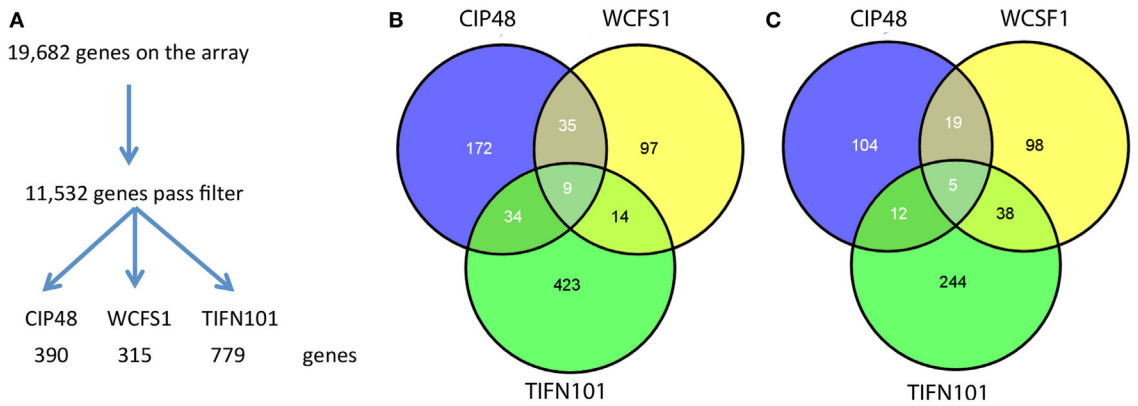

FIGURE 6 | Flowchart of the microarray analysis **(A)** and the number of unique genes regulated in human intestinal biopsies after consumption of three different *Lactobacillus plantarum* strains [*L. plantarum* WCFS1 (WCFS), *L. plantarum* CIP104448 (CIP48), *L. plantarum* TIFN101 (TIFN101)]. Intensity >20 on at least five arrays, interquartile range >0.2, at least seven probes per gene. Venn diagrams of the number of upregulated **(B)** and downregulated **(C)** genes in the intestinal biopsies after consumption of *L. plantarum* and indomethacin.

TABLE 1 | *Lactobacillus plantarum* WCFS1 (WCSF): 10 most upregulated and downregulated genes.

	Gene name	IBMT *p*-value	Mean fold versus placebo
Top 10: upregulated genes placebo versus WCFS			
Kinesin family member 20B	KIF20B	0.01	1.34
microRNA 186	MIR186	0.03	1.31
Guanylate cyclase activator 2A (guanylin)	GUCA2A	0.04	1.31
Integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor)	ITGA4	0.01	1.31
Centromere protein E, 312 kDa	CENPE	0.05	1.30
Putative homeodomain transcription factor 1	PHTF1	0.00	1.30
Spindle and kinetochore-associated complex subunit 2	SKA2	0.01	1.29
Killer cell lectin-like receptor subfamily D, member 1	KLRD1	0.00	1.29
Gamma-aminobutyric acid A receptor, alpha 2	GABRA2	0.02	1.27
Retinitis pigmentosa GTPase regulator	RPGR	0.01	1.27
Top 10: downregulated genes placebo versus WCFS			
Small nucleolar RNA, H/ACA box 16A	SNORA16A	0.02	−1.35
Small nucleolar RNA, C/D box 53	SNORD53	0.04	−1.36
Contactin 3 (plasmacytoma associated)	CNTN3	0.00	−1.38
Small nucleolar RNA, C/D box 6	SNORD6	0.04	−1.39
Small nucleolar RNA, H/ACA box 57	SNORA57	0.00	−1.44
Small nucleolar RNA, H/ACA box 60	SNORA60	0.00	−1.44
Small nucleolar RNA, H/ACA box 14A	SNORA14A	0.03	−1.49
Small nucleolar RNA, H/ACA box 38B (retrotransposed)	SNORA38B	0.01	−1.58
Small nucleolar RNA, H/ACA box 14B	SNORA14B	0.01	−1.58
Small Cajal body-specific RNA 4	SNORA16A	0.01	−1.72

TABLE 2 | *Lactobacillus plantarum* CIP104448 (CIP48): 10 most upregulated and downregulated genes.

	Gene name	IBMT *p*-value	Mean fold versus placebo
Top 10: upregulated genes placebo versus CIP48			
Coiled-coil domain containing 59	CCDC59	0.01	1.33
Aldehyde dehydrogenase 1 family, member L2	ALDH1L2	0.03	1.32
KIAA0125	KIAA0125	0.00	1.31
Phospholipase C, beta 4	PLCB4	0.01	1.31
Coiled-coil domain containing 102B	CCDC102B	0.04	1.29
RAS guanyl releasing protein 3 (calcium and DAG-regulated)	RASGRP3	0.03	1.28
Peptidase domain containing associated with muscle regeneration 1	PAMR1	0.00	1.28
DEP domain containing 1	DEPDC1	0.01	1.28
Phospholipase A2, group IIA (platelets, synovial fluid)	PLA2G2A	0.02	1.28
Heparan sulfate (glucosamine) 3-O-sulfotransferase 3B1	HS3ST3B1	0.04	1.28
Top 10: downregulated genes placebo versus CIP48			
Potassium channel, subfamily K, member 15	KCNK15	0.05	−1.33
Transient receptor potential cation channel, subfamily V, member 6	TRPV6	0.01	−1.33
Long intergenic non-protein coding RNA 282	LINC00282	0.03	−1.35
Ephrin-A1	EFNA1	0.02	−1.38
Matrix metallopeptidase 10 (stromelysin 2)	MMP10	0.05	−1.41
Angiopoietin-like 4	ANGPTL4	0.03	−1.47
Heme oxygenase (decycling) 1	HMOX1	0.00	−1.50
Nuclear factor, interleukin 3 regulated	NFIL3	0.01	−1.52
Major facilitator superfamily domain containing 2A	MFSD2A	0.02	−1.59
Glucose-6-phosphatase, catalytic subunit	G6PC	0.02	−1.65

pairwise comparisons. The datasets generated from microarray profiling experiments have been deposited to the publicly accessible database repository Gene Expression Omnibus (GSE74988).

Pathway Analysis

Gene set enrichment analysis (GSEA[2]) was performed using MADMAX and gene sets with a false discovery rate <0.2 were considered significantly enriched. GSEA takes into account the broader context in which gene products function, namely in physically interacting networks such as biochemical, metabolic, or signal transduction routes, and has the advantage that it is unbiased because no gene selection step is used. Possible transcription factors were identified using upstream regulator analysis in ingenuity pathway analysis (IPA; Ingenuity Systems, Redwood City, CA, USA).

Bacterial Genome Sequencing and Annotation

For DNA preparation, bacteria were pelleted, washed, and resuspended in TES buffer (*N*-[tris(hydroxymethyl)methyl]-2-aminoet hanesulfonic acid). Cells were lysed with lysozyme (360 mg/ml) and mutanolysin (140 U/ml) by incubation for 2 h at 37°C. Subsequently, 300 µl water and 80 µl of a 20% SDS solution were added. The DNA extraction was done using phenol/chloroform (3×). DNA was precipitated with isopropanol and washed with 70% ethanol. Samples were treated with 100 µg/ml RNAse (Sigma) for 1 h at 37°C. DNA paired-end libraries with barcoding were made and sequenced using Illumina technology (Baseclear Leiden). The genome sequences of *L. plantarum* strains CIP104448 and TIFN 101 have been deposited in NCBI/GenBank under accession numbers JSUW00000000 and JSUX00000000, respectively. The contig sequences were submitted to the RAST automatic annotation server,[3] which provided ORF calling and automatic annotation. The annotated contigs of CIP48 and TIFN101 were ordered by comparing them to the circular template genome of *L. plantarum* WCFS1 (33), and comparing them to each other. Contigs/genes that did not match to the WCFS1 genome were annotated in more detail using BLASTP[4] and InterProscan.[5] Ortholog groups (OGs) in the three genomes were determined using OrthoMCL.[6]

Statistics

Flow-cytometry results are expressed as the mean ± SEM. Normal distribution was confirmed by the Kolmogorov–Smirnov test.

[2]http://broad.mit.edu/gsea/.

[3]http://rast.nmpdr.org/.

[4]http://blast.ncbi.nlm.nih.gov/.

[5]http://www.ebi.ac.uk/interpro/.

[6]http://www.orthomcl.org/.

TABLE 3 | *Lactobacillus plantarum* TIFN101 (TIFN101): 10 most upregulated and downregulated genes.

	Gene name	IBMT *p*-value	Mean fold versus placebo
Top 10: upregulated genes placebo versus TIFN101			
Immunoglobulin lambda variable 6–57	IGLV6-57	0.01	1.63
Putative V-set and immunoglobulin domain-containing protein 6-like	LOC642131	0.00	1.55
Immunoglobulin lambda variable 7–46 (gene/pseudogene)	IGLV7-46	0.04	1.48
Heparan sulfate (glucosamine) 3-O-sulfotransferase 3B1	HS3ST3B1	0.00	1.41
Interferon regulatory factor 4	IRF4	0.00	1.40
GDNF family receptor alpha 2	GFRA2	0.00	1.40
CD27 molecule	CD27	0.01	1.40
CD79a molecule, immunoglobulin-associated alpha	CD79A	0.03	1.38
Plasminogen activator, tissue	PLAT	0.00	1.37
Der1-like domain family, member 3	DERL3	0.00	1.37
Top 10: downregulated genes placebo versus TIFN101			
Small nucleolar RNA, H/ACA box 38B (retrotransposed)	SNORA38B	0.04	−1.40
Small nucleolar RNA, H/ACA box 21	SNORA21	0.03	−1.40
Small nucleolar RNA, H/ACA box 60	SNORA60	0.01	−1.41
Small nucleolar RNA, C/D box 53	SNORD53	0.01	−1.43
Ephrin-A1	EFNA1	0.00	−1.45
Small nucleolar RNA, C/D box 6	SNORD6	0.02	−1.46
Small nucleolar RNA, H/ACA box 57	SNORA57	0.00	−1.47
Small nucleolar RNA, H/ACA box 14B	SNORA14B	0.00	−1.73
Family with sequence similarity 5, member C	FAM5C	0.03	−1.97
Small Cajal body-specific RNA 4	SCARNA4	0.00	−2.00

The two-sided Student's *t*-test was used for changes in immune-cell populations after *L. plantarum* treatment. Gene expression data are depicted as the median (range). The two-sided Mann–Whitney *U*-test was used to determine changes in expression profiles after *L. plantarum* treatment *in vivo*. A p-value <0.05 was considered statistically significant.

RESULTS

Cell Frequencies and T-Cell Polarization after Treatment with *L. plantarum* Strains

In peripheral blood samples, we did not observe differences in the frequencies of total $CD3^+$ cells, $CD3^+CD4^+$ cells (naïve or memory), or the $CD3^+CD4^+$ activated memory cells after treatment with any of the *L. plantarum* strains. However, the percentage of $CD4^+Foxp3^+$ cells was significantly decreased following placebo and CIP48 treatment, but not after TIFN101 treatment (**Figure 1**). Moreover, although we did not observe any effect with indomethacin treatment on $CD3^+CD8^+$ (naïve and memory) cells, activated memory-cells exhibited a statistically significantly decrease after CIP48 treatment ($p < 0.05$) (**Figure 2**).

Treatment did not influence percentages of NK-cells or NKT-cells. There was also no change in the percentages of the NK-cell subtypes (i.e., $CD56^{high}$ and $CD56^{dim}$); and the expression of CD161 (KLRB1), mediating cytotoxicity (33, 34), was not affected either by the *L. plantarum* treatments (results not shown).

T-cell polarization was studied after three types of T-cell stimulation: (i) non-specific polyclonal stimulation with PMA/Ca^{2+} or superantigen *Staphylococcus aureus*-enterotoxin B (SEB) to study whether the total responsiveness was influenced by *L. plantarum* treatment, (ii) stimulation with a previously administered vaccine-antigen (TT) to study stimulation of specific memory responses, and (iii) stimulation with cell extracts of the specific *L. plantarum* strains in order to investigate whether specific immune responses against the *L. plantarum* were stimulated.

After non-specific stimulation with PMA/Ca-ionophore or SEB, we quantified the percentage of IFNγ, IL-4, IL-17, or IL-21 positive Th cells and memory Th cells. Treatment with placebo or the administered *L. plantarum* strains did not influence cytokine production of the total population of Th cells or of Th memory cells after non-specific stimulation with PMA/Ca-ionophore. Although no differences were found after SEB-stimulation in cytokine production by the total Th cell population after the three *L. plantarum* treatments (results not shown), we did observe differences in cytokine production of the Th memory cells after *L. plantarum* treatment. After stimulation with SEB, we observed a decreased percentage of IL-17-producing activated memory Th cells following treatment with CIP48 and an increased percentage of IL-17-producing activated memory Th cells after treatment with TIFN101 (**Figure 3**). Moreover, the percentage of IFNγ-producing activated memory Th cells was also increased after TIFN101 treatment (**Figure 3**).

Treatment with *L. plantarum* strains also modulated cytokine production following a more specific stimulation by TT (**Figure 4**). After TIFN101 treatment, the percentage IL-17 and the percentage IFN-γ-producing memory Th cells were significantly increased, while no effect on cytokine production by memory Th cells after TT-stimulation was observed with the other *L. plantarum* strains.

Finally, we stimulated human blood samples with cell extracts of the *L. plantarum* strain that the volunteers had consumed in the study (**Figure 5**). We observed that subjects, who were treated with WCFS, showed a trend toward an increased IL-17 response after stimulation with WCFS cell extracts. Other cytokines were not affected after this treatment. There were no differences in cytokine production in subjects treated with CIP48, when their blood samples were stimulated with CIP48 cell extract. When subjects were treated with TIFN101, their activated memory cells exhibited increased IL-17 and IFN-γ-production following stimulation with TIFN101 cell extract.

Transcriptional Response in Duodenal Mucosa upon Exposure to *L. plantarum* Strains

Differential gene expression profiles were found in the stressed intestinal mucosa of the subjects consuming each bacterial strain; 315 genes were differentially regulated with *L. plantarum* WCFS, 390 with CIP48, and 779 with TIFN101, as compared to the placebo intervention (**Figure 6A**). Of these genes, the different bacterial strains shared only relatively small numbers of upregulated

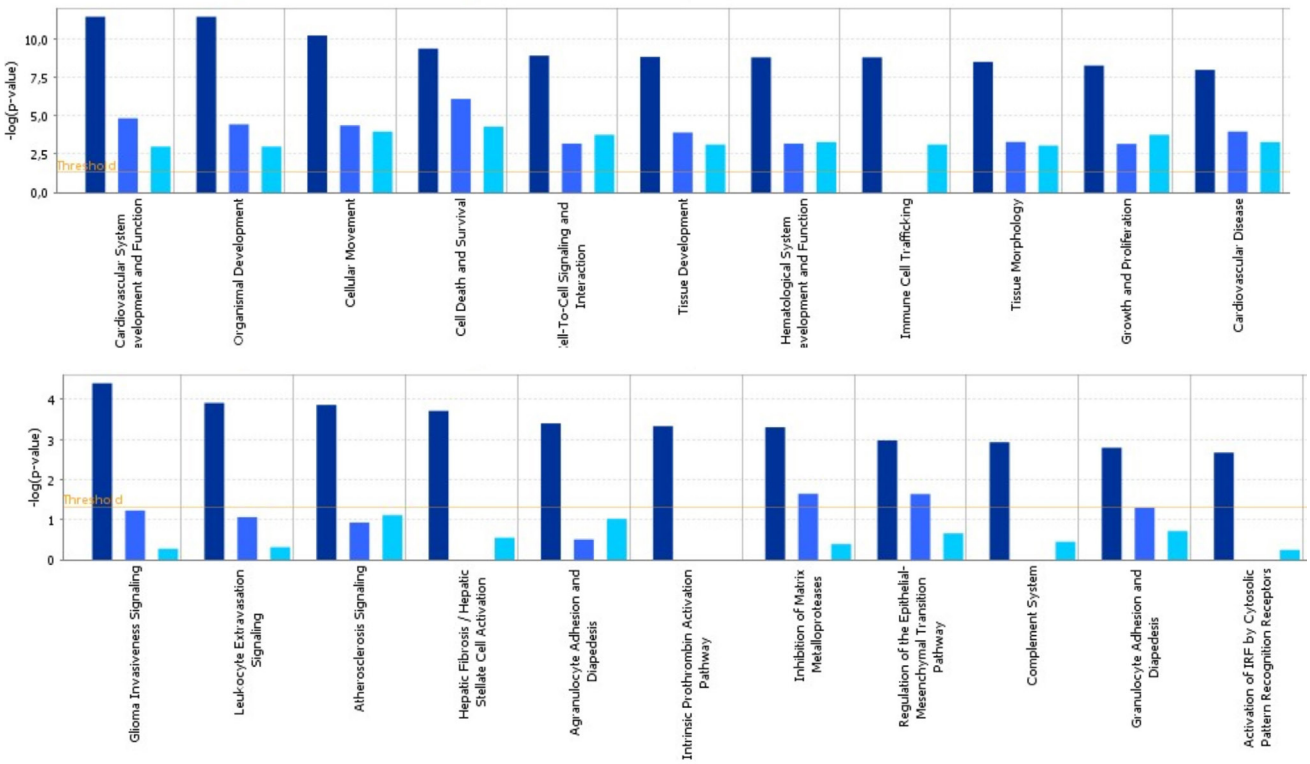

FIGURE 7 | Cellular pathways are significantly more modulated after consumption of either *Lactobacillus plantarum* TIFN101 (dark blue, left column) than after consumption of *L. plantarum* CIP104448 (light blue, middle column) or *L. plantarum* WCFS1 (cyan, right column). Statistical significance of pathway modulation was calculated *via* a right-tailed Fisher's Exact test in ingenuity pathway analysis and represented as −log (*p*-value); −log values exceeding the depicted threshold were significant ($p < 0.05$).

and downregulated genes (**Figures 6B,C**, respectively). Shared genes were mainly involved in general cellular functions and metabolism.

The 10 genes that were most highly up- or downregulated are listed in **Tables 1–3**. Of the most highly induced genes after TIFN101 consumption 80% are related to immunity: immunoglobulin lambda variable 6–57, putative V-set and immunoglobulin domain-containing protein 6-like, immunoglobulin lambda variable 7–46, interferon regulatory factor 4, GDNF family, CD27, CD79a, and plasminogen activator. WCFS and TIFN101 shared the downregulation of six small nucleolar RNAs, i.e., snoRNA (H)C/D(ACA) box 6, 14b, 53, 57, 60, 388, while CIP48 had a complete different profile of up and downregulation.

To identify specific transcription factors and to identify pathways regulated by the strains, IPA was performed (**Figure 7**). TIFN101 induced more changes than CIP48 and WCSF1 in the NSAID-stressed intestine. The most significant set of target-genes in the TIFN101 group were immune response-related genes. TIFN101 upregulated MHC-IIα, while CIP48 and WCFS downregulated MHC-IIβ (**Figure 8**). Another pathway that might contribute to the enhanced responses in TIFN101 is the upregulation of genes involved in leukocyte extravasation (**Figure 9**). TIFN101 enhanced RAPL expression, which is a GTPase involved in regulating integrin affinity. Concomitantly, an upregulation of essential adhesion molecules such as ICAM-1 and Cadherin 5 was seen, illustrating the upregulation of immune-cell migration pathways by TIFN101. Also, some regulation of leukocyte extravasation was observed with CIP48 and WCFS. However, it was much less pronounced than for the TIFN101 intervention.

Differential Gene Content Profiles between the Three *L. plantarum* Strains

Lactobacillus plantarum strains CIP48 and TIFN101 were sequenced, annotated, and compared with the genome (chromosome and plasmids) of *L. plantarum* WCFS1 (35, 36). A total of 3,010 OGs were assigned to the chromosome, based on the ordering of contigs to the template WCFS genome. The three genomes shared 2,455 of the 3,010 chromosomal OGs (=81.5%), which is defined as the core genome for this study. **Figure 10** presents the shared and genes and contigs of the *L. plantarum* strains. When the contigs and OGs/genes are included that do not match to the WCFS chromosome, higher numbers of unique genes were found for the CIP48 and TIFN101 genomes. Many of these extra unique genes are on plasmids (Table S2 in Supplementary Material). All unique genes for *L. plantarum* TIFN101 and *L. plantarum* CIP104448 compared to WCFS1 are listed in Tables S3 and S4 in Supplementary Material, and all absent genes in the two strains are listed in Tables S5 and S6 in Supplementary Material, respectively, as these are potential candidate genes for the biological

effects of the two strains. *L. plantarum* CIP48 lacks the complete plantaricin biosynthesis gene cluster, a large set of genes adjacent to this cluster (i.e., OGs 334–348), and the entire gene cluster for EPS biosynthesis. *L. plantarum* TIFN101 is missing some genes associated with plantaricin biosynthesis as well as genes for exopolysaccharide biosynthesis, many sugar utilization cassettes, and two large LPXTG-anchored mucus-binding proteins (Tables S7A,B in Supplementary Material).

DISCUSSION

The current experimental human study was undertaken to investigate the immunomodulatory and potentially beneficial effects of three *L. plantarum* strains, which were selected *in vitro* for their differential immune stimulating capacity. Indicators of responses of mucosal and systemic immunity were assessed in healthy individuals undergoing a mild, commonly encountered stressor (25, 26) of the intestine and immune system, *i.e.*, the intake of NSAIDs. We show that three *L. plantarum* strains had a highly strain-dependent effect on immunity *in vivo*, which were different to those predicted *in vitro*.

Indicators of systemic immunomodulatory effects were assessed in peripheral blood samples of the study participants. Consumption of indomethacin induced a reduction in $CD4^+$/Foxp3 regulatory cell frequencies, which was prevented by WCFS and TIFN101 administration. This can be considered a beneficial regulatory effect. CIP48, not preventing the reduction of $CD4^+$Foxp3 and reducing the number of memory cells, had effects that suggest a pro-inflammatory effect of consumption of this bacterium. Furthermore, T-cell polarization was studied

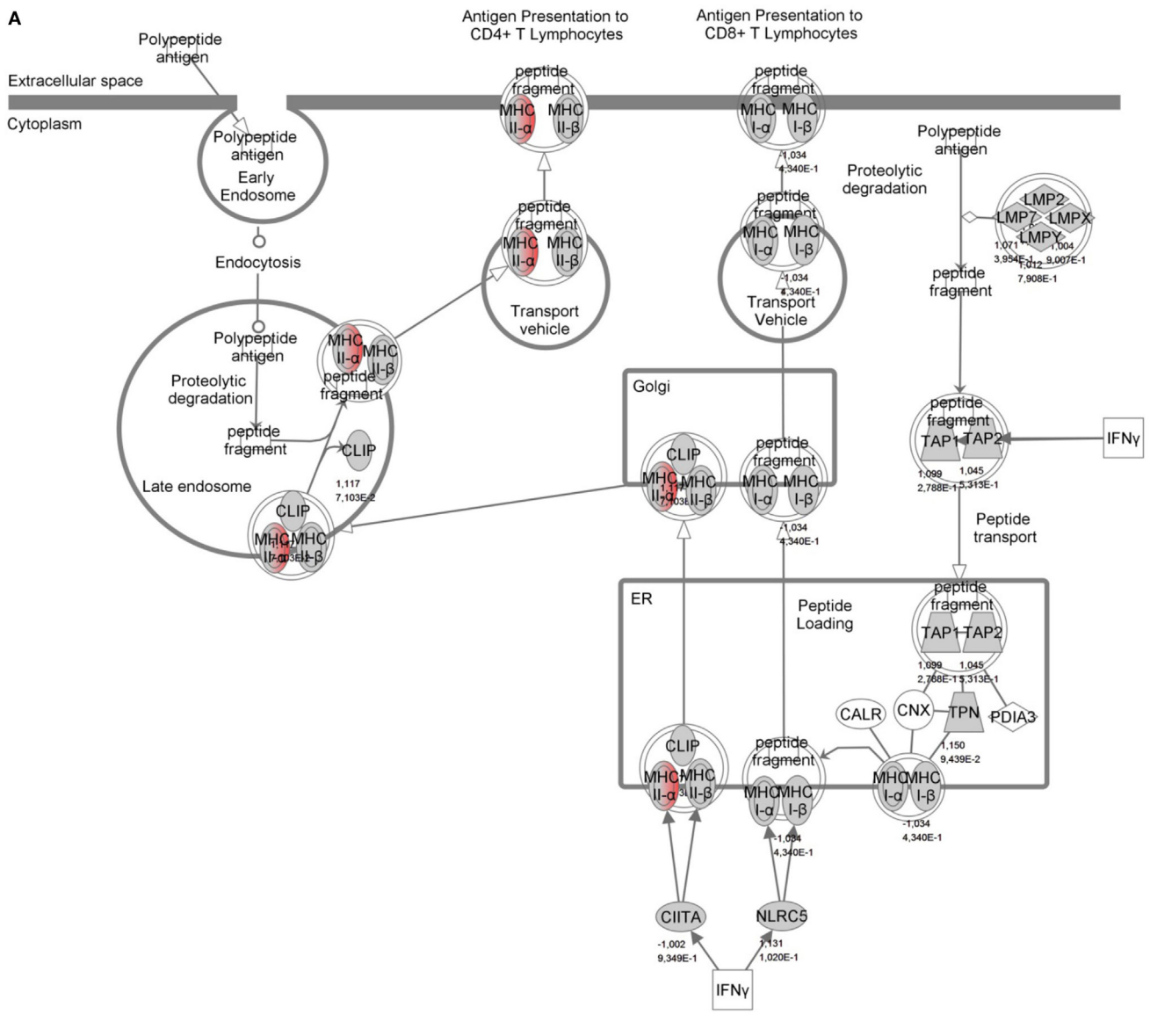

FIGURE 8 | Continued

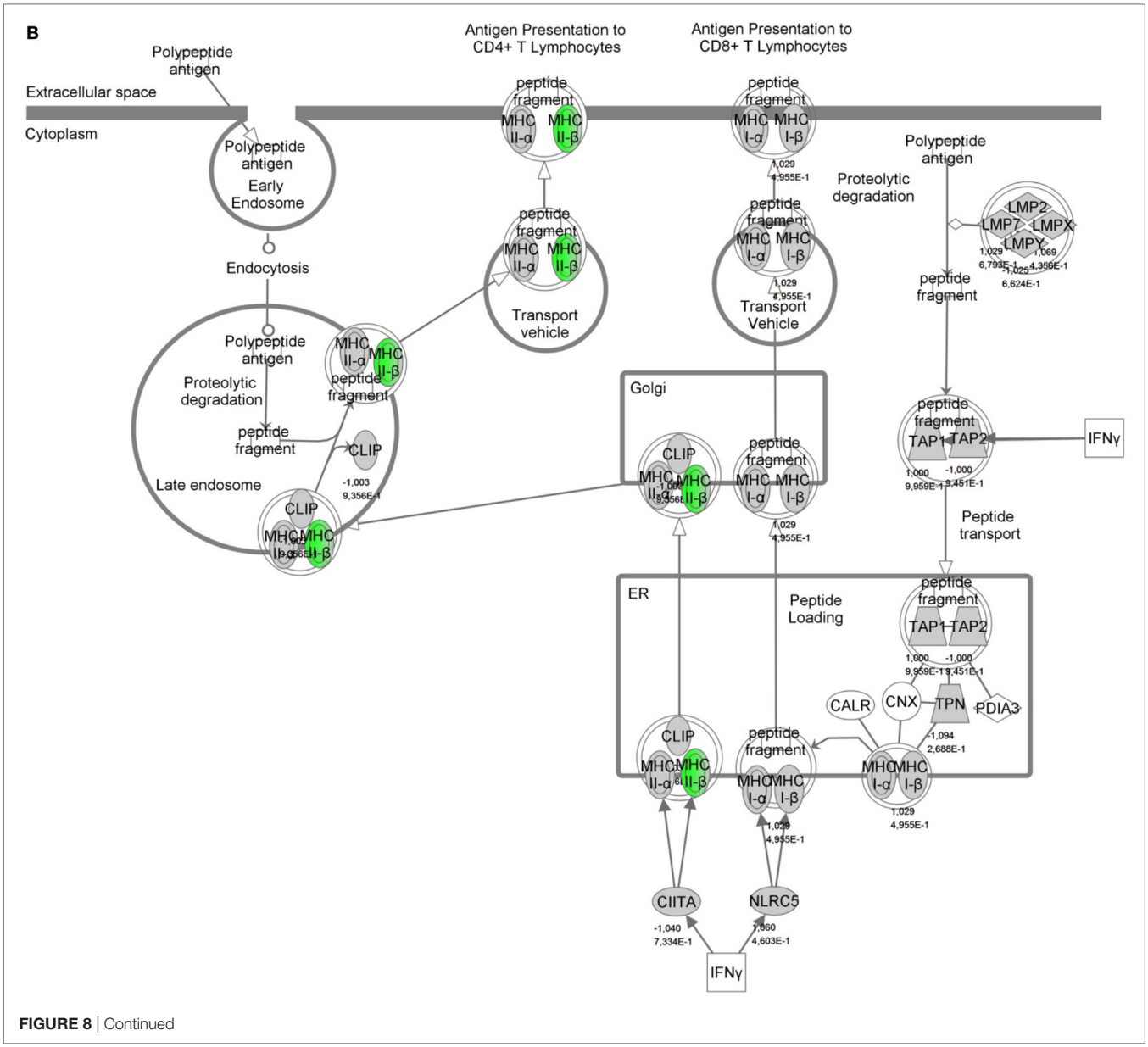

FIGURE 8 | Continued

after different stimuli. The hypothesis was that bacterial cell-wall components might induce immune responses (1) and enhance systemic immunity as a bystander effect. This happened with WCFS and TIFN101 (**Figure 5**). However, the most pronounced stimulator of immunity, i.e., TIFN101, also showed enhanced responses against specific pathogenic antigens such as TT and had an overall enhanced response to the SEB challenge. It is unlikely that the enhanced response against TT in the TIFN101 consumers is caused solely by the response against TIFN101 cell components. This is supported by the analysis of the intestinal biopsies that suggest that TIFN101 stimulate specific processes in the intestinal mucosa. TIFN101, in contrast to the other strains, upregulates mucosal transcription processes associated with T and B cell function and antigen presentation, when compared to placebo. It also had a pronounced effect on CD27 upregulation, which is required for generation and long-term maintenance of T cell immunity (37). Furthermore, TIFN101 enhanced expression of MHC-IIα in intestinal mucosa as well as key regulatory molecules such as RAPL. RAPL enhances integrin affinity and the adhesion of T cells (38, 39). These observations in the mucosa may explain the enhanced memory T cell responses observed with TIFN101 consumption. Furthermore, B cell immunity in the mucosa was enhanced as illustrated by upregulation of immunoglobulin regulatory genes and by CD79a. CD79a is also known as B cell antigen receptor complex-associated protein alpha-chain forming, together with CD79b protein, the B cell antigen receptor complex-associated protein (40). CIP48 and WCFS did not have these effects; in fact, they had a tendency to downregulate processes such as antigen presentation in the mucosa.

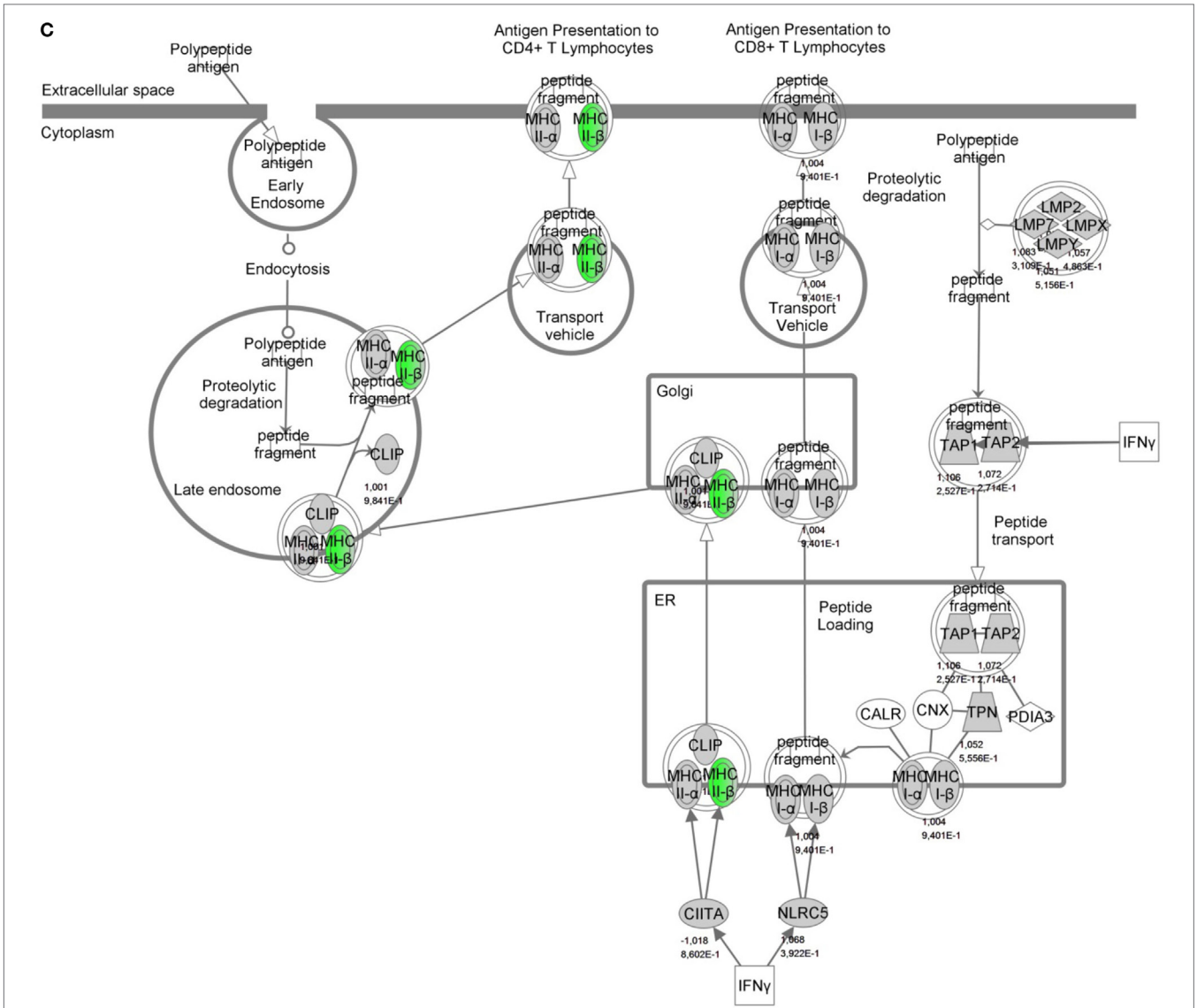

FIGURE 8 | Regulation of genes involved in antigen presentation in non-steroidal anti-inflammatory drug-stressed intestine after consumption of either *Lactobacillus plantarum* TIFN101 **(A)**, *L. plantarum* CIP104448 **(B)**, or *L. plantarum* WCFS1 **(C)**. Red indicates upregulation while green depicts downregulation of the specific gene. TIFN101 upregulated MHC-IIα while CIP48 and WCFS1 downregulated MHC-IIβ.

As a consequence of differences in TLR-binding, *L. plantarum* strains induce upon incubation with monocytes or dendritic cells different quantities of the pro-inflammatory cytokine IL-12 and the regulatory cytokine IL-10 (10). On the basis of these findings, three strains were selected, *L. plantarum* WCFS1 (WCFS), *L. plantarum* CIP104448 (CIP48), and *L. plantarum* TIFN101 (TIFN101), as their induced IL-10/IL-12 ratio *in vitro* were classified as pro-inflammatory, neutral, or anti-inflammatory, respectively. However, the immune responses to these strains *in vivo* were very different to that predicted *in vitro*. Consumption of NSAID induced a reduction in $CD4^+$/Foxp3 regulatory cell frequencies, which was prevented by WCFS1 and TIFN101 administration. This should be considered to be a beneficial regulatory effect. CIP48 did not prevent NSAID-induced reduction of $CD4^+$Foxp3 T cells and had more negative effects. CIP48 reduced the number of memory cells suggesting a pro-inflammatory, worsening effect of consumption of this bacterium. Our data suggest that solely studying the effects of bacteria on a single cell type *in vitro*, such as dendritic cells, has limited value, as it does not provide representative insight into the complex interplay between immune and other mucosal cells *in vivo*.

To our knowledge, this is the first report that bacteria can downregulate snoRNAs in the stressed intestine. SnoRNA are metabolically stable, non-coding RNAs that associate with a set of proteins to form small nucleolar ribonucleoproteins.

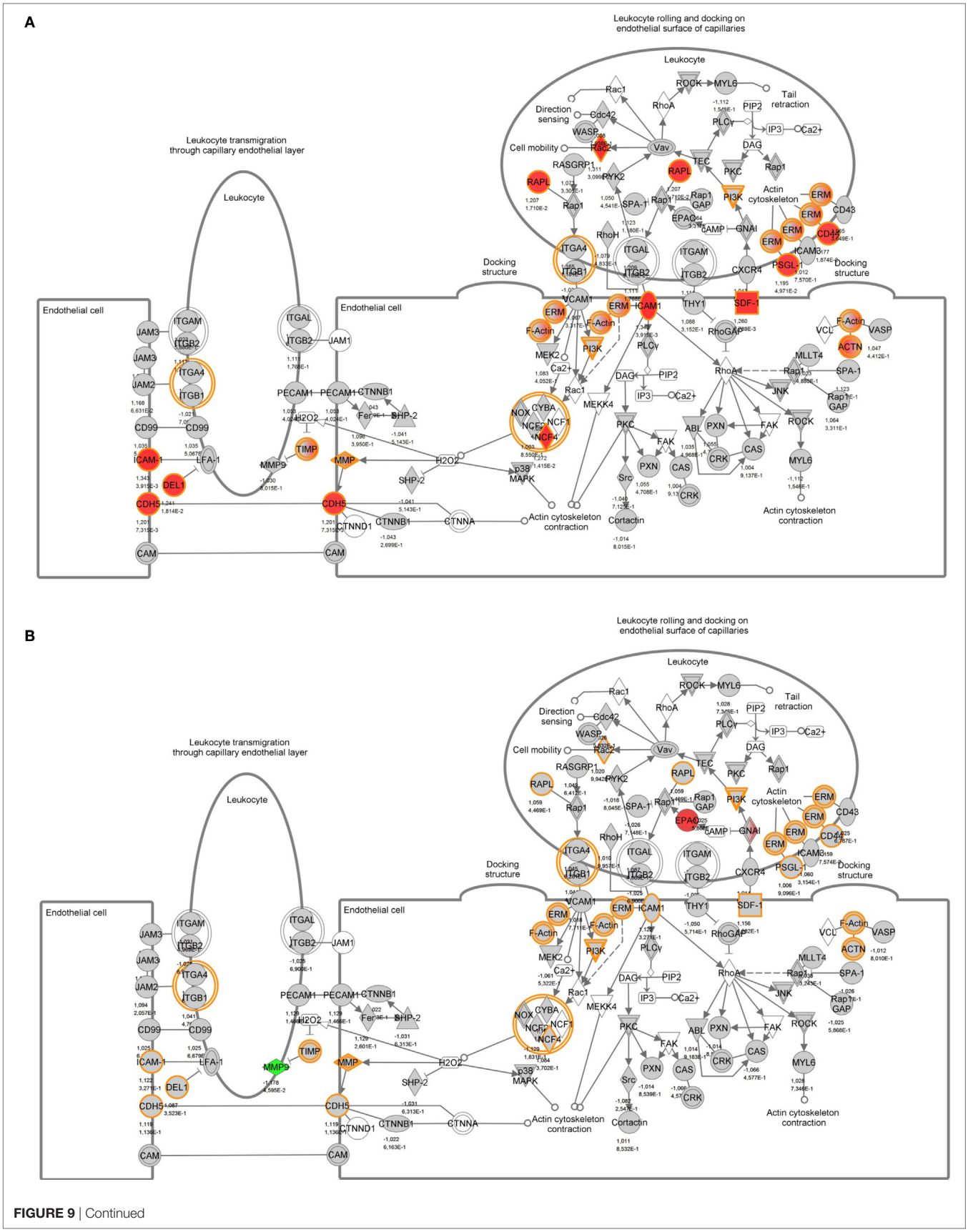

FIGURE 9 | Continued

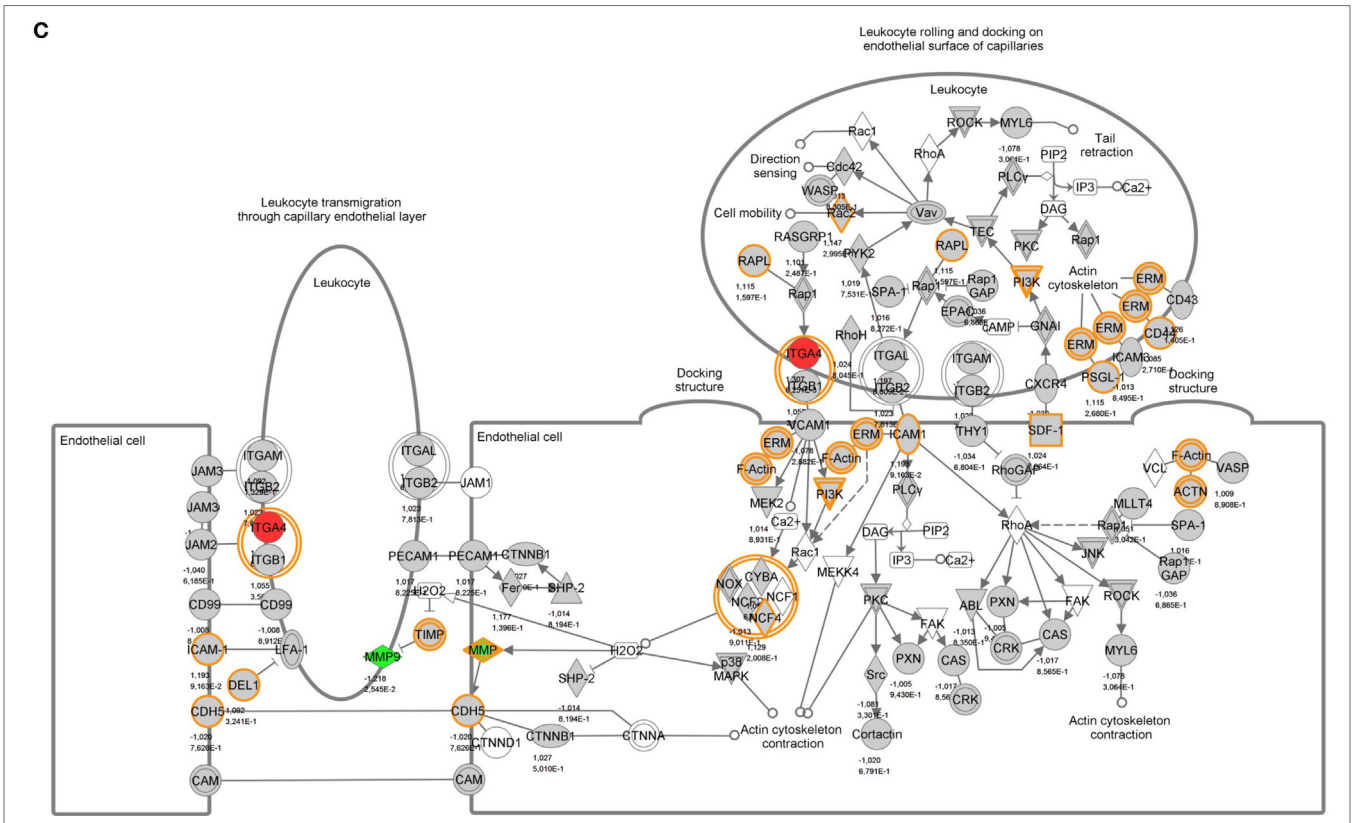

FIGURE 9 | Regulation of genes involved in leukocyte extravasation pathways in non-steroidal anti-inflammatory drug-stressed intestine after consumption of either *Lactobacillus plantarum* TIFN101 **(A)**, *L. plantarum* CIP104448 **(B)**, or *L. plantarum* WCFS1 **(C)**. Red indicates upregulation while green depicts downregulation of the specific gene.

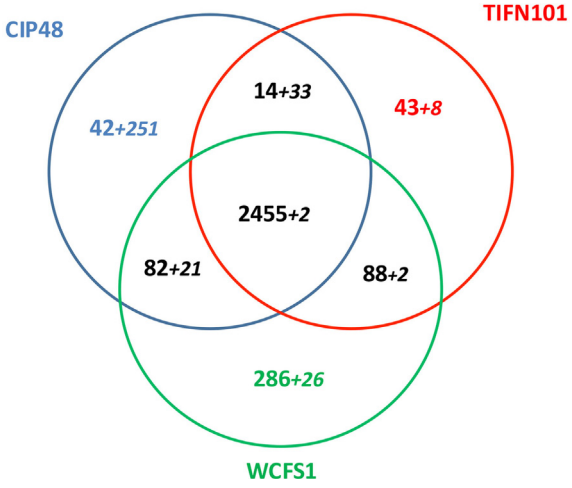

FIGURE 10 | Venn diagram of shared and unique genes in all contigs of *Lactobacillus plantarum* strains [*L. plantarum* WCFS1 (WCFS), *L. plantarum* CIP104448 (CIP48), *L. plantarum* TIFN101]. The smaller numbers in italics represent ortholog groups that do not match to the chromosome of strain WCFS. These numbers do not include the putative prophage genes in each genome.

The majority of snoRNAs function to guide RNAs in the post-transcriptional synthesis of 2′-O-methylated nucleotides and pseudo-uridines in ribosomal RNAs (rRNAs), small nuclear RNAs, and other cellular RNAs, including messenger RNAs (41–43). The relative reduction of several snoRNAs by TIFN101 and WCFS suggest a downregulation of methylation of rRNA (44) and downregulation of 14b diminished pseudo-uridinilation of RNA (45). Usually, this is indicative of a destabilization of cellular processes, but it may also be an indicator of tissue repair (46).

It should be noted that, in the present trial, we used a cross-over design to eliminate potential influences of inter-subject differences. Previous interventions may influence subsequent tests in such a study design. However, a long wash-out period of 4 weeks was chosen and the order of interventions was randomly assigned to each study subject, which should have minimized potential influences between interventions. Furthermore, the impact of *L. plantarum* consumption observed in this study may be caused by direct effects on host immunity; however, indirect effects *via* modulation of the resident microbiota may contribute as well.

Finally, full genome sequencing of *L. plantarum* CIP48 and *L. plantarum* TIFN101 was applied to identify possible gene

clusters that might be responsible for the differential biological effects of the three *L. plantarum* strains. Several 100 novel *L. plantarum* genes were found in strain CIP48 and TIFN101 as compared to the known genome of strain WCFS (35, 36), of which only a small number are shared by both CIP48 and TIFN101. The majority of the novel genes appear to be located on plasmids. CIP48 appears to have several plasmids that are not present in TIFN101 or WCFS. Strain CIP48 has specific genes, such as for lantibiotic biosynthesis and several cell-surface proteins, that might explain its differential effect. Notably, however, there are also numerous genes/gene clusters in *L. plantarum* CIP48 and TIFN101 that are not present in the WCFS genome. Strikingly, CIP48 completely and TIFN101 partially lack the plantaricin biosynthesis clusters. These genes have been linked to strain differences in cytokine production (10, 47), but were shown here not to be associated with immune effects *in vivo*. Also, both strains lack very large regions important for sugar metabolism. These differences have been attributed to adaptations to environmental factors which, in our opinion, are interesting targets genes and possibly associated with probiotic effects (48). Not only the presence but also the absence of genes may enhance immune effects of bacteria (8).

CONCLUSION

The current randomized double-blind placebo-controlled cross-over human trial demonstrated immune modulatory effects by orally consumed *L. plantarum* strains *via* specific responses against bacterial components as well as direct stimulation of specific immunity in the intestine. Specific bacteria can influence and possibly prevent undesired immune responses during commonly encountered stressors of the intestine such as intake of NSAIDs. Strong strain-dependent effects were found that showed *L. plantarum* TIFN101 to have a positive effect on host immunity, while CIP48 and WCFS1 had effects that may to be considered as less beneficial. Caution should be taken in selecting beneficial bacteria as even closely related strains can have very different effects. The observation that some bacteria can enhance specific memory cell populations can be helpful in identifying putative immune active human applicable bacterial strains. Furthermore, the current comparative genomics study provides many leads for follow-up experimental work to identify genes that are responsible for or involved in the observed differences in immune effects in humans.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the University Hospital Maastricht Ethical Committee with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the University Hospital Maastricht Ethical Committee. The study has been registered in the US National Library of Medicine (http://www.clinicaltrials.gov, NCT01456767).

AUTHOR CONTRIBUTIONS

PV co-supervised and co-designed the study and wrote the manuscript; ZM conducted the study and was involved in manuscript writing; BH and RS performed most analyses and reviewed the manuscript; PB provided the bacterial supplements and placebo; MM, JW, and AM co-supervised the study and interpretation of data; MB conducted the transcriptome analyses; and MF and FT co-supervised and co-designed the study.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.01000/full#supplementary-material.

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Probiotic Strain *Lactobacillus casei* BL23 Prevents Colitis-Associated Colorectal Cancer

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The gut microbiota plays a major role in intestinal health, and an imbalance in its composition can lead to chronic gut inflammation and a predisposition to developing colorectal cancer (CRC). Currently, the use of probiotic bacteria represents an emerging alternative to treat and prevent cancer. Moreover, consumption of these beneficial bacteria may also favorably modulate the composition of the gut microbiota, which has been described in several studies to play an important role in CRC carcinogenesis. In this context, the aim of this study was to assess the protective effect of oral treatment with *Lactobacillus casei* BL23, a probiotic strain well known for its anti-inflammatory and anticancer properties. First, CRC was induced in C57BL6 mice by a single intraperitoneal injection with azoxymethane (8 mg/kg), followed by four courses of dextran sodium sulfate (2.5%) in drinking water that were separated by an adjustable recovery period. At the time of sacrifice (day 46), tumor incidence, histological scores, and epithelial proliferation were determined in colon samples. Our results show that *L. casei* BL23 significantly protected mice against CRC development; specifically, *L. casei* BL23 treatment reduced histological scores and proliferative index values. In addition, our analysis revealed that *L. casei* BL23 had an immunomodulatory effect, mediated through the downregulation of the IL-22 cytokine, and an antiproliferative effect, mediated through the upregulation of *caspase-7*, *caspase-9*, and *Bik*. Finally, *L. casei* BL23 treatment tended to counterbalance CRC-induced dysbiosis in mice, as demonstrated by an analysis of fecal microbiota. Altogether our results demonstrate the high potential of *L. casei* BL23 for the development of new, probiotic-based strategies to fight CRC.

Keywords: *Lactobacillus casei* BL23, lactic acid bacteria, probiotic, azoxymethane-dextran sodium sulfate, colorectal cancer, immunomodulation

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INTRODUCTION

Colorectal cancer (CRC) is a major public health problem and is considered the third most common cancer around the world, with nearly 1.2 million new cases every year and a mortality rate of ~40% (1). The incidence of CRC can be associated with a large number of both genetic (2) and environmental factors (3). In particular, one major risk factor for the development of CRC is chronic intestinal inflammation (4); indeed, patients suffering from inflammatory bowel diseases (IBDs) are six times more likely to develop CRC than healthy individuals (5).

Today, the use of probiotics represents a promising strategy for the treatment and prevention of cancer. Probiotics are "live microorganisms, which when administered in adequate amounts confer a health benefit on the host" (6). The most common probiotic strains belong to the genera *Bifidobacterium* and *Lactobacillus*. Interestingly, epidemiological studies have shown a lower incidence of CRC in healthy volunteers who regularly consumed fermented dairy products (containing probiotics), especially yogurt (7–10). However, despite encouraging observations of the anticancer effects of probiotics (which have been accumulating for over 30 years), these clues have thus far been poorly investigated, and even today, the mechanisms underlying these beneficial effects are largely unknown. The beneficial role of probiotic bacteria against CRC onset may be explained by three different mechanisms: (i) modulation of the immune response, (ii) induction of cell apoptosis, or (iii) antioxidant activity [reviewed in Ref. (11)]. As a specific example, the food supplement VSL#3 (a mixture of eight probiotic bacteria) has been shown to modulate the immune response and reduce adenoma development in a model of CRC induced by azoxymethane (AOM) and dextran sodium sulfate (DSS) (12). Furthermore, *Lactobacillus gasseri* and *Bifidobacterium longum* have been shown to inhibit cellular proliferation and increase phagocytic activity in a model of 1,2-dimethylhydrazine (DMH)-associated CRC and thus reduce the multiplicity of aberrant crypt foci as well as tumor size (13). In addition, the probiotic strain *Lactobacillus casei* Shirota is able to suppress chemically induced intradermal tumor onset through both an enhancement of the cytotoxicity of natural killer (NK) cells (14) and IL-12 release by dendritic cells (15).

Beyond having cancer fighting effects, the consumption of probiotics may also favorably modulate the composition of the gut microbiota (16). In this context, several studies have confirmed the important role that the gut microbiota plays in CRC carcinogenesis by generating both biochemical and physiological conditions that may increase the number of colonic preneoplastic lesions (17, 18). However, despite the well-known role of microbiota dysbiosis in CRC pathogenesis, there are conflicting reports about a specific correlation between the bacterial community composition in the gut and susceptibility to CRC (18–20). To date, animal studies have revealed that germ-free mice are more predisposed to developing inflammatory-induced CRC than conventional mice (21). Therefore, manipulation of the gut microbiota could be a promising approach to prevent and/or treat CRC.

We have previously reported anti-inflammatory effects of the probiotic strain *L. casei* BL23 in two different murine models of chemically induced colitis (22–24). Furthermore, we have recently observed that this strain also displays antitumoral properties in a mouse allograft model of human papilloma virus (HPV)-induced cancer and in a DMH-induced CRC model (25). We attributed this antitumoral effect to a modulation of the immune response and, in particular, to the modulation of regulatory T-cells toward a Th17-biased immune response associated with the expression of regulatory cytokines (IL-6, IL-17, IL-10, and TGF-β) (25). However, the beneficial role of *L. casei* BL23 in colitis-associated CRC (such as that induced by AOM-DSS) remains to be assessed.

Thus, keeping in mind the role of the gut microbiota and chronic intestinal inflammation in CRC carcinogenesis, we decided in this study to further investigate the impact of *L. casei* BL23 in a mouse model of CRC induced by AOM and DSS. Our results showed that oral administration of *L. casei* BL23 significantly reduced tumor onset. Histological analyses revealed reduced proliferation in tumor sections, as demonstrated by the upregulation of three genes involved in apoptosis (caspase 7, 9, and Bik) and cytokine IL-22. Illumina sequencing revealed that the gut microbiota of mice treated with *L. casei* BL23 tended to differ in both community composition and community richness from those of PBS-treated mice. Finally, we provide some clues about the host molecular mechanisms involved in the anticancer effects of this beneficial probiotic bacterium.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Lactobacillus casei BL23 (26) was grown in Man, Rogosa, and Sharpe medium at 37°C without agitation overnight. To prepare live bacterial inoculum, strains were washed twice with PBS at 3,000 *g* and suspended in PBS at a concentration of 5×10^9 colony-forming units/ml plus 15% glycerol.

Animals and Experimental Design

All experiments were handled in accordance with institutional ethical guidelines, and the study was approved by the COMETHEA ethics committee ("Comité d'Ethique en Expérimentation Animale") of the Centre INRA of Jouy-en-Josas and AgroParisTech. Female C57BL/6 mice (6–8 weeks old; Janvier SAS, St. Berthevin, France) were maintained in sterile isolators at the INRA animal facility ($n = 5$ per cage) with 12 h light cycles and fed irradiated normal chow (R 03-40, SAFE) and water *ad libitum*.

Temperature and moisture were carefully controlled. Mice were separated into three groups. The first two groups were administered PBS, while the last group was orally administered *L. casei* BL23. As shown in **Figure 1**, administration of PBS or *L. casei* BL23 started a week before tumor induction and was performed every day until sacrifice.

Tumors were induced with a single intraperitoneal injection of 8 mg/kg AOM (Sigma-Aldrich) 6 days before the start of a DSS treatment (with the exception of group 1, the negative control). Mice received a 5-day course of 2.5% DSS (TdB) in sterile drinking water, followed by an adjustable recovery period. This schema was followed four times (**Figure 1**). Intestinal inflammation was assessed daily by measuring the Disease Activity Index (DAI), which included body weight loss (0 = <1%, 1 = 5% > X > 1%, 2 = 10% > X > 5%, 3 = 15% > X > 10%, 4 = > 15%); mouse activity (0 = normal, 2 = hooked back, and 4 = lethargy); stool consistency (0 = absence, 2 = soft and sticky, and 4 = diarrhea); occult/gross rectal bleeding (0 = normal, 1 = occult+, 2 = occult++, 3 = occult+++, and 4 = gross bleeding); and mouse coat state (0 = normal, 2 = ruffed, and 4 = very ruffed). Mice were sacrificed at day 46. Colons were harvested and cleaned with PBS, and tumors were measured with a caliper. Colon sections were

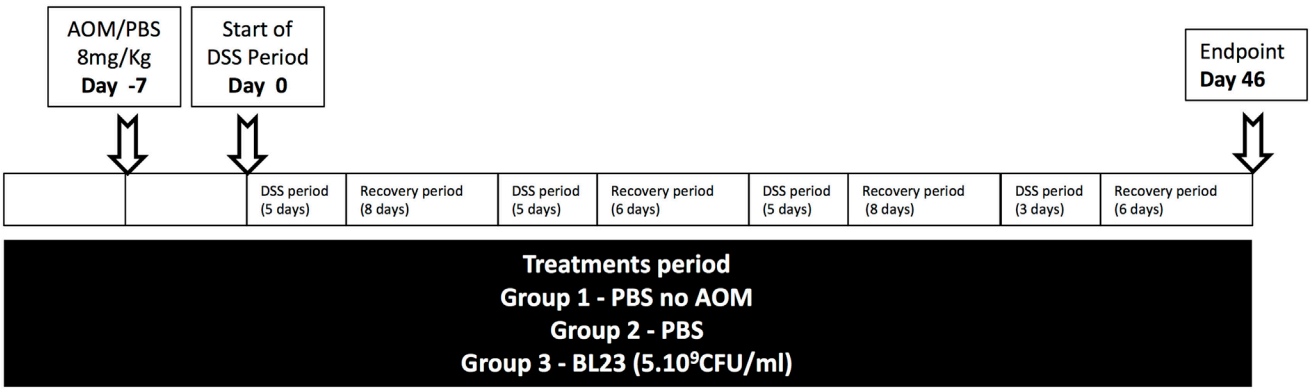

FIGURE 1 | Experimental protocol. AOM, azoxymethane; CFU, colony-forming unit; DSS, dextran sodium sulfate.

stored under conditions appropriate to the subsequent analyses (detailed in the following sections). For each mouse, three sections (~1 cm) were recovered from rectum samples and used for histology, gene expression analysis, and protein analysis. Colic contents were frozen in liquid nitrogen.

Cytokine Analysis

Mononuclear cells were isolated from spleens by gentle extrusion of the tissue through a 50-µm-mesh Nylon cell strainer (BD). Cells were resuspended in DMEM medium that was supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 U/mg penicillin, and 50 U/mg streptomycin (Lonza, Levallois-Perret, France). Erythrocytes were lysed with red blood cell lysing buffer (Sigma-Aldrich).

For stimulation experiments, 2.5×10^6 cells per well were cultured for 48 h (37°C, 10% CO_2) in DMEM medium in P24 plates that were precoated with anti-CD3/CD28 antibodies (4 µg/ml each; eBioscience). Culture supernatant was frozen at −80°C until processing.

Proteins from each colon were extracted with T-PER tissue protein extraction reagent (ThermoFisher Scientific) using a Fastprep instrument at 4,500 *g* for 30 s (two cycles). Samples were centrifuged at 500 *g* for 1 min, and supernatants were harvested for cytokine analysis. A cytometric bead array system (LEGENDplex Mouse Th Cytokine Panel, Biolegend) was used, according to manufacturer's instructions, to determine the levels of the following cytokines: IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-17A, IL-17F, IL-21, IL-22, IFN-γ, and TNF-α.

Tumor Histology

Colon sections were formalin fixed and embedded in paraffin (4%, VWR, France). Epithelial proliferation was assessed by Ki67 staining according to the manufacturer's instructions, using mouse monoclonal anti-Ki67 antibody (MM1, Leica Biosystems; 1:50). The proliferation index was determined by counting the number of Ki67-positive cells per crypt in three well-aligned crypts.

Two-micron colon sections were used for H&E staining. Histological score was determined using a BX43 Olympus microscope in a blinded manner, *via* the observation of three parameters: inflammation/cellular infiltration, epithelial lesions, and regeneration.

Gene and Protein Expression Analysis

Colon sections were stored in RNA later (Ambion) at −80°C. RNAs were extracted using the RNeasy mini-kit (Qiagen, Courtaboeuf, France) following the manufacturer's recommendations. RNA concentration was measured using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). cDNA synthesis was carried out from 1 µg of RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA), according to the manufacturer's instructions. RT-qPCR was carried out in a reaction volume of 25 µl with Taqman probes (β-*actin*: Mm01963702_S1, *caspase-9*: Mm00516563_m1, *caspase-7*: Mm01195085_m1, *Bik*: Mm00476123_m1) (Life Technologies, France) according to the manufacturer's instructions, using an ABI Prism 7700 (Applied Biosystems, USA) thermal cycler. To quantify and normalize the expression data, we used the ΔCt method, using the geometric mean Ct value from β-actin as the endogenous reference gene.

Total proteins were extracted from colon sections using T-PER buffer (Thermoscientific) and protease inhibitor mixture (Roche, Germany), through mechanical lysis with the Precellys homogenizer (Ozyme, France; 2 runs of 4,500 *g* for 30 s). Supernatants were collected, and western blots were performed. Briefly, samples were separated on a mini-PROTEAN TGX precast stain-free gel (4–20%, Biorad). Blots (Trans Blot Turbo Transfer system, Biorad) were incubated with β-actin, caspase-7, and cleaved caspase-7 antibodies (Cell Signaling, Danvers, MA, USA).

Microbial DNA Extraction

DNA was extracted using the Godon technique from stool (T0 = day of AOM treatment) and cecal content (Tf = time final; day of sacrifice) as described by Lamas et al. (27). The DNA pellet was washed with 70% ethanol, dried, and resuspended in 50 µl of Tris–EDTA buffer. DNA suspensions were stored at −20°C until amplification.

16S rDNA Amplification and Gene Analysis

16S rDNA was amplified with primers for the V3 and V4 hypervariable regions (PCR1F_460: 5′-CTTTCCCTACACGACGCTCTTCCGATCTACGGRAGGCAGCAG-3′, PCR1R_460:5′-GGAGTTCAGACGTGTGCTCTTCCGATCTTACCAGGGTATCTAATCCT-3′). The reaction mixture contained 10 ng of genomic DNA, 5 U/µl MTP Taq DNA polymerase (Sigma, France), 0.2 mM dNTP, and 0.5 µM (final concentration) of each primer. Reactions were performed using an annealing temperature of 65°C for 30 cycles in a T100 thermocycler (Biorad, France). Sequencing was performed using 460-bp paired-end reads and an Illumina Miseq protocol on the GeT-PLaGe platform (Toulouse, France). Illumina reads were joined using the fastq-join method. The sequences were demultiplexed and quality filtered using the QIIME (version 1.8.0) software package. The sequences were assigned to OTUs using UCLUST algorithm 41 with a 97% threshold of pairwise identity and classified taxonomically using the Greengenes reference database.

Statistical Analysis

Data were analyzed with Prism software (version 5). All normally distributed data were displayed as mean ± SEM. Comparisons between two groups were performed with a Student's *t*-test.

RESULTS

L. casei BL23 Protects against Tumor Development in a Colitis-Associated CRC Model

To determine the potential beneficial effects of the dairy strain BL23 of *L. casei* on CRC onset, live bacteria were orally administered to mice treated with AOM and DSS. As shown in **Figure 2A**, mice fed with *L. casei* BL23 were protected against tumor development: no mouse in this group developed macroscopic tumors, compared to 67% (6/9) of mice that received only PBS (**Figures 2A,B**). Mice treated with PBS developed a per-mouse average of 2.3 tumors at least 2 mm in diameter (**Figure 2C**). Since this CRC model is related to chronic intestinal inflammation, we then assessed DAI and histological scores and intestinal epithelial damage. As shown in **Figure 3A**, DAI scores increased after each DSS cycle; however, there were no significant differences between any of the treated groups. For the histological scores, mice fed *L. casei* BL23 showed less damage than control mice did ($p = 0.052$, Student's *t*-test; **Figures 3B,C**). In addition, Ki67 levels (which are expressed in proliferating cells) were significantly lower ($p = 0.044$, Student's *t*-test) in mice treated with *L. casei* BL23 than in control mice (**Figures 3D,E**).

L. casei BL23 Displays Antiproliferative Activities

As immunomodulation and the induction of cell apoptosis are among the main probiotic-related protective mechanisms against CRC, we examined changes in both the immune response and apoptosis pathways due to *L. casei* BL23 treatment. We first determined the levels of both local (i.e., colon and mesenteric lymphoid node (MLN) samples) and systemic (i.e., spleen samples) cytokines that are involved in inflammation and carcinogenesis, including IL-22, IFN-γ, IL-10, IL-21, TNF-α, IL-6, and IL-17A (Figures S1–S3 in Supplementary Material). As shown in **Figure 4A**, colonic IL-22 (a cytokine that promotes proliferation of cancer cells) (28) levels were lower ($p = 0.057$, Student's *t*-test) in *L. casei* BL23-treated mice compared to controls. Other cytokines that have been linked to CRC carcinogenesis, such as IFN- γ, TNF-α, IL-6, IL-10, IL-21, and IL-17A, were assessed in MLN (Figure S1 in Supplementary Material), in spleen (Figure S2 in Supplementary Material), and in colon samples (Figure S3 in Supplementary Material), but no significant difference was observed between treated and control mice.

In addition, we performed colic gene expression analysis of genes involved in apoptosis, specifically, *caspase-7*, *caspase-9*, and *Bik*. Our results reveal that *L. casei* BL23 induced a significant increase compared to controls in the expression of the executioner *caspase-7* (**Figure 4B**, $p = 0.017$, Student's *t*-test) and the initiator *caspase-9* (**Figure 4C**, $p = 0.028$, Student's *t*-test), together with

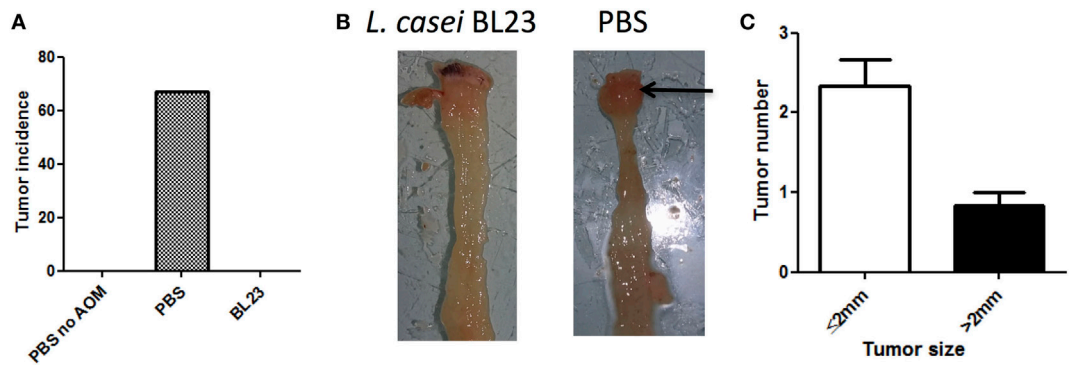

FIGURE 2 | *Lactobacillus casei* BL23 protected against tumor formation. **(A)** Macroscopic colic tumor incidence. Data are represented as the mean of each group ± SEM ($n = 9$ mice) in an *in vivo* experiment. **(B)** Representative view of tumor in a PBS/azoxymethane (AOM)/dextran sodium sulfate (DSS)-treated mouse. **(C)** Colic tumor size in PBS/AOM-treated mice (number of small tumors ≤2 mm and large tumors >2 mm per mouse) in the whole colon. Data are represented as the mean of each group ± SEM ($n = 9$ mice).

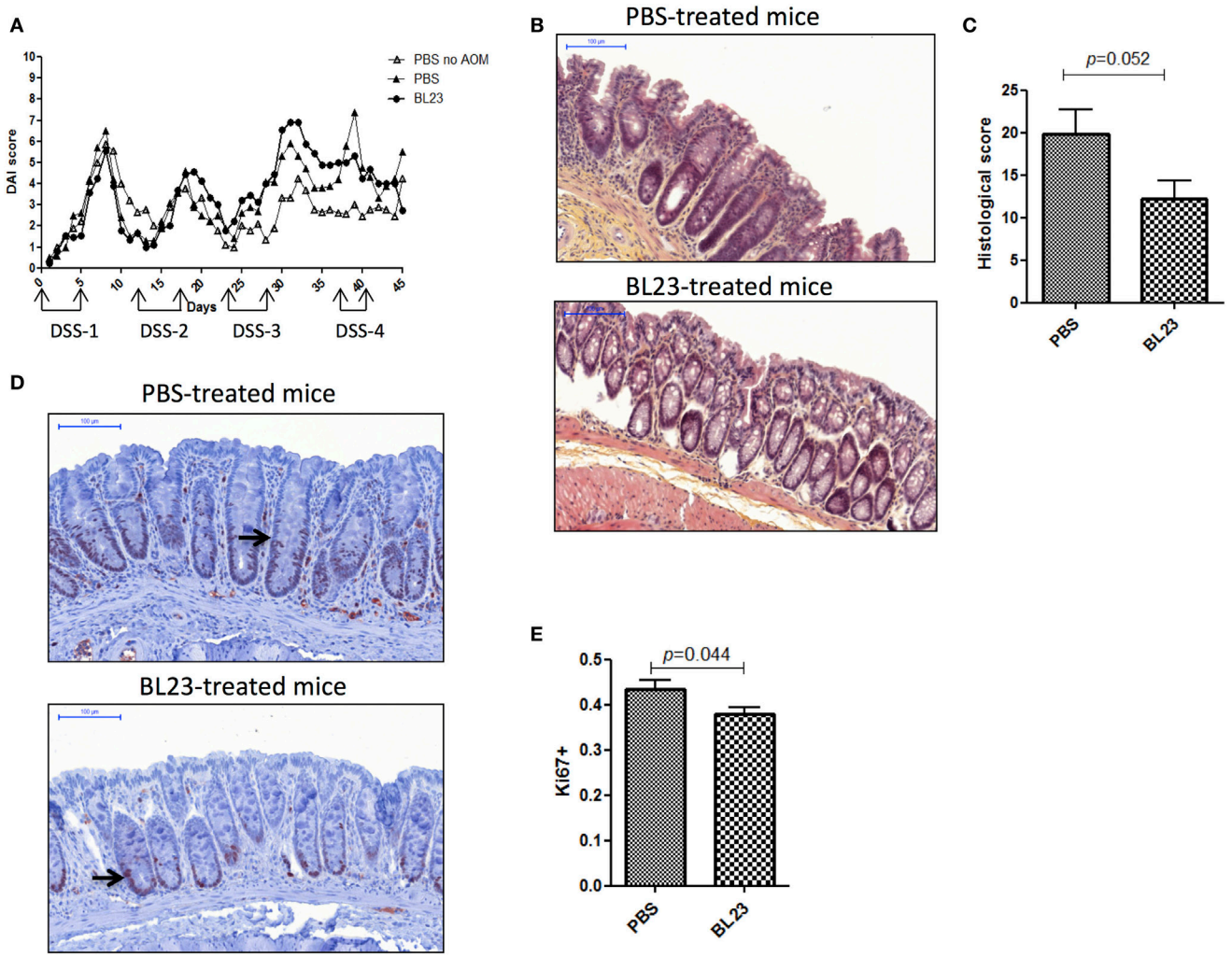

FIGURE 3 | Macroscopic and histological assessment of inflammation and proliferation in mice. **(A)** Disease Activity Index score [dextran sodium sulfate (DSS) treatment periods are indicated on the graph]. **(B)** Representative H&E-stained images of colic tissues from either PBS- or BL23-treated mice at sacrifice; scale bars, 100 µm. **(C)** Semiquantitative scoring of histopathology ($p = 0.044$, Student's *t*-test). **(D)** Representative Ki67-stained images from either PBS- or BL23-treated mice at sacrifice; scale bars, 100 µm; black arrow indicates Ki67 distribution inside the crypt. **(E)** Proliferative assessment ($p = 0.052$, Student's *t*-test). Data are represented as the mean of each group ± SEM ($n = 9$ mice) for each graph. AOM, azoxymethane.

an increase of the apoptotic gene *Bik* (**Figure 4D**, $p = 0.082$, Student's *t*-test). Finally, we determined the level of truncated caspase-7 (which corresponds to the active form of caspase-7 in apoptosis). For this, we selected two mice treated with *L. casei* BL23 and protected against tumor development, which presented high *caspase-7* RNA expression levels, and a mouse treated with PBS (not protected against tumors), which presented low levels of *caspase-7* RNA expression. As shown in **Figure 4E**, western blot results confirmed that the mice protected against tumor onset produced higher levels of the active form of caspase-7 compared to the non-protected mouse.

Altogether, these data suggest that *L. casei* BL23 has an anti-proliferative and apoptotic effect in this CRC model; a detailed proposal of the mechanisms of action of *L. casei* BL23 against CRC is shown in **Figure 5**.

Impact of *L. casei* BL23 Treatment on Microbiota Richness and Diversity

To determine the impact of *L. casei* BL23 on the gut microbiota, we analyzed microbiota richness and diversity after BL23 oral treatment. This analysis provided a total of 1,709,762 high-quality and classifiable reads, with an average of 5,000 ($n = 51$) reads per sample. First, beta diversity (Bray-Curtis distance) was analyzed in microbial samples using principal components analysis, which reduced the dimensionality of the data set. While no grouping was observed before AOM challenge (T0; **Figure 6A**), the microbiota of mice treated with *L. casei* BL23 tended to diverge from those of PBS-treated animals at Tf (sacrifice; **Figure 6B**, $p = 0.1$, Anosim). Then, we estimated community richness (alpha diversity; Shannon and Simpson indexes) at T0 and Tf. As shown in **Figure 6C**, AOM injection resulted in a reduction

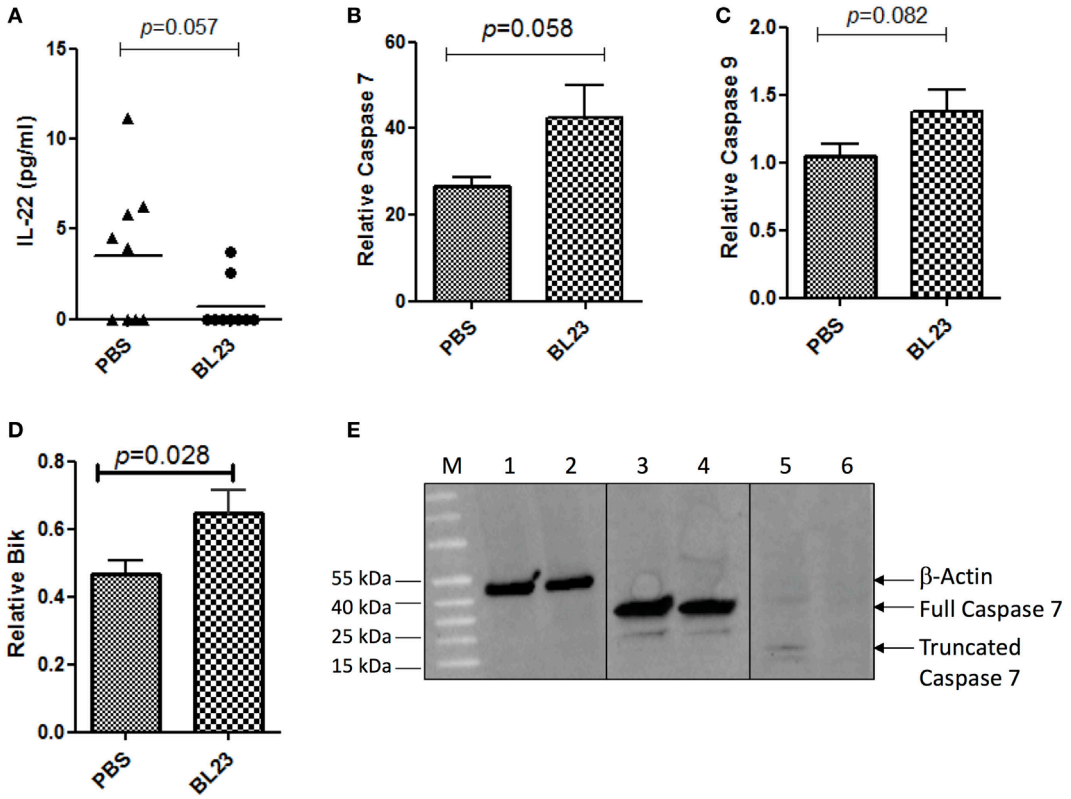

FIGURE 4 | *Lactobacillus casei* BL23 induced antiproliferative activity and increased apoptosis. **(A)** Protein analysis of IL-22 in colon tumor section. **(B)** Real-time PCR analysis of relative expression in colic tumor sections of mRNA of *caspase-7* ($p = 0.058$, Student's *t*-test), **(C)** *caspase-9* ($p = 0.082$, Student's *t*-test), and **(D)** *Bik* ($p = 0.028$, Student's *t*-test). Data are represented as the mean ± SEM of each group ($n = 9$ mice) for each graph. **(E)** Caspase 7 protein expression. Lines 1, 3, and 5 correspond to mice treated with *L. casei* BL23 (and protected against tumors and that expressed high levels of *caspase-7* RNA) and lines 2, 4, and 6 to mice treated with PBS (not protected against tumors). Lines 1 and 2 correspond to samples treated with anti-β-actin antibodies; 3 and 4 with full caspase 7 antibodies; and 5 and 6 with antibodies recognizing the cleaved form of caspase 7.

in alpha diversity with respect to that found in the group treated with PBS but not AOM (Shannon index, 6.4 ± 0.22 in PBS/AOM-treated group versus 6.8 ± 0.46 in PBS/no AOM group, $p = 0.028$, Student's *t*-test). Despite the fact that the effects of *L. casei* strain BL23 were not statistically significant (Simpson index, 483.3 ± 111.8 in BL23/AOM-treated group *versus* 436.8 ± 54.7 in PBS/AOM-treated group, ns, Student's *t*-test; **Figure 6D**), these results provide intriguing clues about this strain's interactions with other members of the gut microbiota.

Finally, bacterial communities were characterized at the phylum level (**Figure 6E**). Firmicutes was the dominant phylum in the *L. casei* BL23/AOM treated group (51 ± 10%), with Bacteroidetes ranked second (45 ± 10%). In contrast, Bacteroidetes was the most abundant phylum in both PBS/AOM and PBS/no AOM treated groups (52 ± 12 and 53 ± 13%, respectively), followed by Firmicutes (44 ± 12 and 43 ± 13%, respectively). The third most abundant phylum in all groups was Proteobacteria, with approximately 3–4% of reads. By using linear discriminant analysis (LDA) coupled with effect size measurements (linear discriminant analysis effect size), we found that *Prevotella*, Ruminococcaceae, and *Lactobacillus* were the key groups that were overrepresented in the *L. casei* BL23-treated mice (**Table 1**). At the species level, only *Lactobacillus zeae* was significantly more abundant ($p = 0.004$, Student's *t*-test) in these mice, with a LDA score of 2.58. This was not surprising, since this species could in fact correspond to strain BL23 of *L. casei*. Indeed, 16S RNA analysis has revealed a close relationship (99% similarity) between our focal strain and *L. zeae* (26).

DISCUSSION

There is now mounting evidence pointing to an important link between both chronic colic and rectal damage (present, for example, in IBD patients) and CRC carcinogenesis. Indeed, the risk of developing colitis-associated cancer increases by ~1% in IBD patients (5). To better understand the mechanisms related to tumor onset, different preclinical animal models of CRC have been developed, such as the AOM-DSS model used in this study (29).

L. casei BL23 has been previously studied for its anti-inflammatory activities in different models of chemically induced colitis (22, 23). Furthermore, *L. casei* has been widely studied in different murine models of cancer (14, 30–32). In particular, *L. casei* BL23 has antiproliferative effects in the mouse allograft model of HPV-induced cancer and protects against DMH-induced CRC (25). Here, we decided to explore the impact of oral administration of *L. casei* strain BL23 in a murine model of CRC induced by

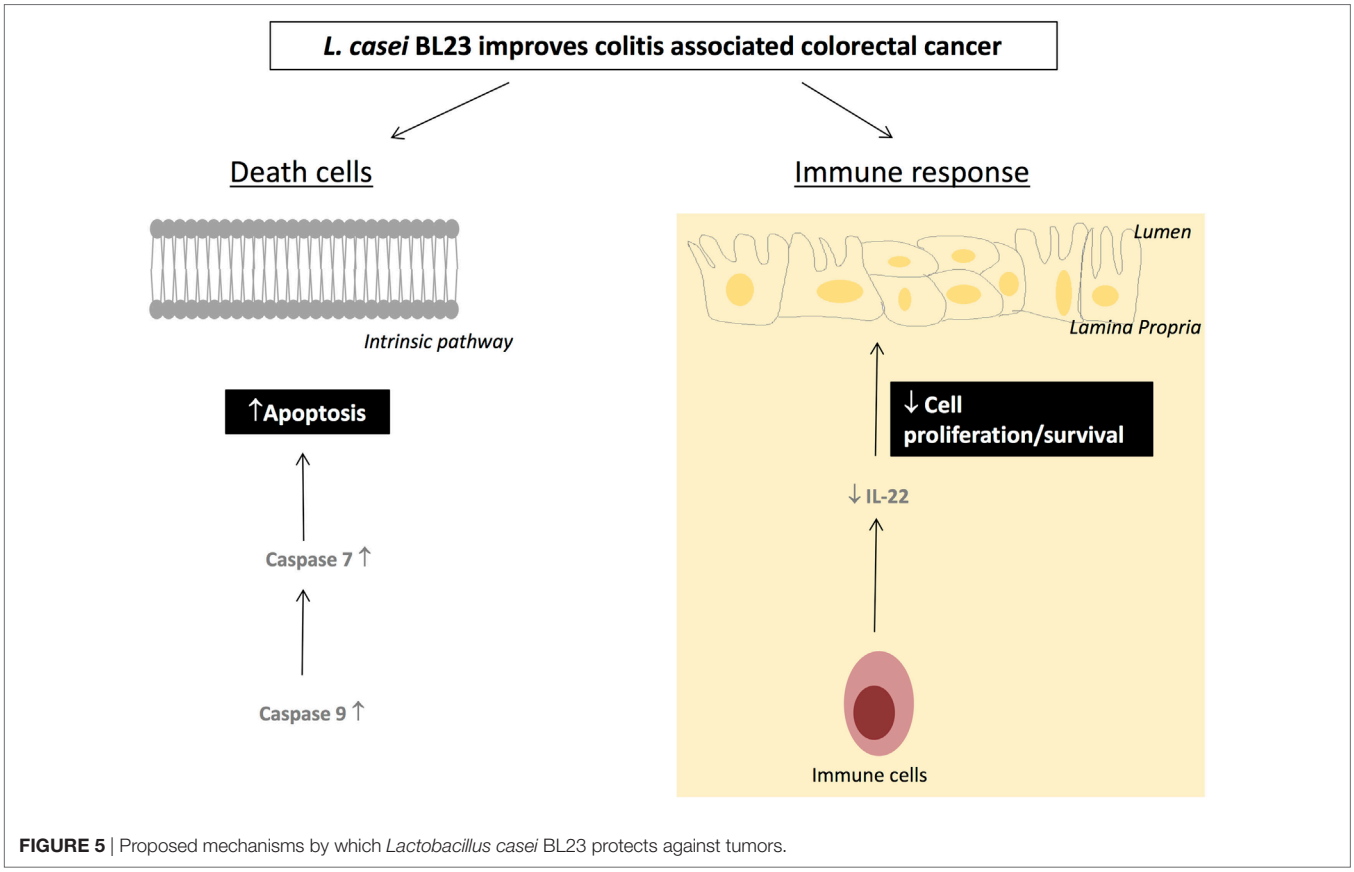

FIGURE 5 | Proposed mechanisms by which *Lactobacillus casei* BL23 protects against tumors.

AOM and DSS. Strikingly, our results revealed that *L. casei* BL23 significantly reduced tumor development, since all treated mice were tumor free at the end of the experiment. In addition, we also found some clues about the molecular mechanisms involved in the protective effect against cancer, and it appears that, in this model, *L. casei* BL23 acts mainly *via* the inhibition of cell proliferation. Indeed, this strain is able to downregulate proliferation, as observed through a decrease in Ki67. In addition, *L. casei* BL23 was also able to increase apoptosis *via* upregulation of *caspase-9*, *caspase-7*, and *Bik*.

Given the reported anti-inflammatory properties of *L. casei* BL23, we also assessed the cytokine profiles of treated mice. With the exception of a reduced histological score, *L. casei* BL23 had no significant effects on cytokine regulation. However, a weak decrease was observed in IL-22 levels in colons from mice treated with BL23. This cytokine has been recently implicated in CRC development in both humans and $APC^{min/+}$ murine model (33). Thus, it appears that IL-22 levels are enhanced in tumor tissues and that mice displaying lower levels of this cytokine are protected from tumorigenesis. It was recently reported that the beneficial effects of a strain of *Lactobacillus reuteri* in a model of CRC induced by AOM-DSS were mediated by a histidine decarboxylase (HDC), which downregulated IL-22 expression (34). We performed *in silico* analyses to search for the nucleotide sequence of the HDC cluster in the *L. casei* BL23 genome, but were unable to find a corresponding sequence region (data not shown). However, it is still possible that another protein produced by this strain may act directly on IL-22 regulation/expression. The main sources of IL-22 are NK cells, γδ T cells, and lymphoid tissue inducer cells, as well as TH17 and TH22 cells. To determine which cell types are affected by *L. casei* BL23 treatment, future experiments will need to examine the correlation between each population of cells in the lamina propria of mice and IL-22 downregulation.

Finally, several reports have described disruption of the composition of the microbiota in CRC (17, 18). Therefore, we analyzed the bacterial diversity (16S rDNA) in fecal samples from our mice and found that *L. casei* BL23 tended to restore the diversity disrupted by AOM injection. In agreement with previous reports (20), Firmicutes was the dominant phylum in the AOM/PBS-treated group. However, the introduction of *L. casei* BL23 reversed the ratio of Firmicutes to Bacteroidetes. Few individual species were affected by *L. casei* treatment, with the exception of *L. zeae*, which is actually considered a synonym of *L. casei* strain BL23 (26). However, future investigations should also consider analyzing the bacterial communities in the colic mucosa from tumor sections, because the assemblages present in stools are not necessarily an accurate reflection of the intestinal environment.

In conclusion, our work revealed that *L. casei* strain BL23 protected against CRC in an AOM/DSS model. Although this bacterium has well-known anti-inflammatory properties, we speculate instead that the protection observed here occurs through a reduction in cell proliferation and the induction of apoptosis.

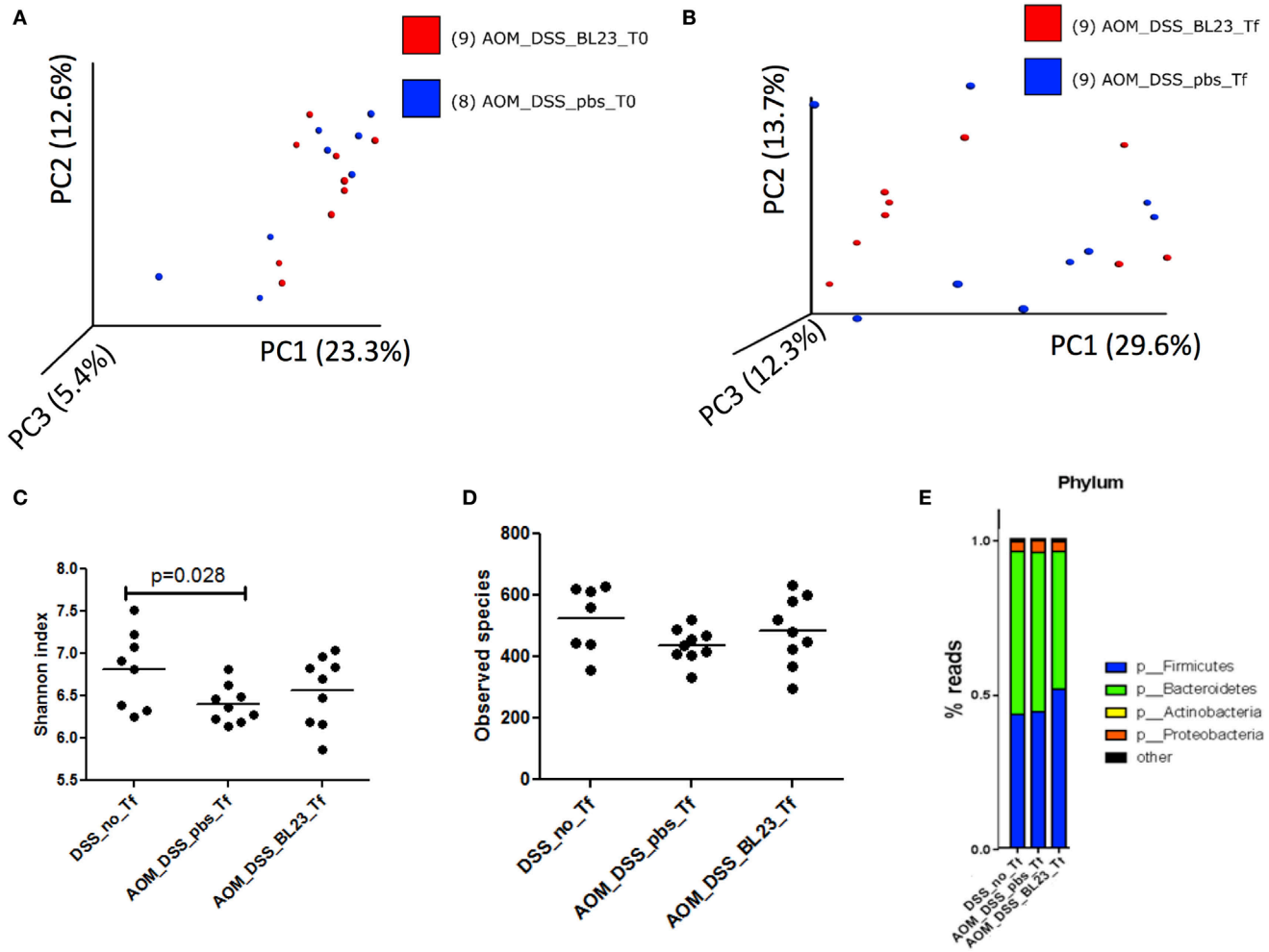

FIGURE 6 | Microbiota analysis. **(A)** Principal components analysis (PCA) of samples at T0. **(B)** PCA at Tf. **(C)** Phylum representation at T0 ($p = 0.028$, Student's *t*-test). **(D)** Phylum representation at Tf. **(E)** Upregulated and downregulated taxa in BL23 group.

TABLE 1 | Linear discriminant analysis effect size in BL23-treated group at Tf.

Taxa	LDA score	*p* Value
Bacteroidetes. Bacteroidia. Bacteroidales. Paraprevotellaceae. Prevotella	3.2891683	0.038
Firmicutes. Clostridia. Clostridiales. Ruminococcaceae	3.01945662	0.012
Firmicutes. Bacilli. Lactobacillales. Lactobacillaceae. *Lactobacillus zeae*	2.58044456	0.004
Tenericutes	2.573562	0.040
Tenericutes. Mollicutes	2.57347147	0.040
Actinobacteria. Actinobacteria. Bifidobacteriales. Bifidobarteriaceae. Bifidobacterium	−0.44318815	0.004
Firmicutes. Clostridia. Clostridiales	−0.43837615	0.011
Actinobacteria. Actinobacteria	−0.42251683	0.018
Actinobacteria. Actinobacteria. Bifidobacteriales. Bifidobacteriaceae. Bifidobacterium	−0.40710059	0.029
Firmicutes. Clostridia. Clostridiales. Clostridiaceae. Clostridium	−0.38186026	0.030
Proteobacteria. Alphaproteobacteria	−0.367064	0.019

ETHICS STATEMENT

All experiments were handled in accordance with institutional ethical guidelines, and the study was approved by the COMETHEA ethics committee ("Comité d'Ethique en Expérimentation Animale") of the Centre INRA of Jouy-en-Josas and AgroParisTech. Female C57BL/6 mice (6–8 weeks old; Janvier SAS, St. Berthevin, France) were maintained in sterile isolators at the INRA animal facility ($n = 5$ per cage) with 12 h light cycles and fed irradiated normal chow (R 03-40, SAFE) and water *ad libitum*.

AUTHOR CONTRIBUTIONS

EJ, LB-H, and FC conceived and designed the study. EJ and HS performed data analysis. EJ and LB-H wrote the manuscript. EJ conducted all experiments. FC provided technical help for the *in vivo* experiments. EJ, LB-H, PL, and HS discussed the experiments and results.

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FUNDING

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/article/10.3389/fimmu.2017.01553/full#supplementary-material.

FIGURE S1 | Cytokine expression levels in mesenteric lymphoid node. **(A)** IFN- γ, **(B)** IL-17A, **(C)** IL-6, **(D)** TNF-α, **(E)** IL-10, and **(F)** IL-22. Medians are represented for each group.

FIGURE S2 | Cytokine expression levels in spleen. **(A)** IFN-γ, **(B)** IL-17A, **(C)** IL-6, **(D)** IL-22, **(E)** TNF-α, **(F)** IL-10, and **(G)** IL-21. Medians are represented for each group.

FIGURE S3 | Cytokine expression levels in colon. **(A)** IFN-γ, **(B)** IL-17A, **(C)** IL-6, **(D)** TNF-α, **(E)** IL-10, and **(F)** IL-21. Medians are represented for each group.

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REVIEW
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Recent Advances on Nutrition in Treatment of Acute Pancreatitis

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Acute pancreatitis (AP) is a common abdominal acute inflammatory disorder and the leading cause of hospital admission for gastrointestinal disorders in many countries. Clinical manifestations of AP vary from self-limiting local inflammation to devastating systemic pathological conditions causing significant morbidity and mortality. To date, despite extensive efforts in translating promising experimental therapeutic targets in clinical trials, disease-specific effective remedy remains obscure, and supportive care has still been the primary treatment for this disease. Emerging evidence, in light of the current state of pathophysiology of AP, has highlighted that strategic initiation of nutrition with appropriate nutrient supplementation are key to limit local inflammation and to prevent or manage AP-associated complications. The current review focuses on recent advances on nutritional interventions including enteral versus parenteral nutrition strategies, and nutritional supplements such as probiotics, glutamine, omega-3 fatty acids, and vitamins in clinical AP, hoping to advance current knowledge and practice related to nutrition and nutritional supplements in clinical management of AP.

Keywords: clinical management of acute pancreatitis, nutritional interventions, probiotics, prebiotics, vitamins, amino acids, omega-3 fatty acids

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INTRODUCTION

Acute pancreatitis is the leading cause of acute hospital admission for gastrointestinal disorders in many countries, and its incidence continues to raise worldwide (1–3). The annual incidence of AP ranges from 13 to 45 cases per 100,000 population with the global estimate of 33.74 cases per 100,000 population, causing uneven burden across the globe. The health-care cost in the United States is reported to be $2.5 billion (1, 4, 5). Gallstones and alcoholism are the long-established two most common etiological factors, and other risk factors such as genetic predisposition, drugs, smoking, type 2 diabetes, and endoscopic retrograde cholangiopancreatography play a part (1, 3, 6). Clinical manifestations of AP vary from a mild edematous form to severe fulminant pancreatitis with potential devastating complications (7). Severity of AP is stratified into three categories: mild, moderately severe, and severe (**Table 1**). The overall mortality ranges from 5 to 20% depending on severity (8, 9). In patients who develop severe necrotizing pancreatitis, mortality is approximately 15%. In cases of infection of pancreatic necrosis and multi-organ failure, mortality

Abbreviations: AP, acute pancreatitis; ERCP, endoscopic retrograde cholangiopancreatography; SIRS, systemic inflammatory response syndrome; MODS, multiple organ dysfunction syndrome; ω-3 FAs, omega-3 fatty acids; PN, parenteral nutrition; EN, enteral nutrition; TPN, total parenteral nutrition.

can be as high as 30% (8). In China, the overall mortality rate of severe AP patients was estimated to be 11.8% (7). Up to date, a major challenge in search of targeted pharmacological therapy specific to AP, despite extensive efforts, is due to heterogeneous etiological factors and varying clinical manifestations associated with this condition (9, 10).

Pathophysiology of AP encompasses complex cascaded events of acinar cell inflammation, involvement of immune system, and systemic pathological outcomes (12) (**Figure 1**). Premature activation of intra-acinar digestive zymogens is one of the early hallmarks of AP. The resultant autodigestion of pancreas leads to release of pro-inflammatory mediators such as tumor necrosis factor-α, interleukin (IL)-1β, IL-6, which intermingle with microcirculation, causing increased vascular permeability, edema, hemorrhage, and necrosis of pancreas (13–15). Profound acinar cell injury and amplified inflammatory responses give rise to systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome (MODS), ultimately responsible for AP-associated mortality (16–18). The immune system is thought to play an important role in the disease pathogenesis of AP. Complex immunological events underlie progression of AP (12, 19). Dysregulated immune responses during AP include increased leukocyte counts, migration and activation of pro-inflammatory innate immune cells (neutrophils and macrophages) as well as depletion of T-lymphocytes and raised levels of plasma pro-inflammatory cytokines (12). Innate immune cells and derived inflammatory mediators as potential therapeutic targets have thus drawn much attention.

Better understanding of the pathophysiology of AP has drawn research efforts to reestablish the immune and organ/tissue homeostasis in clinical AP and toward the development of new intervention strategies (20). With still obscure disease-specific pharmacological therapies, developing managing strategies from randomized clinical trials are critical in the prevention of systemic complications during severe AP. Nutrition support and intervention is an important part of clinical management of patients with AP (21, 22).

TABLE 1 | AP classification.

Classification	Severity	Local complications	Systemic complications			Reference
			TOF	POF	EPC	
Atlanta 2012[a]	Mild	×	×	×	×	(10, 11)
	Moderate	√	√	×	√	
	Severe	√	×	√	√/×	
Determinant based[b]	Mild	×	×	×	N/A	
	Moderate	Sterile	√	×	N/A	
	Severe	Infected	√	√	N/A	
	Critical	Infected	×	√	N/A	

AP, acute pancreatitis; EPC, exacerbation of preexisting comorbidity; N/A, not applicable; POF, persistent organ failure; TOF, transient organ failure; √, yes; ×, no.

[a]In Atlanta 2012, local complications are subcategorized (interstitial edematous, necrotizing pancreatitis, infected necrotizing pancreatitis, other local complications, etc.), whereas systemic complications are defined as TOF or POF or an EPC (organ failure persisting for >48 h; three organ systems = renal, respiratory, cardiovascular; Marshal score ≥2).

[b]Sepsis-related organ failure assessment scoring system is used to define organ failure, and for severe pancreatitis, either POF or infected necrosis is mandatory.

NUTRITIONAL INTERVENTION IN CLINICAL AP

Nutrition and nutritional supplements have demonstrated necessity and importance not only in restoring energy balance but also

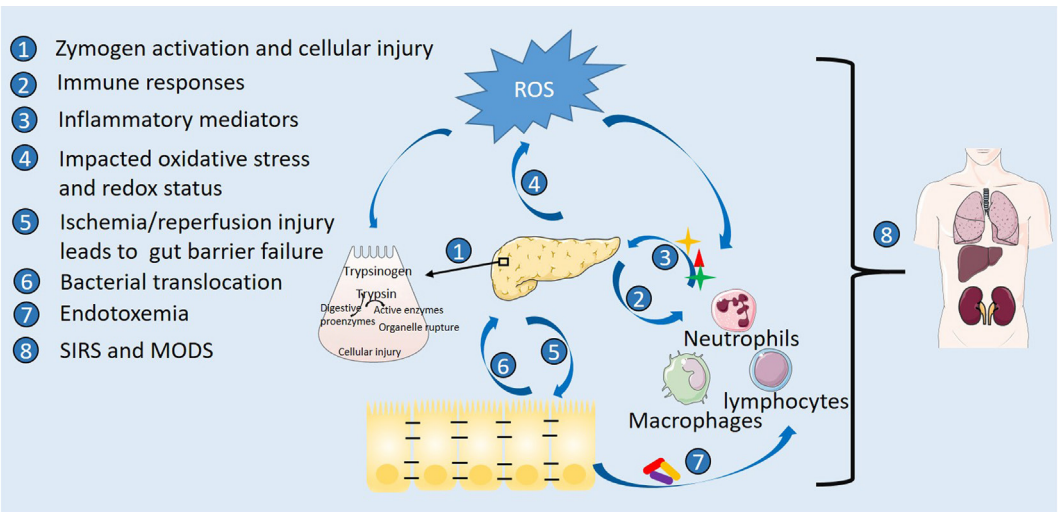

FIGURE 1 | Pathophysiology of acute pancreatitis highlighting sites of action by nutrition. Etiological stress triggers premature activation of digestive zymogens and intra-acinar cellular injury with accompanying oxidative stress. Involvement of immune cells with released inflammatory mediators and amplified oxidative stress exacerbate the inflammatory cascade. Gut inflammation and barrier failure occur following systemic inflammatory responses, vascular disturbance, and ischemia/reperfusion injury secondary to pancreatic inflammation. Disrupted barrier function further leads to bacterial translocation, pancreatic infection and necrosis, and endotoxemia, ultimately responsible for multiple organ dysfunction syndrome (MODS) and death.

in maintaining gut barrier function and providing important immunomodulatory and antioxidant effects (**Figure 2**). The gut is an important secondary organ and also a site of starting severe systemic complications during AP. Intestinal barrier dysfunction is associated with translocation of bacteria and their inflammatory and toxic products, responsible for infection of the necrotic pancreas and systemic inflammatory responses. Therefore, maintaining the integrity of the gut barrier in the small intestine is one of the main goals in early-phase treatment of severe AP (23). Optimal nutritional support in AP has been under debate for decades. Bowl at rest (*nothing by mouth*) strategy has been implemented conventionally to treat AP (24, 25). However, dietary restrictions exacerbate patient's malnutrition due to imbalance between reduced food intake and higher nutritional requirements, leading to further catabolism, bacterial translocation (26), and ultimate mortality (27). Evidence of clinical trials has demonstrated parenteral nutrition (PN) in preventing pancreatic stimulation and many benefits of enteral nutrition (EN). However, in daily practice, it remains challenging to predict whether EN will be tolerated in patients with AP (8).

Strategic approaches to include nutritional supplements have also been attempted to provide additional immune regulatory

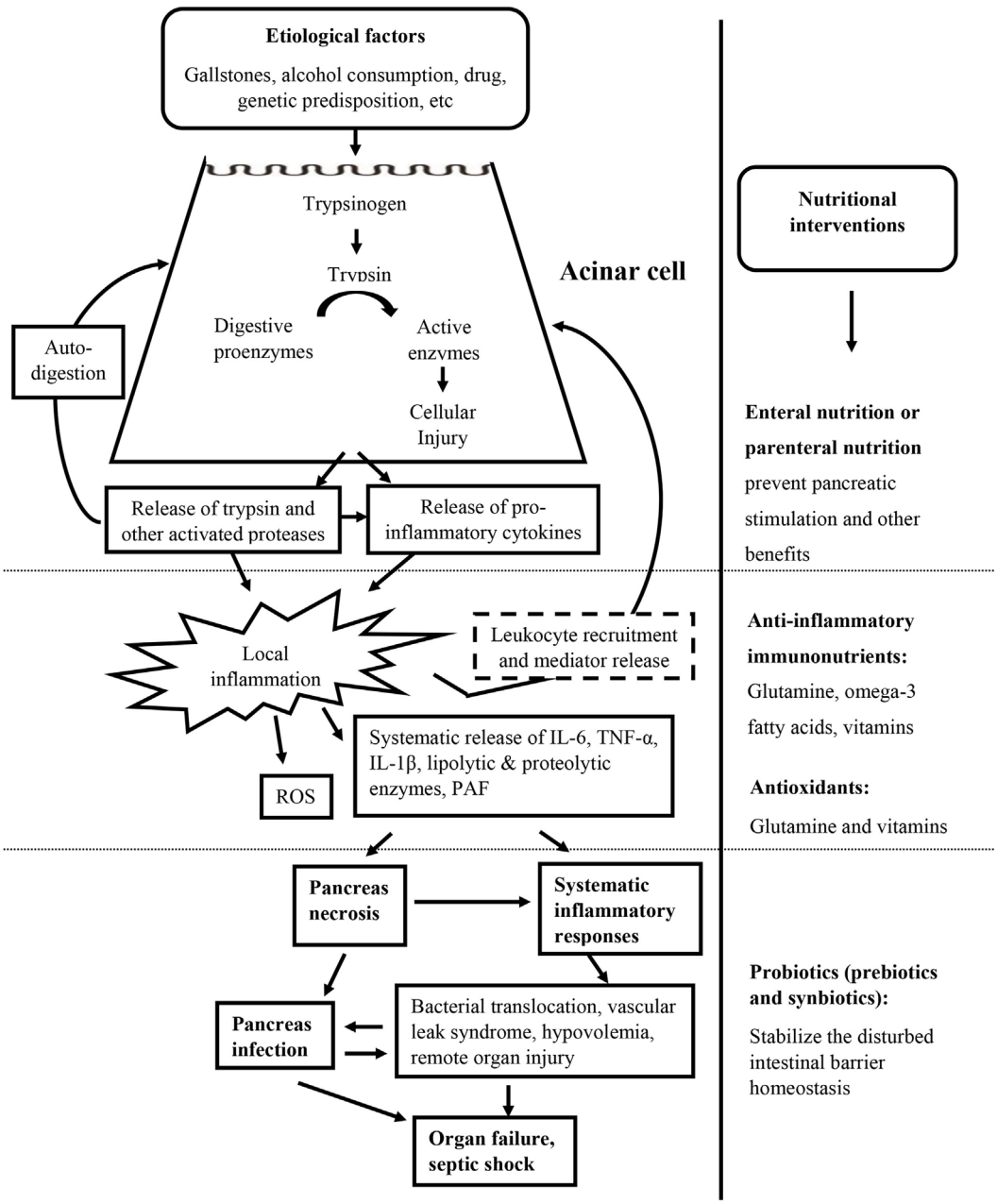

FIGURE 2 | Targeted nutritional interventions during the whole episode of acute pancreatitis. Targeted nutritional interventions: enteral or parental nutrition and nutritional supplements including anti-inflammatory immunonutrients, antioxidants, and probiotics are presented at the administration stage.

and antioxidative effects. Probiotics and prebiotics have been shown to stabilize the disturbed intestinal barrier homeostasis and be beneficial in reducing the infection rate in primary clinical trials (28–31). Due to the immunosuppressive and inflammatory nature of the disease, immunonutrients like glutamine and omega-3 fatty acids (ω-3 FAs) have been added to parenteral or enteral formulas to modulate immune functions, suppress the hyper inflammatory responses, and reestablish tissue and organ homeostasis in clinical practice (21, 32, 33). Supplements with antioxidative properties like glutamine and vitamin C have also been suggested to provide additional beneficial effects (34).

The review aims to provide a comprehensive chronological review on latest clinical trials on EN versus PN strategies and nutritional supplements including probiotics (prebiotics and synbiotics), glutamine, ω-3 FAs, and vitamins, hoping to provide the basis for future development of nutritional strategies in clinical AP.

EN VERSUS PN

Traditionally, AP patients were maintained on *nil per os* or *nothing per mouth* treatment until resolution of pain or normalization of pancreatic enzymes to allow the pancreas to rest (35). Currently, it is widely accepted that early EN may be critical to improve AP-associated malnutrition and the overall outcomes, as bowel rest is associated with intestinal mucosal atrophy and increased infectious complications (9). Gut barrier dysfunction is found in approximately 60% of patients with AP (8, 36). Importantly, EN exerts immunomodulatory effects to preserve gut mucosa integrity, stimulate intestinal motility, and reduce bacterial overgrowth (8, 37). A randomized clinical study demonstrated that immediate oral feeding in patients with mild AP was feasible and safe and accelerated recovery without adverse gastrointestinal events (38). Another randomized controlled trial supported early-stage introduction of initial oral nutrition with either a clear liquid diet or a low-fat solid diet for patients who developed mild AP (39). In these patients, if oral intake is not tolerated, enteral feeding is recommended (9). In patients with severe AP or predicted severe AP, EN with oral or tube feeding thought to preserve the gut barrier function to prevent bacterial translocation is preferred over PN. A multicenter randomized study in the *New England Journal of Medicine* demonstrated that early tube feeding and oral diet after 72 h are equivalent in reducing infection rates or death in AP patients at high risk for complications (40). A Cochrane meta-analysis of eight randomized controlled studies found that EN reduced mortality, systemic infections, and multiorgan failure among patients with AP as compared to PN (41). Another meta-analysis of 381 patients confirmed the benefit of EN versus PN support in patients with severe AP with lower mortality, fewer infectious complications, decreased organ failure and surgical intervention rate (42). Over the optimal route of EN, several trials have suggested the nasogastric route as an alternative to nasoduodenal or nasojejunal routes (43). Multiple randomized controlled trials involving 157 patients with predicted severe AP demonstrated that nasogastric feeding was safe and well tolerated compared with nasojejunal feeding (44). Given its demonstrated beneficial outcomes, it remains challenging to predict whether EN will be tolerated in patients with AP (8). However, as shown by multiple randomized trials that have associated total PN (TPN) with risks of infection and other complications (35), PN should still be minimized unless the enteral route is not available, not tolerated, or not meeting caloric requirements.

NUTRITIONAL SUPPLEMENTS

Probiotics, Prebiotics, and Synbiotics

Changes in intestinal motility and microbiome, immune response, and mucosal barrier function during AP lead to bacterial translocation and subsequent pancreatic necrosis infection, which is one of the principal causes of complications and death in severe AP patients (45). Potential roles of probiotics have been proposed for immunomodulatory and health-promoting benefits to restore the gut integrity, modulate immune responses against invading pathogens, and prevent proliferation of harmful bacteria beyond those of basic nutrition, which have been evaluated in a number of clinical trials (**Table 2**).

An early indication of beneficial effects of synbiotics on severe AP-associated endotoxemia came from a randomized, double-blind clinical trial with 45 patients receiving either live or heat-inactivated *Lactobacillus plantarum 299* with oat fiber supplement as early EN. The results suggested that supplementary combined pre- and probiotics was effective in reducing infected pancreatic necrosis and surgical interventions (46, 47). The findings were subsequently supported and extended by a larger study with 62 patients on the Synbiotic 2000 formulated early EN with four different types of prebiotics (inulin, beta-glucan, resistant starch, and pectin) and probiotics (four different *Lactobacilli* preparations). Patients receiving synbiotic therapy had reduced total incidence of SIRS and lower rates of organ failure, supporting that early EN with synbiotics may prevent organ dysfunctions in the late phase of severe AP (48). The effects of *L. plantarum* only enteral feeding were evaluated in 76 patients with AP. Overall, the patients with ecoimmunonutrition showed attenuated disease severity, improved intestinal permeability, and better clinical outcomes (49). Prebiotic fiber alone supplementation with EN assessed in a randomized, double-blind study with 30 consecutive severe AP patients was found to shorten hospital stay, duration of nutrition therapy, and reduce the acute phase response and overall complications compared to standard EN therapy (50). Probiotic prophylaxis in severe AP has been contraindicated. The Dutch Acute Pancreatitis Study Group reported in PROPATRIA, a multicenter, randomized, double-blind, placebo-controlled trial with in a total of 200 patients with predicted severe AP that multispecies probiotic (Ecologic 641: six probiotic strains) prophylaxis did not reduce the risk of infectious complications and was associated with an increased risk of mortality (55, 56), although overall this combination of probiotic strains reduced bacterial translocation (52). Following studies involving multispecies probiotic supplementation with EN early abandoned after the publication of PROPATRIA study seemed to support the results that no significant trend was identified for an effect of probiotics on gut permeability or endotoxemia in AP (53, 57), although a positive effect was observed with reduced endotoxin levels (57).

TABLE 2 | Characteristics of clinical trials on probiotic treatment in AP.

Reference	Probiotic(s) or prebiotic(s) tested	Comparison groups	Gut barrier permeability		Systemic complications				
			Methods	Results	Infected necrosis	SIRS	MODS	Infection	Mortality
Olah et al. (46)	*Lactobacillus plantarum* 299 plus oat fiber ($10^9 \times 2$/daily dose)	EN + symbiotic + fibers versus EN + heat-inactivated symbiotic + fibers	–	–	No difference	No difference	No difference	↓ pancreatic infection requiring operation in the probiotic arm	No difference
Kecskes et al. (47)	*L. plantarum* 299 plus oat fiber	EN + symbiotic + fibers versus EN + heat-inactivated symbiotic + fibers	–	–	↓ in symbiotic arm	–		–	–
Olah et al. (48)	Multistrain (40×10^9/daily dose) and multifibers	EN + fibers versus EN + fibers + symbiotic	–	–	↓ in symbiotic arm	↓ SIRS + MODS in symbiotic arm		↓ surgical interventions in the probiotic arm	No difference
Qin et al. (49)	*L. plantarum* (unspecified strain) (10^{10}/daily dose)	TPN versus partial PN + EN + probiotics	Lactulose/rhamnose urinary excretion	↓ in the probiotic arm	–	↓ SIRS in the probiotic arm	↓ MODS in the probiotic arm	↓ infective complications in the probiotic arm	No difference
Karakan et al. (50)	Multifibers	EN + multifibers versus EN	–	–	–	No difference	No difference	–	No difference
Besselink et al. (51)	Multistrain product (10^{10}/daily dose) plus maltodextrins and cornstarch	EN + placebo versus EN + probiotics	–	–	No difference	–	↑ MODS in the probiotic arm	No difference	↑ in the probiotic arm due to NOMI
Besselink et al. (52)	Multistrain product (10^{10}/daily dose)	EN + placebo versus EN + probiotics	PEG urinary excretion	No difference	–	–	–	–	–
Sharma et al. (53)	Multistrain product (10^{10}/daily dose)	Placebo versus probiotics (through the current mode of feeding)	Lactulose/rhamnose urinary excretion	No difference	–	–	No difference	↓ endotoxin core antibody IgG, IgM in the probiotic arm	No difference
Cui et al. (54)	Multistrain product 1×10^{11}/12 h	PN versus EN versus EN + probiotics (PN)	–	–	↓ in the EN arm and EN + probiotics arm	–	–	–	No difference

AP, acute pancreatitis; EN, enteral nutrition; MODS, multiple organ dysfunction syndrome; PN, parenteral nutrition; SIRS, systemic inflammatory response syndrome; TPN, total parenteral nutrition.

Recently, a local study of 70 patients with severe AP comparing PN, EN and EN with addition of the probiotic *Bifidobacterium* found that early EN with *Bifidobacterium* resulted in lower levels of pro-inflammatory cytokines, improved gastrointestinal function, reduced complications, and shorter hospital stay in patients with severe AP (54). These data suggest the potential of single specific probiotic strains supplemented, which however should be further evaluated by validated clinical trials before their beneficial effects could be confirmed.

Glutamine

Glutamine is an important constituent of intra and extracellular amino acid pool, with immune modulatory and antioxidant effects, and its depletion has been demonstrated in critical illness (58). Glutamine improves immune cell functions and contributes to antioxidative defenses. It can also support the intestinal integrity and decrease bacterial translocation; hence reduce systemic inflammatory responses and sepsis, which are important in critical illnesses such as AP (33).

An early randomized, controlled study with 28 AP patients received either a standard TPN or an isonitrogen, isocaloric TPN containing 0.3 g/kg L-alanine-L-glutamine demonstrated that glutamine supplementation with TPN was associated with a significant increase of cholinesterase, albumin, and lymphocyte count in AP as well a decrease of C-reactive protein compared to standard TPN. AP patients receiving glutamine was associated with a reduced length of TPN and a trend of reduced length of hospital stay, suggesting that glutamine substitution in TPN is beneficial in patients with AP (59). The effects of glutamine enriched (0.3 g/kg/day) TPN when further evaluated in 40 patients with AP. Beneficial effects of glutamine supplementation to TPN were found on acute pancreatic responses with serum

lipase, amylase activities, and C-reactive protein levels decreased and the prevention of complications in patients with AP (59). Later, the effect of parenteral glutamine on recovery from severe AP was more thoroughly investigated in a randomized trial with 44 patients. L-alanyl-L-glutamine-supplemented PN increased serum IL-10 levels, improved nitrogen balance, and decreased infectious morbidity in patients with severe AP (60). Enterally, supplementation of glutamine and arginine in patients diagnosed of AP and predicted to develop a severe course was found to improve gut barrier function by reducing the gut permeability and decreasing plasma endotoxin level in the early stage of severe AP (61). Other than glutamine supplemented with TPN and EN, intravenously administered glutamine with early nasojejunal nutrition was also evaluated. In a randomized study, 45 patients with severe AP received glutamine or normal amino acid solution together with nasojejunal nutrition. The results demonstrated that the glutamine-receiving group showed signs of improvement in all end-point measurements including the rate of pancreas-specific infectious complications, organ failure, length of hospital stay, and mortality rate; and statistical significant difference was noted only in the length of hospital stay (62). Furthermore, a randomized trial compared early versus late intravenous infusion of alanylglutamine dipeptide in 76 patients with severe AP and demonstrated that early-stage intervention achieved a better clinical outcome: shortened duration of hospitalization, reduced rate of infection, organ dysfunction, need for surgery, and mortality, compared to the late treatment (63). More recently, glutamine supplemented in combination with normal saline and hydroxyethyl starch in resuscitation fluids were more efficient in relieving inflammation and sustaining the intestinal barrier in patients with severe AP (64). Two recent meta-analysis studies of randomized controlled trials demonstrated that glutamine supplementation resulted in significantly reduced mortality and complications (65, 66). Further analysis suggested a clear advantage for glutamine supplementation in patients who received TPN. In contrast, patients with AP who received EN did not require glutamine supplementation (65). Finally, oral glutamine supplementation did not seem to confer any significant effect on gut permeability and endotoxemia in severe AP (67). Characteristics of clinical studies on glutamine supplementation included in this review have been summarized in **Table 3**. Together, while glutamine supplementation with TPN shows promising clinical outcomes, enteral glutamine supplementation needs to be investigated in future.

Omega-3 Fatty Acids

Dietary polyunsaturated fatty acids have known immunomodulatory and other beneficial health-promoting effects. A prospective cohort study on the association of fish consumption and non-gallstone-related AP has suggested that total fish (fatty fish and lean fish combined) consumption may be associated with decreased risk of non-gallstone-related AP (68). A randomized prospective clinical trial assessing enteral formula enriched with ω-3 FAs in the treatment of AP suggested that EN supplemented with ω-3 FAs seemed to have clinical benefits based upon the shortened time of jejunal feeding and hospital stay (69). Subsequently, independent studies evaluated the effects of PN with ω-3 FA supplementation on severe AP. Wang et al. compared in a randomized, double-blind trial a total of 40 severe AP patients receiving PN with the same basal nutrients but different lipid compositions: soybean oil-/fish oil-based fat solutions. The study showed that patients with ω-3 FAs-supplemented PN had increased eicosapentaenoic acid concentrations and decreased pro-inflammatory cytokines, together with improved respiratory function and shortened continuous renal replacement therapy time, suggesting attenuated systemic responses to pancreatic and organ injury (70). A parallel study by the same group enrolling 56 patients who received isocaloric and isonitrogenous PN with fats of all ω-6 FAs or 4:1 ω-6:ω-3 FAs demonstrated that ω-3 FAs-supplemented PN elevated the IL-10 level and human

TABLE 3 | Characteristics of clinical trials on glutamine as the nutritional supplement in AP.

Reference	Subjects/ regions	Dosage (g/kg BW/day)	Method of assessment	AD-EN or PN interval (h)	Duration of EN or PN (days)		Infectious complication (*n*/*N*)		Mortality (*n*/*N*)		DOS (median or days mean ± SD)	
					Cont.	Interv.	Cont.	Interv.	Cont.	Interv.	Cont.	Interv.
Ockenga et al. (59)	28/ Germany	0.3	APACHE CT severity index	<72	10–18	6–16	5/14	4/14	1/14	0/14	25 (19–40)	21 (14–32)
Fuentes-Orozco et al. (60)	44/Mexico	0.4	APACHE CT severity index	24–48	17.5 ± 7.9	19.31 ± 12.62	16/22	9/22	5/22	2/22	26.59 ± 13.3	30.18 ± 10.42
Huang et al. (61)	32/China	0.099	APACHE	<72	–	–	2/18	2/14	0/18	0/14	20 ± 5	22 ± 5
Hajdu et al. (62)	45/ Hungarian	0.5	–	48	–	–	–	–	3/21	0/24	15.9	10.6
Xue et al. (63)	76/China	20 g/day/ person	APACHE CT severity index	<24	–	–	10/38	3/38	8/38	2/38	45.2 ± 27.1	28.8 ± 9.4
Singh et al. (67)	80/India	20 g/day/ person	APACHE CT severity index	<120	7	7	19/39	21/41	6/39	5/41	11 (2–36)	12 (1–101)

AD, the interval between admittance to ICU and start of enteral or parenteral nutrition; AP, acute pancreatitis; APACHE, acute physiology and chronic health evaluation; Cont., control; DOS, duration of hospital stay; EN, enteral nutrition; Interv., intervention; PN, parenteral nutrition.

leukocyte antigen-DR expression in severe AP patients (71). In accordance, during the initial stage of severe AP, parenteral supplementation with ω-3 fish oil emulsion was found to suppress SIRS, modulate the balance of pro-/anti-inflammatory cytokines and thus improve AP-associated severe conditions (72). Clinical studies on ω-3 FA supplementation have been summarized in **Table 4**. Although polyunsaturated FAs remain potential beneficial supplements with EN/PN, further larger trials are needed for formulations and confirmatory beneficial clinical effects.

Vitamins

Oxidative stress is involved in the onset of AP and also in the development of the systemic inflammatory responses, being glutathione depletion, xanthine oxidase activation, and thiol oxidation in proteins critical features of the disease in the pancreas. Vitamins as important immunonutrients and antioxidants have been inversely associated with AP (73). Plasma concentrations of vitamin A and vitamin C were found significantly lower in AP patients than in healthy controls ($P < 0.05$) (74). Recently, vitamin D, mainly from the milk products, has been inversely associated with gallstone-related AP (73). Vitamin supplementation assessed in combination with other antioxidants or in vitamin-only therapy has been evaluated earlier and yielded mixed outcomes. A multicenter randomized, double-blind, placebo clinical trial by Siriwardena et al. concluded that use of intravenous combination antioxidant therapy containing vitamin C (*N*-acetylcysteine, selenium, vitamin C) was not justified to continue in clinical severe AP (75). Subsequently, another group comparing vitamin C, *N*-acetylcysteine, antoxyl forte antioxidant combination with standard medical treatment in early AP patients suggested that antioxidant supplementation could decrease the length of hospital stay and complications in patients with early AP, but this hypothesis needed to be supported by a larger clinical trial (76). With respect of vitamin-only antioxidant therapies, a study involving 84 AP patients and 40 healthy subjects in China on high-dose vitamin C has demonstrated that it has therapeutic

TABLE 4 | Characteristics of clinical trials on ω-3 FAs as the nutritional supplements in AP.

Reference	Subjects/ regions	Dosage (g/kg BW/day)	Method of assessment	AD-EN or PN interval (h)	Duration of EN or PN (days)		Infectious complication (*n*/*N*)		Mortality (*n*/*N*)		DOS (days mean ± SD)	
					Cont.	Interv.	Cont.	Interv.	Cont.	Interv.	Cont.	Interv.
Lasztity et al. (69)	28/ Hungary	3.3 g/day	APACHE CT severity index	<24	17.57 ± 10.52	10.57 ± 6.70	–	–	1/14	2/14	19.28 ± 7.18	13.07 ± 6.70
Wang et al. (70)	40/China	0.2	APACHE	<72	5	5	5/20	3/20	2/20	0/20	70.5 ± 9.1	65.2 ± 7.3
Wang et al. (71)	28/China	0.2	APACHE CT severity index	<72	5	5	9/28	6/28	2/28	0/28	–	–

AD, the interval between admittance to ICU and start of enteral or parenteral nutrition; AP, acute pancreatitis; APACHE, acute physiology and chronic health evaluation; Cont., control; DOS, duration of hospital stay; EN, enteral nutrition; Interv., intervention; PN, parenteral nutrition; ω-3 FAs, omega-3 fatty acids.

TABLE 5 | Characteristics of clinical trials on vitamins as the nutritional supplements in AP.

Reference	Subjects/ region	Vitamin(s) tested	Dosage (g/kg BW/day)	Method of assessment	Duration of EN or PN (days)		Mortality (*n*/*N*)		DOS (days mean ± SD)	
					Cont.	Interv.	Cont.	Interv.	Cont.	Interv.
Siriwardena et al. (75)	43/UK	Vitamin C + *N*-acetylcysteine, selenium	For vitamin C, 2 g/day for 2 days, 1 g/day (continued for up to day 7)	APACHE	7	7	0/21	4/22	14.3 (15.7)	20.4 (24.4)
Sateesh et al.(76)	53/India	Vitamin C, *N*-acetyl cysteine, and antoxyl forte	Vitamin C 500 mg, *N*-acetyl cysteine 200 mg 8 hourly and antoxyl forte 1 capsule hourly	APACHE CT severity index	–	–	0/30	1/23	10.3 ± 7	7.2 ± 5
Du et al. (77)	84/China	Vitamin C	10 or 1 g/day (con)	Detection of clinical, biochemical, and immunological markers	5	5	–	–	13.45 ± 3.21	9.34 ± 4.24
Bansal et al. (78)	39/India	Vitamin A, vitamin E, vitamin C	Vitamin C (1,000 mg in 100 ml saline), vitamin E (200 mg oral), and vitamin A (10,000 IU)	APACHE CT severity index	14	14	2/20	0/19	15.1 ± 5.43	12.8 ± 3.9

AP, acute pancreatitis; APACHE, acute physiology and chronic health evaluation; Cont., control; DOS, duration of hospital stay; EN, enteral nutrition; Interv., intervention; PN, parenteral nutrition.

efficacy on the disease and proposed the potential mechanisms to be promoting anti-oxidizing capability in patients, blocking lipid peroxidation and improving cellular immune function (77). In contrast, multiple vitamins-based antioxidant therapy (vitamin A, vitamin C, and vitamin E) in a single-center randomized study involving 39 patients has not been proven beneficial in patients with established severe AP (78). Collectively, data so far on vitamin therapy in AP (**Table 5**) have been mixed and should be carefully evaluated for dosing and timing of intervention for potential promising outcomes in clinical use.

CONCLUSION AND FUTURE PERSPECTIVES

In most patients, an oral soft or solid diet can be beneficial if tolerated. When oral feeding is not tolerated for a few days, enteral feeding through a nasogastric or nasojejunal feeding tube should be attempted within the first 72 h of administration. PN should be minimized for its risks of infection and other complications. Only if enteral route is not available or tolerated, PN may be considered. Overall, nutritional support plays a critical role in clinical management of severe AP, although the optimal timing remains unclear. Predicting the nutritional tolerance of patients with AP remains challenging as the current evaluation system needs to be improved. Various nutritional supplement(s) together with PN or EN with currently mixed clinical outcomes is a subject of interest for future evaluation and may lead to promising outcomes. In addition, given its heterogeneous etiological factors and varying clinical manifestations, precision medicine, although not much applied in the condition, remains as a temping approach to optimize clinical outcomes on classified individuals based on susceptibility to the condition and its systemic complications.

AUTHOR CONTRIBUTIONS

JS designed the subject content of the review article. L-LP, JL, MS, and JS conducted initial search of literature, drafted the manuscript, and prepared the figures and tables. MB gave the constructive comments and critically reviewed the manuscript. JS had primary responsibility for final content. All authors read and approved the final manuscript.

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Inulin-Type Fructans Modulates Pancreatic–Gut Innate Immune Responses and Gut Barrier Integrity during Experimental Acute Pancreatitis in a Chain Length-Dependent Manner

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Acute pancreatitis (AP) is a common abdominal inflammatory disorder and one of the leading causes of hospital admission for gastrointestinal disorders. No specific pharmacological or nutritional therapy is available but highly needed. Inulin-type fructans (ITFs) are capable of modifying gut immune and barrier homeostasis in a chemistry-dependent manner and hence potentially applicable for managing AP, but their efficacy in AP has not been demonstrated yet. The current study aimed to examine and compare modulatory effects of ITFs with different degrees of fermentability on pancreatic–gut immunity and barrier function during experimentally induced AP in mice. BALB/c mice were fed short (I)- or long (IV)-chain ITFs supplemented diets for up to 3 days before AP induction by caerulein. Attenuating effects on AP development were stronger with ITF IV than with ITF I. We found that long-chain ITF IV attenuated the severity of AP, as evidenced by reduced serum amylase levels, lipase levels, pancreatic myeloperoxidase activity, pancreatic edema, and histological examination demonstrating reduced pancreatic damage. Short-chain ITF I demonstrated only partial protective effects. Both ITF IV and ITF I modulated AP-associated systemic cytokine levels. ITF IV but not ITF I restored AP-associated intestinal barrier dysfunction by upregulating colonic tight junction modulatory proteins, antimicrobial peptides, and improved general colonic histology. Additionally, differential modulatory effects of ITF IV and ITF I were observed on pancreatic and gut immunity: ITF IV supplementation prevented innate immune cell infiltration in the pancreas and colon and tissue cytokine production. Similar effects were only observed in the gut with ITF I and not in the pancreas. Lastly, ITF IV but not ITF I downregulated AP-triggered upregulation of IL-1 receptor-associated kinase 4 (IRAK-4) and phosphor-c-Jun N-terminal kinase (p-JNK), and a net decrease of phosphor-nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) p65 (p-NF-κB p65) nuclear translocation and activation in the pancreas. Our findings demonstrate a clear chain length-dependent effect of inulin on

AP. The attenuating effects are caused by modulating effects of long-chain inulin on the pancreatic–gut immunity via the pancreatic IRAK-4/p-JNK/p-NF-κBp65 signaling pathway and on prevention of disruption of the gut barrier.

Keywords: dietary fibers, inflammation, pancreatic–intestinal immunity, signaling kinases, tight junction proteins, antimicrobial peptides

INTRODUCTION

Acute pancreatitis (AP) is a sudden inflammation of the pancreas caused by inappropriate activation of local digestive enzymes. During the past decades, it has become the leading cause of hospital admission for gastrointestinal disorders in many countries and the worldwide incidence is increasing (1). Although the mild form of AP is self-limited, nearly 20–25% of patients with AP develop severe symptoms with systemic inflammatory responses. The mortality rate in these patients is as high as 30% (2). Up to now, a targeted pharmacological or nutraceutical therapy specific for AP management is lacking (3, 4). Novel insight however in function–effector relationship of dietary components and their anti-inflammatory effects has generated optimism about the possibility to create nutraceutical strategies to control local inflammation and prevent systemic complications of AP (2).

Pathophysiological studies of AP have revealed that AP is caused by unregulated intra-acinar activation of trypsin and other digestive enzymes, leading to autodigestion of the pancreas and local inflammation. The main triggering factors include pancreatic hyperstimulation, gallstones, and alcohol abuse (5). The induced local inflammation is accompanied by activation of endothelial cells and transendothelial migration of leukocytes, neutrophils, and macrophages, leading to release of harmful enzymes and cytokines that amplify the local inflammatory responses. As a consequence, pancreatic complications such as acinar cell necrosis, pseudocyst formation, and abscess development might occur in most severe cases, leading to transmission of inflammation to other remote organs, which is ultimately responsible for AP-associated mortality.

Innate immune activation and acinar cell inflammatory signaling play a pivotal role in the pathogenesis of AP (5–8). Local inflammation is initiated by acinar cell damage and local production of pro-inflammatory cytokines/chemokines by these cells, followed by infiltration of neutrophils and macrophages (9). Neutrophils, macrophages, and dendritic cells with distinct cell-surface and intracellular markers have been associated with the development of AP and severity of the inflammatory conditions (10–13). The balance between pro-inflammatory and anti-inflammatory mediators produced from acini and infiltrated immune cells determines the outcome of the AP. Tumor necrosis factor (TNF)-α, interleukin (IL)-1, and IL-10 are important cytokines in AP (11, 14–16). TNF-α is released by local Ly6C^{hi} monocytes/macrophages in the pancreas and enhances the severity of the experimental disease (11, 17, 18). Regulatory cytokines, such as IL-10, limit the local and systemic consequences of experimental pancreatitis (18). Expression of these cytokines is under the control of the transcription factor NF-κB. NF-κB activation in pancreatic acinar cells is responsible for the expression of a large number of inflammation-related genes (19). NF-κB activation is regulated by its upstream signaling kinases, such as MAP kinases (MAPKs), which may be activated by receptor-associated kinases, such as IL-1 receptor-associated kinase 4 (IRAK-4) and transforming growth factor-β activated kinase (TAK) (20, 21). Consequently, modulation of immune cell activation, inflammatory cytokine production, and pancreatic inflammatory signaling molecules may be rational approaches to alleviate AP symptoms.

Accumulating evidence is available demonstrating that severe AP is associated with changes in the microcirculation, gut permeability/motility, bacterial translocation, and activation of the gut-associated lymphoid tissue. Preventing AP-associated intestinal barrier disruption or inflammation might therefore be a key target for effective therapy (22). A possible nutraceutical approach to prevent intestinal barrier disruption is by intervening with anti-inflammatory food components such as specific dietary fibers. Dietary fibers may be fermented by the gut microbiota to produce health-promoting short-chain fatty acids (SCFAs) and modify intestinal barrier function (23). Therefore, consumption of dietary fibers represents a promising strategy to modulate the progression of AP. We have recently demonstrated that low-methoxyl lemon pectin (LMP) attenuated inflammatory responses and improved intestinal barrier integrity in experimental AP (2). Another family of nutritional molecules with supporting effect on barrier function and anti-inflammatory effect that might be instrumental for management of AP is inulin-type fructans (ITFs) (23–27). ITFs are a family of dietary fibers belonging to beta(2→1) linear fructan-type carbohydrate subgroup and have an impact on gastrointestinal functions, which is largely related to their biochemical and physiological attributes. In the gut, ITFs are rapidly fermented to produce SCFAs that exert some of the local and systemic effects of ITFs. In addition, ITFs have demonstrated clear chemistry or degree of polymerization (DP)-dependent effects (28). Previous studies have demonstrated that DP or chain length of ITFs that affects the magnitude of receptor activation and/or their site of fermentation in the gut determines their prebiotic and immune modulatory effects on immune cells, gut barrier function, and microbiota composition (23–27, 29). ITFs have been implicated in a variety of inflammatory and

Abbreviations: AP, acute pancreatitis; AMPs, antimicrobial peptides; ANOVA, analysis of variance; CRAMP, cathelicidin-related antimicrobial peptide; DEFB1, β-defensin-1; DP, degree of polymerization; ERK, extracellular signal-regulated kinase; H&E, hematoxylin and eosin; IL, interleukin; IRAK, IL-1 receptor associated kinase; ITF, inulin-type fructan; JNK, c-Jun N-terminal kinase; LMP, low-methoxyl pectins; MAPKs, mitogen-activated protein kinases; MPO, myeloperoxidase; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; RT-qPCR, real-time quantitative polymerase chain reaction; SCFAs, short-chain fatty acids; TAK, TGF-β activated kinase; TGF-β, transforming growth factor-β; TJ, tight junction; TNF-α, tumor necrosis factor-α; ZO-1, zonula occludens-1.

immunological dysfunctions. However, their potential effects have not been studied in the management of AP.

In this study, we examined and compared the effects of long-chain ITF IV and the more readily fermentable short-chain ITF I on their protective effects during experimentally induced AP. In a caerulein-induced AP mice model, we studied after ITF treatment severity of pancreatitis, the frequencies of infiltrating neutrophils, macrophages, and dendritic cells in the pancreas and colon as well as production of the cytokines TNF-α, IL-1β, and IL-10. Additionally, we studied colon integrity by determining expression of gut–epithelial tight junction (TJ) modulatory proteins and barrier reinforcing immunomodulatory antimicrobial peptides (AMPs). Finally, we investigated the signaling pathways and kinases modulated by ITFs to gain insight in the mechanisms by which ITFs modulate AP.

MATERIALS AND METHODS

Fibers and Structural Characterization

The applied ITF I (frutalose OFP, $2 < DP < 25$) and ITF IV (FrutafitTEX, $10 < DP < 60$) were extracted from chicory roots (Sensus B.V., Roosendaal, The Netherlands). Their specific chain length profiles (range and distribution) were characterized by high-performance anion exchange chromatography as previously described (23, 24).

Animals

All animal-related experimental protocols were approved by the Institutional Animal Ethics Committee of Jiangnan University in compliance with the recommendations of national and international guidelines for the Care and Use of Laboratory Animals, and were performed in accordance with the guidelines therein. Eight-week-old female BALB/c mice, 20 ± 2 g (Su Pu Si Biotechnology, Suzhou, Jiangsu, China) reared on *ad-libitum* access to standard laboratory chow and water were used in this study. The animals were maintained at *Animal Housing Unit* of the University under controlled temperature (23–25°C), pathogen-free conditions, and at a 12:12 h light:dark cycle.

Experimental Design and AP Induction

One-week time was allowed for the acclimatization of animals before starting the experiment(s). Mice were randomly divided into four groups ($n = 4–6$) according to different diets and fed for 72 h (**Table 1**).

After 12 h of fasting, AP was induced in mice by eight repeated intraperitoneal (i.p.) injections of caerulein (50 μg/kg/h) (Sigma-Aldrich, St. Louis, MO, USA). The littermates in control group were injected (i.p.) with the same volume of normal saline and served as controls.

TABLE 1 | Different groups of animals with their corresponding diet used in this study.

Group	Diet
Control	Normal diet without AP
AP	Normal diet with AP
ITF IV + AP	5% ITF IV (in normal diet, w/w) with AP
ITF I + AP	5% ITF I (in normal diet, w/w) with AP

AP, acute pancreatitis; ITF IV, inulin-type fructan IV (long-chain inulins); ITF I, inulin-type fructan I (short-chain inulins).

Tissues Sampling

Mice were euthanized and sacrificed with a lethal dose of pentobarbitone sodium (100 mg/kg) 1 h after the last caerulein injection. For serum analysis, blood samples were centrifuged at 3,000 × *g* for 15 min, after which serum was collected and stored at −80°C. Tissues, including pancreas and colon, were excised, fixed in 4% paraformaldehyde or snap freeze in liquid nitrogen and stored at −80°C for later analysis.

Serum Amylase and Lipase Measurements

Serum amylase activities were measured by a serum assay kit (Jian Cheng Bioengineering Institute, Nanjing, China) (30). Lipase activities were measured by enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA).

Edema and Myeloperoxidase (MPO) Activity Measurement

A portion of freshly harvested pancreatic tissue was trimmed of fat and weighed. Pancreatic water contents were evaluated by the ratio of initial weight (wet weight) of the pancreas to its weight after drying at 80°C for 48 h (dry weight). MPO activity measurements were determined by an MPO assay kit (Jian Cheng Bioengineering Institute, Nanjing, China).

Histological Evaluation

Fresh pancreatic and colonic tissues were fixed in 4% paraformaldehyde overnight, washed with ddH_2O, and rehydrated with ethanol and embedded in paraffin. The Skiving machine Slicer PM2245 (Leica, Wetzlar, Germany) diced 5-μm sections were stained with hematoxylin and eosin (H&E). For pancreatic injury evaluation, a DM2000 light microscope (Leica, Wetzlar, Germany) was used at ×40 magnification. The examination was carried out based on both infiltrating inflammatory cells and other morphological changes in tissues which are considered to be markers of inflammation/tissue damage.

ELISA Analysis

The levels of cytokines (TNF-α, IL-1β, and IL-10) in serum were measured by ELISA kits (R&D Systems, Minneapolis, MN, USA) following the standard procedure of the manufacturer. To measure the cytokines level of pancreatic and colonic tissues (TNF-α, IL-1β, and IL-10), the tissues were homogenized in a saline solution (1:19, w/v) using a Polytron homogenizer (Scientz-48, Ningbo, Zhejiang, China) at 55 Hz for 1 min. Samples were centrifuged at 4°C, 10,000 × *g* for 10 min. Finally, the supernatant was collected for ELISA analysis.

RNA Isolation and Real-time Quantitative Polymerase Chain Reaction (RT-qPCR) Analysis

Transcription of mRNA of occludin, zonula occludens protein-1 (ZO-1), β-defensin-1 (DEFB1), and cathelicidin-related antimicrobial

peptide (CRAMP) was analyzed by RT-qPCR. Total RNA was isolated from pancreas and colonic tissues using TRIzol (Life Technologies, MA, USA) and was subjected to reverse transcription using Prime-Script RT reagent kit (TaKaRa Bio, Japan) following the manufacturer's instructions. SYBR® Green RT-qPCR was performed using real-time PCR system (BIO RAD CFX Connect, CA, USA). The relative mRNA levels were normalized to mRNA levels of β-actin (housekeeping control) and calculations for fold change of each mRNA were made on comparative cycle threshold method ($2^{-\Delta\Delta Ct}$). The primers used in this study are provided in **Table 2**.

Western Blot Analysis

Pancreatic and colonic tissues were homogenized in ice-cold lysis buffer RIPA (containing cocktail protease inhibitors; Beyotime, Shanghai, China). Samples were centrifuged at 4°C, 10,000 × *g* for 10 min and equal amounts of protein (30 μg), as determined using standard bicinchoninic acid assay (BCA) method by BCA Protein Assay Kit (Beyotime, Shanghai, China). Samples were electrophoresed on blots 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel and transferred to polyvinylidene difluoride membranes. Membranes were blocked with blocking buffer for 1.5 h at room temperature, washed with TBS-Tween 20 (TBST), and finally incubated overnight at 4°C with anti-extracellular signal-regulated kinase (ERK)/phospho-ERK, JNK/phospho-JNK, p38/phospho-p38, IRAK-4, phospho-p65, and GAPDH (housekeeping) antibodies. Incubation with fluorescently labeled secondary horseradish peroxidase (HRP)-conjugated secondary antibodies (1:3,000) was performed for 2 h at room temperature and immunoreactivity was analyzed by Western Lightening Plus enhanced chemiluminescence (PerkinElmer, MA, USA) according to the manufacturer's instructions.

Flow Cytometry Analysis

Freshly harvested pancreatic and colonic tissues were digested in 1.0 mg/mL collagenase-P (Boehringer, Mannheim, Germany) solution at 37°C for 15 min and filtered through 75 μm filters with hank's solution (Beyotime, Shanghai, China). Single-cell suspensions were incubated for 30 min at 4°C in hank's solution with the following mAbs: APC Rat Anti-Mouse CD11b, BV421 Rat Anti-Mouse F4/80, Alexa Fluor 700 Rat Anti-Mouse Ly-6G, PE Hamster Anti-Mouse CD11c, FITC Rat Anti-Mouse MHCII, and PerCP-Cy5.5 Rat Anti-Mouse CD8a (BD Pharmingen, CA, USA). Gating method of fluorescence-activated cell sorting was programmed as $CD11b^+$ $Ly\text{-}6G^+$ (for neutrophils), $CD11b^+$ $F4/80^+$ (for macrophages), $CD11c^+$ $MHCII^+$ (for conventional dendritic cells, cDCs), and $CD8a^+CD11c^+$ $MHCII^+$ (for plasmacytoid dendritic cells, pDCs). Flow cytometer was performed on Attune NxT (Thermo Fisher Scientific, MA, USA). Data were analyzed using ACEA NovoExpress software (Novo Express International, Inc., South San Francisco, CA, USA).

TABLE 2 | List of primers used for RT-qPCR.

Gene	Forward (5′–3′)	Reverse (5′–3′)
β-actin	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT
ZO-1	CTTCTCTTGCTGGCCCTAAAC	TGGCTTCACTTGAGGTTTCTG
Occludin	CACACTTGCTTGGGACAGAG	TAGCCATAGCCTCCATAGCC
DEFB1	CACATCCTCTCTGCACTCTGGAC	CCATCGCTCGTCCTTTATGCCATTC
CRAMP	GCTGTGGCGGTCACTATCAC	TGTCTAGGGACTGCTGGTTGA

DEFB1, β-defensin-1; CRAMP, cathelicidin-related antimicrobial peptide; RT-qPCR, real-time quantitative polymerase chain reaction.

Statistical Analysis

Data are expressed as means ± SEM. The parametric distribution of the results was confirmed using Kolmogorov–Smirnov test. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test using GraphPad Prism (version 5; GraphPad Software Inc., San Francisco, CA, USA). The values of $P < 0.05$ were considered to indicate a statistically significant difference.

RESULTS

ITF IV Supplementation of the Diet Alleviates Severity of Caerulein-Induced AP

Modulatory effects of ITFs were examined in caerulein hyperstimulated mice by supplementing the diets with either ITF I or ITF IV for 3 days before AP induction. While dietary supplementation with 5% ITF I or ITF IV alone without AP induction did not show any effect on pancreatic markers (Figure S1 in Supplementary Material), protective effects of ITF supplementation on AP were observed and the effects were highly ITF chain length dependent. ITF IV had more attenuating effects than ITF I. ITF IV but not ITF I attenuated caerulein-induced increases in serum amylase, serum lipase, pancreatic MPO activities, and edema (**Figures 1A–D**). However, both ITF I and ITF IV modulated serum cytokines. Both types of ITFs reduced serum pro-inflammatory IL-1β levels and increased the regulatory cytokine IL-10 (**Figures 1E,F**). Histological examination of pancreatic sections confirmed an overall better attenuating AP effect of ITF IV as evidenced by generally improved cellular morphology, restored interlobular space expansion, reduced inflammatory infiltrates, and acini necrosis (**Figure 1G**). Taken together, these data indicate that ITF-IV-supplemented diets alleviate the severity of AP in mice, while ITF I diet has a more minor beneficial effect.

ITF IV but Not ITF I Supplementation Strengthening Intestinal Barrier Function by Upregulating TJ Proteins and AMPs

Compromised intestinal barrier integrity and intestinal injury, marked by dysregulated expression of TJ modulatory proteins and barrier reinforcing AMPs, develop as AP progresses (22, 31). Therefore, maintaining intestinal barrier function might be key in the ITF-induced attenuating effects on AP. To determine such an effect of ITFs, we measured in ITF-treated animals, the mRNA expression of major structural proteins such as TJ, occludin and ZO-1, as well as AMPs DEFB1 and CRAMP. All these molecules are involved in the physical and chemical barriers of the mucosa (32, 33). The RT-qPCR analysis

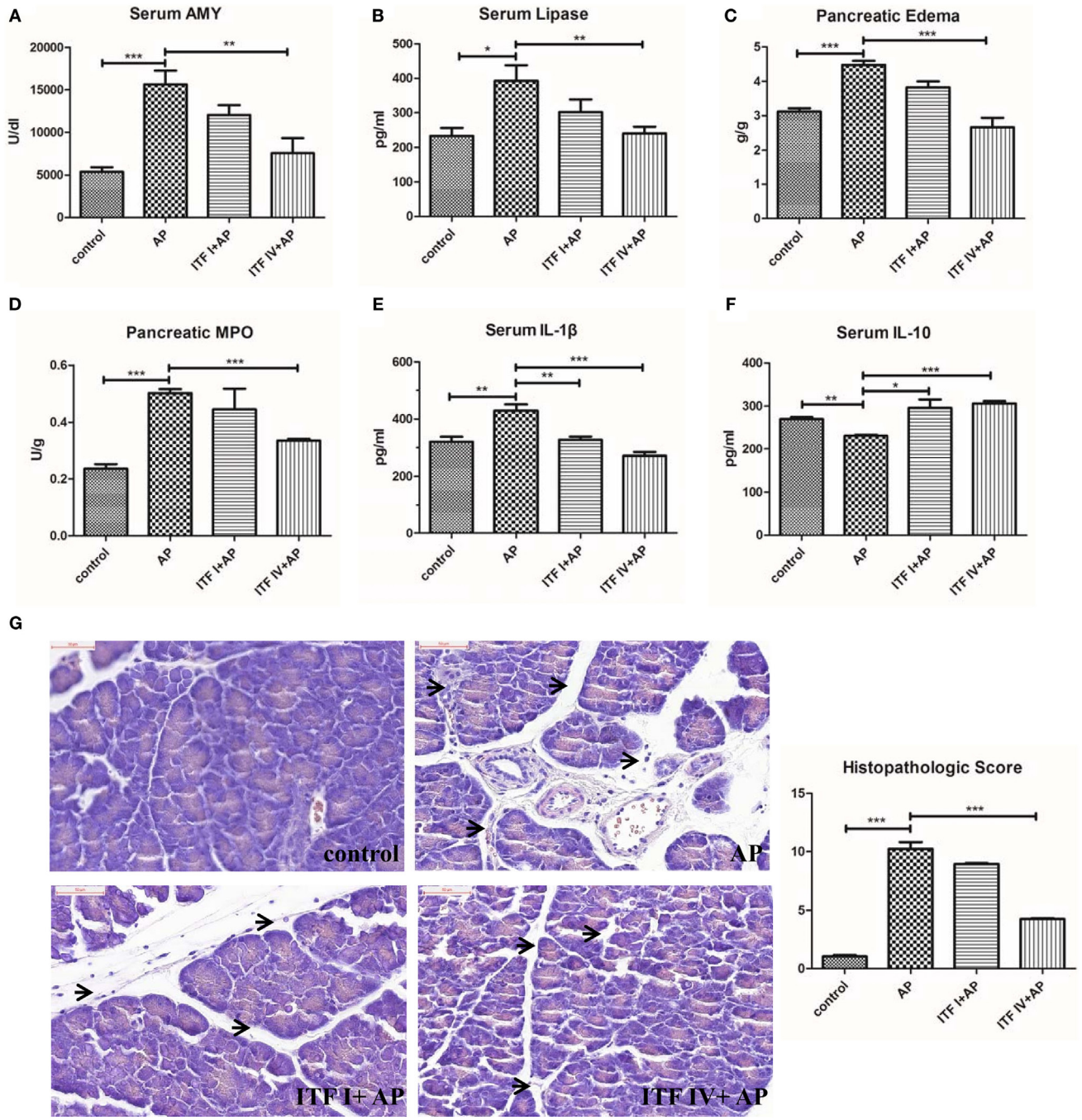

FIGURE 1 | ITF IV attenuating the severity of AP. Mice were fed with 5% ITF I or ITF IV supplemented diets for 72 h before AP induction by caerulein. Serum amylase **(A)**, serum lipase **(B)**, pancreatic edema **(C)**, MPO activity **(D)**, serum IL-1β **(E)**, and IL-10 levels **(F)** were then determined as described in Section "Materials and Methods," respectively. Representative photographs showed histomorphology of pancreatic tissues by H&E staining for the indicated groups (bar = 50 μm) and pancreatitis histopathologic score **(G)**. Data shown are means ± SEM. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. AP, acute pancreatitis; H&E, hematoxylin and eosin; IL, interleukin; ITF, inulin-type fructans; MPO, myeloperoxidase.

showed that ITF IV but not ITF I supplementation prevented caerulein-induced downregulation of occludin and ZO-1 as well as the downregulation of DEFB1 and CRAMP (**Figures 2A–D**). Furthermore, histological examination of colon sections revealed that intestinal mucosal epithelia widened and villous apical epithelium peeled off in mice with AP as compared with control mice. This colonic damage was less severe in mice fed on ITF IV supplemented diet (**Figure 2E**). Together, these findings suggest that ITF IV but not ITF I supplementation prevents AP-associated gut integrity damage and injury.

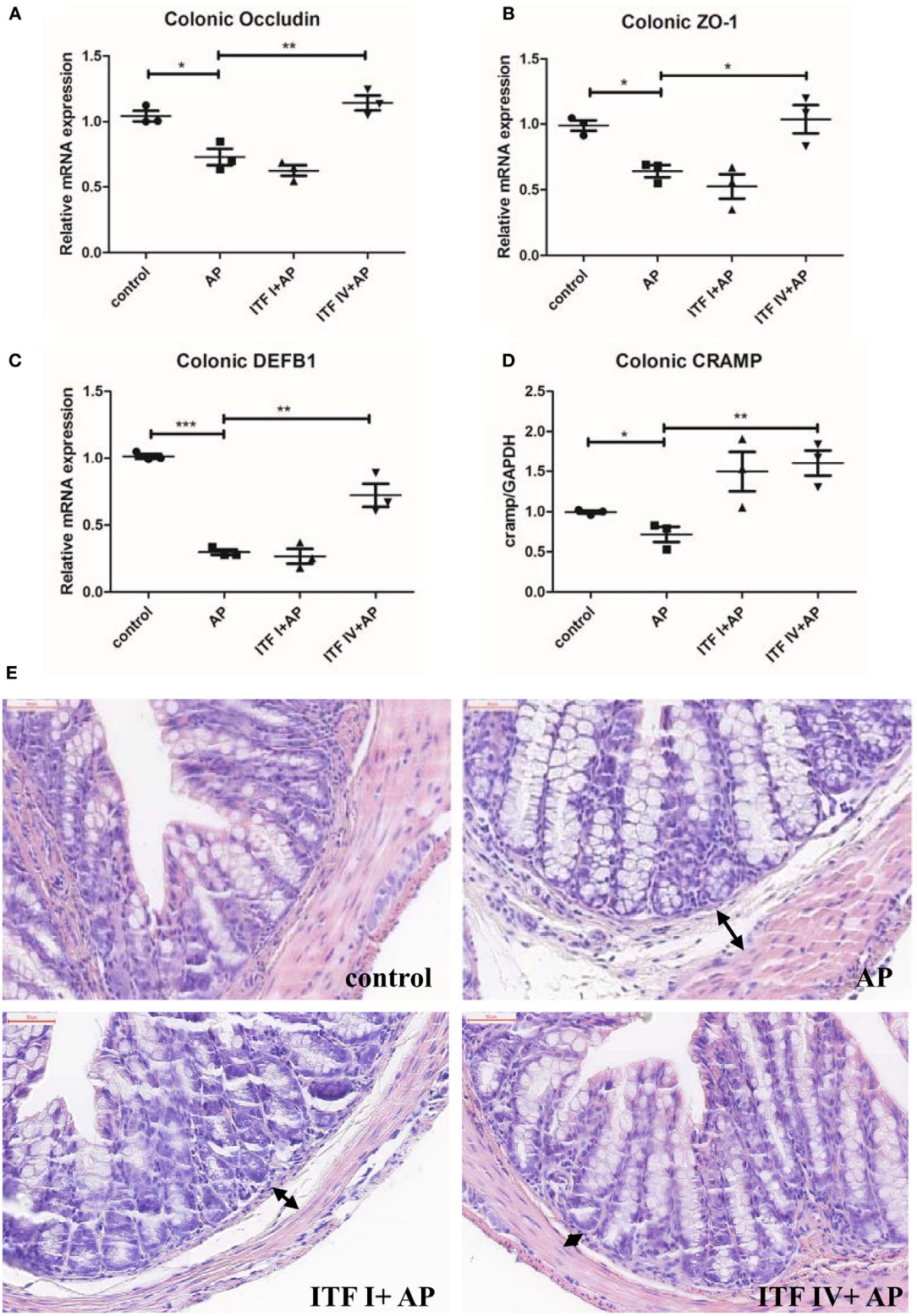

FIGURE 2 | ITF IV upregulating TJ proteins and AMPs in the colon. Mice were fed with 5% ITF I or ITF IV supplemented diets for 72 h before AP induction by caerulein. The mRNA levels of TJ proteins occluding **(A)**, ZO-1 **(B)**, DEFB1 **(C)**, and CRAMP **(D)** in the colon were then determined by RT-qPCR analyses. Representative photographs showed histomorphology of colonic tissues (50 μm) by H&E staining **(E)**. Data shown are means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. AMPs, antimicrobial peptides; AP, acute pancreatitis; CRAMP, cathelicidin-related antimicrobial peptide; DEFB1, β-defensin-1; H&E, hematoxylin and eosin; ITF, inulin-type fructans; RT-qPCR, real-time quantitative polymerase chain reaction; SEM, standard error of mean; TJ, tight junction; ZO-1, zonula occludens-1.

ITF IV Supplementation Reducing Innate Immune Cell Infiltration into the Pancreas and Pancreatic Cytokine Production during AP

Both infiltration of inflammatory immune cells and an imbalance between pro-inflammatory and anti-inflammatory cytokines are key immunological pathophysiological events in AP development (34–36). For this reason, we determined the frequencies of neutrophils ($CD11b^+Ly\text{-}6G^+$), macrophages ($CD11b^+F4/80^+$), cDCs ($CD11c^+MHCII^+$), and pDCs ($CD8a^+$ $CD11c^+MHCII^+$) that infiltrated into the pancreas of AP mice fed with ITFs. ITF IV supplementation profoundly inhibited the infiltration of neutrophils and macrophages (Figure S2 in Supplementary Material). The percentage of $CD8a^+$ DCs or pDCs but not cDCs was lowered with AP but normal in mice fed with ITF IV. ITF I did not have such an effect (Figure S2 in Supplementary Material). The percentages of neutrophil and macrophage in the pancreas robustly increased with AP induction and were significantly less in mice fed with ITF IV (**Figures 3A–C**) but this was still increased in mice fed with ITF I. Additionally, pancreatic cytokine production remained enhanced in ITFI but not in animals fed with ITF IV (**Figure 3D**). Collectively, these data demonstrate the immune attenuating effects of ITF IV.

Both ITF IV and ITF I Supplementation Modulating AP-Induced Innate Immune Cell Infiltration in the Gut and Cytokine Production

Immune compromised gut environment as a result of disrupted barrier function and intestinal injury is associated with major inflammatory cell infiltration and imbalanced inflammatory cytokine production. Therefore, we also investigated infiltration of innate immune cells in the gut during AP. AP induction resulted in robust increases in neutrophils and macrophages in the colon. Both ITF I and ITF IV supplementation reduced the number of infiltrating neutrophils and macrophages (**Figures 4A–C**). The percentage of $CD8a^+$ DCs or pDCs in the colon lowered with AP, but was prevented by ITF IV and not by ITF I (Figure S3 in Supplementary Material). Furthermore, consistent with reduced inflammatory cell infiltration in the gut, both ITFs prevented AP-induced increases in pro-inflammatory TNF-α and IL-1β levels, and decreases in IL-10 level in the colon as compared with untreated AP control (**Figure 4D**). Taken together, these data suggest positive immunomodulatory effects of both ITFs on AP-associated gut inflammation.

ITF IV but Not ITF I Supplementation Modulating Pancreatic IRAK-4/JNK/NF-κB Activation in AP

Lastly, we investigated the signaling molecular pathways underlying differential effects by ITF I and ITF IV supplementation. Activation of NF-κB and its upstream signaling kinases (IRAK, TAK1, and MAPKs) were determined by Western blot. It was found that ITF IV significantly modulated AP-induced IRAK-4, JNK, and NF-κB activation, while p-TAK, p-ERK, and p-p38 remained unaffected by ITF (**Figures 5A–D**). In comparison, ITF I attenuated IRAK-4 and p-JNK activation associated with AP, but did not affect NF-κB nuclear translocation and activation (**Figures 5A–D**).

DISCUSSION

The current study examines and compares effects of different types of ITFs in experimental AP and associated intestinal immune and barrier dysregulation. The effects were ITF type dependent and more pronounced with ITF IV than with ITF I. We observed that dietary long-chain inulin (ITF IV) supplementation mitigated the severity of AP: it suppressed characteristic inflammatory cell infiltration, modulated inflammatory cytokine production in the pancreatic–gut region, and prevented intestinal barrier function integrity. Its attenuating effect was associated with suppression of AP-induced IRAK-4/p-JNK/NF-κB pathway activation. ITF I only demonstrated significant modulatory effects in colonic immune responses and serum inflammatory markers, suggesting that short-chain inulin effects are limited to the gut (**Figure 6**).

Inulin-type fructans have been tested in a variety of inflammatory and immunological disease models and it has been shown that administration of ITF can exert immunomodulatory actions and alleviate acute inflammation (23, 28, 37). To the best of our knowledge, this is the first study in which efficacy of ITF is shown in AP. It has been earlier suggested, however, that dietary fibers are inversely related with pancreatic enzyme activity and can inhibit pancreatic digestive enzyme activities *in vivo* (38). As premature activation of pancreatic zymogens is a triggering event of human AP and inhibited by ITF, this study supports the potential application of ITF in preventing AP.

A particular intriguing observation of this study was that long-chain inulins have a better local pancreatic and gut protective effect in AP than short-chain inulins. Previously, differential prebiotic and immunomodulatory effects have been demonstrated by ITFs of varying chain length. Prolonged fermentation of long-chain inulin (DP > 10) compared with short-chain inulin (DP < 10) ensures more endurable and profound prebiotic and immunomodulatory effects, locally in the colon and systemically in remote organs. Another ITF chain length-dependent mechanism which might explain the difference is the magnitude by which ITFs can activate toll-like receptor 2, which is higher with long-chain than with short-chain ITFs (24). In addition, the preferential sites of fermentation for these two ITFs are different: colon for long-chain and small intestine for short-chain inulins. This may imply a prolonged and different action of ITF IV as compared with ITF I and might explain the observation that ITF IV but not ITF I increased the colonic expression of the TJ proteins ZO-1 and occluding as well as AMP DEFB1 and CRAMP. Differential regulation of TJ proteins has been observed after dietary interventions earlier (39).

Innate immune activations and pancreatic acinar cell inflammatory signaling are characteristic for AP (7). Moreover, AP induction by caerulein hyperstimulation caused neutrophil and

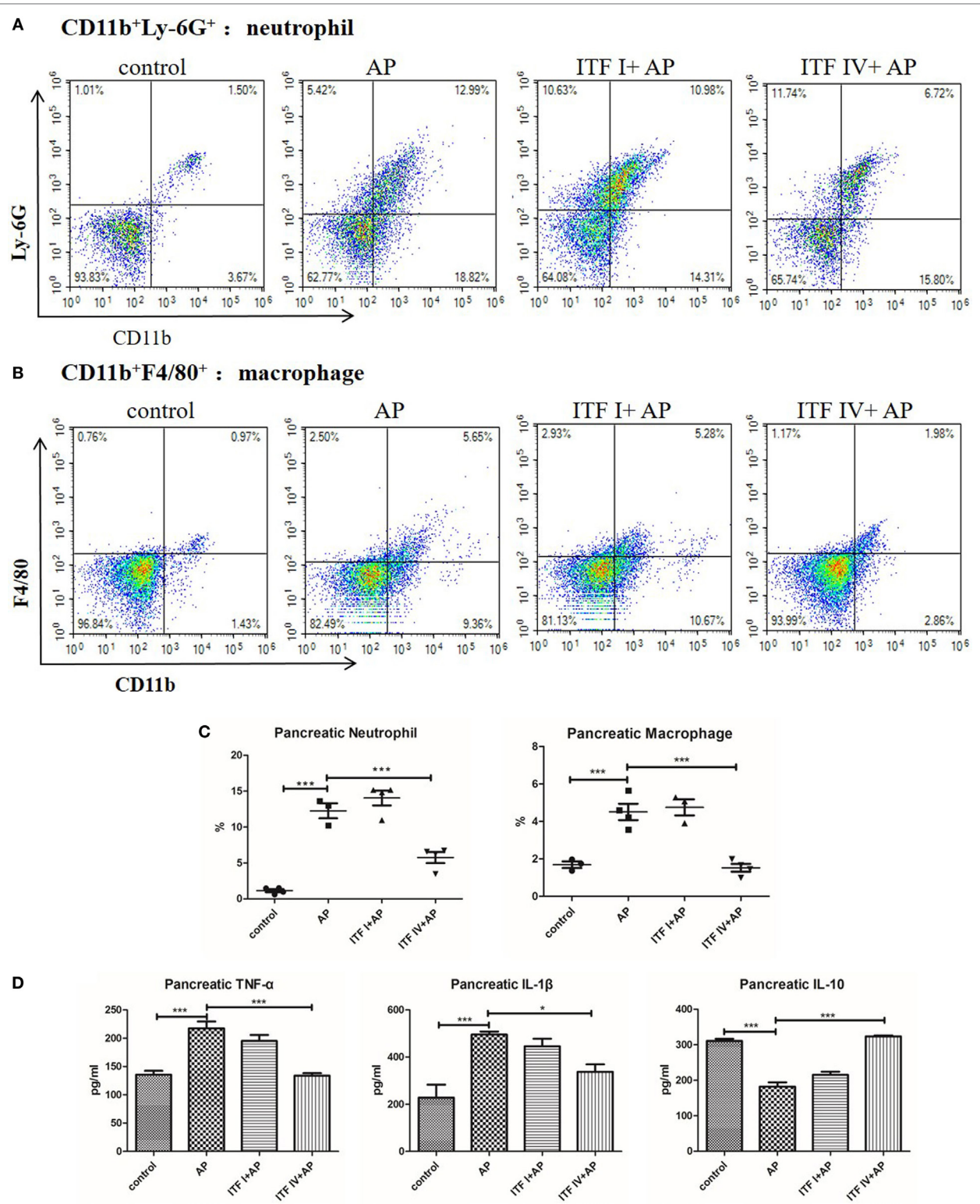

FIGURE 3 | Effects of ITF IV on AP-mediated neutrophil and macrophage infiltration and cytokines production in the pancreas. Mice were fed with 5% ITF I or ITF IV supplemented diets for 72 h before AP induction by caerulein. Neutrophil and macrophage infiltration and cytokine production in the pancreas were determined as described in Section "Materials and Methods," respectively. Representative graphs showed dot plots of Ly-6G$^+$CD11b$^+$ neutrophils **(A)** and F4/80$^+$CD11b$^+$ macrophages **(B)** in the pancreas and quantitative analysis of neutrophils and macrophages infiltration **(C)**. Quantitative analyses of TNF-α, IL-1β, and IL-10 levels in the pancreas were performed by ELISA **(D)**. Data shown are means ± SEM. $^*P < 0.05$ and $^{***}P < 0.001$. AP, acute pancreatitis; ELISA, enzyme-linked immunosorbent assay; IL, interleukin; ITF, inulin-type fructans; TNF-α, tumor necrosis factor-α.

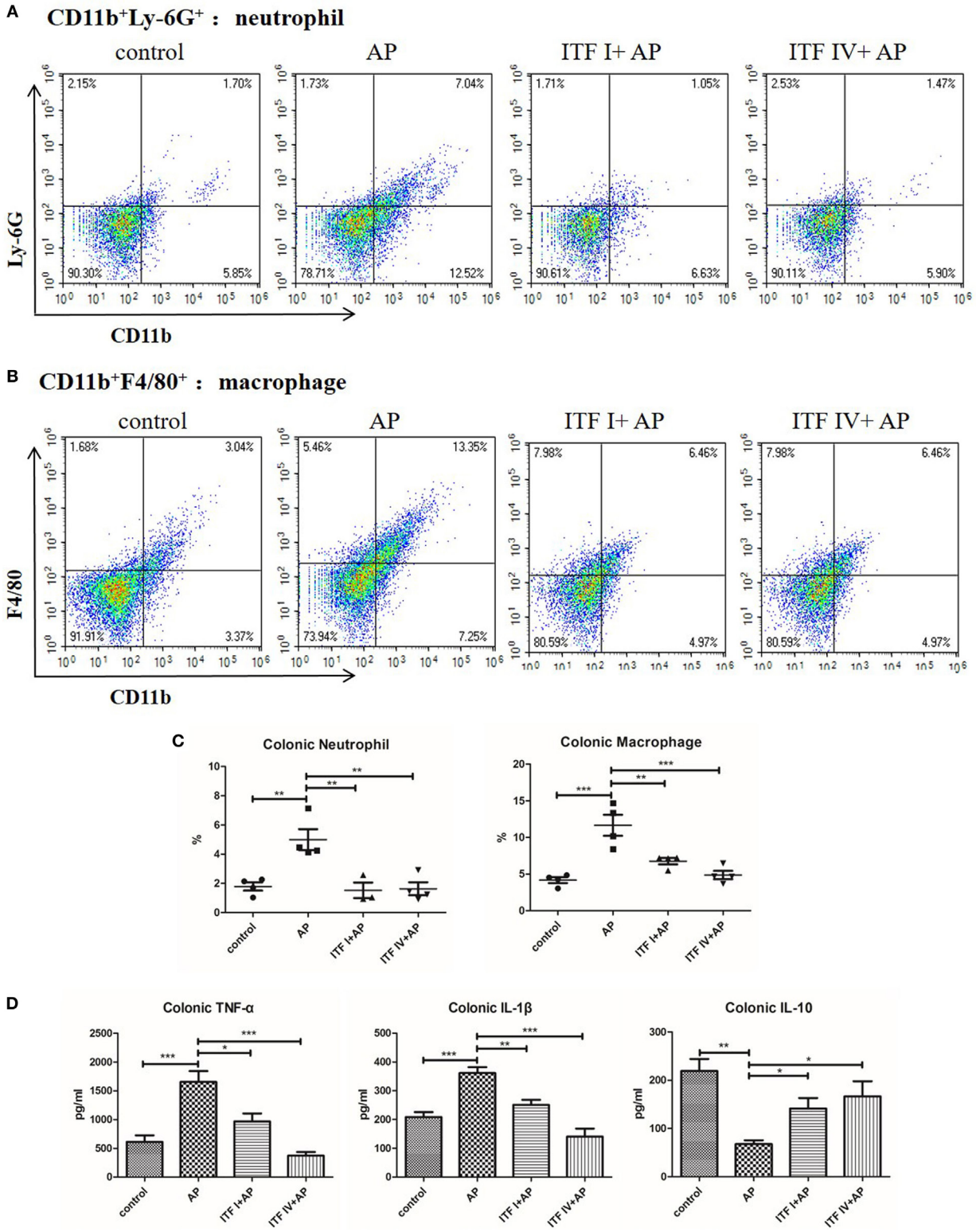

FIGURE 4 | Effects of ITFs on neutrophil and macrophage infiltration and cytokine production in the colon. Mice were fed with 5% ITF I or ITF IV supplemented diets for 72 h before AP induction by caerulein. Neutrophil and macrophage infiltration and cytokine production in the colon were determined as described in Section "Materials and Methods," respectively. Representative graphs showed dot plots of Ly-6G$^+$CD11b$^+$ neutrophils **(A)** and F4/80$^+$CD11b$^+$ macrophages **(B)** in the pancreas and quantitative analysis of neutrophils and macrophages infiltration in the colon **(C)**. Quantitative analyses of TNF-α, IL-1β, and IL-10 levels in the colon were performed by ELISA **(D)**. Data shown are means ± SEM. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. AP, acute pancreatitis; ELISA, enzyme-linked immunosorbent assay; IL, interleukin; ITF, inulin-type fructans; TNF-α, tumor necrosis factor-α.

macrophage infiltration. This infiltration is prevented by dietary ITF IV supplementation. Increased pancreatic Ly6C^{hi} monocytes/macrophages have been found to be positively correlated with the severity of AP and are dependent on TNF-α production by these cells (11). IL-10 production by M2 macrophages has been shown to impair neutrophil recruitment in inflammatory and infectious conditions (40, 41). Along with reduced infiltration of neutrophils and macrophages in ITF IV-fed mice with AP, the production of TNF-α and IL-1β was found to be suppressed and IL-10 levels increased in pancreatic and colonic tissues. While modulatory effects of ITF IV were observed on pancreatic, colonic, and systemic cytokine levels, those of ITF I was only significant in

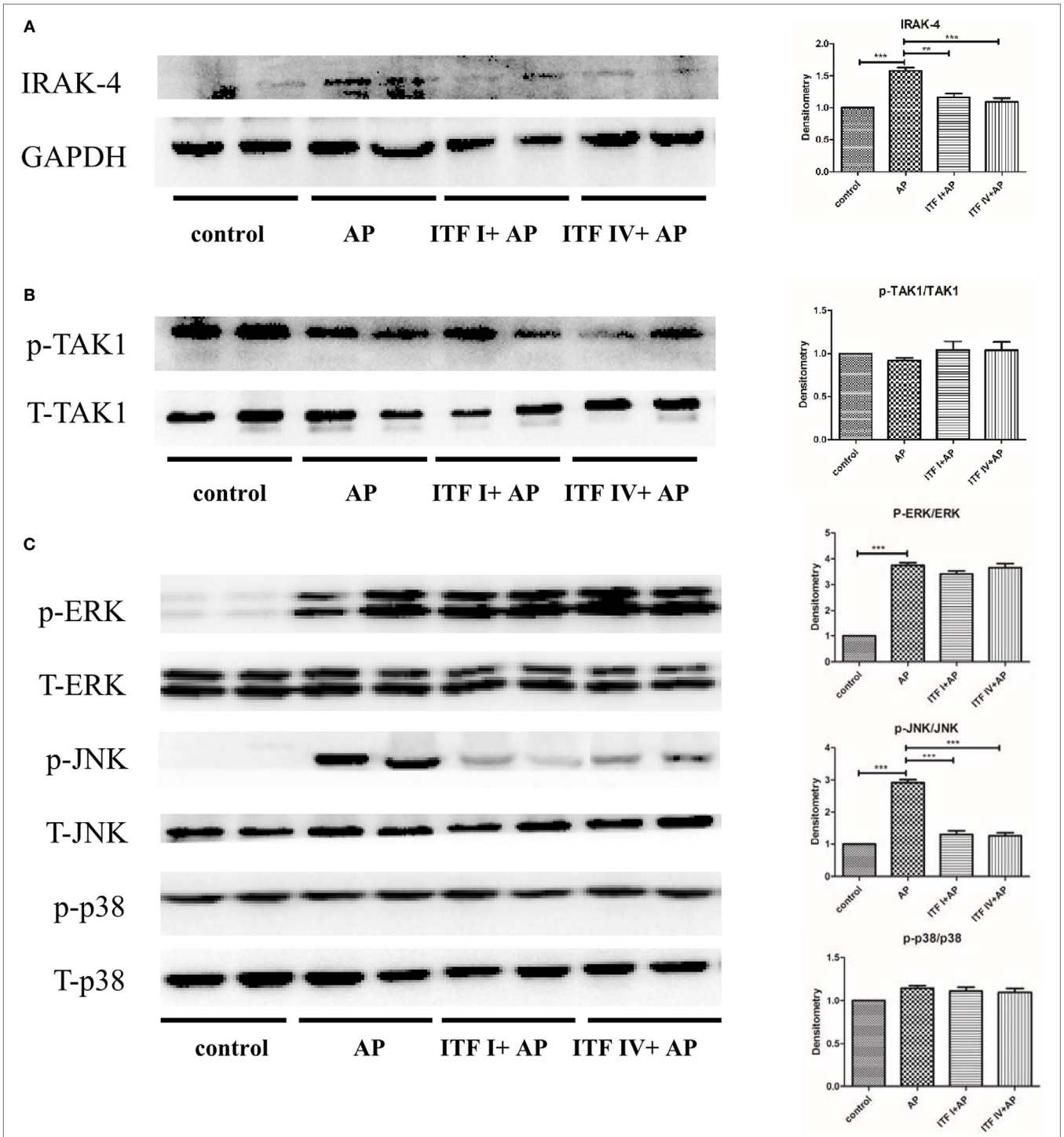

FIGURE 5 | Continued

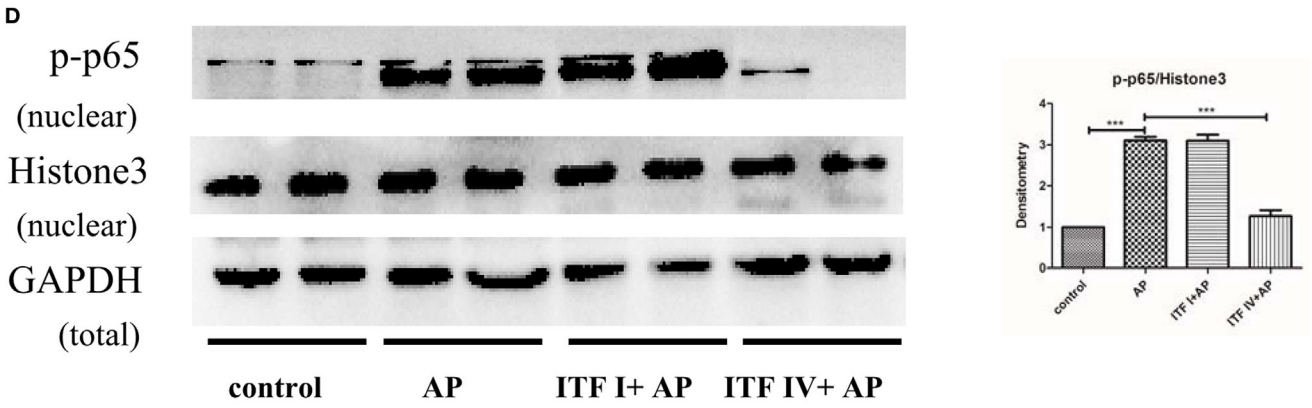

FIGURE 5 | Effects of ITFs on AP-mediated activation of MAPK and NF-κB pathways in the pancreas. Mice were fed with 5% ITF I or ITF IV supplemented diets for 72 h before AP induction by caerulein. The activation/expression of pancreatic IRAK4 **(A)**, p-TAK1 **(B)**, p-ERK, p-JNK, p-p38 **(C)**, and NF-κB p-p65 **(D)** were examined by Western blot. Data shown are means ± SEM. $^{**}P < 0.01$ and $^{***}P < 0.001$. AP, acute pancreatitis; ERK, extracellular signal-regulated kinase; IRAK, IL-1 receptor associated kinase; ITF, inulin-type fructans; JNK, c-Jun N-terminal kinase; MAPKs, mitogen-activated protein kinases; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; TAK, TGF-β activated kinase.

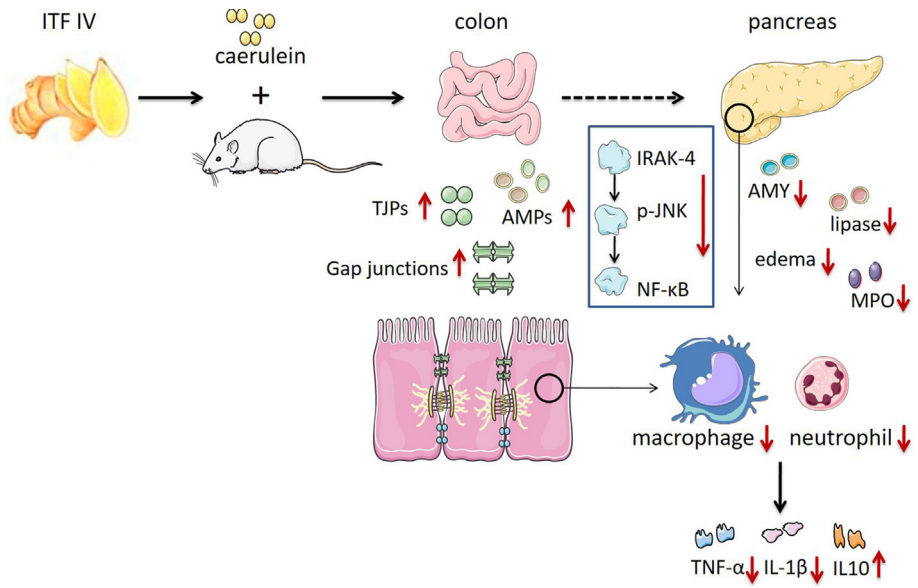

FIGURE 6 | ITF IV protection against AP by reducing pancreatic damage and AP-associated intestinal injury preventing its progression into a systemic inflammatory response. The role of ITF IV on AP in preventing tissues damage and the subsequent inflammatory responses was illustrated. AP, acute pancreatitis; ITF, inulin-type fructans.

colonic and systemic cytokines. Balanced pro-inflammatory and anti-inflammatory cytokine production prevented an augmented inflammatory response from a positive inflammatory feedback circuit (42).

Pancreatic acinar inflammation with activation of MAPKs and of the transcription factor NF-κB is a key pathological event during the development of AP (43–46). AP induction or supraphysiological concentrations of caerulein are associated with MAPK activation in pancreatic acini (47). Overexpression of p65 protein trans-activated NF-κB aggravates acute pancreatic inflammatory responses and MAPKs regulate NF-κB activation by mediating phosphorylation of its inhibitory-κB IκB protein, to allow NF-κB translocation to the nucleus followed by upregulation of inflammatory genes (19, 47, 48). Here, we found that MAPKs JNK but not ERK1/2 or p38 activation was inhibited by ITF IV, concomitant with an attenuated NF-κB p65 nuclear translocation and activation. It might be speculated that different upstream MAPKs are regulated by dietary fibers. Our data suggest that ITF IV interferes with the development of AP, by reducing pancreatic activation of IRAK-4-JNK-NF-κB p65 signaling pathway and inhibiting the release of inflammatory mediators. It has been demonstrated that IRAK-4 has been demonstrated essential in many innate immune

responses and can activate JNK pathway and downstream NF-κB (20, 21, 49). NF-κB translocation in acinar cells increases the severity of pancreatitis in mice, and leads to transcription of cytokines and other inflammatory mediators (19). Our study shows that ITF IV downregulated IRAK-4/p-JNK/NF-κB p65 in the pancreas during AP.

As AP progresses, the inflammatory cytokines produced in the pancreas, including TNF-α and IL-1β, reach the gut by the microcirculation. These cytokines will recruit more leukocytes and induce more inflammatory mediators that ultimately will contribute to intestinal barrier dysfunction and mucosal injury (22, 50, 51). Gut barrier dysfunction and worsened gut permeability in AP are accompanied by reduced expression of TJ reinforcing proteins and AMPs. Lack of physical and chemical barriers of the gut makes the host susceptible to bacterial translocation that contributes to a second golf of inflammatory event in the pancreas, causing excessive inflammation and development of multi-organ dysfunctions. As shown here, dietary ITF IV intervention, by limiting pancreatic–gut inflammation, restoring gut barrier function and integrity to prevent secondary excessive inflammatory responses, represents a promising approach to prevent the progress of AP. Although we have shown the modulatory effects of ITF IV on pancreatic–intestinal immunity and gut barrier function, the detailed mechanisms remain to be fully understood. Future investigation could identify specific receptor involvement and/or examine how their fermentation product SCFAs are regulated to exert protective effects during the condition. Moreover, the translational importance of these safe, value-added prebiotics for clinical AP merits further exploration.

Collectively, our data suggest that dietary ITF IV but not ITF I intake prevents the development of AP by three-graded actions: first, by preventing pancreatic inflammation and damage; second, by preventing AP-associated intestinal barrier dysfunction and intestinal inflammation; and last, by precluding progression of the gut and pancreatic inflammation into a systemic inflammatory response by the two aforementioned mechanisms. The current study provides evidence that nutritional application of long-chain inulins in clinical AP might be efficacious.

ETHICS STATEMENT

All animal-related experimental protocols were approved by the Institutional Animal Ethics Committee of Jiangnan University in compliance with the recommendations of national and international guidelines for the Care and Use of Laboratory Animals, and were performed in accordance with the guidelines therein.

AUTHOR CONTRIBUTIONS

YH, CW, JL, and HL performed experiments and analyzed data. P-LL, ZS, HZ, and PDV provided intellectual inputs, contributed to the data acquisition, and critically reviewed the manuscript. JS and P-LL designed and interpreted experiments. JS, P-LL, YH, and PDV wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.01209/full#supplementary-material.

FIGURE S1 | Effects of dietary ITF supplementation on markers of pancreatic damage. Mice were fed with 5% ITF I or 5% ITF IV supplemented diets for 72 h. Serum amylase **(A)**, pancreatic MPO activity **(B)**, and pancreatic edema **(C)** were then determined as described in Section "Materials and Methods," respectively. Data shown are means ± SEM. ITF, inulin-type fructans; MPO, myeloperoxidase.

FIGURE S2 | Effects of ITF IV on $CD8a^+$ DCs in the pancreas during AP: representative flow cytometry dot plots of $CD11c^+MHCII^+$ DCs **(A)**, histogram **(B)**, and percentage **(C)** of $CD8a^+$ DCs on DC subsets in the pancreas were shown. Data shown are means ± SEM from four independent experiments with three to four pooled mice per group in each experiment. $^{**}P < 0.01$. AP, acute pancreatitis; DCs, dendritic cells; ITF, inulin-type fructans.

FIGURE S3 | Effects of ITF IV on $CD8a^+$ DCs in the colon during AP: representative flow cytometer dot plots of $CD11c^+MHCII^+$ DCs **(A)**, histogram **(B)**, and percentage **(C)** of $CD8a^+$ DCs on DC subsets in the colon were shown. Data shown are means ± SEM from four independent experiments with three to four pooled mice per group in each experiment. $^{*}P < 0.05$ and $^{**}P < 0.01$. AP, acute pancreatitis; DCs, dendritic cells; ITF, inulin-type fructans.

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frontiers in Immunology

REVIEW
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Effects of Food Additives on Immune Cells As Contributors to Body Weight Gain and Immune-Mediated Metabolic Dysregulation

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Food additives are compounds used in order to improve food palatability, texture, and shelf life. Despite a significant effort to assure safety of use, toxicological analysis of these substances, generally, rely on their direct toxicity to target organs (liver and kidney) or their genotoxic effects. Much less attention is paid to the effects of these compounds on cells of the immune system. This is of relevance given that metabolic dysregulation and obesity have a strong immune-mediated component. Obese individuals present a state of chronic low-grade inflammation that contributes to the establishment of insulin resistance and other metabolic abnormalities known as the metabolic syndrome. Obesity and metabolic syndrome are currently recognized as worldwide epidemics that pose a profound socioeconomic impact and represent a concern to public health. Cells of the immune system contribute to both the maintenance of "lean homeostasis" and the metabolic dysregulation observed in obese individuals. Although much attention has been drawn in the past decades to obesity and metabolic syndrome as a result of ingesting highly processed food containing large amounts of fat and simple sugars, mounting evidence suggest that food additives may also be important contributors to metabolic derangement. Herein, we review pieces of evidence from the literature showing that food additives have relevant effects on cells of the immune system that could contribute to immune-mediated metabolic dysregulation. Considering their potential to predispose individuals to develop obesity and metabolic syndrome, their use should be taken with caution or maybe revisited.

Keywords: food additives, type 2 diabetes, metabolic syndrome, metainflammation, microbiota, citrate, immunometabolism

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INTRODUCTION

Obesity and Metabolic Syndrome

Obesity has reached worldwide epidemic proportions in the last decades. This is accompanied by an increased prevalence of comorbidities, such as type II diabetes, cardiovascular diseases, cancer, and other chronic diseases. In fact, 7 out of 10 leading causes of death in the U.S. are chronic disease states of which many have associations with obesity (1–3). Altogether, obesity

and its comorbidities impose high social and economic costs to individuals and society alike. Therefore, understanding the causes of obesity and designing preventive strategies are fundamental demands.

The factors that contribute to obesity are many (2), but it is generally accepted that obesity results from an association of genetic predisposition (which is currently unmodifiable) with a dysregulated energy balance. The World Health Organization (WHO) defines obesity as a body mass index (BMI, weight in kilograms divided by the square of height in meters) greater or equal to 30 kg/m^2 (3). Based on this definition, obesity can be considered a disorder of disproportionate mass of an individual. In that sense, it is reasonable to think of obesity as a result of positive energy balance, that is, increased calorie intake (especially in the form of dense calorie processed food) and decreased calorie expenditure (sedentary lifestyle).

Based on the positive energy balance view, excessive calorie intake and sedentary habits are considered the main modifiable factors contributing to the obesity epidemic. Special attention has been drawn to the often consumed highly processed and energy-dense foods. In fact, many obesity models in experimental animals rely on feeding mice and rats a high-fat or a high-fat, high-sugar diet. Therefore, the sugar and lipid content of foods and drinks are always taken into account when referring to weight gain and obesity.

Metainflammation and Insulin Resistance

The concept of obesity as excessive weight due to increased fat deposition is simplistic. Over the last two decades, cumulating pieces of evidence support the concept that fat tissue hyperplasia and hypertrophy is accompanied by profound alterations in adipose tissue homeostasis. The breakthrough description of elevated tumor necrosis factor α (TNFα) levels in adipose tissue of obese individuals and the contribution of TNFα to the establishment of insulin resistance (4) paved the way for the concept of an inflammatory component of obesity. This inflammatory component is referred to as "metainflammation," a pathological condition of chronic, low-grade inflammation observed in obese subjects (5, 6).

Later studies have shown macrophage infiltration in obese fat tissue and their contribution to pro-inflammatory cytokine production (including TNFα) and insulin resistance. More recently, studies have shown the participation of many immune cell types in adipose tissue homeostasis, dysregulation, and an important contribution of macrophage polarization. In lean individuals, adipose tissue has a prevalence of alternatively activated (or M2) macrophages. These M2 macrophages produce anti-inflammatory cytokines, such as IL-10, and express arginase-1, which metabolizes arginine to polyamines and away from free radical nitric oxide (NO). Various immune cell types maintain M2 polarization in lean adipose tissue; T cells (Tregs) that contribute to leanness (7) and may be a relevant source of IL-10, a cytokine that polarizes macrophages to a M2 phenotype in adipose tissue (8), and eosinophils, which are the main source of the M2-polarizing cytokine IL-4 in adipose tissues (9). These cytokines and M2 macrophages sustain adipose tissue homeostasis and contribute to insulin sensitivity.

In obese adipose tissue, on the other hand, pro-inflammatory mediators such as TNFα and the CC chemokine ligand C–C motif ligand 2 recruit blood monocytes, where these cells become polarized toward the "classical" pro-inflammatory M1 state (10, 11). Interestingly an important contributor to M1 polarization is the activation of TLR4 by free fatty acids (12), which are found in large amounts due to increased lipolysis in obese adipose tissue. In contrast to their M2 counterparts, M1 macrophages produce pro-inflammatory mediators, such as TNF-α, IL-1β, and leukotriene B_4, and express the inducible isoform of NO synthase (iNOS), leading to the production of large amounts of NO from arginine. Altogether, these mediators contribute to adipose tissue insulin resistance and, consequently, a sustained state of lipolysis which contributes to the perpetuation of the local low-grade inflammatory state. Moreover, eosinophil and Treg numbers were shown to lower in obese adipose tissue further favoring M1 polarization.

A macrophage M1–M2 imbalance can also be seen in other target organs, such as the liver (13). In lean individuals, liver-resident macrophages, known as Kupffer cells (KCs) show a M2-like phenotype, sustained by their PPARδ expression (14). Mediators derived from obese adipose tissue, especially TNF-α and FFAs are able to polarize KCs to a M1-like state. This is further complicated by the fact that M1-KCs produce mediators that recruit M1-prone monocytes from the circulation to populate the liver (15). This process is directly related to the establishment of liver insulin resistance and the progress of liver steatosis to cirrhosis and liver failure. In fact, depletion of liver macrophages prevents steatohepatitis and insulin resistance in mice (16, 17). Besides endogenous mediators, KCs can be activated by microbial products derived from the gut (18). The liver is anatomically positioned so that it is the first organ to contact gut-derived products. Actually, this is one proposed mechanism by which disturbances in gut barrier—either directly or through an imbalance of the gut microbiome—can result in liver inflammation and metabolic derangements (18).

In summary, the current view is that the modern lifestyle, characterized by overeating associated with low physical activity, leads to increased weight and fat mass gain through a shifted energy balance. This disturbs the homeostasis of target organs, such as the liver and adipose tissue, likely through mechanisms involving the pro-inflammatory activation of immune cells, leading to peripheral insulin resistance. In the longer term, these alterations will culminate in metabolic syndrome, characterized by abdominal fat, hypertension, dyslipidemia, insulin resistance, and steatohepatitis.

Food Additives As Neglected Players in Obesity and Metabolic Syndrome

In modern societies, the easy access to food, together with the demand for highly palatable and ready-to-serve products lead to the generalized consumption of industrialized processed foods. Processed food lacking in vitamins, fibers, and minerals, and dense in calories in the form of fat and simple sugar are, thus, considered the main villains of modern diets (19). The contribution of excessive calorie intake to the alarming increase in obesity witnessed in the last three decades is undeniable.

However, processed food is composed of not only sugar and fat but also a series of other products that are added in order to increase palatability, modify texture, and prolong shelf life. These products are collectively called food additives. A few of these additives even have well-known beneficial health effects as exemplified by probiotics and prebiotics. These additives are used to directly or indirectly influence gut microbiota and have well-documented benefits for host health and well-being (20). All prospected food additives, however, need to pass a rigorous scrutiny for their potential toxicity and undesired side effects. These toxicity tests are carried out following a series of guidelines, including outcome parameters that must be observed. These parameters comprise analysis of clinical manifestations, biochemical and hematological alterations, and postmortem analysis (21, 22). Based on these results, food additives are allowed to be used in varying amounts and many of them are considered innocuous and safe. However, studies sometimes present evidence that questions food additive safety and asks for a reassessment of their use. In this regard, we present evidence from the current literature that food additives—even some that are generally considered safe—may have relevant effects on immune cells and, thus, could also contribute to the burden of obesity and its related comorbidities. We have selected a few examples to illustrate and base a discussion on effects that food additives may have on cells of the immune system, which may potentially contribute to a series of pathologic and metabolic conditions. These examples are summarized in **Table 1** and discussed in detail in the subsequent sections.

WHEN "NATURAL" MAY NOT BE HEALTHY: THE CASE OF CITRATE

Citrate is a very common food and drink additive widely used by the food industry as a chemical acidifier, flavoring agent and a preservative (36, 37). Since it is found in large amounts in many fruits and vegetables, especially citric fruits, it is generally considered natural and healthy. The Food and Drug Administration (FDA), for example, does not pose a limit for citric acid addition to foods or drinks.

Apart from being a food additive, citrate is also a metabolite involved in carbohydrate and lipid metabolisms. In situations of anabolism, as after meals, citrate is formed in the mitochondria and exported to the cytosol. Cytosolic citrate is metabolized to acetyl-CoA and oxaloacetate by the enzyme ATP:citrate lyase (ACLY) (38). Cytosolic acetyl-CoA then serves as the main building block for fatty acids and cholesterol synthesis. Despite the well-described fate of endogenous citrate, the fate of ingested citrate remains largely unexplored. Citrate enters the cells through the SLC13A5 citrate transporter encoded by the mINDY (mammalian I'm Not Dead Yet) gene (39). In humans, this gene is primarily expressed in the liver, presenting very low but detectable expression in the brain, testes, and ovary (40). Moreover, expression of this citrate transporter is upregulated in the liver of animals submitted to a high-fat, high-sucrose diet (40). Since ACLY is also highly expressed in the liver (41), it is reasonable to assume that food-derived citrate may contribute to postprandial lipid and cholesterol synthesis. Although expression of SLC13A5 in inflammatory cells such as monocytes, has not been reported, it has been reported that exogenous citrate modulates liposaccharide-induced monocyte inflammatory responses in cell culture (29), raising the question of the role of exogenous citrate in inflammation.

We recently addressed this issue by supplementing citrate in the drinking water of mice fed a standard diet (28). Contrary to our expectations, we did not find any increase in weight gain, adipose tissue mass, ectopic lipid deposition, or plasmatic lipid levels. This suggests that citrate supplementation did not contribute significantly to *de novo* lipid synthesis, at least in the medium-term duration (75 days) of our experiment. We currently do not know how citrate supplementation may affect lipid metabolism in the long term. On the other hand, we found a significant effect on glucose homeostasis. Mice receiving citrate plus sucrose showed higher fasting glycemia and diminished glucose tolerance. This

TABLE 1 | Common food additives and their proposed effects on cells of the immune system and metabolic parameters.

Additive	Max. daily intake (FDA)	Described effects	Reference
Sucralose	5 mg/kg	Dysbiosis in rats	(23)
		Dysbiosis and impaired glucose tolerance in mice	(24)
Saccharin	15 mg/kg	Dysbiosis and impaired glucose tolerance in mice	(24)
Aspartame	50 mg/kg	Impaired glucose tolerance	(25, 26)
Carboxymethylcellulose	No limitations	Weight gain	(27)
		Impaired glucose tolerance	
Polysorbate-80	25 mg/kg	Low-grade inflammation increased adiposity	(27)
Citrate	No limitations	Increased fasting glycemia and impaired glucose tolerance	(28)
		Potentiate LPS-induced activation of macrophage cell line (THP-1)	(29)
Sodium	2,400 mg/day	Exacerbates TNBS-induced colitis in mice	(30)
		Favors M1 macrophage polarization	(31)
		Favors Th17 polarization and predispose mice to develop autoimmune disease	(32, 33)
Carrageenan	No limitations	Glucose intolerance in mice	(34)
		Exacerbated glucose intolerance and dyslipidemia induced by HFD in mice	(35)

Food additives, their described effects, and their maximum daily intake recommendations established by Food and Drug Administration.

was accompanied by increased levels of inflammatory cytokines in adipose tissue (28).

The mechanisms by which exogenous citrate may contribute to adipose tissue inflammation and establishment of insulin resistance remains to be determined. In this regard, recent data suggest that citrate may be pro- or anti-inflammatory. For example, citrate administration was shown to protect mice from cerebral and hepatic oxidative damage induced by low-dose LPS (42). Parameters of tissue damage, such as DNA fragmentation and hepatic enzymes in plasma, were significantly diminished in citrate-treated mice. However, this was only observed at lower citrate doses. At the highest dose tested citrate showed no effect or even a potentiating effect. *In vitro*, exogenous citrate was recently shown to potentiate LPS-induced activation of THP-1 monocyte cell line (29). This was dependent on calcium availability, since higher citrate concentrations showed an opposite inhibitory effect that was rescued by calcium supplementation. Potentiation of LPS effects by citrate could be blocked by TCA, an ACLY competitive inhibitor, suggesting the participation of cytosolic citrate metabolism in this effect. Indeed, cytosolic citrate accumulation is a metabolic characteristic of inflammatory macrophages (43) and some effector functions of macrophages depend on citrate metabolism (44). It was recently demonstrated that ACLY is important for macrophage synthesis of lipid mediators and free radical production. Interfering with ACLY expression or activity significantly inhibited prostaglandin synthesis and NO and reactive oxygen species production by LPS- or cytokine-activated macrophage cell lines (45). Moreover, mitochondrial citrate carrier and ACLY expression is upregulated by LPS in a NF-κB-dependent fashion, increasing citrate influx to the cytosol (46).

Furthermore, citrate may also have epigenetic effects on macrophages. ACLY plays a role in epigenetic regulation in diverse mammalian cell types. Histone acetylation is responsive to ACLY-dependent glucose availability (41, 47) and ACLY was shown to localize in the nucleus, where it can contribute to the epigenetic program of adipocyte differentiation as a source of acetyl groups for histone acetylation (48). In kidney mesangial cells, ACLY can also promote histone hyperacetylation and upregulation of fibrogenic genes, contributing to high glucose-triggered diabetic renal fibrosis (49). More specifically in macrophages, a recent report showed that epigenetic modifications underlying IL-4-mediated M2 polarization were dependent on ACLY activation triggered by Akt–mTOR pathway (50). Furthermore, citrate carrier acetylation and inhibition strongly decreases LPS-induced inflammatory response (46).

Finally, the contribution of citrate to obesity and its related comorbidities can be inferred indirectly by pharmacological data. Bempedoic acid, a lipid-regulating compound, was recently described as a potent ACLY inhibitor (51, 52). In mouse model of metabolic dysregulation, bempedoic acid treatment significantly reduced adiposity, plasma lipid levels, and attenuated inflammation onset and atherosclerotic lesion development (53). A phase-II clinical trial showed that bempedoic acid safely lowers LDL-c levels and could be used alone or in association with others therapies (51). Moreover, treatment with hydroxycitrate, which functions as ACLY inhibitor, attenuates weight gain, lipid deposition, and adipose tissue inflammation in spontaneous genetically obese rats (54).

These data support the hypothesis that citrate intake from foods and drinks may promote inflammation. Our results show that citrate contributes to adipose tissue inflammation and glucose intolerance even when mice are fed a regular diet. Although citrate effects may vary depending on experimental settings, i.e., may protect from LPS effects (42) or potentiate LPS effects (29), the abovementioned pieces of evidence suggest that it is important to reassess the concept that citrate is inert. Further studies are needed to address the metabolic effects of citrate supplementation in the long-term as well as in association with high-calorie diets.

SALT: A VIEW BEYOND HYPERTENSION

Salt, especially in the form of sodium chloride, is largely used worldwide to increase food flavor and palatability. High sodium intake correlates with the development of a series of diseases, being hypertension the most popularly known (55). Industrialized and processed foods usually contain high amounts of salt that can be up to 500 times more than a similar home-cooked meal (56). Moreover, associated with their high-sodium amounts, industrialized foods present low or undetectable levels of other minerals, especially those related to beneficial effects, such as magnesium, selenium, zinc, and others (57). The low consumption of these minerals is strongly associated with the prevalence of obesity, type 2 diabetes (T2D) (58), and inflammatory bowel disease (59, 60). The mechanisms underlying the detrimental effects of high salt consumption are still a matter of debate. However, recent data provide pieces of evidence that high salt may have a relevant impact on cells of the immune system.

Two independent studies have shown that a high-salt diet induces T cell polarization toward the pathogenic Th17 phenotype, predisposing mice to develop autoimmune disease (32, 33). This was shown to be dependent on the activation of the serine/threonine kinase serum glucocorticoid kinase (SGK)-1, p38-MAPK, and nuclear factor of activated T cells (NFAT) 5. Concerted activation of these factors leads to increased IL-23R expression and stabilization of the Th17 phenotype. Since Th17 cells play a pathogenic role in obesity and metabolic syndrome (61, 62), it is reasonable to assume that a similar mechanism may predispose individuals consuming large amounts of salt to develop metabolic derangements.

Similar to the findings in Th17 cells, macrophages also had their effector phenotype modulated by extracellular osmolarity (*in vitro*) and high salt consumption (*in vivo*). Through the same p38–NFAT5 pathway triggered in T cells, increased sodium concentrations were able to boost NO production and the leishmanicidal activity of macrophages (31). This suggests that high salt concentrations shift macrophage activation toward the M1 phenotype. In fact, high salt blunted M2 polarization induced by IL-4 and IL-13 and impaired their effector functions (63). This is interesting when considering, for example, the effects of citrate on macrophage activation. High sodium and citrate are commonly found in processed food and both seem to favor M1 polarization of macrophages. This could have profound influence on the

development of insulin resistance and adipose tissue inflammation, especially if a synergic effect could be demonstrated in future studies. Moreover, a recent report described that macrophages are able to sense hyperosmotic stress and activate both the NLRP3 and NLRC4 inflammasomes (64). This was dependent on mitochondrial ROS production and lead to IL-1β secretion that contributed to a biased T cell polarization toward the Th17 phenotype. Given the well-documented participation of NLRP3 inflammasome and IL-1β in insulin resistance (65, 66), this could be a reasonable mechanism for the association between high salt consumption and the metabolic syndrome.

Finally, increased salt concentrations, either *in vitro* or *in vivo*, was shown to impair Treg differentiation and function (67). This effect was also dependent on SGK-1, resulted in loss of Treg suppressive function and increased interferon (IFN)-γ production, thus contributing to the aggravation of both graft-versus-host disease and experimental colitis. Taken together, these studies suggest that the consumption of a high-salt diet would strongly favor a pro-inflammatory immune response with heightened Th17 and M1 activation and decreased Treg and M2 functions. Given the well-documented pathological role of both Th17 T cells and M1 macrophages in obesity, it is reasonable to speculate that high salt consumption would favor obesity and diabetes by an immune-mediated mechanism involving a strong bias toward pro-inflammatory phenotype of both macrophages and T cells. In fact, high salt consumption has been associated with features of the metabolic syndrome, such as obesity, hypertension, and T2D (68). However, the impact of this salt-triggered pro-inflammatory bias to obesity and T2D remains to be determined. Moreover, the effect of chronic exposure to high salt diet has never been tested, neither in obesity nor in autoimmune models. Another open question is the outcome of high salt consumption in association with other additives with potential pro-inflammatory effects, such as citrate (above) or carrageenan and emulsifiers (discussed below). Nevertheless, the straight interrelationship among salt consumption, weight gain, and immune-associated metabolic dysregulation cannot be dismissed.

THE GUT MICROBIOME: A SYMBIOTIC RELATIOSHIP WITH THE IMMUNE SYSTEM AFFECTING WEIGHT GAIN AND TYPE 2 DIABETES

The gut microbiota comprises a diverse community of microbes that inhabits the intestinal tract. It is now well demonstrated that the gut microbiota provides key signals for the full maturation of the immune system and also has important metabolic benefits for the host. On the other hand, disturbances of the microbiota–host relationship are associated with immune-mediated and metabolic-associated chronic diseases, such as diabetes and the metabolic syndrome (27). Mucus at the intestinal surface prevents direct contact between epithelial cells that line the intestine and the gut microbiota (69). Diverse agents (nutritional, chemical, or even an imbalance among the different bacteria species) that interfere with this multilayered mucus barrier might have the potential to promote or to control diseases associated with gut inflammation.

Regardless of the mucus barrier, gut microbiota interacts with immune cells, providing signals that are fundamental to the normal development of the host immune functions (70–72). Development of Th17 cells in the gut, for example, strictly depends on the presence of segmented filamentous bacteria (73, 74). By contrast, Clostridium and *Bacteroides fragilis* favor Tregs development (75, 76). In fact, any dysbiosis affecting the ileum microbiota deeply affect intestinal innate immunity and CD4 T cell homeostasis—particularly, a decrease in Th17 cells. These changes are the initial steps for the onset of metabolic diseases. Certainly, they are sufficient to induce glucose intolerance and insulin resistance.

In the gut, CD4 T cells contribute to immunity by differentiating into various subsets, notably inflammatory and regulatory cells (77, 78). Th17 cells are the most abundant CD4 T cells in mucosal tissues (79). They secrete two isoforms of IL-17 (IL-17A and IL-17F) and/or IL-22, which confer protection against fungi and pathogenic bacteria. Th17 cell differentiation is mediated by the transcription factor retinoid-related orphan receptor gamma t (80), induced by the cytokines TGFβ1, IL-6, IL-1β, and IL-21, and maintained by IL-23 (81).

It is remarkabe that 90% of the human gut microbiome is made up of organisms from two phyla, Firmicutes and Bacteroidetes. Importantly, an imbalance between these phyla has been directly linked to obesity, T2D, and inflammation (82, 83). Most of the studies report the augmentation in Firmicutes abundance and reduction in Bacteroidetes in obese versus lean individuals (84–87).

Corroborating these previous studies, elegant results from Cani et al. (88) and Turnbaugh et al. (89) demonstrated an association between the development of obesity and T2D, and an increase in the Firmicutes to Bacteroidetes ratio in the gut. This imbalance results in increased energy harvesting from food, as well as an increase in the transcription of genes controlling both lipogenesis in the liver and adipose tissue development (86, 89). Changes in the gut microbiota may also directly influence cells of both the innate (macrophages, dendritic cells, and innate lymphoid cells) and adaptive immune systems, thus contributing to development and maintenance of a state of chronic low-grade inflammation in obese individuals (75, 90).

Hydrolyzed carbohydrates and simple sugars can be fermented by gut microorganisms to short-chain fatty acids (SCFA), such as acetate, propionate, and butyrate. This sugar metabolism can result in an added 10% daily dietary energy to the host (91–93). Consumption of Western diets—low or normal in fiber contents and rich in fat and digestible sugars—can alter gut microbiota composition, impacting the production of SCFA and other gut-derived metabolites. Dysbiosis induced by fat- and sugar-rich diets is also associated with a thinner and less protective mucus layer, which results in increased gut permeability, chronic low-grade inflammation, and metabolic disorders (88, 94). Bacteria-derived SCFA have been shown to have anti-inflammatory properties attenuating the production of pro-inflammatory cytokines, such as TNF-α, IL-6, and IFN-γ. Thus, consumption of Western diets that results in decreased abundance of SCFA-producing bacteria could facilitate inflammatory responses in the gut (95).

FOOD ADDITIVES THAT ALTER GUT MICROBIOME: EMULSIFIERS AND SWEETENERS

Recent studies have demonstrated that the consumption of artificial sweeteners and dietary emulsifiers can alter the gut microbiota, resulting in intestinal disturbance and inflammation, favoring the development of the metabolic syndrome (27, 96).

Sweeteners such as high-fructose corn syrup and low-calorie or calorie-free sugar substitutes (sugar alcohols and artificial sweeteners) are among the most widely used food additives worldwide, regularly consumed by lean and obese individuals alike. A series of compounds (sucralose, aspartame, saccharin, steviol, Luo han guo extract, among others) are approved to be used as sweeteners in food, and are generally recognized as safe by the FDA. Cyclamates are banned in the United States but are still allowed in many other countries (97). Moreover, most of the commercial formulations of saccharin, sucralose, or aspartame comprise ~5% sweetener and ~95% glucose, functioning as a sugar source. Despite being considered safe and even beneficial owing to their low caloric content, pieces of evidence in various species, including humans, show that artificial sweeteners can be metabolically active (56, 98–104).

Pieces of evidence from both mice and human studies suggest that the consumption of artificial sweeteners is associated with increased glucose intolerance. This effect seems to result from alterations in the composition and function of the gut microbiota. In fact, several bacterial taxa that changed in response to sweetener consumption were previously associated with T2D development in humans (105, 106). For instance, it is reported that sweetener consumption increases the Bacteroides:Clostridiales ratio by increasing the former and decreasing the later. Indeed, the exposure of rats to Splenda (an artificial sweetener containing 1% sucralose) was associated with both increased weight gain and significant alterations in gut microbiota composition (23). In addition, the exposure to saccharin, sucralose, and especially aspartame-promoted alterations of the gut microbiota leading to an impaired glucose tolerance (24, 26). In fact, individuals consuming aspartame presented elevated fasting glucose and impaired insulin sensitivity, which were associated with gut dysbiosis (25).

Most artificial sweeteners are not chemically modified in the human gastrointestinal tract (107, 108) and, thus, directly encounter the intestinal microbiota. However, aspartame is a target for intestinal esterases and peptidases, which transforms aspartame into amino acids and methanol before reaching the colon (109). These products of aspartame metabolism should not have a significant influence on gut microbiota. Thus, the mechanisms of aspartame-induced dysbiosis remain unclear.

Currently, sucralose is widely used due to its taste (extremely similar to natural sugar) and providing no calories to the human body. Moreover, sucralose does not present the common and undesirable bitter aftertaste present in most of the artificial sweeteners. Therefore, sucralose has been considered a safe food additive mainly for the lack of any toxic effect and noeffect on metabolism (110). However, in a recent study, it was shown that sucralose consumption deeply impacts the gut microbiome composition increasing the release of pro-inflammatory mediators in mice. This effect was accompanied by an increase of inflammatory markers in the liver, such as MMP-2 and iNOS (26).

Saccharin is another sweetener strongly associated with the onset of metabolic diseases. Saccharin decreases the release of glucagon-like peptide-1 (GLP-1), an incretin hormone implicated in the regulation of various physiological processes, such as food intake, glycemic control, and cardiovascular protection (111, 112). Long-term cohort studies of artificial sweetener consumption reported increased risks of adverse outcomes, such as diabetes, cardiovascular diseases, and stroke, a pattern consistent with persistently reduced GLP-1 levels. On the other hand, Daly et al. (113) showed some prebiotic-like effects of saccharin/neohesperidin dihydrochalcone (SUCRAM®). Saccharin significantly increased gut abundance of Lactobacillus in piglets. Lactobacilli are the predominant lactic acid bacteria in the pig intestine and have well-described protective activity in the gastrointestinal tract, influencing gut immune system maturation and regulating intestinal inflammatory responses (113).

Emulsifiers are detergent-like molecules used by food industry as stabilizers for processed foods. Two commonly used emulsifiers are carboxymethylcellulose (CMC) and polysorbate-80 (P80). Toxicological and carcinogenic potential of P80 have been established and P80 is approved by the FDA for use in concentrations of up to 1%. CMC has not been extensively studied but it is used in various processed food at up to 2%. A recent study observed that, in mice, relatively low concentrations of these emulsifiers induced low-grade inflammation, obesity, and metabolic syndrome (27). These effects were associated with emulsifier-induced dysbiosis, bacterial translocation through the mucosal barrier, and increased pro-inflammatory potential. In fact, Roberts et al. (114) have shown that both emulsifiers can increase bacterial translocation across cultured epithelial layer (114). Moreover, bacterial translocation was associated with mucus loss and increased bacterial adherence after chronic exposure to dietary emulsifier (27).

Modest increases in body weight and fasting glycemia were observed in animals given doses as low as 0.1% CMC. Higher doses of 0.5% resulted in low-grade inflammation and increased adiposity. For P80, pieces of evidence of low-grade inflammation and increased adiposity were found in animals consuming amounts of as little as 0.1%. Higher concentrations of 0.5% resulted in a mild increase in fasting glycemia (27). These emulsifier-induced metabolic derangements were long-lasting since they could be observed even 6 weeks after emulsifier consumption withdraw (27).

Emulsifier-induced changes in gut microbiota play a role in driving inflammation and metabolic changes promoted by these food additives. They altered fecal levels of SCFA, including decreased levels of butyrate and bile acids (71, 115). In fact, emulsifiers induce gut barrier alterations and low-grade inflammation. They also result in significantly increased food intake (at least twice as high as the control mice), which drives the development of obesity by increasing fat mass and promoting metabolic alterations. Besides, excessive fat mass accumulation is associated with low-grade inflammation, which plays a crucial role in the onset

of obesity and related metabolic disorders. Cani and Everard (116) reported that as the gut microbiota composition and the fecal SCFA profile are modified in emulsifier treated mice, such changes may also impact food intake. Growing evidence suggests that microbial products, for example, butyrate and propionate, may directly bind to specific G-protein-coupled receptors such as GPR41/43 (117), enriched in enteroendocrine L-cells. The stimulation of these receptors triggers the release of enteroendocrine peptides, such as GLP-1 and peptide YY that reduce food intake *via* a gut/brain axis.

In conclusion, emulsifiers and sweeteners, which are freely used by people controlling caloric intake as well as by obese and patients with diabetes, present a verified effect on gut microbiota. This effect results in dysbiosis that normally is associated with the development of insulin resistance and to weight gain. Therefore, these additives should not be considered as innocuous and must have their use controlled and labeled with regard to possible undesirable effects.

ASSOCIATION OF FOOD ADDITIVES: CARRAGEENAN

Carrageenan is a hydrocolloid extracted from seaweeds and widely used by food industry as thickener and stabilizer. Due to its high molecular weight, it is assumed that carrageenan is not absorbed in the intestine and, thus, can be safely consumed (118). In fact, orally administered carrageenan can be recovered in feces in substantial amounts (119). Recovered carrageenan behaves similarly to the original in chromatographic assays, suggesting that it is stable at low pH and resistant to bacterial degradation, not being substantially altered or metabolized in the gastrointestinal tract (119).

However, recent studies demonstrate that oral carrageenan administration can lead to insulin resistance. This suggests that either there is a mechanism of immune cell activation in the gut or that the absorption of even trace amounts of carrageenan is sufficient to trigger pro-inflammatory responses systemically.

Carrageenan has been widely used as an inducer of inflammation and inflammatory pain (120). Its pro-inflammatory potential results from TLR4 activation, an innate immune receptor also involved in obesity and insulin resistance (12). This renders carrageenan as a potential to trigger for cellular processes that would contribute to the establishment of metabolic syndrome. For example, intratumoral carrageenan administration was recently reported to inhibit tumor progression by shifting macrophages toward a M1 phenotype (121).

In that sense, recent studies report the effect of administering carrageenan in water on metabolic parameters in mice. It was first shown that carrageenan consumption—in doses equivalent to those in foodstuff—leads to glucose intolerance *via* increased IRS-1 serine phosphorylation and interference with insulin-triggered PI3K–Akt pathway (34). This was also observed *in vitro*, using the human hepatocyte cell line HepG2, suggesting that a similar mechanism may be operating in human obesity. Further studies by the same group demonstrated that interference with insulin signaling by carrageenan was triggered *via* a signaling pathway involving TLR4, Bcl10, and mitochondrial ROS, which was further sustained by GRB10 (122). GRB10 is an adaptor protein that associates with and inhibits IRS-1 and was upregulated by carrageenan exposure both *in vitro* and *in vivo* (122).

Interestingly, carrageenan consumption exacerbated glucose intolerance and dyslipidemia induced by high-fat diet feeding (35). Of note, carrageenan did not show any effect on lipid profile of mice fed a normal diet, but induced a substantial increase in non-HDL cholesterol levels. This suggests that carrageenan consumption, especially in association with fat rich food (as seen in industrialized foods), may predispose individuals to develop atherosclerosis and increase cardiovascular risk.

The safety of carrageenan consumption is still a matter of debate. Arguments in favor of its safety rely on toxicological studies showing no significant intestinal absorption after oral administration. In these studies, there was no evidence of carrageenan traces in the liver or cells of the gastrointestinal tract of non-human primates and rats (123, 124), although the analytical method applied were not very sensitive. By using a more sensitive method, administering radiolabeled carrageenan, it was observed the presence of absorbed carrageenan in cecal lymph node, Peyer's patches, and the intestinal wall, mainly associated with macrophages (125). Other authors showed the presence of macrophage-borne carrageenan in intestinal lamina propria and the liver, suggesting that sampling of intestinal content by mucosal macrophages may provide a route for carrageenan entry (126, 127).

Despite the lack of consensus on whether carrageenan is absorbed or not, its pro-inflammatory activity is undeniable. Active absorption or passive entry of even trace amounts of carrageenan could have significant effects on metabolic homeostasis, especially with chronic exposure. Given that a high-fat diet or food additives such as emulsifiers were shown to interfere with intestinal mucosal permeability, it is important to reconsider the toxicological properties of carrageenan. For example, the addition of carrageenan (as a thickener) to foods rich in fat could facilitate the extravasation of carrageenan due to high-fat-induced intestinal permeability. Likewise, association of carrageenan with emulsifiers (as seen in many processed foods) would facilitate carrageenan absorption after disruption of the epithelial intestinal barrier. Therefore, in light of these new data showing that the intestinal barrier homeostasis may be disrupted by dietary components, it becomes necessary to reassess the toxicological properties of this widely used food additive.

CONCLUSION

Modern societies have witnessed an alarming increase in cases of obesity, T2D, and other comorbidities associated with poor eating habits. In addition, the incorporation of processed and industrialized food into everyday eating correlates with this phenomenon. In an attempt to tackle that threat, much attention has been paid to the amount of calories present in food. Specifically, the amount of fat and simple sugar is considered the main villains of modern processed food. Although the detrimental effects of fat and sugar are undeniable, industrialized food is composed of many other

substances of which the metabolic effects are usually ignored. Emulsifiers, thickeners, artificial sweeteners, and preservatives may have direct and indirect effects on cells of the immune system, contributing to metabolic dysregulation. Moreover, safety evaluation and estimates of food additives toxicity are usually based on tests of single compounds, thus underestimating the effects of associating two or more additives. Therefore, policies that aim at curbing the evolution of obesity must take into account not only calorie content of food but also all the other components and their effects. Whenever possible, people should avoid ready-to-serve, additive rich, processed food and prefer healthier food choices or home-cooked meals.

AUTHOR CONTRIBUTIONS

HN conceived the text and wrote the manuscript. PA, LG, JL, PZ, and MS-P wrote the manuscript.

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High-Salt Diet Induces IL-17-Dependent Gut Inflammation and Exacerbates Colitis in Mice

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Excess intake of sodium is often associated with high risk for cardiovascular disease. More recently, some studies on the effects of high-salt diets (HSDs) have also demonstrated that they are able to activate Th17 cells and increase severity of autoimmune diseases. The purpose of the present study was to evaluate the effects of a diet supplemented with NaCl in the colonic mucosa at steady state and during inflammation. We showed that consumption of HSD by mice triggered a gut inflammatory reaction associated with IL-23 production, recruitment of neutrophils, and increased frequency of the IL-17-producing type 3 innate lymphoid cells (ILC3) in the colon. Moreover, gut inflammation was not observed in IL-17–/– mice but it was present, although at lower grade, in RAG$^{-/-}$ mice suggesting that the inflammatory effects of HSD was dependent on IL-17 but only partially on Th17 cells. Expression of SGK1, a kinase involved in sodium homeostasis, increased 90 min after ingestion of 50% NaCl solution and decreased 3 weeks after HSD consumption. Colitis induced by oral administration of either dextran sodium sulfate or 2,4,6-trinitrobenzenesulfonic acid was exacerbated by HSD consumption and this effect was associated with increased frequencies of RORγt$^+$ CD4$^+$ T cells and neutrophils in the colon. Therefore, our results demonstrated that consumption of HSD *per se* triggered a histologically detectable inflammation in the colon and also exacerbated chemically induced models of colitis in mice by a mechanism dependent on IL-17 production most likely by both ILC3 and Th17 cells.

Keywords: sodium chloride, gut inflammation, IL-17, Th17 cells, ILC3

INTRODUCTION

Sodium is an indispensable nutrient for proper cell functions in living animals when consumed in appropriate amounts (1). It is essential for maintenance of osmotic pressure, normal pH, distribution of body fluids, and for most of metabolic processes. The influx of sodium ions across plasma membrane is required for action potential involved in nerve impulses and muscle contraction. Sodium chloride is readily available in processed food and ingested in large amounts as part of Western

diets (2). Excess intake of sodium is often associated with high risk for cardiovascular diseases (2–4).

It has been reported recently that immune cells play an important role in mediating the detrimental effects associated with consumption of high concentration of sodium. Elevated concentration of sodium chloride was found to induce pro-inflammatory IL-17-producing helper T (Th17) cells *via* p38/MAPK/nuclear factor of activated T cells 5 pathway and the serum/glucocorticoid-regulated kinase 1/forkhead box protein 1 (SGK1) pathway both *in vitro* and *in vivo* (5, 6). These studies have demonstrated that high-salt diets (HSDs) exacerbate autoimmune encephalomyelitis in mice in a SGK1-dependent fashion (5, 6). In addition, hypertension and renal injury induced by HSD in salt-sensitive rats are accompanied by increased infiltration of T cells in kidneys (7, 8).

Intestinal mucosa is the major surface of contact with the external environment and the first tissue to interact with all types of food components including salt. The gastrointestinal tract is in constant interaction with the microbiota and the antigens from diet. Under normal conditions, exposure to these antigenic proteins would induce oral tolerance. However, any failure in intestinal homeostasis may result in inflammatory reactions toward microbiota and food antigens (9). These reactions are especially present during inflammatory bowel diseases (IBDs) such as Crohn's disease (CD) and ulcerative colitis (UC). It has been proposed that IBD results from the imbalance between regulatory T cells (Treg) and inflammatory Th1, Th2, and Th17 cells (10). Several studies have demonstrated an increased expression of Th17-related cytokines in both UC and CD, although it is not clear whether these cytokines are only produced by T cells (11).

Dietary composition has been long seen as a potential risk factor for the increased incidence of autoimmune diseases and IBD. A specific factor that has changed in the Western diet over time is the increasing amount of sodium-containing salt intake. Some recent studies have demonstrated that diets containing high amounts of sodium chloride can also induce an increase in Th17 cells and aggravation of experimental models of colitis by the activation of SGK1 kinase (12–14). These studies highlighted the importance of SGK1/Th17 axis but did not explore appropriately the possibility that IL-17 could be produced by other sources such as ILC3. It is clear that several cell types other than lymphocytes are important in intestinal homeostasis and also in the onset of inflammatory diseases (15).

Innate lymphoid cells (ILCs) represent a heterogeneous group of hematopoietic cells of the innate immune system that have a common lymphoid progenitor. While ILCs lack rearranged antigen-specific receptors, they express many of the transcription factors and effector molecules expressed by $CD4^+$ T helper (Th) cell populations, suggesting that ILCs may be an evolutionary precursor of cells of the adaptive immune system. They also have homologous functions with the Th cells. ILC1 express the transcription factor t-bet and is homologous to Th1 cells; ILC2 expresses GATA 3 and are homologous to Th-2 cells and ILC3 express ROR-γt and are homologous to Th-17 cells (16, 17). Considering that Th17 cells have been demonstrated to be pivot cells in pathogenicity of HSD in encephalomyelitis (5, 6) as well as colitis (12–14) models, that ILC3 are very abundant in the gut mucosa and that they are increased in the colon of individuals during the course of IBD (17, 18), it is likely that the effect of HSDs in colitis development is dependent on IL-17 production by both Th17 cells and ILC3.

This study aims to investigate the direct effect of a sodium chloride-rich diet in the gut mucosa as well as in the development of chemically induced colitis. Our working hypothesis is that sodium chloride may have an inflammatory effect *per se* and that both T cells and ILCs may play a role in this effect. In addition, we sought to investigate the synergistic effect of HSD in the inflammatory events taking place during experimental colitis induced by administration of 1% dextran sodium sulfate (DSS) in C57BL/6 mice and in the colitis induced by 2,4,6-trinitrobenzenesulfonic acid (TNBS) in BALB/c mice.

MATERIALS AND METHODS

Animals

Female BALB/c and C57BL/6 mice were purchased from CeBio (Centro de Bioterismo da Universidade Federal de Minas Gerais). Eight-week-old animals were used. IL-$17^{-/-}$ and $RAG^{-/-}$ mice were kindly provided by Dr. João Santana from Universidade de São Paulo, Ribeirão Preto, Brazil, and by Dr. Ricardo Gazzinelli, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil, and maintained in our animal facility located at Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais. Animals were bred and housed in microisolators. All procedures were approved by the local ethical committee for animal research (CEUA-UFMG, Brazil, protocol 50/2014). Experiments were performed in accordance with guidelines and regulation established by Conselho Nacional de Controle de Experimentação Animal (CONCEA), Brazil.

High-Salt Diet

Mice received either AIN93G diet as a control diet (15) or modified AIN93G supplemented with 4% NaCl (HSD) throughout the experiments.

Hemodynamic Measurements Using the Tail-Cuff Method

Mean arterial pressure (MAP) was evaluated by a volume pressure recording sensor and an occlusion tail-cuff, which measures mice's tail blood pressure noninvasively (Kent Scientific Corporation, Torington, CT, United States) (16). Mice were acclimated to restraint and tail-cuff inflation for 2 days before the beginning of experiments. The restraint platform was maintained at 31–34°C. In each session, mice were placed in an acrylic retainer, and the tail was inserted into a compression cuff that measured the blood pressure eight times. Following the measurement cycle, the average of these values was considered for each mouse. All parameters were evaluated weekly, 1 day before the beginning of dietary consumption as well as 7, 14, and 21 days thereafter.

Intestinal Permeability Test

Intestinal permeability was determined by measuring radioactivity diffusion in the blood after oral administration of diethylenetriamine pentaacetic acid (DTPA) labeled with

^{99m}-technetium (^{99m}Tc). After 21 days of diet consumption, animals received 0.1 mL of a DTPA solution labeled with 18.5 mebequerel of ^{99m}Tc by gavage. Four hours later, mice were anesthetized; their blood was collected, weighed, and placed in appropriate tubes for radioactivity determination. Blood radioactivity levels were determined using an automated gamma counter (Perkin Elmer Wallac Wizard 1470–020 Gamma Counter; PerkinElmer Inc., Waltham, MA, USA). Results obtained were compared with the standard dose and calculated as a percentage of the dose per gram of blood using the following equation:

$$\%\ \text{dose/g blood} = \left(\text{cpm in g of blood/cpm dose of standard}\right) \times 100,$$

where cpm represents the counts of radioactivity per minute.

Flow Cytometry Analyses

Following euthanasia, colon was harvested, and mesenteric lymph nodes were removed. Intestines were then cut open longitudinally and drawn through a pair of curved forceps while applying gentle pressure to remove intestinal contents. Tissues were cut into 2–4 cm fragments, washed twice to remove feces in calcium- and magnesium-free HBSS containing 2% FCS (at 4°C). Tissues were placed in 50 ml tubes and washed three times in HBSS containing 2% FCS at 4°C. Tissues were transferred to 25 cm^3 tissue culture flasks and incubated at 37°C in HBSS containing 10% FCS, 0.2 mmol/l EDTA, 1 mmol/l DTT, 100 U/ml penicillin, and 100 µg/ml streptomycin. After 20 min, flasks were shaken vigorously for 30 s, and supernatant containing IEL was separated from the tissue fragments using a stainless steel sieve. To isolate lamina propria lymphocytes, the remaining tissue was washed three times with RPMI, and intestinal pieces were subsequently incubated for 30 min at 37°C in RPMI supplemented with 100 U/mL liberase (Roche). Cells were separated from tissue debris by purification through a 70 µm nylon filter. This step was repeated with a 40 µm nylon filter. Cell suspensions were adjusted to 10^6 cells/ml.

Flow cytometry analysis for neutrophils was performed using the following antibodies: CD11c-APC, CD103-Pacific Blue, MHC (IA IE)-PE, Ly6C-Percp, Ly6G-FITC, CD45-APC-Cy7, and F4/80-PE-Cy7. For T lymphocyte analysis, the following antibodies were used: CD4-FITC, ROR-γt-PE, IL-23R-PercepCy5.5, CD45-APC-Cy7. For ILC analysis, the following antibodies were used: Pacific Blue: CD11b, CD11c, or FceR1a; BV421: CD16 or CD19; PERCP-CY5.5: CD8; PE/CY7: CD4; BV570: CD45; BV605: CD117; APC: CD127; F700: CD3; PE-CF594: ROR-γt; PE: t-bet; AF488: GATA 3. All antibodies were purchased from Biolegend (San Diego, CA, USA). Strategies used to analyze these cell populations are shown in Figures S1 and S2 in Supplementary Material. For surface antigen detection, cells were labeled with monoclonal antibodies for 30 min at 4°C. For intracellular labeling, a fixing/permeabilization kit (e-Bioscience, San Diego, CA, USA) was used after this step. Samples were then incubated for 30 min with a solution containing the appropriate antibodies. After washing with PBS containing 0.5% FBS, samples were fixed with 3% paraformaldehyde for 30 min, washed and stored in PBS at 4°C. Cells were acquired using a FACSCanto II cytometer (Becton Dickinson, East Rutherford, NJ, USA) and data was analyzed by FlowJo software (Tree Star, Ashland, OR, USA).

Intestinal Tissue Preparation for Cytokine Measurement

Colon samples were weighted and homogenized in PBS containing 0.05% Tween-20, 0.1 mM phenylmethylsulphonyl fluoride, 0.1 mM benzethonium chloride, 10 mM EDTA, and 20 KIU Aprotinin A using a tissue homogenizer (100 mg tissue/ml buffer). Suspensions were centrifuged at 12.000g for 20 min at 4°C and the supernatants were transferred to microtubes. Supernatants were collected for cytokine assay levels of IFN-γ and IL-23 by capture ELISA. Briefly, plates were coated with purified monoclonal antibodies reactive with the cytokines IFN-γ and IL-23 (BD-Pharmingen, Franklin, NJ, USA) overnight at 4°C. In the following day, wells were washed, supernatants and standards were added, and plates were incubated overnight at 4°C. In the third day, biotinylated monoclonal antibodies against cytokines were added and plates were incubated for 2 h at room temperature. Color reaction was developed at room temperature with 100 µL/well orthophenylenediamine (1 mg/mL), 0.04% H_2O_2 substrate in sodium citrate buffer. Reaction was interrupted by the addition of 20 µL/well of 2 N H_2SO_4. Absorbance was measured at 492 nm by ELISA reader (Bio-Rad, Philadelphia, PA, USA).

RNA Isolation, Reverse Transcription, and Quantitative Real-time PCR (qPCR)

Total RNA extraction from frozen colon of C57BL/6 mice that received either standard diet (control group) or HSD group for 3 weeks, 5 days, 3 days, 120 h, 48 h, 36 h, or 24 h and colon of mice that received a 50% NaCl solution by oral gavage and were euthanized after 90 min thereafter, was performed using TRIzol® reagent (Invitrogen, USA), followed by treatment with RQ1 RNase-Free DNase (Promega, USA), and cDNA synthesis using RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, UK). qPCR assays were performed to evaluate the mRNA levels of *Sgk1* (serum/glucocorticoid-regulated kinase 1) gene. *Gapdh* (glyceraldehyde-3-phosphate dehydrogenase) gene was used for the normalization of the data. Primers were designed using Primer3 program, version 0.4.0 (http://frodo.wi.mit.edu/) and analyses of parameters such as GC content and formation of homo-dimer, hetero-dimer, and hairpins structures were performed using OligoAnalyzer 3.1 software (https://www.idtdna.com/calc/analyzer). The specificity of the primers was confirmed using Primer-BLAST program (http://www.ncbi.nlm.nih.gov/tools/primer-blast/), to ensure that the primers amplified only the coding region (CDS) of interest. The sequences of the forward and reverse primers used for amplification of *Sgk1* (GenBank: NM_001161847.2) and *Gapdh* (GenBank: GU214026.1) were 5′-CAAATCAACCTGGGTCCGTC-3′ (Sgk1-F), 5′-TCCAAAACTGCCCTTTCCG-3′ (Sgk1-R); 5′-CATCTTCCAGGAGCGAGACC-3′ (Gapdh-F), and 5′-GAAGGGGCGGAGATGATGAC-3′ (Gapdh-R). Each qPCR reaction contained 5 µL KAPA SYBR® FAST qPCR Master Mix 2× (Kapa Biosystems, USA); 0.2 µL of ROX High 50×; 200 ng of cDNA; each forward and reverse primer at the optimized

concentrations (0.4 μM (F)/0.4 μM (R) for Sgk1 and 0.3 μM (F)/0.3 μM (R) for Gapdh) and water up to a final volume of 10 μL. The reaction profile was an initial step of denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 3 s and combined annealing and extension at 60°C for 30 s. A "no template control" was made in all the qPCR reactions for each pair of primers containing all the reagents except cDNA. Reaction specificity was confirmed with melting curves analysis and agarose gel electrophoresis experiments (*Sgk1* amplicon size = 87 bp; *Gapdh* amplicon size = 150 bp). Standard curves were generated with series of log dilutions of cDNA to calculate the amplification efficiency (*Sgk1*: Eff = 98.98%, $R^2 = 0.99$; *Gapdh*: Eff = 96.28%, $R^2 = 0.99$). The qPCR reactions were performed using ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems, UK), and the data were processed by SDS Software, version 2.4 (Applied Biosystems, UK). The calculation of gene expression was performed using the $2^{-\Delta Ct}$ method, where $\Delta C_t = C_t$ value of target gene $- C_t$ value of reference gene.

Colitis Induction

Dextran sodium sulfate-induced colitis was triggered by administration of 1% dextran sulfate sodium in the drinking water to C57BL/6 mice for 7 days (19). TNBS-induced colitis was triggered by administration of 2.5 mg TNBS in 35% alcohol intrarectally to BALB/c mice 7 days before euthanasia (19).

Clinical Score and Histological Assessment of Colitis

Colons were excised and colonic inflammation assessed using a previously defined scoring system (20), which includes macroscopic features such as the presence or absence of adhesions, strictures, and diarrhea (diarrhea was defined as loose, watery stool). Samples of colon were fixed in formalin and processed for microscopic analysis. Hematoxylin-eosin-stained sections were blindly scored based on a semiquantitative scoring system that includes the main alterations observed: goblet cell depletion, muscular layer (erosion/ulceration) destruction, cellular infiltration, and edema; each parameter could receive 0–3 in the score index.

Colitis clinical severity (Disease Activity Index) were evaluated by a scoring system described previously (20) where the following features were graded: extent of destruction of normal mucosal architecture (0: normal; 1, 2, and 3: mild, moderate, and extensive damage, respectively), presence and degree of cellular infiltration (0: normal; 1, 2, and 3: mild, moderate, and transmural infiltration, respectively), extent of muscle thickening (0: normal; 1, 2, and 3: mild, moderate, and extensive thickening, respectively), presence or absence of crypt abscesses (0: absent; 1: present) and the presence or absence of goblet cell depletion (0: absent; 1: present). Scores for each feature were summed up to a maximum possible score of 11.

Measurement of the Activity of Myeloperoxidase (MPO)

Samples were first homogenized in buffer solution (NaCl 0.1 M, Na_3PO_4 0.02 M, and Na_2EDTA 0.015 M) and then centrifuged. Supernatant was discarded and the pellet resuspended and centrifuged. Supernatant was discarded and the pellet resuspended in another buffer (Na_3PO_4 and 0.5% w/v HETAB) solution and then stored at room temperature in a ratio of 1.9 ml/100 mg. Samples were homogenized and half the volume was withdrawn for NAG enzyme activity measurement. The other half of the homogenate was used for the determination of MPO activity. From this step on, the samples received different treatments.

Evaluation of NAG activity was performed separately. Salina/Triton (Saline 0.9% and Triton X-100 0.1%) was added to the homogenate, samples were homogenized, and then centrifuged at 4°C for 10 min at 3000 RPM. Hundred microliters of the supernatant were collected and diluted in citrate phosphate buffer (0.1 M citric acid and 0.1 M Na_2HPO_4) for NAG measurement. In the microplate, 100 μL of each diluted sample was pipetted, and 100 μL of the substrate *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide diluted in phosphate citrate buffer was added to each well. Samples were incubated. Then, 100 μL of 0.2 M glycine buffer was added to the samples to stop the reaction. The absorbance was red at 400 nm. The mean of the values was obtained from each duplicate and was used to determine the activity of the enzyme.

Statistical Analysis

All results were expressed as the mean ± SD of the mean. Significance of differences among groups was determined by either Student's *t*-test or analysis of variance (ANOVA). Most of the data represent results from two or three independent experiments with five mice per group as indicated in the legend of figures. Means were considered statistically different when $p < 0.05$.

RESULTS

High-Salt Diet Induced Augmented Arterial Pressure in Mice

To confirm that 4% sodium chloride diet was able to increase the blood pressure, as expected for a HSD, we performed a plethysmography (a technique used to measure changes in body blood volume) in mice. After 3 weeks of HSD consumption, we observed an increase in mean arterial pressure (MAP) in mice fed HSD when compared to mice fed a standard diet (**Figure 1A**).

High-Salt Diet Induced Histological Signs of Colonic Inflammation and Increase in Intestinal Permeability

Clinical signs of colitis such as weight loss, diarrhea, rectal bleeding, and reduced colon's length were not observed in mice that received HSD when compared to mice fed standard diet (**Figure 1B**). Intestinal permeability was also measured as a more sensitive method to evaluate intestinal damage. **Figure 1C** shows that mice fed HSD had an increased intestinal permeability than control mice. Histological sections where obtained to access microscopic signs of inflammation. HSD-fed mice presented depletion of goblet cells, cellular infiltration in the colonic mucosa, erosion, ulceration, and destruction in *muscularis mucosa* layer, as well as edema in submucosal layer (**Figure 1D**). Histological scores were calculated and it showed inflammatory alterations in the HSD-fed mice (**Figure 1E**).

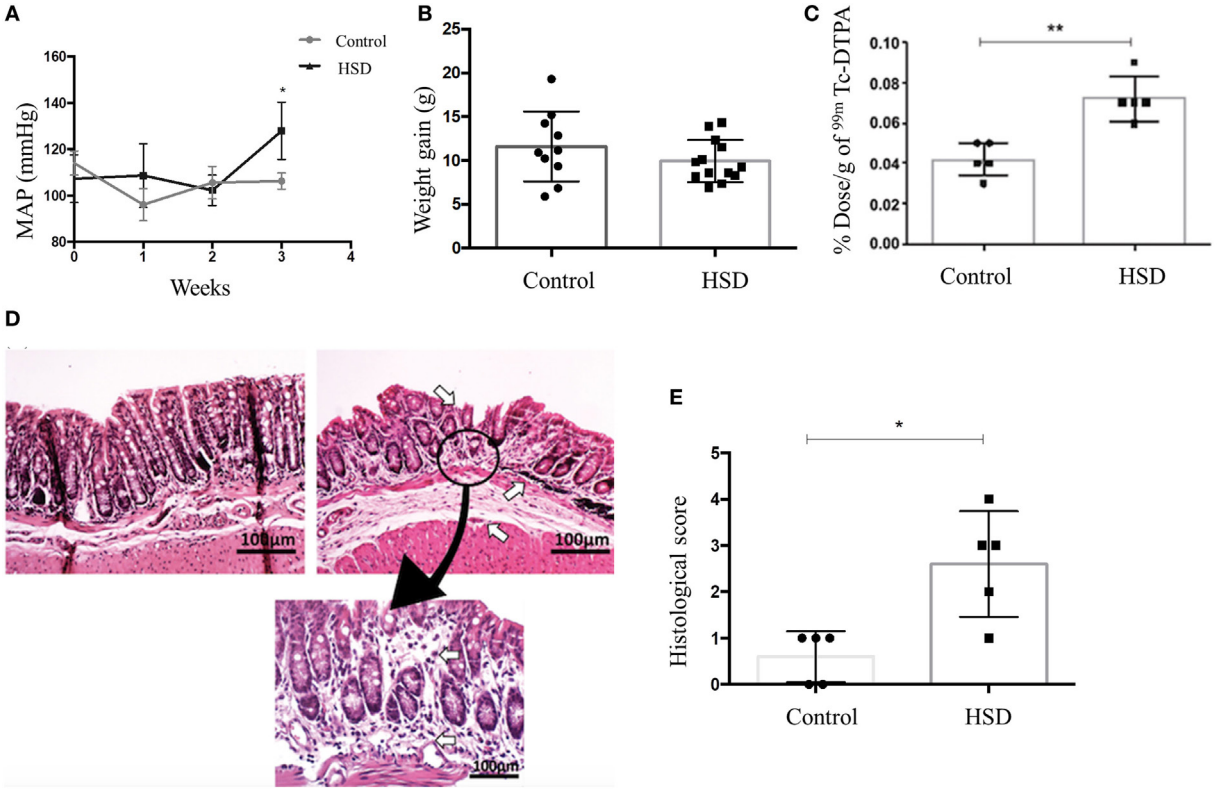

FIGURE 1 | High-salt diet (HSD) consumption increased arterial pressure and intestinal permeability, and led to gut inflammation. **(A)** Blood volume measured in a plethysmograph to obtain the mean arterial pressure (MAP) of mice fed either standard diet (control) (n = 5–15) or HSD (n = 5–15) during 3 weeks. **(B)** Weight gain; **(C)** intestinal permeability; **(D)** representative histological image of colon of control mice (left), HSD fed mice (right) after 3 weeks of diet consumption (100× magnification). Area of gut inflammatory reaction of HSD fed mice colon is shown at 200× magnification; **(E)** Histological score comparing control and HSD fed mice after 3 weeks of diet consumption. Score represents the sum of edema, inflammation area, and erosion area. Graphs are representative of three independent experiments with five mice per group. Statistical analysis between control and HSD fed mice at the end time point of each experimental was performed using Student's t-test for parametric data and Mann–Whitney for non-parametric data. Differences over time were evaluated using two-way analysis of variance test. $^*p < 0.05$.

High-Salt Diet Increased the Production of IL-23 and the Frequency of IL-23R$^+$ CD4$^+$ T Cells

Next, we investigated local factors that could lead to intestinal inflammation in HSD-fed mice. Colon extracts were examined for their content of IL-23, a cytokine involved in Th17 and type 3 innate lymphoid cell (ILC3) differentiation. IFN-γ, a typical Th1 cytokine, was also measured. HSD-fed mice presented increased levels of IL-23, but normal levels of IFN-γ in their colons when compared with the control group (**Figures 2A,B**, respectively). An increased frequency of CD4$^+$ T cells expressing IL-23R$^+$ in the colonic tissues (**Figure 2C**) was also observed. Th17 are known to secrete IL-17 and this inflammatory cytokine is involved in recruitment of neutrophils, ultimately leading to mucosal inflammation (21). To verify whether increased levels of IL-23 could be related to neutrophil infiltration in the colon, the frequency of these cells was analyzed by flow cytometry. **Figure 2D** shows an increased frequency of neutrophils in the colon of mice fed HSD for 3 weeks. This result was confirmed by the histological analysis of the colonic mucosa showing a significant infiltration of neutrophils in the colons of HSD-fed mice (**Figure 2E**).

High-Salt Diet Led to Increase in the Frequency of ILC 3 in the Colon

Type 3 innate lymphoid cells (ILC3) are important cells for gut homeostasis but are also involved in the development of intestinal inflammatory diseases (16–18). Considering that IL-23 cytokine, that is elevated in our model, is closely associated with ILC3 differentiation, the frequency of colonic ILC populations where analyzed. ILC1 population was decreased in the colon after 3 weeks of HSD consumption (**Figure 3A**). ILC2 frequency also decreased after 2 and 3 weeks of HSD consumption (**Figure 3B**). However, ILC3 frequency was increased in the colon of mice fed HSD for 2 weeks (**Figure 3C**).

High-Salt Diet Lead to Gut Inflammation in RAG$^{-/-}$ Mice but Not in IL-17$^{-/-}$ Mice

Since we found that consumption of HSD was associated with IL-23 production and increased frequencies of ILC3

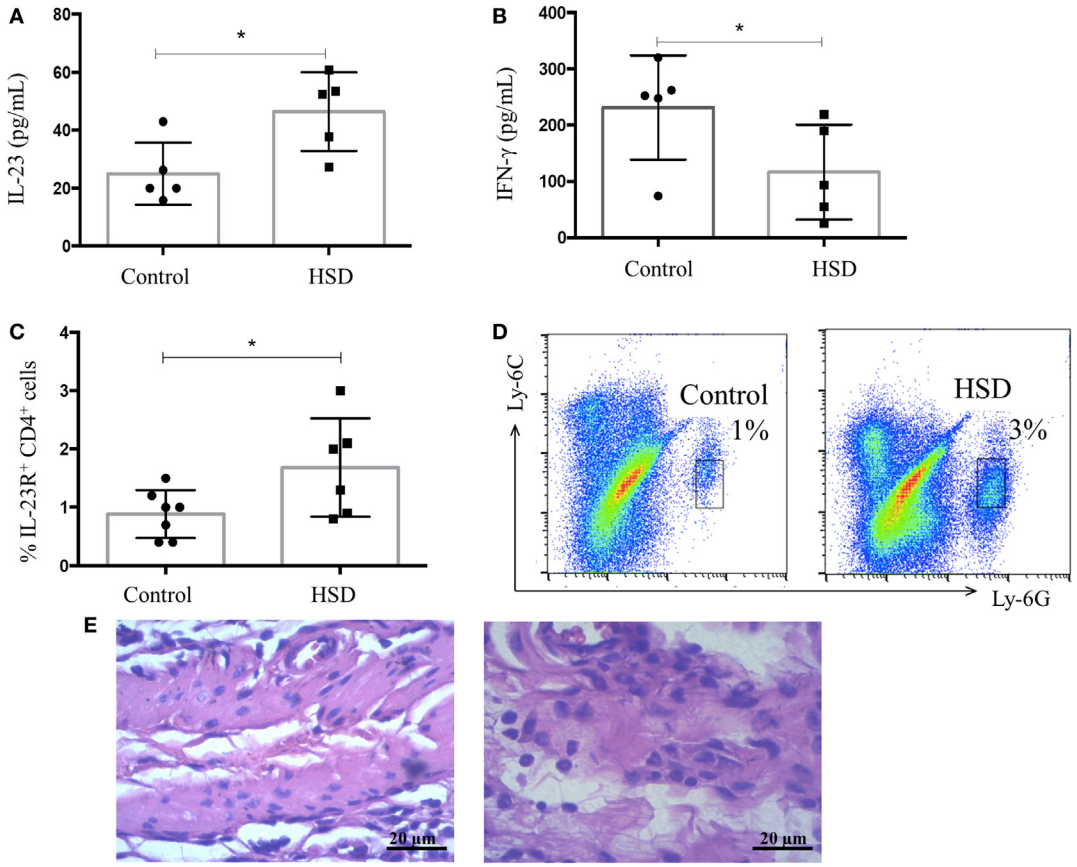

FIGURE 2 | IL-23 concentration and frequency of neutrophils in colonic mucosa increased after high-salt diet (HSD) consumption. C57BL/6 mice received control diet (n = 5–15) or HSD (n = 5–15) for 3 weeks. **(A)** IL-23 concentration in colon extract of mice fed either control diet or HSD; and **(B)** IFN-γ concentration in colon extract of control diet- or HSD-fed mice measured by ELISA; **(C)** $CD4^+$ IL-$23R^+$ cell frequency obtained by flow cytometry; and **(D)** representative dot plot of neutrophils in colons of control group (left) and HSD group (right) analyzed by flow cytometry. **(E)** Representative histological image of colon of control (left) or HSD fed mice (right) after 3 weeks of diet consumption (200× magnification). Graphs are representative of two independent experiments with five mice per group. Statistical analysis between groups was performed using Student's t-test. $^*p < 0.05$.

(important cells for IL-17 production), we examined next the role of IL-17 as a trigger of inflammation. **Figures 4A,B** show the histological section from colon of wild-type (WT) mice fed either control or HSD, respectively. To evaluate the role of IL-17 in the inflammatory process driven by HSD, we analyzed the histological scores of $IL\text{-}17^{-/-}$ mice that received HSD diet for 3 weeks and no inflammatory infiltration in their colons was found (**Figure 4D**) suggesting the effects of HSD consumption were dependent on IL-17.

Since **Figure 3C** showed an augmented frequency of ILC3 in the colon of mice treated with HSD, we decided to investigate further if the colonic inflammation was dependent exclusively on T lymphocytes. To test this, $RAG^{-/-}$ mice (that lack T and B lymphocytes) were fed HSD for 3 weeks. **Figure 4E** ($RAG^{-/-}$ mice fed control diet) and **Figure 4F** ($RAG^{-/-}$ mice fed HSD) show that HSD-fed $RAG^{-/-}$ mice also developed histological signs of colonic inflammation when compared to their controls, although at a statistically significant lesser extent than HSD-fed WT mice (**Figure 4G**). These results suggest that the inflammation caused by HSD is dependent on IL-17 but only partially dependent on Th17 cells.

High-Salt Consumption Lead to a Trend to Increase *sgk1* Expression in the Colon Shortly after NaCl Ingestion but Not Later

Sgk1 is a kinase associated with Na^+ absorption. An increase in its expression was previously associated with activation of Th17 cells and inflammation caused by high concentrations of salt in the western diet (5, 6). To verify whether there was any difference in *sgk1* expression in colon of HSD-fed mice, expression of this gene was evaluated either 90 min after oral administration (by gavage) of a solution containing high concentration of NaCl (50%) by gavage (**Figure 5A**) or 24, 36, 48, 72, and 120 h (**Figure 5B**) as well as 3 weeks (**Figure 5C**) after HSD consumption. There was no difference in the expression of *sgk1* up to 72 h post HSD consumption, but we could observe reduction in expression of sgk1 after 120 h and after 3 weeks of consumption. At an earlier

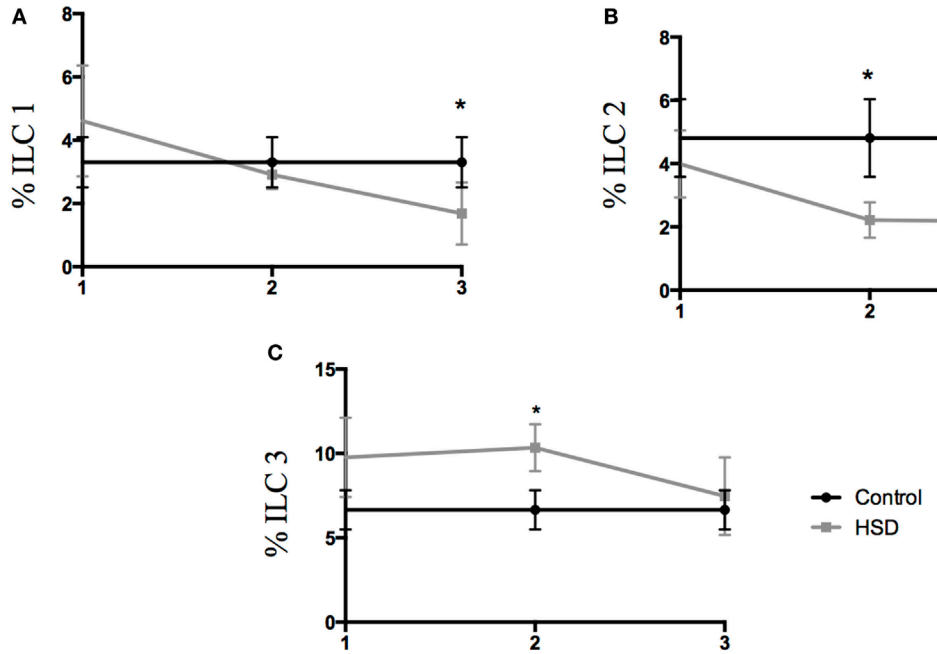

FIGURE 3 | Frequency of innate lymphoid cell populations changed during high-salt diet (HSD) consumption. Frequencies of ILCs were analyzed by flow cytometry in the colonic *lamina propria* of mice that received either control diet (n = 5–10) or HSD (n = 5–10) during 1, 2, and 3 weeks. **(A)** ILC1 (Lin-t-bet$^+$); **(B)** ILC2 (Lin-GATA3$^+$); **(C)** ILC3 (Lin- RORγt$^+$). Graphs are representative of two independent experiments with five mice per group. Statistical analysis between groups was performed using Student's t-test. *$p < 0.05$.

time point, 90 min, there was a trend to increase in the expression of sgk1 ($p < 0.06$).

High-Salt Diet Worsened DSS-Induced Colitis in C57BL/6 Mice

After observing that HSD led to inflammation in the colon, we sought to investigate whether HSD could also worsen colitis inflammation. C57BL/6 mice were fed HSD for 3 weeks and in the last week they also received 1% DSS in the drinking water (19). Mice that received HSD did not show any weight gain (**Figure 6A**) and presented more severe clinical scores (**Figure 6B**) (diarrhea, weight loss, shortening of colon) and more severe shortening of the colon (**Figure 6C**) when compared to control mice challenged with DSS. Histological analysis (**Figures 6D,E**) also showed a more prominent inflammatory reaction in the colon of HSD-fed mice. Gut inflammation was associated with higher frequency of neutrophils (**Figure 6F**) and increased MPO activity (**Figure 6G**).

High-Salt Diet Worsened TNBS-Induced Colitis in BALB/c Mice

2,4,6-trinitrobenzenesulfonic acid-induced colitis is a model of IBD triggered by Th1/Th17 cells (19). In order to evaluate the effects of HSD in TNBS-induced colitis, mice were fed HSD for 3 weeks, and in day 14 received TNBS diluted in alcohol by intrarectal injection. HSD-fed mice had a more pronounced weight loss (**Figure 7A**), decreased survival (**Figure 7B**), and increased clinical scores (**Figure 7C**) when compared to control TNBS-treated mice. Histological analyses also showed signs of a more severe gut inflammation (**Figure 7D**) and higher histological scores (**Figure 7E**) in HSD-fed mice.

Analysis of cells in the *lamina propria* of the colons from TNBS-treated mice showed an increased frequency of CD4$^+$ ROR-γt$^+$ T cells (**Figure 7F**) and CD4$^+$ T cells expressing IL-23R (**Figure 7G**).

Of note, administration of diets rich in other salts such as magnesium and potassium chloride (data not shown) also resulted in worsening of DSS-induced colitis suggesting that pathways different from SGK1 may also lead to gut inflammation.

DISCUSSION

It is known that a high consumption of salt leads to lipid disorders, target kidney damage with hypertension, and decline in renal function (22), but little is known about the influence of HSD in immunological features. It has been demonstrated that increase in dietary NaCl concentration lead to Th17 cell differentiation and worsening of experimental autoimmune encephalomyelitis (EAE) (5, 6). In the present study, we sought to determine the consequences of consumption of HSD in the gut mucosa since this is the first tissue to contact all food components. Some recent studies demonstrated that consumption of HSD induces Th17 cells and exacerbates experimental colitis in mice thorough a SKG1 signaling pathway (12–14). However, none of these studies examined the direct damage caused by salt in the colonic mucosa without another source of insult. Moreover, they all

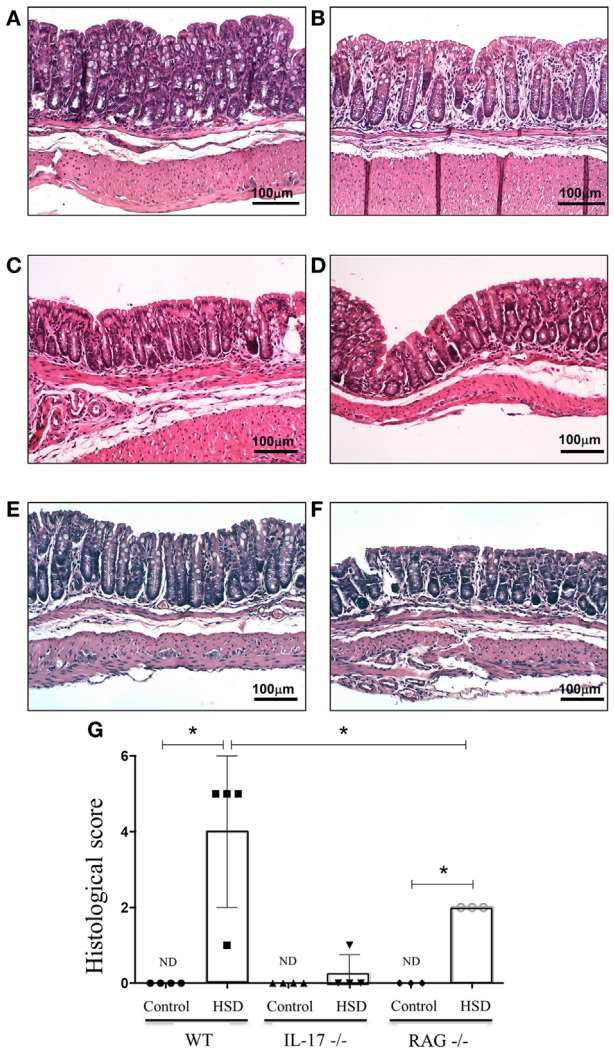

FIGURE 4 | Consumption of high-salt diet (HSD) induced gut inflammation in RAG$^{-/-}$ mice but not in IL-17$^{-/-}$ mice. C57BL/6 wild type (WT), IL-17$^{-/-}$ and RAG$^{-/-}$ mice received either control diet (n = 3) or HSD (n = 3) for 3 weeks. For histological score, goblet cell depletion, muscular layer (erosion/ulceration) destruction, cellular infiltration, and edema were considered, each parameter received 0–3 in the score index. **(A,B)** Representative images of colon sections from WT mice fed either control diet or HSD, respectively; **(C,D)** representative histological image of colons of IL-17$^{-/-}$ mice fed either control diet or fed HSD, respectively; **(E,F)** representative histological images of colon from RAG$^{-/-}$ mice fed either control diet or HSD, respectively. **(G)** Histological score comparing IL-17$^{-/-}$ and RAG$^{-/-}$ with their respective WT littermates. Statistical analysis between groups was performed using Student's t-test. *p < 005. Images are shown at 100× magnification.

proposed Th17 cells as the sole responsible for the IL-17 induced by high concentrations of sodium chloride.

We observed that consumption of a diet supplemented with 4% NaCl for 3 weeks led to a histologically identifiable inflammation in the colonic mucosa of mice with depletion of goblet cells, cellular infiltration, erosion, ulceration, and destruction in *muscularis mucosa* layer, as well as edema in submucosal layer. Other typical signs of mucosal inflammation such as increase in intestinal permeability were also present.

Inflammatory events triggered by HSD included production of high levels of IL-23, but not INF-γ, increased frequency of CD4$^+$ IL-23R$^+$ T cells and neutrophils in the colonic mucosa suggesting that the IL-17 axis was activated. Indeed, no histological sign of gut inflammation was detected in IL-17$^{-/-}$ mice confirming that the HSD effect was dependent on this cytokine. Another recent study by Wei and coworkers also showed that exacerbation of colitis by HSD was dependent on IL-17 production (12).

Moreover, the inflammatory reaction induced by HSD consumption involved significant increase in the frequency of type 3 innate lymphoid cells (ILC3) in the colonic *lamina propria*. The decrease observed in ILC1 and ILC2 frequencies may have occurred as a compensatory balance after ILC3 increase. It has been reported that ILC3 have homologous functions to Th17 and that these cells are very abundant in the gut mucosa. They are also shown to be involved in intestinal homeostasis as they are producers of IL-22 and/or IL-17 in response to IL-23 and IL-1β (18). To examine whether the inflammation observed in HSD-fed mice was dependent exclusively on T cells or whether innate cells such as ILC3 could also be involved, we used RAG$^{-/-}$ mice. These mice developed a histologically identifiable inflammation in their colonic mucosa after 3 weeks of HSD consumption indicating that T cells are not necessary for triggering gut inflammation. Indeed, the role of ILCs in the pathogenesis of IBDs has been widely acknowledged, and these cells are considered as potential therapeutic targets (15, 16, 18). The role of ILCs is also described in experimental models of intestinal inflammation associated with epithelium damage and IL-1β secretion by intestinal epithelial cells. Excessive amounts of sodium chloride in the diet may act directly in intestinal epithelial cells stimulating IL-1β secretion. This cytokine has been reported to promote innate immune pathology in *Helicobacter hepaticus*-triggered intestinal inflammation by augmenting the recruitment of granulocytes and the accumulation as well as activation of innate lymphoid cells (ILCs). In this study, IL-1β promoted Th17 responses from both CD4$^+$ Th17 cells and ILC3 in the intestine, and a synergistic interaction between IL-1β and IL-23 signals sustained innate and adaptive inflammatory responses in the gut (23). In our case, the participation of Th17 cells in the effects of HSD is clear since the histological scores of RAG$^{-/-}$ mice were lower than the ones of WT mice. However, our results also show that the IL-17-dependent gut inflammation occurred in the absence of lymphocytes. There was a significant difference in histological scores between WT and RAG$^{-/-}$ mice suggesting other cells are involved. Therefore, it is likely that both cell types (Th17 cells and ILC3) participated in triggering inflammation upon high-salt exposure.

IL-17 induces the release of chemokines and other chemoattractants from epithelial and endothelial cells that enhance the inflammatory response through the recruitment of neutrophils (21). Protective effects of IL-17, in IBD, for example, are also reported. This dichotomy was recently explained by the observation that IL-17 promotes epithelial barrier function in the absence of IL-23 (24, 25). Hence, IL-23-independent IL-17 production is protective, whereas IL-23-dependent IL-17 production might be deleterious. In our study, gut inflammation caused by HSD was

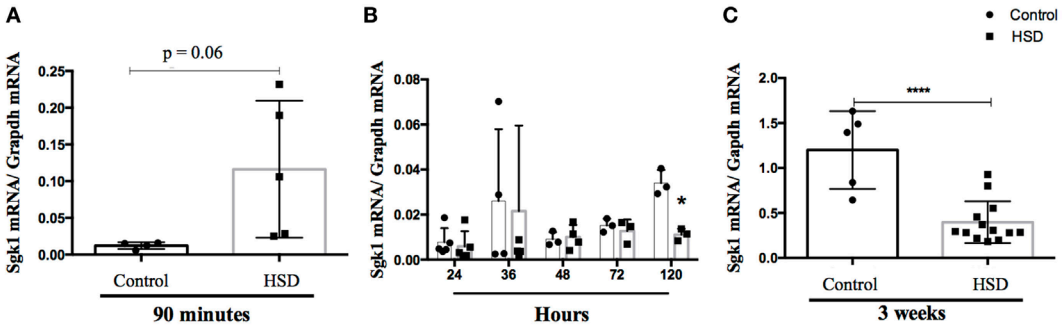

FIGURE 5 | Consumption of high-salt diet (HSD) altered expression of SGK-1. mRNA was extracted from colon of C57BL/6 mice **(A)** 90 min after they received a 50% NaCl solution by gavage; **(B)** 24 h, 36 h, 48 h, 72 h, and 120 h after consumption of either control diet (n = 5–10) or HSD (n = 5–10) and **(C)** after 3 weeks of control diet (n = 5–10) or HSD consumption (n = 5–10). *Sgk-1* relative expression was quantified by quantitative real-time PCR in the colon extracts. Graphs are representative of three independent experiments with five mice per group. Statistical analysis between groups was performed using Student's t-test. *$p < 0.05$.

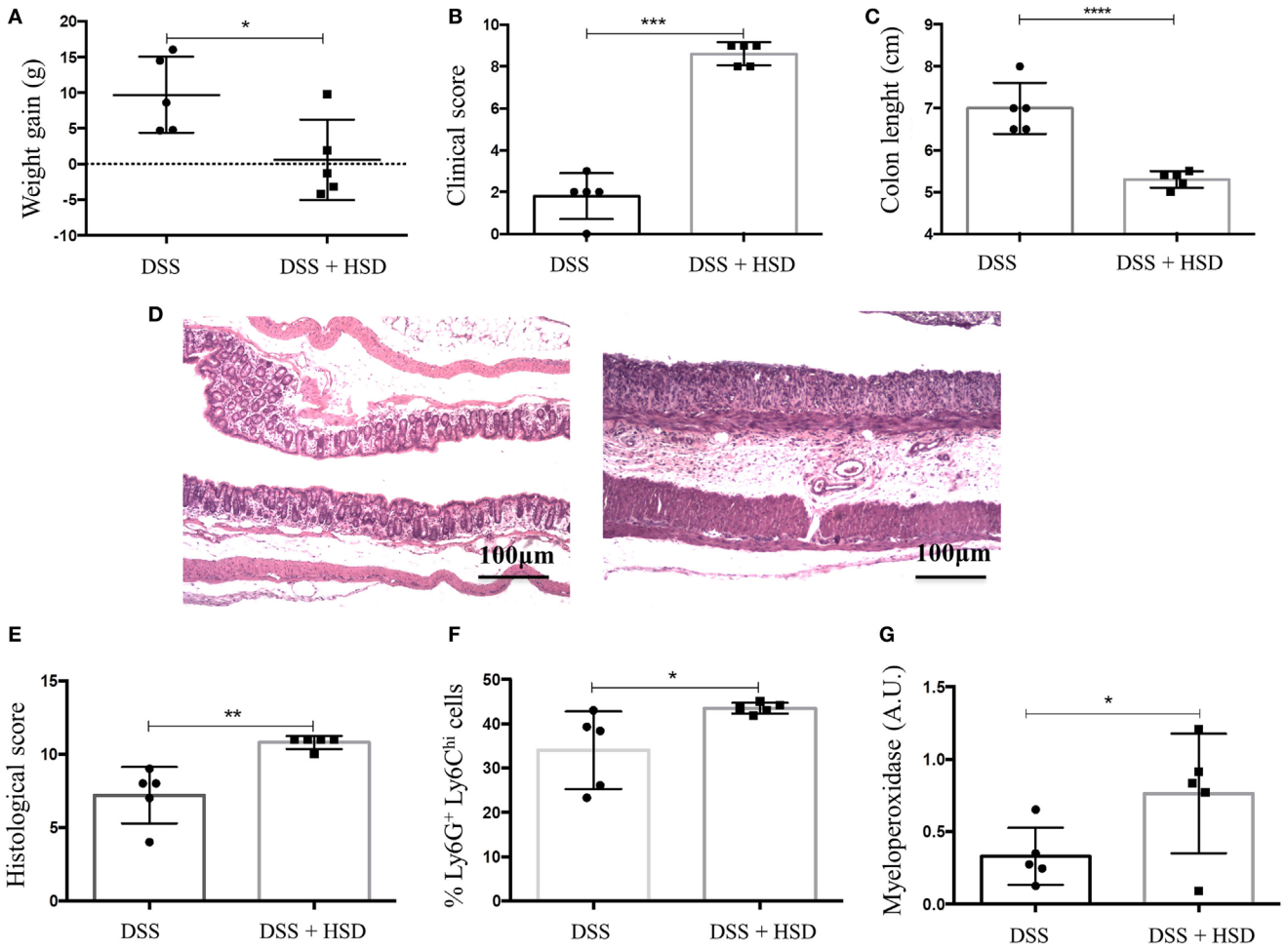

FIGURE 6 | Consumption of high-salt diet (HSD) worsened dextran sodium sulfate (DSS)-induced colitis. C57BL/6 mice were fed either control diet or HSD for 3 weeks; mice received 1% DSS in drinking water in the last 7 days of the experiment. **(A)** Weight gain of DSS (n = 5–10) and DSS + HSD (n = 5–15) groups; **(B)** clinical scores of DSS and DSS+ HSD groups; **(C)** Colon lengths in centimeters of DSS and DSS+ HSD groups; **(D)** representative histological image from colon of DSS (left) and DSS+ HSD (right) groups; **(E)** histological score of DSS and DSS+ HSD groups; **(F)** Ly6G+Ly6C^{hi} cell frequency in the colonic *lamina propria* measured by flow cytometry; **(G)** myeloperoxidase activity measured in colonic extract. For histological score, goblet cell depletion, muscular layer (erosion/ulceration) destruction, cellular infiltration, and edema were considered, each parameter received 0–3 in the score index. Graphs are representative of three independent experiments with five mice per group. Statistical analysis between groups was performed using Student's t test. *$p < 0.05$.

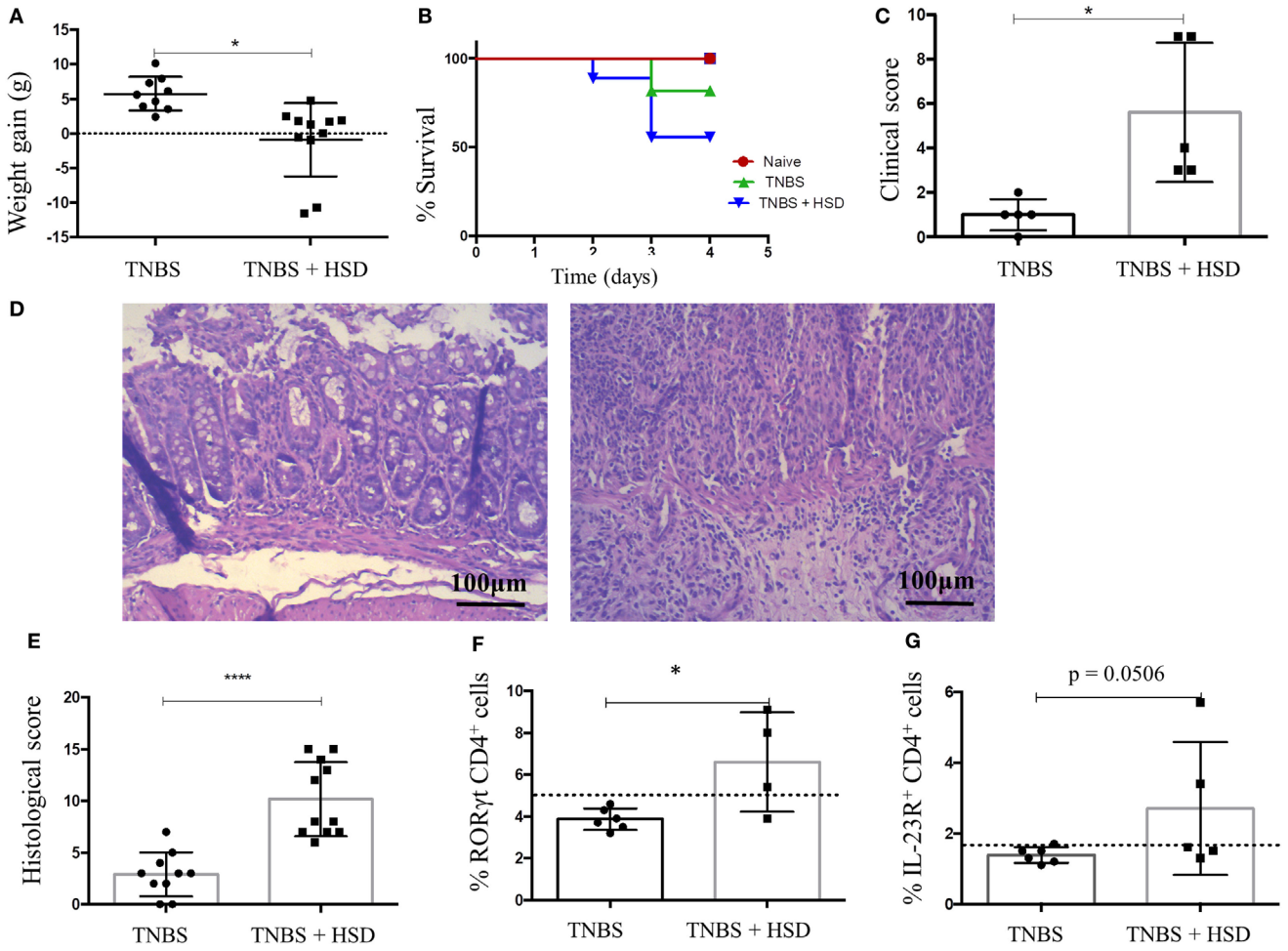

FIGURE 7 | Consumption of high-salt diet (HSD) worsened 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis in BALB/c mice. BALB/c mice were fed either control diet or HSD for 3 weeks. TNBS-induced colitis was induced by administration of 2.5 mg TNBS in 35% alcohol by intrarectal injections in mice 7 days before euthanasia. TNBS group (n = 5–15) received control diet for 21 days and TNBS$^+$ HSD group (n = 5–15) received HSD throughout the experiment. **(A)** Weight gain during colitis; **(B)** survival curve; **(C)** clinical score; **(D)** representative histology images of TNBS (left) and TNBS$^+$ HSD (right) groups; **(E)** histological score; **(F)** frequency of CD4$^+$ RORγt$^+$ cells in the colonic *lamina propria* of mice measured by flow cytometry; and **(G)** frequency of CD4$^+$ IL-23R$^+$ cells in the colonic *lamina propria* measured by flow cytometry. For histological score, goblet cell depletion, muscular layer (erosion/ulceration) destruction, cellular infiltration, and edema were considered; each parameter received 0–3 in the score index. Graphs are representative of three independent experiments with five mice per group. Statistical analysis between groups was performed using Student's t-test. *$p < 0.05$.

associated with increase in IL-23 production and in the frequency of CD4$^+$ T cells bearing IL-23R, suggesting that these cells have an inflammatory role in colon of mice. Of note, no alteration was observed in the frequencies of CD4$^+$ CD25$^+$ Foxp3$^+$ T cells in the colonic mucosa of HSD-fed mice (data not shown).

It has been described that Th17 cells are induced by HSD through SGK1 signaling pathway (5, 6). We tested SGK1 expression 90 min after oral administration of NaCl-containing solution and at several time points after HSD consumption (24, 36, 48, 72, 120 h, and 3 weeks after the beginning of feeding). We found no increase in sgk1 expression up to 72 h, despite a tendency for elevation in its expression at an earlier time point (90 min) and a decrease after 3 weeks of diet consumption. Since SGK-1 is an early gene, the expression of which increases to a steady state within hours (26), this early elevation right after high-salt ingestion and the decrease of its expression to normal levels after HSD consumption suggests that SGK1 and other signaling pathways may participate in the inflammatory effect triggered by high salt, especially when ILCs are involved. Moreover, SGK-1 expression could be increased in the first minutes after high-salt concentration and this elevation might be overturned by a compensatory mechanism later on. Results on SGK-1 involvement upon HSD consumption are ambiguous in other studies. In studies using rats, the authors detected a reduction in SGK-1 expression upon high-salt intake. In another study by the same group, rats fed a HSD for 5 weeks showed no change in SGK1 expression (26, 27). SGK-1 was decreased in

the colon after adrenalectomy, but remained unchanged after sodium depletion or aldosterone injection (28), while other studies reported an increased induction upon these very same interventions (29, 30).

Our data also showed that consumption of HSD was also able to worsen colitis induced by both DSS and TNBS in C57BL/6 and BALB/c, respectively. Further investigation revealed that colitis exacerbation was associated with differentiation of Th17 cells, which occurred with an increase in the expression of IL-23R and the transcription factor RORγt by $CD4^+$ T cells, as previously described by others (12, 14). It is plausible that the initial inflammatory event triggered by HSD depended on both ILC3 and Th17 cells. This early insult would predispose the colonic mucosa to increased production of IL-17 by a secondary trigger such as DSS or TNBS.

Of note, consumption of diets containing high concentrations of other salts such as potassium chloride also exacerbated DSS-induced colitis (Figure S3 in Supplementary Material) suggesting that sodium chloride and SGK1 activation may not be the only triggers of inflammation by HSDs.

Interestingly, a recent study showed that high-salt intake induces Th17 activation and worsening of EAE by affecting the gut microbiome in mice, particularly by depleting *Lactobacillus murinus* (31). Therefore, more than one mechanism seems to be triggered by high concentrations of salts resulting in inflammatory IL-17-dependent events. We cannot rule out the possibility that a change in the gut microbiota by HSD consumption also acted to induce gut inflammation and exacerbation of colitis.

Our findings indicate that, in addition to worsening colitis, HSD consumption itself was able to cause inflammation in the colonic mucosa of mice. The alterations induced by HSD were associated with increased frequencies of $IL\text{-}23R^+$ $CD4^+$ T cells and type 3 innate lymphoid cells (ILC3) and were dependent on IL-17 but not exclusively on T cells.

ETHICS STATEMENT

All procedures were approved by the local ethical committee for animal research (CEUA-UFMG, Brazil, 50/2014). Experiments were performed in accordance with guidelines and regulation established by Conselho Nacional de Controle de Experimentação Animal (CONCEA), Brazil.

AUTHOR CONTRIBUTIONS

SA performed the experiments, discussed the results, and wrote the manuscript; MM helped performing the experiments and writing the manuscript; MG helped with colitis experiments; HS and CQ supervised and discussed the results of experiments with ILCs; PC performed SGK1 expression analysis; DC supervised and discussed the results of histological analysis; GF and AJF performed and discussed the results of the blood pressure analysis; PB performed the measurement of intestinal permeability with the supervision of VC; TM co-supervised the experiments, helped with discussion of the results and wrote the manuscript; AMCF supervised all the experiments, discussed the results, and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fimmu.2017.01969/full#supplementary-material.

FIGURE S1 | Flow cytometry strategy analyses for innate lymphoid cells. The lymphocyte gate was defined considering SSC-height and FCS-height; within the lymphocyte gate, Lin^- $CD45^+$ cells were selected, then $CD3^+$ cells, next $CD45^+$ $CD127^+$ cells and finally each ILC was defined considering the expression of different transcriptional factors: (a) ILC1 were t-bet^+ cells; (b) ILC2 were $GATA3^+$ cells and (c) ILC3 were $ROR\gamma t^+$ cells. Lin^- = CD3, CD4, CD8, CD16, CD19, CD11c, FceR1a. Pacific Blue-labeled anti-CD11b, BV421-labeled anti-CD16, BV421-labeled anti-CD19, Pacific Blue-labeled ant-CD11c, Pacific Blue-labeled anti-FceR1a, PERCP-Cy5.5-labeled anti-CD8, PE-Cy7-labeled anti-CD4, BV570-labeled anti-CD45, BV605-labeled anti-CD117, APC-labeled anti-CD127, AF700-labeled anti-CD3, PE-CF594-labeled anti-RORγt, PE-labeled anti-t-Bet, and AF488-labeled anti-GATA3 were used. Samples were acquired in FACSfortessa and data analyzed in FlowJo software 7.0.

FIGURE S2 | Flow cytometry strategy analyses for neutrophils. First, the granulocyte gate was defined considering FCS-height and SSC-height, then $CD11b^+$ cells were selected and $F4/80^+$ cells excluded. Finally, $Ly6C^{Hi}$ $Ly6G^{Int\text{-}Low}$ analyzed as neutrophils. PERCP-labeled anti-CD11b, APC-labeled anti-F4/80, PE-labeled anti-Ly6C, and FITC-labeled anti-Ly6G antibodies were used. Samples were acquired in FACScan and data were analyzed by FlowJo software 7.0.

FIGURE S3 | Hemoglobin and Clinical Score in dextran sodium sulfate (DSS)-induced colitis in mice fed NaCl-supplemented diet and KCl-supplemented diet. C57BL/6 mice were divided into six groups: control group received control diet and water during 3 weeks of experiment; high-salt diet (HSD) group received HSD containing 4% NaCl for 3 weeks; KCl group received diet containing 4% KCl during 3 weeks; DSS group received control diet and DSS 1% in the drinking water in the last 7 days of dietary consumption; HSD^+ DSS received HSD with 4% of NaCl for 3 weeks and DSS 1% in the last 7 days of the experiment; HSD^+ DSS received HSD with 4% KCl for 3 weeks of experiment and DSS 1% in the last 7 days of the experiment. **(A)** Hemoglobin concentration in peripheral blood and **(B)** Clinical score of colitis. Statistical analysis among groups was performed using one-way ANOVA. $^*p < 0.05$.

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L-Arginine-Dependent Epigenetic Regulation of Interleukin-10, but Not Transforming Growth Factor-β, Production by Neonatal Regulatory T Lymphocytes

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A growing number of diseases in humans, including trauma, certain cancers, and infection, are known to be associated with L-arginine deficiency. In addition, L-arginine must be supplemented by diet during pregnancy to aid fetal development. In conditions of L-arginine depletion, T cell proliferation is impaired. We have previously shown that neonatal blood has lower L-arginine levels than adult blood, which is associated with poor neonatal lymphocyte proliferation, and that L-arginine enhances neonatal lymphocyte proliferation through an interleukin (IL)-2-independent pathway. In this study, we have further investigated how exogenous L-arginine enhances neonatal regulatory T-cells (Tregs) function in relation to IL-10 production under epigenetic regulation. Results showed that cord blood mononuclear cells (CBMCs) produced higher levels of IL-10 than adult peripheral blood mononuclear cells (PBMCs) by phytohemagglutinin stimulation but not by anti-CD3/anti-CD28 stimulation. Addition of exogenous L-arginine had no effect on transforming growth factor-β production by PBMCs or CBMCs, but enhanced IL-10 production by neonatal $CD4^+CD25^+FoxP3^+$ Tregs. Further studies showed that IL-10 promoter DNA hypomethylation, rather than histone modification, corresponded to the L-arginine-induced increase in IL-10 production by neonatal $CD4^+$ T cells. These results suggest that L-arginine modulates neonatal Tregs through the regulation of IL-10 promoter DNA methylation. L-arginine supplementation may correct the Treg function in newborns with L-arginine deficiency.

Keywords: L-arginine, neonate, regulatory T-cells, interleukin-10, DNA hypomethylation

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INTRODUCTION

L-Arginine is a semi-essential amino acid; it can be synthesized from glutamine, glutamate, and proline by enzymes in the human intestinal–renal axis, but must be supplemented in the diet at times of physiological or pathological stress, including during pregnancy to aid fetal development (1). Both experimental and clinical studies have shown that certain human conditions, such as infertility, poor fetal growth, necrotizing enterocolitis in infants, cancer, trauma, and certain liver diseases, are associated with L-arginine deficiency (1–4).

L-Arginine can be metabolized to the cytotoxic and antimicrobial effector molecule nitric oxide through inducible nitric oxide synthase (iNOS). L-arginine can also be hydrolyzed by the enzyme arginase to ornithine and urea (1, 5), which depletes L-arginine. Arginase and iNOS compete for L-arginine. Induction of iNOS or arginase alone will result in reversible suppression of T cell proliferation. When both enzymes are induced, iNOS will generate peroxynitrites, which induce apoptosis of activated T cells. Therefore, relative changes in iNOS and arginase 1 activities may affect L-arginine metabolism and control specific types of T cell responses (6). L-arginine is also required for host defense against various pathogens and malignant cells. L-arginine modulates immune responses; it is critical for expression of the ζ-chain subunit of the T cell receptor complex, production of antibodies by B cells, and development of memory B cells (4, 7). Recently, L-arginine was also shown to regulate glycolysis and mitochondrial activity and enhance T cell survival and antitumor responses (8). Polymorphonuclear granulocytes and myeloid-derived suppressor cells have been shown to suppress T and natural killer cell proliferation and responses through arginase-mediated L-arginine depletion during activation (5, 9, 10). Moreover, dietary L-arginine supplementation in tumor-bearing or septic rats can increase thymus weight; interleukin (IL)-2/IL-2 receptor-mediated lymphocyte proliferation; cytotoxicity of T lymphocytes, macrophages, and natural killer cells; and delayed-type hypersensitivity responses (1).

Helper T cells are generally categorized based on their cytokine secretion profiles and their functions within the immune system (11–14). Th1 cells produce interferon-γ and play an important role in intracellular defense against microorganisms. Th2 cells produce IL-4, IL-5, and IL-13 and are responsible for allergic reactions and responses to parasitic infections (15). Regulatory T cells (Tregs), which produce IL-10 and transforming growth factor (TGF)-β, play key roles in the regulation of Th1/Th2-immune responses and peripheral tolerance (13, 14). Tregs suppress activated effector T cells and prevent pathological self-reactivity (16). They control effector T cell activation, proliferation, differentiation, and effector functions (17). Tregs can suppress effector T cells through the secretion of regulatory cytokines such as TGF-β and IL-10 (18). Other possible mechanisms of Treg-mediated T cell suppression include IL-2 deprivation *via* high surface CD25 expression and release of soluble CD25 (19). Although Tregs play a crucial role in immune responses, few studies have investigated their status and plasticity in neonatal blood.

Few reports have described L-arginine nutrition in neonates, especially preterm infants with L-arginine deficiency (20). We have previously shown that, compared to adults, neonates have lower plasma arginine levels and more abundant arginase 1 expression in leukocytes, which are associated with reduced lymphocyte proliferation (21). Additionally, we provided evidence that L-arginine modulates neonatal lymphocyte proliferation through an IL-2-independent pathway (22). Thus, L-arginine has distinct immune regulatory effects on neonatal and adult lymphocytes. In this study, we further investigated how L-arginine affects neonatal Tregs. Understanding the biological effects of L-arginine deficiency on T cell function may enable the design of novel treatments for neonatal immunodeficiency.

MATERIALS AND METHODS

Collection of Human Umbilical Cord Blood (CB) and Adult Peripheral Blood

Human umbilical CB was collected at the time of elective cesarean section or normal spontaneous delivery from healthy mothers, after informed consent was obtained from the subjects as previously described (22). Adult peripheral blood samples were obtained from healthy volunteers aged 20–40 years. The leukocyte separation protocol has been described previously (23). In brief, leukocytes and red blood cells were separated from whole blood using 4.5% (w/v) dextran sedimentation. Polymorphonuclear cells and mononuclear cells (MNCs) were further separated by density gradient centrifugation with Ficoll-Paque™ (Amersham Pharmacia, Biotech AB, Uppsala, Sweden). This protocol was approved by the Institutional Review Board of Chang Gung Memorial Hospital, Kaohsiung Medical Center (104-7809C1) and the study was carried out in accordance with their recommendations.

Induction of Cytokine Release by MNCs

We performed the cytokine induction protocol as previously described (24, 25). In brief, adult peripheral blood mononuclear cells (PBMCs) and cord blood mononuclear cells (CBMCs) (2×10^6 cells/mL) were stimulated with or without purified phytohemagglutinin (PHA) (10 μg/mL), or 1 μg/mL anti-CD3 (HIT3a, Cat. #300314, BioLegend, San Diego, CA, USA) in combination with 1 μg/mL anti-CD28 (CD28.2, Cat. #302914, BioLegend), in 1-cm tissue culture plates with L-arginine-free medium (SILAC R1780 SIGMA, RPMI-1640 Medium) supplemented with 10% heat-inactivated fetal bovine serum, 1 mM glutamine, 1 mM sodium pyruvate, 50 mg/L L-leucine, 40 mg/L L-lysine, and 1× non-essential amino acids (Gibco cat. # 11140-035), 100 IE/mL penicillin, and indicated L-arginine (Sigma-Aldrich, St. Louis, MO, USA). After 72 h, cell-free culture supernatants were collected and assayed for cytokine production by enzyme-linked immunosorbent assay: TGF-β1 (R&D systems Inc., MN, USA) and IL-10 (R&D Systems).

Isolation of $CD4^+$ T Cells

$CD4^+$ T cells were separated from MNCs using an IMag™ Cell Separation System (BD Biosciences, San Jose, CA, USA) as previously reported (26). In brief, MNC pellets were incubated with anti-human CD4 magnetic particles (BD Biosciences) for 30 min.

Then, the labeled cells were resuspended in BD IMag™ buffer and isolated using a BD IMagnet™ (BD Biosciences).

RNA Isolation and Real-time Reverse Transcription-Polymerase Chain Reaction (RT-qPCR) Analysis

Total RNA was extracted from cells using TRIzol® Reagent (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions. Purified RNA was quantified by assessment of optical density at 260 nm using a NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific) and qualitatively analyzed using a 2100 Bioanalyzer with RNA 6000 Nano Labchip Kit (Agilent Technologies, Palo Alto, CA, USA). RT-qPCR was then conducted as previously reported (22). In brief, a total of 200 ng of RNA was mixed with dNTPs and oligo-dTs (Invitrogen, San Diego, CA, USA) for 5 min at 65°C to reverse transcribe cDNA. The cDNA products were subjected to PCR amplification with specific primers (Table S1 in Supplementary Material), followed by SYBR Green quantification in an ABI Prism 7500 sequence detection system (Applied Biosystems, Foster City, CA, USA). For the relative quantification of gene expression, we employed the comparative threshold cycle (C_t) method (27).

Flow Cytometric Analysis of Intracellular FoxP3 and IL-10 Expression

We assessed the phenotype of Tregs by flow cytometry. CBMCs and PBMCs were cultured with PHA and the indicated concentration of L-arginine at 37°C for 48 h, then washed with fluorescence-activated cell sorting (FACS) buffer prior to evaluation of their Treg subsets. At the end of the stimulation, the cells were stained with anti-CD4-PerCP (BD Biosciences, Franklin Lakes, NJ, USA) and anti-CD25-FITC (Beckman Coulter, Brea, CA, USA) for 30 min, then fixed with paraformaldehyde/phosphate-buffered saline (PBS), and permeabilized using FACS permeabilizing solution (Sigma-Aldrich, St. Louis, MO, USA). For intracellular staining of FoxP3 and IL-10, cells were stained with anti-human FoxP3-PE (eBioscience, San Diego, CA, USA) and IL-10-APC (BD Biosciences). We analyzed the percentages of human $CD4^+CD25^-$, $CD4^-CD25^+$, and $CD4^+CD25^+$ cells, and the intracellular expression of FoxP3 and IL-10 in the subsets, using a FACSCalibur flow cytometer (BD Biosciences).

Chromatin Immunoprecipitation (ChIP) Assay

Chromatin immunoprecipitation assays were performed as previously described (28). In brief, cell pellets (1×10^7) were fixed with warmed 1% formaldehyde at 37°C, washed with ice-cold PBS, then assayed using an EZ-Magna ChIP™ A Kit (Millipore, Billerica, MA, USA), according to the manufacturer's instructions. After the DNA was sheared to an average length of 200–1,000 bp by sonication, 5 µL of the supernatant (the input) was removed and stored at 4°C. The supernatant was then incubated overnight at 4°C with 5 µg of the indicated antibodies for immunoprecipitation. The antibodies used in the immunoprecipitation were as follows: acetyl histone H3 (Millipore), acetyl histone H3 lysine 4 (Cell Signaling, Danvers, MA, USA), monomethyl histone H3 lysine 4 (Abcam, Cambridge, UK), acetyl histone H3 lysine 9 (Cell Signaling), trimethyl-histone H3 lysine 36 (Abcam), and trimethyl-histone H3 lysine 4 (Abcam). The immunoprecipitated DNA was eluted and quantitated by RT-qPCR, performed at an annealing temperature of 57°C for 45 cycles with the indicated primers (Table S1 in Supplementary Material). The C_t values of the diluted input were adjusted to 100% of the input by subtracting 3.322 cycles (log 2 of 10) from the C_t value of the diluted input. One percent of starting chromatin was used as input. The amount of DNA precipitated by the indicated antibodies was calculated as percentage of the input using the following formula: % of input $= 2\Delta C_t \times 100$, where $\Delta C_t = C_t$ input—C_t IP.

Genomic DNA Extraction, Bisulfite Conversion, and Pyrosequencing

Genomic DNA was isolated from $CD4^+$ T cells using the PUREGENE® DNA Purification Kit (Gentra Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. Bisulfite conversion of genomic DNA was performed using the EZ DNA Methylation™ Kit (Zymo Research, Orange, CA, USA), according to the manufacturer's instructions. In brief, 500 ng of genomic DNA was added to M-Dilution Buffer, which was adjusted to a total volume of 50 µL with water, and incubated for 15 min at 37°C. CT Conversion Reagent was then added to the denatured DNA and incubated for 16 h at 50°C. We washed and eluted the DNA by centrifugation for 30 s, then performed PCR amplification of the target regions using a PyroMark PCR Kit (Qiagen, Valencia, CA, USA). Each PCR mix (25 µL) contained 2 × PyroMark PCR Master Mix, 10 × CoralLoad Concentrate, 5 µL Q-Solution, 5 µM primers, and 100 ng bisulfite-treated DNA. The cycling protocol was as follows: 95°C for 15 min, 45 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s, followed by a final extension of 72°C for 10 min. The biotin-labeled PCR products were captured by Streptavidin Sepharose high performance beads (Amersham Pharmacia). We purified the bead-bound PCR products, which were made single-stranded using a Pyrosequencing Vacuum Prep Tool (Qiagen). The sequencing primers were annealed to the single-stranded PCR products and pyrosequencing was performed using the PyroMark Q24 system (Qiagen). Quantitation of cytosine methylation was determined using PyroMark Q24 Software 2.0.6 (Qiagen). PCR amplification conditions were determined and sequencing primers were designed using PyroMark Assay Design Software (Qiagen). The primer sequences are listed in Table S1 in Supplementary Material.

Statistics

Differences in parameters between PBMCs and CBMCs were analyzed using the Mann–Whitney *U*-test. Differences in parameters between different L-arginine conditions were analyzed using a one-way analysis of variance with Fisher's least significant difference *post hoc* test. Data are expressed as the mean ± SEM. The correlation between IL-10 promoter DNA methylation levels and IL-10 mRNA levels in CB $CD4^+$ T cells at the indicated concentrations of L-arginine was calculated as Pearson's correlations.

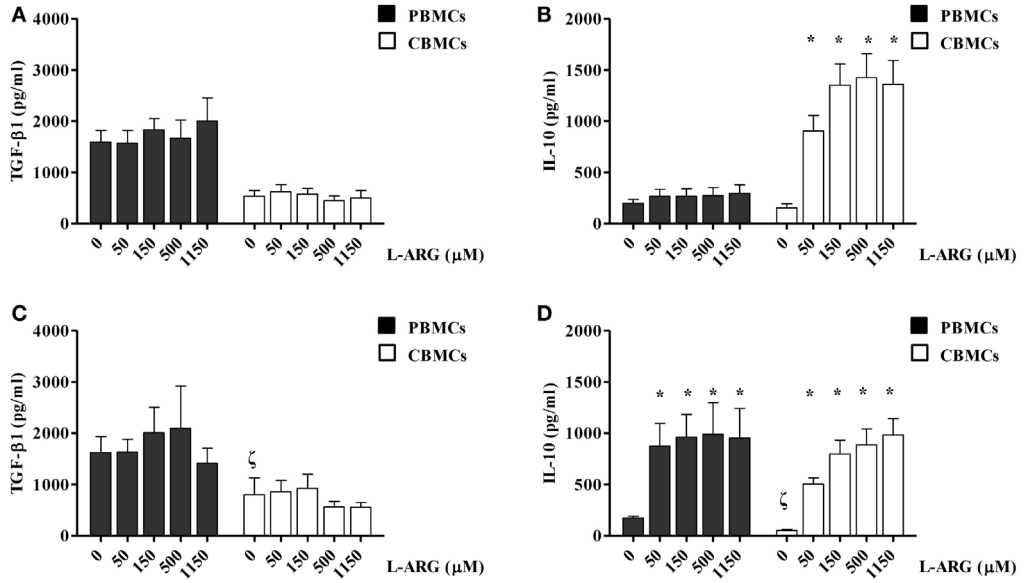

FIGURE 1 | Effects of L-arginine supplementation on transforming growth factor (TGF)-β and interleukin (IL)-10 production by adult and neonatal mononuclear cells (MNCs). MNCs isolated from adult peripheral blood and cord blood (CB) were suspended at a concentration of 2×10^6/mL in 24-well plates and treated with 10 μg of phytohemagglutinin and the indicated concentrations of L-arginine for 72 h. The culture supernatants were collected and then levels of **(A)** TGF-β and **(B)** IL-10 were determined by enzyme-linked immunosorbent assay (ELISA). In a separate experiment, MNCs were treated with anti-CD3 (1 μg/mL) in combination with anti-CD28 (1 μg/mL) and the indicated concentrations of L-arginine for 72 h. The culture supernatants were collected and the levels of **(C)** TGF-β and **(D)** IL-10 were determined by ELISA (n = 6–9 for adult and 8–14 for CB as indicated). *$p < 0.05$ compared to the control with 0 μM L-arginine by ANOVA. $^{\zeta}p < 0.05$ compared to the adult control without addition of L-arginine by Mann–Whitney U-test.

TABLE 1 | CD4 and CD25 cell surface expression upon L-arginine treatment.

PBMCs	$CD4^+CD25^-$	$CD4^-CD25^+$	$CD4^+CD25^+$
L-ARG 0 μM	6.95 ± 0.32	36.83 ± 7.73	15.58 ± 1.49
L-ARG 50 μM	7.08 ± 3.07	33.15 ± 9.83	14.45 ± 2.20
L-ARG 150 μM	6.98 ± 3.26	34.65 ± 10.30	14.33 ± 2.37
L-ARG 500 μM	6.43 ± 3.63	39.20 ± 9.51	14.85 ± 2.48
L-ARG 1,150 μM	6.55 ± 3.43	35.95 ± 10.00	14.25 ± 2.29
CBMCs	**$CD4^+CD25^-$**	**$CD4^-CD25^+$**	**$CD4^+CD25^+$**
L-ARG 0 μM	1.40 ± 0.46	35.13 ± 10.80	23.87 ± 7.62
L-ARG 50 μM	1.00 ± 0.54	39.30 ± 12.15	41.20 ± 12.14*
L-ARG 150 μM	0.77 ± 0.38	38.40 ± 11.50	48.93 ± 14.32*
L-ARG 500 μM	0.83 ± 0.41	38.90 ± 11.59	48.70 ± 14.18*
L-ARG 1,150 μM	0.80 ± 0.42	38.37 ± 11.40	48.67 ± 14.17*

Analysis of CD4 and CD25 expression on peripheral blood mononuclear cells (PBMCs) and cord blood mononuclear cells (CBMCs) by flow cytometry.
**p < 0.005 compared to expression with 0 μM L-arginine level by analysis of variance.*

Differences with a p-value of less than 0.05 were considered statistically significant in all tests. All statistical tests were performed using SPSS 15.0 for Windows XP (SPSS, Chicago, IL, USA).

RESULTS

Effects of L-Arginine on IL-10 and TGF-β Production by CBMCs

To clarify the modulatory effects of L-arginine on the Treg response, we determined the levels of TGF-β and IL-10 production by CBMCs and PBMCs, which were exposed to differential L-arginine levels in their respective origins, upon PHA stimulation. As shown in **Figure 1**, CBMCs produced less TGF-β1, but an equivalent amount of IL-10, compared to PBMCs in L-arginine-free culture medium. The addition of different concentrations of L-arginine had no effect on the production of TGF-β by CBMCs or PBMCs (**Figure 1A**). Thus, TGF-β production by human MNCs was L-arginine-independent (**Figure 1A**). Interestingly, exogenous L-arginine enhanced IL-10 production by neonatal, but not adult, MNCs in a dose-dependent manner (**Figure 1B**) upon PHA stimulation. Given that PHA stimulates leukocytes through CD2-mediated activation (29), we also used anti-CD3/anti-CD28 to specifically stimulate T cells (30). TGF-β1 production by PBMCs and CBMCs upon anti-CD3/CD28 stimulation was similar to that upon PHA stimulation. Anti-CD3/CD28-induced TGF-β1 production by human MNCs was also L-arginine-independent (**Figure 1C**). However, PBMCs and CBMCs behave in similar manner concerning the IL-10 production upon anti-CD3/CD28 stimulation (**Figure 1D**). The reason why CBMCs produce higher levels of IL-10 than PBMCs by PHA stimulation but not by anti-CD3/anti-CD28 stimulation may be because that PHA stimulation, which has been shown to mediate through CD2 activation (29), may be different from the combination of anti-CD3 and anti-CD28 stimulation (31). In order to explore the unique L-arginine-dependent PHA-induced IL-10 production of CBMCs, further studies were conducted on the cellular and molecular mechanisms of neonatal Tregs in CBMCs stimulated by PHA.

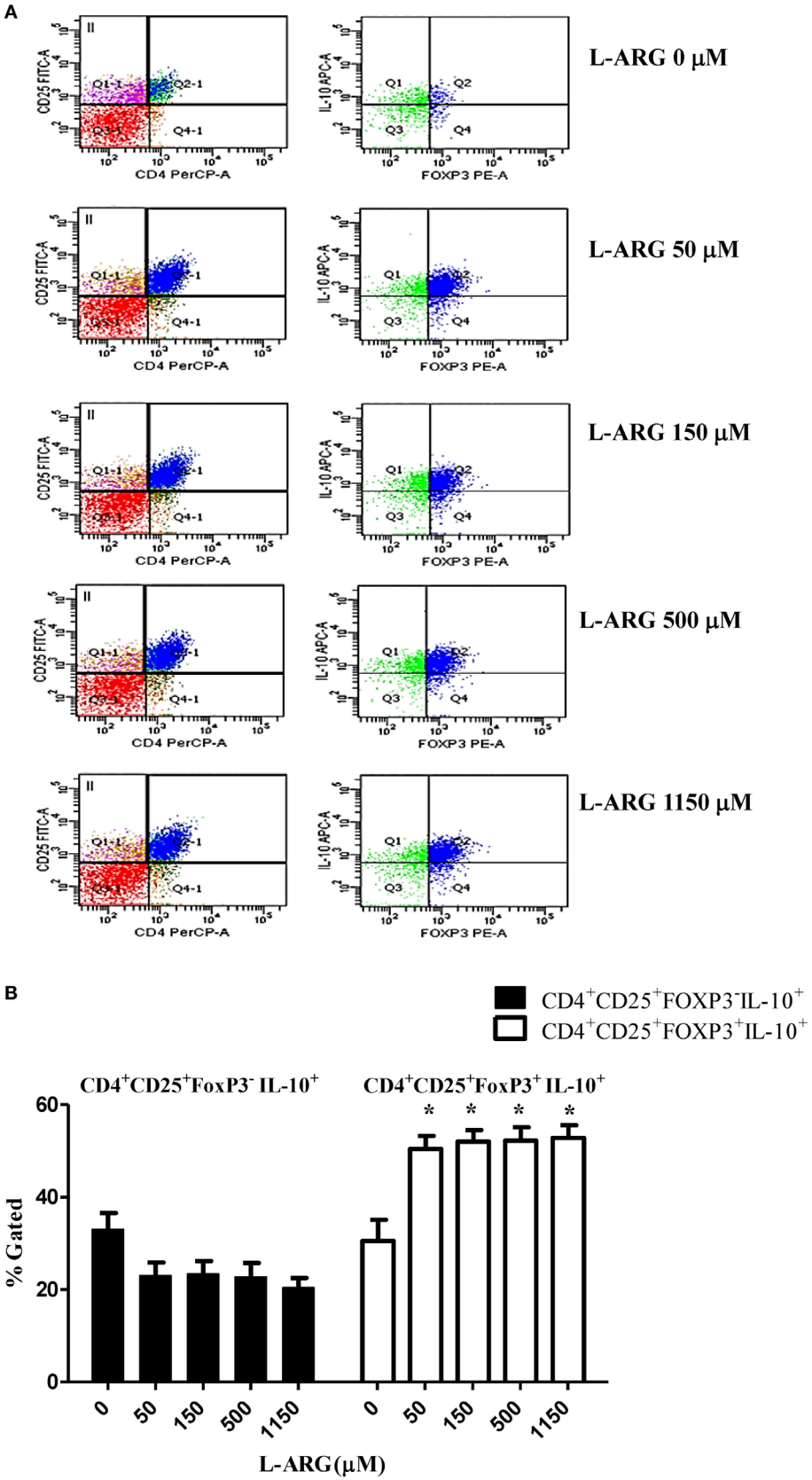

FIGURE 2 | **Continued**

FIGURE 2 | Continued
Expression of intracellular FoxP3 and interleukin (IL)-10 in $CD4^+CD25^+$ T cells. Mononuclear cells isolated from adult peripheral blood and cord blood were treated with 10 μg of phytohemagglutinin and the indicated concentrations of L-arginine for 48 h, then samples were stained with PerCP- and FITC-labeled antibodies specific for CD4 and CD25 cell surface markers, respectively. The cells were then permeabilized and stained with sheep antibodies specific for human FoxP3 and IL-10. **(A)** The flow plots show the effects of L-arginine supplementation on cell surface CD4 and CD25 expression in cord blood mononuclear cells, and the intracellular FoxP3 and IL-10 expression of $CD4^+CD25^+$ cells. The results are representative of four replicate experiments. **(B)** The bars illustrate the percentage of $CD4^+CD25^+FoxP3^-IL\text{-}10^+$ cells and $CD4^+CD25^+FoxP3^+IL\text{-}10^+$ cells in the presence of the indicated concentrations of L-arginine ($n = 6$ for each group). *$p < 0.05$ compared to the control without L-arginine by analysis of variance.

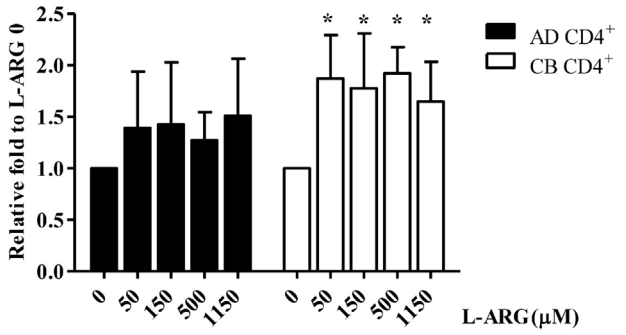

FIGURE 3 | Quantitative reverse transcription-polymerase chain reaction (RT-qPCR) analysis of interleukin (IL)-10 mRNA expression in CB and adult blood $CD4^+$ T cells. $CD4^+$ T cells isolated from adult peripheral blood and CB were suspended at a concentration of 2×10^6/mL in 24-well plates, then treated with 10 μg of phytohemagglutinin and the indicated concentrations of L-arginine for 48 h. Cell pellets were subjected to RT-qPCR analysis. IL-10 mRNA levels shown are relative to levels in 0 μM L-arginine ($n = 6$ for each group). AD $CD4^+$, adult peripheral blood $CD4^+$ T cells; CB $CD4^+$, cord blood $CD4^+$ T cells. *$p < 0.05$ compared to the control without L-arginine by ANOVA.

L-Arginine-Induced IL-10 Production by Neonatal Tregs (Tregs)

To study the modulatory effects of L-arginine on Tregs, we assessed the expression of cell surface CD4 and CD25, and intracellular expression of the transcription factor FoxP3, in cells cultured in L-arginine. As shown in **Table 1**, neonates had a higher proportion of $CD4^+CD25^+$ lymphocytes than adults, and L-arginine supplementation enhanced the proportion of CBMCs expressing CD4 and CD25. We evaluated the expression of intracellular FoxP3 by flow cytometry. Exposure to L-arginine was associated with higher MFI expression of FoxP3 in neonatal $CD4^+CD25^+$ T cells (**Figure 2A**). In order to identify the cell population associated with IL-10 production, $CD4^+CD25^+$ cells were gated for intracellular FoxP3 and IL-10. As shown in **Figure 2B**, $CD4^+CD25^+FoxP3^+$ cells were responsible for the majority of IL-10 production in $CD4^+CD25^+$ CBMCs that were supplemented with L-arginine.

L-Arginine-Induced IL-10 Production by Neonatal $CD4^+$ T Cells Correlated with IL-10 Promoter Hypomethylation but Not Histone Modification

To investigate the mechanism of Treg IL-10 production, we treated isolated adult and CB $CD4^+$ T cells with L-arginine and PHA as indicated, and studied their expression of IL-10 mRNA. As shown in **Figure 3**, consistent with IL-10 protein production, CB $CD4^+$ T cells had significantly higher IL-10 mRNA levels in L-arginine-supplemented conditions than in the non-supplemented condition. However, L-arginine supplementation did not significantly enhance IL-10 mRNA expression by adult $CD4^+$ T cells.

Given that DNA methylation and histone modification can influence the expression of IL-10 mRNA, we next studied the epigenetic regulation of IL-10. First, we investigated the effect of L-arginine treatment on IL-10 promoter histone modification. We performed ChIP assays on nuclear extracts of CB $CD4^+$ T cells using antibodies directed against acetyl-histone H3 lysine 9 (Ac-H3K9), Ac-H3K14, Ac-H3K27, Ac-H4K5, phospho-histone H3 serine 10, and trimethyl-histone H3K4, which are promoter activation markers (32, 33). We then used RT-PCR to determine the amount of IL-10 promoter input DNA that was bound to each protein. We designed four pairs of IL-10 primers for RT-PCR analysis (**Figure 4A**). As shown in **Figure 4B**, there were no differences in IL-10 promoter binding to these active histone markers in any of the L-arginine treatment conditions. We also investigated if repression of histone modification altered L-arginine-induced IL-10 transcription. ChIP assays were performed on the nuclear extracts of CB $CD4^+$ T cells using antibodies directed against H3K9me3 and H3K27me3 (34). As with the other histone markers, there were no differences in H3K9me3 and H3K27me3 association with the IL-10 promoters among the L-arginine treatment conditions (Figure S1 in Supplementary Material). Thus, we did not detect either activating or repressing histone modifications of the IL-10 promoters in CB $CD4^+$ T cells with exogenous L-arginine treatment.

To test whether changes in DNA methylation contributed to IL-10 expression in neonatal $CD4^+$ T cells, we analyzed the methylation content of CG pairs in the 192-bp CpG island (position +3119 to +3310) within the intron 4 enhancer element of the IL-10 gene, which contains the transcriptional activator STAT5-binding site (35, 36). **Figure 5A** shows the average methylation content of eight CG sites in the IL-10 promoter from 10 CB and 10 adult $CD4^+$ T cell samples. The position +3281 CG site was hypomethylated in both adult and CB $CD4^+$ T cells. However, the methylation content of the other seven CG sites (positions +3144, +3162, +3170, +3200, +3229, +3261, and +3265) was significantly greater in CB $CD4^+$ T cells than in adult $CD4^+$ T cells. The methylation content of six of the CG sites (positions +3144, +3162, +3229, +3261, +3265, and +3281) decreased significantly in CB $CD4^+$ T cells at the indicated exogenous L-arginine concentrations (**Figure 5B**). Moreover, we found that the average degree of methylation of the eight methylated CG pairs within the intron 4 CpG islands negatively correlated with the levels of IL-10 mRNA expression in CB $CD4^+$ T cells (**Figure 5C**).

DISCUSSION

Although it is well known that L-arginine modulates T cell proliferation and immune responses, its mechanism of action is still not fully understood. Tregs can control improper activation, proliferation, and differentiation of effector T cells through several mechanisms in addition to the IL-2 signaling pathway (18). In previous studies, we have found that L-arginine modulates neonatal lymphocyte proliferation through an IL-2-independent pathway (22). In this study, we investigated how exogenous

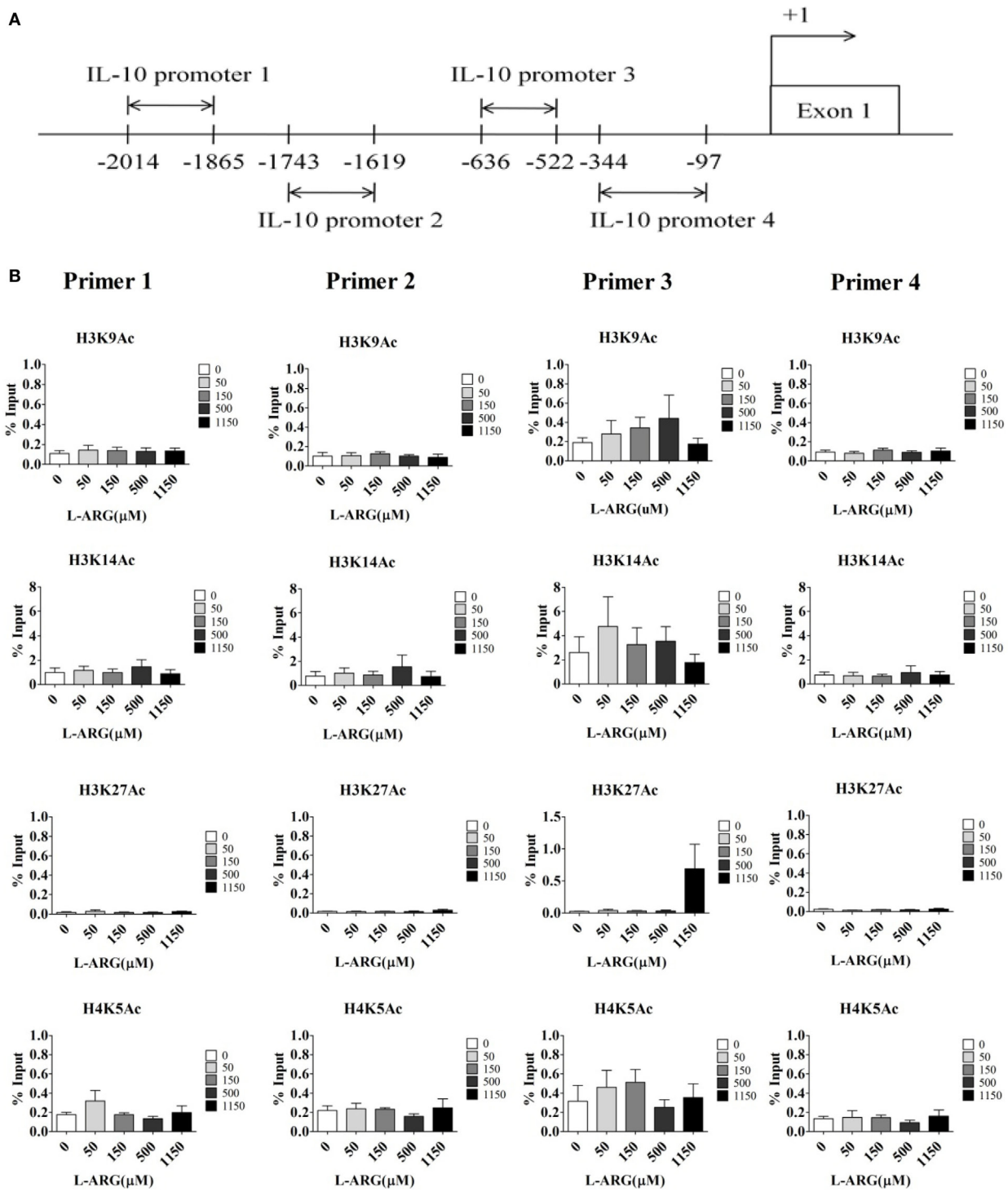

FIGURE 4 | **Continued**

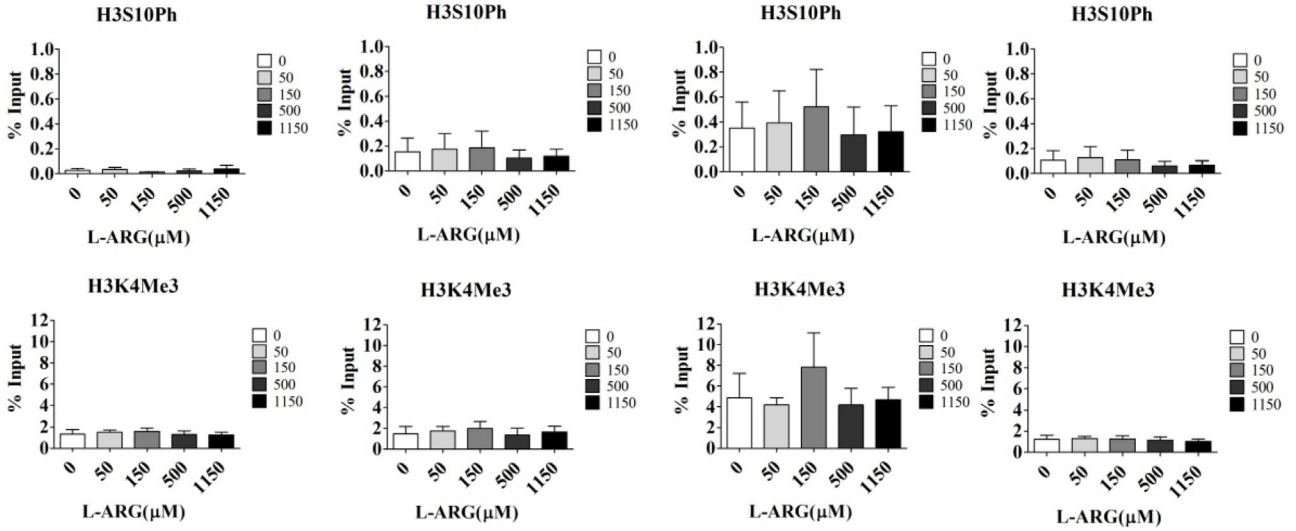

FIGURE 4 | Assessment of histone activation markers at the interleukin (IL)-10 promoters in cord blood (CB) CD4⁺ T cells with and without L-arginine supplementation. CD4⁺ T cells isolated from CB were treated with 10 μg of phytohemagglutinin and the indicated concentrations of L-arginine for 48 h. **(A)** The diagram indicates the primer positions in the IL-10 promoters; "+1" indicates a transcription initiation site. IL-10 promoter 1 was 2,014–1,865 bp upstream of the transcription site. **(B)** Chromatin in the cell pellet was immunoprecipitated using anti-acetyl-histone H3 lysine 9 (ac-H3K9), anti-ac-H3K14, anti-ac-H3K27, anti-ac-H4K5, anti-phospho-histone H3 serine 10 (ph-H3S10), or anti-trimethyl-histone H3 lysine 4 antibodies. The bar graphs show the levels of the indicated histone markers at the various IL-10 promoters. Results are expressed as percentage of the input (mean ± SEM; $n = 4$).

L-arginine enhances neonatal Tregs function in relation to IL-10 production and epigenetic regulation under PHA and anti-CD3/anti-CD28 stimulations. L-Arginine supplementation has been reported not to affect Treg frequency or IL-10 production by mouse splenocytes (37). To our knowledge, no experiments have focused on the modulatory mechanisms of L-arginine on human Tregs. In this study, we found that neonates, in contrast to adults, had impaired TGF-β, but not IL-10-related Treg responses. For both adults and neonates, the production of TGF-β1 was L-arginine-independent. IL-10 production by neonatal MNCs was mainly dependent on L-arginine-induced FoxP3 expression and hypomethylation of the IL-10 promoter in CB $CD4^+CD25^+$ T cells. Thus, we concluded that exogenous L-arginine modulates IL-10 production by neonatal Treg through demethylation of the IL-10 promoter.

The importance of L-arginine in the immune system was initially identified by the association of impaired T cell function and low-serum L-arginine levels in trauma patients (4). This low L-arginine-related T cell dysfunction could be rapidly reversed by L-arginine supplementation (4). L-Arginine depletion by arginase 1 and/or nitric oxide produced by granulocytes and myeloid-derived suppressor cells has been observed in certain cancers and infections (9, 38). The existence of myeloid-derived suppressor cells that produce arginase in cancer patients suggests that arginase production is a tumor evasive mechanism (39). L-Arginine depletion can result in inhibition of T lymphocyte proliferation, interferon-γ production, and CD3ζ downregulation, leading to impaired adaptive immune responses (7, 9). In addition to T lymphocytes, L-arginine depletion also suppresses the proliferation of natural killer cells and their production of IL-12/IL-18-mediated interferon-γ (5). Here, we provide evidence of L-arginine modulation of Tregs through epigenetic regulation. This is a novel finding, which may enable pharmacological regulation of Treg functions by inhibition of DNA methylation.

Interleukin-10 is an anti-inflammatory cytokine. It regulates innate and adaptive immune responses and limits immunopathologies (40). IL-10 is produced by many different immune cells, including dendritic cells, macrophages, and Tregs (40). Tregs include diverse immune cell populations that regulate the adaptive immune response. $CD4^+CD25^+FoxP3^+$ T cells, natural Tregs (nTregs), are thymically derived. The two major types of induced Tregs are Treg type 1 cells and Th3 Treg cells (41). Treg type 1 cells have a $CD4^+CD25^+FoxP3^-$ phenotype and specialize in IL-10 production (41, 42). Th3 cells express FoxP3 in the periphery and secrete TGF-β and IL-10 (41). Different conditions induce the various Treg subsets to produce IL-10. Induced Treg cells are known to be the major producers of IL-10 in filarial infections (43), whereas $CD4^+CD25^+$ Tregs produce IL-10 in *Leishmania major* and several other infections (40, 44, 45). In our study, we found that $CD4^+CD25^+FoxP3^+$ cells were responsible for the majority of IL-10 production. There is no specific marker to differentiate nTregs from Th3 cells, as both show a $CD4^+CD25^+FoxP3^+$ phenotype (41, 46); however, the IL-10-producing $CD4^+CD25^+FoxP3^+$ T cells that responded to L-arginine supplementation in CBMCs were likely nTregs because Th3 differentiation is induced by TGF-β, and we did not observe a change in TGF-β production with L-arginine treatment.

In a previous report, we had illustrated that the L-arginine levels in CB plasma (about 50 μM) were less than those in adult plasma (about 100–150 μM) (22). CBMCs presenting overexpression of arginase significantly upregulated surface CD25, the

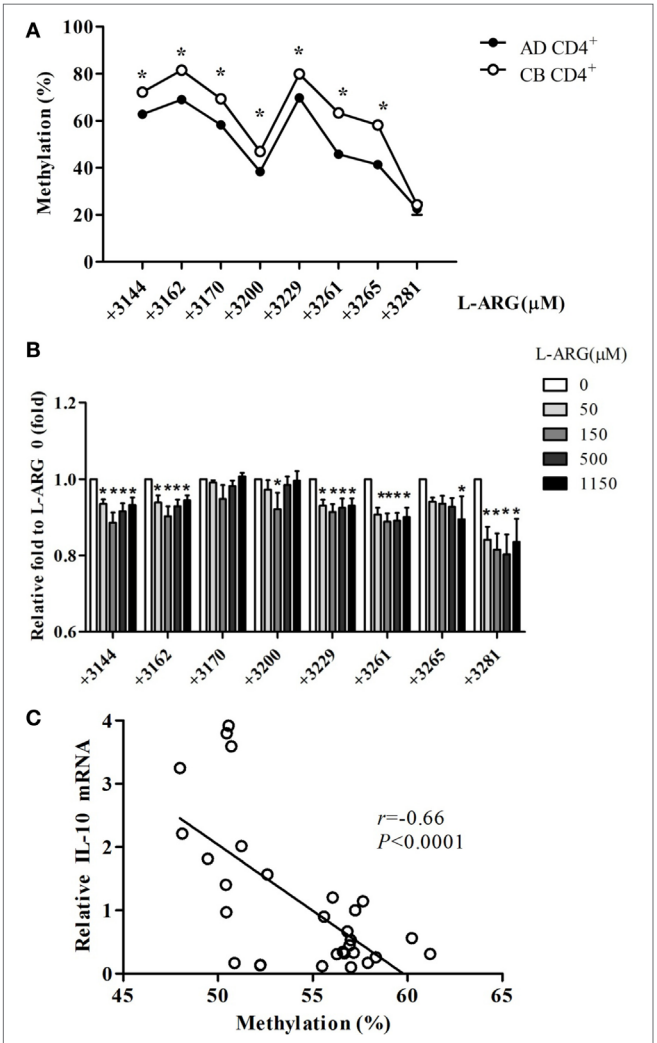

FIGURE 5 | Interleukin (IL)-10 enhancer methylation patterns in CD4+ T cells from adult and cord blood (CB). (A) The mean level of methylation for each of the eight CG pairs in intron 4 of the IL-10 locus in CD4+ T cells from adult (AD) and CB ($n = 10$). *$p < 0.05$ in a comparison between AD and CB by Mann–Whitney *U*-test. **(B)** The average level of methylation for the eight CG pairs (positions +3144, +3162, +3170, +3200, +3229, +3261, +3265, and +3281) in IL-10 intron 4 in CB CD4+ T cells with exposure to the indicated concentrations of L-arginine for 48 h ($n = 6$). *$p < 0.05$ compared to expression by cells treated with 0 μM L-arginine by analysis of variance (ANOVA). **(C)** The average levels of IL-10 promoter DNA methylation correlated with the relative IL-10 mRNA levels in CB CD4+ T cells at the indicated concentrations of L-arginine ($r = -0.66$; $p < 0.0001$). *$p < 0.05$ compared to the expression without addition of L-arginine by ANOVA.

IL-2 receptor, upon PHA stimulation after the exogenous supplementation with L-arginine (22). In this study, we have further determined that the increase of surface CD25 on CBMCs is mainly on CD4+ T cells, associated with higher IL-10 production through the regulation of IL-10 promoter DNA hypomethylation, but not histone modification. We also demonstrated that TGF-β1 production by PBMCs or CBMCs was L-arginine independent. The reason why CBMCs produce higher levels of IL-10 than PBMCs by PHA stimulation but not by the anti-CD3/anti-CD28 stimulation may be because that PHA stimulation, which has been shown to mediate through CD2 activation (29), may be different from the combination of anti-CD3 and anti-CD28 stimulation (31). Further studies to clarify the distinct signal transductions for IL-10 production by PBMCs and CBMCs between PHA and anti-CD3/CD28 stimulations are needed.

Our study is the first to show the effect of L-arginine on IL-10 promoter DNA methylation in neonatal CD4+CD25+ T cells, although the mechanism driving IL-10 DNA hypomethylation with L-arginine supplementation has not been determined. DNA methylation of CpG dinucleotides is an important epigenetic mechanism for gene regulation. Protein arginine methylation is a common posttranslational modification that regulates protein function in the cell. DNA methylation and protein arginine methylation are catalyzed by DNA methyltransferases and protein arginine methyltransferases, respectively (47, 48). Methyltransferases utilize S-adenosylmethionine (SAM) as a methyl group donor to form S-adenosylhomocysteine (SAH) and methylated substrates (including DNA and proteins). SAM treatment has been shown to increase global DNA methylation in human macrophages (49). Supplementation with L-arginine, a cofactor for SAM, can increase the production of SAH from SAM through the methylation-dependent generation of creatine (50). As SAH is a competitive inhibitor of SAM-dependent methyltransferases (51), L-arginine supplementation may cause IL-10 DNA hypomethylation through methyl group consumption and SAH production.

In conclusion, we studied the modulatory effects of L-arginine on Tregs in adults and neonates in depth. For both adult and neonatal MNCs, the production of TGF-β1 was L-arginine-independent. Neonatal MNCs produced higher levels of IL-10 than adult MNCs upon PHA stimulation. The frequency of IL-10-producing CD4+CD25+FoxP3+ T cells was increased by L-arginine supplementation in newborns. L-Arginine may modulate neonatal Treg IL-10 production through DNA hypomethylation. Given that Treg activation is a major mechanism for establishing immune regulation, L-arginine supplementation has the potential to correct the Treg function in newborns with L-arginine deficiency.

AUTHOR CONTRIBUTIONS

H-RY, T-YH, H-CH, KY, J-YW, H-CK, and L-SC contributed to designed the work; H-RY, C-CT, H-HC, J-MS, Y-HH, and K-SH contributed to data acquisition; H-RY, T-YH, L-SC, H-CH, H-CK, J-MS, and C-CT performed data analysis and interpretation; H-RY, KY, J-YW, and K-SH drafted the manuscript; H-RY, C-CT, Y-HH, and T-YH finalized the article. All authors have read and approved the final manuscript and agreed to be accountable for all aspects of the work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.00487/full#supplementary-material.

FIGURE S1 | Assessment of histone repression markers at the interleukin (IL)-10 promoter in cord blood (CB) CD4$^+$ T cells with and without L-arginine supplementation. CD4$^+$ T cells isolated from CB were treated with 10 μg of phytohemagglutinin and the indicated concentrations of L-arginine for 48 h. The primer positions in the IL-10 promoters are shown in **Figure 4**. Chromatin from the cell pellet was immunoprecipitated using anti-trimethyl-histone H3 lysine 9 (H3K9me3) or H3K27me3 antibodies. The bar graphs show the levels of histone activation markers at the different IL-10 promoters. Results are expressed as percentage of the input (mean ± SEM; n = 4).

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Sexual Dimorphic Responses in Lymphocytes of Healthy Individuals after *Carica papaya* Consumption

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Sexual dimorphism in immune response is widely recognized, but few human studies have observed this distinction. Food with endo-immunomodulatory potential may reveal novel sex-biased *in vivo* interactions. Immunomodulatory effects of *Carica papaya* were compared between healthy male and female individuals. Volunteers were given fixed meals supplemented with papaya for 2 days. Changes in blood immune profiles and hormone levels were determined. In females, total natural killer (NK) cell percentages decreased (12.7 ± 4.4 vs 14.6 ± 5.8%, $p = 0.018$, $n = 18$) while B cells increased (15.2 ± 5.5 vs 14.5 ± 5.0, $p = 0.037$, $n = 18$) after papaya consumption. Increased 17β-estradiol (511.1 ± 579.7 vs 282.7 ± 165.0 pmol/l, $p = 0.036$, $n = 9$) observed in females may be crucial to this change. Differentiation markers (CD45RA, CD69, CD25) analyzed on lymphocytes showed naïve ($CD45RA^+$) non-$CD4^+$ lymphocytes were reduced in females (40.7 ± 8.1 vs 46.8 ± 5.4%, $p = 0.012$, $n = 8$) but not males. A general suppressive effect of papaya on $CD69^+$ cells, and higher percentage of $CD69^+$ populations in females and non-CD4 lymphocytes, may be relevant. $CD107a^+$ NK cells were significantly increased in males (16.8 ± 7.0 vs 14.7 ± 4.8, $p = 0.038$, $n = 9$) but not females. Effect in females may be disrupted by the action of progesterone, which was significantly correlated with this population ($R = 0.771$, $p = 0.025$, $n = 8$) after papaya consumption. In males, total T helper cells were increased (33.4 ± 6.4 vs 32.4 ± 6.1%, $p = 0.040$, $n = 15$). Strong significant negative correlation between testosterone and $CD25^+CD4^+$ lymphocytes, may play a role in the lower total $CD4^+$ T cells reported in males. Thus, dissimilar immune profiles were elicited in the sexes after papaya consumption and may have sex hormone influence.

Keywords: natural killer cells, T cells, CD25, CD69, CD107a, sex hormones, *Carica papaya*

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INTRODUCTION

Sexual dimorphism in immune response of the innate and adaptive systems has been extensively reviewed in literature and manifested in differential resistance to infections. Females in general are better than males in defense against a variety of bacterial, viral, and parasitic infestations (1, 2). Dimorphism in severity and pathogenesis are also apparent as certain infectious and parasitic diseases increase mortality in females but not males (3). Sex hormones and sex chromosome-related

genes such as toll-like receptors, cytokine receptors involved in T-cell and B-cell activity in the X chromosome and inflammatory pathway genes in the Y chromosome (4, 5) are expected to be major contributors to this disparity.

Sex differences in non-communicable diseases are also observed in particular autoimmune diseases (6) as well as metabolic diseases, hypertension, cardiovascular diseases, psychiatric, neurological disorders, and cancer (7). More than 80% of all patients with autoimmune disease are women. Sex as a risk factor in coronary artery disease is observed in incidence rate and also age of onset, progression, treatment efficacy, morbidity, and mortality (8). These dissimilarities are attributed to genetic as well as hormonal differences and interactions and responses to environmental factors including infection, diet, drugs, stress, as well as behavior. Host hormone interactions with commensal gut microbiome are suggested to shape the microbiome composition (9), which is essential in immune homeostasis. Thus, sex matters and must be a consideration when decisions around therapeutic intervention strategies are being developed (9). Substantial data have accumulated from many epidemiological studies. *In vitro* studies demonstrating effects of sex hormones on immune cell subsets are well documented. However, *in vivo* human studies are still lacking.

Immunomodulatory potentials of phytochemicals and purified components of natural products are well studied. Whole food and its nutrients also have immunomodulatory effects, health healing potential, and play a role in homeostatic maintenance of the immune system but are less investigated. Grape juice consumption mobilized gamma–delta T cells and maintained immunity in healthy humans (10). A study on mice showed ginseng berry extract injected into mice exerted immunostimulatory effect by increasing pro-inflammatory molecules in dendritic cells from spleen after 24 h treatment (11). In diseased models, polysacccharide fractions from *Momorica charantia,* an edible medicinal vegetable, significantly increased various immune indexes to normal control levels in cyclophosphamide-induce immunosuppressed mice (12). Feijoa sellowiana Berg var. coolidge fruit juice consumption was shown to have anti-inflammatory activity on edema-induced mice within first hour of treatment (13) while agipenin, a natural flavonoid reduced neuroinflammation by protection against damage from dendritic cells stimulated T cells in experimental autoimmune encephlalomyelitis mouse models (14). Dietary polyphenols were found to exert a regulatory role on dendritic cell function. Researches in human are few and still new but represent an area of scientific need, opportunity, and challenge (15).

Carica papaya fruit is commonly consumed worldwide. It has high antioxidant activity (16) and rich in phytochemicals such as flavonoids (17). Different plant parts such as fruit, leaf, seed, root, bark, and flowers have been used as health treatments in tropical countries where it is grown. The seeds of papaya, however, have contraceptive effect on male fertility as well as manifest antifertility, anti-implantation, and abortifacient activity in female rats (18) suggesting a possibility to alter sex hormone levels.

We examined the potential of papaya fruit to modulate immune profiles and sex hormones in healthy male and female individuals. We observed differential immune profiles in sexes after papaya consumption, which may be influenced by sex hormones.

METHODOLOGY

Subjects

Apparently healthy individuals, age 18–35 years old, with no history of chronic or acute illness, no recent history of vaccination, piercing or blood transfusion, and not on medication or supplements were included. A total of 33 subjects, 15 males and 18 females, were recruited and underwent a papaya supplementation experiment. Subsequent lab investigations, however, were not conducted on all samples collected. Female subjects were enlisted during their second or third week after onset of menstruation and determined not on oral contraceptive. This study was approved by the Medical Research Ethics Committee, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia. All procedures complied with the principles of the Declaration of Helsinki. Informed written consents were obtained from participants.

Papaya Supplementation Experimental Design

A 5-day experiment was designed. Two subjects, one male and one female, were randomly selected at a time. Food intake was controlled with provision of standard meals consisting of bread/rice/noodle, chicken, vegetables, and liquid. The menu for day 3 and day 4 were replicates of day 1 and day 2, respectively. A pre-exposure period of 2 days (without papaya) was followed by 2 days with 100 g of fresh papaya fruit (fruit color index 4) in the day's three major meals. Daily dietary recall was conducted to confirm that the fruit provided each time was completely consumed while a medical call was carried out to determine no adverse effects. A peripheral blood sample (20 ml) was collected in K_2EDTA vacutainers in the morning before meal of day 3 (0 h) and day 5 (48 h). Either whole blood or peripheral blood mononuclear cells (PBMCs) was used in the experiments. Whole blood was used directly after withdrawal. PBMC was isolated by density centrifugation with Ficoll-Paque (GE Healthcare, USA) and stored over liquid nitrogen until further use. Plasma was collected and stored at −80°C for measurement of sex hormones levels.

Lymphocyte Subset Enumeration

Percentages and absolute counts of lymphocyte subsets in whole blood were determined with the BD Multitest™ IMK kit (BD Biosciences, USA) containing antibodies against CD45, CD3, CD4, CD8, and CD16$^+$CD56 together with BD Trucount tubes, according to procedures provided by manufacturer. All samples were tested. Cells were acquired on a BD FACSCanto flow cytometer (BD) and analyzed with FACSCanto Clinical Software (BD).

Expression of CD45RA, CD69, and CD25 on CD4$^+$ T Cells and Non-CD4$^+$ Lymphocytes

From here on, only nine paired samples from males and nine paired samples from females were tested. Subsequent missing samples were due to loss of data during a transition period. For surface marker studies, heparinized whole blood sample (100 µl) was incubated with monoclonal antibodies to CD4-PerCP,

CD45RA-FITC, CD25-APC, and CD69-PE purchased from Becton Dickinson (USA), following standard procedures. Briefly, after 20 min incubation in dark at 4°C, red blood cells were lysed with 1× lysing solution (Becton Dickinson, USA). After washing with 1× PBS, cells were re-suspended in 500 µl of 2% paraformaldehyde. Ten thousand events gated on $CD4^+$ bright population were acquired on a flow cytometer (BD LSR-Fortessa) and analyzed using FACSDiva (Becton Dickinson, USA).

Surface Marker Expression of Interleukin (IL) Receptors (IL-12Rβ2, IL-15Rα, IL-21R) on $CD8^+$ T and NK Cells

Whole blood (600 µl) diluted with equal volume of RPMI 1640 medium without FBS was dispensed in BD Falcon polystrene tubes and incubated with 400 ng/ml phorbol myristate acetate (PMA) (Sigma-Aldrich, USA) together with calcium ionophore (Sigma-Aldrich, USA) and golgi stop containing monensin (Becton Dickinson, USA) for 6 h at 37°C and 5% CO_2. After incubation, four-color staining (FITC/PE/APC/PerCP) for lineage markers, CD3, CD8, and CD56 and one of the surface IL receptor, IL-12Rβ2-PE, IL-15Rα-PerCP, or IL-21R-PE was performed. Subsequently, RBC was lysed with 1× lysing solution (Becton Dickinson, USA) following manufacturer's protocol and then fixed with 2% paraformaldehyde before analysis using BD FACSDiva software on LSR-Fortessa flow cytometer (BD).

Intracellular Cytokine Staining for Interferon-γ (IFN-γ)

The same stimulation procedure as above (cytokine receptors) was carried out. After 6 h incubation, cell surface staining for lineage specific markers (CD3, CD8, CD56) was performed. To detect IFN-γ secretion, cells were fixed with 2% paraformaldehyde followed by permeabilization with BD Perm/Wash solution before staining for intracellular IFN-γ PE-labeled antibody. Cells were analyzed on BD LSR-Fortessa flow cytometer (BD).

CD107a Degranulation Assay on $CD8^+$ T and NK Cells

Peripheral blood mononuclear cell (1×10^6 cells/ml) from volunteers were re-suspended in 500 µl of complete RPMI 1640 medium in BD Falcon polystrene tubes and incubated with 100 ng/ml PMA with calcium ionophore and golgi stop-containing monensin. PBMC was also incubated with monoclonal antibody to CD107a. Tubes were vortexed gently and incubated for 5 h in dark at 37°C with 5% CO_2. Subsequently, cells were washed with PBS, stained with monoclonal antibodies specific for CD3, CD8, and CD56 and analyzed on BD LSR-Fortessa flow cytometer (BD, USA).

Sex Hormone Assay

Measurement of sex hormone levels was outsourced to a local pathology laboratory for detection of 17β-estradiol, progesterone, and testosterone serum levels using System ARCHITECT ci8200 together with respective kits. Normal ranges were provided with the kits. Levels of sex hormones (17β-estradiol, progesterone, and testosterone) were then correlated with immune profiles determined in the study.

Statistical Analysis

The Shapiro–Wilk and Kolmogorov–Sminov tests showed non-normal distribution of the data collected here; therefore, non-parametric Wilcoxon matched pair test was used to compare paired groups and Spearman's correlation test was performed to determine associations from changes in variables that occurred after papaya consumption. Statistical analysis was performed using SPSS (version 22.0). $p < 0.05$ was considered significant. Results were presented as mean ± SD.

RESULTS

Only NK Cells and a $CD69^+$ Subpopulation Were Significantly Different between the Sexes

Comparison between males and females for all parameters combined for the two time points showed significantly lower percentages of total CD3-CD56/16^+ NK cells in females. Interestingly, a non-CD4 lymphocyte subpopulation with activated features ($CD45RA^-CD69^+CD25^-$) was significantly higher (10.4 ± 9.4 vs 5.3 ± 2.3, $p = 0.032$, $n = 32$) in females compared to males. As expected, the sex hormones levels were significantly different between males and females (**Table 1**).

17β-Estradiol and Progesterone Levels Were Significantly Increased in Females after Papaya Consumption

Plasma sex hormone levels of 17β-estradiol ($p = 0.036$, $n = 9$) and progesterone ($p = 0.039$, $n = 9$) were significantly increased in females after papaya consumption (**Figure 1**; **Table 1**). Even though the experiment was designed to be carried out during the follicular phase of the female menstrual cycle, two subjects showed luteal phase levels for progesterone. These samples were not excluded as this study analyzed pre- and post-levels. None of the hormones tested demonstrated significant change in males after *C. papaya* consumption.

Total NK Cell Percentages Were Significantly Reduced in Females while Total $CD4^+$ T Cell and Total B Cell Were Significantly Increased in Males and Females, Respectively, after Papaya Consumption

Total NK cells from peripheral blood were significantly down-regulated ($p = 0.018$, $n = 18$), while total B cell percentages were significantly increased ($p = 0.037$, $n = 18$) in females after papaya consumption (**Figure 2**). Total $CD4^+$ T cells was significantly increased ($p = 0.040$, $n = 15$) in males (**Table 1**).

A negative association was detected between change in 17β-estradiol levels and change in NK cell percentages in females ($R = -0.586$, $p = 0.097$, $n = 9$) suggesting increased 17β-estradiol may play a role in downregulating NK cells. No significant

TABLE 1 | Mean ± SD values of sex hormones and immune parameters in healthy males and females, combined (all samples) and pre- and post-papaya consumption.

	All samples					Pre-papaya vs post-papaya										
						All subjects			Male			Female		All	M	F
	Male	*n*	Female	*n*	M vs F	Pre-	Post-	*n*	Pre-	Post-	*n*	Pre-	Post-	*n*	(Pre- vs Post-)	
Sex hormone																
Estradiol (pmol/l)	98.1 ± 26.5	18	396.9 ± 429.9	18	$P = 0.000$	191.5 ± 147.9	303.5 ± 451.9	18	100.3 ± 23.9	95.8 ± 30.1	9	282.7 ± 165.0	511.1 ± 579.7	9	$p = 0.107$	$p = 0.594$	$p = 0.036$
Progesterone (nmol/l)	0.3 ± 0.0	18	6.4 ± 9.7	18	$p = 0.010$	2.8 ± 6.8	4.0 ± 8.1	18	0.3 ± 0.0	0.3 ± 0.0	9	5.2 ± 9.2	7.61 ± 0.5	9	$p = 0.039$	$p = 1.000$	$p = 0.039$
Testosterone (nmol/l)	7.5 ± 2.5	18	0.8 ± 0.5	18	$p = 0.000$	4.3 ± 4.0	4.0 ± 3.8	18	7.8 ± 2.5	7.2 ± 2.7	9	0.8 ± 0.4	0.8 ± 0.7	9	$p = 0.248$	$p = 0.129$	$p = 1.000$
Lymphocyte subpopulation																
$CD3^+CD4^+$ %	32.9 ± 6.1	30	37.1 ± 8.9	36	$p = 0.056$	34.8 ± 8.3	35.6 ± 7.8	33	32.4 ± 6.1	33.4 ± 6.4	15	36.8 ± 9.4	37.5 ± 8.5	18	$p = 0.009$	$p = 0.040$	$p = 0.107$
$CD3^+CD4^+$ cnt	858.5 ± 264.2	30	980.1 ± 543.2	36	$p = 0.643$	927.6 ± 485.2	922.0 ± 397.6	33	857 ± 254.6	860.0 ± 282.5	15	986.4 ± 617.8	973.7 ± 475.0	18	$p = 0.688$	$p = 0.910$	$p = 0.647$
$CD3^+CD8^+$ %	28.1 ± 9.1	30	28.7 ± 6.7	36	$p = 0.610$	28.4 ± 8.0	28.5 ± 7.7	33	28.3 ± 9.3	28.0 ± 9.1	15	28.5 ± 7.0	29.0 ± 6.6	18	$p = 0.712$	$p = 0.694$	$p = 0.217$
$CD3^+CD8^+$ cnt	748.4 ± 352.0	30	719.0 ± 295.1	36	$p = 0.872$	741.2 ± 345.0	723.6 ± 297.9	33	766.8 ± 372.7	730.1 ± 342.0	15	719.8 ± 329.6	718.2 ± 265.8	18	$p = 0.761$	$p = 0.551$	$p = 0.327$
$CD3^-CD16^+$ 56%	16.8 ± 5.9	30	13.6 ± 5.2	36	$p = 0.031$	15.7 ± 6.0	14.4 ± 5.5	33	17.1 ± 6.0	16.4 ± 6.1	15	14.6 ± 5.8	12.7 ± 4.4	18	$p = 0.010$	$p = 0.233$	$p = 0.018$
$CD3^-CD16^+56$ cnt	465.3 ± 274.9	30	343.5 ± 202.5	36	$p = 0.053$	413.2 ± 245.1	384.5 ± 245.9	33	474.6 ± 264.9	456.1 ± 293.5	15	362.1 ± 221.7	324.8 ± 185.9	18	$p = 0.183$	$p = 0.650$	$p = 0.157$
$CD3^-CD19^+$ %	13.6 ± 3.5	30	14.9 ± 5.2	36	$p = 0.728$	14.1 ± 4.4	14.4 ± 4.7	33	13.7 ± 3.8	13.5 ± 3.4	15	14.5 ± 5.0	15.2 ± 5.5	18	$p = 0.216$	$p = 0.368$	$p = 0.037$
$CD3^-CD19^+$ cnt	362.8 ± 151.7	30	373.1 ± 199.3	36	$p = 0.880$	370.0 ± 194.3	366.8 ± 163.1	33	371.4 ± 153.7	354.2 ± 154.7	15	368.9 ± 227.1	377.4 ± 173.6	18	$p = 0.531$	$p = 0.460$	$p = 0.149$
Expression of differentiation markers in lymphocyte subpopulations																
CD4+ helper T cells																
$CD4^+CD45RA^+$	14.3 ± 4.3	16	16.2 ± 6.4	16	$p = 0.515$	15.6 ± 5.4	15.0 ± 5.7	16	14.9 ± 5.1	13.8 ± 3.5	8	16.3 ± 5.8	16.2 ± 7.4	8	$p = 0.277$	$p = 0.161$	$p = 0.889$
$CD69^-CD25^-$	52.5 ± 12.6	16	53.1 ± 12.5	16	$p = 0.838$	52.5 ± 11.8	53.1 ± 13.3	16	51.6 ± 11.9	53.5 ± 14.0	8	53.4 ± 12.3	52.7 ± 13.4	8	$p = 0.535$	$p = 0.263$	$p = 0.779$
$CD69^-CD25^+$	42.7 ± 13.6	16	40.3 ± 13.0	16	$p = 0.539$	41.4 ± 12.5	41.6 ± 14.1	16	43.4 ± 13.2	42.1 ± 14.8	8	39.3 ± 12.4	41.2 ± 14.4	8	$p = 0.836$	$p = 0.483$	$p = 0.183$
$CD69^+CD25^-$	2.8 ± 2.5	16	4.0 ± 3.5	16	$p = 0.323$	3.7 ± 3.5	3.0 ± 2.6	16	3.0 ± 2.8	2.6 ± 2.2	8	4.5 ± 4.1	3.5 ± 3.0	8	$p = 0.013$	$p = 0.233$	$p = 0.028$
$CD69^+CD25^+$	2.0 ± 2.0	16	2.7 ± 2.1	16	$p = 0.184$	2.5 ± 2.1	2.2 ± 2.0	16	2.1 ± 2.1	1.9 ± 2.0	8	2.9 ± 2.2	2.6 ± 2.2	8	$p = 0.046$	$p = 0.171$	$p = 0.149$
$CD4^+CD45RA^-$	13.9 ± 3.0	16	16.3 ± 4.8	16	$p = 0.184$	15.3 ± 4.1	14.9 ± 4.2	16	13.9 ± 2.7	14.0 ± 3.4	8	16.7 ± 4.9	15.9 ± 5.0	8	$p = 0.778$	$p = 0.866$	$p = 0.735$
$CD69^-CD25^-$	46.9 ± 12.2	16	46.3 ± 12.3	16	$p = 0.590$	46.1 ± 11.4	47.0 ± 13.1	16	45.8 ± 11.6	48.0 ± 13.5	8	46.5 ± 11.9	46.1 ± 13.5	8	$p = 0.501$	$p = 0.326$	$p = 0.779$
$CD69^-CD25^+$	51.8 ± 12.3	16	51.6 ± 12.4	16	$p = 0.780$	52.0 ± 11.6	51.4 ± 13.1	16	52.8 ± 11.8	50.8 ± 13.5	8	51.2 ± 12.1	52.0 ± 13.5	8	$p = 0.605$	$p = 0.327$	$p = 0.575$
$CD69^+CD25^-$	0.6 ± 0.3	16	0.9 ± 0.8	16	$p = 0.239$	0.8 ± 0.7	0.6 ± 0.6	16	0.6 ± 0.3	0.5 ± 0.3	8	1.1 ± 0.8	0.8 ± 0.9	8	$p = 0.007$	$p = 0.066$	$p = 0.041$
$CD69^+CD25^+$	0.7 ± 0.3	16	1.2 ± 1.0	16	$p = 0.305$	1.0 ± 0.8	0.9 ± 0.8	16	0.8 ± 0.4	0.7 ± 0.3	8	1.3 ± 1.0	1.1 ± 1.0	8	$p = 0.045$	$p = 0.121$	$p = 0.180$
CD4– lymphocytes																
$CD4^-CD45RA^+$	47.6 ± 5.4	16	43.7 ± 7.3	16	$p = 0.119$	47.5 ± 5.1	43.9 ± 7.6	16	48.2 ± 5.1	47.1 ± 5.9	8	46.8 ± 5.4	40.7 ± 8.1	8	$p = 0.009$	$p = 0.401$	$p = 0.012$
$CD69^-CD25^-$	91.4 ± 7.2	16	90.4 ± 7.0	16	$p = 0.491$	90.2 ± 7.7	91.6 ± 6.3	16	90.7 ± 8.3	92.2 ± 6.4	8	89.7 ± 7.6	91.1 ± 6.7	8	$p = 0.039$	$p = 0.161$	$p = 0.093$
$CD69^+CD25^-$	8.0 ± 7.0	16	9.1 ± 7.1	16	$p = 0.539$	9.3 ± 7.7	7.8 ± 6.4	16	8.8 ± 8.0	7.2 ± 6.3	8	9.8 ± 7.9	8.3 ± 6.8	8	$p = 0.016$	$p = 0.093$	$p = 0.093$
$CD4^-CD45RA^-$	24.1 ± 5.6	16	23.8 ± 10.3	16	$p = 0.402$	21.7 ± 5.7	26.2 ± 9.7	16	23.0 ± 5.6	25.2 ± 5.8	8	20.3 ± 5.8	27.3 ± 12.8	8	$p = 0.019$	$p = 0.123$	$p = 0.123$
$CD69^-CD25^-$	93.5 ± 2.1	16	89.0 ± 9.5	16	$p = 0.094$	91.2 ± 6.2	91.3 ± 8.2	16	93.3 ± 2.0	93.7 ± 2.3	8	89.1 ± 8.2	88.9 ± 11.2	8	$p = 0.836$	$p = 0.484$	$p = 0.779$
$CD69^+CD25^-$	5.3 ± 2.3	16	10.4 ± 9.4	16	$p = 0.032$	7.7 ± 6.4	8.0 ± 8.2	16	5.2 ± 2.4	5.5 ± 2.4	8	10.3 ± 8.2	10.6 ± 11.1	8	$p = 0.918$	$p = 0.889$	$p = 1.000$
Expression of activation markers in cytotoxic lymphocytes																
$CD8^+$ cytotoxic T cells																
$CD3^+CD8^+IFN^+$	6.0 ± 4.8	16	7.1 ± 5.7	16	$p = 0.468$	7.0 ± 5.4	6.1 ± 5.0	16	6.7 ± 5.7	5.3 ± 3.8	8	7.2 ± 5.5	7.0 ± 6.2	8	$p = 0.427$	$p = 0.779$	$p = 0.398$
$CD3^+CD8^+IL\text{-}12R^+$	4.6 ± 1.7	16	5.4 ± 2.2	16	$p = 0.590$	5.0 ± 2.1	5.0 ± 2.0	16	4.7 ± 2.0	4.6 ± 1.5	8	5.2 ± 2.3	5.5 ± 2.3	8	$p = 0.660$	$p = 0.944$	$p = 0.441$
$CD3^+CD8^+IL\text{-}15R^+$	8.4 ± 4.5	18	9.6 ± 5.3	16	$p = 0.422$	9.5 ± 4.9	8.4 ± 4.9	17	8.6 ± 4.5	8.2 ± 4.7	9	10.5 ± 5.4	8.7 ± 5.4	8	$p = 0.218$	$p = 0.678$	$p = 0.182$
$CD3^+CD8^+IL\text{-}21R^+$	7.2 ± 2.8	16	8.6 ± 3.6	16	$p = 0.341$	8.1 ± 3.5	7.7 ± 3.0	16	7.1 ± 3.5	7.2 ± 2.1	8	9.0 ± 3.5	8.2 ± 3.8	8	$p = 0.642$	$p = 0.575$	$p = 0.208$
$CD3^+CD8^+CD107a^+$	5.8 ± 2.3	18	5.3 ± 1.8	16	$p = 0.851$	5.7 ± 2.0	5.5 ± 2.2	18	6.0 ± 2.1	5.6 ± 2.7	9	5.3 ± 2.0	5.3 ± 1.8	9	$p = 1.000$	$p = 0.767$	$p = 0.799$
Natural killer cells																
$CD3^-CD56^+IFN^+$	21.1 ± 2.1	14	23.1 ± 15.7	14	$p = 0.946$	21.9 ± 14.5	22.2 ± 13.5	14	22.6 ± 13.4	19.5 ± 11.5	7	21.3 ± 16.7	24.9 ± 15.7	7	$p = 0.683$	$p = 0.176$	$p = 0.612$
$CD3^-CD56^+IL12R^+$	11.2 ± 5.3	16	13.2 ± 6.5	16	$p = 0.838$	11.9 ± 5.7	12.5 ± 6.3	16	11.0 ± 5.3	11.5 ± 5.7	8	12.8 ± 6.2	13.5 ± 7.2	8	$p = 0.642$	$p = 0.889$	$p = 0.674$
$CD3^-CD56^+IL15R^+$	17.9 ± 11.5	18	20.0 ± 9.9	18	$p = 0.355$	20.3 ± 12.9	17.6 ± 7.7	18	18.4 ± 14.8	17.3 ± 7.6	9	22.1 ± 11.3	17.8 ± 8.3	9	$p = 0.356$	$p = 0.889$	$p = 0.214$
$CD3^-CD56^+IL\text{-}21R^+$	17.8 ± 6.9	16	21.7 ± 10.2	16	$p = 0.381$	20.1 ± 8.5	19.3 ± 9.4	16	16.9 ± 5.8	18.6 ± 8.2	8	23.4 ± 9.8	20.0 ± 11.0	8	$p = 0.679$	$p = 0.484$	$p = 0.327$
$CD3^-CD56^+CD107a^+$	15.7 ± 5.9	18	16.1 ± 6.9	16	$p = 0.746$	15.1 ± 6.5	16.7 ± 6.2	17	14.7 ± 4.8	16.8 ± 7.0	9	15.6 ± 8.4	16.5 ± 5.7	8	$p = 0.044$	$p = 0.038$	$p = 0.397$

M, male; F, female; n, number of samples.

Statistical significance achieved where $p < 0.05$.

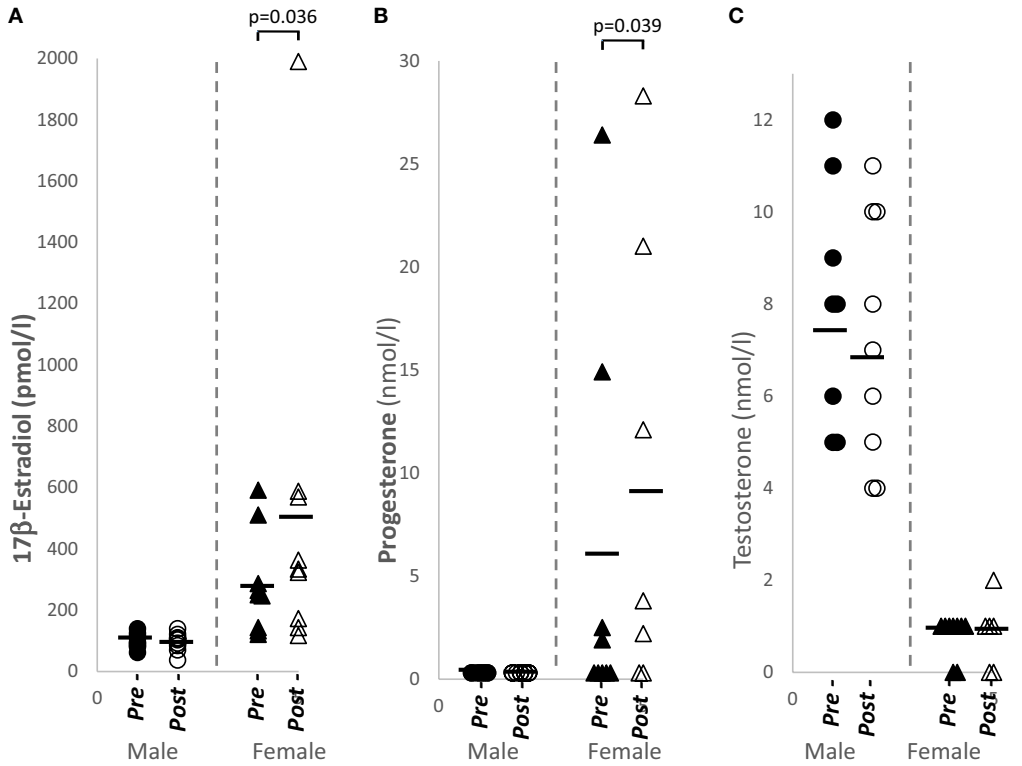

FIGURE 1 | Distribution of sex hormone levels in healthy human males ($N = 9$) and females ($N = 9$), pre- and post-papaya consumption. **(A)** 17β-estradiol, **(B)** progesterone, and **(C)** testosterone. *Statistical significance achieved where $p < 0.05$.

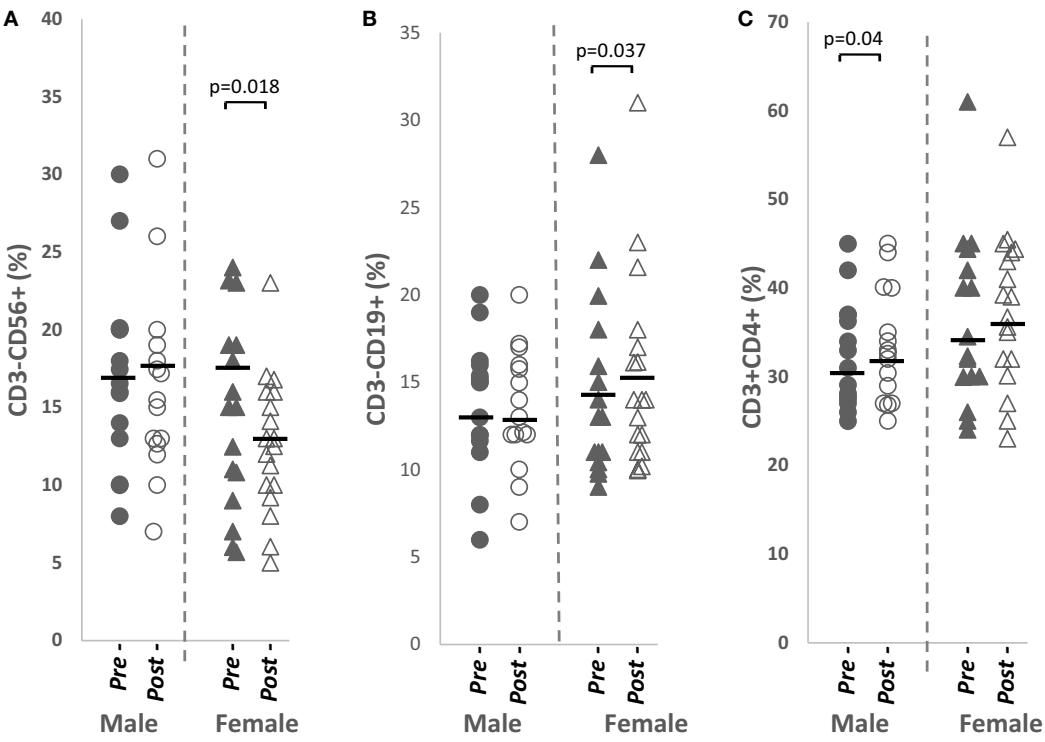

FIGURE 2 | Distribution of **(A)** total $CD3^-CD56^+$ NK cells, **(B)** total $CD3^-CD19^+$ B cells, and **(C)** total $CD3^+CD4^+$ helper T cells percentages in healthy males ($n = 15$) and females ($n = 18$) pre- and post-papaya consumption. *Statistical significance achieved where $p < 0.05$.

correlation was observed between sex hormone and total B cells in females or total $CD4^+$ T cells in males.

$CD69^+$ T Cells Were Significantly Reduced after Papaya Consumption

Three differentiation markers (CD45RA, CD69, and CD25) were selected from literature based on their use as naïve and activated/effector markers (**Figure 3A**). **Table 1** shows comparable percentages of naïve, $CD4^+CD45RA^+$ and non-naïve, $CD4^+CD45RA^-$ T helper cells. Among naïve cells, the double negative, $CD69^-CD25^-$ subpopulation made up a major proportion followed by $CD69^-CD25^+$ single positive cells. Within non-naive cells, mean percentage of the double negative subpopulation was lower while $CD69^-CD25^+$ cells were higher (**Table 1**).

CD69 expression ($CD25^+/CD25^-$) was observed on only a small fraction (2.0–4.0%) of naïve cells and was lower (0.6–1.2%) among non-naïve T helper cells (**Table 1**). The non-naïve ($CD45RA^-$) component consisted of activated, effector, memory as well as regulatory cells, thus these cells may also be referred as activated cells here.

A relatively large mean percentage of $CD25^+$ cells was observed in the naïve component (40–45%) and was higher in activated $CD4^+$ T cells (**Table 1**).

All CD69-expressing T cells, either single $CD69^+CD25^-$ or double positive $CD69^+CD25^+$ were in general, significantly downregulated in naïve and activated subpopulations after papaya consumption (**Figure 3B**; **Table 1**). Correlation analysis with sex hormones revealed negative associations [$R \leq (-)0.591$] with progesterone in females and testosterone in males but these associations were insignificant ($p \geq 0.116$).

Correlation analysis between sex hormones and CD25-expressing cells, however, revealed significant strong negative associations between changes in testosterone levels and percentages of CD25-expressing T helper cells, in a naïve $CD4^+CD45RA^+CD69^-CD25^+$ ($R = -0.899$, $p = 0.002$, $n = 8$) and the activated subpopulations, $CD4^+CD45RA^-CD69^-CD25^+$ ($R = -0.894$, $p = 0.003$, $n = 8$) and $CD4^+CD45RA^-CD69^+CD25^+$ ($R = -0.852$, $p = 0.007$, $n = 8$), in males following papaya consumption (**Figure 2**). A "mirror image" significant strong positive correlations were observed between testosterone with double-negative ($CD69^-CD25^-$) naïve ($R = 0.894$, $p = 0.003$, $n = 8$) and activated ($R = 0.899$, $p = 0.002$, $n = 8$) $CD4^+$ T cells (**Table 1**).

Progesterone also had an apparent suppressive effect on $CD25^+$ cells in females, as negative correlations were observed with single positive, naïve $CD45RA^+CD69^-CD25^+$ ($R = -0.524$, $p = 0.183$, $n = 8$) and activated $CD45RA^-CD69^-CD25^+$ ($R = -0.600$, $p = 0.116$, $n = 8$) T helper cells, following papaya consumption. The relation, however, was not as strong as testosterone in males.

Total Naïve Non-$CD4^+$ Lymphocytes Were Significantly Reduced while Total Activated Non-$CD4^+$ Lymphocytes Significantly Increased after Papaya Consumption

Non-$CD4^+$ ($CD4^-$) lymphocytes were a mixed population consisting of CD8 T cells, NK, B and NKT subsets. CD25 positivity was very low among these cells, <1% (data not shown) and excluded from further analysis.

The majority of non-$CD4^+$ lymphocytes, were double negative ($CD69^-CD25^-$). Compared to $CD4^+$ lymphocytes where expression of CD69 was found on 0.6–4.0%, a larger population of $CD69^+$ cells was observed among the non-$CD4^+$ lymphocytes forming average percentages of 8.0–9.1% in $CD45RA^+$ and 5.3–10.4% in $CD45RA^-$ lymphocytes (**Table 1**).

Total naïve non-$CD4^+$ lymphocytes were significantly reduced while the activated populations were significantly increased after papaya consumption (**Table 1**). Distribution of pre- and post-papaya consumption percentages are shown in **Figure 3C**. Among naïve cells, the $CD69^+CD25^-$ subpopulations were significantly reduced while the corresponding double negative ($CD69^-CD25^-$) subpopulations were significantly increased (**Table 1**). Within the activated population, changes were not significant as modulations were more heterogeneous between individual subjects which resulted in less obvious total effect. Interestingly, $CD69^+CD25^-$ subpopulations were larger in females, particularly the significantly higher activated $CD4^-CD45RA^-CD69^+CD25^-$ subpopulation (**Table 1**). Negative correlations were observed between naïve and activated $CD69^+$ subpopulations and testosterone in males ($R = -0.728$, $p = 0.041$, $n = 8$ and $R = -0.664$, $p = 0.073$, $n = 8$, respectively) as well as progesterone in females ($R = -0.434$, $p = 0.282$, $n = 8$ and $R = -0.668$, $p = 0.070$. $n = 8$, respectively), though the majority of these correlations were not significant.

Interesting also to note, CD69 expression was associated with two divergent levels of CD45RA expression, i.e., $CD45RA^{hi}CD69^+$ and $CD45RA^-CD69^+$ (**Figure 3D**). CD69 was not expressed on cells with intermediate levels of CD45RA. These may be useful in differentiating circulating regulatory cells/early activated cells before cell division, and memory/effector cells/migrating Tregs, respectively.

No Significant Change in $CD8^+$ T Cell Subsets Expressing Effector Markers after Papaya Consumption

The distinctly increased activated non-$CD4^+$ cells after papaya consumption prompted a closer examination of this population, consisting of $CD8^+$ T cells, B cells, NK cells, or NKT cells. We opted for the cytotoxic component for further analysis and selected several activation markers associated with these cells. Effector markers analyzed were IFN-γ, IL-12R2β, IL-15Rα, IL-21R, and degranulation marker, CD107a.

No significant changes were observed in $CD8^+$ T cells expressing any of these markers (**Table 1**).

Significantly Increased $CD107a^+$ NK Cells after Papaya Consumption

The same effector markers were analyzed on NK cells ($CD3^-CD56^+$). By comparison, these markers were expressed on a larger percentage of NK cells compared to $CD8^+$ T cells (**Table 1**).

Overall, a significant upregulation of $CD107a^+$ NK cells was observed after papaya consumption (**Figure 4B**; **Table 1**)

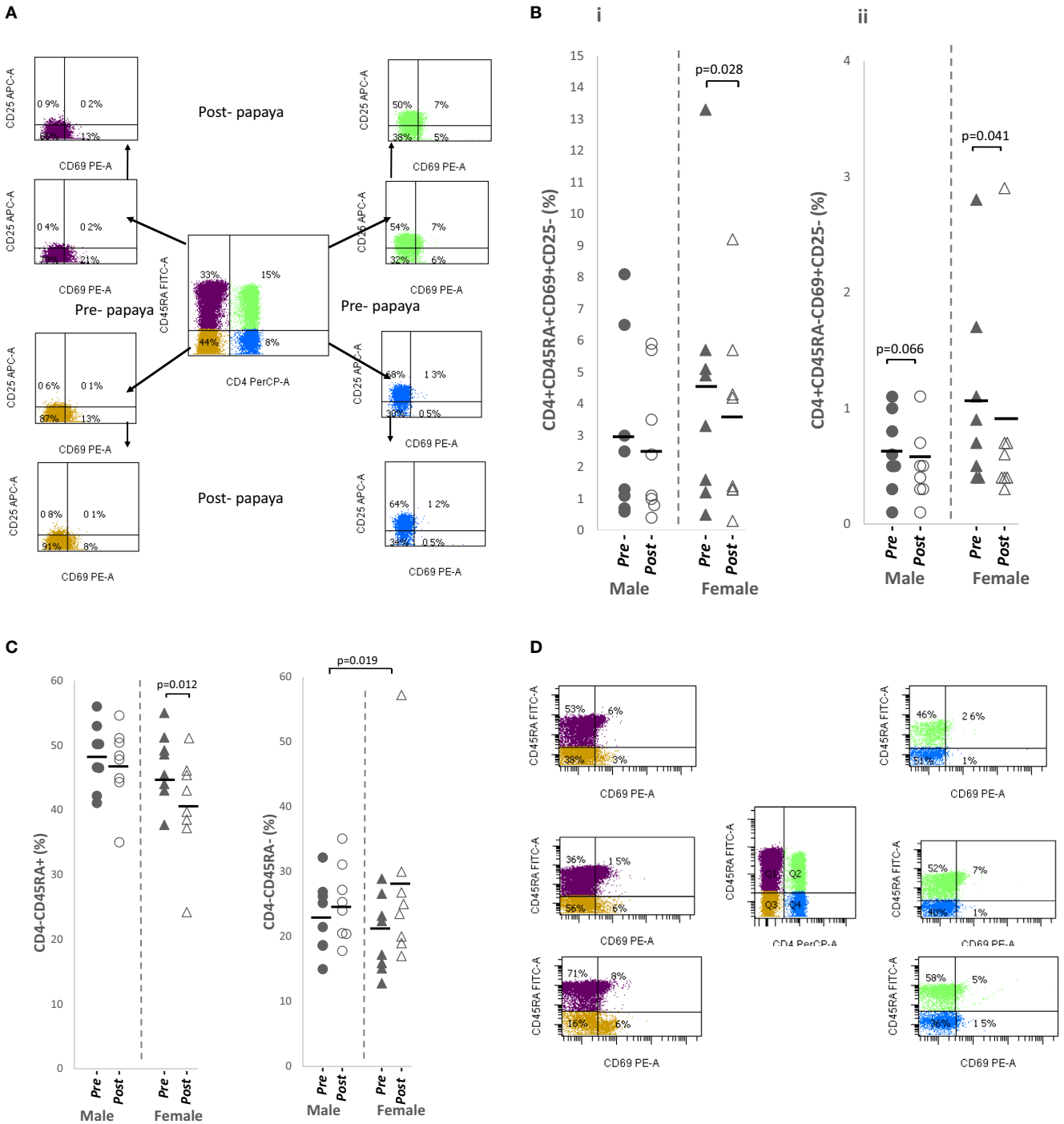

FIGURE 3 | **(A)** Flow cytometry gating strategy for analysis of differentiation markers expressed on CD4⁺ T cells/non-CD4⁺ lymphocytes in lysed whole blood. Initial gating was on lymphocytes with low SSC/low FSC. Distribution of **(B)** CD69⁺ in CD4⁺ T cell subpopulations i. naïve, $CD4^+CD45RA^+CD69^+CD25^-$ and ii. activated, $CD4^+CD45RA^-CD69^+CD25^-$, and **(C)** non-CD4⁺ lymphocytes i. total naïve $CD4^+CD45RA^+$ and ii. total activated $CD4^+CD45RA^-$ in healthy males ($n = 8$) and females ($n = 8$) pre- and post-papaya consumption. **(D)** Representative flow cytometry plots showing CD69⁺ fractions in naïve, CD45RA⁺ and activated $CD45RA^-$ CD4⁺ T cells and non-CD4⁺ lymphocytes. *Statistical significance achieved where $p < 0.05$.

particularly in males. Representative flow cytometry plots demonstrating gating strategy to detect CD107a expression on cytotoxic cells are shown in **Figure 4A**.

Correlation analysis revealed CD107a⁺ NK cells no strong correlation with testosterone levels in males ($R = 0.520$, $p = 0.151$, $n = 9$) but demonstrated strong positive association ($R = 0.771$,

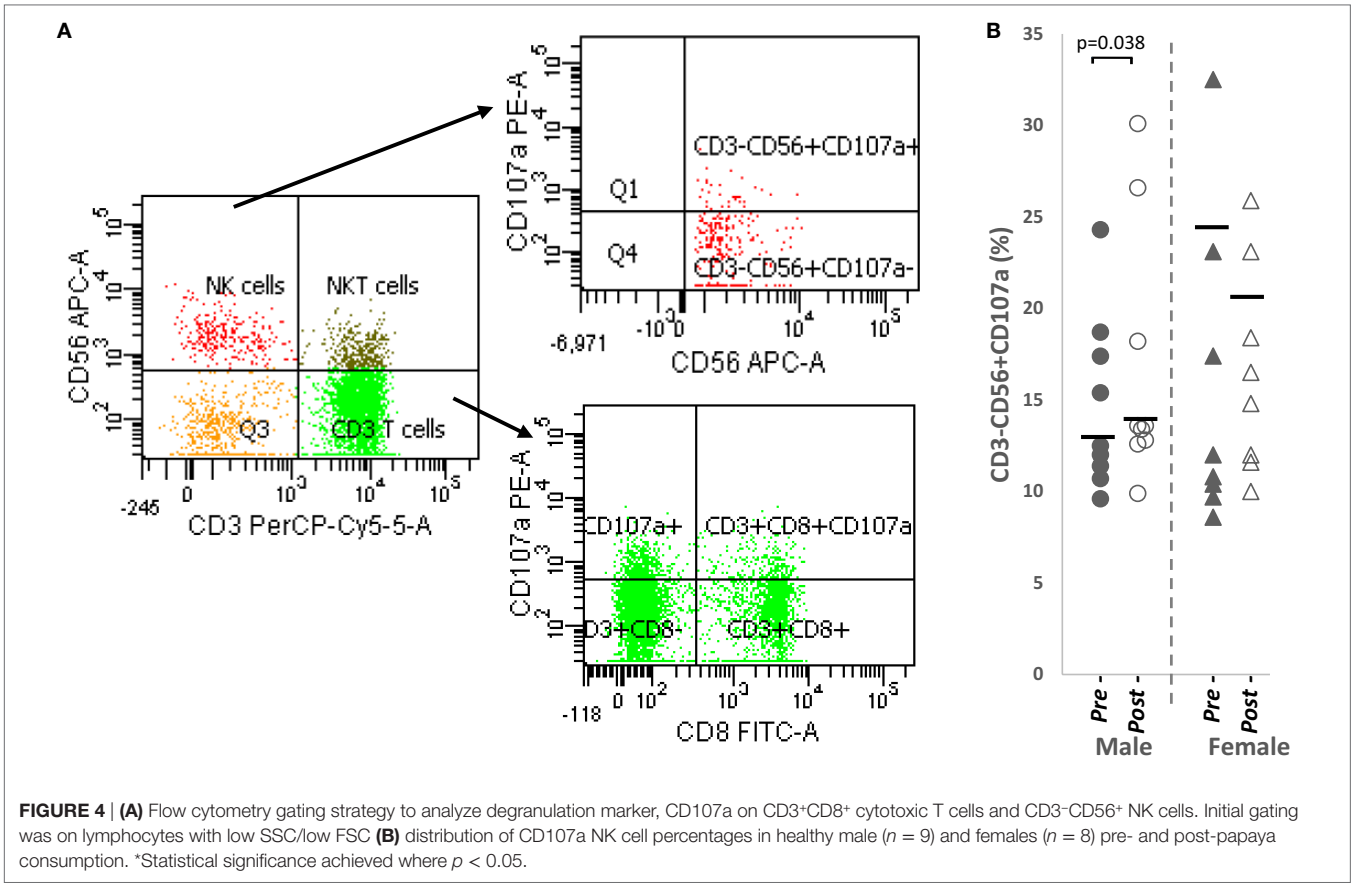

FIGURE 4 | **(A)** Flow cytometry gating strategy to analyze degranulation marker, CD107a on $CD3^+CD8^+$ cytotoxic T cells and $CD3^-CD56^+$ NK cells. Initial gating was on lymphocytes with low SSC/low FSC **(B)** distribution of CD107a NK cell percentages in healthy male ($n = 9$) and females ($n = 8$) pre- and post-papaya consumption. *Statistical significance achieved where $p < 0.05$.

$p = 0.025$, $n = 8$) with progesterone in females. Thus, while papaya generally induced NK cell degranulation, in females, this occurred only in increased progesterone.

DISCUSSION

In this study, the feasibility of detecting endo-immunomodulation by dietary intake of *Carica papaya* fruit was explored in an oral sensory receptive model involving a small population of apparently healthy individual. This preliminary, 2-day short-term exposure revealed interesting results.

Exogenous supplementation from plant-based hormones may affect outcomes in the study as fruits and vegetables contain a myriad of phytochemicals including phytoestrogens. However, a study on premenopausal women given isoflavone-rich diets was not shown to affect serum estradiol or progesterone concentrations (19).

We observed significantly increased 17β-estradiol (E2) and progesterone (P4) in females after papaya consumption. Researches on effects of whole fruits on sex hormones in premenopausal women are limited. In the BioCycle Study on healthy premenopausal women, increased intake of citrus fruit juice did not alter estradiol levels but increased progesterone levels. No significant changes were observed, however, with increased intake of non-citrus fruit juice (20). In healthy postmenopausal women, whole grapefruit significantly increased estrone-3-sulfate (E1S) while fresh juice, bottled juice, and soda intake significantly lowered estradiol (E2) (21). Pomegranate juice reduced estrone (E1) and testosterone in normal weight postmenopausal women (22). Thus, our results support evidence of potential sex hormone alterations from intake of fruits in women.

Estrogen receptor (ER) and progesterone receptors are expressed on various lymphocytes [reviewed in Ref. (23)]. Total NK cell percentages significantly reduced in females consuming papaya in this study was shown to have a negative association with 17β-estradiol. Several conditions have shown that NK cell counts are decreased by estrogen. Peripheral NK cells are reduced during pregnancy (24). *In vivo* application of ethinyl estradiol in transsexual male resulted in significant decrease in percentages of NK cells (25). These studies support our observation and suggest increased estradiol levels in females may have contributed to reduced percentage of NK cells in females.

Other researchers found estrogen replacement therapy in postmenopausal women especially increased B-lymphocyte numbers and decreased pro-inflammatory cytokine production (26, 27). ERβ is upregulated in B cells (23). The increased 17β-estradiol levels and total B cells seen here may be similar responses as reported.

Differentiation markers such as CD45RA, CD69, and CD25 are extensively used in literature but comparison across lymphocyte subpopulations in the system is few. Human naive and memory T cells have been identified by the reciprocal expression

of the CD45RA and CD45RO isoforms. The peripheral blood reportedly, contains a comparable proportion of $CD45RO^+$ and $CD45RA^+$ subsets (28), as was similarly observed here.

CD69 and CD25 are regarded as early and late activation markers, respectively, as an early peak in expression (24 h) of CD69 and a later (48 h) peak in expression of CD25 after *in vitro* phytohemagglutination (PHA) stimulation of CD45RA/CD45RO $CD4^+$ T cell subsets were observed (29). Since then, CD69 expression has been induced *in vitro* on cells of most hematopoietic lineages, including T and B lymphocytes, NK cells, murine macrophages, neutrophils, and eosinophils, while it is constitutively expressed on human monocytes, platelets, and epidermal Langerhans cells (30).

Even though, the specific ligand for CD69 has not been identified and the role of CD69 is currently intensively investigated. CD69-expressing T cells, $CD4^+CD69^+CD25^-$ has been proposed as a novel regulatory cell type defined by TGF-β1 activity (31). The suppressive function of Tregs is dependent on CD69 expression, which forms approximately fifty percent of total $CD4^+CD25^+FoxP3^+$ Tregs in thymus and secondary lymphoid organs (32). Another major function, however, is involvement in immune cell migration. CD69 is expressed at high levels on approximately 10–15% of thymocytes and play a role in selection and maturation processes in the thymus (33). This may explain the small fraction of $CD69^+$ cells ($CD25\pm$) found among naïve ($CD45RA^+$) T helper cells in this study. A study on tissues acquired from deceased organ donors, however, showed $CD45RA^+$ T cells were predominantly CD69 negative, including 100% of naive T cells in blood (28).

Brenchley et al. (34) examined expressions of differentiation markers in activated, proliferated and effector T cells after *in vitro* stimulation. CD25 and CD45RO become expressed on activated cells prior to cell division and maintained thereafter. CD45RA and CD69 were both down-modulated in a cell division dependent fashion, i.e., mitotic dilution, after stimulation. An expansion period for activated naïve T-cells may be more important before acquisition of effector function, after which some differentiate into resting, antigen-experienced T-cells. These results correspond with observations here where CD69 expression was lower in the non-naïve/activated population. This also suggested that activated cells ($CD69^+$) were in the "naïve" populations. Furthermore, migratory role of CD69 has also been demonstrated following activation. In inflamed lymph nodes, CD69 is upregulated on lymphocytes, which control its movements within (35). A small fraction (1–20%) of circulating memory ($CD45RO^+$) cells express CD69 (28). CD69 mediates homing and retention of $CD4^+$ T memory cells in the bone marrow (36).

Expression of CD25 has typically been associated with activated cells. However, we observed a large fraction of $CD25^+$ cells (40–45%) among naïve T helper cells. Using an extensive number of activation, differentiation, and exhaustion markers combined with microarray analysis, Pekalski et al. (37) confirmed existence of a subset of naive $CD4^+CD45RA^+$ T cells that express CD25. The percentage of $CD4^+CD25^+$ naive T cells was strongly associated with increasing age and were also detected in cord blood, indicating that acquisition of CD25 expression by naive CD4 T cells begins prior to birth (25). CD25 induction in naïve cells occurs through TCR signaling which, however, are not strong enough to lead to T cell activation and loss or acquisition of markers characterizing effector or memory cells but is important for expansion in the periphery of a naïve TCR repertoire particularly after the period of thymic involution. These cells respond faster and better to low dose IL-2 compared to their $CD25^-$ counterpart (37). CD25 is also highly expressed on regulatory T cells. It is now apparent that the naive $CD45RA^+$ subpopulation of $CD4^+CD25^{hi}FoxP3^{lo}$ T cells in blood is the most suitable target population for *in vitro* expansion of regulatory T cells (38). These resting Treg cells are induced into activated Tregs to become $CD45RA^-FoxP3^{hi}$ cells, a population also observed in peripheral blood of healthy individuals (39).

$CD25^+$ percentages are higher in non-naïve T helper cells as was seen here. Resting memory T-cells may be $CD25^-$, i.e., late differentiated cells that respond to antigens associated with chronic immune responses. The majority however, are CD25(INT) memory T cells that respond to antigens associated with recall responses, produce a greater array of cytokines, and are less dependent on co-stimulation for effector responses due to their expression of CD25 (40).

Many studies have shown levels of $CD4^+$ T cells are lower in males compared to females [reviewed in Ref. (23)] as was also observed here. We detected strong negative associations between testosterone and CD25-expressing T cells. These were consistent with another report showing suppressive effect of testosterone on $CD25^+CD45RA^+$ and $CD25^+CD45RO^+$ T cells (41). Medical castration reduced testosterone levels and increased $CD4^+CD25^+$ cells (42). Thus, testosterone appeared to target the $CD25^+$ marker. The action of testosterone on the $CD25^+$ cells may be to induce cell death as testosterone is shown to induce apoptosis in T cells (43). We also observed decreased percentages of the major populations of $CD25^+$ cells ($CD4^+CD45RA^+CD69^-CD25^+$/$CD4^+CD45RA^+CD69^-CD25^+$) in males (but not in females) as the majority of male subjects maintained/increased testosterone levels after papaya consumption.

In reverse, $CD25^-$ cells, both naïve and activated were increased in males (but not females) after papaya consumption. This may be a homeostatic response [discussed in Ref. (44)] in an effort to return T cells to normal levels by inducing proliferation of naïve cells ($CD4^+CD45^+CD69^-CD25^-$), which are dominant and robust having indefinite life span. In the presence of androgen, CD4 T cell differentiation inhibition was also demonstrated by significantly reduced levels of Tbet and IFN-γ (45) possibly mediated through upregulation of $CD4^+CD25^+Foxp3^+$ regulatory T cells (46). In an earlier study, we also demonstrated increased regulatory T cells after papaya consumption (47). The accumulative effect may have been to increase slightly the percentage of $CD4^+$ T cells in males, observed here.

The low expression of CD25 on non-$CD4^+$ lymphocytes is consistent with other reports; immature B and certain NKT subsets may express low levels of CD25. Mature B cells, NK cells, and NKT are absent for CD25 (48). The alpha (CD25) chain is one of three subunits that make up the IL-2 receptor, the other two being beta (CD122), and gamma (gammac) chains. $CD8^+$

T cells preferentially express CD122 and naturally occurring $CD8^+CD122^+$ T cells maintain T cell homeostasis as well as Treg function. Murine $CD8^+CD122^+$ Tregs carry CD122 or IL-2Rβ, but not CD25, while $CD4^+CD25^+$ Tregs do not express CD122, although both subsets of Tregs are $CD44^{high}$, $CD62L^{high}$ and mostly CD127-negative [reviewed in Ref. (49)].

Consumption of papaya in general induced a suppressive effect on $CD69^+$ cells, particularly $CD4^+$ T helper cells as well as the naïve non-$CD4^+$ lymphocytes. The potential of fruits to inhibit CD69 expression has been shown in the *in vitro* administration of auraptene, a citrus fruit-derived coumarin (50) and cactus pear fruit extract (51) on activated lymphocytes.

However, this effect was not similarly observed in activated non-$CD4^+$ lymphocytes. Individual responses were heterogeneous and mean percentage was, in reverse, slightly increased after papaya consumption. Negative correlations were generally observed between $CD69^+$ subpopulations with testosterone in males and progesterone in females. In fact, the negative correlation with this activated non-$CD4^+$ lymphocyte was the strongest in females. The selective nature of progesterone is in concordance with reported evidence of progesterone suppression of uterine natural killer (NK) cells in human and spleen cells in mice expressing CD69 (52). In females, this population of cells appeared to be responsive to the effect of progesterone and resulted in reduced percentages in subjects increased for progesterone. Since not all subjects increased progesterone levels, overall effect of suppression was not significant. More importantly, in subjects with low levels of progesterone, percentages of these cells increased and were not affected by the presumed suppressive effect of papaya. In females, non-naïve $CD69^+$non-$CD4^+$ lymphocytes formed a significantly larger population compared to males. This may also be due to the majority of females being in the follicular phase of the menstrual cycle with relatively lower levels of progesterone. We were unable to locate literature studying effect of testosterone on $CD69^+$ lymphocytes.

The significantly increased NK cell degranulation ($CD107a^+$) in males after papaya consumption appeared to be unaffected by sex hormone changes. Other studies strengthen this observation. NK cell activity of peripheral mononuclear cells against target K562 cells measured by the 51Cr release assay did not differ between patients with idiopathic hypogonadotropic hypogonadism (with significantly lower mean plasma testosterone) and healthy adults. Most importantly, this activity did not change during hormonal treatment, which normalized plasma testosterone levels in the patients (53). NK cells from normal donors exhibiting K562 lysis are shown to be $CD56^+CD69^-$. $CD56^+CD69^+$ cells did not significantly increase cytotoxicity even though PMA stimulation increases CD69 expression on NK cells (54). Thus, CD107a NK cells may be CD69 negative. In an earlier study, we also observed significantly increased percentages of NK cells in males (but not females) after *in vitro* PHA activation. The lower response in females did not appear to involve $CD69^+$ cells, indirectly confirming no sex hormone effects in the *in vitro* study (55).

In females, increased NK degranulation activity was only observed when progesterone levels were also increased in subjects after papaya consumption. *In vitro*, no effect of progesterone on NK activity was demonstrated but women on oral contraceptive and fertile females in the luteal phase of the cycle have lower NK cell activity than males or post-menopausal women. During the follicular phase, these differences were not apparent. However, the effect may be either from estrogen or progesterone [reviewed in Ref. (56)]. Our results differ from reported evidence, as we observed progesterone may have stimulatory effect on NK activity. Thus, this remains controversial but the action of hormones may be dependent on status of cell activation.

Fruit extracts have been shown to modulate the immune system significantly even within a day of treatment (11, 13). Nevertheless, studies with long term treatments, e.g., of 33 days (12) to 70 days (10) also provide similar evidence of immunomodulatory responses. Thus, regular supplementation may continually induce an immune-related change. However, whether this is a desired change will be dependent on the purported outcome.

The inability to elicit similar sex hormonal changes in all subjects resulting in heterogeneous responses may be due to individual variability, insufficient stimulation with 2 days exposure or observations were just random changes to the physiological environment. However, the inclusion of the sex hormone markers in this study has clarified many dimorphism seen in immune responses that would not have been otherwise understood.

CONCLUSION

The vast knowledge available on the immune system allowed us to better interpret complex changes from normal exposures. The short-term papaya consumption experiment revealed sexual dimorphic changes in the immune system. Both stimulatory and suppressive effects were observed in lymphocyte subsets of healthy individuals after papaya consumption. Stimulation of $CD4^+$ T cell percentages and NK cell activity in males suggest a beneficial potential from papaya consumption in this subset of individuals. Increased B cell percentages and reduced percentages of NK cells are characteristics of the female immune profile. It is not clear if "exacerbation" of these situations with papaya consumption may not be advantageous. Similarly, decreased naïve non-$CD4^+$ lymphocytes seen in females may not be desirable. This study also revealed endocrine–immune system interactions, in particular, the possible suppressive effect of testosterone on CD25. Furthermore, low progesterone levels, e.g., during the follicular phase appeared to promote activated $CD69^+$ non-$CD4^+$ lymphocytes but led to non-responsiveness in NK degranulation inducible by external factors such as papaya consumption, as observed here.

Due to a spectrum in expression of these markers across normal individuals, an overlap of phenotypes did occur between sexes, thus no strict "sex-labeled" boundaries existed. However, sex-biased responses were still distinguishable and sex hormone levels were able to provide a guide. The ability to measure immune response *in vivo* fulfills an important facet in the overall evaluation of immune health. The limitations of this study were the short supplementation period and the small number of samples analyzed. A larger number of subjects and a longer period of supplementation will be required to confirm these results.

ETHICS STATEMENT

This study was approved by Medical Research Ethics Committee, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia. All procedures complied with the principles of the Declaration of Helsinki. Informed consents were obtained.

AUTHOR CONTRIBUTIONS

MA, ZS, RJ, and WK contributed to the conception and design of the study. NJ, CY, and MA contributed to acquisition of data, analysis, and interpretation of data. MA and NJ drafted the article and revised it critically for important intellectual content. All authors approved the final the version to be submitted.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.00680/full#supplementary-material.

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