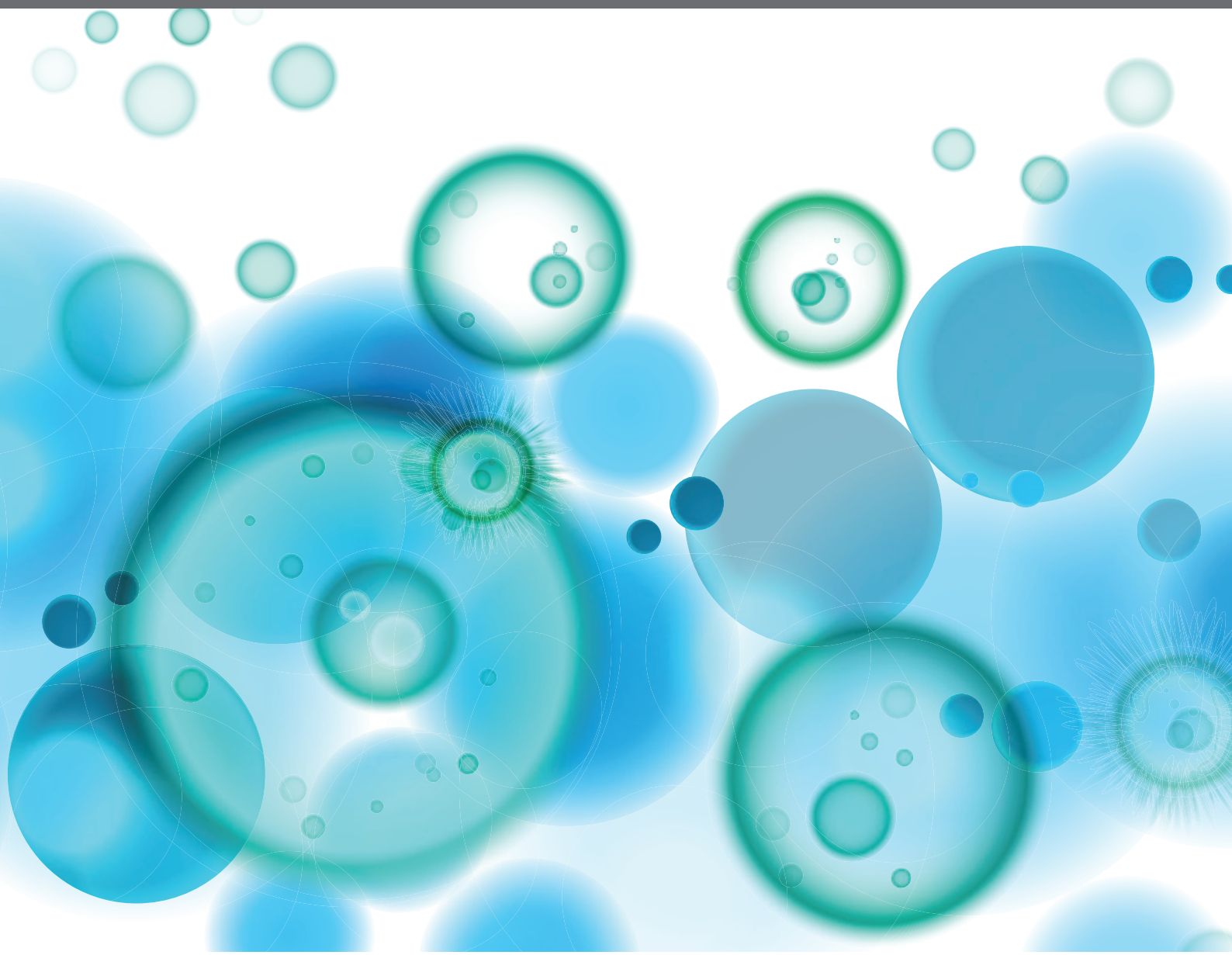
GUT MICROBIOTA-BRAIN AXIS IN ENTERIC AND CENTRAL NEURONAL FUNCTIONS IN HEALTH AND NEUROPSYCHIATRIC DISORDERS

EDITED BY: Francesca Ronchi, Raffaella Gozzelino and Lloyd Kasper
PUBLISHED IN: Frontiers in Immunology



frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88976-439-6
DOI 10.3389/978-2-88976-439-6

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: frontiersin.org/about/contact

GUT MICROBIOTA-BRAIN AXIS IN ENTERIC AND CENTRAL NEURONAL FUNCTIONS IN HEALTH AND NEUROPSYCHIATRIC DISORDERS

Topic Editors:
Francesca Ronchi, University of Bern, Switzerland
Raffaella Gozzelino, Technical University of Atlantic, Cape Verde
Lloyd Kasper, Dartmouth College, United States

Topic Editor Dr. Kasper is co-founder of Symbiotix Biotherapeutics. The other Topic Editors declare no competing interests with regards to the Research Topic theme.

Citation: Ronchi, F., Gozzelino, R., Kasper, L., eds. (2022). Gut Microbiota-Brain Axis in Enteric and Central Neuronal Functions in Health and Neuropsychiatric Disorders. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88976-439-6

Table of Contents

frontiers in Immunology

REVIEW
published: 19 October 2020
doi: 10.3389/fimmu.2020.510113

A Gut Feeling: The Importance of the Intestinal Microbiota in Psychiatric Disorders

Javier Ochoa-Repáraz[1]*, *Christina C. Ramelow*[1] and *Lloyd H. Kasper*[2]

[1] Department of Biology, Eastern Washington University, Cheney, WA, United States, [2] Department of Microbiology and Immunology, Geisel School of Medicine at Dartmouth College, Hanover, NH, United States

The intestinal microbiota constitutes a complex ecosystem in constant reciprocal interactions with the immune, neuroendocrine, and neural systems of the host. Recent molecular technological advances allow for the exploration of this living organ and better facilitates our understanding of the biological importance of intestinal microbes in health and disease. Clinical and experimental studies demonstrate that intestinal microbes may be intimately involved in the progression of diseases of the central nervous system (CNS), including those of affective and psychiatric nature. Gut microbes regulate neuroinflammatory processes, play a role in balancing the concentrations of neurotransmitters and could provide beneficial effects against neurodegeneration. In this review, we explore some of these reciprocal interactions between gut microbes and the CNS during experimental disease and suggest that therapeutic approaches impacting the gut-brain axis may represent the next avenue for the treatment of psychiatric disorders.

Keywords: microbiome, neurological disorders, gut/brain axis, neuroinflammation, immunoregulation

OPEN ACCESS

Edited by:
Scott S. Zamvil, University of California, San Francisco, United States

Reviewed by:
Anne-Katrin Pröbstel, University Hospital of Basel, Switzerland
Maria Grazia Cifone, University of L'Aquila, Italy

***Correspondence:**
Javier Ochoa-Repáraz
jochoareparaz@ewu.edu

Specialty section:
This article was submitted to Multiple Sclerosis and Neuroimmunology, a section of the journal Frontiers in Immunology

Received: *02 June 2020*
Accepted: *30 September 2020*
Published: *19 October 2020*

Citation:
Ochoa-Repáraz J, Ramelow CC and Kasper LH (2020) A Gut Feeling: The Importance of the Intestinal Microbiota in Psychiatric Disorders. Front. Immunol. 11:510113. doi: 10.3389/fimmu.2020.510113

INTRODUCTION

Charles Darwin kept a diary where he would annotate feelings and symptoms, often describing his trouble with the gastrointestinal (GI) tract and anxiety (1). In one of his letters to his medical advisors, he noted the "nervousness" when his wife Emma would depart that would trigger "intensely acid, slimy (sometimes bitter) vomit". He also wrote in The Expression of the Emotions in Man and Animals (1872): "The manner in which the secretions of the alimentary canal and certain other organs … are affected by strong emotions, is another excellent instance of the direct action of the sensorium on these organs, independently of the will or of any serviceable associated habit" (2), as was recently discussed in the context of the gut/brain axis (3), the topic of this review article.

Is there a scientific basis for the adage, "my gut tells me?" Reading Darwin's notes, one would consider that emotions and GI tract functions are directly connected. As the most recent works demonstrate, the intestinal tract is home for a heterogeneous microbial ecosystem dominated by bacteria but also comprised of viruses, archaea, and other eukaryotic microorganisms. Although the existence of this complex intestinal ecosystem was first alluded to by the Nobel laureate Eli Mechnikov over a century ago, the biological relevance of the large variety of microbial species that colonize our intestines soon after birth has been understudied and certainly unappreciated. With

increasing attention worldwide, the potential impact that the gut microbiome has on human health and disease has now begun to be understood.

Multiple factors affect the composition of the gut microbiome, diet being possibly the most prominent (4). Other environmental exposures such as pollution, antibiotic over-usage, lifestyle habits, vitamin D skin exposure, and a range of other factors such as host genetic composition have been shown to promote changes in the intestinal microbiome (5). Because of microbiome and host's multifactorial interactions, these changes can lead to significant functional changes on the immune, metabolic or neuroendocrine systems, that could potentially result in disease. Dysbiosis, the mechanism of disease triggered by the microbial imbalance of normal gut microbial colonization, may be the source for a wide range of human conditions (6). Increasing number of reports now show that alterations of the gut microbiome, in some cases observed at the lowest taxonomic levels, are detected in stool samples obtained from patients suffering from metabolic (7–9) and autoimmune diseases, including those affecting the CNS, such as multiple sclerosis (MS) (10–15) or neuromyelitis optica (NMO) (16, 17). **Figure 1** summarizes some of the proposed mechanisms for the reciprocal interactions that occur between the intestinal microbiota and the brain. In this review, we discuss some of the most recent findings that suggest the multifactorial nature of the gut/brain axis in the context of neurological diseases; a topic that has been reviewed in recent years by other authors [for a recent review: (18)]. The manuscript also highlights some key components of the interface between the gut microbiota and the CNS of multiple sclerosis (MS) patients (19). Our review extends the discussions by providing some potential novel avenues for treating neuropsychiatric diseases based on the conditions' neuroinflammatory components and the influential role of the gut microbiota regulating inflammatory processes.

GUT MICROBES AND MICROBIAL PRODUCTS AS THERAPEUTICS AGAINST NEUROINFLAMMATION

The intestinal epithelium is a single layer of different cell types that promote communication between the host and the intestinal lumen. Enterocytes serve as absorptive cells that line the epithelium that play a critical role in digestion through the uptake of water, ions, and nutrients. Goblet cells are known for

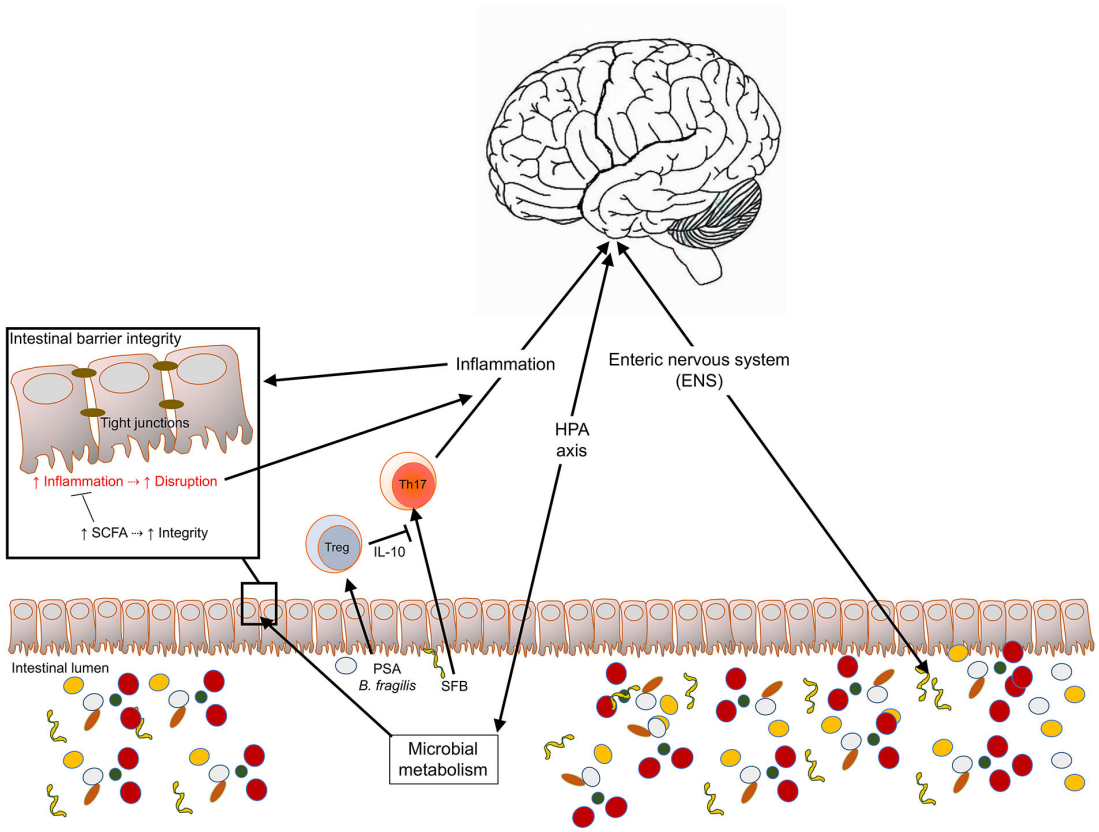


FIGURE 1 | Multifactorial interactions occurring between the intestinal microbiota and products and the CNS. The CNS and the gut microbiota are connected reciprocally through the hypothalamic-pituitary-adrenal (HPA) axis, the immune system, and the neuronal system. These interactions have critical impacts on immune homeostasis, intestinal barrier permeability and the integrity of the blood brain barrier and can regulate CNS inflammation and disease.

producing mucin proteins that provide a protective layer from the lumen and facilitates the exchange of molecules between the epithelium and luminal environment (20). At the base of the crypts of Lieberkün, Paneth cells secrete antimicrobial peptides and proteins *via* the release of granules (21). In addition, enteroendocrine cells produce neuroendocrine molecules (i.e., gut hormones) that modulate physiological processes within and outside the gut (22). Within the Peyer's patches, microfold cells (M-cells) surveil the intestinal lumen sampling microbes and microbial components and aid in transporting bacteria to gut-associated lymphoid tissues (GALT) (23). The GALT is the reservoir for almost two-thirds of the total lymphocyte populations within humans and other mammals (24, 25). Because the gut lumen harbors as many as 1 x 10^{13} bacteria as well as a wide range of possible pathogenic species, the presence of vigorous and effective immune cells is imperative for our well-being. As a result, the gut microbiota is an essential regulator of the immune system's function and the nervous system and may be critical in the control of neuroinflammation (26). The distal small intestine is a reservoir for immune cells expressing and secreting the proinflammatory cytokine interleukin 17 (IL-17). T helper (Th) 17 (Th17) cells are essential in controlling extracellular pathogens and orchestrating neutrophil infiltration; however, they also promote tissue inflammation mediated by IL-23 (27, 28). Intestinal bacteria have been identified as an essential trigger for the differentiation, upon activation, of naïve Th cells into Th17 cells by activating the Retinoic-acid-receptor-related orphan nuclear receptor gamma (ROR-γt) master regulator and the production of proinflammatory cytokines of the IL-17 cytokine superfamily such, IL-22, or granulocyte-macrophage colony-stimulating factor (GM-CSF), among others. Th17 are a necessary adaptive immune cell population against extracellular pathogens by their ability to recruit neutrophils to infection sites. Significant roles for Th17 cells have been described for several autoimmune conditions, including human MS (29), in response to the microbiota (30). Th17 are critical in the induction of many experimental models of human diseases such as experimental autoimmune encephalomyelitis (EAE), the experimental model of multiple sclerosis and other disease models such as rheumatoid arthritis and experimental uveitis (31).

Among the microbes capable of initiating a Th17 profile, the most widely studied is the *Segmented filamentous bacterium* (SFB). SFB act as commensal symbionts by inducing Th17 cells in the small intestines (32, 33) and other immune compartments where autoimmune inflammation occurs (34, 35). During "leaky gut", when the intestinal barrier is compromised, microbial products disseminate systemically, leading to activation of the IL-23 inflammatory pathway promoting Th17 responses and barrier repair (36). Leaky gut may be caused in part by specific metabolic, toxic product (fragylysin) that is produced by gut commensal bacteria, in particular, *Bacteroides fragilis* (37, 38). Fragylysin produced by enterotoxigenic strains *B. fragilis* is a zinc metalloproteinase with effects in the intestinal barrier integrity as it functions as an endotoxin, promoting sepsis in mice (37). The cleavage of E-cadherin by fragylysin was shown to be the underlying mechanism triggering leaky gut but pathogenic *B. fragilis*. As the lipid A present in LPS produced by other Gram-negative bacteria, fragylysin promotes strong pro-inflammatory responses that can result in neuroinflammatory processes and associated with CNS diseases, such as Alzheimer's disease (39–41).

Alternatively, the gut microbiome is involved in the critical role of immune homeostatic balance, *via* the promotion of Th cells that induce peripheral T regulatory (iTregs) expressing the transcription factor forkhead box P3 (Foxp3). Tregs produce various antiinflammatory cytokines including IL-10 and Transforming growth factor-beta (TGF-β), and are recognized for their ability to control inflammatory cell subsets' proliferation. Tregs appear to be critical in both the protective response in experimental EAE and dysfunctional in those with MS patients with reduced suppressive potency (42) and frequencies in the peripheral blood obtained from MS patients, as well as in Alzheimer's disease patients (43). Several bacterial taxa promote the expansion of iTreg populations. Polysaccharide A (PSA)-producing *B. fragilis* (44), Lactobacilli (45), specific clusters of Clostridia (46), or *Prevotella histicola* (47), among others, expand iTregs and have been associated with neuroprotection in experimental models of disease (47–50).

One key mechanism of protection observed in different CNS disease models is the production of interleukin 10 (IL-10). IL-10 is an antiinflammatory cytokine member of the class II superfamily found as a homodimer produced by monocytes, NK cells, B cells, and different subsets of activated T cells, including Tregs. Tregs induced in response to intestinal microbes may contribute significantly to the control of CNS inflammatory diseases. Experimentally, much of the work done to elucidate the microbiota's impact on IL-10-mediated control of CNS inflammation is in experimental autoimmune encephalomyelitis (EAE) in mice or rats. EAE is induced either passively by the adoptive transfer of autoreactive T cells or actively by subcutaneous injection of self-antigens. The induction of disease disrupts peripheral tolerance, which results in the proliferation of self-antigen-specific T cells that differentiate into effector, pathogenic Th1 and Th17 cells within the secondary lymphoid tissues. From there, Th1 and Th17 cells circulate through lymphatics to the bloodstream. T cell activation increases the expression of surface integrins that allow immune driven cells to cross the blood-brain barrier into the CNS parenchyma. In the parenchyma, autoreactive, pathogenic T cells are reactivated by resident antigen-presenting cells, activation that results in exacerbated production of proinflammatory cytokines. Other cells, such as monocytes, neutrophils, dendritic cells, and resident glial cells, contribute to the pathogenesis and progression of the disease by mechanisms yet to be elucidated entirely (51). Germ-free mice exhibit reduced disease severity that is reversed upon colonization with SFB (34).

In CNS inflammatory processes, such as those observed in patients suffering from multiple sclerosis (MS), the suppressive capacity of IL-10 has been associated with dysbiosis (14, 15). Experimentally, IL-10 elicited by regulatory cells upon exposure to intestinal microbes such as *B. fragilis* PSA promotes protective

responses against neuroinflammation (49, 50, 52). The induction of IL-10 by PSA has been also shown in human *in vitro* systems in mechanisms dependent on antigen presentation by dendritic cells and induction of Tregs (53), in CNS autoimmunity (54). More recently, the protective effects of IL-10 induced in response to the oral treatment with PSA were confirmed against viral encephalitis (55). In their work, Ramakrishna and colleagues show that the polysaccharide is protective against Herpes Virus Encephalitis (HVE) mediated neuroinflammation induced in mice through IL-10 produced by $CD4^+$ T cells with a regulatory phenotype ($ICOS^+CD39^+CD37^+$), $CD37^+CD8^+$ T cells, and IL-10-producing B cells. PSA's protective effects were observed after early treatment against neuroinflammation and were also observed in HVE mice treated orally with PSA-producing *B. fragilis* (55). An accumulation of IL-10-producing regulatory cells was observed in the cervical lymph nodes of treated mice when compared with PBS-treated sham controls (55). Rojas et al. found that commensal-reactive immunoglobin A (IgA)-producing plasmablasts and/or plasma cells are reduced in EAE mice's gut and act as an essential source of IL-10, suggesting that IL-10 expression is required to attenuate EAE symptoms (56). Further, the study demonstrates that IgA-producing B cells in the gut can enter the periphery and then infiltrate inflamed CNS tissues, providing evidence for the reduction in IgA targeted fecal bacteria during MS relapses (56).

The experimental evidence gathered in recent years using various animal models of CNS disease highlight the importance of regulating the immune responses to gut microbes. Understanding the balance of effector Th cells, such as Th17, Th1 cells and Tregs, and regulatory B cells, during homeostatic and immune responses in the intestinal microbiome may shed light on psychiatric disorders.

CLINICAL EVIDENCE THAT SUGGEST THE VALUE OF EXPLORING THE MICROBIOME IN THE CONTEXT OF PSYCHIATRIC DISORDERS

The apparent rise in mental health concerns constitutes a broad socioeconomic and clinical problem in developed and underdeveloped countries (57). The impact of neurological disorders affecting mood and behavior impacts interpersonal relationships. Although these diseases' etiological nature is mostly unknown, it is appreciated the CNS of those patients suffering from a psychiatric disease have both neurological alterations and an inflammatory component that may trigger or exacerbate these conditions (58).

Among the broad list of symptoms that characterize psychiatric disorders, gastrointestinal tract dysfunction is not uncommon. The enteric nervous system (ENS), as a fundamental part of the autonomic nervous system, plays a crucial role in human physiology by regulating the GI's autonomous actions. Although the autonomous nervous system innervates it, the ENS can function independently using afferent and efferent neurons and interneurons, forming what is known as the second brain. The ENS is critical to regulating peristalsis, enzyme production, and the synthesis of neurotransmitters such as serotonin, dopamine, or acetylcholine, among many others (59). In turn, these physical and chemical factors serve as modulators for microbial growth: for instance, peristaltic movements regulate nutrient flow through the GI tract, influence the nutrient and oxygen availability for gut microbes.

Notably, the ENS can directly communicate with the CNS through the parasympathetic and sympathetic nervous systems. Recent studies show that enteroendocrine cells of the gut epithelium secrete hormones and communicate with the brain through electrochemical signals. By having axon-like basal formations that contain neurofilaments (60, 61), enteroendocrine cells through synapses and the secretion of glutamate, connect with vagal nerve cells and stimulate nutrients to the brain (62). More recent work demonstrates that the system is associated with dopamine activity and reward circuits in rats linking the gut vagal nerves with the striatum in the brain (63). The neuroendocrine system is another mechanism for reciprocal control of the gut-brain axis, including the hypothalamic-pituitary-adrenal (HPA) axis. Through the HPA axis, environmental factors such as stress regulate intestinal physiology, the immune system, and metabolic system (64). Human cortisol produced in response to the HPA axis activation is a major modulator of the intestinal microbiota and a regulator of intestinal permeability (65). In turn, inflammation and imbalanced levels of neurotransmitters have been proposed as inhibitors of the negative regulation that controls the release of cortisol (66).

Co-morbidity of functional gastrointestinal disorders is more frequent in patients that suffer from psychiatric disorders than those considered mentally healthy (67). Inflammatory bowel syndrome (IBS) and inflammatory bowel diseases (IBD) have been linked to mental health disorders over the last decades, as evidenced in a review discussing ten published case-control studies conducted by Shah and colleagues indicating a higher prevalence of IBS and ulcerative colitis (UC) in patients suffering from anxiety or depression than in control individuals (68). Moreover, the study showed that the association between the mental disorder and the GI dysfunction appears to be reciprocal since the severity of the mood diseases' symptoms increases in IBS and UC patients (31). The review highlights a fundamental question that remains to be answered. Are GI diseases cause or consequence of the bidirectional interaction between the brain and the gut?

Another symptom associated with mental health disorders that suggest a reciprocal interaction between the brain and the gut is functional constipation (69, 70). The reduction in the flow of fecal content within the gut impacts significantly the environmental conditions that directly affect microbial growth and could affect the composition of the gut microbiota. As a result, the CNS pathological process would determine the proportions of aerobes versus anaerobes or the relative abundance of microbes based on nutritional requirements within the large intestine that may be considered a vital

constipation factor. The interactions with the gut epithelium and underlying immune system could then affect inflammatory processes, including those within the CNS. Furthermore, the microbiota composition changes could modify the composition of metabolites, including short-chain fatty acids (SCFAs) and neurotransmitter consumption and production, and others. The link between constipation and psychiatric diseases could be another instance of the bidirectional association between the CNS and the gut, as previously hypothesized by us and others (71). Using the EAE model of inflammatory demyelination, our group demonstrated the reciprocal interactions in the context of the gut/brain axis and disease (72). While EAE induction altered the composition of the gut microbiota at the early stages of the disease, alterations that correlated with disease severity levels, changes in the microbiota with a high dose of a mixture of a broad spectrum of antibiotics resulted in reductions in the severity of EAE (72). Others had previously shown that the disease induction increased intestinal permeability and proinflammatory responses in the gut (73). Increased intestinal permeability, often referred to as leaky gut, can be reversed with the probiotic treatments (74), and in the context of disease through fecal transplantation (75). Although the mechanisms linking disease and intestinal permeability remain elusive, it has been demonstrated that gut dysbiosis promotes leaky gut (76). In turn, a commensal microbe such as *B. fragilis* can secrete neurotoxic (fragilysin) that can lead to a biologically "leaky gut" (38). An increased serum IgG and IgA levels produced in response to LPS were observed in the context of depression, when 112 diagnosed patients were compared with 28 healthy individuals suggest the translocation of microbial-derived metabolites from the intestinal lumen to circulation, enhanced in the context of mental diseases (77). These findings suggest, once again, the multifactorial nature of the changes that occur in the intestinal ecosystem in health, but more intriguingly, during CNS disease.

Although the experimental findings suggest an essential association between GI dysfunction and brain diseases, the clinical evidence has not yet been solidified. One significant pitfall that case-control studies face when comparing the microbiota of patients of psychiatric disorders with healthy controls is precisely determining a healthy individual for comparison. Considering that inflammation and neuroinflammation might be considered an essential modulating or even triggering factor for mental health diseases, identifying mentally healthy individuals constitutes a significant challenge due to our constant exposure to inflammatory environmental and internal factors that could potentially promote changes in the microbiota. Additionally, the permanent exposure to confounding factors such as pollution, stress, diet, and many others, result in major difficulties grouping individuals when inclusion and exclusion parameters are considered, such as age, sex, pharmacologic therapies or use of antibiotics in the studies. Nevertheless, despite limitations such as sample sizes, the clinical data gathered to date suggests a link between mental health diseases and microbiota. A large study involving 871 mothers and children performed by interview and questionnaire evaluated the impact of using antibiotics in children during the first year of age and from year one to three and a half, and their neurocognitive outcomes (78). Approximately 70% of the children used antibiotics during the time-period studied. Intelligence scores obtained at ages 3 ½, 7, and 11 indicated an increased rate of behavioral difficulties and depression-related symptoms in those receiving antibiotic treatment (78). The results obtained in this study imply that early disruption of the intestinal microbiota could result in neurological disorders associated with psychiatric conditions. This bidirectional effect of dysbiosis on the gut-brain axis has been assessed in several affective and psychiatric disorders, as discussed below.

Autism Spectrum Disorder

Autism affects mental development that impacts communication and behavioral patterns, which may appear first in children aged two or younger. As noted in the Diagnostic and Statistical Manual of Mental Disorders and derived works, the patient has "difficulty with communication and interaction with other people, restricted interests and repetitive behaviors, and symptoms that hurt the person's ability to function properly in school, work, and other areas of life" (79). Although genetics is a risk factor, the causes of the disorder remain unknown. Evidence suggests that germ-free mice have social and repetitive behavior deficits, indicating that the gut microbiota composition is required for social development (80, 81). Environmental triggers, including changes in the intestinal microbiota, are currently the focus of investigation (82). As discussed previously with mental disorders, ASD has a known association with GI dysfunction (83).

Recent studies in experimental ASD support a link with the gut microbiota (84), and microbial products of intestinal fermentation such as SCFA (85, 86), maternal diet (87), and immune dysfunction (88). Colonization with *B. fragilis* discussed earlier, corrects symptoms of maternal immune activation (MIA) used as a model for ADS by restoring intestinal integrity and re-balancing the composition of the dysbiotic following MIA induction (84). The gut/brain link in ASD is also strongly supported by findings using a genetic model of the disease that suggest the importance of the microbiota regulating the disease, as results showed that in the diseased animals, a reduction in *Bifidobacterium* and *Blautia* species impacted tryptophan and bile-acids metabolism that in turn resulted in increased intestinal permeability and exacerbated ASD behavioral symptoms (89). Moreover, the treatment of ASD mice with *Lactobacillus reuteri* reversed social deficits in a mechanism controlled by the vagus nerve's stimulation and depended on the expression of oxytocin receptors (90).

While experimental models of the disorders suggest the existence of the link between the microbiota and disease, clinically mixed results support the possible crosstalk between the intestinal microbiota and ASD. Some of those studies reported different taxonomical profiles in the microbiota of ASD patients compared with non-ASD children (75, 76, 79, 82–84), while others did not support the association when comparing the microbiota of ASD children with siblings sharing environment (91, 92). The presence of *Clostridium perfringens* was also used to link ASD with the microbiota. In children with ASD, 49 isolates of *C. perfringens* were detected (29 children), versus 30 that were isolated from 17

healthy individuals and 32 detected in 24 obese children (93). Moreover, the *cpb2* gene expression that encodes *C. perfringens*' Beta2 toxin was significantly enhanced in ASD children (93). Despite the discrepancies, reduced alpha diversity of the microbiota and reduced beta abundance of specific bacterial species have been reported, and as it will be described later, promising results have been obtained in one fecal microbiota transplantation (FMT) pilot project (94). The experimental evidence may also support the use of probiotics or prebiotics. An intervention based on the use of prebiotics and exclusion diet was recently tested in 30 ASD children, resulting in expected microbiota changes, but more importantly, reduced abdominal pain and reduced bowel movement (95). A probiotic intervention reduced plasma myeloperoxidase (MPO) levels, an inflammatory mediator produced by polymorphonuclear leukocytes during oxidative burst, in ASD children (96). These findings could provide further evidence for the link between the control of intestinal inflammation and disease in ASD (96).

Schizophrenia Spectrum Disorders

Schizophrenia is a mental health disorder that impacts the individual's ability to interact with the environment and others. The disease is considered chronic and severe, with symptoms that may arise during puberty or later until about age 30. It appears that an imbalance in the brain's neurotransmitter profiles is present in schizophrenic patients (97). Although family history is a risk for the disease, environmental factors, including viral and parasitic infections, are under scrutiny (98). Neuroinflammation has also been recently proposed to play a role in the pathology of schizophrenic brains (99, 100), including the impact of Th17 cells in 22q11.2 deletion syndrome (22q11.2DS) patients suffering from schizophrenia spectrum disorders (SSD) and psychotic symptoms (101). A positive correlation between IgG levels to dietary wheat gluten and bovine milk casein in serum and cerebrospinal fluid of schizophrenic patients was found (102). In patients suffering from early-stage schizophrenia, the combination of risperidone with the antibiotic minocycline resulted in improved outcomes in Assessment of Negative Symptoms (SANS) and Positive and Negative Syndrome Scale (PANSS), indicating the potential of supplementing the treatments with antibiotics as a new therapeutic avenue for schizophrenia (103).

The importance of the intestinal microbiota regulating immune homeostasis could then be a relevant factor to consider in schizophrenia (104). A recent study examined the composition of the fecal microbiota of 28 first-episode psychosis (FEP) patients, 14 of which were clinically diagnosed with schizophrenia. Results suggested that bacterial numbers, specifically Lactobacilli, *Lachnospiraceae*, *Ruminococcaceae*, and *Bacteroides* spp. were correlated with symptom severity in patients, with predominant bacteria strongly correlated to negative symptoms and poorer function. Moreover, *Lactobacillaceae*, *Halothiobacillaceae*, *Brucellaceae*, and *Micrococcineae* were increased, and *Veillonellaceae* were decreased in FEP patients compared to controls (105). Because FEP patients are vulnerable to relapse, understanding intestinal microbiota's role in immune function may be useful for the development of precluding strategies and novel treatment targets. When comparing serum cytokine profiles of schizophrenic patients to healthy controls, schizophrenic patients displayed elevated serum levels of inflammation, as seen with an overall decrease in the anti-inflammatory IL-2 and an increase in IL-6, IL-8, and TNF-α (106).

Using a rodent model of schizophrenic-like behavior known as subchronic phenylcycline (subPCP) treatment, Jørgensen and colleagues investigated the possible effect of subPCP on the gut microbiota and found slightly significant alterations in the core microbiome of subPCP and vehicle-treated rats. Additionally, differences in microbiota profiles were associated with poor object recognition memory performance, suggesting gut dysbiosis impacts cognition (107). Furthermore, studies suggest that patients with schizophrenia have increased soluble CD14 (102) and increased intestinal permeability (108), demonstrating evidence of bacterial translocation and intestinal inflammation. Another study introducing a probiotic treatment inhibited pro-inflammatory pathways and ameliorated gut dysbiosis caused by yeast (109). These data provide profound evidence that alterations to the gut microbiome may impact immune responses and be an alternative avenue to explore future therapeutic interventions. Mechanistically, a recent study provides further evidence for the hypothesized gut/brain connection in the context of schizophrenia. In mice, the transplantation of fecal content from schizophrenia patients to a murine model of the disease resulted in exacerbated symptoms associated with the disease, such as psychomotor hyperactivity and learning and memory dysfunction (110). Remarkably, the authors of the study highlighted the importance of tryptophan-kynurenine metabolic pathways associated with disease-driving microbiota.

Post-Traumatic Stress Disorder

PTSD is a mental health disease caused by trauma and severe stress, by exposure or witnessing, characterized by symptoms that severely affect negatively social behavior and social interactions. In a study using samples from a South African cohort, although alpha- and beta-diversity of the intestinal microbiota of trauma-exposed control individuals and PTSD patients were not significantly different three phyla were found to be reduced in PTSD stool samples: *Actinobacteria*, *Lentisphaerae*, and *Verrucomicrobia* (111).

Depression

Depression is a debilitating psychiatric illness that affects individuals of any age and significantly impacts the brain, the immune system, and the endocrine system (112). Clinical depression, also known as major depressive disorder (MDD), causes immense feelings of sadness and hopelessness, anxiety, lack of energy, cognitive impairment, and other symptoms. Monoamine neurotransmitters such as serotonin, dopamine, and noradrenaline play a role in mood changes, anxiety, and depression (113). Antidepressants are at the forefront of treatment by increasing levels of these neurotransmitters to reduce depressive symptoms. Because there are limitations to the response on antidepressants (114), other factors may play a role in the disease. Traditionally, depression studies have focused on behavioral, genetic, and neurological pieces of the disease. More recently, environmental factors, synaptic dysfunction, hyperactive

HPA axis, neuroinflammation, and gut dysbiosis have been explored. Elevated serum levels of IL-6, a potent activator of HPA and a pleiotropic inflammatory cytokine, in MDD patients supports that immune homeostasis is involved (115).

Recent findings support that the composition of the gut microbiota of patients with depression is different from that of healthy individuals. A preclinical and clinical study showed that with an orally administered probiotic formulation (*Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175), psychological distress was mitigated, suggesting that the gut microbiota play a role in depression, anxiety, and stress (116). The microbiota of patients with depression displays a low-diversity dysbiosis with elevated levels of Bacteroidetes and Proteobacteria, and lower levels of Firmicutes (29).

Anxiety and Stress

As previously introduced, the HPA axis is a significant regulator of the mammalian physiology. Through the HPA axis, the neuroendocrine system modifies the secretion levels of cortisol (in humans, corticosterone in rodents) in response to stress levels. Stress triggers the secretion of corticotropin-releasing factor (CRF) in the hypothalamus. CRF stimulation of the anterior pituitary gland promotes adrenocorticotropic hormone (ACTH) secretion that stimulates the release of cortisol in the adrenal cortex. The release of cortisol increases with stress for several hours until reaching a serum concentration that triggers a negative feedback response shutting down CRF release in the hypothalamus. Cortisol release depends on factors such as sex and age, but other confounding factors have been identified, such as inflammation, obesity, drugs, and other factors (117), also known to affect the composition of the microbiota. Some of those factors, such as inflammation and neurotransmitter balances are also induced by alterations of the microbiota.

DISCUSSION AND NOVEL THERAPEUTIC DIRECTIONS

The experimental and clinical data summarized above provide increasing evidence for a multifactorial connection between the intestinal microbiota and the CNS in health and disease. However, uncertainties that remain to be addressed. Whether a disease is preceded by changes in the microbiota or alternatively dysbiosis results from ongoing CNS disease is an essential question that requires further exploration to be answered. Furthermore, whether the microbiota is altered differently at different disease stages, periods of relapses and remissions need to be addressed. Exploring this question could provide insights into whether the observed microbial changes' relevance as a biomarker approach to predict clinical outcomes or even responsiveness and unresponsiveness to therapies affecting the microbiome.

The search for most appropriate microbiome-based therapeutic approaches and whether the approach is disease-specific needs further investigation. Targeting the microbiota for the treatment of CNS inflammation has been previously used as minocycline (118). Treatment with minocycline reduced T cell activation, proliferation, and production of inflammatory cytokines (119, 120), reduced microglial activation (121), and reduced axonal loss and neuroprotection in animal models of CNS inflammatory demyelination (122). Interestingly, minocycline inhibits the production of matrix metalloproteinases, mediators of intestinal disruption, or "leaky gut" (123). In EAE, the treatment with minocycline is protective (123–125), and several trials were done to determine the efficacy of the treatment against MS [for a review, (118)]. Different therapeutic approaches are currently being explored experimentally (126); This includes treatment with probiotics, purified microbial compounds, fecal microbiota transplantations (FMT), phage therapies, dietary changes, and dietary habits are being evaluated.

Prebiotics and Probiotics

As noted previously, Eli Metchnikov in the late 1800s suggested that yogurt would be an essential aid in the control of human anxiety. The potential of probiotics was proposed over a century ago in two different clinical studies published in 1909 (127) and 1910 (128). Both studies evaluated the effects of the treatment of melancholia, or depression, with orally administered lactic acid bacteria. Prebiotics, nutritional supplements that favor the growth of proposed beneficial microbes, is now postulated as a novel mechanism to treat behavioral diseases (129). Although probiotics for the treatment of psychiatric disorders have been proposed and discussed (126, 130–134), few studies are sufficiently powered to assess their benefit in humans fully (126). As highlighted in the previous section, a probiotic mixture administered for 30 days to healthy volunteers in a double-blind group comparison with placebo study showed reduced psychological distress quantified by significant reductions in global severity index, somatization, depression, anger-hostility, as well as anxiety-related markers (116). A subsequent addendum to the previous study reported that the administration of the probiotic formulation of *Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175 to 25 subjects with low urinary free cortisol (UFC) levels (less than 50 ng/ml at baseline) and on psychological distress resulted in similar improved parameters as those identified previously (135). *Bifidobacterium longum* 1714 has also been tested in healthy volunteers and reported beneficial effects against depression and increased memory (136). A recent randomized trial performed in 75 children that received either *Lactobacillus rhamnosus* GG or placebo showed promising evidence that early supplementation with probiotics (during the first 6 months of life) could reduce later development of attention deficit hyperactivity disorder and autism spectrum disorder diagnosed at age 13 (137). Although encouraging, the sample size is limited and larger groups are needed to be conclusive, as indicated by the authors of the study (137). Other studies reported beneficial effects of different probiotic formulations against stress (138, 139), while others failed to provide positive outcomes of mental health in healthy individuals (126, 140).

Purified Microbial Components and Metabolites

One observation made clear in light of all recent efforts to characterize the intestinal microbiota taxonomically and functionally is its complexity. The intraindividual and interindividual variability of the microbiota is high and changes over time due to, therapeutic administration, infection, and exposure to many other environmental factors. Moreover, some microbial species within our intestines form intrinsic functional networks that, when disassembled, results in functional loss. Because of that, a general therapeutic approach that does not consider this diversity in taxa and function might not be sufficient to promote protective effects against a given condition or stage of the disease. A possible alternative would be selecting a known microbial compound, expressed or produced and secreted, capable of inducing a response with a defined mechanism of action against neuroinflammation and neurodegeneration. It this scenario, the intestinal microbiota is considered a new source for novel therapeutics (141). For example, PSA produced by *B. fragilis* and purified in the laboratory has shown protective effects after oral treatment against neuroinflammation in different experimental disease models by inducing antiinflammatory IL-10-producing regulatory T and B cells (49, 55, 142). The zwitterionic capsular polysaccharide is taken by antigen-presenting cells, dendritic cells (128), and plasmacytoid dendritic cells (143) through, at least partially, recognition by toll-like receptor 2 (TLR2) (44, 142). Dendritic cells then promote T cells' differentiation with regulatory phenotypes and B cells that produced enhanced levels of IL-10 (44, 49, 55). Although most PSA studies are in experimental models of disease, PSA's immunomodulatory effects have also been described using human cells (53), and more significantly, cells isolated from patients suffering from neuroinflammation such as in MS (54).

SCFA produced by fermenting bacteria in response to complex carbohydrates has also shown compelling evidence for their potential use against neuroinflammation. It has been shown that dietary habits with increased fiber consumption significantly impact the plasma concentration of proinflammatory cytokines (144). As a result of the microbial metabolism, fermented fibers by the intestinal microbiota result in lactate, formate, butyrate, acetate, butyrate, propionate, among others. Although dietary fibers are the main source for SCFA, mucin produced by intestinal goblet cells is also a source (145). The SCFA are recognized by Free Fatty Acid receptors 2, and 3 (FFA-2/3) expressed in many different cells of different systems, including the immune system, playing a role in various metabolic and immune processes (146). The effects of SCFA have also been observed in the microglia's maturation, indicating their importance regulating CNS' function (147). Different mechanisms of action that could lead to protection against neuroinflammation have been described for SCFAs. Butyrate is an essential regulator of the intestinal barrier's integrity, an anatomical fence that, when disrupted, could impact systemic immune homeostasis (148) and promote disease (149). The reconstitution of germ-free mice with fermenting bacteria that produce SCFA restores the integrity of the blood-brain barrier (BBB) that appears reduced in the absence of microbiota (150). Also, SCFA promotes a regulatory phenotype on T cells by their signaling through G protein-coupled receptors (GPCRs), such as GPR109A, expressed in adipocytes and immune cells. SCFA promotes differentiation of colonic Tregs (151–153) through the inhibitor of histone deacetylases enhancing Foxp3 expression when supplemented with TGF-β stimulation. Butyrate stimulates intestinal epithelial cells to produce TGF-β that, at least in part, could promote Treg differentiation (154). The effects of SCFA in inflammation are thus multifactorial, and although anti-inflammatory functions have been described, more profound evaluation is needed to fully understand their impact on systemic inflammation and neuroinflammation (155).

Other microbial metabolites produced in the intestine might constitute novel targets for therapeutic interventions against neuroinflammation. For example, the levels of tryptophan and serotonin levels are impacted in germ-free animals when compared with conventionally-housed counterparts with significant effects on depressive behavior (156). In this study, the investigators evaluated the depressive disorder model in response to acute tryptophan depletion in the brain, which results in low levels of both tryptophan and serotonin, in the absence or presence of intestinal microbiota. When no microbiota is present, the intervention resulted in an enhanced depressive phenotype, suggesting a link between the microbiota and tryptophan homeostasis in the context of psychiatric disorders (156). Brain-derived neurotrophic factor levels are also reduced in germ-free mice and show increased stress responses than conventional animals, a phenotype that is reversed by monocolonization with *Bifidobacterium infantis* (157). The intestinal microbiota could also be a novel source for therapeutics based on the modulation of neurotransmitter metabolism. For instance, both γ-aminobutyric acid (GABA)-producers and consumers are found in the GI tract. One hypothesis would be that dysbiosis could promote an imbalance on GABA producers and consumers' levels, thereby promoting an exacerbation of neurological symptoms (158–160). For instance, reduced serum GABA levels have been found in patients suffering from secondary-progressive MS (161), while exogenous GABA administration results in the protection against CNS inflammatory demyelination in EAE mice (162).

Fecal Microbiota Transplantations and Microbiota Transfer Therapy

One open-label clinical study showed that FMT reduced the symptoms of autism in ASD children between ages 7 and 17 (94). The study showed that in ASD children, the intestinal microbiota diversity was reduced at baseline compared to controls. The study included the pre-FMT treatment of patients with antibiotics for 2 weeks and subsequent doses with microbiota for 10 weeks and a follow-up evaluation 8 weeks after the end of the treatment. The use of vancomycin as a pre-treatment was used to, according to the authors, suppress pathogenic bacteria. The alterations of the intestinal microbiota using antibiotics were

previously shown to promote significant improvement in ASD-associated symptoms. The potential benefit of antibiotics has been demonstrated when vancomycin was used to treat a small cohort of ASD children that showed short term improvement in ASD symptoms (163).

In the study of Kang et al., the transplanted microbiota consisted of a standardized human microbiota preparation previously described for the treatment of recurrent *Clostridium* infections (151). At the end of the treatment, the microbial diversity of ASD children treated with the standardized microbiota increased in 16 out of 18 patients during treatment. Interestingly, two patients remained unresponsive to FMT. The study reported an 80% reduction in GI symptoms that included constipation, diarrhea, or abdominal pain, and improved behavioral ASD symptoms in patients receiving the FMT treatment (94). More recently, Kang and colleagues performed a follow-up study 2 years after the Microbiota Transfer Therapy (MTT) with the same 18 participants and concluded that improvements to GI distress were sustained, and autistic-like behaviors were improved (164). The individuals with ASD also exhibited significant increases in bacterial diversity and relative abundances of *Bifidobacteria* and *Prevotella*, providing evidence for MTT as a potential therapy for children with ASD and GI issues (164). The authors acknowledge the study's limitations in terms of sample size, use of antibiotics and pre-treatment with proton pump inhibitors, and the nature of the comparisons as an open-label analysis, with no placebo control. Other work by Sharon and colleagues found that fecal transplantation from human donors with autism spectrum disorders into germ-free mice promoted hallmark autistic behaviors (165). Nevertheless, the results provided are encouraging and suggest a potential mechanism for the treatment of behavioral disorders.

CONCLUSIONS

Increasing experimental and clinical evidence suggest that the intestinal microbiota and the CNS are connected by a reciprocal gut-brain axis with multifactorial components. Despite the difficulty of understanding the complexity of the interactions between our metabolic, immune, and nervous systems and the microbiota, and between the enormously diverse microbial population of the gut, it is now appreciated the relevance of the interactions for health and disease. Studies in germ-free animals, animals treated with antibiotics, the use of probiotics, monocolonization studies, or fecal transplantation, among other approaches, show that the alteration of the microbiota may result in changes in behavioral outcomes. Many questions remain to be answered regarding the mechanisms of actions by which gut microbes could impact mental health. However, the impact on neuroinflammation appears to be a key route for the regulation of disease. In this context, it is now well established that intestinal microbes, and microbial products, modulate the extend of pro- and anti-inflammatory responses that could exacerbate or reduce the immunopathological parameters associated with the disease. Nevertheless, a more profound analysis is necessary to understand the exact mechanism of control, whether the mechanisms are disease-specific and varies with disease stages, or even if the changes quantified in the composition of the microbiota precede disease are a result of it or co-occur. Despite the limitations of what it is known, the gut/brain axis is a complex but exciting area of investigation that has begun to expose intriguing biological interactions between our body and the microbes that inhabit each of us.

AUTHORS CONTRIBUTIONS

JO-R, CR, and LK contributed equally to the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

Research supported by the National Institute of Neurological Disorders and Stroke (NINDS), of the National Institutes of Health under award number 1R15NS107743.

ACKNOWLEDGMENTS

The authors wish to thank the Department of Biology at Eastern Washington University for their support.

REFERENCES

1. Bergman DJ. *The Dark Side of Charles Darwin*. Green Forest, Ark: New Leaf Publishing Group (2011).
2. Darwin C, Ekman P, Prodger P. *The Expression of the Emotions in Man and Animals, 3rd edn*. London: Harper Collins (1998).
3. Cryan JF, Dinan TG. Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. *Nat Rev Neurosci* (2012) 13:701–12. doi: 10.1038/nrn3346
4. Conlon MA, Bird AR. The Impact of Diet and Lifestyle on Gut Microbiota and Human Health. *Nutrients* (2014) 7:17–44. doi: 10.3390/nu7010017
5. Ochoa-Repáraz J, Kasper LH. Gut microbiome and the risk factors in central nervous system autoimmunity. *FEBS Lett* (2014) 588:4214–22. doi: 10.1016/j.febslet.2014.09.024
6. DeGruttola AK, Low D, Mizoguchi A, Mizoguchi E. Current Understanding of Dysbiosis in Disease in Human and Animal Models. *Inflammation Bowel Dis* (2016) 22:1137–50. doi: 10.1097/MIB.0000000000000750
7. Turnbaugh PJ, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, Ley RE, et al. A core gut microbiome in obese and lean twins. *Nature* (2009) 457:480–4. doi: 10.1038/nature07540
8. Sonnenburg JL, Bäckhed F. Diet-microbiota interactions as moderators of human metabolism. *Nature* (2016) 535:56–64. doi: 10.1038/nature18846
9. Harsch IA, Konturek PC. The Role of Gut Microbiota in Obesity and Type 2 and Type 1 Diabetes Mellitus: New Insights into "Old" Diseases. *Med Sci (Basel)* (2018) 6:32–28. doi: 10.3390/medsci6020032
10. Tremlett H, Fadrosh DW, Faruqi AA, Hart J, Roalstad S, Graves J, et al. Associations between the gut microbiota and host immune markers in pediatric multiple sclerosis and controls. *BMC Neurol* (2016) 16:1–9. doi: 10.1186/s12883-016-0703-3

11. Chen J, Chia N, Kalari KR, Yao JZ, Novotna M, Soldan MMP, et al. Multiple sclerosis patients have a distinct gut microbiota compared to healthy controls. *Sci Rep* (2016) 6:28484. doi: 10.1038/srep28484
12. Jangi S, Gandhi R, Cox LM, Li N, von Glehn F, Yan R, et al. Alterations of the human gut microbiome in multiple sclerosis. *Nat Commun* (2016) 7:12015. doi: 10.1038/ncomms12015
13. Miyake S, Kim S, Suda W, Oshima K, Nakamura M, Matsuoka T, et al. Dysbiosis in the Gut Microbiota of Patients with Multiple Sclerosis, with a Striking Depletion of Species Belonging to Clostridia XIVa and IV Clusters. *PloS One* (2015) 10:e0137429. doi: 10.1371/journal.pone.0137429
14. Berer K, Gerdes LA, Cekanaviciute E, Jia X, Xiao L, Xia Z, et al. Gut microbiota from multiple sclerosis patients enables spontaneous autoimmune encephalomyelitis in mice. *Proc Natl Acad Sci U S A* (2017) 145:201711233–6. doi: 10.1073/pnas.1711233114
15. Cekanaviciute E, Yoo BB, Runia TF, Debelius JW, Singh S, Nelson CA, et al. Gut bacteria from multiple sclerosis patients modulate human T cells and exacerbate symptoms in mouse models. *Proc Natl Acad Sci U S A* (2017) 363:201711235–6. doi: 10.1073/pnas.1711235114
16. Cree BAC, Spencer CM, Varrin-Doyer M, Baranzini SE, Zamvil SS. Gut microbiome analysis in neuromyelitis optica reveals overabundance of Clostridium perfringens. *Ann Neurol* (2016) 80:443–7. doi: 10.1002/ana.24718
17. Zamvil SS, Spencer CM, Baranzini SE, Cree BAC. The Gut Microbiome in Neuromyelitis Optica. *Neurotherapeutics* (2018) 15:92–101. doi: 10.1007/s13311-017-0594-z
18. Cryan JF, O'Riordan KJ, Sandhu K, Peterson V, Dinan TG. The gut microbiome in neurological disorders. *Lancet Neurol* (2020) 19:179–94. doi: 10.1016/S1474-4422(19)30356-4
19. Pröbstel A-K, Baranzini SE. The Role of the Gut Microbiome in Multiple Sclerosis Risk and Progression: Towards Characterization of the "MS Microbiome." *Neurotherapeutics* (2018) 15:126–34. doi: 10.1007/s13311-017-0587-y
20. Johansson MEV, Hansson GC. Immunological aspects of intestinal mucus and mucins. *Nat Rev Immunol* (2016) 16:639–49. doi: 10.1038/nri.2016.88
21. Bevins CL, Salzman NH. Paneth cells, antimicrobial peptides and maintenance of intestinal homeostasis. *Nat Rev Microbiol* (2011) 9:356–68. doi: 10.1038/nrmicro2546
22. Gribble FM, Reimann F. Enteroendocrine Cells: Chemosensors in the Intestinal Epithelium. *Annu Rev Physiol* (2016) 78:277–99. doi: 10.1146/annurev-physiol-021115-105439
23. Dillon A, Lo DD. M Cells: Intelligent Engineering of Mucosal Immune Surveillance. *Front Immunol* (2019) 10:1499. doi: 10.3389/fimmu.2019.01499
24. Suzuki C, Kimoto-Nira H, Kobayashi M, Nomura M, Sasaki K, Mizumachi K. Immunomodulatory and cytotoxic effects of various Lactococcus strains on the murine macrophage cell line J774.1. *Int J Food Microbiol* (2008) 123:159–65. doi: 10.1016/j.ijfoodmicro.2007.12.022
25. Dasgupta S, Kasper DL. Traffic control at the "Gut-GALT crossroads". *Cell Res* (2013) 23:590–1. doi: 10.1038/cr.2013.41
26. Yoo BB, Mazmanian SK. The Enteric Network: Interactions between the Immune and Nervous Systems of the Gut. *Immunity* (2017) 46:910–26. doi: 10.1016/j.immuni.2017.05.011
27. Harrington LE, Hatton RD, Mangan PR, Turner H, Murphy TL, Murphy KM, et al. Interleukin 17–producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol* (2005) 6:1123–32. doi: 10.1038/ni1254
28. Park H, Li Z, Yang XO, Chang SH, Nurieva R, Wang Y-H, et al. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol* (2005) 6:1133–41. doi: 10.1038/ni1261
29. Compston A, Coles A. Multiple sclerosis. *Lancet* (2008) 372:1502–17. doi: 10.1016/S0140-6736(08)61620-7
30. Cosorich I, Dalla-Costa G, Sorini C, Ferrarese R, Messina MJ, Dolpady J, et al. High frequency of intestinal TH17 cells correlates with microbiota alterations and disease activity in multiple sclerosis. *Sci Adv* (2017) 3:e1700492. doi: 10.1126/sciadv.1700492
31. Bedoya SK, Lam B, Lau K, Larkin J3. Th17 Cells in Immunity and Autoimmunity. *Clin Dev Immunol* (2013) 2013:1–16. doi: 10.1155/2013/986789
32. Ivanov II, Atarashi K, Manel N, Brodie EL, Shima T, Karaoz U, et al. Induction of Intestinal Th17 Cells by Segmented Filamentous Bacteria. *Cell* (2009) 139:485–98. doi: 10.1016/j.cell.2009.09.033
33. Gaboriau-Routhiau V, Rakotobe S, Lécuyer E, Mulder I, Lan A, Bridonneau C, et al. The Key Role of Segmented Filamentous Bacteria in the Coordinated Maturation of Gut Helper T Cell Responses. *Immunity* (2009) 31:677–89. doi: 10.1016/j.immuni.2009.08.020
34. Lee YK, Menezes JS, Umesaki Y, Mazmanian SK. Proinflammatory T-cell responses to gut microbiota promote experimental autoimmune encephalomyelitis. *Proc Natl Acad Sci U S A* (2011) 108 Suppl 1:4615–22. doi: 10.1073/pnas.1000082107
35. Wu H-J, Ivanov II, Darce J, Hattori K, Shima T, Umesaki Y, et al. Gut-Residing Segmented Filamentous Bacteria Drive Autoimmune Arthritis via T Helper 17 Cells. *Immunity* (2016) 32:1–13. doi: 10.1016/j.immuni.2010.06.001
36. Shih VF-S, Cox J, Kljavin NM, Dengler HS, Reichelt M, Kumar P, et al. Homeostatic IL-23 receptor signaling limits Th17 response through IL-22-mediated containment of commensal microbiota. *Proc Natl Acad Sci U S A* (2014) 111:13942–7. doi: 10.1073/pnas.1323852111
37. Choi VM, Herrou J, Hecht AL, Teoh WP, Turner JR, Crosson S, et al. Activation of Bacteroides fragilis toxin by a novel bacterial protease contributes to anaerobic sepsis in mice. *Nat Med* (2016) 22:563–7. doi: 10.1038/nm.4077
38. Wu S, Lim K-C, Huang J, Saidi RF, Sears CL. *Bacteroides fragilis* enterotoxin cleaves the zonula adherens protein, E-cadherin. *Proc Natl Acad Sci U S A* (1998) 95:14979–84. doi: 10.1073/pnas.95.25.14979
39. Zhao Y, Lukiw WJ. Microbiome-generated amyloid and potential impact on amyloidogenesis in Alzheimer's disease (AD). *J Nat Sci* (2015) 1(7):e138.
40. Zhao Y, Dua P, Lukiw W. Microbial Sources of Amyloid and Relevance to Amyloidogenesis and Alzheimer's Disease (AD). *J Alzheimers Dis Parkinsonism* (2015) 5:177. doi: 10.4172/2161-0460.1000177
41. Zhan X, Stamova B, Jin L-W, DeCarli C, Phinney B, Sharp FR. Gram-negative bacterial molecules associate with Alzheimer disease pathology. *Neurology* (2016) 87:2324–32. doi: 10.1212/WNL.0000000000003391
42. Fletcher JM, Lonergan R, Costelloe L, Kinsella K, Moran B, O'Farrelly C, et al. CD39+Foxp3+ Regulatory T Cells Suppress Pathogenic Th17 Cells and Are Impaired in Multiple Sclerosis. *J Immunol* (2009) 183:7602–10. doi: 10.4049/jimmunol.0901881
43. Ciccocioppo F, Lanuti P, Pierdomenico L, Simeone P, Bologna G, Ercolino E, et al. The Characterization of Regulatory T-Cell Profiles in Alzheimer's Disease and Multiple Sclerosis. *Sci Rep* (2019) 9:8788. doi: 10.1038/s41598-019-45433-3
44. Round JL, Mazmanian SK. Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota. *Proc Natl Acad Sci U S A* (2009) 107:12204–9. doi: 10.1073/pnas.0909122107
45. Wang K, Dong H, Qi Y, Pei Z, Yi S, Yang X, et al. Lactobacillus casei regulates differentiation of Th17/Treg cells to reduce intestinal inflammation in mice. *Can J Vet Res* (2017) 81:122–8.
46. Atarashi K, Tanoue T, Shima T, Imaoka A, Kuwahara T, Momose Y, et al. Induction of Colonic Regulatory T Cells by Indigenous Clostridium Species. *Science* (2011) 331:337–41. doi: 10.1126/science.1198469
47. Mangalam A, Shahi SK, Luckey D, Karau M, Marietta E, Luo N, et al. Human Gut-Derived Commensal Bacteria Suppress CNS Inflammatory and Demyelinating Disease. *Cell Rep* (2017) 20:1269–77. doi: 10.1016/j.celrep.2017.07.031
48. Shahi SK, Freedman SN, Murra AC, Zarei K, Sompallae R, Gibson-Corley KN, et al. Prevotella histicola, A Human Gut Commensal, Is as Potent as COPAXONE® in an Animal Model of Multiple Sclerosis. *Front Immunol* (2019) 10:462:462. doi: 10.3389/fimmu.2019.00462
49. Ochoa-Repáraz J, Mielcarz DW, Wang Y, Begum-Haque S, Dasgupta S, Kasper DL, et al. A polysaccharide from the human commensal Bacteroides fragilis protects against CNS demyelinating disease. *Mucosal Immunol* (2010) 3:487–95. doi: 10.1038/mi.2010.29
50. Wang Y, Begum-Haque S, Telesford KM, Ochoa-Reparaz J, Christy M, Kasper EJ, et al. A commensal bacterial product elicits and modulates migratory capacity of CD39(+) CD4 T regulatory subsets in the suppression of neuroinflammation. *Gut Microbes* (2014) 5:552–61. doi: 10.4161/gmic.29797

51. Dendrou CA, Fugger L, Friese MA. Immunopathology of multiple sclerosis. *Nat Rev Immunol* (2015) 15:545–58. doi: 10.1038/nri3871
52. Ochoa-Reparaz J, Mielcarz DW, Ditrio LE, Burroughs AR, Begum-Haque S, Dasgupta S, et al. Central nervous system demyelinating disease protection by the human commensal *Bacteroides fragilis* depends on polysaccharide A expression. *J Immunol* (2010) 185:4101–8. doi: 10.4049/jimmunol.1001443
53. Telesford KM, Yan W, Ochoa-Reparaz J, Pant A, Kircher C, Christy MA, et al. A commensal symbiotic factor derived from *Bacteroides fragilis* promotes human CD39(+)Foxp3(+) T cells and Treg function. *Gut Microbes* (2015) 6:234–42. doi: 10.1080/19490976.2015.1056973
54. Burgess JN, Pant AB, Kasper LH, Colpitts Brass S. CD4+ T cells from multiple sclerosis patients respond to a commensal-derived antigen. *Ann Clin Transl Neurol* (2017) 4:825–9. doi: 10.1002/acn3.465
55. Ramakrishna C, Kujawski M, Chu H, Li L, Mazmanian SK, Cantin EM. *Bacteroides fragilis* polysaccharide A induces IL-10 secreting B and T cells that prevent viral encephalitis. *Nat Commun* (2019) 10:2153. doi: 10.1038/s41467-019-09884-6
56. Rojas OL, Pröbstel A-K, Porfilio EA, Wang AA, Charabati M, Sun T, et al. Recirculating Intestinal IgA-Producing Cells Regulate Neuroinflammation via IL-10. *Cell* (2019) 177:492–3. doi: 10.1016/j.cell.2019.03.037
57. Thakur KT, Albanese E, Giannakopoulos P, Jette N, Linde M, Prince MJ, et al. Neurological Disorders. In: V Patel, D Chisholm, T Dua, R Laxminarayan, ME Medina-Mora, editors. *Mental, Neurological, and Substance Use Disorders: Disease Control Priorities, 3rd ed., vol. 4.* Washington (DC): The International Bank for Reconstruction and Development / The World Bank (2016). doi: 10.1596/978-1-4648-0426-7_ch5
58. Schain M, Kreisl WC. Neuroinflammation in Neurodegenerative Disorders-a Review. *Curr Neurol Neurosci Rep* (2017) 17:25. doi: 10.1007/s11910-017-0733-2
59. Martinucci I, Blandizzi C, de Bortoli N, Bellini M, Antonioli L, Tuccori M, et al. Genetics and pharmacogenetics of aminergic transmitter pathways in functional gastrointestinal disorders. *Pharmacogenomics* (2015) 16:523–39. doi: 10.2217/pgs.15.12
60. Bohórquez DV, Shahid RA, Erdmann A, Kreger AM, Wang Y, Calakos N, et al. Neuroepithelial circuit formed by innervation of sensory enteroendocrine cells. *J Clin Invest* (2015) 125:782–6. doi: 10.1172/JCI78361
61. Bohórquez DV, Samsa LA, Roholt A, Medicetty S, Chandra R, Liddle RA. An enteroendocrine cell-enteric glia connection revealed by 3D electron microscopy. *PloS One* (2014) 9:e89881. doi: 10.1371/journal.pone.0089881
62. Kaelberer MM, Buchanan KL, Klein ME, Barth BB, Montoya MM, Shen X, et al. A gut-brain neural circuit for nutrient sensory transduction. *Science* (2018) 361:eaat5236. doi: 10.1126/science.aat5236
63. Han W, Tellez LA, Perkins MH, Perez IO, Qu T, Ferreira J, et al. A Neural Circuit for Gut-Induced Reward. *Cell* (2018) 175:887–8. doi: 10.1016/j.cell.2018.10.018
64. Farzi A, Fröhlich EE, Holzer P. Gut Microbiota and the Neuroendocrine System. *Neurotherapeutics* (2018) 15:5–22. doi: 10.1007/s13311-017-0600-5
65. Kelly JR, Kennedy PJ, Cryan JF, Dinan TG, Clarke G, Hyland NP. Breaking down the barriers: the gut microbiome, intestinal permeability and stress-related psychiatric disorders. *Front Cell Neurosci* (2015) 9:392. doi: 10.3389/fncel.2015.00392
66. Tafet GE, Nemeroff CB. The Links Between Stress and Depression: Psychoneuroendocrinological, Genetic, and Environmental Interactions. *J Neuropsychiatry Clin Neurosci* (2015) 28:77–88. doi: 10.1176/appi.neuropsych.15030053
67. Henningsen P, Zimmermann T, Sattel H. Medically unexplained physical symptoms, anxiety, and depression: a meta-analytic review. *Psychosom Med* (2003) 65:528–33. doi: 10.1097/01.psy.0000075977.90337.e7
68. Shah E, Rezaie A, Riddle M, Pimentel M. Psychological disorders in gastrointestinal disease: epiphenomenon, cause or consequence? *Ann Gastroenterol* (2014) 27:224–30.
69. van Dijk M, Benninga MA, Grootenhuis MA, Last BF. Prevalence and associated clinical characteristics of behavior problems in constipated children. *Pediatrics* (2010) 125:e309–17. doi: 10.1542/peds.2008-3055
70. Hosseinzadeh ST, Poorsaadati S, Radkani B, Forootan M. Psychological disorders in patients with chronic constipation. *Gastroenterol Hepatol Bed Bench* (2011) 4:159–63. doi: 10.1177/1756283X11401773
71. Ochoa-Reparaz J, Magori K, Kasper LH. The chicken or the egg dilemma: intestinal dysbiosis in multiple sclerosis. *Ann Transl Med* (2017) 5:145–5. doi: 10.21037/atm.2017.01.18
72. Colpitts SL, Kasper EJ, Keever A, Liljenberg C, Kirby T, Magori K, et al. A bidirectional association between the gut microbiota and CNS disease in a biphasic murine model of multiple sclerosis. *Gut Microbes* (2017) 8:561–73. doi: 10.1080/19490976.2017.1353843
73. Nouri M, Bredberg A, Weström B, Lavasani S. Intestinal barrier dysfunction develops at the onset of experimental autoimmune encephalomyelitis, and can be induced by adoptive transfer of auto-reactive T cells. *PloS One* (2014) 9:e106335. doi: 10.1371/journal.pone.0106335
74. Kozakova H, Schwarzer M, Tuckova L, Srutkova D, Czarnowska E, Rosiak I, et al. Colonization of germ-free mice with a mixture of three lactobacillus strains enhances the integrity of gut mucosa and ameliorates allergic sensitization. *Cell Mol Immunol* (2015) 13:1–12. doi: 10.1038/cmi.2015.09
75. Kuethe JW, Armocida SM, Midura EF, Rice TC, Hildeman DA, Healy DP, et al. Fecal Microbiota Transplant Restores Mucosal Integrity in a Murine Model of Burn Injury. *Shock* (2016) 45:647–52. doi: 10.1097/SHK.0000000000000551
76. Thevaranjan N, Puchta A, Schulz C, Naidoo A, Szamosi JC, Verschoor CP, et al. Age-Associated Microbial Dysbiosis Promotes Intestinal Permeability, Systemic Inflammation, and Macrophage Dysfunction. *Cell Host Microbe* (2017) 21:455–466.e4. doi: 10.1016/j.chom.2017.03.002
77. Maes M, Kubera M, Leunis J-C, Berk M. Increased IgA and IgM responses against gut commensals in chronic depression: further evidence for increased bacterial translocation or leaky gut. *J Affect Disord* (2012) 141:55–62. doi: 10.1016/j.jad.2012.02.023
78. Slykerman RF, Thompson J, Waldie KE, Murphy R, Wall C, Mitchell EA. Antibiotics in the first year of life and subsequent neurocognitive outcomes. *Acta Paediatr* (2017) 106:87–94. doi: 10.1111/apa.13613
79. Drislane LE, Sellbom M, Brislin SJ, Strickland CM, Christian E, Wygant DB, et al. Improving characterization of psychopathy within the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5), alternative model for personality disorders: Creation and validation of Personality Inventory for DSM-5 Triarchic scales. *Pers Disord* (2019) 10:511–23. doi: 10.1037/per0000345
80. Desbonnet L, Clarke G, Shanahan F, Dinan TG, Cryan JF. Microbiota is essential for social development in the mouse. *Mol Psychiatry* (2014) 19:146–8. doi: 10.1038/mp.2013.65
81. Luczynski P, McVey Neufeld K-A, Oriach CS, Clarke G, Dinan TG, et al. Growing up in a Bubble: Using Germ-Free Animals to Assess the Influence of the Gut Microbiota on Brain and Behavior. *Int J Neuropsychopharmacol* (2016) 19:pyw020. doi: 10.1093/ijnp/pyw020
82. Cryan JF, Wit H. The gut microbiome in psychopharmacology and psychiatry. *Psychopharmacol (Berl)* (2019) 236:1407–9. doi: 10.1007/s00213-019-05288-y. (2019)1–3.
83. Adams JB, Johansen LJ, Powell LD, Quig D, Rubin RA. Gastrointestinal flora and gastrointestinal status in children with autism–comparisons to typical children and correlation with autism severity. *BMC Gastroenterol* (2011) 11:22. doi: 10.1186/1471-230X-11-22
84. Hsiao EY, McBride SW, Hsien S, Sharon G, Hyde ER, McCue T, et al. Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders. *Cell* (2013) 155:1451–63. doi: 10.1016/j.cell.2013.11.024
85. Macfabe DF. Short-chain fatty acid fermentation products of the gut microbiome: implications in autism spectrum disorders. *Microb Ecol Health Dis* (2012) 23:1. doi: 10.3402/mehd.v23i0.19260
86. Liu S, Li E, Sun Z, Fu D, Duan G, Jiang M, et al. Altered gut microbiota and short chain fatty acids in Chinese children with autism spectrum disorder. *Sci Rep* (2019) 9:287. doi: 10.1038/s41598-018-36430-z
87. Buffington SA, Di Prisco GV, Auchtung TA, Ajami NJ, Petrosino JF, Costa-Mattioli M. Microbial Reconstitution Reverses Maternal Diet-Induced Social and Synaptic Deficits in Offspring. *Cell* (2016) 165:1762–75. doi: 10.1016/j.cell.2016.06.001

88. Hsiao EY, McBride SW, Chow J, Mazmanian SK, Patterson PH. Modeling an autism risk factor in mice leads to permanent immune dysregulation. *Proc Natl Acad Sci U S A* (2012) 109:12776–81. doi: 10.1073/pnas.1202556109
89. Golubeva AV, Joyce SA, Moloney G, Burokas A, Sherwin E, Arboleya S, et al. Microbiota-related Changes in Bile Acid & Tryptophan Metabolism are Associated with Gastrointestinal Dysfunction in a Mouse Model of Autism. *EBioMedicine* (2017) 24:166–78. doi: 10.1016/j.ebiom.2017.09.020
90. Sgritta M, Dooling SW, Buffington SA, Momin EN, Francis MB, Britton RA, et al. Mechanisms Underlying Microbial-Mediated Changes in Social Behavior in Mouse Models of Autism Spectrum Disorder. *Neuron* (2019) 101:246–59. doi: 10.1016/j.neuron.2018.11.018
91. Gondalia SV, Palombo EA, Knowles SR, Cox SB, Meyer D, Austin DW. Molecular characterisation of gastrointestinal microbiota of children with autism (with and without gastrointestinal dysfunction) and their neurotypical siblings. *Autism Res* (2012) 5:419–27. doi: 10.1002/aur.1253
92. Son JS, Zheng LJ, Rowehl LM, Tian X, Zhang Y, Zhu W, et al. Comparison of Fecal Microbiota in Children with Autism Spectrum Disorders and Neurotypical Siblings in the Simons Simplex Collection. *PloS One* (2015) 10:e0137725. doi: 10.1371/journal.pone.0137725
93. Góra B, Gofron Z, Grosiak M, Aptekorz M, Kazek B, Kocelak P, et al. Toxin profile of fecal Clostridium perfringens strains isolated from children with autism spectrum disorders. *Anaerobe* (2018) 51:73–7. doi: 10.1016/j.anaerobe.2018.03.005
94. Kang D-W, Adams JB, Gregory AC, Borody T, Chittick L, Fasano A, et al. Microbiota Transfer Therapy alters gut ecosystem and improves gastrointestinal and autism symptoms: an open-label study. *Microbiome* (2017) 5:10–6. doi: 10.1186/s40168-016-0225-7
95. Grimaldi R, Gibson GR, Vulevic J, Giallourou N, Castro-Mejía JL, Hansen LH, et al. A prebiotic intervention study in children with autism spectrum disorders (ASDs). *Microbiome* (2018) 6:133. doi: 10.1186/s40168-018-0523-3
96. Russo AJ. Decreased plasma myeloperoxidase associated with probiotic therapy in autistic children. *Clin Med Insights Pediatr* (2015) 9:13–7. doi: 10.4137/CMPed.S17901
97. Conio B, Martino M, Magioncalda P, Escelsior A, Inglese M, Amore M, et al. Opposite effects of dopamine and serotonin on resting-state networks: review and implications for psychiatric disorders. *Mol Psychiatry* (2019) 25:82–93. doi: 10.1038/s41380-019-0406-4
98. Severance EG, Prandovszky E, Castiglione J, Yolken RH. Gastroenterology issues in schizophrenia: why the gut matters. *Curr Psychiatry Rep* (2015) 17:27. doi: 10.1007/s11920-015-0574-0
99. Sanders AR, Drigalenko EI, Duan J, Moy W, Freda J, Göring HHH, et al. Transcriptome sequencing study implicates immune-related genes differentially expressed in schizophrenia: new data and a meta-analysis. *Transl Psychiatry* (2017) 7:e1093–3. doi: 10.1038/tp.2017.47
100. Pouget JG. The Emerging Immunogenetic Architecture of Schizophrenia. *Schizophr Bull* (2018) 44:993–1004. doi: 10.1093/schbul/sby038
101. Vergaelen E, Schiweck C, Van Steeland K, Counotte J, Veling W, Swillen A, et al. A pilot study on immuno-psychiatry in the 22q11.2 deletion syndrome: A role for Th17 cells in psychosis? *Brain Behav Immun* (2018) 70:88–95. doi: 10.1016/j.bbi.2018.03.022
102. Severance EG, Gressitt KL, Alaedini A, Rohleder C, Enning F, Bumb JM, et al. IgG dynamics of dietary antigens point to cerebrospinal fluid barrier or flow dysfunction in first-episode schizophrenia. *Brain Behav Immun* (2015) 44:148–58. doi: 10.1016/j.bbi.2014.09.009
103. Liu F, Guo X, Wu R, Ou J, Zheng Y, Zhang B,X, et al. Minocycline supplementation for treatment of negative symptoms in early-phase schizophrenia: a double blind, randomized, controlled trial. *Schizophr Res* (2014) 153:169–76. doi: 10.1016/j.schres.2014.01.011
104. Nguyen TT, Kosciolek T, Eyler LT, Knight R, Jeste DV. Overview and systematic review of studies of microbiome in schizophrenia and bipolar disorder. *J Psychiatr Res* (2018) 99:50–61. doi: 10.1016/j.jpsychires.2018.01.013
105. Schwarz E, Maukonen J, Hyytiäinen T, Kieseppä T, Orešič M, Sabunciyan S, et al. Analysis of microbiota in first episode psychosis identifies preliminary associations with symptom severity and treatment response. *Schizophr Res* (2018) 192:398–403. doi: 10.1016/j.schres.2017.04.017
106. Boerrigter D, Weickert TW, Lenroot R, O'Donnell M, Galletly C, Liu D. t al. Using blood cytokine measures to define high inflammatory biotype of schizophrenia and schizoaffective disorder. *J Neuroinflammation* (2017) 14:188. doi: 10.1186/s12974-017-0962-y
107. Pyndt Jørgensen B, Krych L, Pedersen TB, Plath N, Redrobe JP, Hansen AK, et al. Investigating the long-term effect of subchronic phencyclidine-treatment on novel object recognition and the association between the gut microbiota and behavior in the animal model of schizophrenia. *Physiol Behav* (2015) 141:32–9. doi: 10.1016/j.physbeh.2014.12.042
108. Dickerson F, Severance E, Yolken R. The microbiome, immunity, and schizophrenia and bipolar disorder. *Brain Behav Immun* (2017) 62:46–52. doi: 10.1016/j.bbi.2016.12.010
109. Severance EG, Gressitt KL, Stallings CR, Katsafanas E, Schweinfurth LA, Savage CLG, et al. Probiotic normalization of Candida albicans in schizophrenia: A randomized, placebo-controlled, longitudinal pilot study. *Brain Behav Immun* (2017) 62:41–5. doi: 10.1016/j.bbi.2016.11.019
110. Zhu F, Guo R, Wang W, Ju Y, Wang Q, Ma Q, et al. Transplantation of microbiota from drug-free patients with schizophrenia causes schizophrenia-like abnormal behaviors and dysregulated kynurenine metabolism in mice. *Mol Psychiatry* (2019). doi: 10.1038/s41380-019-0475-4
111. Hemmings SMJ, Malan-Müller S, van den Heuvel LL, Demmitt BA, Stanislawski MA, Smith DG, et al. The Microbiome in Posttraumatic Stress Disorder and Trauma-Exposed Controls. *Psychosom Med* (2017) 79:936–46. doi: 10.1097/PSY.0000000000000512
112. Belmaker RH, Agam G. Major Depressive Disorder. *N Engl J Med* (2008) 358:55–68. doi: 10.1056/NEJMra073096
113. Stahl SM. *Stahl"s essential psychopharmacology: neuroscientific basis and practical application. 4th ed.* Cambridge: New York: Cambridge University Press (2013).
114. Liu B, Liu J, Wang M, Zhang Y, Li L. From Serotonin to Neuroplasticity: Evolvement of Theories for Major Depressive Disorder. *Front Cell Neurosci* (2017) 11:305. doi: 10.3389/fncel.2017.00305
115. Alesci S, Martinez PE, Kelkar S, Ilias I, Ronsaville DS, Listwak SJ, et al. Major Depression Is Associated with Significant Diurnal Elevations in Plasma Interleukin-6 Levels, a Shift of Its Circadian Rhythm, and Loss of Physiological Complexity in Its Secretion: Clinical Implications. *J Clin Endocrinol Metab* (2005) 90:2522–30. doi: 10.1210/jc.2004-1667
116. Messaoudi M, Lalonde R, Violle N, Javelot H, Desor D, Nejdi A, et al. Assessment of psychotropic-like properties of a probiotic formulation (*Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175) in rats and human subjects. *Br J Nutr* (2011) 105:755–64. doi: 10.1017/S0007114510004319
117. Veldhuis JD, Sharma A, Roelfsema F. Age-Dependent and Gender-Dependent Regulation of Hypothalamic-Adrenocorticotropic-Adrenal Axis. *Endocrinol Metab Clin North Am* (2013) 42:201–25. doi: 10.1016/j.ecl.2013.02.002
118. Chen X, Ma X, Jiang Y, Pi R, Liu Y, Ma L. The prospects of minocycline in multiple sclerosis. *J Neuroimmunol* (2011) 235:1–8. doi: 10.1016/j.jneuroim.2011.04.006
119. Kloppenburg M, Verweij CL, Miltenburg AM, Verhoeven AJ, Daha MR, Dijkmans BA, et al. The influence of tetracyclines on T cell activation. *Clin Exp Immunol* (1995) 102:635–41. doi: 10.1111/j.1365-2249.1995.tb03864.x
120. Kloppenburg M, Brinkman BM, de Rooij-Dijk HH, Miltenburg AM, Daha MR, Breedveld FC, et al. The tetracycline derivative minocycline differentially affects cytokine production by monocytes and T lymphocytes. *Antimicrob Agents Chemother* (1996) 40:934–40. doi: 10.1128/AAC.40.4.934
121. Yrjänheikki J, Keinänen R, Pellikka M, Hökfelt T, Koistinaho J. Tetracyclines inhibit microglial activation and are neuroprotective in global brain ischemia. *Proc Natl Acad Sci U S A* (1998) 95:15769–74. doi: 10.1073/pnas.95.26.15769
122. Garrido-Mesa N, Zarzuelo A, Gálvez J. Minocycline: far beyond an antibiotic. *Br J Pharmacol* (2013) 169:337–52. doi: 10.1111/bph.12139
123. Brundula V, Rewcastle NB, Metz LM, Bernard CC, Yong VW. Targeting leukocyte MMPs and transmigration: minocycline as a potential therapy for multiple sclerosis. *Brain* (2002) 125:1297–308. doi: 10.1093/brain/awf133

124. Popovic N, Schubart A, Goetz BD, Zhang S-C, Linington C, Duncan ID. Inhibition of autoimmune encephalomyelitis by a tetracycline. *Ann Neurol* (2002) 51:215–23. doi: 10.1002/ana.10092
125. Nessler S, Dodel R, Bittner A, Reuss S, Du Y, Hemmer B, et al. Effect of minocycline in experimental autoimmune encephalomyelitis. *Ann Neurol* (2002) 52:689–690; author reply 690. doi: 10.1002/ana.10353
126. Evrensel A, Önen Ünsalver B, Ceylan ME. Therapeutic Potential of the Microbiome in the Treatment of Neuropsychiatric Disorders. *Med Sci (Basel)* (2019) 7(2):21. doi: 10.3390/medsci7020021
127. Norman HJ. Lactic Acid Bacilli In The Treatment Of Melancholia. *Br Med J* (1909) 1:1234–5.
128. Phillips JGP. The Treatment of Melancholia by the Lactic Acid Bacillus. *J Ment Sci* (1910) 56:422–30. doi: 10.1192/bjp.56.234.422
129. Burokas A, Arboleya S, Moloney RD, Peterson VL, Murphy K, Clarke G, et al. Targeting the Microbiota-Gut-Brain Axis: Prebiotics Have Anxiolytic and Antidepressant-like Effects and Reverse the Impact of Chronic Stress in Mice. *Biol Psychiatry* (2017) 82:472–87. doi: 10.1016/j.biopsych.2016.12.031
130. Evrensel A, Ceylan ME. The Gut-Brain Axis: The Missing Link in Depression. *Clin Psychopharmacol Neurosci* (2015) 13:239–44. doi: 10.9758/cpn.2015.13.3.239
131. Borre YE, O'Keeffe GW, Clarke G, Stanton C, Dinan TG, Cryan JF. Microbiota and neurodevelopmental windows: implications for brain disorders. *Trends Mol Med* (2014) 20:509–18. doi: 10.1016/j.molmed.2014.05.002
132. Bastiaanssen TFS, Cowan CSM, Claesson MJ, Dinan TG, Cryan JF. Making Sense of \ldots the Microbiome in Psychiatry. *Int J Neuropsychopharmacol* (2018) 22:37–52. doi: 10.1093/ijnp/pyy067
133. Dinan TG, Cryan JF. Melancholic microbes: a link between gut microbiota and depression? *Neurogastroenterol Motil* (2013) 25:713–9. doi: 10.1111/nmo.12198
134. Dinan TG, Stanton C, Cryan JF. Psychobiotics: a novel class of psychotropic. *Biol Psychiatry* (2013) 74:720–6. doi: 10.1016/j.biopsych.2013.05.001
135. Messaoudi M, Violle N, Bisson J-F, Desor D, Javelot H, Rougeot C. Beneficial psychological effects of a probiotic formulation (Lactobacillus helveticus R0052 and *Bifidobacterium longum* R0175) in healthy human volunteers. *Gut Microbes* (2011) 2:256–61. doi: 10.4161/gmic.2.4.16108
136. Allen AP, Hutch W, Borre YE, Kennedy PJ, Temko A, Boylan G, et al. *Bifidobacterium longum* 1714 as a translational psychobiotic: modulation of stress, electrophysiology and neurocognition in healthy volunteers. *Transl Psychiatry* (2016) 6:e939. doi: 10.1038/tp.2016.191
137. Pärtty A, Kalliomäki M, Wacklin P, Salminen S, Isolauri E. A possible link between early probiotic intervention and the risk of neuropsychiatric disorders later in childhood: a randomized trial. *Pediatr Res* (2015) 77:823–8. doi: 10.1038/pr.2015.51
138. Kato-Kataoka A, Nishida K, Takada M, Kawai M, Kikuchi-Hayakawa H, Suda K, et al. Fermented Milk Containing Lactobacillus casei Strain Shirota Preserves the Diversity of the Gut Microbiota and Relieves Abdominal Dysfunction in Healthy Medical Students Exposed to Academic Stress. *Appl Environ Microbiol* (2016) 82:3649–58. doi: 10.1128/AEM.04134-15
139. Sashihara T, Nagata M, Mori T, Ikegami S, Gotoh M, Okubo K, et al. Effects of *Lactobacillus gasseri* OLL2809 and α-lactalbumin on university-student athletes: a randomized, double-blind, placebo-controlled clinical trial. *Appl Physiol Nutr Metab* (2013) 38:1228–35. doi: 10.1139/apnm-2012-0490
140. Kelly JR, Allen AP, Temko A, Hutch W, Kennedy PJ, Farid N, et al. Lost in translation? The potential psychobiotic Lactobacillus rhamnosus (JB-1) fails to modulate stress or cognitive performance in healthy male subjects. *Brain Behav Immun* (2017) 61:50–9. doi: 10.1016/j.bbi.2016.11.018
141. Ochoa-Reparaz J, Kasper LH. The Microbiome and Neurologic Disease: Past and Future of a 2-Way Interaction. *Neurotherapeutics* (2018) 173:1714. doi: 10.1007/s13311-018-0604-9
142. Wang Y, Telesford KM, Ochoa-Reparaz J, Haque-Begum S, Christy M, Kasper EJ, et al. An intestinal commensal symbiosis factor controls neuroinflammation via TLR2-mediated CD39 signalling. *Nat Commun* (2014) 5:4432. doi: 10.1038/ncomms5432
143. Dasgupta S, Erturk-Hasdemir D, Ochoa-Reparaz J, Reinecker H-C, Kasper DL. Plasmacytoid dendritic cells mediate anti-inflammatory responses to a gut commensal molecule via both innate and adaptive mechanisms. *Cell Host Microbe* (2014) 15:413–23. doi: 10.1016/j.chom.2014.03.006
144. Ma Y, Hébert JR, Li W, Bertone-Johnson ER, Olendzki B, Pagoto SL, et al. Association between dietary fiber and markers of systemic inflammation in the Women's Heath Initiative Observational Study. *Nutrition* (2008) 24:941–9. doi: 10.1016/j.nut.2008.04.005
145. Derrien M, Vaughan EE, Plugge CM, de Vos WM. *Akkermansia muciniphila* gen. nov., sp. nov., a human intestinal mucin-degrading bacterium. *Int J Syst Evol Microbiol* (2004) 54:1469–76. doi: 10.1099/ijs.0.02873-0
146. Morrison DJ, Preston T. Formation of short chain fatty acids by the gut microbiota and their impact on human metabolism. *Gut Microbes* (2016) 7:189–200. doi: 10.1080/19490976.2015.1134082
147. Erny D, Hrabě de Angelis AL, Jaitin D, Wieghofer P, Staszewski O, David E, et al. Host microbiota constantly control maturation and function of microglia in the CNS. *Nat Neurosci* (2015) 18:965–77. doi: 10.1038/nn.4030
148. Wang H-B, Wang P-Y, Wang X, Wan Y-L, Liu Y-C. Butyrate Enhances Intestinal Epithelial Barrier Function via Up-Regulation of Tight Junction Protein Claudin-1 Transcription. *Dig Dis Sci* (2012) 57:3126–35. doi: 10.1007/s10620-012-2259-4
149. Liu B, Qian J, Wang Q, Wang F, Ma Z, Qiao Y. Butyrate Protects Rat Liver against Total Hepatic Ischemia Reperfusion Injury with Bowel Congestion. *PloS One* (2014) 9:e106184. doi: 10.1371/journal.pone.0106184
150. Braniste V, Al-Asmakh M, Kowal C, Anuar F, Abbaspour A, Tóth M, et al. The gut microbiota influences blood-brain barrier permeability in mice. *Sci Transl Med* (2014) 6:263ra158–263ra158. doi: 10.1126/scitranslmed.3009759
151. Furusawa Y, Obata Y, Fukuda S, Endo TA, Nakato G, Takahashi D, et al. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. *Nature* (2013) 504:1–8. doi: 10.1038/nature12721
152. Smith PM, Howitt MR, Panikov N, Michaud M, Gallini CA, Bohlooly –M, et al. The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. *Science* (2013) 341:569–73. doi: 10.1126/science.1241165
153. Arpaia N, Campbell C, Fan X, Dikiy S, van der Veeken J, deRoos P, et al. Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. *Nature* (2013) 504:451–5. doi: 10.1038/nature12726
154. Martin-Gallausiaux C, Béguet-Crespel F, Marinelli L, Jamet A, Ledue F, Blottière HM, et al. Butyrate produced by gut commensal bacteria activates TGF-beta1 expression through the transcription factor SP1 in human intestinal epithelial cells. *Sci Rep* (2018) 8:1–13. doi: 10.1038/s41598-018-28048-y
155. Li M, van Esch BCAM, Wagenaar GTM, Garssen J, Folkerts G, Henricks PAJ. Pro- and anti-inflammatory effects of short chain fatty acids on immune and endothelial cells. *Eur J Pharmacol* (2018) 831:52–9. doi: 10.1016/j.ejphar.2018.05.003
156. Lukić I, Getselter D, Koren O, Elliott E. Role of Tryptophan in Microbiota-Induced Depressive-Like Behavior: Evidence From Tryptophan Depletion Study. *Front Behav Neurosci* (2019) 13:123. doi: 10.3389/fnbeh.2019.00123
157. Sudo N, Chida Y, Aiba Y, Sonoda J, Oyama N, Yu X-N, et al. Postnatal microbial colonization programs the hypothalamic-pituitary-adrenal system for stress response in mice. *J Physiol* (2004) 558:263–75. doi: 10.1113/jphysiol.2004.063388
158. Auteri M, Zizzo MG, Serio R. GABA and GABA receptors in the gastrointestinal tract: from motility to inflammation. *Pharmacol Res* (2015) 93:11–21. doi: 10.1016/j.phrs.2014.12.001
159. Mazzoli R, Pessione E. The Neuro-endocrinological Role of Microbial Glutamate and GABA Signaling. *Front Microbiol* (2016) 7:1934:1934. doi: 10.3389/fmicb.2016.01934
160. Hyland NP, Cryan JF. A Gut Feeling about GABA: Focus on GABAB Receptors. *Front Pharmacol* (2010) 01:124. doi: 10.3389/fphar.2010.00124
161. Cao G, Edden RAE, Gao F, Li H, Gong T, Chen W, et al. Reduced GABA levels correlate with cognitive impairment in patients with relapsing-remitting multiple sclerosis. *Eur Radiol* (2018) 28:1140–8. doi: 10.1007/s00330-017-5064-9
162. Bhat R, Axtell R, Mitra A, Miranda M, Lock C, Tsien RW, et al. Inhibitory role for GABA in autoimmune inflammation. *Proc Natl Acad Sci U S A* (2010) 107:2580–5. doi: 10.1073/pnas.0915139107

163. Sandler RH, Finegold SM, Bolte ER, Buchanan CP, Maxwell AP, Väisänen ML, et al. Short-term benefit from oral vancomycin treatment of regressive-onset autism. *J Child Neurol* (2000) 15:429–35. doi: 10.1177/088307380001500701
164. Kang D-W, Adams JB, Coleman DM, Pollard EL, Maldonado J, McDonough-Means S, et al. Long-term benefit of Microbiota Transfer Therapy on autism symptoms and gut microbiota. *Sci Rep* (2019) 9:5821. doi: 10.1038/s41598-019-42183-0
165. Sharon G, Cruz NJ, Kang D-W, Gandal MJ, Wang B, Kim Y-M, et al. Human Gut Microbiota from Autism Spectrum Disorder Promote Behavioral Symptoms in Mice. *Cell* (2019) 177:1600–18.e17. doi: 10.1016/j.cell.2019.05.004

Conflict of Interest: JO-R serves as a consultant for Symbiotics Biotherapies. LK is a co-founder of Symbiotix Biotherapies.

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

frontiers in Immunology

ORIGINAL RESEARCH
published: 11 December 2020
doi: 10.3389/fimmu.2020.578648

Human Commensal *Prevotella histicola* Ameliorates Disease as Effectively as Interferon-Beta in the Experimental Autoimmune Encephalomyelitis

Shailesh K. Shahi[1], *Samantha N. Jensen*[1,2], *Alexandra C. Murra*[1], *Na Tang*[1], *Hui Guo*[1], *Katherine N. Gibson-Corley*[1], *Jian Zhang*[1], *Nitin J. Karandikar*[1,2,3], *Joseph A. Murray*[4,5] *and Ashutosh K. Mangalam*[1,2,3]*

[1] Department of Pathology, University of Iowa, Iowa City, IA, United States, [2] Graduate Program in Immunology, University of Iowa, Iowa City, IA, United States, [3] Graduate Program in Molecular Medicine, University of Iowa, Iowa City, IA, United States, [4] Department of Immunology, Mayo Clinic, Rochester, MN, United States, [5] Division of Gastroenterology and Hepatology, Mayo Clinic, Rochester, MN, United States

OPEN ACCESS

Edited by:
Lloyd Kasper,
Dartmouth College, United States

Reviewed by:
Daniel Mielcarz,
Dartmouth College, United States
Enrico Castroflorio,
Medical Research Council Harwell (MRC), United Kingdom

***Correspondence:**
Ashutosh K. Mangalam
Ashutosh-mangalam@uiowa.edu

Specialty section:
This article was submitted to Multiple Sclerosis and Neuroimmunology, a section of the journal Frontiers in Immunology

Received: 30 June 2020
Accepted: 04 November 2020
Published: 11 December 2020

Citation:
Shahi SK, Jensen SN, Murra AC, Tang N, Guo H, Gibson-Corley KN, Zhang J, Karandikar NJ, Murray JA and Mangalam AK (2020) Human Commensal Prevotella histicola Ameliorates Disease as Effectively as Interferon-Beta in the Experimental Autoimmune Encephalomyelitis. Front. Immunol. 11:578648. doi: 10.3389/fimmu.2020.578648

Gut microbiota has emerged as an important environmental factor in the pathobiology of multiple sclerosis (MS), an inflammatory demyelinating disease of the central nervous system (CNS). Both genetic and environmental factors have been shown to play an important role in MS. Among genetic factors, the human leukocyte antigen (HLA) class II allele such as HLA-DR2, DR3, DR4, DQ6, and DQ8 show the association with the MS. We have previously used transgenic mice expressing MS susceptible HLA class II allele such as HLA-DR2, DR3, DQ6, and DQ8 to validate significance of HLA alleles in MS. Although environmental factors contribute to 2/3 of MS risk, less is known about them. Gut microbiota is emerging as an imporatnt environmental factor in MS pathogenesis. We and others have shown that MS patients have distinct gut microbiota compared to healthy control (HC) with a lower abundance of *Prevotella*. Additionally, the abundance of *Prevotella* increased in patients receiving disease-modifying therapies (DMTs) such as Copaxone and/or Interferon-beta (IFNβ). We have previously identified a specific strain of *Prevotella* (*Prevotella histicola*), which can suppress experimental autoimmune encephalomyelitis (EAE) disease in HLA-DR3.DQ8 transgenic mice. Since Interferon-β-1b [IFNβ (Betaseron)] is a major DMTs used in MS patients, we hypothesized that treatment with the combination of *P. histicola* and IFNβ would have an additive effect on the disease suppression. We observed that treatment with *P. histicola* suppressed disease as effectively as IFNβ. Surprisingly, the combination of *P. histicola* and IFNβ was not more effective than either treatment alone. *P. histicola* alone or in combination with IFNβ increased the frequency and number of $CD4^+FoxP3^+$ regulatory T cells in the gut-associated lymphoid tissue (GALT). Treatment with *P. histicola* alone, IFNβ alone, and in the combination decreased frequency of pro-inflammatory IFN-γ and IL17-producing $CD4^+$ T cells in the CNS. Additionally, *P. histicola* alone or IFNβ alone or the combination

treatments decreased CNS pathology, characterized by reduced microglia and astrocytic activation. In conclusion, our study indicates that the human gut commensal *P. histicola* can suppress disease as effectively as commonly used MS drug IFNβ and may provide an alternative treatment option for MS patients.

Keywords: experimental autoimmune encephalomyelitis, human leukocyte antigen transgenic mice, multiple sclerosis, interferon beta, *Prevotella histicola*

INTRODUCTION

Multiple sclerosis (MS), an inflammatory and demyelinating disease of the central nervous system (CNS), is a multifactorial disease where the interaction between genetic and environmental factors play an important role in disease pathogenesis. Among various genetic factors (more than 50 genetic polymorphisms), human leukocyte antigen (HLA) class II haplotypes such as DR2/DQ6, DR3/DQ2, and DR4/DQ8 show the strongest association with MS (1, 2). Previously, we have characterized and validated HLA-DR3.DQ8 double transgenic mice as an animal model to study MS (3–6). HLA-DR3.DQ8 transgenic mice expressing the human class II genes HLA-DR3 (DRB1*0301) and DQ8 (DQB1*0302), lacking endogenous mouse class II genes (I-A and I-E) were used in this study.

Accumulating evidence suggests that environmental factors play an important role in a individual's susceptibility to MS (7). However, specificity of an environmental factor contributing to MS susceptibility or resistance remains elusive. We and others have shown that the gut microbiota of MS patients are distinct from healthy controls (HC), suggesting the gut microbiota is an important environmental factor that contributes to MS pathogenesis (8–13). A number of MS microbiome studies have shown that genus *Prevotella* is either depleted or has a lower abundance among gut bacteria from MS patient, compared to HC (8–10, 14). Additionally, MS patients on disease-modifying therapies such as Copaxone or IFNβ showed a higher abundance of *Prevotella* compared to untreated MS patients (9, 15). We have previously identified a specific strain of *Prevotella*, *Prevotella histicola*, which can suppress proteolipid protein $(PLP)_{91\text{-}110}$-induced EAE disease in the HLA-DR3.DQ8 transgenic mouse (5).

Interferon-β-1b [IFNβ (Betaseron)] is a major disease-modifying drug used in MS patients (16). The Food and Drug Administration (FDA)-approved an expanded repertoire of IFNβ use, namely intramuscular (IM) IFNβ-1a (Avonex, Biogen), subcutaneous (SC) IFNβ-1a (Rebif, EMD Serono), and PEGylated IFN (PEGIFN)- β-1a (Plegridy, Biogen) for the treatment of MS (17). Although IFNβ has been used as a first-line drug over the years in relapsing-remitting MS (RRMS), IFNβ alone is ineffective in 7–49% of patients with RRMS (18). Therefore, there is a need to develop additional therapeutic options that can either be used alone or in combination with IFNβ to improve the treatment for MS.

In the present study, we investigated whether a combination of *P. histicola* and IFNβ is more effective than either drug alone utilizing EAE in HLA-DR3.DQ8 transgenic mice. We found that *P. histicola* alone was as effective as IFN-β in suppressing $PLP_{91–110}$-induced EAE in HLA-DR3.DQ8 transgenic mice. Additionally, we observe that naïve HLA-DR3.DQ8 transgenic mice treated with *P. histicola*, either alone or in combination with IFNβ, led to an increased frequency and number of $CD4^+Foxp3^+$ regulatory T cells (Treg) in the gut-associated lymphoid tissue (GALT). Treatment with *P. histicola* alone, IFNβ alone, and the combination of both in the EAE induction phase of disease also decreased frequency of pro-inflammatory IFN-γ and IL17-producing $CD4^+$ T cells. Furthermore, we observed that *P. histicola* and/or IFNβ treatments suppressed microglia and astrocytes activation in the CNS. Altogether, our results suggest that *P. histicola* is as effective as IFNβ in suppressing EAE by boosting anti-inflammatory immune responses and inhibiting pro-inflammatory immune responses.

MATERIALS AND METHODS

Mice

Human leukocyte antigen (HLA)-DR3.DQ8 double transgenic [DQ8 (DQA1*0103, DQB1*0302)-DR3 (DRB1*0301)] mice used in this study has been previously characterized and validated by our group (3–6, 19). The HLA-DR3.DQ8 mouse expresses the human class II genes HLA-DR3 (DRB1*0301) and DQ8 (DQB1*0302), and lack endogenous murine major histocompatibility complex (MHC) class II genes I-A, I-E ($AE^{-/-}$). For the simplicity these mice will be referred as HLA-DR3.DQ8 transgenic mice throughout the text. Both male and female mice (8–12 weeks of age) were utilized in this study. Mice were bred and maintained in the University of Iowa animal facility in accordance with NIH and institutional guidelines. All experiments were approved by the Institutional Animal Care and Use Committee at the University of Iowa.

Disease Induction in HLA-DR3.DQ8 Transgenic Mice and Scoring

HLA-DR3.DQ8 transgenic mice (8 to 12 weeks old) were immunized subcutaneously in both flanks with 25 μg of $PLP_{91–110}$ emulsified in complete Freund's adjuvant (CFA) containing *Mycobacterium tuberculosis* H37Ra (100 μg/mouse; Becton, Dickinson and Company, Sparks, MD, USA). Pertussis toxin (PTX) (Sigma Chemicals, St. Louis, MO, USA; 100 ng) was administered i.p. at days 0 and 2 post immunization. HLA-DR3.DQ8 transgenic mice were scored daily for clinical

symptoms using the standard 0–5 scoring system described previously (5). Briefly, 0 for no disease; 1 for loss of tail tone; 2 for hind limb weakness; 3 for hind limb paralysis; 4 for hind limb paralysis and forelimb paralysis or weakness; and 5 for morbidity/death.

Isolation, Characterization, and Identification of *Prevotella histicola*

Isolation, characterization, and identification (based on 16SrRNA-specific PCR) of *P. histicola* has been described previously (5). Briefly, *P. histicola* was grown at 37°C for 3 days in trypticase soy broth (TSB) (Hardy Diagnostics Santa Maria, USA) in an anaerobic jar with an AnaeroPack system (Mitsubishi Gas Chemical America) (5).

Treatment of Mice With *Prevotella histicola* and IFNβ

We used two protocols for Interferon-beta (IFNβ) (Betaseron, Bayer HealthCare Pharmaceuticals) treatment: In the first protocol (prophylactic), IFNβ treatment was given every alternate day for 2 weeks to naïve HLA-DR3.DQ8 transgenic mice before the EAE induction and in second protocol IFNβ treatment was given during the disease induction phase (7 days post EAE induction).

In a prophylactic setting, HLA-DR3.DQ8 transgenic mice were divided into four groups (*P. histicola* alone, IFNβ alone, *P. histicola* and IFNβ, and media alone). IFNβ alone, *P. histicola* and IFNβ group of mice received 10,000 IU of IFNβ (20) in 100 µl of phosphate buffer saline (PBS) every other day for a total of seven doses. *P. histicola* alone and *P. histicola* plus IFNβ combination group of mice were orally gavaged with live *P. histicola* (10^8 CFUs) every other day for a total of seven doses. Mice in the control group were orally gavaged with TSB media every other day for a total of seven doses.

In the second protocol, we treated mice in the induction phase (at disease onset) of the disease. Mice received 1st dose of *P. histicola* treatment at day 7 post-immunization as the HLA-DR3.DQ8 transgenic mice develop the disease around day 7 (4, 6). Mice were divided into four groups (*P. histicola* alone, IFNβ alone, *P. histicola* plus IFNβ, and media alone) and treated on an alternate day with *P. histicola*, IFNβ, *P. histicola* plus IFNβ, or media as described above. All mice were evaluated for EAE scores till the duration of the experiment.

Pathology

Brains and spinal cords from mice treated with *P. histicola* alone, IFNβ alone, a combination of both *P. histicola* plus IFNβ, or TSB media alone were fixed in 10% neutral buffered formalin, routinely processed and stained with Hematoxylin and Eosin (HE). Brains and spinal cords sections were analyzed for pathology, specially inflammation and demyleination, by a board-certified veterinary pathologist, specifically but not limited to the cortex, corpus callosum, hippocampus, brainstem, straitum, and cerebellum regions as described previously (4, 21).

Immunohistochemistry (IHC)

Antigen retrieval was performed on freshly cut paraffin sections in a decloaking chamber for 5 min at 125°C in citrate buffer (pH 6.0). Endogenous peroxidase was blocked by incubation with 3% peroxide at room temperature for 8 min. For GFAP antibody staining (ab16997Abcam) the primary antibody was applied at 1:100 in Dako (Dako Agilent, Santa Clara, CA, USA) diluent for 1 h at room temperature after blocking with Dako Background Buster. IBA-1 immunostaining was performed with the antibody (019-10741, Wako Chemicals) diluted at 1:500 after blocking with both avadin/biotin block (Vector Laboratories) and 10% goat serum in Dako buffer. Bound antibody was detected using EnvisonTM + HRP, rabbit (Dako) for 30 min at room temperature followed by incubation with diaminobenzidene substrate (DAB) for 5 min at room temperature. Slides were counterstained with hematoxylin and evaluated by a board-certified veterinary pathologist (KGC).

Western Blot Analysis

The brain and spinal cord's tissues from mice were homogenized, and lysed in radioimmuno-precipitation assay (RIPA) buffer (25 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 1% sodium deoxycholate, 0.5% SDS, 100 µ M Na3VO4, 1 mM NaF, 1 mM PMSF, 10 µ g/ml aprotonin, 10 µg/ml leupeptin) (22). Protein concentrations in the tissue lysates were determined using NanoDrop Spectrophotometer (ThermoFisher; Waltham, MA, USA). Tissue lysates were normalized for protein concentration levels. Two hundred µg proteins were separated by SDSPAGE, and transferred to nitrocellulose membranes (Hybond C Super, cytiva, Marlborough, MA, USA). Membranes were blocked for 1 h at room temperature in TBST containing 5% milk, and then incubated overnight with anti-Iba1 (1:1,000; 016-20001, FUJIFILM Wako Pure Chemical Corporation; Richmond, VA, USA) or anti-GFAP (1:5,000; ab-254082, Abcam) at 4°C. After washing three times with TBS containing 0.05% Tween-20, membranes were incubated with HRP-conjugated mouse anti-rabbit IgG (1:30,000; #31464, ThermoFisher; Waltham, MA, USA), and visualized by Bio-Rad ChemiDocTM Touch Imaging System (Hercules, CA, USA).

Flow Cytometry

Mononuclear infiltrating cells from the CNS (brain and spinal cord) were isolated using a percoll density gradient separation method as described previously (23). Mice in each treatment group were stained with antibodies to detect surface expression of CD4 (GK1.5) and CD25 (PC61) (BD Biosciences, Franklin Lakes, NJ, USA), whereas intracellular expression of FoxP3$^+$ were stained using an anti-Mouse/Rat FoxP3 (FJK-16s) staining kit (eBiosciences, San Diego, CA, USA). Intracellular staining for IL17, IFNγ, GM-CSF, and IL10 were performed using the intracellular fixation permeabilization kit and anti-mouse IL17 (TC11-18H10.1), IFNγ (XMG1.2), GM-CSF (MP1-22E9), and IL10 (FES5-16E3) specific antibodies from eBioscience™.

Cells were also stained with antibodies to detect surface expression of CD45 (30-F11) and CD4 (clone GK1.5) to gate on the leukocyte population. Gut-associated lymphoid cells were isolated and stained with antibodies as per the method described previously (24).

Statistical Analysis

Differences in the frequency of regulatory T cells or cytokine-producing CD4 T cells among different treatment groups were assessed by Mann-Whitney U test. Average clinical EAE scores and cumulative EAE scores were compared using 2-way ANOVA with multiple comparisons of the means and non-parametric Mann-Whitney U test respectively. Statistical analyses were done with GraphPad Prism 8 (GraphPad Software, La Jolla, CA, USA). A value of $p \leq 0.05$ was considered significant.

RESULTS

P. histicola Suppresses EAE in Mice as Effectively as IFNβ

First, we examined whether the combination treatment of *P. histicola* and IFNβ can work in an additive manner to ameliorate disease in HLA-DR3.DQ8 transgenic mice. We started treating mice from day 7 postimmunization (disease induction phase) with *P. histicola* alone, IFNβ alone, and the combination of *P. histicola* and IFNβ. Treatment with *P. histicola* alone or IFNβ alone resulted in a lower average daily EAE score (**Figure 1A**) and a lower cumulative EAE score compared with mice who received media (**Figure 1B**). The combination treatment group had a similar average daily clinical score (**Figure 1A**) and average cumulative EAE score (**Figure 1B**) compared to the groups receiving *P. histicola* or IFNβ alone. Thus, our data indicate that *P. histicola* is effective at suppressing EAE when administered alone or in combination with IFNβ and is as effective as treatment with IFNβ alone.

Treatment With *P. histicola* or IFNβ Reduces Inflammation in the CNS

To determine whether disease suppression was accompanied with less severe CNS pathology, we analyzed brain and spinal cord tissues from all four groups by performing semi-quantitative analyses. HLA-DR3.DQ8 transgenic mice treated with *P. histicola* alone, or the combination of *P. histicola* and IFNβ had less inflammatory cell infiltrates in the brain and spinal cord compared to mice treated with media alone (**Figure 2**). Brain tissue sections from HLA-DR3.DQ8 transgenic mice treated with *P. histicola* alone, IFNβ alone, and the combination of both exhibited relatively less inflammation in the meningeal and stratum region compared with media group (**Figure 2**). Similarly, spinal cord tissue sections from mice that received *P. histicola* alone, or IFNβ alone and the combination of both, showed lower inflammation whereas spinal cord sections from mice treated with media showed severe inflammation (**Figure 2**). As mice received *P. histicola* orally, we sought to determine if *P. histicola* causes any histopathology (HE sections) of the gastrointestinal system. We found that neither of these treatment (*P. histicola* alone, IFNβ alone, and the combination of both) or the media control group caused any overt pathology in the stomach, small intestine, and large intestine (**Supplementary Figure 1**). In summary, we observed that treatment with *P. histicola* alone, IFNβ alone, or the combination of *P. histicola* plus IFNβ caused reduced CNS pathology in mice induced for EAE.

Treatment With *P. histicola* or IFNβ Reduces Microglia and Astrocyte Activation

The CNS resident microglia and astrocytes mediate myelin injury through enhanced phagocytosis and promoting autoreactive T-cell responses by functioning as antigen presenting cells (25, 26). Therefore, to determine whether *P. histicola* or IFNβ influence microglia and/or astrocyte activation, we stained CNS tissue with microglia specific anti-Iba-1 antibody and astrocyte specific GFAP antibody post-EAE induction. We found that the Iba-1 positive microglia cells were lower in the perivascular spaces of the brain cerebellar region and white matter of the spinal cord in treated group than those treated with media alone (**Figure 3**, **Supplementary Figure 2**). Additionally, a lower GFAP positive astrocytes were observed in cerebrum region of brain (**Figure 4**, **Supplementary Figure 3**) and white matter of the spinal cord (**Figure 4**) of the mice groups treated with *P. histicola* alone, IFNβ alone, or the combination of *P. histicola* plus IFNβ compared to the group treated with media alone. Besides IHC, expression of Iba-1 and GFAP at the protein level were confirmed in spinal cords by western blot using their specific antibodies. We observed that the Iba-1 and GFAP were lower at the protein levels in the spinal cord tissue from mice groups treated with *P. histicola* alone, IFNβ alone, or the combination of *P. histicola* plus IFNβ compared to the group treated with media alone (**Supplementary Figure 4**). Thus, treatment with *P. histicola* alone, IFNβ alone, or the combination of *P. histicola* plus IFNβ can reduce CNS pathology by reducing microglia and astrocytes activation in mice induced for EAE.

P. histicola Alone and in Combination With IFNβ Induces $CD4^+FoxP3^+$ Regulatory T Cells in Gut-Associated Lymphoid Tissue of Mice

We and others have shown that $CD4^+FoxP3^+$ Treg cells play a significant role in suppressing EAE disease (5, 6, 27). As *P. histicola* is a gut commensal, it may modulate the immune system through influencing the immune compartment of the intestinal tract. Therefore, we analyzed levels of $CD4^+FoxP3^+$ Treg cells and $CD4^+$ $IL10^+$ T cells in the GALT. Mice treated with *P. histicola* alone showed a higher frequency and number of $CD4^+FoxP3^+$ Treg cells compared to mice treated with media

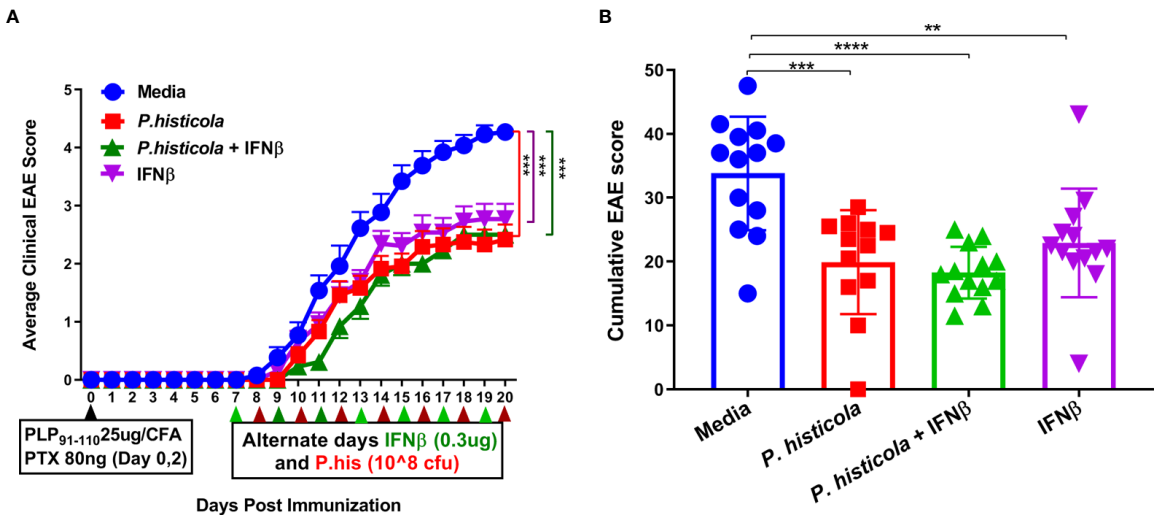


FIGURE 1 | P. *histicola* suppresses $PLP_{91–110}$-induced EAE in HLA-DR3.DQ8 transgenic mice as effectively as treatment with Interferon-Beta (IFNβ). **(A)** Mice were immunized with $PLP_{91–110}$/CFA plus pertussis toxin on days 0 and 2 of the disease induction and 1 week later mice were treated with IFNβ, *P. histicola*, or a combination of both (with treatment administered on alternate days for a total of 14 doses, 7 doses of IFNβ and 7 doses of *P. histicola*) for 2 weeks. Clinical scores were assessed daily for the duration of the experiment. **(B)** Cumulative EAE scores of mice treated as in A. The data presented represent two of four experiments performed at different time points (n ≥ 12 mice per group). Two asterisks indicates p ≤ 0.01, three asterisks indicate p ≤ 0.001, and four asterisks indicate p ≤ 0.0001 when compared to the medium treated group. 2way ANOVA Dunnett's multiple comparisons test were used to calculate *p*-value in average clinical EAE score **(A)** and Mann- Whitney unpaired U test was used to calculate *p*-value in cumulative EAE score **(B)**.

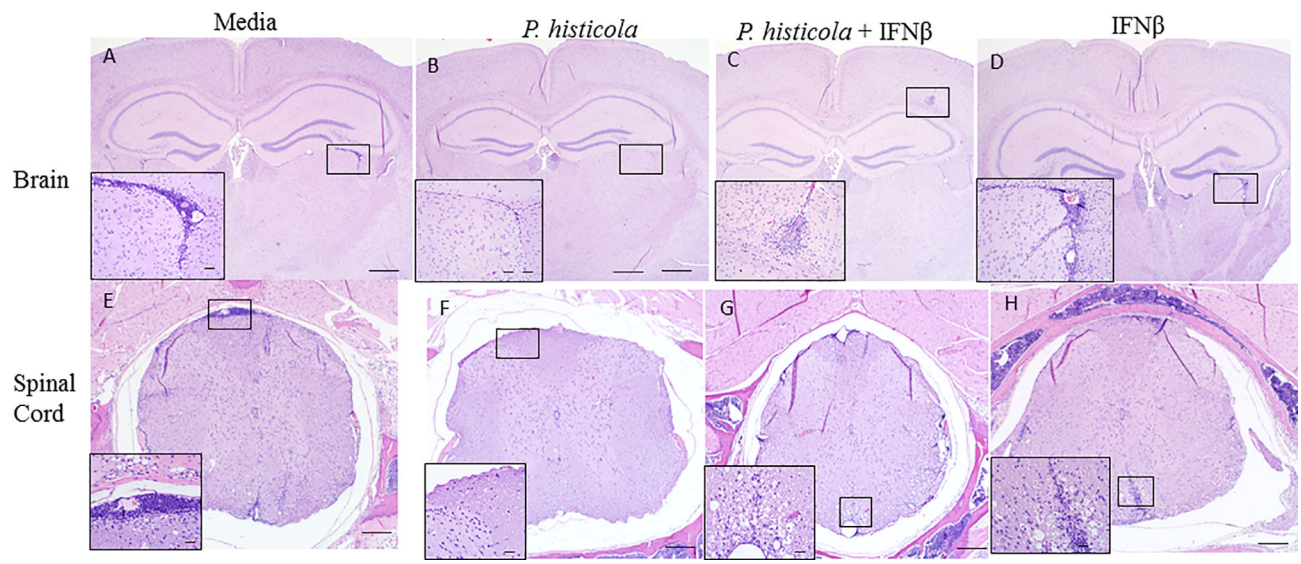


FIGURE 2 | Treatment with *P. histicola* alone, IFNβ alone, or the combination of *P. histicola* and IFNβ resulted in decreased inflammation in the brain and spinal cord of mice induced with EAE. Representative HE stained images of the brain from mice treated with media **(A)**, *P. histicola* alone **(B)** or in combination with IFNβ **(C)**, and IFNβ alone **(D)**. Representative HE stained images of the lumbar spinal cord *in situ* from mice treated with media **(E)**, *P. histicola* alone **(F)** or in combination with IFNβ **(G)**, and IFNβ alone **(H)**. Insets in **(A–D)** identify areas of inflammatory cell infiltration and/or demyelination in brain (Bars = 500 um, inset bars = 50 um) and insets in E–H identify areas of inflammatory cell infiltration and/or demyelination in spinal cord when present (Bars = 200 um, inset bars = 20 um). The data presented represent one of two experiments performed at different time points (n ≥ 3 mice per group).

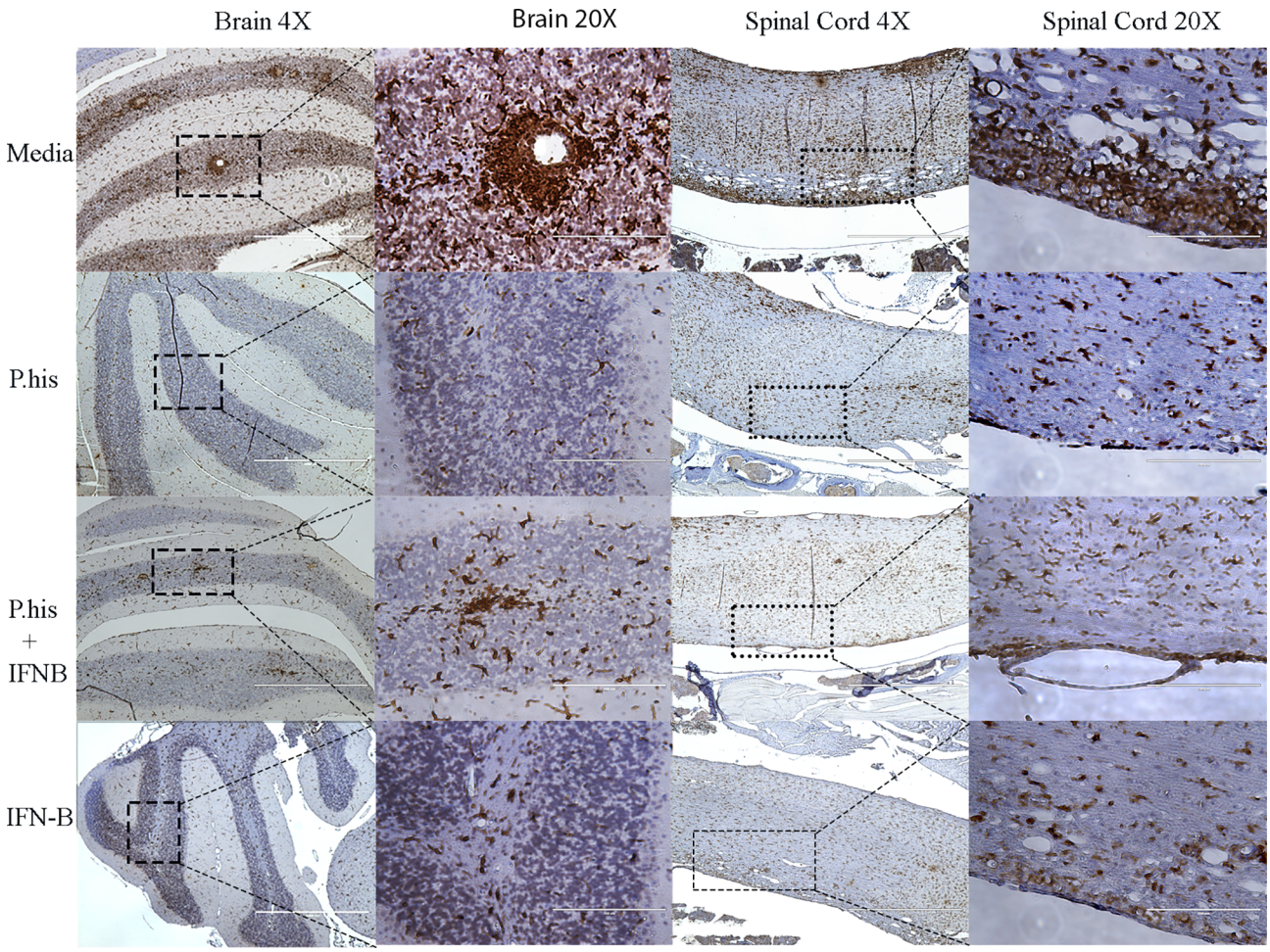


FIGURE 3 | Treatment with *P. histicola* alone, IFNβ alone, or the combination of *P. histicola* and IFNβ resulted in decreased microglial activation in the brain and spinal cord of mice with EAE. Representative ionized calcium-binding adaptor protein-1 (Iba-1) staining of the brain and spinal cord sections from mice induced with EAE that received treatment with either *P. histicola* alone, IFNβ alone, the combination of both treatments, or media. The data presented represent one of two experiments performed at different time points (n ≥ 3 mice per group).

(% mean 21.53 *vs.* 32.05 ± 3.87, $p = 0.037$, number mean 139,241 *vs.* 245,810 ± 35,924, $p = 0.04$) (**Figures 5A–C**). Mice treated with a combination of *P. histicola* and IFNβ also showed the higher number but not the frequency of $CD4^+FoxP3^+$ Treg cells compared to media treated group (% mean 21.53 *vs.* 28.65 ± 3.87, $p = 0.09$, # mean 139,241 *vs.* 237,258 ± 35,924, $p = 0.02$) (**Figures 5A–C**). We did not observe any change in $CD4^+FoxP3^+$ Treg cells in the IFNβ alone treated mice group compared to media control group (% mean 21.53 *vs.* 30.73 ± 0, $p = 0.11$, # mean 139,241 *vs.* 279,087 ± 38,803, $p = 0.057$) (**Figures 5A–C**). Additionally, we found that the *P. histicola* alone or in combination with IFNβ treatment led to a higher number and frequency of $CD4^+$ IL-10 T cells compared with media control group (**Supplementry Figure 5**). We did not observe any difference in IL-17, IFN-γ, and GM-CSF, producing $CD4^+$ T cells in the GALT (**Figures 6A–F**). Thus, our data indicate that *P. histicola* alone or in combination with IFNβ, ameliorates disease and is associated with an induction of $CD4^+FoxP3^+$ regulatory T cells and $CD4^+$ $IL10^+$ T cells in the gut. However, IFN-γ alone had no effect on either $CD4^+FoxP3^+$ regulatory T cells or $CD4^+$ IL-10 T cells in the gut.

Treatment With *P. histicola* and/or IFNβ Reduces Antigen-Specific Th1 and Th17 Cytokines in the CNS of Mice Induced With EAE

Next, we analyzed whether *P. histicola*, IFNβ, or the combination of both suppresses disease through influencing Th1 and Th17 cytokines. We isolated mononuclear cells from the brain and spinal cord of EAE mice from all groups and stimulated with the PLP_{91-110} peptide plus Brefeldin A for 14 h (28). HLA-DR3.DQ8

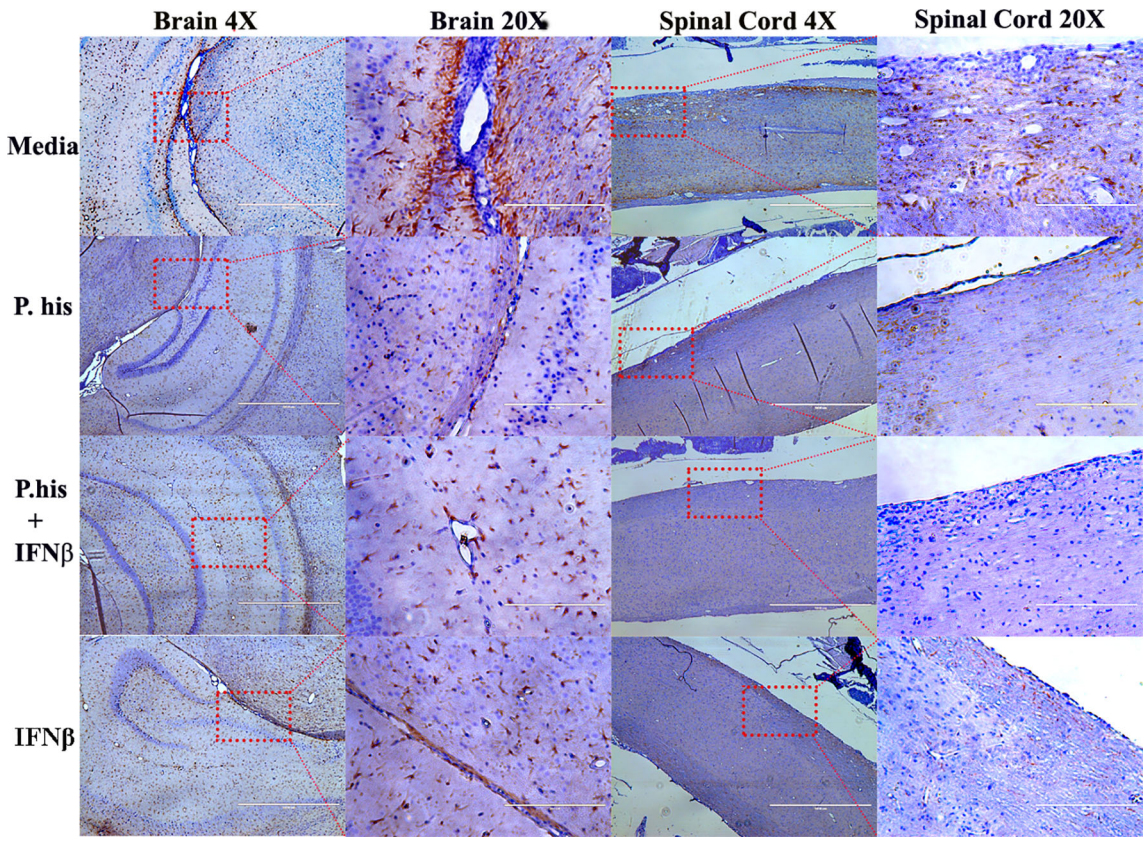


FIGURE 4 | Treatment with *P. histicola* alone, IFNβ alone, or the combination of *P. histicola* and IFNβ reduced astrocytes activation in the brain and spinal cord of mice with EAE. Representative staining glial fibrillary acidic protein (GFAP) of the brain and spinal cord sections from mice induced with EAE that received treatment with either *P. histicola* alone, IFNβ alone, the combination with IFNβ, or media only. The data presented represent one of two experiments performed at different time points (n ≥ 3 mice per group).

transgenic mice treated with *P. histicola* alone, IFNβ alone, the combination of *P. histicola* and IFNβ had a lower frequency of $CD4^+IL17^+$ T cells (**Figures 7A, B**) and $CD4^+IFN\gamma^+$ T cells (**Figures 7C, D**) compared to those treated with media alone. Thus, our data suggest that treatment with *P. histicola* alone, IFNβ alone, the combination of *P. histicola* and IFNβ decreases the frequency of $IFN\gamma^+$ and $IL17^+$ producing $CD4^+$ T cells in the CNS of mice with EAE.

DISCUSSION

In the present study, we showed that *P. histicola* alone was as effective as the disease-modifying drug IFNβ in suppressing EAE in HLA-DR3.DQ8 transgenic mice. We also observed that the combination of *P. histicola* and IFNβ was not more effective compared to either treatment alone. The treatment with *P. histicola* alone or in combination IFNβ caused an increase in $CD4^+FoxP3^+$ Treg cells in the GALT of naïve HLA-DR3.DQ8 transgenic mice. Furthermore, we observed that *P. histicola* and/or IFNβ treatments effectively reduced microglia, astrocytes activation, and the frequency of IFN-γ and IL17- pro-inflammatory cytokine producing $CD4^+$ T cells in the CNS of EAE mice. Thus, this study for the first time, showed that gut commensal *P. histicola* can suppress disease in HLA-DR3.DQ8 transgenic mice model of MS as effectively as IFNβ and both *P. histicola* and IFNβ utilize some common regulatory pathways including reduced activation of microglia, astrocytes and downregulation of pro-inflammatory immune response in the CNS.

The importance of *Prevotella* in MS can be highlighted by a number of studies showing a lower abundance of *Prevotella* in untreated MS patient, compared to healthy control (8–10, 14). Additionally, the abundance of *Prevotella* was increased in MS patients treated with disease-modifying drugs such as IFNβ and Copaxone (9, 15). We observed that a specific strain of *Prevotella* (*P. histicola*) can suppress disease in EAE mice. This is in line with our previous study, where we showed that *P. histicola* suppressed disease in a dose dependent manner (5). Our finding is further supported by the study showing that

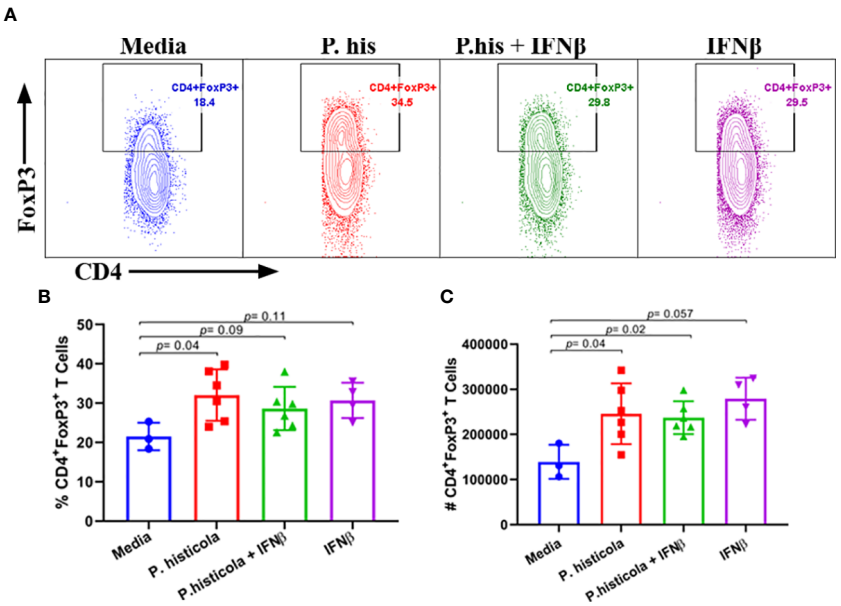


FIGURE 5 | Pre-treatment with *P. histicola* alone or in combination with IFNβ increases $CD4^+FoxP3^+$ regulatory T cells in the gut-associated lymphoid tissue (GALT). **(A)** Naïve mice were treated with IFNβ (seven doses), *P. histicola* (seven doses), or a combination of both (with treatment administered on alternate days for a total of 14 doses). Gut-associated lymphoid cells were isolated from treated and the control group of mice and stained with CD45, CD4, and FoxP3 antibodies. Representative flow cytometric plots to demonstrate $CD4^+FoxP3^+$ regulatory T cells in GALT of mice treated with *P. histicola* alone, IFNβ alone, *P. histicola* and IFNβ, or media. **(B)** Frequency of $CD4^+FoxP3^+$ regulatory T cells from mice treated IFNβ, *P. histicola*, *P. histicola* plus IFNβ, and media. **(C)** Quantification of the number of $CD4^+FoxP3^+$ regulatory T cells in mice treated as in **(A)**. Error bars are presented as the standard error of the mean. The *p*-value determined by the Mann-Whitney unpaired U test for comparing each group to media. Criteria for setting positive gates for specific flourochrome and gating strategy for different cell populations had been provided in **Supplementary Figure 6**. The data presented represent one of three experiments performed at different time points (n ≥ 3 mice per group).

P. histicola can also suppress disease in an animal model of rheumatoid arthritis (29). Our study, for the first-time report that IFNβ can suppress disease in HLA-DR3.DQ8 transgenic mice. This is in agreement with earlier studies showing disease suppressive role of IFNβ in C57BL/6 (30) and SJL mice (31). Interestingly, we observed that a combination of *P. histicola* and IFNβ was not more effective in suppressing disease than either treatment alone. Previously, using Copaxone, we also did not observe any additive effect of *P. histicola* and Copaxone (6). One potential explanation for this phenomenon is that *P. histicola*, and IFNβ have the same or a similar mechanism of action, thus their effect may be convergent rather than synergistic.

CNS pathology is the hallmark of EAE/MS. Reduced inflammation and demyelination in *P. histicola* alone, IFNβ alone, and combination group (*P. histicola* plus IFNβ) indicate that disease suppression was accompanied with reduced CNS pathology. Lower disease in treatment groups was accompanied by lower microglia and astrocytes activation in the CNS. Enhanced activation of CNS resident microglia has been shown to play an important role in pathogenesis of EAE by promoting autoreactive T-cell responses through its ability to function as antigen presenting cells and neutralization of microglial activation can suppressed the development of EAE (25, 32–34). Similarly, reactive astrocytes are the main source of pro-inflammatory cytokine that plays a critical role in breaching the blood brain barrier (BBB) and induce recruitment of pathogenic immune cells into the CNS during MS and EAE (35–37). Astrocytes depletion is associated with reduced inflammation and demyelination in EAE mice (38). IFNβ and/or *P. histicola* can modulate neuroinflammatory properties of microglia and astrocytes by reducing Th17 cells infiltration into the CNS, as a higher CNS infiltration of Th17 cells can augment their neuroinflammatory properties of microglia and astrocytes (26). Thus, our data suggest that IFNβ and/or *P. histicola* induced anti-inflammatory environment might reduce microglia and astrocytes activation resulting in disease suppression.

MS and EAE are mediated by pro-inflammatory Th1 and Th17 T-cells (39–43). Reduced level of $IL17^+$, and $IFN\gamma^+$ CD4 T cell in the CNS of EAE mice are in line with current hypothesis that Th1 and Th17 play a pathogenic role in EAE (40, 44). Previously we have shown that *P. histicola* alone or combination with disease modifying drug Copaxone decreased $IL17^+$ and $IFN\gamma^+$ $CD4^+$ T cells infiltrating the CNS of HLA-DR3.DQ8 mice (6). Further, IFNβ treatment had been shown to reduce Th1 and Th17 cells as well as other inflammatory cytokines (45). Additionally, IFNβ treatment

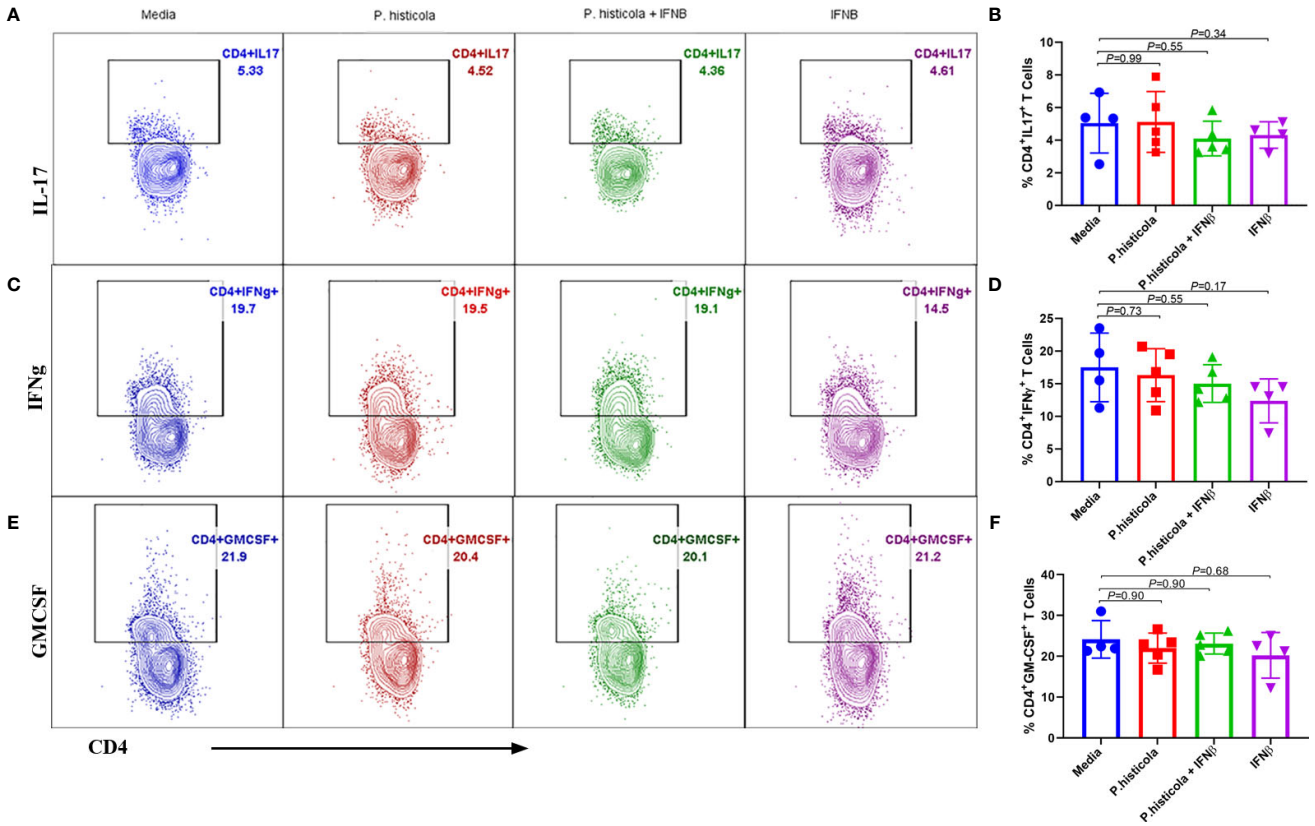


FIGURE 6 | Treatment with *P. histicola* alone, IFNβ alone or *P. histicola* plus IFNβ do not modulate $CD4^+IL17^+$, $CD4^+IFN\gamma^+$, and $CD4^+GM\text{-}CSF^+$ cells frequency in the GALT of naïve mice. Mice were treated with IFNβ (seven doses), *P. histicola* (seven doses), or a combination of both (with treatment administered on alternate days for a total of 14 doses, 7 doses of IFNβ and 7 doses of *P. histicola*). Flow cytometric were done to study $IL17^+$, $IFN\gamma^+$, and $GM\text{-}CSF^+$ expressing CD4 T cells that were isolated from gut-associated lymphoid tissue of mice treated as mentioned. Cells were previously gated on lymphocytes and singlets. **(A)** Representative flow cytometric plots to demonstrate $CD4^+IL17^+$ T cells in the GALT of mice treated with *P. histicola* alone, IFNβ alone, *P. histicola* and IFNβ, or media. **(B)** Frequency of $CD4^+IL17^+$ T cells from mice treated as in **(A)**. **(C)** Representative flow cytometric plots to demonstrate $CD4^+$ $IFN\gamma^+$ T cells in the GALT of mice treated as in **(A)**. **(D)** Frequency of $CD4^+$ $IFN\gamma^+$ T cells from mice treated as in **(A)**. **(E)** Representative flow cytometric plots to demonstrate $CD4^+GM\text{-}CSF^+$ T cells in the GALT of mice treated as in **(A)**. **(F)** Frequency of $CD4^+GM\text{-}CSF^+$ T cells from mice treated as in **(A)**. Gating strategy for different population had been provided in **Supplementary Figure 7**. Error bars presented as standard error of the mean. *P*-value determined by Mann-Whitney unpaired t-test for comparing each group to media.

can inhibits IL-17 differentiation and induces IL-10 secretion in the T cells from the MS patients (46). Thus, our study suggests that *P. histicola* and IFNβ both suppressed disease by reducing pathogenic Th1 and Th17 cells in the CNS of EAE mice.

A number of therapeutic interventions in EAE had been shown to work through induction of Tregs and IL-10. We observed that *P. histicola* alone or in combination with IFNβ induced $CD4^+FoxP3^+$ Treg cells in the GALT of mice. Previous studies have shown that a single bacterium *P. histicola*, or *B. fragilis*, or a mixture *of Lactobacillus* species, or a mixture of *Clostridium* species can suppress EAE disease by inducing $CD4^+FoxP3^+$ Treg cells (5, 6, 47–49). Interestingly, we found that IFNβ alone treatment does not affect Treg cells in the gut. While IFNβ is associated with an increase in anti-inflammatory cytokines and reduced trafficking across the BBB, IFNβ is not associated with a modulation of Treg populations in mice (50). This is in contrast with effect of IFNβ in RRMS patients, where IFNβ treatment caused an increase in $PD1^-$ Treg cells compared with $PD1^+$ Treg in peripheral blood and CSF (51). The difference in our findings and Saresella et al. may be due to physiological differences between human and mice. Altogether, our study suggests that *P. histicola* can mediate disease suppressive effect through the induction of $CD4^+FoxP3^+$ Treg cells. Although the mechanism through which *P. histicola* induce Treg is not well understood. We hypothesize that *P. histicola* can induce Tregs in the gut through metabolism of dietary compounds (52).

In summary, our study suggests that *P. histicola* and IFNβ possesses both overlapping as well as non-overlapping modes of action in HLA-DR3.DQ8 transgenic mice. For example, *P. histicola* or IFNβ or combined treatment reduced the level of pro-inflammatory Th1 and Th17 cells, and reduced microglia and astrocytes activation in CNS of EAE mice but only groups

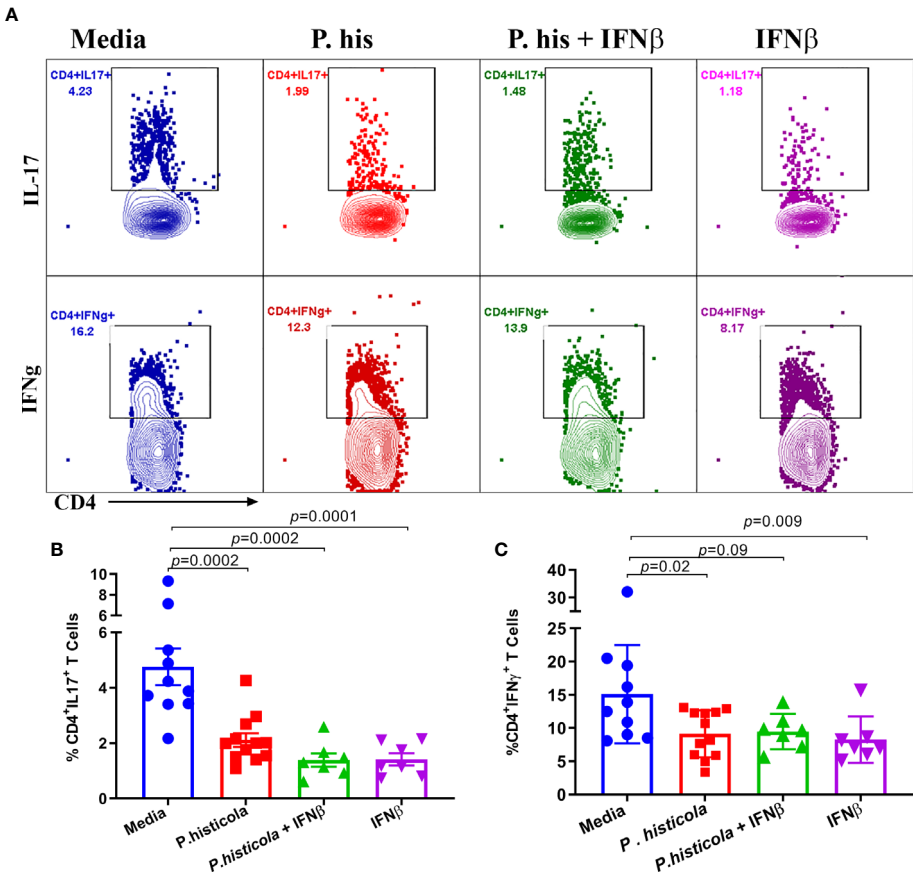


FIGURE 7 | Treatment with *P. histicola* alone, IFNβ alone or *P. histicola* plus IFNβ modulate $CD4^+IL17^+$, and $CD4^+IFN\gamma^+$, T cells frequency in the CNS of mice. Mice were immunized with PLP_{91-110}/CFA plus pertussis toxin on days 0 and 2 of the disease induction and 1 week later mice were treated with IFNβ (seven doses), *P. histicola* (seven doses), or a combination of both (with treatment administered on alternate days for a total of 14 doses, 7 doses of IFNβ and 7 doses of *P. histicola*). Clinical scores were assessed daily for the duration of the experiment. Flow cytometric plots of $IL17^+$ or $IFN\gamma^+$-expressing mononuclear lymphoid cells were isolated from the brain and spinal cord of mice from all groups. Cells were isolated and stimulated with antigen (PLP_{91-110}) plus Brefeldin A for 12 h. **(A)** Repregentative flow cytometric plots to demonstrate $CD4^+IL17^+$T cells, and $CD4^+IFN\gamma^+$ T cells in the CNS of mice treated as above. **(B)** Quantification of the frequency of $CD4^+IL17^+$T cells, and $CD4^+IFN\gamma^+$ T cells **(C)** from mice treated as in A. Cells were first gated on lymphocytes, singlets, and $CD4^+$ cells. Gating strategy for different population had been provided in **Supplementary Figure 8**. The data presented are the average of two independent experiments with n ≥ 4 mice per group. The *p*-value determined by Mann-Whitney unpaired U test.

receiving *P. histicola* induced anti-inflammatory Treg cells in the GALT. In conclusion, our present study, for the first time report that human gut commensal bacteria *P. histicola* suppresses EAE in HLA-DR3.DQ8 transgenic mice as effectively as commonly used MS drug IFNβ. As the gut microbiota seems to play an important role in the pathobiology of MS, beneficial gut bacteria such as *P. histicola* can provide additional treatment option for MS as well as other autoimmune inflammatory diseases.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee at the University of Iowa. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

AKM conceptualized the study, designed and performed the experiments, and gave final approval of the manuscript to be published. SKS designed and performed the experiments, analyzed the data, and wrote the manuscript; SNJ helped with experimental design and performing experiment. ACM performed mouse genotyping, KG-C performed all histopathology and

immunostaining. NT and HG performed western blot experiments. JZ, JM, and NK helped with the study design and interpretation of the data. All authors contributed to the article and approved the submitted version.

FUNDING

The authors acknowledge funding from the National Multiple Sclerosis Society (RG 5138A1/1T), NIAID/NIH (1R01AI137075-01), a Carver Trust Medical Research Initiative Grant, and the University of Iowa Environmental Health Sciences Research Center, NIEHS/NIH (P30 ES005605). SF was supported on an institutional training grant (T32AI007485 to Dr. Gail Bishop) and diversity supplement award to AKM on patent 1R01AI137075.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.578648/full#supplementary-material

REFERENCES

1. Barcellos LF, Oksenberg JR, Begovich AB, Martin ER, Schmidt S, Vittinghoff E, et al. HLA-DR2 dose effect on susceptibility to multiple sclerosis and influence on disease course. *Am J Hum Genet* (2003) 72(3):710–6. doi: 10.1086/367781
2. Dyment DA, Herrera BM, Cader MZ, Willer CJ, Lincoln MR, Sadovnick AD, et al. Complex interactions among MHC haplotypes in multiple sclerosis: susceptibility and resistance. *Hum Mol Genet* (2005) 14(14):2019–26. doi: 10.1093/hmg/ddi206
3. Das P, Drescher KM, Geluk A, Bradley DS, Rodriguez M, David CS. Complementation between specific HLA-DR and HLA-DQ genes in transgenic mice determines susceptibility to experimental autoimmune encephalomyelitis. *Hum Immunol* (2000) 61(3):279–89. doi: 10.1016/S0198-8859(99)00135-4
4. Mangalam A, Luckey D, Basal E, Jackson M, Smart M, Rodriguez M, et al. HLA-DQ8 (DQB1*0302)-restricted Th17 cells exacerbate experimental autoimmune encephalomyelitis in HLA-DR3-transgenic mice. *J Immunol* (2009) 182(8):5131–9. doi: 10.4049/jimmunol.0803918
5. Mangalam A, Shahi SK, Luckey D, Karau M, Marietta E, Luo N, et al. Human Gut-Derived Commensal Bacteria Suppress CNS Inflammatory and Demyelinating Disease. *Cell Rep* (2017) 20(6):1269–77. doi: 10.1016/j.celrep.2017.07.031
6. Shahi SK, Freedman SN, Murra AC, Zarei K, Sompallae R, Gibson-Corley KN, et al. Prevotella histicola, A Human Gut Commensal, Is as Potent as COPAXONE(R) in an Animal Model of Multiple Sclerosis. *Front Immunol* (2019) 10:462. doi: 10.3389/fimmu.2019.00462
7. Ascherio A, Munger KL. Epidemiology of Multiple Sclerosis: From Risk Factors to Prevention-An Update. *Semin Neurol* (2016) 36(2):103–14. doi: 10.1055/s-0036-1579693
8. Chen J, Chia N, Kalari KR, Yao JZ, Novotna M, Paz Soldan MM, et al. Multiple sclerosis patients have a distinct gut microbiota compared to healthy controls. *Sci Rep* (2016) 6:28484. doi: 10.1038/srep28484
9. Jangi S, Gandhi R, Cox LM, Li N, von Glehn F, Yan R, et al. Alterations of the human gut microbiome in multiple sclerosis. *Nat Commun* (2016) 7:12015. doi: 10.1038/ncomms12015
10. Miyake S, Kim S, Suda W, Oshima K, Nakamura M, Matsuoka T, et al. Dysbiosis in the Gut Microbiota of Patients with Multiple Sclerosis, with a Striking Depletion of Species Belonging to Clostridia XIVa and IV Clusters. *PloS One* (2015) 10(9):e0137429. doi: 10.1371/journal.pone.0137429
11. Tremlett H, Fadrosh DW, Faruqi AA, Hart J, Roalstad S, Graves J, et al. Gut microbiota composition and relapse risk in pediatric MS: A pilot study. *J Neurol Sci* (2016) 363:153–7. doi: 10.1016/j.jns.2016.02.042
12. Colpitts SL, Kasper EJ, Keever A, Liljenberg C, Kirby T, Magori K, et al. A bidirectional association between the gut microbiota and CNS disease in a biphasic murine model of multiple sclerosis. *Gut Microbes* (2017) 8(6):561–73. doi: 10.1080/19490976.2017.1353843
13. Ochoa-Reparaz J, Kasper LH. Gut microbiome and the risk factors in central nervous system autoimmunity. *FEBS Lett* (2014) 588(22):4214–22. doi: 10.1016/j.febslet.2014.09.024
14. Cosorich I, Dalla-Costa G, Sorini C, Ferrarese R, Messina MJ, Dolpady J, et al. High frequency of intestinal TH17 cells correlates with microbiota alterations and disease activity in multiple sclerosis. *Sci Adv* (2017) 3(7):e1700492. doi: 10.1126/sciadv.1700492
15. Castillo-Alvarez F, Perez-Matute P, Oteo JA, Marzo-Sola ME. The influence of interferon beta-1b on gut microbiota composition in patients with multiple sclerosis. *Neurologia* (2018) S0213-4853(18)30158-0. doi: 10.1016/j.nrl.2018.04.006
16. Marziniak M, Meuth S. Current perspectives on interferon Beta-1b for the treatment of multiple sclerosis. *Adv Ther* (2014) 31(9):915–31. doi: 10.1007/s12325-014-0149-1
17. Weinstock-Guttman B. An update on new and emerging therapies for relapsing-remitting multiple sclerosis. *Am J Manag Care* (2013) 19(17 Suppl):s343–54.
18. Rio J, Nos C, Tintore M, Tellez N, Galan I, Pelayo R, et al. Defining the response to interferon-beta in relapsing-remitting multiple sclerosis patients. *Ann Neurol* (2006) 59(2):344–52. doi: 10.1002/ana.20740
19. Shahi SK, Freedman SN, Dahl RA, Karandikar NJ, Mangalam AK. Scoring disease in an animal model of multiple sclerosis using a novel infrared-based automated activity-monitoring system. *Sci Rep* (2019) 9(1):19194. doi: 10.1038/s41598-019-55713-7
20. Agasing AM, Gawde S, Kumar G, Turner E, Axtell RC. B cell function impacts the efficacy of IFN-beta therapy in EAE. *J Neuroimmunol* (2020) 338:577106. doi: 10.1016/j.jneuroim.2019.577106
21. Mangalam AK, Luo N, Luckey D, Papke L, Hubbard A, Wussow A, et al. Absence of IFN-gamma increases brain pathology in experimental autoimmune encephalomyelitis-susceptible DRB1*0301.DQ8 HLA transgenic mice through secretion of proinflammatory cytokine IL-17 and induction of pathogenic monocytes/microglia into the central nervous system. *J Immunol* (2014) 193(10):4859–70. doi: 10.4049/jimmunol.1302008
22. Zhang J, Bardos T, Li D, Gal I, Vermes C, Xu J, et al. Cutting edge: regulation of T cell activation threshold by CD28 costimulation through targeting Cbl-b for ubiquitination. *J Immunol* (2002) 169(5):2236–40. doi: 10.4049/jimmunol.169.5.2236
23. Pino PA, Cardona AE. Isolation of brain and spinal cord mononuclear cells using percoll gradients. *J Vis Exp* (2011) 2(48):2348. doi: 10.3791/2348
24. Couter CJ, Surana NK. Isolation and Flow Cytometric Characterization of Murine Small Intestinal Lymphocytes. *J Vis Exp* (2016) 8(111):54114. doi: 10.3791/54114
25. Fabriek BO, Van Haastert ES, Galea I, Polfliet MM, Dopp ED, Van Den Heuvel MM, et al. CD163-positive perivascular macrophages in the human CNS express molecules for antigen recognition and presentation. *Glia* (2005) 51(4):297–305. doi: 10.1002/glia.20208
26. Prajeeth CK, Kronisch J, Khorooshi R, Knier B, Toft-Hansen H, Gudi V, et al. Effectors of Th1 and Th17 cells act on astrocytes and augment their neuroinflammatory properties. *J Neuroinflammation* (2017) 14(1):204. doi: 10.1186/s12974-017-0978-3
27. O'Connor RA, Anderton SM. Foxp3+ regulatory T cells in the control of experimental CNS autoimmune disease. *J Neuroimmunol* (2008) 193(1-2):1–11. doi: 10.1016/j.jneuroim.2007.11.016
28. Itani FR, Sinha S, Brate AA, Pewe LL, Gibson-Corley KN, Harty JT, et al. Suppression of autoimmune demyelinating disease by preferential stimulation of CNS-specific CD8 T cells using Listeria-encoded neuroantigen. *Sci Rep* (2017) 7(1):1519. doi: 10.1038/s41598-017-01771-8

29. Marietta EV, Murray JA, Luckey DH, Jeraldo PR, Lamba A, Patel R, et al. Suppression of Inflammatory Arthritis by Human Gut-Derived Prevotella histicola in Humanized Mice. *Arthritis Rheumatol* (2016) 68(12):2878–88. doi: 10.1002/art.39785
30. Wang D, Ghosh D, Islam SM, Moorman CD, Thomason AE, Wilkinson DS, et al. IFN-beta Facilitates Neuroantigen-Dependent Induction of CD25+ FOXP3+ Regulatory T Cells That Suppress Experimental Autoimmune Encephalomyelitis. *J Immunol* (2016) 197(8):2992–3007. doi: 10.4049/jimmunol.1500411
31. Yu M, Nishiyama A, Trapp BD, Tuohy VK. Interferon-beta inhibits progression of relapsing-remitting experimental autoimmune encephalomyelitis. *J Neuroimmunol* (1996) 64(1):91–100. doi: 10.1016/0165-5728(95)00160-3
32. Gordon EJ, Myers KJ, Dougherty JP, Rosen H, Ron Y. Both anti-CD11a (LFA-1) and anti-CD11b (MAC-1) therapy delay the onset and diminish the severity of experimental autoimmune encephalomyelitis. *J Neuroimmunol* (1995) 62(2):153–60. doi: 10.1016/0165-5728(95)00120-2
33. Heppner FL, Greter M, Marino D, Falsig J, Raivich G, Hovelmeyer N, et al. Experimental autoimmune encephalomyelitis repressed by microglial paralysis. *Nat Med* (2005) 11(2):146–52. doi: 10.1038/nm1177
34. Kant R, Halder SK, Fernandez JA, Griffin JH, Milner R. Activated Protein C Attenuates Experimental Autoimmune Encephalomyelitis Progression by Enhancing Vascular Integrity and Suppressing Microglial Activation. *Front Neurosci* (2020) 14:333. doi: 10.3389/fnins.2020.00333
35. Wang D, Ayers MM, Catmull DV, Hazelwood LJ, Bernard CC, Orian JM. Astrocyte-associated axonal damage in pre-onset stages of experimental autoimmune encephalomyelitis. *Glia* (2005) 51(3):235–40. doi: 10.1002/glia.20199
36. Tanuma N, Sakuma H, Sasaki A, Matsumoto Y. Chemokine expression by astrocytes plays a role in microglia/macrophage activation and subsequent neurodegeneration in secondary progressive multiple sclerosis. *Acta Neuropathol* (2006) 112(2):195–204. doi: 10.1007/s00401-006-0083-7
37. Brambilla R. The contribution of astrocytes to the neuroinflammatory response in multiple sclerosis and experimental autoimmune encephalomyelitis. *Acta Neuropathol* (2019) 137(5):757–83. doi: 10.1007/s00401-019-01980-7
38. Allnoch L, Baumgartner W, Hansmann F. Impact of Astrocyte Depletion upon Inflammation and Demyelination in a Murine Animal Model of Multiple Sclerosis. *Int J Mol Sci* (2019) 20(16):3922. doi: 10.3390/ijms20163922
39. McFarland HF, Martin R. Multiple sclerosis: a complicated picture of autoimmunity. *Nat Immunol* (2007) 8(9):913–9. doi: 10.1038/ni1507
40. O'Connor RA, Prendergast CT, Sabatos CA, Lau CW, Leech MD, Wraith DC, et al. Cutting edge: Th1 cells facilitate the entry of Th17 cells to the central nervous system during experimental autoimmune encephalomyelitis. *J Immunol* (2008) 181(6):3750–4. doi: 10.4049/jimmunol.181.6.3750
41. Tzartos JS, Friese MA, Craner MJ, Palace J, Newcombe J, Esiri MM, et al. Interleukin-17 production in central nervous system-infiltrating T cells and glial cells is associated with active disease in multiple sclerosis. *Am J Pathol* (2008) 172(1):146–55. doi: 10.2353/ajpath.2008.070690
42. Compston A, Coles A. Multiple sclerosis. *Lancet* (2002) 359(9313):1221–31. doi: 10.1016/S0140-6736(02)08220-X
43. Damsker JM, Hansen AM, Caspi RR. Th1 and Th17 cells: adversaries and collaborators. *Ann N Y Acad Sci* (2010) 1183:211–21. doi: 10.1111/j.1749-6632.2009.05133.x
44. Domingues HS, Mues M, Lassmann H, Wekerle H, Krishnamoorthy G. Functional and pathogenic differences of Th1 and Th17 cells in experimental autoimmune encephalomyelitis. *PloS One* (2010) 5(11):e15531. doi: 10.1371/journal.pone.0015531
45. Zhao Q, Cheng W, Xi Y, Cao Z, Xu Y, Wu T, et al. IFN-beta regulates Th17 differentiation partly through the inhibition of osteopontin in experimental autoimmune encephalomyelitis. *Mol Immunol* (2018) 93:20–30. doi: 10.1016/j.molimm.2017.11.002
46. Ramgolam VS, Sha Y, Jin J, Zhang X, Markovic-Plese S. IFN-beta inhibits human Th17 cell differentiation. *J Immunol* (2009) 183(8):5418–27. doi: 10.4049/jimmunol.0803227
47. Lavasani S, Dzhambazov B, Nouri M, Fak F, Buske S, Molin G, et al. A novel probiotic mixture exerts a therapeutic effect on experimental autoimmune encephalomyelitis mediated by IL-10 producing regulatory T cells. *PloS One* (2010) 5(2):e9009. doi: 10.1371/journal.pone.0009009
48. Ochoa-Reparaz J, Mielcarz DW, Wang Y, Begum-Haque S, Dasgupta S, Kasper DL, et al. A polysaccharide from the human commensal Bacteroides fragilis protects against CNS demyelinating disease. *Mucosal Immunol* (2010) 3(5):487–95. doi: 10.1038/mi.2010.29
49. Atarashi K, Tanoue T, Oshima K, Suda W, Nagano Y, Nishikawa H, et al. Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota. *Nature* (2013) 500(7461):232–6. doi: 10.1038/nature12331
50. Kay M, Hojati Z, Dehghanian F. The molecular study of IFNbeta pleiotropic roles in MS treatment. *Iran J Neurol* (2013) 12(4):149–56.
51. Saresella M, Marventano I, Longhi R, Lissoni F, Trabattoni D, Mendozzi L, et al. CD4+CD25+FoxP3+PD1- regulatory T cells in acute and stable relapsing-remitting multiple sclerosis and their modulation by therapy. *FASEB J* (2008) 22(10):3500–8. doi: 10.1096/fj.08-110650
52. Freedman SN, Shahi SK, Mangalam AK. The "Gut Feeling": Breaking Down the Role of Gut Microbiome in Multiple Sclerosis. *Neurotherapeutics* (2018) 15(1):109–25. doi: 10.1007/s13311-017-0588-x

Conflict of Interest: AKM and JM are inventors of a technology claiming the use of *Prevotella histicola* for the treatment of autoimmune diseases. The patent for the technology is owned by Mayo Clinic, who has given exclusive license to Evelo Biosciences. AKM and JM received royalties from Mayo Clinic (paid by Evelo Biosciences).

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

frontiers in Immunology

ORIGINAL RESEARCH
published: 16 December 2020
doi: 10.3389/fimmu.2020.590783

Alterations of the Fecal Microbiota in Chinese Patients With Multiple Sclerosis

Zongxin Ling[1†], Yiwen Cheng[1†], Xiumei Yan[2†], Li Shao[3,4†], Xia Liu[5†], Dajin Zhou[2], Lijuan Zhang[2], Kunqiang Yu[2] and Longyou Zhao[2*]*

[1] Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, National Clinical Research Center for Infectious Diseases, the First Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou, China, [2] Department of Laboratory Medicine, Lishui Second People's Hospital, Lishui, China, [3] Hangzhou Normal University, Hangzhou, China, [4] Institute of Translational Medicine, The Affiliated Hospital of Hangzhou Normal University, Hangzhou, China, [5] Department of Intensive Care Unit, the First Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou, China

OPEN ACCESS

Edited by:
Lloyd Kasper,
Dartmouth College, United States

Reviewed by:
Ashutosh K. Mangalam,
The University of Iowa, United States
Gurumoorthy Krishnamoorthy,
Max Planck Institute of Biochemistry, Germany

***Correspondence:**
Zongxin Ling
lingzongxin@zju.edu.cn
Longyou Zhao
zly8897@126.com

[†]These authors have contributed equally to this work

Specialty section:
This article was submitted to Multiple Sclerosis and Neuroimmunology, a section of the journal Frontiers in Immunology

Received: 24 August 2020
Accepted: 16 November 2020
Published: 16 December 2020

Citation:
Ling Z, Cheng Y, Yan X, Shao L, Liu X, Zhou D, Zhang L, Yu K and Zhao L (2020) Alterations of the Fecal Microbiota in Chinese Patients With Multiple Sclerosis. Front. Immunol. 11:590783. doi: 10.3389/fimmu.2020.590783

Mounting evidence indicates that alterations in the intestinal microbiota may be associated with neurological disorders such as multiple sclerosis (MS). MS is a putative autoimmune disease of the central nervous system. However, it has not been determined whether the intestinal microbiota and host immune status are altered in Chinese patients with stable MS. In our study, 22 Chinese patients with stable MS and 33 healthy controls were enrolled for fecal microbiota analysis and host immunity evaluation. The microbial diversity and composition, bacterial co-occurrence correlations, predictive functional profiles, and microbiota-cytokine correlations between the two groups were compared. We observed that while the overall structure of the fecal microbiota did not change significantly, the abundances of several key functional bacteria, primarily *Faecalibacterium*, decreased remarkably. *Faecalibacterium* and *Granulicatella* could be used to distinguish between patients with MS and healthy controls with an area under the curve of 0.832. PiCRUSt analysis revealed that genes associated with fructose, mannose, and fatty acid metabolism were significantly enriched in the MS microbiota. In addition, we also observed that the levels of several pro- and anti-inflammatory cytokines and chemokines, such as IL-1ra, IL-8, IL-17, and TNF-α changed observably, and the abundances of key functional bacteria like butyrate producers correlated with the changes in the cytokine levels. Our present study indicated that altered composition of the fecal microbiota might play vital roles in the etiopathogenesis of MS by regulating host immunity, which suggests that microbiota-targeting patient-tailored early intervention techniques might serve as novel therapeutic approaches for MS.

Keywords: multiple sclerosis, *Faecalibacterium*, IL-17, fecal microbiota, butyrate

INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system with an autoimmune mechanism of development, which is one of the leading causes of disability in young adults. MS affects approximately 2.3 million people worldwide, with a prevalence of 50–300/100,000 (1). The prevalence of MS has increased substantially in many regions since 1990, including China (2). Thus far, there are no comprehensive studies available on the national prevalence of MS. However, several regional studies have investigated the prevalence of MS in China and have indicated increasing prevalence over time (3–5). A study conducted by Cheng et al. revealed that the crude MS prevalence rate was 1.39 per 100,000 individuals in Shanghai in 2004 to 2005 and the female-to-male ratio was 1.8 (6). Another study estimated the prevalence of MS to be 3.7 and 6.7 cases per 100,000 individuals among males and females, respectively, based on hospital data for 2013 in Shandong Province (7). To date, more than 100,000 validated cases of MS have been diagnosed in China (2). Recently, there has been significant progress in the understanding of genetic and environmental factors underlying the condition. Compared to genetic factors, environmental factors, such as infection with Epstein-Barr virus, cigarette smoking, and low vitamin D levels owing to insufficient exposure to sunlight, play a greater role in susceptibility to the condition (8). However, the underlying cause of this disease remains elusive.

In humans, a complex cross-talk is required between the gut microbiota and the host immune system to maintain host homeostasis. The gut microbiota can influence the development of host immunity, and consequently, the immune system regulates the microbiota through gut barrier maintenance and immune exclusion. Mounting evidence suggests that the gut microbiota plays vital roles in various autoimmune disorders, including MS, which can contribute significantly to both susceptibility and protection (9–11). Studies using the experimental autoimmune encephalomyelitis model, which is most commonly used as an animal model of MS, have successfully confirmed that alterations in the gut microbiota are a potential risk factor for autoimmune diseases such as MS. Yokote et al. reported that the disruption of gut microbiota upon the administration of oral antibiotics reduces the severity of conventional experimental autoimmune encephalomyelitis (12). Moreover, using a relapsing-remitting mouse model of spontaneously developing experimental autoimmune encephalomyelitis, Berer et al. demonstrated that transgenic SJL/J mice raised in germ-free conditions are protected from the disease, while re-colonization with indigenous bacteria in the gut restored its susceptibility (13). These studies using animal models indicate that dysbiosis of the gut microbiota may play a central role in the development of MS.

Recently, several groups have profiled the fecal microbiota of patients with MS from western developed countries and have shown that patients with MS exhibit gut microbial dysbiosis with both depletion and enrichment of certain bacteria compared to healthy subjects. Jangi et al. reported an increased abundance of *Methanobrevibacter* and *Akkermansia* and decreased abundance of *Prevotella* in patients with MS. After successful treatment by disease-modifying therapy, the abundance of *Prevotella* increased significantly (10). Other studies also showed that the abundance of genus *Prevotella* decreases in patients with MS (9, 11). *Prevotella copri* has previously been found to be enriched in patients with new-onset rheumatoid arthritis, which is also an autoimmune disease (14). Therefore, the reduced abundance of *Prevotella* observed across multiple MS microbiome studies conducted in different geographical locations suggests that this bacterium might play an important role in MS. Miyake et al. (11) and Cantarel et al. (15) also observed that the abundance of *Fecalibacterium* was low in patients with MS compared to that in healthy controls. *Faecalibacterium prausnitzii* is an anti-inflammatory commensal bacterium identified in the gut microbiota analysis of patients with Crohn's disease (16). In addition, Jangi et al. also observed alterations in the human gut microbiome in MS that correlate with changes in the host immune transcriptome and treatment. The gut microbiota can modulate host immune gene expression by establishing direct contact with cell wall components or by secreting factors such as short-chain fatty acids (SCFAs) (17, 18). Collectively, altered gut microbiota is considered to be related to MS pathogenesis and may serve as an important consideration in novel preventive or therapeutic strategies for MS in future.

To date, there have been no studies on the examination of gut microbiota in the Chinese MS-affected population. The environmental conditions, economic status, ethnic background, lifestyles, and long-term diets in China are distinct from those in western developed countries, which would affect the overall structure and composition of the gut microbiota and its roles and mechanisms in the pathogenesis of MS. In our study, we compared fecal microbiota specimens from patients with stable MS to those from healthy controls using high-throughput sequencing technique and attempted to explore the potential key functional fecal microbiota that were associated with MS in the patients studied. We observed that the abundance of several predominant genus-level taxa known to include butyrate producers decreased in the MS cohort, which indicated that altered composition of the gut bacteria might participate actively in the development of MS. Our study sheds light on the roles and mechanisms underlying the function of gut microbiota in MS pathogenesis, which will help identify novel microbiota-targeted biomarkers for noninvasive diagnosis and MS treatment.

MATERIALS AND METHODS

Subjects' Enrollment

The protocols for the present study were reviewed and approved by the Ethics Committee of Lishui Second People's Hospital (Zhejiang, China). Informed written consent was obtained from each participant before enrollment. Twenty-two patients with MS, who were diagnosed based on the 2005 McDonald criteria (19), were recruited from Zhejiang province (China) from February 2019 to July 2019, and thirty-three age- and sex-matched healthy subjects were recruited as controls (**Table 1**). The cases were stable, and not of new onset or active relapse, and had not been treated with steroids, beta-interferon/glatiramer acetate, or other immunosuppressive medications in the preceding 3 months. The following exclusion criteria were established: age < 20 years; body mass index (weight in

TABLE 1 | Summary of the study subjects' characteristics.

Characteristics	Control (n = 33)	Patients (n = 22)
Age (means ± SD)	34.5 ± 8.2	35.0 ± 7.1
Gender(Female/male)	21/12	14/8
BMI(means ± SD)	23.85 ± 3.56	24.18 ± 3.24
Complications, no		
Hypertension	0	0
Diabetes mellitus	0	0
Hyperlipidemia	0	1
Irritable bowel syndrome	0	0
Autoimmune liver disease	0	0
Active infections	0	0
Antibiotics use within 1 month, no	0	0
Yogurt use within 1 month, no	0	0
Immunosuppressive medications within 3 months	0	0

BMI, body mass index; SD, standard deviation.

kilograms divided by height in meters squared) > 30; pregnancy; hypertension; diabetes mellitus; use of antibiotics, probiotics, prebiotics, or synbiotics in the previous month; known active infections such as bacterial, fungal, chlamydial, or viral infections; and other diseases such as irritable bowel syndrome, inflammatory bowel disease, or other autoimmune diseases.

Fecal Sample Collection and DNA Extraction

Approximately 2 g of a fresh fecal sample was collected in a sterile plastic cup, and stored at −80°C after preparation within 15 min until use. Bacterial genomic DNA was extracted from 300 mg of homogenized feces using a QIAamp® DNA Stool Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions, with additional glass-bead beating steps on a Mini-beadbeater (FastPrep; Thermo Electron Corporation, Boston, MA, USA). The amount of DNA was determined using a NanoDrop ND-1000 spectrophotometer (Thermo Electron Corporation); the integrity and size were checked by 1.0% agarose gel electrophoresis containing 0.5 mg/ml ethidium bromide. All DNA was stored at −20°C before further analysis.

Amplicon Library Construction and Sequencing

Amplicon libraries were constructed with Illumina sequencing-compatible and barcode-indexed bacterial PCR primers 319F/806R, which target the V3–V4 regions of 16S rRNA gene (20). All PCR reactions were performed with KAPA HiFi HotStart ReadyMix using the manufacturer's protocol (KAPA Biosystems) and approximately 50 ng of extracted DNA per reaction. Thermocycling conditions were set at 95°C for 1 min, 55°C for 1 min, then 72°C for 1 min for 30 cycles, followed by a final extension at 72°C for 5 min. All PCR reactions were performed in 50 µl triplicates and combined after PCR. The amplicon library was prepared using a TruSeq™ DNA sample preparation kit (Illumina Inc, San Diego, CA, USA). Prior to sequencing, the PCR products were extracted with the MiniElute® Gel Extraction Kit (QIAGEN) and quantified on a NanoDrop ND-1000 spectrophotometer (Thermo Electron Corporation) and Qubit 2.0 Fluorometer (Invitrogen). The purified amplicons were then pooled in equimolar concentrations and the final concentration of the library was determined by Qubit (Invitrogen). Negative DNA extraction controls (lysis buffer and kit reagents only) were amplified and sequenced as contamination controls. Sequencing was performed on a MiSeq instrument (Illumina) using a 300 × 2 V3 kit together with PhiX Control V3 (Illumina) (21, 22).

Bioinformatic Analysis

The 16S rRNA gene sequence data set generated from the MiSeq run were first merged and demultiplexed into per samples using the QIIME version 1.9.0 with default parameters (23). Chimera sequences were detected and removed using the USEARCH software based on the UCHIME algorithm (24). Open-reference operational taxonomic unit (OTU) pick was then performed with USEARCH V7 referenced against Greengenes database version 13.8 at 97% sequence similarity (25, 26). OTUs with a number of sequences <0.005% of the total number of sequences were discarded as recommended (27). The result was an OTU table, which was used for subsequent downstream analysis.

For taxonomic assignment, the most abundant sequences were chosen as the representative sequences of corresponding OTUs. Taxonomic assignment of individual datasets were classified against the Greengenes database version 13.8 using both RDP classifier and UCLUST version 1.2.22 methods implemented in QIIME (26, 28). Any sequences that were identified as members of Eukarya, Archaea, Mitochondria, Chloroplasts, and Cyanobacteria lineages, were removed. Alpha diversity was calculated with QIIME software with Python scripts base on the sequence similarity at 97% level, including index of observed species, abundance-based coverage estimator (ACE), Chao1 estimator, Shannon, Simpson, Evenness, and PD whole tree. Sequence coverage was assessed in mothur by rarefaction curves and Good's coverage (29, 30). Beta diversity was measured by jaccard, bray-curtis, unweighted UniFrac, and weighted UniFrac distance calculated with 10 times of subsampling by QIIME. These distances were visualized by principal coordinate analysis (PCoA) (31). Hierarchical clustering was performed and heatmap was generated using a Spearman's rank correlation coefficient as a distance measure and a customized script developed in the R statistical package. The output file was further analyzed using Statistical Analysis of Metagenomic Profiles software package (STAMP) version 2.1.3 (32).

For the predictive functional analyses, PiCRUSt software package version 1.0.0 was used to identify predicted gene families and associated pathways from inferred metagenomes of taxa of interest identified from the compositional analyses, which was based on the fact that phylogeny and function are closely linked (33). Predicted functional genes were categorized into Clusters of Orthologous Groups (COG) and into Kyoto Encyclopedia of Genes and Genome (KEGG) orthology (KO), and compared across patient groups using STAMP. Pathways and enzymes were assigned using KEGG database options built into the pipeline. The pathways that were nonprokaryotic, had fewer than two sequences in each cohort, or had a difference in mean proportions less than 0.1% were excluded from analysis. The characterization of microorganismal features differentiating the gastric microbiota was performed using the linear discriminant analysis (LDA) effect size

(LEfSe) method (http://huttenhower.sph.harvard.edu/lefse/) for biomarker discovery, which emphasizes both statistical significance and biological relevance (34). With a normalized relative abundance matrix, LEfSe uses the Kruskal-Wallis rank sum test to detect features with significantly different abundances between assigned taxa and performs LDA to estimate the effect size of each feature. A significant alpha at 0.05 and an effect size threshold of 2 were used for all biomarkers discussed in this study.

Correlation analysis was performed using sparse compositional correlation (SparCC) algorithm on the complete OTU table collapsed to the genus level, which was introduced by Friedman and Alm and was known for its robustness to the compositional effects that are influenced by the diversity and sparsity of correlation in human microbiome data sets (35). SparCC was employed to represent co-abundance and co-exclusion networks between OTUs. For SparCC, 1000 bootstrap replicates were used to calculate significance values, and considered correlation coefficients greater or less than 0.2 and −0.2, respectively, and p-values <0.05. This set of iterative procedures were applied separately to normal, peritumor and tumor data sets to infer the basis correlation values within and/or between paired sampling sites. Visualization of the network was achieved using Cytoscape version 3.4.1.

Systemic Inflammatory Cytokines Analysis

Serum samples from these participants were obtained using their fasting blood in the early morning. Using a 27-plex magnetic bead based immunoassay kit (Bio-Rad, CA, USA), the following cytokines were quantified: interleukin-1β (IL-1β), IL-1 receptor antagonist (IL-1ra), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12(p70), IL-13, IL-15, IL-17, Eotaxin, Fibroblast growth factor-basic (FGF-basic), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophages colony-stimulating factor (GM-CSF), interferon gamma (IFN-γ), interferon gamma-inducible protein 10 (IP-10), monocyte chemotactic protein-1 (MCP-1), macrophages inflammatory protein-1α (MIP-1α), platelet-derived growth factor (PDGF-bb), MIP-1β, regulated upon activation normal T-cell expressed and secreted (RANTES), tumor necrosis factor-alpha (TNF-α), and vascular endothelial growth factor (VEGF). The Bio-Plex 200 system was utilized for the analysis of Bio-Rad 27-plex human group I cytokines and the Bio-Plex assay (Bio-Rad) was performed according to the manufacturer's directions. The results expressed as picogram per milliliter (pg/ml) using standard curves integrated into the assay and Bio-Plex Manager v6.0 software with reproducible intra- and inter-assay CV values of 5% to 8%.

Statistical Analysis

For continuous variables, independent *t*-test, White's nonparametric *t*-test, and Mann-Whitney *U*-test were applied. For categorical variables between groups, Pearson chi-square or Fisher's exact test was used, depending on assumption validity. For correlation analyses, Spearman's rank correlation test was used. Statistical analysis was performed using the SPSS V19.0 (SPSS Inc., Chicago, IL) and STAMP V2.1.3 (32). GraphPad Prism version 6.0 (San Diego, CA) was used for preparation of graphs. All tests of significance were two sided, and $p < 0.05$ or corrected $p < 0.05$ was considered statistically significant.

Accession Number

The sequence data from this study are deposited in the GenBank Sequence Read Archive with the accession number SRP258890.

RESULTS

Overall Bacterial Diversity in Fecal Microbiota in Patients With Stable Multiple Sclerosis

In our present study, the possible confounders of microbiota analyses, such as sex, age, and BMI between patients with stable MS and healthy controls did not differ significantly ($p > 0.05$). To investigate the alterations in fecal microbiota between the patients and healthy controls, we obtained 1,360,209 high-quality reads with an average of 24,731 reads per sample for the subsequent microbiota analysis. Good's estimator of coverage was 99.33%, which indicated that the identified reads represented the majority of bacterial sequences (909 OTUs identified) present in the fecal microbiota. The diversity indices and richness indices were used to assess the overall differences in microbial community structure between patients with MS and healthy controls. We observed that the alpha-diversity indices, such as Shannon and Simpson indices, did not differ significantly between patients with MS and healthy controls (**Supplementary Figures 1A–C**), and ACE and Chao1 for the observed species in patients with MS were similar to those in healthy controls (**Supplementary Figures 1D, E**). Owing to significant inter-individual variations, the two groups could not be divided into different clusters using principal coordinate analyses based on Jaccard, Bray-Curtis, unweighted UniFrac, and weighted UniFrac algorithms (**Supplementary Figures 1F–I**). However, the Adonis test yielded p-values of 0.009 for Jaccard and 0.012 for Bray-Curtis analyses, which indicated significant microbial differences between the two groups. The Venn diagram showed that 728 OTUs with a total richness of 909 were shared between the two groups, with less unique OTUs in patients with MS (**Supplementary Figure 1J**). The rarefaction and rank abundance curves for patients with MS were also similar to those for healthy controls (**Supplementary Figures 1K, L**). The alpha - and beta-diversity analyses indicated that the overall diversity of MS-associated fecal microbiota remained unaltered; however, a lower number of OTUs and phylotypes were observed in patients with MS.

Altered Composition of Fecal Microbiota in Patients With Multiple Sclerosis

With the RDP classifier, sequences from the fecal microbiota could be classified among nine phyla; Firmicutes and Bacteroidetes were the predominant phyla in both groups. **Figure 1** shows that the abundances of Proteobacteria, Lentisphaerae, Synergistetes, and Verrucomicrobia, which were the non-dominant phyla, differed significantly between patients with MS and healthy controls ($p < 0.05$), while the ratio of Firmicutes/Bacteroidetes did not differ significantly between the two groups. In addition, 62 families and

149 genera were identified in the fecal microbiota specimens from both groups. **Supplementary Figure 2** shows a heatmap of bacterial families present in the microbiota in patients with MS and healthy controls, which represents the relative percentages of most families identified in each sample. Our data indicated that there were no significant differences in the heatmap between the two groups. Discriminant analyses using LEfSe showed that 21 bacterial phylotypes differed significantly between the two groups (LDA score > 2.0, $p < 0.05$, **Figure 2A**). Among the differentially functional bacterial taxa, only two taxa, including *Blautia* and *Flavonifractor* (belonging to Firmicutes) were enriched in patients with MS, while the abundances of other taxa, such as *Faecalibacterium*, *Roseburia*, *Haemophilus*, *Bilophila*, *Dorea*, *Butyricicoccus*, *Gemella*, *Clostridium* XIVb, and *Granulicatella*, decreased significantly in patients with MS (**Figure 2B**). Notably, *Faecalibacterium*, *Roseburia*, *Dorea*, *Butyricicoccus*, and *Clostridium* XIVb (all belonging to Firmicutes) can produce various SCFAs, such as butyrate. The present data indicates that the compositional abnormalities observed in the fecal microbiota in patients with MS are associated with a reduced capacity for producing SCFAs, especially butyrate, in the presence of different substrates, which indicates that functional dysbiosis becomes more obvious as MS progresses.

The overall structure of the fecal microbiota results from dynamic interactions between members of the microbial community. A SparCC algorithm with false discovery rate adjustments was employed to generate correlation-based microbial interaction networks based on the relative abundance of OTUs between the two groups (**Figure 3**). We observed that the interaction networks in healthy controls are more complicated than those in MS patients. There were more positive correlations among the bacteria in healthy controls, while more negative correlations were observed in those in patients with MS.

We also assessed the potential of using fecal microbiota as biomarkers to distinguish between patients with MS and healthy controls. First, using a single differential bacteria as a predictor, we found that the area under the receiver operating characteristic curve ranged from 0.225 to 0.659 (**Figure 4**). Further, multivariable stepwise logistic regression analysis was performed using the MS-associated genera to identify taxa that helped create the most significant distinction between patients with MS and controls. We observed that the best and simplest combination was that of *Faecalibacterium* and *Granulicatella*, which could help distinguish between patients with MS and healthy controls with an area under the curve (AUC) of 0.832. However, the other combinations did not significantly improve the predictive performance (AUC ≤ 0.832).

Functional Changes in Multiple Sclerosis-Associated Fecal Microbiota

To study the functional and metabolic changes in the microbial communities between MS and controls, we retrieved the metagenomes from the 16S rRNA data and analyzed the functional potential of the fecal microbiota using PiCRUSt based on closed-reference OTU picking. We compared 64 Kyoto

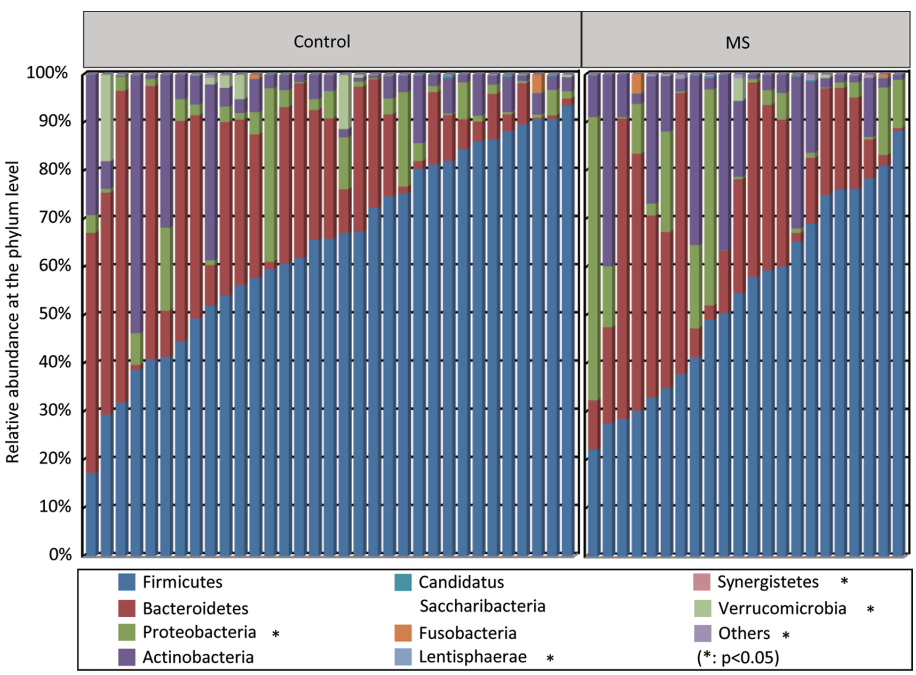


FIGURE 1 | Variations in fecal microbial composition in Chinese patients with multiple sclerosis (MS). Relative proportions of bacterial phyla in patients with MS (n = 22) and in healthy controls (n = 33).

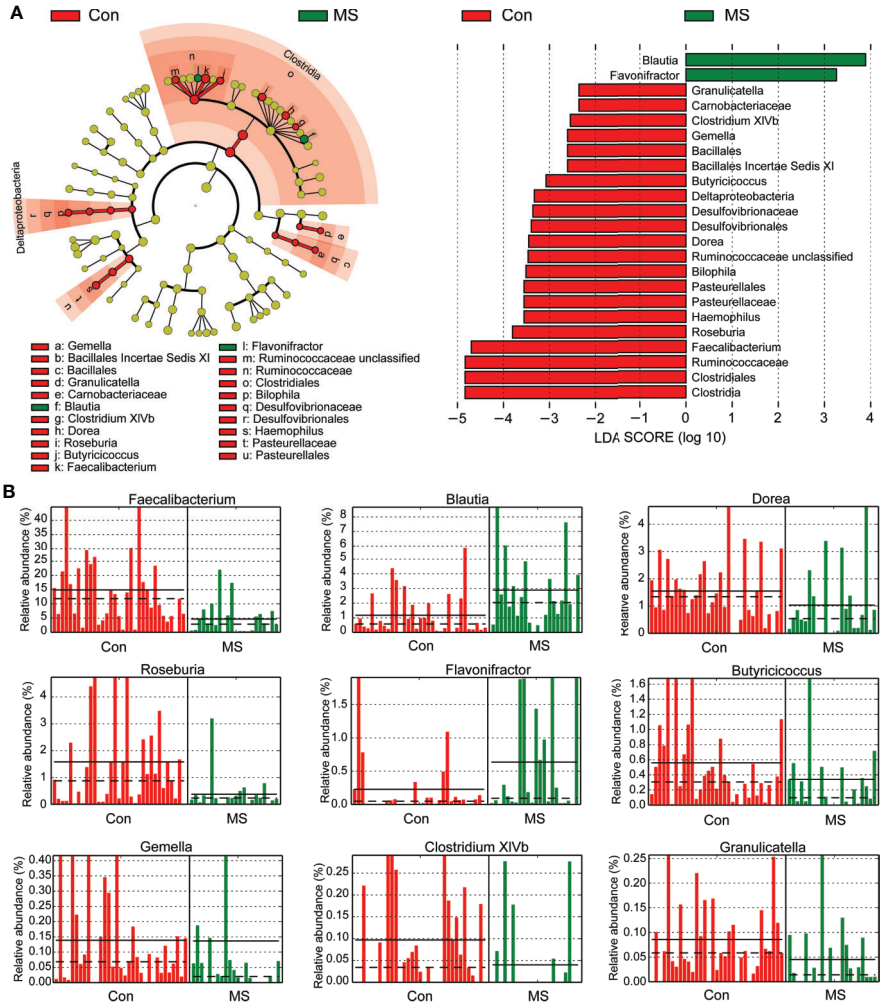


FIGURE 2 | Different bacterial taxa between Chinese patients with multiple sclerosis (MS) and healthy controls. LEfSe identifies the taxa with the greatest differences in abundances between Chinese patients with MS and healthy controls. Only the taxa that meet a significant LDA threshold value of > 2 are shown **(A)**. Nine differentially abundant bacterial taxa were identified between the two groups **(B)**.

Encyclopedia of Genes and Genome (KEGG) pathways at level 2 and identified three KEGG categories, carbohydrate metabolism, environmental adaptation, and immune system, with significantly different abundances between patients with MS and healthy controls ($p < 0.05$; **Figure 5**). Specifically, fructose and mannose metabolism, ubiquinone and other terpenoid-quinone biosynthesis, vitamin B6 metabolism, ascorbate and aldarate metabolism, galactose metabolism, glycolysis/gluconeogenesis, taurine and hypotaurine metabolism, fatty acid metabolism, aminobenzoate degradation, and retinol metabolism were significantly enriched at level 3 in the fecal microbiota of MS patients, while plant-pathogen interaction, epithelial cell signaling in *Helicobacter pylori* infection, and NOD-like receptor signaling pathway were significantly inhibited ($p < 0.05$). Collectively, these functional changes in the fecal microbiota may be associated with MS pathogenesis.

Correlations Between Fecal Microbiota and Systemic Inflammation Among Patients With Multiple Sclerosis

The findings of the Bio-Plex Pro™ human cytokine group I panel 27-plex analysis revealed that the concentrations of several cytokines changed significantly between healthy controls and patients with MS (**Figure 6**). In patients with MS, the levels of inflammatory cytokines, such as TNF-α and IL-17, increased significantly, while those of anti-inflammatory mediators IL-1ra and the chemokines IL-8, Eotaxin, RANTES, MIP-1a, MIP-1b, and MCP-1 decreased. In addition, the levels of growth factors such as bFGF, G-CSF, PDGF-bb, and pleiotropic cytokine IL-9 also decreased significantly in patients with MS. We also evaluated the correlations between the altered cytokine levels and the abundances of key functional bacteria among Chinese patients with MS (**Figure 7**). Notably, we observed that the

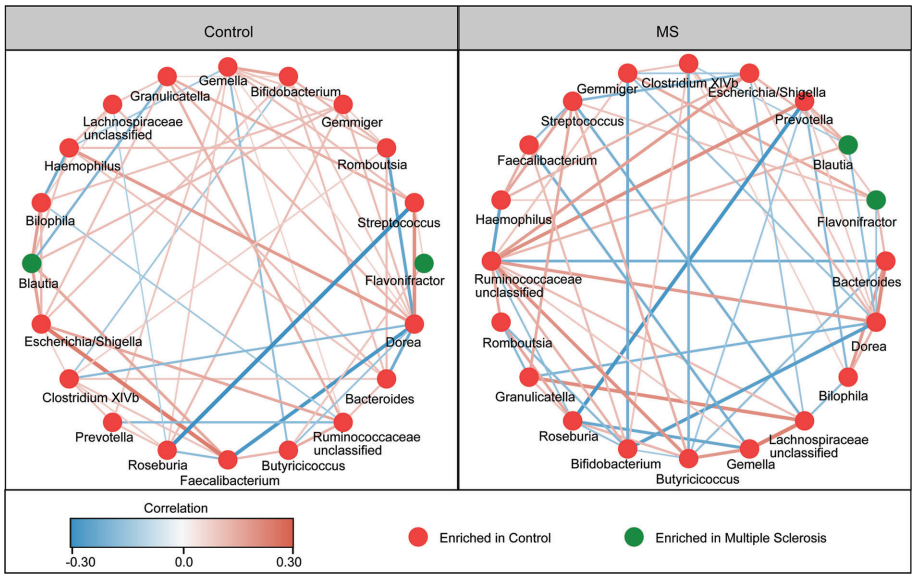


FIGURE 3 | Correlation strengths of the abundant fecal microbiota in Chinese patients with multiple sclerosis (MS) and healthy controls. Correlation network of the abundant fecal microbiota in healthy controls and in patients with MS. The correlation coefficients were calculated using the Sparse Correlations for Compositional data (SparCC) algorithm. Cytoscape version 3.4.0 was used for network construction. The red and blue lines represent positive and negative correlations, respectively. The correlation networks were observed to become simpler.

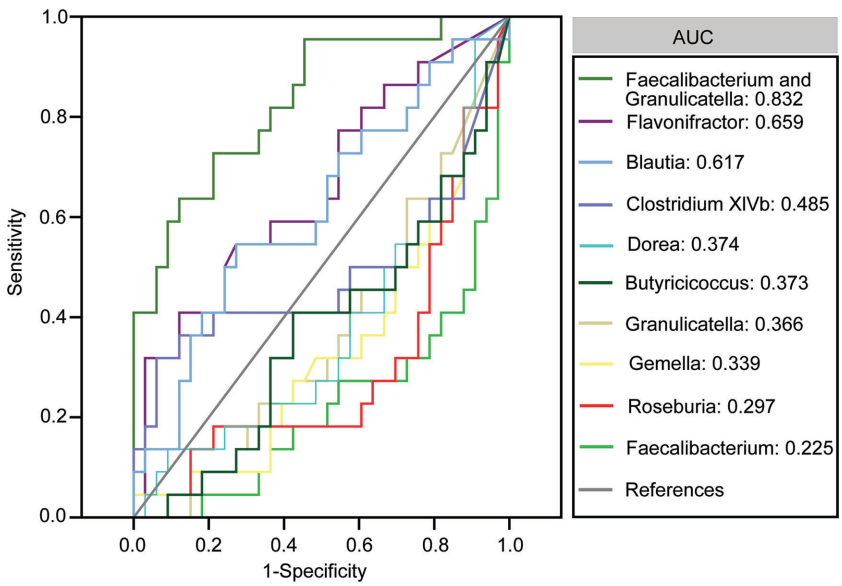


FIGURE 4 | Receiver operating characteristic curves for differentially abundant genera such as *Flavonifractor, Blautia, Clostridium* XlVb, *Dorea, Butyricicoccus, Granulicatella, Gemella, Roseburia* and *Faecalibacterium, Streptococcus, Halomonas, Shewanella*, and *Ruminococcus* and *Dialister* that were used to distinguish between patients with multiple sclerosis and healthy controls.

abundance of butyrate-producing *Faecalibacterium* correlated positively with the levels of chemokines such as IL-8 ($r = 0.315$; $p = 0.019$) and MIP-1a ($r = 0.333$; $p = 0.013$), while it correlated negatively with those of inflammatory cytokines such as TNF-α ($r = -0.310$; $p = 0.021$). The abundance of *Flavonifractor*, which is enriched in MS, correlated positively with the levels of TNF-α ($r = 0.332$; $p = 0.013$), whereas the abundance of *Roseburia* correlated negatively with the levels of TNF-α ($r = -0.331$; $p = 0.013$). The abundance of *Granulicatella*, which is generally low in patients with MS, was also observed to

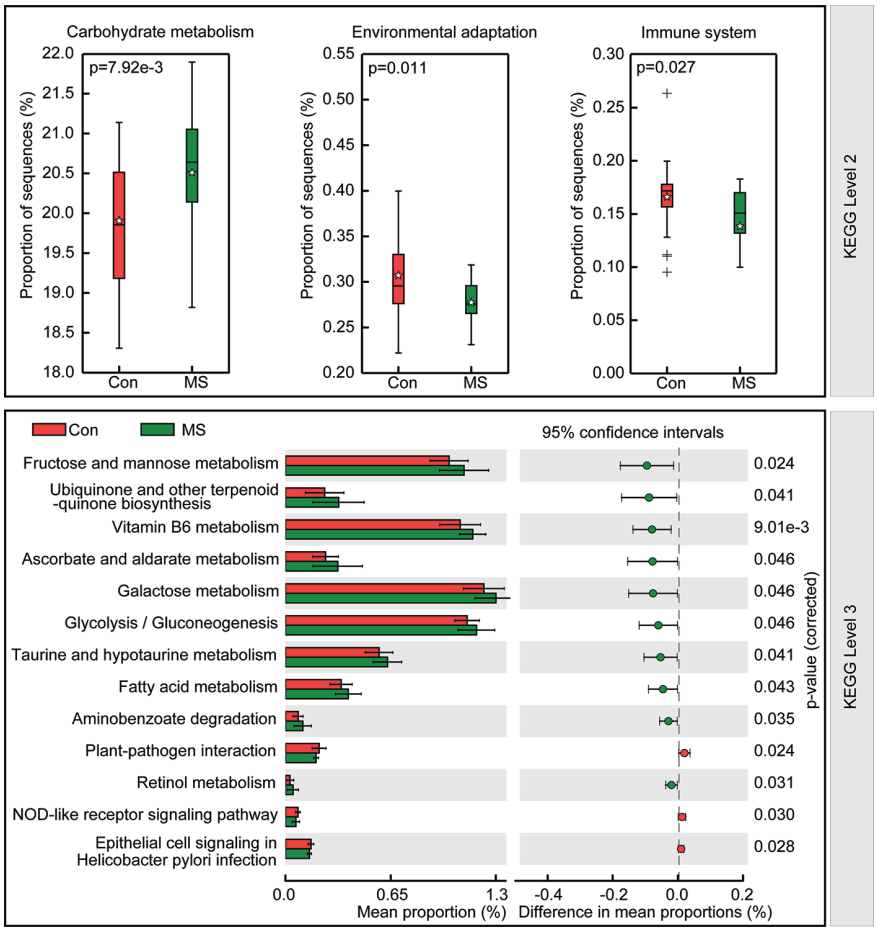


FIGURE 5 | PiCRUSt-based study of fecal microbiome in Chinese patients with multiple sclerosis (MS) and healthy controls. The different bacterial functions were evaluated and compared between the two groups based on two-sided Welch's *t*-test. Comparisons between the two groups for each KEGG functional category (levels 2 and 3) are indicated by the percentage. The Benjamini-Hochberg method was used for multiple testing correction based on the false discovery rate determined by STAMP.

correlate positively with the levels of MIP-1b (r = 0.352; p = 0.008). The abundance of the beneficial bacteria *Bifidobacterium* did not change significantly in Chinese patients with MS; however, it correlated negatively with the levels of IL-8 (r = −0.286; p = 0.034) and MIP-1b (r = −0.428; p=0.001). These correlations indicate that the key functional bacteria with altered abundances might regulate the inflammatory status in MS, at least partially, and consequently, may play a vital role in the progression, remission, and prognosis of MS.

DISCUSSION

Mounting evidence indicates that commensal microbiota that colonize the gastrointestinal tract play a vital role in regulating host immunity and maintaining host immune homeostasis. The host gut microbiota, which co-evolve tightly with the immune system, can modulate development and regulate innate and adaptive immunity functions, whereas dysbiosis of the gut microbiota facilitates abnormal immunological development (36). Research over the past decade has helped us appreciate the importance of commensal microbiota in the development of autoimmune diseases, such as inflammatory bowel disease, psoriasis/psoriatic arthritis, rheumatoid arthritis, systemic lupus erythematosus, celiac disease, and type 1 diabetes (37–43). As the most common autoimmune disease, MS targets the central nervous system and can lead to chronic disability, including cognition impairment, loss of motor control, and sensitivity (44, 45). MS is a multifactorial complex disease, and both genetic and environmental factors contribute to its pathogenesis (46). Similar to other gut-brain disorders, alterations in the relative proportions of gut microbiota in newly or active MS have been reported in several recent studies (9–11, 18, 46–49). Therefore, MS is also considered a type of gut-brain disorder.

In our previous studies, we reported that gut-brain disorders such as depression, Parkinson's disease, Alzheimer's disease, cerebral ischemia/reperfusion injury, and vascular dementia are associated with the dysbiosis of gut microbiota among Chinese

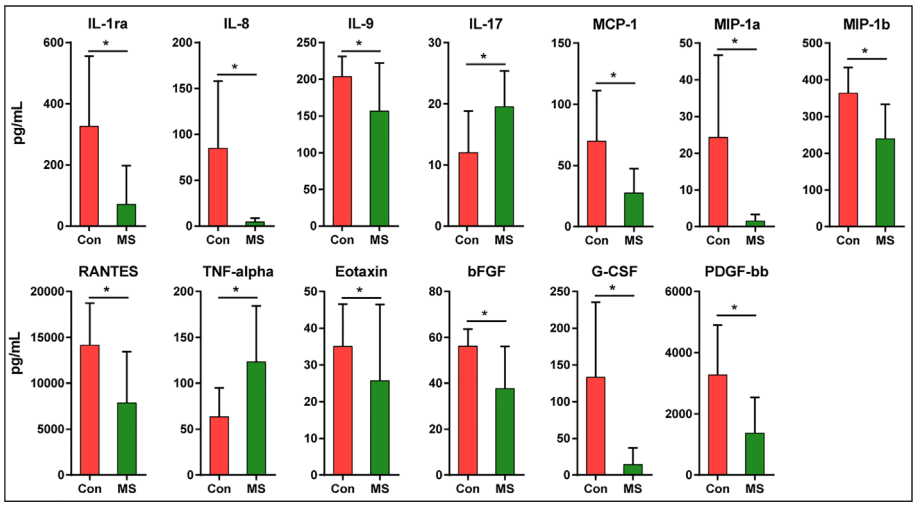


FIGURE 6 | Mean (SEM) concentrations (pg/ml) of 27 pro- and anti-inflammatory cytokines and chemokines in Chinese patients with MS and in healthy controls determined using Bio-Plex immunoassays. The concentrations of TNF-α and IL-17 increased significantly in Chinese patients with MS, while those of IL-1ra, IL-8, IL-9, MCP-1, MIP-1a, MIP-1b, RANTES, Eotaxin, bFGF, G-CSF, and PDGF-bb decreased significantly. *p < 0.05.

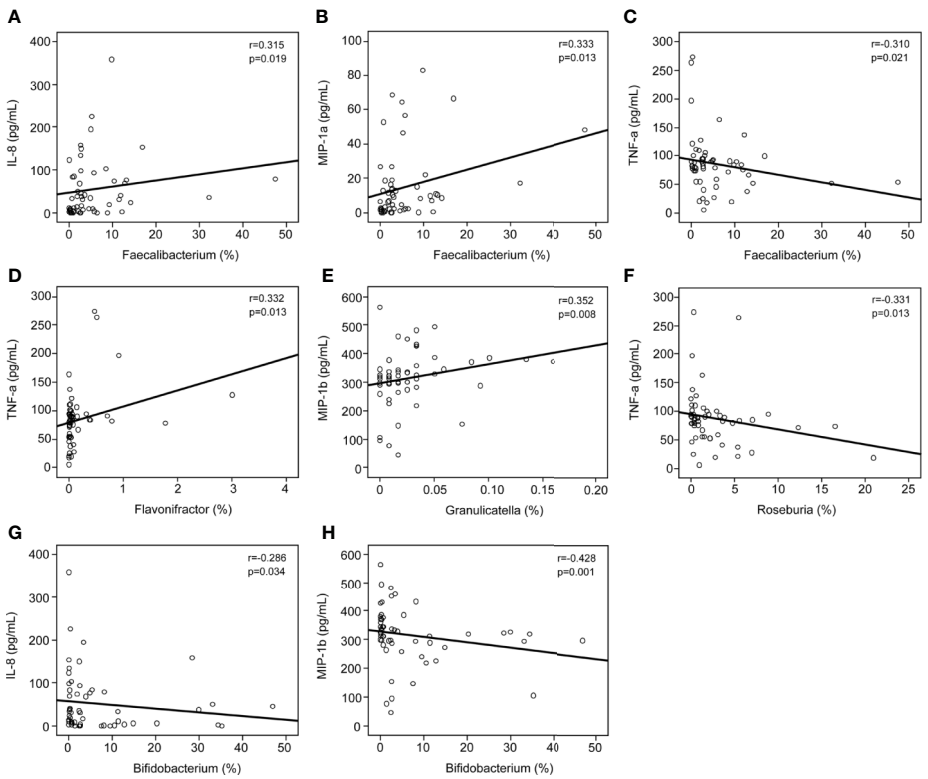


FIGURE 7 | Correlations between pro- and anti-inflammatory cytokines and chemokines with altered concentrations and the relative abundance of the key functional genera. Correlation between the relative abundance of *Faecalibacterium* and the levels of IL-8 **(A)**, MIP-1a **(B)**, and TNF-α **(C)**; the relative abundance of *Flavonifractor* and the levels of TNF-α **(D)**; the relative abundance of *Granulicatella* and the levels of MIP-1b **(E)**; the relative abundance of *Roseburia* and the levels of TNF-α **(F)**; and the relative abundance of *Bifidobacterium* and the levels of IL-8 **(G)** and MIP-1b **(H)**. Spearman's rank correlation (r) and probability (p) were determined to evaluate the statistical importance.

individuals and animal models, which provides novel targets for the study of their pathogenesis and sheds light on novel intervention strategies (18, 50–57). The majority of studies on the "MS microbiome" conducted earlier primarily focused on Caucasian populations from western developed countries. However, there are several potential confounders, such as natural environmental conditions, economic status, ethnic background, lifestyles, and long-term diets that influence the bacterial composition in the gut; moreover, dietary patterns among Chinese populations, characterized by low calorie, low fat, and high dietary fiber, are different from those among Caucasian populations. Given that different populations have diverse gut microbiota, studies that target a specific population are necessary to decipher the mechanism underlying pathogenesis and identify therapeutic strategies more precisely. Therefore, the specific differences in gut microbiota that result from different environmental factors such as dietary habits may interfere with the development of MS, and we speculate that there may be specific changes in the gut microbiota in Chinese patients with stable MS. In the present study, we observed that the overall bacterial diversity in the fecal microbiota did not change significantly between the healthy cohort and MS patient cohort. The alpha-diversity indices, such as the Shannon and Simpson indices, did not undergo obvious changes, and the richness indices, such as observed OTUs, ACE, and Chao1, were also not altered significantly. In addition, the groups could not be divided into different clusters based on the beta-diversity indices. Consistent with the findings of a previous study by Jangi et al. (10), the present study showed that the overall bacterial diversity in the fecal microbiota was unaltered between healthy controls and Chinese patients with MS, which suggests that the overall structure of the fecal microbiota remained relatively stable among Chinese patients with MS.

Although the overall bacterial diversity of the fecal microbiota remained unaltered among Chinese patients with MS in this study, based on statistical analysis, compositional changes in the fecal microbiota were observed to be associated with MS development. At the phylum level, the abundances of several non-abundant phyla such as Proteobacteria, Lentisphaerae, Synergistetes, and Verrucomicrobia were observed to be altered, while the ratio of Firmicutes/Bacteroidetes, the two most prevalent phyla, did not change significantly. Lopez et al. demonstrated that a reduced Firmicutes/Bacteroidetes ratio was observed in patients with systemic lupus erythematosus (58), which indicates that gut dysbiosis is a major feature in this autoimmune disease. However, the composition of the fecal microbiota at the family and genus levels changed significantly in Chinese patients with MS. We also observed a more complex network of interactions among the differentially abundant bacteria in healthy controls than in patients with MS. According to our findings, the most important features of the specific "MS microbiota" among Chinese patients were characterized by a reduction in the abundances of bacteria belonging to *Faecalibacterium*, *Roseburia*, *Dorea*, *Butyricicoccus*, and *Clostridium* XIVb, which were potent producers of SCFAs, especially butyrate, and ferment indigestible carbohydrates to produce SCFAs. Although we did not measure the altered levels of SCFAs in the fecal samples directly, the altered profiles of the fecal microbiota provided some clues that fecal SCFAs might involve in the development of MS. In fact, the types and concentrations of SCFAs in the gut depend on the composition of the microbiota, the intestinal transit time, and the fiber content in the host diet. In addition to their role as local energy carriers for the gut microbiota and gut epithelial cells, SCFAs also play roles in a broad array of functions that influence gastrointestinal physiology, peripheral immunity, liver metabolism, and blood-brain barrier integrity, which could indirectly contribute to effects on the brain (59). SCFAs can promote health at a steady state by increasing intestinal epithelial cell integrity, pathogen-specific antibody responses, and the number of colonic Tregs, which can act as important signals that physically bridge the gap between the commensal microbiota and the mucosal immune system (60–64). Tregs play a critical role in immune homeostasis and in suppressing excessive immune responses elicited by the host. Kim et al. found that SCFAs are able to induce metabolic and epigenetic reprogramming linked to the suppression of inflammatory immune responses (65), which are essential for the development of autoimmune diseases. Mizuno et al. demonstrated that the oral administration of SCFAs ameliorates the severity of systemic autoimmune inflammatory conditions mediated by lymphocytes, such as experimental autoimmune encephalitis and collagen-induced arthritis. Amelioration of a disease is associated with a reduction in the number of Th1 cells and an increase in those of Treg cells (66). Tregs, the regulation of which is driven by the transcription factor Foxp3, are particularly important for limiting autoimmunity and chronic inflammation (67, 68). Therefore, alterations in the production of potent SCFAs and their metabolites by the fecal microbiota might contribute significantly to MS development, and could be used as a potential therapeutic target for MS in future studies.

In fact, gut dysbiosis and the consequent increase in gut permeability can lead to the disruption of immune homeostasis, following the development of gut inflammation, which is increasingly considered to be the ultimate source of systemic immune activation and Th17/Treg cell imbalance, and possibly of neurological disturbances as well. Consistent with the findings of a previous study, the relative abundance of *Faecalibacterium* (a member of *Clostridium* cluster IV) reduced significantly, which can convert acetate and lactate into butyrate (10). The most important properties of *Faecalibacterium* are reported to be their anti-inflammatory characteristics and the ability to attenuate inflammation in mouse models of colitis, which is mediated by the modulation of the mucosal T cell response (69). A previous study reported that the supernatant of *Faecalibacterium* culture, primarily the metabolite butyrate, can maintain Th17/Treg balance and ameliorate colorectal colitis by inhibiting histone deacetylase 1 (HDAC1) (70); this is of significance because Th17/Treg cell imbalance has been implicated in the pathogenesis of most common autoimmune diseases, including MS (71). Furusawa et al. reported that treatment of naïve T cells with butyrate, which is an HDAC inhibitor, enhanced the acetylation of Foxp3, and consequently promoted the differentiation of Treg cells (72). The abundance of *Clostridium* XIVb, which is another butyrate producer, was observed to decrease significantly in Chinese

patients with MS. In our previous study, we observed a reduction in the relative abundance of *Clostridium* XIVb in patients with major depressive disorder, which correlated negatively with the serum BDNF levels (55). Inconsistent with our present findings, the abundance of *Clostridium* XIVb was observed to increase in various diseases such as juvenile idiopathic arthritis and Rett syndrome (73, 74). Similar to *Clostridium* cluster IV, *Clostridium* XIVb was the most effective in generating high levels of Treg cells in the $CD4^+$ T cell population, which are essential for maintaining immune tolerance and abrogating chronic inflammatory or autoimmune diseases (75). In addition, our study also demonstrated the reduction in the abundance of *Roseburia*, a member of the *Clostridium* cluster XIVa, in Chinese patients with MS. Similar to *Faecalibacterium*, the butyrate producer, *Roseburia*, was also detected in patients with Crohn's disease. Machiels et al. reported that the abundances of both *Faecalibacterium* and *Roseburia* correlate inversely with disease activity in ulcerative colitis (76). *Roseburia* has been shown to exert beneficial effects in colitis model mice and Caco-2 cells by enhancing the anti-inflammatory response, which could reduce the DAI score by enhancing colonic Treg cell differentiation and the levels of the anti-inflammatory cytokines TSLP, IL-10, and TGF-β in the intestinal mucosa of colitis model mice (77). Therefore, *Roseburia* also played a beneficial probiotic role in alleviating inflammation in autoimmune diseases. *Dorea*, which is also a member of the *Clostridium* cluster XIVa, has been considered to form a part of healthy gut microbiota. Chen et al. found that the relative abundance of *Dorea* was higher among American patients with MS, which was inconsistent with the findings of the present study (9). The anti-inflammatory properties of *Dorea* might be associated with the development of MS. *Butyricicoccus*, which is also a butyrate producer, is reportedly a beneficial bacterium that suppresses inflammatory bowel diseases, which has a strong negative correlation with the levels of the target cytokines and cell types (78). Shi et al. observed that the abundance of *Butyricicoccus* decreased significantly in patients with severe and active Graves' orbitopathy compared to that in controls (79). Everard et al. demonstrated that the abundance of *Butyricicoccus*, which can improve gut barrier integrity, correlated negatively with the levels of pathogenic Aβ42 in the brain (80). Therefore, our present study revealed that altered composition of the fecal microbiota especially those SCFAs producers, which regulate host immune homeostasis, might play vital roles in the development of MS.

In fact, systemic inflammation can also affect disease expression in MS. The progression of MS is associated with the loss of immune homeostasis, increased systemic inflammation, and disruption of the blood-brain barrier. A previous study has revealed that $CD4^+$ T cells mediate inflammation in the central nervous system, which triggers demyelination and axonal degeneration, and this has been shown to play a major role in MS pathogenesis (81). Consistent with previous studies, our present study also revealed obvious systemic inflammation in Chinese patients with MS, characterized by increased levels of IL-17 and TNF-α and decreased levels of IL-1ra, IL-8, IL-9, MCP-1, MIP-1a, and MIP-1b. The changes in the levels of inflammatory mediators such as inflammatory and anti-inflammatory cytokines, chemokines, and growth factors might constitute the primary characteristics of systemic inflammation in MS patients, and current MS therapies aim to restore host immunity to a healthy status. Kürtüncü et al. observed that increased IL-17 levels can be reduced significantly after IFN-β treatment, which indicates that Th17-type immunity plays a crucial role in the pathogenesis of MS. Recently, the chemokine IL-9 has been shown to play a major role in regulating autoimmune responses in experimental autoimmune encephalomyelitis, which is an animal model of MS. IL-9 has emerged as a key cytokine involved in the regulation of the balance between Th17 and Tregs levels in MS (82). The intestinal microbiota can influence host immunity in the intestinal tract as well as at distal sites. The intestinal microbiota affect systemic immune responses by modulating several key pathways: expansion of extra-intestinal T cell populations, production of SCFAs, development of oral tolerance, and control of inflammation (83). In our study, we found that the abundance of *Faecalibacterium* correlated positively with the levels of IL-8 and MIP-1a. IL-8 possesses potent neutrophil chemoattractant and activating properties. A previous study showed that butyrate, mainly metabolites of *Faecalibacterium*, can induce Caco-2 cells to secrete IL-8 in response to lipopolysaccharide (84). However, recent study found that the supernatant of *Faecalibacterium* culture can NF-κB activation and the production of the pro-inflammatory cytokine IL-8 *in vitro*, which is contrary to our present findings (85). The abundances of *Faecalibacterium* and *Roseburia* correlated negatively with the levels of the pro-inflammatory cytokine TNF-α, while that of *Flavonifractor*, which is an MS-enriched taxa, correlated positively with the levels of TNF-α. TNF-α can mediate monocyte infiltration into the intestinal tissues resulting in tissue damage, which is one signs of inflammation. Interestingly, *Faecalibacterium* and its supernatant can suppress the expression TNF-α, and then alleviate the inflammation. Evidence has shown that *Roseburia* plays an important role in maintaining gut health by improving the gut ecosystem and exhibiting anti-inflammatory effects (86, 87). The correlations between *Roseburia* and TNF-α in our present findings might also link with its metabolite such as butyrate. *Flavonifractor* has been reported to participate in the metabolism of catechin in the gut (88). However, the biological regulatory effects of *Flavonifractor* in autoimmune diseases are still unclear. We postulated that *Flavonifractor* might influence the expression of TNF-α directly or *via* its metabolites. Of course, the systemic immune response in MS was impacted by the bacterial mixtures of these key functional bacteria together. The specific roles of these key functional bacteria alone should be explored in MS animal models in the future. Interestingly, numerous studies have demonstrated that the metabolites of these key functional bacteria, SCFAs, have been demonstrated to influence systemic autoimmune responses and participate in different steps of the inflammatory process (89, 90). SCFAs have been observed to regulate the functions of almost every type of immune cell by altering gene expression, differentiation, chemotaxis, proliferation, and apoptosis. Previous studies have shown that compared to acetate and propionate, butyrate exhibits strong anti-inflammatory properties, which exert regulatory effects on inflammatory processes by maintaining the balance of Th17/Treg cells and the levels of pro- and anti-inflammatory cytokines (91). These data

indicate that the disturbed intestinal microbiota, especially the SCFA-producing key intestinal functional bacteria that are variably abundant, could modulate host immunity, induce systemic inflammation, and participate in the progression of MS. Therefore, this is a useful strategy that targets systemic inflammation by modulating the abundances of specific intestinal key functional bacteria, and may provide novel avenues for MS-modifying therapy.

However, our present study is limited in some ways. First, the number of MS patients was relatively small in our present microbiota analysis. More MS patients enrolled from different regions of China might make our results more solid and reasonable. Second, our 16S rRNA amplicon rather than metagenomic sequencing limited the finding of specific bacteria related to MS at the species level. Third, the functional and metabolic changes in the microbial communities were mostly speculative and there was no functional validation of the microbiome findings. Future culturomics should be used to obtain the MS-associated bacteria, and cell and animal experiments could help determine the cause-effect relationship between these key functional bacteria and the pathogenesis of MS.

CONCLUSION

In summary, our study revealed alterations in the fecal microbiota and altered host immune response in Chinese patients with stable MS. The characterization and manipulation of the MS-associated microbiome may have significant potential as diagnostic and therapeutic strategies, respectively. In addition, the abundances of key functional bacteria that were altered significantly correlated with the levels of pro- and anti-inflammatory cytokines and chemokines, which indicates that intestinal microbiota might play vital roles in the development of MS *via* the regulation of host immunity. Therefore, the modulation of intestinal microbiota through personalized diet or beneficial microbial intervention may be a potential strategy for patient-tailored early intervention of MS. However, the role of those key functional bacteria especially butyrate producers in systemic inflammation in Chinese patients with MS in the present study remains unclear. Further studies are necessary to decode the sophisticated bidirectional dialogue between butyrate-producing bacteria and host immunity associated with the development, remission, and prognosis of Chinese patients with MS.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Lishui Second People's Hospital (Zhejiang, China). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ZL, XY, and LYZ conceived and designed the experiments. ZL, XY, YC, SL, XL, DZ, LJZ, and KQ performed the experiments. ZL, XY, LS, and XL analyzed the data. ZL, YC, and LYZ wrote the paper and edited the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This present work was funded by the grants of the National S&T Major Project of China (2018YFC2000500), the National Natural Science Foundation of China (81771724, 31700800, 81790631), the S&T Major Project of Lishui (2017ZDYF15), and the Foundation of China's State Key Laboratory for Diagnosis and Treatment of Infectious Diseases. The authors thank all of the participants who recruited patients in this study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.590783/full#supplementary-material

SUPPLEMENTARY FIGURE 1 | The diversity and richness of the fecal microbiota in MS. The diversity indices, such as Shannon **(A)**, and Simpson **(B)**, and the richness indices, such as observed species **(C)** ACE **(D)**, and Chao1 **(E)**, were used to evaluate the overall structure of the fecal microbiota in Chinese MS patients and healthy controls. Data are presented as mean ± standard deviation. Unpaired *t* tests (two-tailed) were used to analyze variation between the two groups. Principal coordinate analysis (PCoA) plots of individual fecal microbiota based on jaccard **(F)**, Bray-Curtis **(G)**, unweighted **(H)** and weighted (**I**) UniFrac distance in Chinese MS patients and healthy controls. Each symbol represents a sample. The Venn diagram illustrates the overlap of OTUs in the fecal microbiota between the two groups **(J)**. Rarefaction curves were used to estimate the richness (at a 97% level of similarity) of the fecal microbiota between the two groups **(K)**. The vertical axis shows the number of OTUs expected after sampling the number of tags or sequences shown on the horizontal axis. Rank abundance curves of bacterial OTUs derived from the two groups, which indicated that the majority of the OTUs were present at low abundance in the fecal microbiota samples with greater sequencing depth **(L)**.

SUPPLEMENTARY FIGURE 2 | Heatmap of the family-level taxa in the fecal microbiota of Chinese MS patients and healthy controls. The colour of the spots in the panel represents the relative abundance (normalized and log10-transformed) of the family in each sample. The relative abundance of the bacteria in each family is indicated by a gradient of colour from blue (low abundance) to red (high abundance). The taxonomic classifications of the family are shown on the right. The corresponding Shannon index in each sample is shown under the heatmap.

REFERENCES

1. Thompson AJ, Baranzini SE, Geurts J, Hemmer B, Ciccarelli O. Multiple sclerosis. *Lancet* (2018) 391(10130):1622–36. doi: 10.1016/s0140-6736(18)30481-1
2. GBD2016MultipleSclerosisCollaborators. Global, regional, and national burden of multiple sclerosis 1990-2016: a systematic analysis for the Global Burden of Disease Study 2016. *Lancet Neurol* (2019) 18(3):269–85. doi: 10.1016/S1474-4422(18)30443-5
3. Makhani N, Morrow SA, Fisk J, Evans C, Beland SG, Kulaga S, et al. MS incidence and prevalence in Africa, Asia, Australia and New Zealand: A systematic review. *Mult Scler Relat Disord* (2014) 3(1):48–60. doi: 10.1016/j.msard.2013.06.015
4. Eskandarieh S, Heydarpour P, Minagar A, Pourmand S, Sahraian MA. Multiple Sclerosis Epidemiology in East Asia, South East Asia and South Asia: A Systematic Review. *Neuroepidemiology* (2016) 46(3):209–21. doi: 10.1159/000444019
5. Cheong WL, Mohan D, Warren N, Reidpath DD. Multiple Sclerosis in the Asia Pacific Region: A Systematic Review of a Neglected Neurological Disease. *Front Neurol* (2018) 9:432. doi: 10.3389/fneur.2018.00432
6. Cheng Q, Miao L, Zhang J, Ding SJ, Liu ZG, Wang X, et al. A population-based survey of multiple sclerosis in Shanghai, China. *Neurology* (2007) 68(18):1495–500. doi: 10.1212/01.wnl.0000260695.72980.b7
7. Liu X, Cui Y, Han J. Estimating epidemiological data of Multiple sclerosis using hospitalized data in Shandong Province, China. *Orphanet J Rare Dis* (2016) 11(1):73. doi: 10.1186/s13023-016-0457-4
8. Ascherio A, Munger KL. Environmental risk factors for multiple sclerosis. Part II: Noninfectious factors. *Ann Neurol* (2007) 61(6):504–13. doi: 10.1002/ana.21141
9. Chen J, Chia N, Kalari KR, Yao JZ, Novotna M, Soldan MMP, et al. Multiple sclerosis patients have a distinct gut microbiota compared to healthy controls. *Sci Rep* (2016) 6:28484. doi: 10.1038/Srep28484
10. Jangi S, Gandhi R, Cox LM, Li N, von Glehn F, Yan R, et al. Alterations of the human gut microbiome in multiple sclerosis. *Nat Commun* (2016) 7:12015. doi: 10.1038/ncomms12015
11. Miyake S, Kim S, Suda W, Oshima K, Nakamura M, Matsuoka T, et al. Dysbiosis in the Gut Microbiota of Patients with Multiple Sclerosis, with a Striking Depletion of Species Belonging to Clostridia XIVa and IV Clusters. *PloS One* (2015) 10(9):e0137429. doi: 10.1371/journal.pone.0137429
12. Yokote H, Miyake S, Croxford JL, Oki S, Mizusawa H, Yamamura T. NKT cell-dependent amelioration of a mouse model of multiple sclerosis by altering gut flora. *Am J Pathol* (2008) 173(6):1714–23. doi: 10.2353/ajpath.2008.080622
13. Berer K, Mues M, Koutrolos M, Rasbi ZA, Boziki M, Johner C, et al. Commensal microbiota and myelin autoantigen cooperate to trigger autoimmune demyelination. *Nature* (2011) 479(7374):538–41. doi: 10.1038/nature10554
14. Scher JU, Sczesnak A, Longman RS, Segata N, Ubeda C, Bielski C, et al. Expansion of intestinal Prevotella copri correlates with enhanced susceptibility to arthritis. *Elife* (2013) 2:e01202. doi: 10.7554/eLife.01202
15. Cantarel BL, Waubant E, Chehoud C, Kuczynski J, DeSantis TZ, Warrington J, et al. Gut microbiota in multiple sclerosis: possible influence of immunomodulators. *J Invest Med* (2015) 63(5):729–34. doi: 10.1097/JIM.0000000000000192
16. Sokol H, Pigneur B, Watterlot L, Lakhdari O, Bermudez-Humaran LG, Gratadoux JJ, et al. Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. *Proc Natl Acad Sci U S A* (2008) 105(43):16731–6. doi: 10.1073/pnas.0804812105
17. Levy M, Thaiss CA, Elinav E. Metagenomic cross-talk: the regulatory interplay between immunogenomics and the microbiome. *Genome Med* (2015) 7:120. doi: 10.1186/s13073-015-0249-9
18. Zeng Q, Gong JL, Liu XY, Chen C, Sun XB, Li HJ, et al. Gut dysbiosis and lack of short chain fatty acids in a Chinese cohort of patients with multiple sclerosis. *Neurochem Int* (2019) 129:104468. doi: 10.1016/J.Neuint.2019.104468
19. Polman CH, Reingold SC, Edan G, Filippi M, Hartung HP, Kappos L, et al. Diagnostic criteria for multiple sclerosis: 2005 revisions to the "McDonald Criteria". *Ann Neurol* (2005) 58(6):840–6. doi: 10.1002/ana.20703
20. Fadrosh DW, Ma B, Gajer P, Sengamalay N, Ott S, Brotman RM, et al. An improved dual-indexing approach for multiplexed 16S rRNA gene sequencing on the Illumina MiSeq platform. *Microbiome* (2014) 2(1):6. doi: 10.1186/2049-2618-2-6
21. Liu X, Shao L, Ji F, Mei Y, Cheng Y, Liu F, et al. Alterations of gastric mucosal microbiota across different stomach microhabitats in a cohort of 276 patients with gastric cancer. *EBioMedicine* (2019) 40:336–48. doi: 10.1016/j.ebiom.2018.12.034
22. Ling Z, Shao L, Liu X, Cheng Y, Yan C, Mei Y, et al. Regulatory T Cells and Plasmacytoid Dendritic Cells Within the Tumor Microenvironment in Gastric Cancer Are Correlated With Gastric Microbiota Dysbiosis: A Preliminary Study. *Front Immunol* (2019) 10:533. doi: 10.3389/fimmu.2019.00533
23. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* (2010) 7(5):335–6. doi: 10.1038/nmeth.f.303
24. Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* (2011) 27(16):2194–200. doi: 10.1093/bioinformatics/btr381
25. McDonald D, Price MN, Goodrich J, Nawrocki EP, DeSantis TZ, Probst A, et al. An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *ISME J* (2012) 6(3):610–8. doi: 10.1038/ismej.2011.139
26. Edgar RC. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* (2010) 26(19):2460–1. doi: 10.1093/bioinformatics/btq461
27. Navas-Molina JA, Peralta-Sanchez JM, Gonzalez A, McMurdie PJ, Vazquez-Baeza Y, Xu ZJ, et al. Advancing Our Understanding of the Human Microbiome Using QIIME. *Method Enzymol* (2013) 531:371–444. doi: 10.1016/B978-0-12-407863-5.00019-8
28. Wang Q, Garrity GM, Tiedje JM, Cole JR. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol* (2007) 73(16):5261–7. doi: 10.1128/Aem.00062-07
29. Good IJ. The Population Frequencies of Species and the Estimation of Population Parameters. *Biometrika* (1953) 40(3-4):237–64. doi: 10.2307/2333344
30. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, et al. Introducing mothur: Open-Source, Platform-Independent, Community-Supported Software for Describing and Comparing Microbial Communities. *Appl Environ Microbiol* (2009) 75(23):7537–41. doi: 10.1128/Aem.01541-09
31. Lozupone C, Knight R. UniFrac: a new phylogenetic method for comparing microbial communities. *Appl Environ Microbiol* (2005) 71(12):8228–35. doi: 10.1128/AEM.71.12.8228-8235.2005
32. Parks DH, Tyson GW, Hugenholtz P, Beiko RG. STAMP: statistical analysis of taxonomic and functional profiles. *Bioinformatics* (2014) 30(21):3123–4. doi: 10.1093/bioinformatics/btu494
33. Langille MG, Zaneveld J, Caporaso JG, McDonald D, Knights D, Reyes JA, et al. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nat Biotechnol* (2013) 31(9):814–21. doi: 10.1038/nbt.2676
34. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, et al. Metagenomic biomarker discovery and explanation. *Genome Biol* (2011) 12(6):R60. doi: 10.1186/gb-2011-12-6-r60
35. Friedman J, Alm EJ. Inferring correlation networks from genomic survey data. *PloS Comput Biol* (2012) 8(9):e1002687. doi: 10.1371/journal.pcbi.1002687
36. Hooper LV, Littman DR, Macpherson AJ. Interactions Between the Microbiota and the Immune System. *Science* (2012) 336(6086):1268–73. doi: 10.1126/science.1223490
37. Shamriz O, Mizrahi H, Werbner M, Shoenfeld Y, Avni O, Koren O. Microbiota at the crossroads of autoimmunity. *Autoimmun Rev* (2016) 15(9):859–69. doi: 10.1016/j.autrev.2016.07.012
38. Vanaclocha F, Crespo-Erchiga V, Jimenez-Puya R, Puig L, Sanchez-Carazo JL, Ferran M, et al. Immune-mediated inflammatory diseases and other comorbidities in patients with psoriasis: baseline characteristics of patients in the AQUILES study. *Actas Dermosifiliogr* (2015) 106(1):35–43. doi: 10.1016/j.ad.2014.06.003
39. Chassaing B, Darfeuille-Michaud A. The commensal microbiota and enteropathogens in the pathogenesis of inflammatory bowel diseases. *Gastroenterology* (2011) 140(6):1720–8. doi: 10.1053/j.gastro.2011.01.054

40. Zhang X, Zhang D, Jia H, Feng Q, Wang D, Liang D, et al. The oral and gut microbiomes are perturbed in rheumatoid arthritis and partly normalized after treatment. *Nat Med* (2015) 21(8):895–905. doi: 10.1038/nm.3914
41. He Z, Shao T, Li H, Xie Z, Wen C. Alterations of the gut microbiome in Chinese patients with systemic lupus erythematosus. *Gut Pathog* (2016) 8:64. doi: 10.1186/s13099-016-0146-9
42. Ou G, Hedberg M, Horstedt P, Baranov V, Forsberg G, Drobni M, et al. Proximal small intestinal microbiota and identification of rod-shaped bacteria associated with childhood celiac disease. *Am J Gastroenterol* (2009) 104 (12):3058–67. doi: 10.1038/ajg.2009.524
43. Mullaney JA, Stephens JE, Costello ME, Fong C, Geeling BE, Gavin PG, et al. Type 1 diabetes susceptibility alleles are associated with distinct alterations in the gut microbiota. *Microbiome* (2018) 6(1):35. doi: 10.1186/s40168-018-0417-4
44. Reich DS, Lucchinetti CF, Calabresi PA. Multiple Sclerosis. *New Engl J Med* (2018) 378(2):169–80. doi: 10.1056/Nejmra1401483
45. Yong H, Chartier G, Quandt J. Modulating inflammation and neuroprotection in multiple sclerosis. *J Neurosci Res* (2018) 96(6):927–50. doi: 10.1002/jnr.24090
46. Probstel AK, Baranzini SE. The Role of the Gut Microbiome in Multiple Sclerosis Risk and Progression: Towards Characterization of the "MS Microbiome". *Neurotherapeutics* (2018) 15(1):126–34. doi: 10.1007/s13311-017-0587-y
47. Shahi SK, Freedman SN, Mangalam AK. Gut microbiome in multiple sclerosis: The players involved and the roles they play. *Gut Microbes* (2017) 8(6):607–15. doi: 10.1080/19490976.2017.1349041
48. Mirza A, Mao-Draayer Y. The gut microbiome and microbial translocation in multiple sclerosis. *Clin Immunol* (2017) 183:213–24. doi: 10.1016/j.clim.2017.03.001
49. Tremlett H, Waubant E. The multiple sclerosis microbiome? *Ann Transl Med* (2017) 5(3):53. doi: 10.21037/atm.2017.01.63
50. Sun J, Xu J, Yang B, Chen K, Kong Y, Fang N, et al. Effect of Clostridium butyricum against Microglia-Mediated Neuroinflammation in Alzheimer's Disease via Regulating Gut Microbiota and Metabolites Butyrate. *Mol Nutr Food Res* (2020) 64(2):e1900636. doi: 10.1002/mnfr.201900636
51. Sun J, Liu S, Ling Z, Wang F, Ling Y, Gong T, et al. Fructooligosaccharides Ameliorating Cognitive Deficits and Neurodegeneration in APP/PS1 Transgenic Mice through Modulating Gut Microbiota. *J Agric Food Chem* (2019) 67(10):3006–17. doi: 10.1021/acs.jafc.8b07313
52. Sun J, Wang F, Ling Z, Yu X, Chen W, Li H, et al. Clostridium butyricum attenuates cerebral ischemia/reperfusion injury in diabetic mice via modulation of gut microbiota. *Brain Res* (2016) 1642:180–8. doi: 10.1016/j.brainres.2016.03.042
53. Sun J, Ling Z, Wang F, Chen W, Li H, Jin J, et al. Clostridium butyricum pretreatment attenuates cerebral ischemia/reperfusion injury in mice via anti-oxidation and anti-apoptosis. *Neurosci Lett* (2016) 613:30–5. doi: 10.1016/j.neulet.2015.12.047
54. Liu J, Sun J, Wang F, Yu X, Ling Z, Li H, et al. Neuroprotective Effects of Clostridium butyricum against Vascular Dementia in Mice via Metabolic Butyrate. *BioMed Res Int* (2015) 2015:412946. doi: 10.1155/2015/412946
55. Jiang H, Ling Z, Zhang Y, Mao H, Ma Z, Yin Y, et al. Altered fecal microbiota composition in patients with major depressive disorder. *Brain Behav Immun* (2015) 48:186–94. doi: 10.1016/j.bbi.2015.03.016
56. Sun J, Xu J, Ling Y, Wang F, Gong T, Yang C, et al. Fecal microbiota transplantation alleviated Alzheimer's disease-like pathogenesis in APP/PS1 transgenic mice. *Transl Psychiatry* (2019) 9(1):189. doi: 10.1038/s41398-019-0525-3
57. Liu J, Wang F, Liu S, Du J, Hu X, Xiong J, et al. Sodium butyrate exerts protective effect against Parkinson's disease in mice via stimulation of glucagon like peptide-1. *J Neurol Sci* (2017) 381:176–81. doi: 10.1016/j.jns.2017.08.3235
58. Lopez P, de Paz B, Rodriguez-Carrio J, Hevia A, Sanchez B, Margolles A, et al. Th17 responses and natural IgM antibodies are related to gut microbiota composition in systemic lupus erythematosus patients. *Sci Rep* (2016) 6:24072. doi: 10.1038/srep24072
59. Fung TC, Olson CA, Hsiao EY. Interactions between the microbiota, immune and nervous systems in health and disease. *Nat Neurosci* (2017) 20(2):145–55. doi: 10.1038/nn.4476
60. Macia L, Tan J, Vieira AT, Leach K, Stanley D, Luong S, et al. Metabolite-sensing receptors GPR43 and GPR109A facilitate dietary fibre-induced gut homeostasis through regulation of the inflammasome. *Nat Commun* (2015) 6:6734. doi: 10.1038/ncomms7734
61. Arpaia N, Rudensky AY. Microbial metabolites control gut inflammatory responses. *Proc Natl Acad Sci U S A* (2014) 111(6):2058–9. doi: 10.1073/pnas.1323183111
62. Bollrath J, Powrie F. Immunology. Feed your Tregs more fiber. *Science* (2013) 341(6145):463–4. doi: 10.1126/science.1242674
63. Zeng H, Chi H. Metabolic control of regulatory T cell development and function. *Trends Immunol* (2015) 36(1):3–12. doi: 10.1016/j.it.2014.08.003
64. Luu M, Pautz S, Kohl V, Singh R, Romero R, Lucas S, et al. The short-chain fatty acid pentanoate suppresses autoimmunity by modulating the metabolic-epigenetic crosstalk in lymphocytes. *Nat Commun* (2019) 10(1):760. doi: 10.1038/s41467-019-08711-2
65. Kim M, Qie Y, Park J, Kim CH. Gut Microbial Metabolites Fuel Host Antibody Responses. *Cell Host Microbe* (2016) 20(2):202–14. doi: 10.1016/j.chom.2016.07.001
66. Mizuno M, Noto D, Kaga N, Chiba A, Miyake S. The dual role of short fatty acid chains in the pathogenesis of autoimmune disease models. *PloS One* (2017) 12(2):e0173032. doi: 10.1371/journal.pone.0173032
67. Vignali DAA, Collison LW, Workman CJ. How regulatory T cells work. *Nat Rev Immunol* (2008) 8(7):523–32. doi: 10.1038/nri2343
68. Tao R, de Zoeten EF, Ozkaynak E, Chen CX, Wang LQ, Porrett PM, et al. Deacetylase inhibition promotes the generation and function of regulatory T cells. *Nat Med* (2007) 13(11):1299–307. doi: 10.1038/nm1652
69. Rossi O, van Berkel LA, Chain F, Tanweer Khan M, Taverne N, Sokol H, et al. Faecalibacterium prausnitzii A2-165 has a high capacity to induce IL-10 in human and murine dendritic cells and modulates T cell responses. *Sci Rep* (2016) 6:18507. doi: 10.1038/srep18507
70. Zhou L, Zhang M, Wang Y, Dorfman RG, Liu H, Yu T, et al. Faecalibacterium prausnitzii Produces Butyrate to Maintain Th17/Treg Balance and to Ameliorate Colorectal Colitis by Inhibiting Histone Deacetylase 1. *Inflammation Bowel Dis* (2018) 24(9):1926–40. doi: 10.1093/ibd/izy182
71. Yang J, Sundrud MS, Skepner J, Yamagata T. Targeting Th17 cells in autoimmune diseases. *Trends Pharmacol Sci* (2014) 35(10):493–500. doi: 10.1016/j.tips.2014.07.006
72. Furusawa Y, Obata Y, Fukuda S, Endo TA, Nakato G, Takahashi D, et al. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. *Nature* (2013) 504(7480):446–50. doi: 10.1038/nature12721
73. Di Paola M, Cavalieri D, Albanese D, Sordo M, Pindo M, Donati C, et al. Alteration of Fecal Microbiota Profiles in Juvenile Idiopathic Arthritis. Associations with HLA-B27 Allele and Disease Status. *Front Microbiol* (2016) 7:1703. doi: 10.3389/fmicb.2016.01703
74. Strati F, Cavalieri D, Albanese D, De Felice C, Donati C, Hayek J, et al. Altered gut microbiota in Rett syndrome. *Microbiome* (2016) 4(1):41. doi: 10.1186/s40168-016-0185-y
75. Atarashi K, Tanoue T, Shima T, Imaoka A, Kuwahara T, Momose Y, et al. Induction of colonic regulatory T cells by indigenous Clostridium species. *Science* (2011) 331(6015):337–41. doi: 10.1126/science.1198469
76. Machiels K, Joossens M, Sabino J, De Preter V, Arijs I, Eeckhaut V, et al. A decrease of the butyrate-producing species Roseburia hominis and Faecalibacterium prausnitzii defines dysbiosis in patients with ulcerative colitis. *Gut* (2014) 63(8):1275–83. doi: 10.1136/gutjnl-2013-304833
77. Shen ZH, Zhu CX, Quan YS, Yang JM, Yuan W, Yang ZY, et al. Insights into Roseburia intestinalis which alleviates experimental colitis pathology by inducing anti-inflammatory responses. *J Gastroen Hepatol* (2018) 33 (10):1751–60. doi: 10.1111/jgh.14144
78. Devriese S, Eeckhaut V, Geirnaert A, Van den Bossche L, Hindryckx P, Van de Wiele T, et al. Reduced Mucosa-associated Butyricicoccus Activity in Patients with Ulcerative Colitis Correlates with Aberrant Claudin-1 Expression. *J Crohns Colitis* (2017) 11(2):229–36. doi: 10.1093/ecco-jcc/jjw142
79. Shi TT, Xin Z, Hua L, Zhao RX, Yang YL, Wang H, et al. Alterations in the intestinal microbiota of patients with severe and active Graves' orbitopathy: a cross-sectional study. *J Endocrinol Invest* (2019) 42(8):967–78. doi: 10.1007/s40618-019-1010-9
80. Everard A, Belzer C, Geurts L, Ouwerkerk JP, Druart C, Bindels LB, et al. Cross-talk between Akkermansia muciniphila and intestinal epithelium controls diet-induced obesity. *P Natl Acad Sci USA* (2013) 110(22):9066–71. doi: 10.1073/pnas.1219451110

81. Kurtuncu M, Tuzun E, Turkoglu R, Petek-Balci B, Icoz S, Pehlivan M, et al. Effect of short-term interferon-beta treatment on cytokines in multiple sclerosis: Significant modulation of IL-17 and IL-23. *Cytokine* (2012) 59 (2):400–2. doi: 10.1016/j.cyto.2012.05.004
82. Elyaman W, Khoury SJ. Th9 cells in the pathogenesis of EAE and multiple sclerosis. *Semin Immunopathol* (2017) 39(1):79–87. doi: 10.1007/s00281-016-0604-y
83. Samuelson DR, Welsh DA, Shellito JE. Regulation of lung immunity and host defense by the intestinal microbiota. *Front Microbiol* (2015) 6:1085. doi: 10.3389/fmicb.2015.01085
84. Fusunyan RD, Quinn JJ, Ohno Y, MacDermott RP, Sanderson IR. Butyrate enhances interleukin (IL)-8 secretion by intestinal epithelial cells in response to IL-1 beta and lipopolysaccharide. *Pediatr Res* (1998) 43(1):84–90. doi: 10.1203/00006450-199801000-00013
85. Martin R, Miquel S, Benevides L, Bridonneau C, Robert V, Hudault S, et al. Functional Characterization of Novel Faecalibacterium prausnitzii Strains Isolated from Healthy Volunteers: A Step Forward in the Use of F. prausnitzii as a Next-Generation Probiotic. *Front Microbiol* (2017) 8:1226. doi: 10.3389/Fmicb.2017.01226
86. Seo B, Jeon K, Moon S, Lee K, Kim WK, Jeong H, et al. Roseburia spp. Abundance Associates with Alcohol Consumption in Humans and Its Administration Ameliorates Alcoholic Fatty Liver in Mice. *Cell Host Microbe* (2020) 27(1):25–40. doi: 10.1016/j.chom.2019.11.001
87. Hoffmann TW, Pham HP, Bridonneau C, Aubry C, Lamas B, Martin-Gallausiaux C, et al. Microorganisms linked to inflammatory bowel disease-associated dysbiosis differentially impact host physiology in gnotobiotic mice. *ISME J* (2016) 10(2):460–77. doi: 10.1038/ismej.2015.127
88. Ozdal T, Sela DA, Xiao JB, Boyacioglu D, Chen F, Capanoglu E. The Reciprocal Interactions between Polyphenols and Gut Microbiota and Effects on Bioaccessibility. *Nutrients* (2016) 8(2):78. doi: 10.3390/Nu8020078
89. Vinolo MA, Rodrigues HG, Hatanaka E, Sato FT, Sampaio SC, Curi R. Suppressive effect of short-chain fatty acids on production of proinflammatory mediators by neutrophils. *J Nutr Biochem* (2011) 22 (9):849–55. doi: 10.1016/j.jnutbio.2010.07.009
90. Lin MY, de Zoete MR, van Putten JP, Strijbis K. Redirection of Epithelial Immune Responses by Short-Chain Fatty Acids through Inhibition of Histone Deacetylases. *Front Immunol* (2015) 6:554. doi: 10.3389/fimmu.2015.00554
91. Asarat M, Apostolopoulos V, Vasiljevic T, Donkor O. Short-Chain Fatty Acids Regulate Cytokines and Th17/Treg Cells in Human Peripheral Blood Mononuclear Cells in vitro. *Immunol Invest* (2016) 45(3):205–22. doi: 10.3109/08820139.2015.1122613

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

frontiers in Immunology

MINI REVIEW
published: 28 January 2021
doi: 10.3389/fimmu.2020.584165

Helicobacter and the Potential Role in Neurological Disorders: There Is More Than *Helicobacter pylori*

Nina Gorlé[1,2], Eva Bauwens[3], Freddy Haesebrouck[3†], Annemieke Smet[4†] and Roosmarijn E. Vandenbroucke[1,2†]*

[1] VIB Center for Inflammation Research, Ghent, Belgium, [2] Department of Biomedical Molecular Biology, Faculty of Sciences, Ghent University, Ghent, Belgium, [3] Department of Pathology, Bacteriology and Avian Diseases, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium, [4] Laboratory of Experimental Medicine and Pediatrics, Faculty of Medicine and Health Sciences, University of Antwerp, Antwerp, Belgium

OPEN ACCESS

Edited by:
Francesca Ronchi, University of Bern, Switzerland

Reviewed by:
Jorge Tolivia, University of Oviedo, Spain
Yoshiro Ohara, Kanazawa Medical University, Japan

***Correspondence:**
Roosmarijn E. Vandenbroucke
Roosmarijn.Vandenbroucke@irc.VIB-UGent.be

†These authors have contributed equally to this work and share senior authorship

Specialty section:
This article was submitted to Multiple Sclerosis and Neuroimmunology, a section of the journal Frontiers in Immunology

Received: *16 July 2020*
Accepted: *04 December 2020*
Published: *28 January 2021*

Citation:
Gorlé N, Bauwens E, Haesebrouck F, Smet A and Vandenbroucke RE (2021) Helicobacter and the Potential Role in Neurological Disorders: There Is More Than Helicobacter pylori. Front. Immunol. 11:584165. doi: 10.3389/fimmu.2020.584165

Trillions of symbiotic microbial cells colonize our body, of which the larger part is present in the human gut. These microbes play an essential role in our health and a shift in the microbiome is linked to several diseases. Recent studies also suggest a link between changes in gut microbiota and neurological disorders. Gut microbiota can communicate with the brain *via* several routes, together called the microbiome–gut–brain axis: the neuronal route, the endocrine route, the metabolic route and the immunological route. *Helicobacter* is a genus of Gram-negative bacteria colonizing the stomach, intestine and liver. Several papers show the role of *H. pylori* in the development and progression of neurological disorders, while hardly anything is known about other *Helicobacter* species and the brain. We recently reported a high prevalence of *H. suis* in patients with Parkinson's disease and showed an effect of a gastric *H. suis* infection on the mouse brain homeostasis. Here, we discuss the potential role of *H. suis* in neurological disorders and how it may affect the brain *via* the microbiome–gut–brain axis.

Keywords: *Helicobacter pylori*, *Helicobacter suis*, microbiome–gut–brain axis, gut microbiota, neurological disorders

INTRODUCTION

The human microbiota contains trillions of symbiotic microbial cells that live in and on our body of which the vast majority are present in the human gut (1–4). These commensal microbes perform several functions essential to our health and survival, including food digestion (5, 6), activation of certain drugs (4), prevention of infections (7–9), and they might play a role in the maturation of our immune system (10, 11).

Already for a few decades, changes in the gastrointestinal microbiota have been associated with a wide range of health problems including rheumatoid arthritis, inflammatory bowel diseases, asthma, and cancer, et cetera (12–17). Moreover, it has been shown that gastrointestinal changes are able to influence neurological disorders such as depression, anxiety, Alzheimer's disease, Parkinson's disease, and multiple sclerosis (MS) (18–29). Recently, it became clear that the gut microbiome can signal to the brain *via* several pathways, together called the microbiome–gut–brain axis (30–34). In general, communication

between microbiota and the brain is divided into four categories: the neuronal route (enteric nervous system and vagus nerve), the endocrine route (*e.g.* cortisol), the metabolic route (*e.g.* short chain fatty acids (SCFAs) and tryptophan), and the immunological route (*e.g.* cytokines and immune cells) (35, 36). Bacteria can also affect the composition of the gut microbiota, thereby indirectly affecting gut-brain signaling [Cryan and Dinan (35)].

HELICOBACTER PYLORI AND NEUROLOGICAL DISORDERS

A gastric spiral-shaped, Gram-negative microorganism, called *H. pylori*, colonizes the stomach of more than half of the world's human population albeit with large geographical variations. Next to gastritis, peptic ulcer disease, mucosa-associated lymphoid tissue (MALT) -lymphoma, and adenocarcinoma, *H. pylori* infection has also been associated with neurological diseases.

Even though both innate and acquired immune responses are activated in individuals infected with *H. pylori*, the host is unable to eradicate the bacteria, leading to a chronic lifelong infection (37, 38). To escape the host's immune response and to survive in the hostile conditions found in the stomach, *H. pylori* has developed several strategies, including manipulating innate immune receptors and inhibiting effector T-cell responses (39, 40). The mechanism to evade the immune system depends on the presence or absence of certain bacterial virulence factors (39). The evoked immune response by the host can lead to the local secretion of various inflammatory mediators, such as interleukin (IL) 8, -6, -1β, -10, and -12, tumor necrosis factor (TNF) and interferon (IFN) γ, which might reach the circulation causing a systemic effect (41, 42). The persistence of noticeable local and systemic concentrations of these pro-inflammatory factors can induce neuroinflammation and -toxicity (41). Next to this, *H. pylori* infection leads to the release of several neurotransmitters, such as acetylcholine, adrenaline, noradrenaline, serotonin, and dopamine (43, 44). Moreover, *H. pylori* infection might lead to axonal/neuronal damage, production of free radicals, and changes in neuropeptide expression, such as vasoactive intestinal peptide (VIP) and c-fos (43). Lastly, *H. pylori* infection is associated with changes in the composition of the gastrointestinal microbiome (43, 45). These changes, illustrated in **Figure 1A**, can potentially alter the outcome of neurological disorders.

Indeed, seropositivity for *H. pylori* has been associated with poor cognition (46), neurologic impairment (47), and cerebrovascular disease (48) and is recognized as a significant

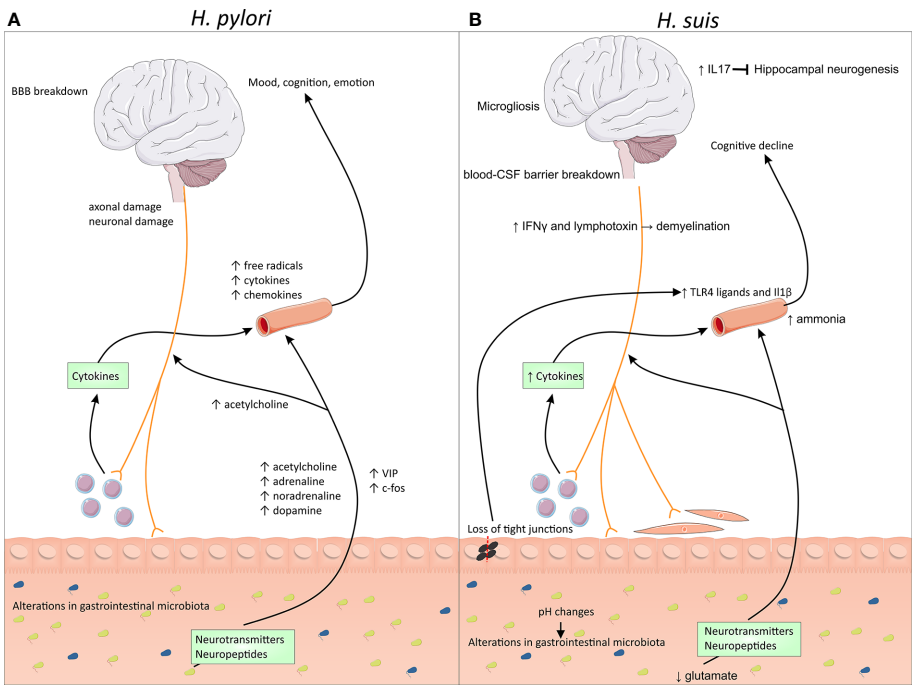


FIGURE 1 | Changes at the microbiome–gut–brain axis during *Helicobacter pylori* and Non-*H. pylori Helicobacter* (NHPH) infection. **(A)** *H. pylori* is associated with persistent local inflammation, which might lead to systemic inflammation, characterized by increased levels of free radicals, cytokines and chemokines in the blood. Infection also leads to the release of several neurotransmitters, such as acetylcholine, adrenaline, noradrenaline and dopamine, but also increased levels of neuropeptides, such as vasoactive intestinal peptide (VIP) and c-fos. Moreover, *H. pylori* can lead to blood–brain barrier breakdown and axonal/neuronal damage. **(B)** *H. suis* is associated with inflammation of the stomach, associated with loss of the gastrointestinal barrier function, leading to leakage of TLR4 ligands into the blood. This leads to the breakdown of the blood-CSF barrier, combined with microgliosis and cognitive decline. *H. suis*-induced changes in the pH possibly leads to changes in the gastrointestinal microbiome. Moreover, lower levels of glutamate are present, which could influence the production of several neurotransmitters. Higher levels of IL-17 can block hippocampal neurogenesis while IFN-γ and lymphotoxins could lead to demyelination. BBB, blood–brain barrier; CSF, cerebrospinal fluid; IL, interleukin; IFN, interferon.

risk factor for the development of dementia (21, 49). Next to an association of *H. pylori* with Parkinson's disease (50), it has also been shown that infection with *H. pylori* increases the risk of developing Parkinson's disease (41, 51, 52). Moreover, it has been shown that eradication of *H. pylori* improves the motor symptoms associated with Parkinson's disease (53, 54). Interestingly, *H. pylori* might influence the bioavailability of L-3,4-dihydroxyphenylalaline (L-DOPA), the most common treatment for Parkinson's disease (52, 55).

H. pylori might also play a role in Alzheimer's disease as discussed in a review by Doulberis et al. (56). *H. pylori* infection is associated with mild cognitive impairment, a prodromal phase of Alzheimer's disease (57, 58) and with Alzheimer's disease itself (59). Higher levels of neuro-inflammation have been found in Alzheimer's disease patients infected with *H. pylori*, which correlated with cognitive decline (60, 61), whereas eradication of *H. pylori* improved the cognitive and functional abilities (62, 63).

In multiple sclerosis (MS), however, *H. pylori* is found less in patients compared to control ones (64) and infection is even thought to be beneficial (65). Lower clinical signs were found in mice infected with *H. pylori* compared to control animals (66).

THERE IS MORE IN THE STOMACH THAN *HELICOBACTER PYLORI*

Since the description of *H. pylori*, many other gastric species in the genus *Helicobacter* have been described. These gastric non-*H. pylori Helicobacter* (NHPH) species have been reported in the stomach of various hosts, including pigs, dogs, cats, and non-human primates and some of them have a zoonotic potential (67, 68). The most prevalent gastric NHPH species in humans is *Helicobacter suis* which naturally colonizes the stomach of pigs and non-human primates (67, 68). The bacterium is of zoonotic importance, infecting 0.2–6% of the human population, causing gastritis, peptic ulcers, and MALT lymphoma (67). However, since some infections with this microorganism remain subclinical, their true prevalence in humans is probably underestimated (67). Furthermore, these spiral-shaped bacteria are not always found in the human stomach after investigation of a small biopsy sample due to their focal and patchy colonization pattern (67, 69–71). Like *H. pylori*, *H. suis* may lead to a life-long infection, associated with a tolerogenic immune response (24, 72).

In literature, hardly any data is available on the association between an infection with NHPH species and neurological disorders. Indeed, there are no papers describing the association of NHPH with neurodegenerative or -immunological disorders like amyotrophic lateral sclerosis, spinocerebellar degeneration, acute disseminated encephalomyelitis, and Guillain-Barré syndrome. One study showed that mice infected with *Helicobacter felis* display both gastric and neuroinflammation (73). In another study, a remarkable high presence of *H. suis* DNA (27%) was found in gastric biopsies from idiopathic Parkinson's disease patients compared to a control group without clinical symptoms of Parkinson's disease (2%) (74). This was not the case for other zoonotically important gastric NHPH species. Additionally, *H. suis* DNA was found in a blood sample of a patient simultaneously affected by Parkinson's and Alzheimer's disease. After eradication of the *H. suis* infection, the patient's gastric and neurological symptoms improved remarkably (74). Moreover, *H. suis* infection in Parkinson's patients has recently been linked with higher mortality (75). To our knowledge, there are no other papers describing a role for *H. suis* in neurological disorders. Here, we will discuss several possible ways *H. suis* might influence the brain. These changes are summarized in **Figure 1B**.

HELICOBACTER SUIS AND THE MICROBIOME–GUT–BRAIN AXIS

In the first part, inflammatory changes in the stomach and how they might affect the brain *via* the systemic circulation are discussed. In the second part, changes due to virulence factors of *H. suis* and the effect on the microbiome are discussed.

Inflammatory Changes and Gastrointestinal Barrier Functioning

Infection with *H. suis* in pigs and mice is associated with increased inflammation in the stomach, characterized by the higher expression of IL-8, -10, -1β, and -4, keratinocyte chemoattractant (KC), lipopolysaccharide-induced CXC chemokine (LIX), and macrophage inflammatory protein (MIP2) depending on the host (72, 76–78). This leads to the infiltration of B- and T-cells and macrophages in mice, inducing a Th2 response.

Gastritis is accompanied by mucosal edema (67) and gastric epithelial cell death (79), all of which could compromise the integrity of the gastrointestinal barrier. The gastrointestinal barrier consists of two layers: the epithelial cell layer, connected by tight junctions, and a mucus layer. In pigs, significant downregulation of claudin 18 (CLDN18) was found in the stomach of *H. suis* infected animals (72). In a recent mouse study, we found increased permeability of the gastrointestinal barrier after *H. suis* infection, accompanied by increased expression of mucine 13 (Muc13) and aberrant localization of zonula occludens 1 (ZO1) (77). This further progressed to systemic inflammation, characterized by the leakage of TLR4 ligands into the blood, affecting the brain homeostasis *via* the blood–cerebrospinal fluid barrier (77). Next to TLR4 ligands, also IL1β was found in the serum of *H. suis*-infected mice, which is shown to induce inflammatory gene expression in the hippocampus and hypothalamus associated with sickness behavior (80). As discussed below, also other molecules that are observed in the stomach upon *H. suis* infection might affect the brain when reaching the systemic circulation due to a leaky gut.

Next to the Th2 response, also a Th17 response has been associated with *H. suis* infection in the different hosts (mice, gerbils, pigs, and humans), characterized by the presence of Th17 cells and/or increased levels of IL-17 in the stomach (76, 78, 81, 82). IL-17 is known to block adult hippocampus neurogenesis (83) and is linked to depression in MS (84). In gerbils, but not mice, also increased levels of IFN-γ were found in the stomach of *H. suis* infected animals (81). IFN-γ is shown to be a regulator of the neural precursor pool in the non-inflamed brain (85) but is

also linked with demyelination due to the reduced proliferation and viability of oligodendroglial cells (86, 87).

H. suis is also associated with increased levels of lymphotoxin (LT)-α and -β in the stomach of mice (88). These cytokines are not only involved in the generation of follicular dendritic cells (89), but also regulate neuronal and glial lineage differentiation (90). Lymphotoxins have been shown to play a role in MS, causing demyelination due to oligodendrocyte toxicity (91). Blocking lymphotoxin in experimental autoimmune encephalomyelitis (EAE), a mouse model of MS, reduces disease symptoms, which is accompanied with lower levels of the chemokine CXCL13 (92). This chemokine plays a role in the recruitment of B-cells and its expression is increased in the stomach after *H. suis* infection in both pigs, mice, and gerbils (72, 81), as are other chemokines such as C-X-C motif chemokine receptor (CXCR) 7, 15 and 4, C-C motif chemokine ligand (CCL) 19 and 21, and C-X-C motif chemokine ligand 12 (CXCL12) (88). In MS, higher levels of CXCL13 have been observed in B-cell aggregates in the inflamed meninges (92) and correlate with demyelination, neural cell loss, and rapid disease progression (93). Thus, higher levels of CXCL13 caused by a *H. suis* infection can potentially lead to accelerated disease progression.

Changes Due to Virulence Factors, Metabolism and Microbiome

H. suis affects the presence of glutamine and glutathione by its virulence factor γ-glutamyl transpeptidase (GGT), in this way damaging epithelial cells (81, 82, 94). Glutamine and glutathione are not only important for the health of gastrointestinal tissue (95), they are also precursors for the neurotransmitters glutamate, aspartate, and γ-amino butyric acid (GABA), which are important neurotransmitters. Depletion of glutamine, caused by *H. suis* infection, could thus lead to changes in these neurotransmitters, affecting gut–brain signaling.

Urea is converted by *H. suis* to ammonia by the presence of urease (96, 97). High levels of ammonia are linked to encephalopathy, associated with neuropsychiatric and neurological symptoms (98, 99). Although it is unlikely that an *H. suis* infection leads to high levels of ammonia, the continuous exposure of slightly higher levels could also interfere with normal brain functioning.

Parietal cells are also affected by *H. suis*-associated inflammation. This leads to changes in the expression and functioning of H^+/K^+-ATPase and subsequent changes in pH, which is associated with more fluid gastric content (72). These changes can subsequently influence the gastric microbiota. Indeed, more *Fusobacterium gastrosuis* was found in *H. suis* infected pigs (100). Infection with *H. felis*, another NHPH known to infect humans, is associated with a decrease in *Lactobacillus* and an increase in *Clostridium*, *Bacteroidetes*, *Prevotella*, *Eubacterium*, *Ruminococcus*, *Streptococcus*, and *E. coli* in the stomach (94, 101). *Lactobacillus* has been shown to secrete acetylcholine, which is important in regulating memory, attention, and learning, and has therapeutic effects in mental illnesses, reducing anxiety and depression (102). Lower numbers of *Lactobacillus* due to *H. suis* could thus possibly affect mood. Increased levels of *Clostridium* has been linked to autism (103), indicating that increased presence of *Clostridium* in *H. suis*-infected animals might affect brain homeostasis.

CONCLUSION

Numerous studies have been published about the possible effect of a *H. pylori* infection on neurological diseases, while other *Helicobacter* species have hardly been studied. However, recent studies report on a possible link between *H. suis* infection and Parkinson's disease. Here, we describe several possible pathways in the microbiome–gut–brain axis which could be influenced by *H. suis* infection. Altogether, this highlights the importance of gaining more insights in the role of *non-Helicobacter pylori Helicobacter* species in neurological diseases.

AUTHOR CONTRIBUTIONS

NG wrote the manuscript. EV, RV, AS, and FH advised and reviewed the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

NG is supported by FWO Vlaanderen. Research in the author's lab is sponsored by FWO Vlaanderen, Ghent University, VIB, and the Baillet Latour Fund.

ACKNOWLEDGMENTS

We thank the Servier Medical Art for the use of Powerpoint images from their images bank.

REFERENCES

1. Kverka M, Tlaskalova-Hogenova H. Intestinal Microbiota: Facts and Fiction. *Dig Dis* (2017) 35(1-2):139–47. doi: 10.1159/000449095
2. Sender R, Fuchs S, Milo R. Revised Estimates for the Number of Human and Bacteria Cells in the Body. *PLoS Biol* (2016) 14(8):e1002533. doi: 10.1371/journal.pbio.1002533
3. Ursell LK, Metcalf JL, Parfrey LW, Knight R. Defining the human microbiome. *Nutr Rev* (2012) 70 Suppl 1:S38–44. doi: 10.1111/j.1753-4887.2012.00493.x
4. Moos WH, Faller DV, Harpp DN, Kanara I, Pernokas J, Powers WR, et al. Microbiota and Neurological Disorders: A Gut Feeling. *Biores Open Access* (2016) 5(1):137–45. doi: 10.1089/biores.2016.0010
5. Bergman EN. Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. *Physiol Rev* (1990) 70(2):567–90. doi: 10.1152/physrev.1990.70.2.567
6. Hill MJ. Intestinal flora and endogenous vitamin synthesis. *Eur J Cancer Prev* (1997) 6 Suppl 1:S43–5. doi: 10.1097/00008469-199703001-00009
7. Brandl K, Plitas G, Mihu CN, Ubeda C, Jia T, Fleisher M, et al. Vancomycin-resistant enterococci exploit antibiotic-induced innate immune deficits. *Nature* (2008) 455(7214):804–7. doi: 10.1038/nature07250
8. Royet J, Gupta D, Dziarski R. Peptidoglycan recognition proteins: modulators of the microbiome and inflammation. *Nat Rev Immunol* (2011) 11(12):837–51. doi: 10.1038/nri3089

9. Spitz JC, Ghandi S, Taveras M, Aoys E, Alverdy JC. Characteristics of the intestinal epithelial barrier during dietary manipulation and glucocorticoid stress. *Crit Care Med* (1996) 24(4):635–41. doi: 10.1097/00003246-199604000-00015
10. Cebra JJ. Influences of microbiota on intestinal immune system development. *Am J Clin Nutr* (1999) 69(5):1046S–51S. doi: 10.1093/ajcn/69.5.1046s
11. Cebra JJ, Logan AC, Weinstein PD. The preference for switching to expression of the IgA isotype of antibody exhibited by B lymphocytes in Peyer's patches is likely due to intrinsic properties of their microenvironment. *Immunol Res* (1991) 10(3-4):393–5. doi: 10.1007/BF02919728
12. Wu X, He B, Liu J, Feng H, Ma Y, Li D, et al. Molecular Insight into Gut Microbiota and Rheumatoid Arthritis. *Int J Mol Sci* (2016) 17(3):431. doi: 10.3390/ijms17030431
13. Abraham C, Cho JH. Inflammatory bowel disease. *N Engl J Med* (2009) 361 (21):2066–78. doi: 10.1056/NEJMra0804647
14. Halfvarson J, Brislawn CJ, Lamendella R, Vazquez-Baeza Y, Walters WA, Bramer LM, et al. Dynamics of the human gut microbiome in inflammatory bowel disease. *Nat Microbiol* (2017) 2:17004. doi: 10.1038/nmicrobiol.2017.4
15. Huang YJ, Boushey HA. The microbiome in asthma. *J Allergy Clin Immunol* (2015) 135(1):25–30. doi: 10.1016/j.jaci.2014.11.011
16. Hahn DL, Dodge RW, Golubjatnikov R. Association of Chlamydia pneumoniae (strain TWAR) infection with wheezing, asthmatic bronchitis, and adult-onset asthma. *JAMA* (1991) 266(2):225–30. doi: 10.1001/jama.266.2.225
17. Trinchieri G. Cancer and inflammation: an old intuition with rapidly evolving new concepts. *Annu Rev Immunol* (2012) 30:677–706. doi: 10.1146/annurev-immunol-020711-075008
18. Bercik P, Denou E, Collins J, Jackson W, Lu J, Jury J, et al. The intestinal microbiota affect central levels of brain-derived neurotropic factor and behavior in mice. *Gastroenterology* (2011) 141(2):599–609, e1-3. doi: 10.1053/j.gastro.2011.04.052
19. Bravo JA, Forsythe P, Chew MV, Escaravage E, Savignac HM, Dinan TG, et al. Ingestion of Lactobacillus strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. *Proc Natl Acad Sci USA* (2011) 108(38):16050–5. doi: 10.1073/pnas.1102999108
20. Dinan TG, Cryan JF. Gut-brain axis in 2016: Brain-gut-microbiota axis - mood, metabolism and behaviour. *Nat Rev Gastroenterol Hepatol* (2017) 14 (2):69–70. doi: 10.1038/nrgastro.2016.200
21. Hu X, Wang T, Jin F. Alzheimer's disease and gut microbiota. *Sci China Life Sci* (2016) 59(10):1006–23. doi: 10.1007/s11427-016-5083-9
22. Mulak A, Bonaz B. Brain-gut-microbiota axis in Parkinson's disease. *World J Gastroenterol* (2015) 21(37):10609–20. doi: 10.3748/wjg.v21.i37.10609
23. Parracho HM, Bingham MO, Gibson GR, McCartney AL. Differences between the gut microflora of children with autistic spectrum disorders and that of healthy children. *J Med Microbiol* (2005) 54(Pt 10):987–91. doi: 10.1099/jmm.0.46101-0
24. Zhang YJ, Li S, Gan RY, Zhou T, Xu DP, Li HB. Impacts of gut bacteria on human health and diseases. *Int J Mol Sci* (2015) 16(4):7493–519. doi: 10.3390/ijms16047493
25. Boulange CL, Neves AL, Chilloux J, Nicholson JK, Dumas ME. Impact of the gut microbiota on inflammation, obesity, and metabolic disease. *Genome Med* (2016) 8(1):42. doi: 10.1186/s13073-016-0303-2
26. Clemente JC, Ursell LK, Parfrey LW, Knight R. The impact of the gut microbiota on human health: an integrative view. *Cell* (2012) 148(6):1258–70. doi: 10.1016/j.cell.2012.01.035
27. Dinan TG, Cryan JF. The Microbiome-Gut-Brain Axis in Health and Disease. *Gastroenterol Clin North Am* (2017) 46(1):77–89. doi: 10.1016/j.gtc.2016.09.007
28. Walters WA, Xu Z, Knight R. Meta-analyses of human gut microbes associated with obesity and IBD. *FEBS Lett* (2014) 588(22):4223–33. doi: 10.1016/j.febslet.2014.09.039
29. Selber-Hnatiw S, Rukundo B, Ahmadi M, Akoubi H, Al-Bizri H, Aliu AF, et al. Human Gut Microbiota: Toward an Ecology of Disease. *Front Microbiol* (2017) 8:1265. doi: 10.3389/fmicb.2017.01265
30. Mayer EA, Knight R, Mazmanian SK, Cryan JF, Tillisch K. Gut microbes and the brain: paradigm shift in neuroscience. *J Neurosci* (2014) 34(46):15490–6. doi: 10.1523/JNEUROSCI.3299-14.2014
31. Martin CR, Osadchiy V, Kalani A, Mayer EA. The Brain-Gut-Microbiome Axis. *Cell Mol Gastroenterol Hepatol* (2018) 6(2):133–48. doi: 10.1016/j.jcmgh.2018.04.003
32. Galland L. The gut microbiome and the brain. *J Med Food* (2014) 17 (12):1261–72. doi: 10.1089/jmf.2014.7000
33. Kelly JR, Minuto C, Cryan JF, Clarke G, Dinan TG. Cross Talk: The Microbiota and Neurodevelopmental Disorders. *Front Neurosci* (2017) 11:490:490. doi: 10.3389/fnins.2017.00490
34. Cryan JF, O'Riordan KJ, Cowan CSM, Sandhu KV, Bastiaanssen TFS, Boehme M, et al. The Microbiota-Gut-Brain Axis. *Physiol Rev* (2019) 99 (4):1877–2013. doi: 10.1152/physrev.00018.2018
35. Cryan JF, Dinan TG. Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. *Nat Rev Neurosci* (2012) 13(10):701–12. doi: 10.1038/nrn3346
36. Grenham S, Clarke G, Cryan JF, Dinan TG. Brain-gut-microbe communication in health and disease. *Front Physiol* (2011) 2:94. doi: 10.3389/fphys.2011.00094
37. Abadi ATB. Strategies used by helicobacter pylori to establish persistent infection. *World J Gastroenterol* (2017) 23(16):2870–82. doi: 10.3748/wjg.v23.i16.2870
38. Robinson K, Kaneko K, Andersen LP. Helicobacter: Inflammation, immunology and vaccines. *Helicobacter* (2017) 22 Suppl 1. doi: 10.1111/hel.12406
39. Mejias-Luque R, Gerhard M. Immune Evasion Strategies and Persistence of Helicobacter pylori. *Curr Top Microbiol Immunol* (2017) 400:53–71. doi: 10.1007/978-3-319-50520-6_3
40. Lundgren A, Suri-Payer E, Enarsson K, Svennerholm AM, Lundin BS. Helicobacter pylori-specific CD4+ CD25high regulatory T cells suppress memory T-cell responses to H. pylori in infected individuals. *Infect Immun* (2003) 71(4):1755–62. doi: 10.1128/IAI.71.4.1755-1762.2003
41. Alvarez-Arellano L, Maldonado-Bernal C. Helicobacter pylori and neurological diseases: Married by the laws of inflammation. *World J Gastrointest Pathophysiol* (2014) 5(4):400–4. doi: 10.4291/wjgp.v5.i4.400
42. Peek RM Jr., Fiske C, Wilson KT. Role of innate immunity in Helicobacter pylori-induced gastric malignancy. *Physiol Rev* (2010) 90(3):831–58. doi: 10.1152/physrev.00039.2009
43. Budzynski J, Klopocka M. Brain-gut axis in the pathogenesis of Helicobacter pylori infection. *World J Gastroenterol* (2014) 20(18):5212–25. doi: 10.3748/wjg.v20.i18.5212
44. Meng WP, Wang ZQ, Deng JQ, Liu Y, Deng MM, Lu MH. The Role of H. pylori CagA in Regulating Hormones of Functional Dyspepsia Patients. *Gastroenterol Res Pract* (2016) 2016:7150959. doi: 10.1155/2016/7150959
45. Engstrand L, Lindberg M. Helicobacter pylori and the gastric microbiota. *Best Pract Res Clin Gastroenterol* (2013) 27(1):39–45. doi: 10.1016/j.bpg.2013.03.016
46. Beydoun MA, Beydoun HA, Shroff MR, Kitner-Triolo MH, Zonderman AB. Helicobacter pylori seropositivity and cognitive performance among US adults: evidence from a large national survey. *Psychosom Med* (2013) 75 (5):486–96. doi: 10.1097/PSY.0b013e31829108c3
47. Kimura A, Matsubasa T, Kinoshita H, Kuriya N, Yamashita Y, Fujisawa T, et al. Helicobacter pylori seropositivity in patients with severe neurologic impairment. *Brain Dev* (1999) 21(2):113–7. doi: 10.1016/S0387-7604(98)00086-2
48. Tsang KW, Lam SK. Helicobacter pylori and extra-digestive diseases. *J Gastroenterol Hepatol* (1999) 14(9):844–50. doi: 10.1046/j.1440-1746.1999.01974.x
49. Roubaud Baudron C, Letenneur L, Langlais A, Buissonniere A, Megraud F, Dartigues JF, et al. Does Helicobacter pylori infection increase incidence of dementia? The Personnes Agees QUID Study. *J Am Geriatr Soc* (2013) 61 (1):74–8. doi: 10.1111/jgs.12065
50. Shen X, Yang H, Wu Y, Zhang D, Jiang H. Meta-analysis: Association of Helicobacter pylori infection with Parkinson's diseases. *Helicobacter* (2017) 22(5). doi: 10.1111/hel.12398
51. Nielsen HH, Qiu J, Friis S, Wermuth L, Ritz B. Treatment for Helicobacter pylori infection and risk of Parkinson's disease in Denmark. *Eur J Neurol* (2012) 19(6):864–9. doi: 10.1111/j.1468-1331.2011.03643.x
52. Mridula KR, Borgohain R, Chandrasekhar Reddy V, Bandaru V, Suryaprabha T. Association of Helicobacter pylori with Parkinson's Disease. *J Clin Neurol* (2017) 13(2):181–6. doi: 10.3988/jcn.2017.13.2.181

53. Dobbs SM, Dobbs RJ, Weller C, Charlett A, Bjarnason IT, Lawson AJ, et al. Differential effect of Helicobacter pylori eradication on time-trends in brady/hypokinesia and rigidity in idiopathic parkinsonism. *Helicobacter* (2010) 15 (4):279–94. doi: 10.1111/j.1523-5378.2010.00768.x
54. Bjarnason IT, Charlett A, Dobbs RJ, Dobbs SM, Ibrahim MA, Kerwin RW, et al. Role of chronic infection and inflammation in the gastrointestinal tract in the etiology and pathogenesis of idiopathic parkinsonism. Part 2: response of facets of clinical idiopathic parkinsonism to Helicobacter pylori eradication. A randomized, double-blind, placebo-controlled efficacy study. *Helicobacter* (2005) 10(4):276–87. doi: 10.1111/j.1523-5378.2005.00330.x
55. Hashim H, Azmin S, Razlan H, Yahya NW, Tan HJ, Manaf MR, et al. Eradication of Helicobacter pylori infection improves levodopa action, clinical symptoms and quality of life in patients with Parkinson's disease. *PLoS One* (2014) 9(11):e112330. doi: 10.1371/journal.pone.0112330
56. Doulberis M, Kotronis G, Thomann R, Polyzos SA, Boziki M, Gialamprinou D, et al. Review: Impact of Helicobacter pylori on Alzheimer's disease: What do we know so far? *Helicobacter* (2018) 23 (1). doi: 10.1111/hel.12454
57. Kountouras J, Tsolaki M, Boziki M, Gavalas E, Zavos C, Stergiopoulos C, et al. Association between Helicobacter pylori infection and mild cognitive impairment. *Eur J Neurol* (2007) 14(9):976–82. doi: 10.1111/j.1468-1331.2007.01827.x
58. Han ML, Chen JH, Tsai MK, Liou JM, Chiou JM, Chiu MJ, et al. Association between Helicobacter pylori infection and cognitive impairment in the elderly. *J Formos Med Assoc* (2018) 117(11):994–1002. doi: 10.1016/j.jfma.2017.11.005
59. Kountouras J, Doulberis M, Polyzos SA, Katsinelos T, Vardaka E, Kountouras C, et al. Impact of Helicobacter pylori and/or Helicobacter pylori-related metabolic syndrome on incidence of all-cause and Alzheimer's dementia. *Alzheimers Dement* (2019) 15(5):723–5. doi: 10.1016/j.jalz.2019.01.008
60. Kountouras J, Boziki M, Gavalas E, Zavos C, Deretzi G, Grigoriadis N, et al. Increased cerebrospinal fluid Helicobacter pylori antibody in Alzheimer's disease. *Int J Neurosci* (2009) 119(6):765–77. doi: 10.1080/00207450902782083
61. Roubaud-Baudron C, Krolak-Salmon P, Quadrio I, Megraud F, Salles N. Impact of chronic Helicobacter pylori infection on Alzheimer's disease: preliminary results. *Neurobiol Aging* (2012) 33(5):1009.e11–9. doi: 10.1016/j.neurobiolaging.2011.10.021
62. Kountouras J, Boziki M, Gavalas E, Zavos C, Deretzi G, Chatzigeorgiou S, et al. Five-year survival after Helicobacter pylori eradication in Alzheimer disease patients. *Cognit Behav Neurol* (2010) 23(3):199–204. doi: 10.1097/WNN.0b013e3181df3034
63. Kountouras J, Boziki M, Gavalas E, Zavos C, Grigoriadis N, Deretzi G, et al. Eradication of Helicobacter pylori may be beneficial in the management of Alzheimer's disease. *J Neurol* (2009) 256(5):758–67. doi: 10.1007/s00415-009-5011-z
64. Li W, Minohara M, Su JJ, Matsuoka T, Osoegawa M, Ishizu T, et al. Helicobacter pylori infection is a potential protective factor against conventional multiple sclerosis in the Japanese population. *J Neuroimmunol* (2007) 184(1-2):227–31. doi: 10.1016/j.jneuroim.2006.12.010
65. Kira J. Helicobacter pylori infection might prove the hygiene hypothesis in multiple sclerosis. *J Neurol Neurosurg Psychiatry* (2015) 86(6):591–2. doi: 10.1136/jnnp-2014-309759
66. Cook KW, Crooks J, Hussain K, O'Brien K, Braitch M, Kareem H, et al. Helicobacter pylori infection reduces disease severity in an experimental model of multiple sclerosis. *Front Microbiol* (2015) 6:52. doi: 10.3389/fmicb.2015.00052
67. Haesebrouck F, Pasmans F, Flahou B, Chiers K, Baele M, Meyns T, et al. Gastric helicobacters in domestic animals and nonhuman primates and their significance for human health. *Clin Microbiol Rev* (2009) 22(2):202–23. doi: 10.1128/CMR.00041-08. Table of Contents.
68. Flahou B, Haesebrouck F, Smet A. Non-Helicobacter pylori Helicobacter Infections in Humans and Animals. In: S Backert and Y Yamaoka, editors. *Helicobacter pylori Research: From Bench to Bedside*. Tokyo: Springer Japan (2016). p. 233–69.
69. Yakoob J, Abbas Z, Khan R, Naz S, Ahmad Z, Islam M, et al. Prevalence of non Helicobacter pylori species in patients presenting with dyspepsia. *BMC Gastroenterol* (2012) 12:3. doi: 10.1186/1471-230X-12-3
70. Trebesius K, Adler K, Vieth M, Stolte M, Haas R. Specific detection and prevalence of Helicobacter heilmannii-like organisms in the human gastric mucosa by fluorescent in situ hybridization and partial 16S ribosomal DNA sequencing. *J Clin Microbiol* (2001) 39(4):1510–6. doi: 10.1128/JCM.39.4.1510-1516.2001
71. De Groote D, Van Doorn LJ, Van den Bulck K, Vandamme P, Vieth M, Stolte M, et al. Detection of non-pylori Helicobacter species in "Helicobacter heilmannii"-infected humans. *Helicobacter* (2005) 10(5):398–406. doi: 10.1111/j.1523-5378.2005.00347.x
72. De Witte C, Devriendt B, Flahou B, Bosschem I, Ducatelle R, Smet A, et al. Helicobacter suis induces changes in gastric inflammation and acid secretion markers in pigs of different ages. *Vet Res* (2017) 48(1):34. doi: 10.1186/s13567-017-0441-6
73. Albaret G, Sifre E, Floch P, Laye S, Aubert A, Dubus P, et al. Alzheimer's Disease and Helicobacter pylori Infection: Inflammation from Stomach to Brain? *J Alzheimers Dis* (2020) 73(2):801–9. doi: 10.3233/JAD-190496
74. Blaecher C, Smet A, Flahou B, Pasmans F, Ducatelle R, Taylor D, et al. Significantly higher frequency of Helicobacter suis in patients with idiopathic parkinsonism than in control patients. *Aliment Pharmacol Ther* (2013) 38(11-12):1347–53. doi: 10.1111/apt.12520
75. Augustin AD, Savio A, Nevel A, Ellis RJ, Weller C, Taylor D, et al. Helicobacter suis Is Associated With Mortality in Parkinson's Disease. *Front Med (Lausanne)* (2019) 6:188. doi: 10.3389/fmed.2019.00188
76. Bosschem I, Bayry J, De Bruyne E, Van Deun K, Smet A, Vercauteren G, et al. Effect of Different Adjuvants on Protection and Side-Effects Induced by Helicobacter suis Whole-Cell Lysate Vaccination. *PLoS One* (2015) 10(6):e0131364. doi: 10.1371/journal.pone.0131364
77. Gorle N, Blaecher C, Bauwens E, Vandendriessche C, Balusu S, Vandewalle J, et al. The choroid plexus epithelium as a novel player in the stomach-brain axis during Helicobacter infection. *Brain Behav Immun* (2017) 69:35–47. doi: 10.1016/j.bbi.2017.12.010
78. Flahou B, Deun KV, Pasmans F, Smet A, Volf J, Rychlik I, et al. The local immune response of mice after Helicobacter suis infection: strain differences and distinction with Helicobacter pylori. *Vet Res* (2012) 43:75. doi: 10.1186/1297-9716-43-75
79. Flahou B, Haesebrouck F, Chiers K, Van Deun K, De Smet L, Devreese B, et al. Gastric epithelial cell death caused by Helicobacter suis and Helicobacter pylori gamma-glutamyl transpeptidase is mainly glutathione degradation-dependent. *Cell Microbiol* (2011) 13(12):1933–55. doi: 10.1111/j.1462-5822.2011.01682.x
80. Skelly DT, Hennessy E, Dansereau MA, Cunningham C. A systematic analysis of the peripheral and CNS effects of systemic LPS, IL-1beta, [corrected] TNF-alpha and IL-6 challenges in C57BL/6 mice. *PLoS One* (2013) 8(7):e69123. doi: 10.1371/journal.pone.0069123
81. Zhang G, Ducatelle R, De Bruyne E, Joosten M, Bosschem I, Smet A, et al. Role of gamma-glutamyltranspeptidase in the pathogenesis of Helicobacter suis and Helicobacter pylori infections. *Vet Res* (2015) 46:31. doi: 10.1186/s13567-015-0163-6
82. Vermoote M, Van Steendam K, Flahou B, Smet A, Pasmans F, Glibert P, et al. Immunization with the immunodominant Helicobacter suis urease subunit B induces partial protection against H. suis infection in a mouse model. *Vet Res* (2012) 43:72. doi: 10.1186/1297-9716-43-72
83. Liu Q, Xin W, He P, Turner D, Yin J, Gan Y, et al. Interleukin-17 inhibits adult hippocampal neurogenesis. *Sci Rep* (2014) 4:7554. doi: 10.1038/srep07554
84. Waisman A, Hauptmann J, Regen T. The role of IL-17 in CNS diseases. *Acta Neuropathol* (2015) 129(5):625–37. doi: 10.1007/s00401-015-1402-7
85. Li L, Walker TL, Zhang Y, Mackay EW, Bartlett PF. Endogenous interferon gamma directly regulates neural precursors in the non-inflammatory brain. *J Neurosci* (2010) 30(27):9038–50. doi: 10.1523/JNEUROSCI.5691-09.2010
86. Hansen-Pupp I, Harling S, Berg AC, Cilio C, Hellstrom-Westas L, Ley D. Circulating interferon-gamma and white matter brain damage in preterm infants. *Pediatr Res* (2005) 58(5):946–52. doi: 10.1203/01.PDR.0000182592.76702.E8

87. Baerwald KD, Popko B. Developing and mature oligodendrocytes respond differently to the immune cytokine interferon-gamma. *J Neurosci Res* (1998) 52(2):230–9. doi: 10.1002/(SICI)1097-4547(19980415)52:2<230::AID-JNR11>3.0.CO;2-B
88. Zhao WJ, Tian ZB, Yao SS, Yu YN, Zhang CP, Li XY, et al. High-fat-diet-induced obesity upregulates the expression of lymphoid chemokines and promotes the formation of gastric lymphoid follicles after Helicobacter suis infection. *Pathog Dis* (2017) 75(8). doi: 10.1093/femspd/ftx101
89. Oldstone MB, Race R, Thomas D, Lewicki H, Homann D, Smelt S, et al. Lymphotoxin-alpha- and lymphotoxin-beta-deficient mice differ in susceptibility to scrapie: evidence against dendritic cell involvement in neuroinvasion. *J Virol* (2002) 76(9):4357–63. doi: 10.1128/JVI.76.9.4357-4363.2002
90. Xiao X, Putatunda R, Zhang Y, Soni PV, Li F, Zhang T, et al. Lymphotoxin beta receptor-mediated NFkappaB signaling promotes glial lineage differentiation and inhibits neuronal lineage differentiation in mouse brain neural stem/progenitor cells. *J Neuroinflamm* (2018) 15(1):49. doi: 10.1186/s12974-018-1074-z
91. Lock C, Oksenberg J, Steinman L. The role of TNFalpha and lymphotoxin in demyelinating disease. *Ann Rheum Dis* (1999) 58 Suppl 1:I121–8. doi: 10.1136/ard.58.2008.i121
92. Huber AK, Irani DN. Targeting CXCL13 During Neuroinflammation. *Adv Neuroimmune Biol* (2015) 6(1):1–8. doi: 10.3233/NIB-150101
93. Haugen M, Frederiksen JL, Degn M. B cell follicle-like structures in multiple sclerosis-with focus on the role of B cell activating factor. *J Neuroimmunol* (2014) 273(1-2):1–7. doi: 10.1016/j.jneuroim.2014.05.010
94. De Witte C, Taminiau B, Flahou B, Hautekiet V, Daube G, Ducatelle R, et al. In-feed bambermycin medication induces anti-inflammatory effects and prevents parietal cell loss without influencing Helicobacter suis colonization in the stomach of mice. *Vet Res* (2018) 49(1):35. doi: 10.1186/s13567-018-0530-1
95. Shibayama K, Kamachi K, Nagata N, Yagi T, Nada T, Doi Y, et al. A novel apoptosis-inducing protein from Helicobacter pylori. *Mol Microbiol* (2003) 47(2):443–51. doi: 10.1046/j.1365-2958.2003.03305.x
96. Burne RA, Chen YY. Bacterial ureases in infectious diseases. *Microbes Infect* (2000) 2(5):533–42. doi: 10.1016/S1286-4579(00)00312-9
97. Eaton KA, Brooks CL, Morgan DR, Krakowka S. Essential role of urease in pathogenesis of gastritis induced by Helicobacter pylori in gnotobiotic piglets. *Infect Immun* (1991) 59(7):2470–5. doi: 10.1128/IAI.59.7.2470-2475.1991
98. Braissant O, McLin VA, Cudalbu C. Ammonia toxicity to the brain. *J Inherit Metab Dis* (2013) 36(4):595–612. doi: 10.1007/s10545-012-9546-2
99. Bosoi CR, Rose CF. Identifying the direct effects of ammonia on the brain. *Metab Brain Dis* (2009) 24(1):95–102. doi: 10.1007/s11011-008-9112-7
100. De Witte C, Flahou B, Ducatelle R, Smet A, De Bruyne E, Cnockaert M, et al. Detection, isolation and characterization of Fusobacterium gastrosuis sp. nov. colonizing the stomach of pigs. *Syst Appl Microbiol* (2017) 40(1):42–50. doi: 10.1016/j.syapm.2016.10.001
101. Schmitz JM, Durham CG, Schoeb TR, Soltau TD, Wolf KJ, Tanner SM, et al. Helicobacter felis–associated gastric disease in microbiota-restricted mice. *J Histochem Cytochem* (2011) 59(9):826–41. doi: 10.1369/0022155411416242
102. Liu L, Zhu G. Gut-Brain Axis and Mood Disorder. *Front Psychiatry* (2018) 9:223:223. doi: 10.3389/fpsyt.2018.00223
103. Argou-Cardozo I, Zeidan-Chulia F. Clostridium Bacteria and Autism Spectrum Conditions: A Systematic Review and Hypothetical Contribution of Environmental Glyphosate Levels. *Med Sci (Basel)* (2018) 6(2). doi: 10.3390/medsci6020029

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

frontiers in Immunology

ORIGINAL RESEARCH
published: 19 February 2021
doi: 10.3389/fimmu.2021.623451

Lactobacillus acidipiscis Induced Regulatory Gamma Delta T Cells and Attenuated Experimental Autoimmune Encephalomyelitis

Saisai Ren[2†], *Xiaorong Zhang*[1†], *Hongbing Guan*[1*], *Lihong Wu*[1], *Miao Yu*[1], *Dan Hou*[1], *Yongyong Yan*[1] *and Xuechun Fang*[2]

[1] *Guangzhou Key Laboratory of Basic and Applied Research of Oral Regenerative Medicine, Department of Basic Science of Stomatology, Affiliated Stomatology Hospital of Guangzhou Medical University, Guangzhou, China,* [2] *Guangzhou Medical University, Guangzhou, China*

Multiple sclerosis is a chronic autoimmune disease involving the central nervous system, and shows a high disability rate. Its pathogenesis is complicated, and there is no good treatment. In recent years, with in-depth studies on the regulation of gastrointestinal flora, the relationship between the mammalian immune system and the intestinal flora has been extensively explored. Changes in the composition and structure of the gastrointestinal flora can affect the characteristics and development of the host immune system and even induce a series of central nervous system inflammation events. The occurrence and development of multiple sclerosis are closely related to the continuous destruction of the intestinal barrier caused by intestinal dysbacteriosis. In this study, we analyzed *Lactobacillus acidipiscis* in a mouse model of experimental autoimmune encephalomyelitis (EAE). We found that the amount of *L. acidipiscis* in the intestinal tract was inversely proportional to the progress of EAE development. In addition, the number of $CD4^+$ $FOXP3^+$ regulatory T cells in the mesenteric lymph nodes of mice increased significantly after the mice were fed with *L. acidipiscis*, and the differentiation of $CD4^+$ T cells to Th1 and Th17 cells was inhibited. However, the protective effect of *L. acidipiscis* was lost in γδ T cell-deficient mice and hence was concluded to depend on the presence of regulatory γδ T cells in the intestinal epithelium. Moreover, including *L. acidipiscis* enhanced the development of $V\gamma1^+$ γδ T cells but suppressed that of $V\gamma4^+$ γδ T cells. In summary, our results demonstrated the ability of *L. acidipiscis* to induce generation of regulatory γδ T cells that suppress the development of the encephalomyelitic Th1 and Th17 cells and the progress of EAE.

Keywords: ***Lactobacillus acidipiscis***, **γδ T cells, regulatory T cells, T helper cells, multiple sclerosis, experimental autoimmune encephalomyelitis**

OPEN ACCESS

Edited by:
Lloyd Kasper,
Dartmouth College, United States

Reviewed by:
Iain Comerford,
University of Adelaide, Australia
Cristina Ulivieri,
University of Siena, Italy

***Correspondence:**
Hongbing Guan
hongbing2015@qq.com

†These authors have contributed equally to this work

Specialty section:
This article was submitted to Multiple Sclerosis and Neuroimmunology, a section of the journal Frontiers in Immunology

Received: *30 October 2020*
Accepted: *27 January 2021*
Published: *19 February 2021*

Citation:
Ren S, Zhang X, Guan H, Wu L, Yu M, Hou D, Yan Y and Fang X (2021) Lactobacillus acidipiscis Induced Regulatory Gamma Delta T Cells and Attenuated Experimental Autoimmune Encephalomyelitis. Front. Immunol. 12:623451. doi: 10.3389/fimmu.2021.623451

INTRODUCTION

More than 2.5 million people worldwide suffer from multiple sclerosis (MS), which is a chronic autoimmune disease affecting the central nervous system (CNS) and mainly occurring in young women with a high disability rate (1, 2). The pathogenesis of MS is very complex and its exact mechanism is unclear. Inflammation of the CNS and demyelination of the nerves are

the main signs of MS. At present, MS is generally believed to be caused by autoreactive immune cells infiltrating the blood-brain barrier (BBB) with abnormal responses to autoantigens of the CNS, and myelin-specific $CD4^+$ T cells are key to the occurrence of this disease (3). $CD4^+$ helper T (Th) cells are considered to play the most important role in the pathogenesis of MS. Th1 and Th17 cells promote inflammation of the CNS, while Th2 and regulatory T (Treg) cells inhibit it. Experimental autoimmune encephalomyelitis (EAE) is a disease model mediated by specific sensitized $CD4^+$ T cells, and is constructed by immunizing experimental animals with myelin sheath protein. This model is consistent with the induction method of MS, specifically combining myelin oligodendrocyte glycoprotein residues 35–55 (MOG_{35-55}) with immunostimulant to induce the generation of pathogenic Th1 and Th17 cells (4).

Changes of intestinal microflora may lead to maladjustments of the immune response in the intestinal tract and other peripheral lymphoid sites including the CNS. There is increasing evidence that signal communication on the microbe-gut-brain response axis is closely related to the occurrence of MS, Parkinson's disease, Alzheimer's disease, depression, and other CNS diseases (5). Intestinal symbiotic bacteria that induce the generation of $CD4^+$ T cells have been shown to change the severity of the demyelination of the CNS, and changing some bacterial groups in the intestinal tract could lead to a proinflammatory state, which in turn may lead to the development of autoimmune diseases, especially MS. Oral treatment of mice with antibiotics has been shown to reduce the severity of EAE by reducing inflammation and increasing accumulation of FOXP3 in mesenteric and cervical lymph nodes (6). Our results in 2016 demonstrated that the attenuation of EAE seen following CD44 gene deletion in mice, i.e., in CD44 knockout (CD44KO) mice, may result from alterations in the gut microbiota and short-chain fatty acids (SCFAs). Furthermore, our studies also demonstrated that phenotypes of gene knock-out animals in general may be shaped by gut microbiota (7).

Lactobacillus acidipiscis is an aerobic gram-positive lactobacillus acidophilus, and was recently discovered to be present in the lungs of healthy people, with this presence related to the function of pulmonary γδ T cells (8). In the intestinal epithelial lymphocytes (IELs) of mice, specifically in the duodenum and jejunum, the percentage of T cells consisting of γδ T cells was observed to be as high as 70%, much higher than that of αβ T cells (9). More interestingly, γδ T cells constitute the main target of intestinal bacteria. Aerobic bacteria or facultative anaerobes can activate γδ T cells more effectively than can anaerobes. One of the effectors produced by γδ T cells has been found to be IL-17, and hence these T cells are called IL-17-producing γδ T (γδ17 T) cells. In EAE, γδ17 T cells have shown both positive and negative effects, namely on the one hand aggravating the disease by increasing the quantity of Th 17 cells, but also on the other hand alleviating the disease by promoting apoptosis of Th17 cells and enhancing the function of Treg cells. The positive and negative effects of γδ17 T cells may be related to the heterogeneity of γδ T cells. The expression of CCR5 in $V\gamma1^+$ γδ17 T cells can enhance the function of Treg cells and induce the apoptosis of αβ T cells through FAS-FASL signal transduction. However, the expression of CCR6 in $V\gamma4^+$ γδ17 T cells promotes the generation of pathological Th17 cells (10, 11). Therefore, inducing protective regulatory γδ T cells has become a new strategy for treating MS. Studies have shown that intestinal bacteria can stimulate macrophages and dendritic cells to produce IL-1 and IL-23, and activate γδ17 T cells via the guanosine exchange factor 1 (VAV1) signaling pathway. According to the composition of T cell receptors, the peripheral γδ T cells in mice can be divided into two main subsets: $V\gamma1^+$ and $V\gamma4^+$ (12). However, $V\gamma1^+$ γδ T cells have been shown to produce more Th2-type cytokines such as IL-4, while $V\gamma4^+$ γδ T cells have been shown to preferentially produce IL-17A. In an experiment involving PMA/ionomycin-activated $V\gamma1^+$ and $V\gamma4^+$ γδ T cells, 20 differentially expressed genes were identified in the two cell subtypes, with most of these genes related to cytokines, cell differentiation, transcription and translation (13). In addition, intestinal probiotics were shown to produce protective short-chain fatty acids (SCFAs) such as acetate, propionate and butyrate acids (14), which have been shown to regulate the immune balance of intestinal and extraintestinal lymphoid tissues and organs, induce the differentiation of $CD4^+$ T cells from Th1/Th17 cells to Th2/Treg cells, and induce the production of regulatory dendritic cells (DCs) and migration of Treg cells to gut-related lymphoid tissues (GALTs).

In rodent models, discrepancies in gut microbiota were found to be associated with in some cases susceptibility to EAE and other cases resistance to EAE (15). Previous studies from our laboratory showed that CD44 may also regulate inflammation, in as much as CD44 deficiency inhibits proinflammatory Th1 and Th17 cells while promoting $CD4^+$ Th2 and Treg cell differentiation (16). In fact, CD44 deficiency led to decreased inflammation and amelioration of an experimental form of EAE. In those studies, CD44 gene deletion led to the alteration of gut microbiome and attenuation of EAE with simultaneously increase of Treg as well as decrease of Th17 (7).

In our current study, we found that *L. acidipiscis* became significantly more abundant in the intestinal flora of the EAE-resistant (CD44KO) mice, having become the predominant lactobacillus, and the number of γδ T cells in the intestinal tissue increased significantly. *L. acidipiscis* induced resistance of otherwise EAE-susceptible mice to EAE by inducing the proliferation of protective Treg cells and inhibiting the differentiation to Th1 and Th17 cells. In addition, *L. acidipiscis* was shown to be able to induce the generation of $V\gamma1^+$ γδ T cells having inhibitory effects on MS, with these T cells denoted as regulatory γδ T cells. In the absence of these regulatory γδ T cells, *L. acidipiscis* did not protect mice from EAE. Neither a clinical correlation between *L. acidipiscis* and MS nor its application in the clinical treatment of MS was investigated in the current work, but the experimental results that were obtained should provide the basis for further research in this field.

MATERIALS AND METHODS

Mice and Reagents

Specific-pathogen-free (SPF) C57BL/6 female mice that were 6–8 weeks old were purchased from Guangdong Medical

Laboratory Animal Center. Also, SPF CD44KO female mice that were 6–8 weeks old were purchased from Jackson Laboratory and TCR $\delta^{-/-}$ female mice that were 6–8 weeks old were purchased from Shanghai Model Organisms. All experimental animals were maintained under specific pathogen-free conditions at Guangzhou Medical University. All animal experiments were conducted under the protocols approved by and in accordance with the guidelines of the Institute Animal Care and Use Committee of the University. Myelin oligodendrocyte glycoprotein (MOG_{35-55}) peptide was purchased from GL Biochem (Shanghai, China). Pertussis toxin was purchased from Tocris. Incomplete Freund's adjuvant was purchased from Sigma. Antibodies and isotypes were purchased from eBioscience and BioLegend. Cell stimulation cocktail (plus protein transport inhibitors, 500×) was purchased from eBioscience.

Microbial Analysis in the Intestinal and *Lactobacillus acidipiscis* Culture

Fecal samples of EAE mice, specifically of CD44KO (KO) mice and C57BL/6 wild-type (WT) mice, were collected according to the procedure described previously (17). The microbial community of mouse intestinal contents was further analyzed using 16S rDNA. 16S rRNA amplicons were generated for the V3-V4 hypervariable regions of the fecal samples. Moreover, the *Lactobacillus acidipiscis* 10851 strain and *Escherichia coli* (*E. coli*) used in this study were obtained from the China Center of Industrial Culture Collection (CICC, Beijing, China). The strains were amplified in MRS liquid bacterial medium at 37°C for 24 h, and *L. acidipiscis* 10851 or *E. coli* solution was obtained. The solution was subjected to centrifugation at 3,000 g for 5 min, and the resulting supernatant was discarded to obtain the *L. acidipiscis* 10851 or *E. coli* precipitate. Each precipitate was suspended in sterile phosphate-buffered saline (PBS, pH 7.4), and subjected to another centrifugation at 3,000 g for 5 min. Then, the resulting supernatant was discarded, and the number of bacteria in the remaining liquid was counted after repeated washing. Final bacterial dilutions were carried out according to bacterial colony forming units (cfu/mL) at the indicated bacterial cell densities in different buffers or media.

Collection of Feces and Analysis of SCFAs

The fecal contents were collected from C57BL/6 mice fed with *E. coli* or *L. acidipiscis* before being subjected to MOG_{35-55} peptide immunization. Feces were collected on day 15 of the immunization as described previously (18). Fecal contents (100 mg) were acidified with 25% metaphosphoric acid for 30 min on ice and then centrifuged at 12,000 g for 15 min at 4°C. Supernatants were filtered using Ultra free MC columns (0.22 μm GVDurapore, ThermoFisher Scientific) at 12,000 g for 4 min at 4°C. And then the eluates were analyzed using a GC-FID instrument.

Probiotic Protection and EAE Induction

C57BL/6 mice (20 animals) were randomly divided into four groups: C57BL/6 mice that received fecal transfer from C57BL/6 mice, those from CD44 KO mice, and *E. coli* and *L. acidipiscis* protection groups (using 10851 strain). Moreover, TCR $\delta^{-/-}$ mice (10 animals) were randomly assigned to two groups: *E. coli* and *L. acidipiscis* protection groups.

Before carrying out the fecal transfers, recipient mice were treated with streptomycin and ampicillin to deplete endogenous gut microbiota. As described previously (19), all mice were given antibiotics (penicillin and streptomycin, 1 mg/mL aqueous solution, 100 μl) by carrying out oral gavage for two consecutive days. Twenty-four hours after the second antibiotic feeding, C57BL/6 mice were fed fecal solution (200 μl) collected from C57BL/6 or CD44KO mice for two consecutive days. Using a procedure similar to that used for the fecal transfer groups, some of the mice were given *E. coli* and others *L. acidipiscis* (6×10^8 cfu/mL, 200 μl) using oral gavage for two consecutive days. At the same time, mice deficient in γδ T cells (TCR $\delta^{-/-}$) were fed *E. coli* or *L. acidipiscis* 10851 in the same manner.

EAE was observed to be induced in all mice by immunizing them with 150 μg of MOG_{35-55} in CFA-containing heat-killed *Mycobacterium tuberculosis* (strain H37Ra, 6 mg/mL) as described previously (20). Then, on days 0 and 2 post-immunization, the mice were treated intraperitoneally with, respectively, 200 and 400 ng of pertussis toxin. Mice were analyzed every day, and severity of EAE was scored using the 0–5 scoring grade: 0, asymptomatic; 1, tail tension loss; 2, unilateral hind limb paralysis; 3, paralysis of hind legs on both sides; 4, paralysis of forelimb; and 5, moribund.

Detection of Th Subsets *in vivo* After *L. acidipiscis* Feeding

On the 15th day after the C57BL/6 mice were immunized with the MOG_{35-55} peptide, cells were isolated from the mesenteric lymph nodes of C57BL/6 mice after they were fed *E. coli* or *L. acidipiscis*. To investigated the frequency of Treg cells in the mesenteric lymph nodes, cells were stained with FITC-conjugated anti-mouse-CD4 (GK1. 5, eBioscience) and APC-conjugated anti-mouse FOXP3 antibody (FJK-16s, eBioscience). In addition, part of the cells isolated from the mesenteric lymph nodes were re-stimulated with MOG_{35-55} *in vitro* to observe the differentiation of Th1 and Th17. These encephalitogenic $CD4^+$ T cells were cultured in RPMI1640 medium (Gibco BRL) with 10% FCS and MOG_{35-55} (30 μg/mL). Twenty-four hours later, the cells were collected and stained with FITC-conjugated anti-mouse-CD4 (GK1. 5, eBioscience), PE-conjugated anti-mouse IL-17A antibody (eBio17B7, eBioscience) and PerCP-conjugated anti-mouse IFN-γ antibody (XMG1. 2, eBioscience). The concentrations of IFN-γ, IL-17A, IL-10 and IL-13 in the supernatant were determined by using a commercial ELISA kit.

γδT Activation and $CD4^+$ T Differentiation *in vitro* With *L. acidipiscis* Stimulation

For γδT activation, flat-bottom 12-well plates were coated with 500 μl of purified anti-mouse TCR γ/δ antibody (UC7–13D5, 1 μg/ml, BioLegend) at 4°C overnight as described previously (21). Splenocytes were collected from EAE-C57BL/6 mice on

day 15 post-immunization when EAE symptoms peaked, and B cells were removed from the population of splenocytes by carrying out magnetic separation using an EasySep FITC Selection Kit (Stemcell). The remaining cells were added to the Ab-coated wells and cultured in RPMI1640 medium (Gibco BRL) supplemented with 10% fetal calf serum and IL-2 (200 IU/ml) for 8 days. The resulting cells (2×10^6 cells) were co-cultured with *E. coli* or *L. acidipiscis* (1×10^7 cfu) at a ratio of 5:1 splenocyte for 3 days. Cells were collected and stained with FITC-conjugated anti-mouse-TCR Vγ1.1/ Cr4 antibody (2.11, Biolegend), and APC-conjugated anti-mouse TCR Vγ2 antibody (UC3-10A6, Biolegend).

$CD4^+$ T differentiation *in vitro* was performed as previously described with minor modification (16). Briefly, splenocytes were prepared from EAE-C57BL/6 mice on day 15 post-immunization, co-cultured with *E. coli* or *L. acidipiscis* (1×10^7 cfu) at a ratio of 5:1 splenocyte and 30 μg/ml of MOG_{35-55} for 24 h, followed by stimulation with Cell Stimulation Cocktail (plus protein transport inhibitors, 500×, eBioscience) for 5 h. Production of IL-4 and IL-17A in the $CD4^+$ T cells were then detected by intracellular staining and flow cytometry.

Intracellular Staining, Flow Cytometry and Cytokine Assays

For staining of intracellular IFN-γ, FOXP3, IL-4 and IL-17A, cells were stimulated for 5–6 h with Cell Stimulation Cocktail (plus protein transport inhibitors, 500×, eBioscience), which is a cocktail of phorbol 12-myristate 13-acetate (PMA) and ionomycin. The cells were harvested, washed twice with PBS, and analyzed for the presence of Treg and Th17 cells. The cells were then fixed, permeabilized, and stained with IFN-γ-PerCP, FOXP3-APC, IL-4-PE-Cyanine7 (11B11, eBioscience) and IL-17A-PE antibodies. Fluorescence signals were detected using a BD FACS Canto II flow cytometer (BD Biosciences, San Jose, CA,

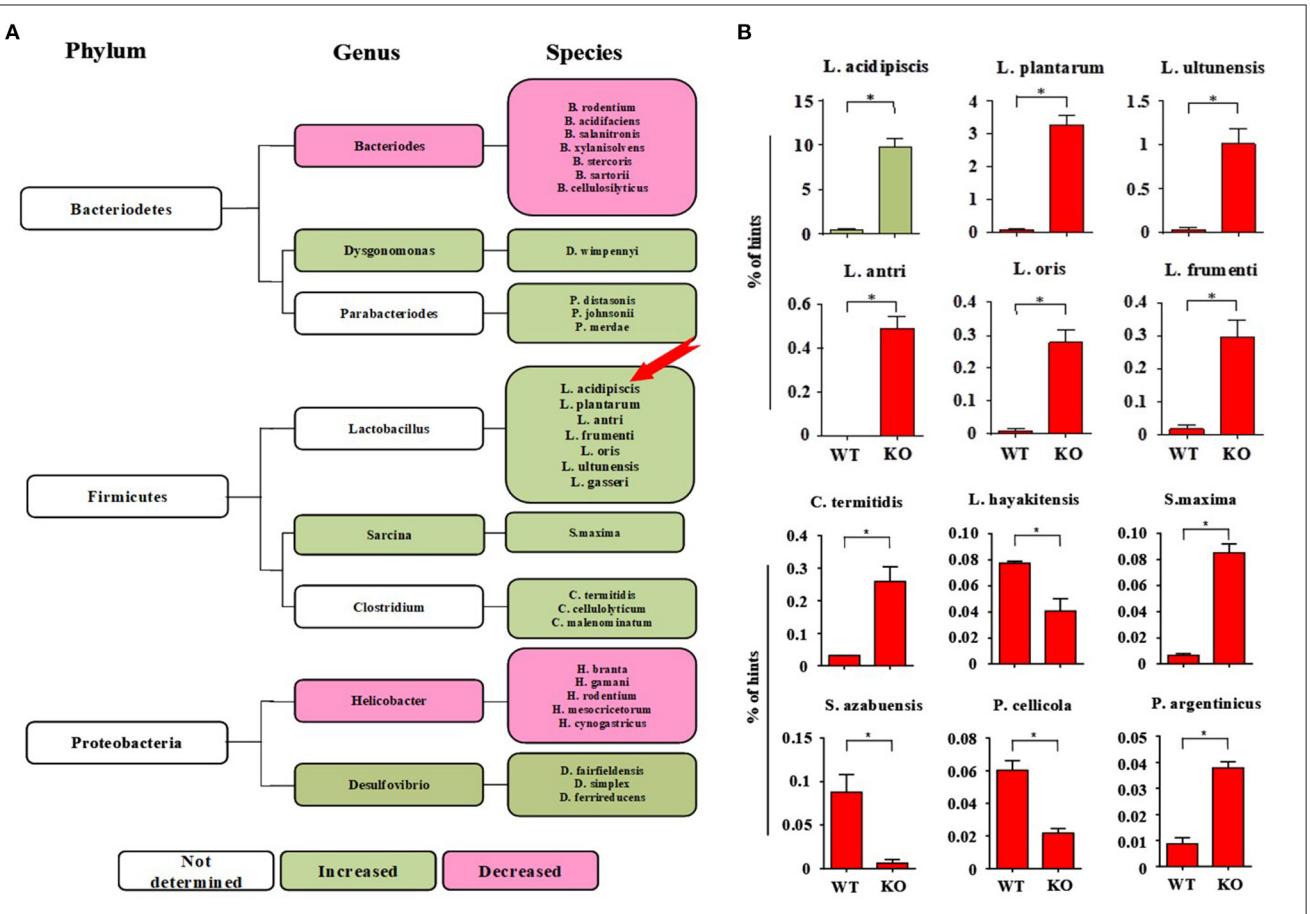


FIGURE 1 | The number of *L. acidipiscis* bacteria was significantly increased in EAE- resistant mice. **(A)** Plots of the intestinal flora compositions of CD44KO and WT mice. The number of lactobacilli and number of desulphurized vibrio bacteria were higher than of WT mice in the intestinal tract of CD44KO mice, while those of bacteroidetes and helicobacter were lower. **(B)** The number of *L. acidipiscis* bacteria was significantly increased in CD44KO mice, and *L. acidipiscis* was the predominant lactobacillus in the intestinal flora of EAE- resistant mice. C57BL/6 wild-type and CD44KO mice are labeled as WT and KO, respectively. Data are shown as mean ± SD and are from a single experiment representative with three mice per experimental group, comparisons were made between EAE WTs ($n = 3$) vs. EAE KOs ($n = 3$). $^*P < 0.05$, Student's *t*-test.

USA). Data were analyzed by using FlowJo (Tree Star, Ashland, OR, USA) software.

Determination of Concentrations of Cytokines Using ELISA

The concentrations of IFN-γ, IL-17A, IL-10 and IL-13 in supernatants were determined by using a commercial ELISA kit (BioLegend) according to the manufacturer's instructions.

Histopathology

Intestinal tissues were removed from mice as a result of subjecting heart to heparin-PBS perfusion and fixed in 10% paraformaldehyde. These intestinal tissues were then treated with GL3 antibody stain, and examined under an optical microscope in order to visualize γδ T cells.

Statistical Analysis

Statistical difference between different groups was analyzed by performing the Student's *t*-test using Graph Pad Prism 6.2 software (GraphPad Software Inc, San Diego, CA, USA). The nonparametric data (EAE scoring) were analyzed using the Mann-Whitney U test. Values of $P < 0.05$ were considered to indicate statistical significance. The statistical analysis data are presented in the manuscript as mean ± SD or mean ± SEM.

RESULTS

The Number of *L. acidipiscis* Bacteria Was Significantly Increased in EAE-Resistant Mice

On the 15th day after mice were immunized with MOG_{35-55} peptide, 16s rRNA V4 sequencing analysis of the intestinal flora of the mice was performed. We found that the intestinal flora composition of CD44KO mice was significantly different from that of WT. The number of *L. acidipiscis* in the intestines of the CD44KO mice was much higher than that in C57BL/6 mice (**Figure 1A**) ($p < 0.05$). The part of the intestinal flora compositions of the EAE-resisitant and WT mice are shown in **Figure 1B**. Significantly different compositions of intestinal bacteria strains in the CD44KO and WT group were observed. Also, significantly more *L. acidipiscis* bacteria than other lactobacilli were found in the CD44KO group, and *L. acidipiscis* was the predominant lactobacillus in the intestinal flora of the CD44KO mice.

L. acidipiscis Induced the Production of Intestinal SCFA

To explore the effect of *L. acidipiscis* on the content of SCFAs in the intestinal tracts of C57BL/6 mice that had been fed *L. acidipiscis*, an EAE model was established by immunizing

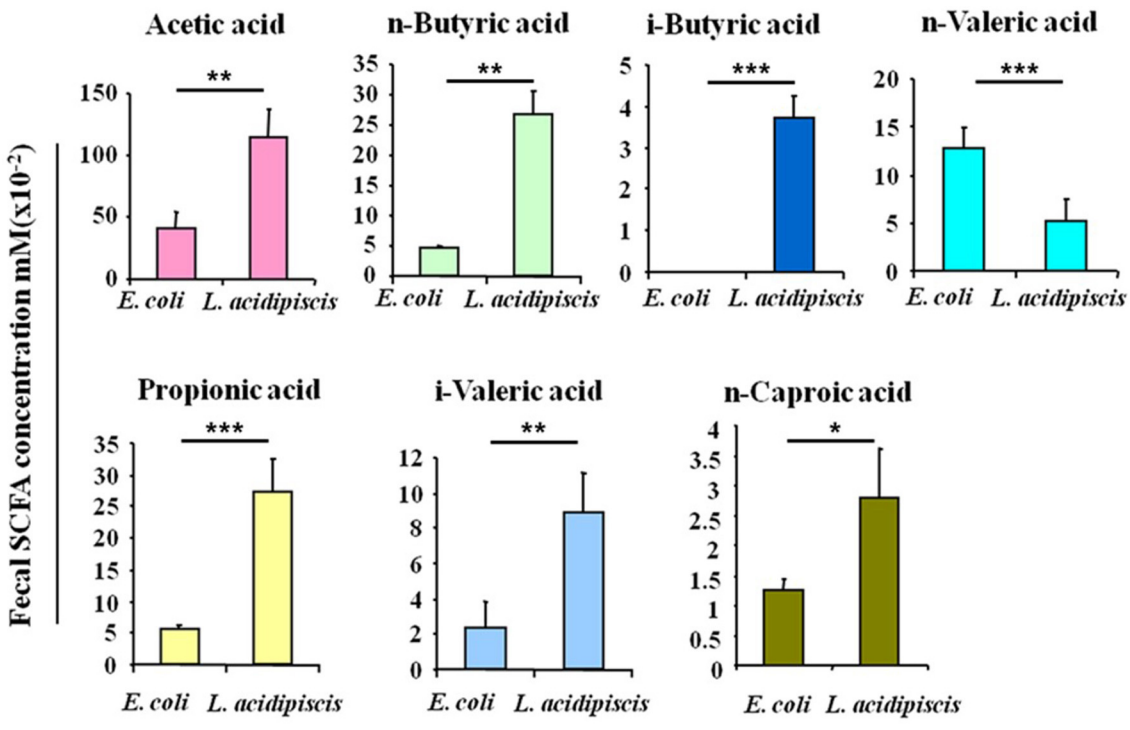


FIGURE 2 | *L. acidipiscis* induced the production of intestinal SCFA. Concentrations of SCFAs in the fecal contents of EAE-WT mice that had been fed *E. coli* or *L. acidipiscis*. Data shown are mean ± SD (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, Student's *t*-test) and are from a single experiment representative with five mice per experimental group.

some of the C57BL/6 mice with the MOG_{35-55} peptide, and treating other mice with *E. coli* as the control group. Mouse feces were collected on day 15 after the immunization with the MOG_{35-55} peptide, and the SCFA content in mouse feces was determined using the GC-FID method. The abundances and concentrations of acetic acid, n-butyric acid, i-butyric acid, n-valeric acid, propionic acid, i-valeric acid and n-caproic acid in each sample are shown in **Figure 2**. The amounts of acetic acid ($p < 0.01$), n-butyric acid ($p < 0.01$), i-butyric acid ($p < 0.001$), propionic acid ($p < 0.001$), i-valeric acid ($p < 0.01$) and n-caproic acid ($p < 0.05$) in the feces of C57BL/6 mice fed with *L. acidipiscis* were significantly higher than for those fed with *E. coli*. Furthermore, the data showed a significant difference ($p < 0.001$) in the concentration of n-valeric acid between the EAE-WT mice that received *E. coli* and those receiving *L. acidipiscis*, indicating that *L. acidipiscis* could induce protective immunophenotypes by synthesizing acetic acid and other SFCAs in the intestinal tracts of EAE-susceptible mice.

L. acidipiscis Induced Resistance to EAE in Susceptible Mice

Seven days before being immunized with the MOG_{35-55} peptide, WT (**Figures 3A,B**) and TCR$\delta^{-/-}$ (**Figure 3C**) mice were inoculated with various materials: some mice were inoculated with fecal material of CD44KO mice (**Figure 3A**), others with *L. acidipiscis* (**Figures 3B,C**), and still others with the fecal material of WT mice (**Figure 3A**) or *E. coli* bacteria (**Figures 3B,C**), respectively, as controls. Feeding CD44KO mouse feces or an *L. acidipiscis* bacteria suspension to WT mice significantly inhibited the occurrence of EAE and reduced the degree of disease (**Figures 3A,B**). However, the occurrence of EAE was not significantly inhibited when *L. acidipiscis* was fed to mice with a deficiency of γδT cells (TCR$\delta^{-/-}$) (**Figure 3C**), suggesting that the protective effect of *L. acidipiscis* on susceptible mice requires the presence of γδT cells.

L. acidipiscis Induced Treg Cell Development and Inhibited Pathological Th1 Cell and Th17 Cell Differentiation

On the 15th day after mice were immunized with MOG_{35-55} peptide, the number of Treg cells from mesenteric lymph nodes of C57BL/6 mice that were fed *L. acidipiscis* was significantly greater than that for C57BL/6 mice that were instead fed control material, and the differentiation of $CD4^+$ T cells to Th1 and Th17 cells was inhibited. The production of protective IL-10 and that of IL-13 were each increased, while the production of pathological IFN-γ and that of IL-17A were each significantly decreased, indicating that *L. acidipiscis* may regulate the differentiation of cerebrospinal inflammatory $CD4^+$ T cells and induce a deviation of the protective T cell immune response (**Figure 4**).

The Number of γδ T Cells Increased Significantly in the Intestines of EAE-Resistance Mice

We also set out to compare the numbers of γδ T cells in EAE-resistant mice and EAE-susceptible mice. For this purpose on day 15 post-immunization of mice with the MOG_{35-55} peptide, immunohistograms were acquired to detect the presence of γδ T cells (GL3 antibody staining positive) in the intestinal tissues of

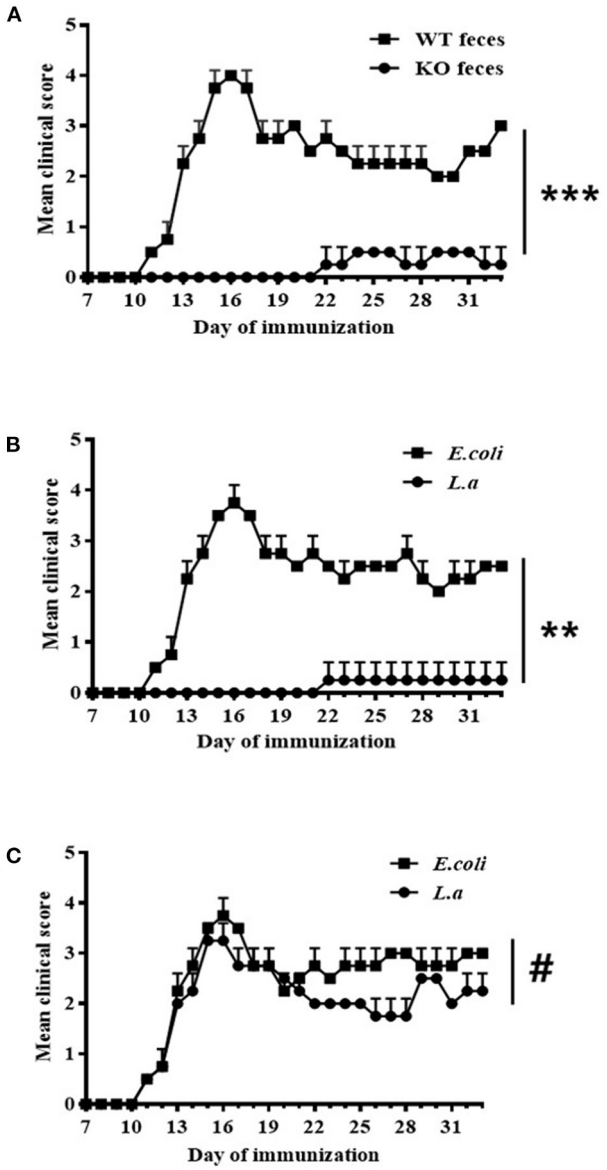


FIGURE 3 | *L. acidipiscis* induced resistance to EAE in susceptible mice. **(A)** Results showing feces of CD44KO mice having induced resistance to EAE in WT receptor mice (WT feces: WT mice with EAE and that received feces from WT mice, $n = 5$; KO feces: WT mice with EAE and that received feces from CD44KO mice, $n = 5$). **(B)** Results showing *L. acidipiscis* having induced resistance to EAE in WT mice (*E. coli*: WT mice with EAE and that were fed *E. coli*, $n = 5$; *L. a*: WT mice with EAE and that were fed *L. acidipiscis*, $n = 5$). **(C)** *L. acidipiscis* did not produce resistance to EAE in TCR$\delta^{-/-}$ mice (*E. coli*: TCR$\delta^{-/-}$ mice with EAE and that were fed *E. coli*, $n = 5$; *L. a*: TCR$\delta^{-/-}$ mice with EAE and that were fed *L. acidipiscis*, $n = 5$). This result suggested the presence of γδT cells to be required for realizing the protective effect of *L. acidipiscis*. Clinical scores were recorded daily after EAE induction and fecal transfer. The values are shown as mean ± SEM. Data were analyzed using the Mann–Whitney *U* test. $^{**}P < 0.01$, $^{***}P < 0.001$, $\#P > 0.05$.

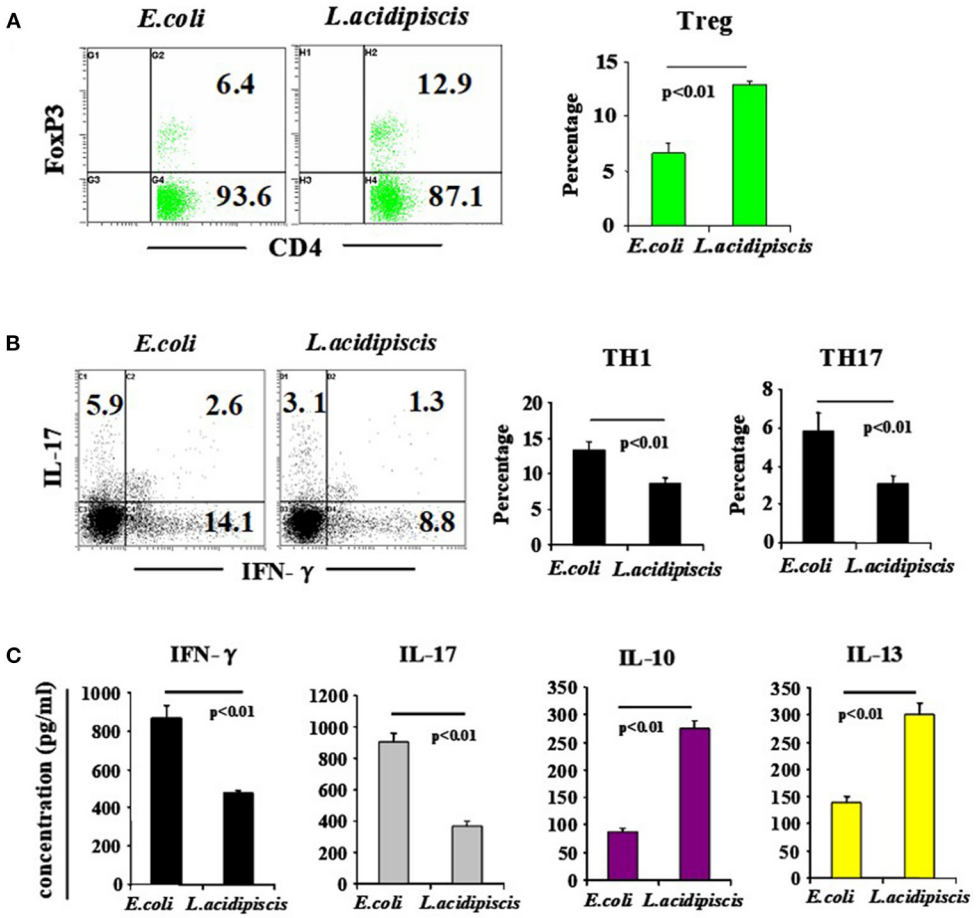


FIGURE 4 | *L. acidipiscis* induced the development of Treg cells and inhibited pathological differentiation into Th1 and Th17 cells. Percentages of $CD4^+$ T cells consisting of Treg, Th1 and Th17 cells in mesenteric lymph nodes of C57BL/6 mice fed with bacteria were determined on day 15 when EAE symptoms peaked. **(A)** FACS detection of $CD4^+$ $FOXP3^+$ Treg cells, and percentage of $CD4^+$ T cells consisting of Treg cells. **(B)** FACS detection of Th1 and Th17 cells, and percentage in $CD4^+$ T cells. **(C)** Cytokine concentration in cell culture supernatant. Representative flow cytometry plots were derived from a single experiment with 5 mice per experimental group and average percentage of each subset was expressed as mean ± SEM from three independent experiments. Statistical differences were determined by using Student's *t*-test, and data with $P < 0.01$ represent significant differences between the two groups.

EAE-resistant mice (CD44KO-EAE) and EAE-susceptible mice (WT-EAE) (arrow in **Figure 5**). Significantly more γδ T cells were found in the intestinal tracts of CD44KO-EAE mice than in those of WT-EAE mice. We confirmed the resistance of CD44KO mice to EAE to be closely related to the presence of γδ T cells in the small intestinal epithelium of mice.

L. acidipiscis Promoted the Development of $V\gamma1^+$ γδ T Cells and Inhibited $V\gamma4^+$ γδ T Cells *in vitro*

To investigate the differential induction of $V\gamma1^+$/ $V\gamma4^+$ γδ T cells and $CD4^+$ Th cells by *L. acidipiscis*, we examined the differentiation of γδ T cells and encephalitogenic $CD4^+$ T cells from the splenocytes of EAE mice. γδ T and $CD4^+$ T cells were co-cultured with or without *L. acidipiscis* strains for 3 days (**Figure 6**). The *L. acidipiscis* strain induced a significant increase in the proportion of $V\gamma1^+$ γδ T cells, and it inhibited the proportion of $V\gamma4^+$ γδ T cells. In addition, the differentiation of Th2 cells was significantly enhanced after they were co-cultured with *L. acidipiscis* (**Figure 6B**), whereas the development of Th17 cells was significantly inhibited after they were co-cultured with *L. acidipiscis* (**Figure 6C**).

DISCUSSION

The cause of multiple sclerosis (MS) is unclear, and there are no effective methods for preventing and treating this disease. Immunology, genetics and histopathology data of patients with MS support the concept that autoimmunity plays a major role in the pathogenesis of the disease. Our current understanding of the pathogenesis of MS and that of its disease-causing mechanisms have mainly derived from the results of investigations using

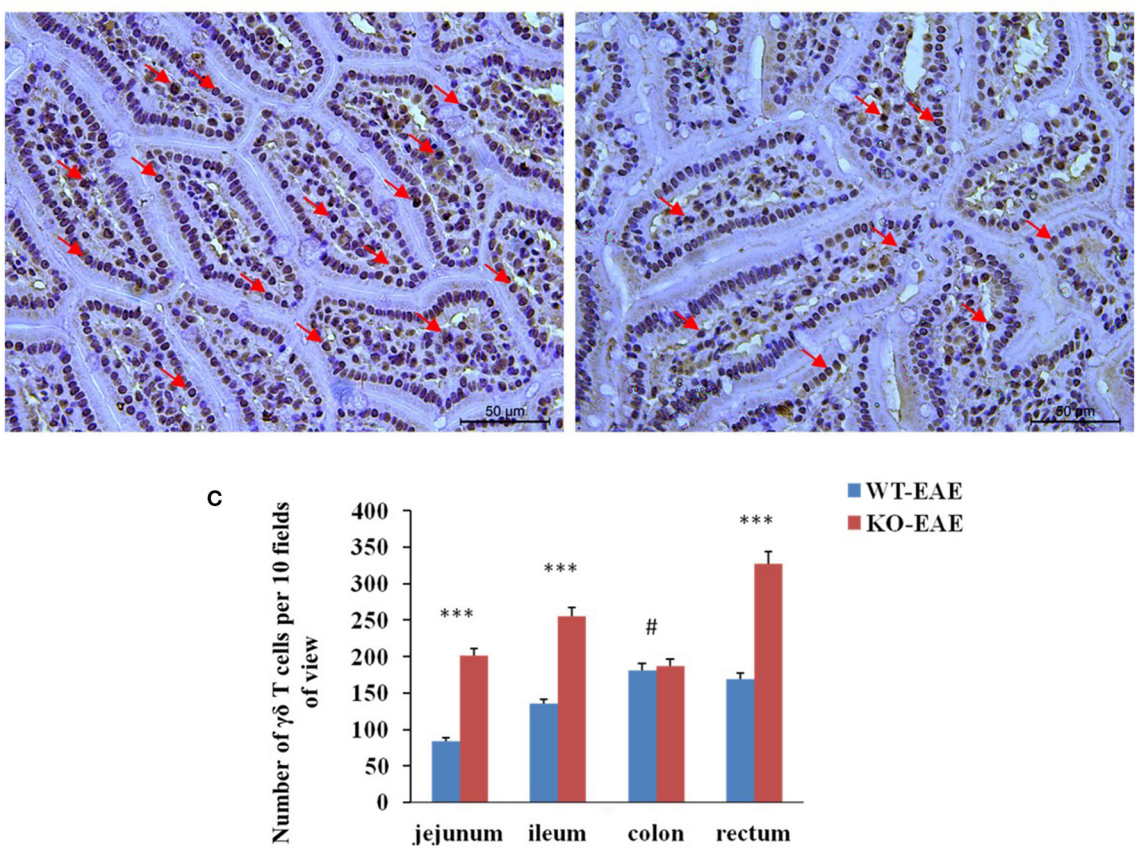


FIGURE 5 | Significantly more γδ T cells were observed in the intestines of EAE-resistant mice. Representative histological appearances of jejunal tissues of CD44-EAE and WT-EAE mice on day 15 post-immunization, parts of γδ T cells with GL3 positive staining were pointed with red arrows. **(A)** Jejunum of a CD44KO-EAE mice. **(B)** Jejunum of a WT-EAE mice. **(C)** Mean numbers of γδ T cells in the jejunum, ileum, colon and rectum of 10 high-power field. Data from three separate experiments with five mice/group are presented as mean ± SEM. Data were analyzed with Student's *t*-test: ***$P < 0.001$, #$P > 0.05$.

a classical mouse model; here, EAE is induced by performing subcutaneous immunization with an emulsion composed of a myelin component, such as MOG peptide, and complete Freund's adjuvant together with an administration of pertussis toxin. The activation of an autoimmune reaction and the production of myelin-specific $CD4^+$ T cells have been identified as being key to the development of MS. Therefore, inducing protective immune phenotype constitutes an important strategy for treatment of MS.

Highly heterogeneous microbial populations reside in the gastrointestinal tracts of mammals, and are essential for the immune systems of the hosts to completely develop. Intestinal microbes determine the development of the host microbial population and immune system, which are in a complex balance; the genetic material of such microbes has recently been coined as the "microbiome." Models for spontaneous EAE were found to be particularly useful for research on the role of gut microbiota in the induction of brain inflammation (22). The results of such research have indicated the signal communication of the gut bacteria-gut-brain response axis to be closely related to the occurrence of MS: when such animals are kept under germ-free conditions, no disease develops; but when their intestines are occupied by normal intestinal flora, disease is triggered. This model can be used to identify the bacterial components of intestinal flora, which can trigger and expand the pro-inflammatory T cells (Th1 and Th17 cells). Intestinal microorganisms come into contact with antigen-presenting cells (APCs) in the intestinal lumen or Peyer's patches. The APCs further present antigen to the naive T cells in the lamina propria. Naive T cells are further activated after migrating to mesenteric lymph nodes. The activated T cells diffuse through the blood and distal lymph nodes and migrate to the ileum propria (23). These results can help explain the mechanisms of chronic brain inflammation induced by antigen diffusion. Generally speaking, intestinal probiotics play an important role in MS, and can prevent and alleviate MS by inhibiting and treating inflammatory cells associated with this disease.

Gut-associated lymphoid tissue (GALT) is an important component of the immune system. Treg and Th17 cells induced

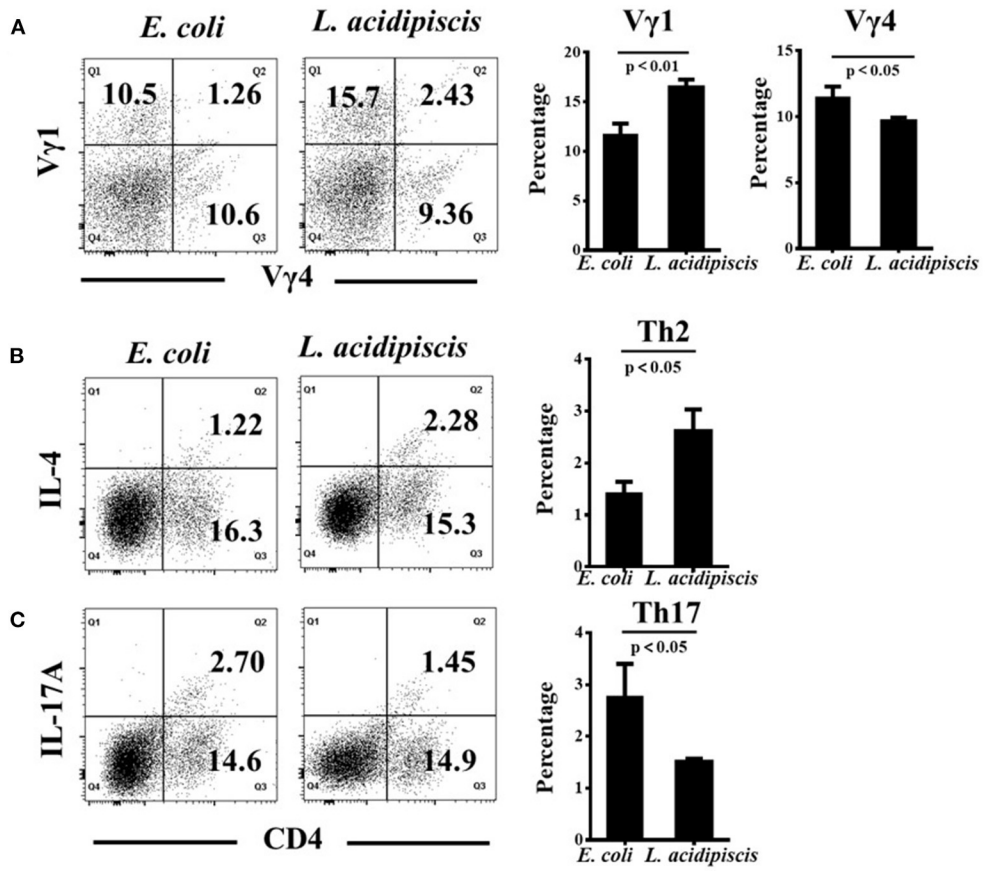


FIGURE 6 | *L. acidipiscis* promoted the development of Vγ1$^+$ γδ T cells and inhibited Vγ4$^+$ γδ T cells *in vitro*. **(A)** The development of Vγ1$^+$ γδ T cells was significantly enhanced while the development of Vγ4$^+$ γδ T cells was significantly suppressed after they were co-cultured with *L. acidipiscis*. **(B)** The development of Th2 cells was significantly enhanced after they were co-cultured with *L. acidipiscis*. **(C)** The differentiation of Th17 cells was significantly inhibited after co-cultured with *L. acidipiscis*. Representative flow cytometry plots were derived from a single experiment with three mice per experimental group and average percentage of each subset was expressed as mean ± SEM from three independent experiments. Data were analyzed with Student's *t*-test, and data with $P < 0.01$, $P < 0.05$ represent significant differences between the two groups.

by the intestinal tract are the characteristic T cells of the intestinal immune network (24). While IL-10 is responsible for maintaining the expression and function of FOXP3 in Treg cells (25). *B. adolescentis* IF1-03 has been shown to stimulate maturation of macrophages, producing higher levels of IL-10 and lower levels of IL-6 and TGF-β, features consistent with the upregulation of Treg cells in DSS-colitis mice *in vivo* and splenocytes *in vitro*. On the other hand, *B. adolescentis* IF1-11 has been shown to stimulate macrophages to secrete higher levels of IL-6 and TGF-β, and lower levels of IL-10, which promoted the differentiation of CD4$^+$ T cells into Th17 cells (26). Therefore, as far as health and disease are concerned, specific bacterial species have been proven to have a significant impact on the differentiation of immune cell subsets and innate immune maturation (27). Specifically, probiotics and prebiotics have been reported to have positive immuno-equilibrium restorative effects. Probiotics contribute to the balance of cytokines, and can positively influence the progress of allergic and inflammatory diseases. Our results showed that compared with *E. coli*, *L. acidipiscis* increased the production of CD4$^+$ FOXP3$^+$ Treg cells, IL-10 and IL-13, and inhibited the production of Th1, Th17, IFN-γ and IL-17A. Clinical observations have shown beneficial clinical effects of probiotics, and probiotics strains may be used to treat inflammatory disease. Evidence has been presented to show that probiotics such as bifidobacterium and lactobacillus in the host can be involved in immune regulation by skewing of naive T cells toward Treg cells (28).

γδ T cells appear earlier than do αβ T cells in the development of thymus, mainly in the early stages of fetal development (29). Compared with αβ T cells, γδ T cells only represent a small number of T cell subsets (1–10%) in the peripheral blood, and are mainly present in epithelial tissue in the form of intraepithelial lymphocytes (IELs). γδ T cells have their own distinct characteristics, such as relatively low TCR diversity and being able to directly recognize antigen without the requirement of a presentation of the antigen that are distinct from those of αβ

T cells (30). In addition, Benakis et al. demonstrated a reduction in ischemic brain damage in mice as a result of antibiotic-induced changes in the intestinal flora, and that the effects could be transmitted through fecal transplantation. Furthermore, intestinal dysbiosis has been found to alter immune homeostasis in the small intestine, leading to an increase in the number of Treg cells and a reduction in IL-17-positive γδ T cells through altered dendritic cell (DC) activity (31). These studies have focused on the role of the intestinal flora and gut-brain axis and IL-17 (+) γδ T cells in MS and EAE as both pathogenic and protective, their role in the CNS, the types of subsets and a possible role in Th17 inflammation.

The heterogeneity of phenotype and function of γδ T cells is not clearly demonstrated so far. Studies have shown activated $V\gamma1^+$ γδ T cells expressing relatively high levels of IL-4 and IL-5 (32), and $V\gamma4^+$ γδ T cells secreting relatively high amounts of IL-17A, IL-17F and IFN-γ (33). $V\gamma1^+$ γδ17 T cells and $V\gamma4^+$ γδ17 T cells are common subtypes of γδ17 T cells. Both cell types maintain the phenotype of producing IFN-γ, TNF-α, TGF-β and IL-10. While $V\gamma1^+$ γδ T cells produce more Th2-type cytokines such as IL-4 and IL-5, $V\gamma4^+$ γδ T cells preferentially produce IL-17 (34). These positive and negative effects may be related to the heterogeneity of γδ T cells. Therefore, the induction of protective regulatory γδ T cells by intestinal probiotics constitutes a new strategy for treating MS. Overall, our results provided evidence that *L. acidipiscis* could influnce the differention of γδ T cells and $CD4^+$ T cells into diffrent subsets *in vitro,* altough the data was limited and lack of *in vivo* evidence. The further study was needed in clarification of the related questions.

CONCLUSIONS

In this study, we found a negative correlation between *L. acidipiscis* in the intestinal tract and the progress of EAE. The resistance of CD44KO mice to EAE was related to the stimulation and activation of γδ T cells in small intestine epithelial tissues by *L. acidipiscis*. Here, we showed the presence of significantly more γδ T cells in the intestinal tracts of CD44KO-EAE mice than in those of WT-EAE mice. The resistance of CD44KO mice to EAE could be transmitted by intragastric administration of *L. acidipiscis* or feces transplanted from CD44KO mice. We analyzed the composition of intestinal flora and the levels of *L. acidipiscis* in EAE. Our results showed a negative correlation between the amount of intestinal *L. acidipiscis* and the progression of EAE, and showed the regulation of encephalitogenic $CD4^+$ T cell differentiation by *L. acidipiscis* to be related to γδ T cells. Meanwhile in our experiments, *L. acidipiscis* suppressed *in vitro* the proliferation of Th1 and Th17 cells as well as the secretion of IFN-γ and IL-17A, and clearly promoted the development of Treg and Th2 cells. In contrast, EAE was not significantly inhibited when *L. acidipiscis* was fed to $TCR\delta^{-/-}$ mice. In summary, we have provided evidence for *L. acidipiscis* being a critical factor in the development of mouse Treg cells. In our experiments, *L. acidipiscis* was used to induce regulatory T cells to differentiate into protective cell subsets, thus EAE-susceptible mice obtaining the resistance to EAE. In addition, our results demonstrated an association between progression of MS and a decreased proportion of *L. acidipiscis* in the host intestinal tract, and demonstrated the ability of *L. acidipiscis* to target $V\gamma1^+$ and $V\gamma4^+$ γδ T cells and to interfere with the expression of IL-10, IL-13, IFN-γ and IL-17A.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of Guangzhou Medical University.

AUTHOR CONTRIBUTIONS

HG contributed to conception and design of the study. SR and XZ performed the experiments. LW and DH carried out data analysis. All authors participated in drafting of the manuscript and critical revision of the draft and contributed to the article and approved the submitted version.

FUNDING

We gratefully acknowledge the financial support from the Guangzhou Science, Technology and Innovation Commission (grant number: 201707010026), Guangdong Science and Technology Department (grant number: 2017A020215141), and Guangzhou Municipal Health and Family Planning Commission (grant number: 20181A011101).

REFERENCES

1. Yamout BI, Alroughani R. Multiple sclerosis. *Semin Neurol.* (2018) 38:212–25. doi: 10.1055/s-0038-1649502
2. Mowry EM, Glenn JD. The dynamics of the gut microbiome in multiple sclerosis in relation to disease. *Neurol Clin.* (2018) 36:185–96. doi: 10.1016/j.ncl.2017.08.008
3. Gran B, Rostami A. T cells, cytokines, and autoantigens in multiple sclerosis. *Curr Neurol Neurosci Rep.* (2001) 1:263–70. doi: 10.1007/s11910-001-0029-3
4. Zhou L, Chong MMW, Littman DR. Plasticity of CD4+ T cell lineage differentiation. *Immunity.* (2009) 30:646–55. doi: 10.1016/j.immuni.2009.05.001
5. Fang X, Zhou X, Miao Y, Han Y, Wei J, Chen T. Therapeutic effect of GLP-1 engineered strain on mice model of Alzheimer's disease and Parkinson's disease. *AMB Express.* (2020) 10:80. doi: 10.1186/s13568-020-01014-6
6. Kadowaki A, Miyake S, Saga R, Chiba A, Mochizuki H, Yamamura T. Gut environment-induced intraepithelial autoreactive CD4(+) T cells suppress central nervous system autoimmunity via LAG-3. *Nat Commun.* (2016) 7:11639. doi: 10.1038/ncomms11639

7. Chitrala KN, Guan H, Singh NP, Busbee B, Gandy A, Mehrpouya-Bahrami P, et al. CD44 deletion leading to attenuation of experimental autoimmune encephalomyelitis results from alterations in gut microbiome in mice. *Eur J Immunol.* (2017) 47:1188–99. doi: 10.1002/eji.201646792
8. Jin C, Lagoudas GK, Zhao C, Bullman S, Bhutkar A, Hu B, et al. Commensal microbiota promote lung cancer development via γδ T cells. *Cell.* (2019) 176:998–1013.e16. doi: 10.1016/j.cell.2018.12.040
9. Ogata M, Itoh T. Gamma/delta intraepithelial lymphocytes in the mouse small intestine. *Anat Sci Int.* (2016) 91:301–12. doi: 10.1007/s12565-016-0341-2
10. McKenzie DR, Kara EE, Bastow CR, Tyllis TS, Fenix KA, Gregor CE, et al. IL-17-producing γδ T cells switch migratory patterns between resting and activated states. *Nat Commun.* (2017) 8:15632. doi: 10.1038/ncomms15632
11. Papotto PH, Reinhardt A, Prinz I, Silva-Santos B. Innately versatile: γδ17 T cells in inflammatory and autoimmune diseases. *J Autoimmun.* (2018) 87:26–37. doi: 10.1016/j.jaut.2017.11.006
12. Welte T, Lamb J, Anderson JF, Born WK, O'Brien RL, Wang T. Role of two distinct γδ T cell subsets during West Nile virus infection. *FEMS Immunol Med Microbiol.* (2008) 53:275–83. doi: 10.1111/j.1574-695X.2008.00430.x
13. Dong P, Zhang S, Cai M, Kang N, Hu Y, Cui L, et al. Global characterization of differential gene expression profiles in mouse Vγ1+ and Vγ4+ γδ T cells. *PLoS ONE.* (2014) 9:e112964. doi: 10.1371/journal.pone.0112964
14. Casanova MR, Azevedo-Silva J, Rodrigues LR, Preto A. Colorectal cancer cells increase the production of short chain fatty acids by propionibacterium freudenreichii impacting on cancer cells survival. *Front Nutr.* (2018) 5:44–. doi: 10.3389/fnut.2018.00044
15. Stanisavljevic S, Lukic J, Momcilovic M, Miljkovic M, Jevtic B, Kojic M, et al. Gut-associated lymphoid tissue, gut microbes and susceptibility to experimental autoimmune encephalomyelitis. *Benef Microbes.* (2016) 7:363–73. doi: 10.3920/BM2015.0159
16. Guan H, Nagarkatti PS, Nagarkatti M. CD44 reciprocally regulates the differentiation of encephalitogenic Th1/Th17 and Th2/regulatory T cells through epigenetic modulation involving DNA methylation of cytokine gene promoters, thereby controlling the development of experimental autoimmune encephalomyelitis. *J Immunol.* (2011) 186:6955–64. doi: 10.4049/jimmunol.1004043
17. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N, et al. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J.* (2012) 6:1621–4. doi: 10.1038/ismej.2012.8
18. Zhao G, Nyman M, Jonsson JA. Rapid determination of short-chain fatty acids in colonic contents and faeces of humans and rats by acidified water-extraction and direct-injection gas chromatography. *Biomed Chromatogr.* (2006) 20:674–82. doi: 10.1002/bmc.580
19. Muller PA, Koscso B, Rajani GM, Stevanovic K, Berres ML, Hashimoto D, et al. Crosstalk between muscularis macrophages and enteric neurons regulates gastrointestinal motility. *Cell.* (2014) 158:300–13. doi: 10.1016/j.cell.2014.04.050
20. Guan H, Fan D, Mrelashvili D, Hao H, Singh NP, Singh UP, et al. MicroRNA let-7e is associated with the pathogenesis of experimental autoimmune encephalomyelitis. *Eur J Immunol.* (2013) 43:104–14. doi: 10.1002/eji.201242702
21. Kang N, Tang L, Li X, Wu D, Li W, Chen X, et al. Identification and characterization of Foxp3(+) gammadelta T cells in mouse and human. *Immunol Lett.* (2009) 125:105–13. doi: 10.1016/j.imlet.2009.06.005
22. Berer K, Mues M, Koutrolos M, Rasbi ZA, Boziki M, Johner C, et al. Commensal microbiota and myelin autoantigen cooperate to trigger autoimmune demyelination. *Nature.* (2011) 479:538–41. doi: 10.1038/nature10554
23. Ochoa-Repáraz J, Mielcarz DW, Begum-Haque S, Kasper LH. Gut, bugs, and brain: role of commensal bacteria in the control of central nervous system disease. *Ann Neurol.* (2011) 69:240–7. doi: 10.1002/ana.22344
24. Atarashi K, Umesaki Y, Honda K. Microbiotal influence on T cell subset development. *Semin Immunol.* (2011) 23:146–53. doi: 10.1016/j.smim.2011.01.010
25. Neumann C, Scheffold A, Rutz S. Functions and regulation of T cell-derived interleukin-10. *Semin Immunol.* (2019) 44:101344. doi: 10.1016/j.smim.2019.101344
26. Yu R, Zuo F, Ma H, Chen S. Exopolysaccharide-Producing Bifidobacterium adolescentis strains with similar adhesion property induce differential regulation of inflammatory immune response in Treg/Th17 Axis of DSS-colitis mice. *Nutrients.* (2019) 11:782. doi: 10.3390/nu11040782
27. Rooks MG, Garrett WS. Gut microbiota, metabolites and host immunity. *Nat Rev Immunol.* (2016) 16:341–52. doi: 10.1038/nri.2016.42
28. Laiño J, Villena J, Kanmani P, Kitazawa H. Immunoregulatory effects triggered by lactic acid bacteria exopolysaccharides: new insights into molecular interactions with host cells. *Microorganisms.* (2016) 4:27. doi: 10.3390/microorganisms4030027
29. Bonneville M, O'Brien RL, Born WK. γδ T cell effector functions: a blend of innate programming and acquired plasticity. *Nat Rev Immunol.* (2010) 10:467–78. doi: 10.1038/nri2781
30. Brenner MB, McLean J, Dialynas DP, Strominger JL, Smith JA, Owen FL, et al. Pillars article: identification of a putative second T-cell receptor. Nature. 1986. 322: 145-149. *J Immunol.* (2016) 196:3509–13. doi: 10.1038/322145a0
31. Benakis C, Brea D, Caballero S, Faraco G, Moore J, Murphy M, et al. Commensal microbiota affects ischemic stroke outcome by regulating intestinal γδ T cells. *Nat Med.* (2016) 22:516–23. doi: 10.1038/nm.4068
32. Wehrmann F, Lavelle JC, Collins CB, Tinega AN, Thurman JM, Burnham EL, et al. γδ T cells protect against LPS-induced lung injury. *J Leukoc Biol.* (2016) 99:373–86. doi: 10.1189/jlb.4A0115-017RR
33. Sun G, Yang S, Cao G, Wang Q, Hao J, Wen Q, et al. γδ T cells provide the early source of IFN-gamma to aggravate lesions in spinal cord injury. *J Exp Med.* (2018) 215:521–35. doi: 10.1084/jem.20170686
34. Blink SE, Caldis MW, Goings GE, Harp CT, Malissen B, Prinz I, et al. γδ T cell subsets play opposing roles in regulating experimental autoimmune encephalomyelitis. *Cell Immunol.* (2014) 290:39–51. doi: 10.1016/j.cellimm.2014.04.013

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

frontiers in Immunology

ORIGINAL RESEARCH
published: 19 February 2021
doi: 10.3389/fimmu.2020.603594

Altered Gut Microbiota Related to Inflammatory Responses in Patients With Huntington's Disease

Gang Du[1,2], *Wei Dong*[1,2], *Qing Yang*[1], *Xueying Yu*[2], *Jinghong Ma*[3], *Weihong Gu*[4] *and Yue Huang*[1,2,5*]

[1] China National Clinical Research Center for Neurological Diseases, Beijing Tiantan Hospital, Capital Medical University, Beijing, China, [2] Centre for Neurological Diseases, Beijing Tiantan Hospital, Capital Medical University, Beijing, China, [3] Neurology Department, XuanWu Hospital, Capital Medical University, Beijing, China, [4] Neurology Department, China-Japan Friendship Hospital, Beijing, China, [5] School of Medical Sciences, Faculty of Medicine, University of New South Wales, Sydney, NSW, Australia

OPEN ACCESS

Edited by:
Raffaella Gozzelino,
New University of Lisbon, Portugal

Reviewed by:
Yanfei Chen,
Zhejiang University, China
Bahtiyar Yilmaz,
Bern University Hospital, Switzerland

***Correspondence:**
Yue Huang
yue.huang@ncrcnd.org.cn

Specialty section:
This article was submitted to Multiple Sclerosis and Neuroimmunology, a section of the journal Frontiers in Immunology

Received: 13 October 2020
Accepted: 30 December 2020
Published: 19 February 2021

Citation:
Du G, Dong W, Yang Q, Yu X, Ma J, Gu W and Huang Y (2021) Altered Gut Microbiota Related to Inflammatory Responses in Patients With Huntington's Disease. Front. Immunol. 11:603594. doi: 10.3389/fimmu.2020.603594

Emerging evidence indicates that gut dysbiosis may play a regulatory role in the onset and progression of Huntington's disease (HD). However, any alterations in the fecal microbiome of HD patients and its relation to the host cytokine response remain unknown. The present study investigated alterations and host cytokine responses in patients with HD. We enrolled 33 HD patients and 33 sex- and age- matched healthy controls. Fecal microbiota communities were determined through 16S ribosomal DNA gene sequencing, from which we analyzed fecal microbial richness, evenness, structure, and differential abundance of individual taxa between HD patients and healthy controls. HD patients were evaluated for their clinical characteristics, and the relationships of fecal microbiota with these clinical characteristics were analyzed. Plasma concentrations of interferon gamma (IFN-γ), interleukin 1 beta (IL-1β), IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, and tumor necrosis factor alpha were measured by Meso Scale Discovery (MSD) assays, and relationships between microbiota and cytokine levels were analyzed in the HD group. HD patients showed increased α-diversity (richness), β-diversity (structure), and altered relative abundances of several taxa compared to those in healthy controls. HD-associated clinical characteristics correlated with the abundances of components of fecal microbiota at the genus level. Genus *Intestinimonas* was correlated with total functional capacity scores and IL-4 levels. Our present study also revealed that genus *Bilophila* were negatively correlated with proinflammatory IL-6 levels. Taken together, our present study represents the first to demonstrate alterations in fecal microbiota and inflammatory cytokine responses in HD patients. Further elucidation of interactions between microbial and host immune responses may help to better understand the pathogenesis of HD.

Keywords: Huntington's disease, gut microbiota, 16S rDNA, cytokines, neuroinflammation

INTRODUCTION

Genetic components are the dominant factors in the pathogeneses of monogenic neurodegenerative diseases. At present, increased attention has been focused on the role of the gut–brain axis and its related humoral response in the development of neurodegenerative diseases. However, the role of the gut–brain axis in monogenic neurodegenerative disease remains unclear.

Huntington's disease (HD) is a monogenic, fully penetrant, progressive neurodegenerative disorder characterized by motor, cognitive, and psychiatric disturbances. HD is caused by the expansion of CAG trinucleotide repeats in exon 1 of the huntingtin (HTT) gene on chromosome 4, and HTT is widely expressed in the brain and in peripheral tissues such as skeletal muscles and the gut (1–4). The mutant huntingtin (mHTT) protein, which is expressed in the gastrointestinal (GI) tract, causes GI dysfunction, including impaired gut motility, diarrhea, and malabsorption of food (5). Malabsorption correlates with the amount of weight loss that is a hallmark of HD, both in patients with HD (6–9) and in several transgenic mouse models of HD (10). A recent study has suggested that gut dysbiosis may play a regulatory role in the onset age and progression of HD symptoms (11). In addition, mHTT is expressed in peripheral myeloid cells, including monocytes and macrophages (12, 13). Furthermore, monocytes from the blood of HD patients produce increased levels of cytokines ex vivo when stimulated (13, 14).

Gut microbiota may play a crucial role in the bidirectional gut–brain axis that affects brain activity under both physiological and pathological conditions (15). A growing number of studies suggests that gut microbiota exhibit physiological functions associated with neurodevelopment, brain function, and behavior (16–19). Maladaptive changes in the composition of gut microbiota, referred to as gut dysbiosis, have been linked to a number of gastrointestinal and metabolic diseases, including inflammatory bowel disease (IBD), obesity, and diabetes (20–22). In addition, gut dysbiosis has been implicated in various neurological and psychiatric diseases, such as autism spectrum disorder (ASD), major depression, amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD), and Alzheimer's disease (AD) (23–28). Furthermore, R6/1 transgenic HD mice exhibit weight loss and gut-microbiota dysbiosis, the latter of which has been confirmed via 16S ribosomal RNA (rRNA) gene sequencing (11). Notably, alterations in circulating metabolites related to gut microbiota in HD patients and transgenic animals have suggested that changes in gut microbiota may occur before the onset of HD (29, 30).

However, there are currently no studies that have reported the composition of gut microbiota and related host cytokine responses in HD patients. Therefore, in the present study, we analyzed and compared the microbiota communities and peripheral cytokine levels of HD patients with those of healthy controls. Additionally, we analyzed the relationships between fecal microbiota and clinical characteristics in HD patients.

MATERIALS AND METHODS

Study Subjects

All HD patients and controls in this study were recruited through a longitudinal study for aging and neurodegeneration project, which was conducted by the neurogenetic group at the China National Clinical Research Center for Neurological Diseases from September 2018 following international standard Enroll HD protocol (31), recommended by China HD Network (CHDN). All participating subjects signed informed consents prior to enrollment. This study was approved by the Research Ethics Committee at Beijing Tiantan Hospital, Capital Medical University, Beijing, China.

By October 2019, 33 HD patients (24 manifest, 9 premanifest) and 33 sex- and age-matched healthy controls were recruited from 14 provinces across China. A study flow chart is presented in **Figure 1**. Each HD patient eligible for the present study received a diagnosis of HD according to a confirmed family history, positive genetic test, and motor disturbance as defined by the Unified HD Rating Scale (UHDRS) total motor score (TMS) diagnostic confidence score. The exclusion criteria were defined as severe chronic diseases such as diabetes, heart failure, liver cirrhosis, malignancy, hematological/autoimmune diseases, irritable bowel syndrome, and other movement disorders such as Wilson disease and chorea-acanthocytosis. The healthy controls were neurologically normal individuals and close relatives of the HD patients, such as neurologically normal spouse or parents, to ensure they shared similar environmental and dietary factors. If the patient's offspring or siblings were included, genetic testing was conducted to confirm the offspring or siblings were not mutant HTT gene carriers. Individuals taking antibiotics or probiotic supplements within one month prior to sample collection were also excluded.

Clinical Data Collection

Clinical data of subjects were collected *via* face-to-face interviews with HD researchers during the enrollment process. The HD researchers had already undertaken clinical-scales training under the instruction of the CHDN, which provided the Enroll HD clinical assessment package. The weight and height of each participant were measured, and body mass index (BMI) was calculated. HD clinical characteristics included disease duration and CAG repeats, as well as the motor section of the Unified Huntington's Disease Rating Scale (UHDRS-M), the Functional Assessment Scale (FAS), Total Functional Capacity (TFC), Category Fluency Test (CFT), Symbol Digit Modalities Test (SDMT), Stroop Interference Test (SIT), Mini-Mental State Examination (MMSE), and Beck Depression Inventory II (BDI-II). To elaborate the quantitative clinical measures further, UHDRS-M ranges from 0 to 124 points with higher scores indicating more severe motor symptoms (32). FAS is a more detailed measure of functional capacity evaluation test and consists of 25 yes/no questions about specific functional abilities. The FAS examines tasks related to occupation (e.g., accustomed work, volunteer work), finances (e.g., cash transactions, financial management), activities of daily living (e.g., driving, hygiene),

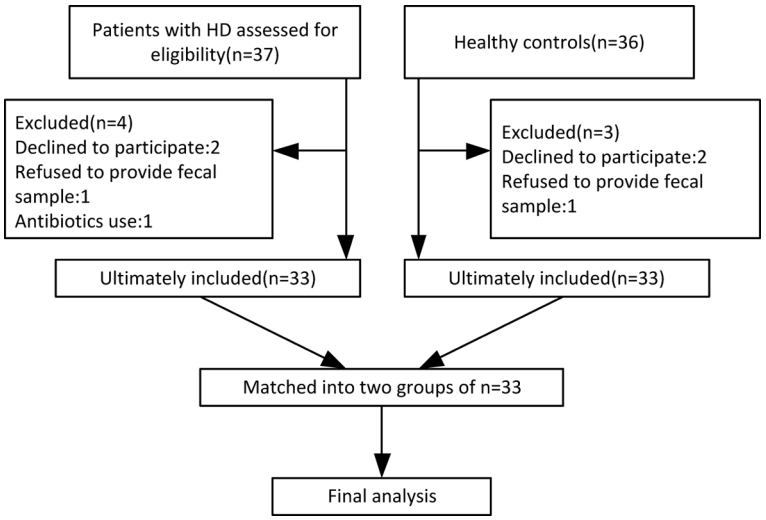


FIGURE 1 | Flow chart of enrolled participants based on our exclusion and inclusion criteria.

domestic chores (e.g., home maintenance, laundry), level of care (e.g., home or supervised care), and physical abilities (e.g., walking, getting out of bed, falls). FAS scores range from 0 to 25 with higher scores indicating greater functionality (33). The TFC provides a measure of broad functional capacity and consists of five global items that assess occupation, finances, domestic chores, activities of daily living, and care level. The scores on each item range from 0 to either 2 or 3. TFC total scores range from 0 to 13 with higher scores indicating greater functioning (33). The CFT requires the subject to name as many examples of the category "animal" as possible within 1 min (34). The SDMT requires participants to make as many symbol–number associations as possible within 90 s, and provides a measure of speed in information processing (35). SIT is a component of the Stroop Test that provides a measure of Executive Function (EF) including cognitive flexibility and resistance to interference. Scores reflect correct number in 45 s and higher scores indicate better performance (36). The MMSE provides a measure of general cognitive functioning (37). The BDI-II contains 21 items used to assess the intensity of the depression in clinically depressed or nondepressed patients. Each component is scored on a 4-point scale from 0 to 3 with higher scores indicating a more severe depressed mood (38).

Sample Collection and DNA Extraction

Fecal samples were collected in tubes (SARSTEDT, Germany) with fecal preservation solution at home by the study participants according to our instructions and transported to our laboratory. Then, all samples were frozen immediately and stored at −80°C until further analysis. The following experiments were carried out commercially by Realbio Genomics Institute (Shanghai, China). DNA was extracted from each fecal sample *via* an improved protocol according to the manual of the QIAamp Fast DNA Stool Mini Kit (Qiagen, Germany). The concentration of genomic DNA in each fecal sample was quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, MA, U.S.). The integrities and sizes of fecal DNA samples were assessed using 1% agarose gel electrophoresis.

Blood samples of 6 ml of venous blood were drawn from each participant at enrollment and were centrifuged (1,000 g for 15 min) within 1 h of collection. The plasma was aliquoted into cryotubes following centrifugation and stored at −80°C until further use for cytokine analysis.

16S rDNA Gene Amplicons and Sequencing

The V3–V4 regions of bacterial 16S rDNA genes were amplified by PCR (95°C for 3 min, followed by 30 cycles at 98°C for 20 s, 58°C for 15 s, and 72°C for 20 s, as well as a final extension at 72°C for 5 min) using universal primers (341F and 806R) linked with indices and sequencing adaptors. PCR amplification was performed in a 30-μL mixture containing 15 μL of 2 × KAPA Library Amplification ReadyMix, 1 μL of each primer (10 μM), 50 ng of template DNA, and ddH_2O. Amplicons were extracted from 2% agarose gels and purified using an AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, U.S.) according to the manufacturer's instructions. Purified amplicons were quantified using Qubit 2.0 (Invitrogen, U.S.). All quantified amplicons were sequenced using an Illumina NovaSeq PE250.

Processing of Sequencing Data

Assembled tags, trimmed barcodes, and primers were further checked in terms of their rest lengths and average base qualities. 16S tags were restricted between 220–500 bp so that the average Phred score of bases was no worse than 20 (Q20) and no more than 3 ambiguous N. The copy number of tags was enumerated and redundancy of repeated tags was removed. Only tags with a

frequency greater than 1, which tend to be more reliable, were clustered into operational taxonomic units (OTUs), each of which had a representative tag. OTUs were clustered based on 97% similarities using UPARSE and chimeric sequences were identified and removed using USEARCH (version 7.0.1090). Each representative tag was assigned to a taxa by the RDP Classifier (http://rdp.cme.msu.edu/) against the RDP database (http://rdp.cme.msu.edu/) using a confidence threshold of 0.8. The α-diversity and β-diversity indices were calculated based on the rarefied OTU counts *via* Qiime v1.9.1. Specifically, α-diversity represents an analysis of diversity in a single sample reflected by parameters including good coverage, Chao 1, PD whole tree, Shannon index, and Simpson index, using Qiime (39). The Wilcox test in R software was used to compare each α-diversity index. β-diversity is used as a measure of the microbiota structure between groups. The results of both the weighted and unweighted Unifrac distance matrices were plotted in the principal coordinate analysis (PCoA), and Adonis was performed using R software. Microbial features used to distinguish fecal microbiotas specific to HD were identified using the linear discriminant analysis (LDA) effect size (LEfSe) method (http://huttenhower.sph.harvard.edu/lefse/) with an alpha cutoff of 0.05 and an effect-size cutoff of 2.0.

Measurement of Plasma Cytokine Levels

The V-PLEX proinflammatory Panel 1 human kit [Meso Scale Discovery (MSD)] was used to measure plasma concentrations of IFN-γ, IL-10, IL-12p70, IL-13, IL-1β, IL-2, IL-4, IL-6, IL-8, and TNF-α according to the manufacturer protocol.

Statistical Analysis

The SPSS (ver. 21.0, SPSS Inc., Chicago, IL, USA) and R software (ver. 3.5.1, the R Project for Statistical Computing) were used for statistical analysis. Differences between groups were determined *via* Student's t-test and Pearson's Chi-squared tests for quantitative and categorical variables, respectively. Variables that violated the assumptions of normality were compared *via* nonparametric Mann-Whitney U tests. Correlations among components of fecal microbiota with clinical parameters and cytokines were determined *via* Spearman correlation analysis. Statistical significance was set at $p < 0.05$.

RESULTS

Demographic and Clinical Characteristics of HD Patients and Healthy Controls

The demographic characteristics of HD patients and healthy controls in this study are presented in **Table 1**. There were no significant differences between the HD and controls in terms of age or gender (**Table 1**), but the age at assessment in the premanifest HD is much younger compared to the manifested HD (**Supplementary Table 1**). The median duration of HD patients at enrollment was 4 years, and the median number of CAG repetitions was 42. HD patients has a significantly lower BMI compared to that of healthy controls (**Table 1**).

TABLE 1 | Demographics and clinical characteristics of HD patients and healthy controls.

	HD group	Healthy control group	*p* value
N	33	33	NA
M:F	15:18	15:18	NA
Age (y/o)	42.6 (12.7)[a]	48.0 (13.5)[a]	0.0967*
BMI (kg/m^2)	21.3 (3.7)[a]	24.0 (3.6)[a]	0.003*
HD duration(y)	4.0 (7.0)[b]	NA	NA
CAG repeat number	42.0 (3.5)[b]	NA	NA
UHDRS-M	35.0 (70.0)[b]	NA	NA
FAS scores	21.0 (11.0)[b]	NA	NA
TFC scores	12.0 (10.0)[b]	NA	NA
SIT scores	18.0 (16.0)[b]	–	NA
SDMT scores	21.0 (25.0)[b]	–	NA
CFT scores	11.0 (11.0)[b]	–	NA
BDI-II scores	6.0 (15.0)[b]	–	NA
MMSE scores	28.0 (5.0)[b]	–	NA

**Unpaired t-test; the data are presented as the mean (SD)[a] or median (IQR)[b]; HD, Huntington's disease; NA, not applicable; SD, standard deviation; IQR, interquartile range; M:F, male: female; y, years; y/o, years old; BMI, body mass index; UHDRS-M, motor section of Unified Huntington's Disease Rating Scale; FAS, Functional Assessment Scale; TFC, Total Functional Capacity; SIT, Stroop Interference Test; SDMT, Symbol Digit Modalities Test; CFT, Category Fluency Test; BDI-II, Beck Depression Inventory II; MMSE, Mini-Mental State Examination.*

Alpha and Beta Diversity Between HD Patients and Healthy Controls

The dilution curves of α-diversity indices were plotted to demonstrate that the sample size in this study is adequate for valid analysis (**Supplementary Figure 1**). The Chao 1, observed species, and PD whole tree of the HD group were significantly higher than those of the healthy control group, whereas there were no significant differences between these two groups in terms of the Shannon and Simpson index (**Figures 2A–E**). These results indicate that the richness of the gut microbiota in the HD group was significantly higher than that of the healthy control group. Significant differences were also found in β-diversity based on the unweighted (qualitative, Adonis R^2 = 0.03, p = 0.02) but not the weighted (quantitative, Adonis R^2 = 0.022, p = 0.197). UniFrac between HD and healthy control groups (**Figures 2F, G**), indicating that the fecal microbial structure, but not the abundance, in the HD group was significantly different from that of the healthy control group.

Compositions of Microbial Taxa at Multiple Phylogenetic Ranks Between HD Patients and Healthy Controls

The top-20 taxa at multiple phylogenetic ranks were analyzed. For example, at the genus level, *Bacteroides* and *Prevotella* constituted two common dominant genera in both the HD group and healthy control group (*Bacteroides*: 27.36 vs. 34.93%, respectively; *Prevotella*: 22.80 vs. 19.39%), which accounted for 50.16 and 54.32% of the total sequencing number. In addition, the average ratios of *Bacteroides* and *Prevotella* between the HD patients and healthy controls were 0.78 and 1.18, respectively (**Figure 3**). The microbial species profiling histogram of the HD cohort were also demonstrated according to the disease duration (**Supplementary Figures 2–6**).

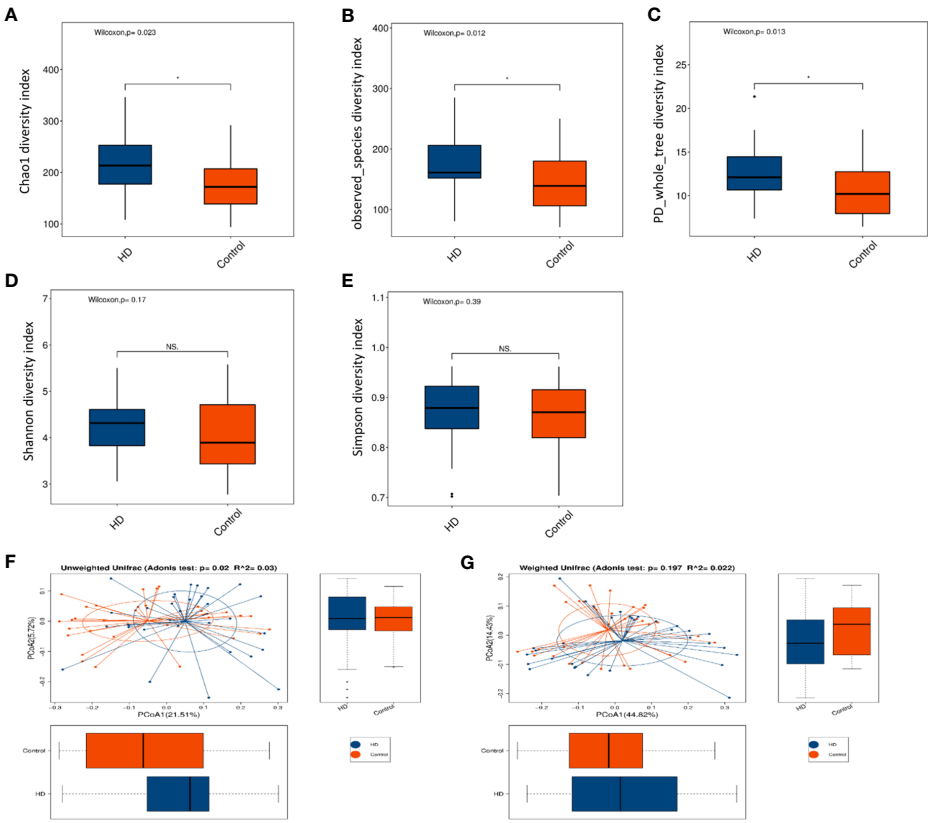


FIGURE 2 | Comparisons of α-diversity indexes (richness and evenness) of the fecal microbiota between HD and healthy controls. **(A–C)**, Comparisons of richness (Chao 1 index, observed species index, and PD whole tree index) between HD and healthy controls. **(D, E)**, Comparisons of evenness (Shannon index and Simpson index) between HD patients and healthy controls. *$p < 0.05$; "NS." means no significant difference. β-diversity analyses using Adonis and unweighted **(F)** and weighted **(G)** PCoA based on the distance matrix of UniFrac dissimilarity of the fecal microbiota in HD patients and healthy controls.

Altered Microbiota Between HD Patients and Healthy Controls

To determine the significantly increased bacteria in the HD group or healthy control group, supervised comparisons *via* LEfSE (LDA > 2.0) were performed. This LEfSe analysis revealed many significant differences in the fecal microbiota between the HD group and healthy control group. Specifically, the following relative abundances in the HD group were significantly higher than those in the healthy control group: *Actinobacteria* at the phylum level; *Deltaproteobacteria* and *Actinobacteria* at the class level; *Desulfovibrionales* at the order level; *Oxalobacteraceae*, *Lactobacillaceae*, and *Desulfovibrionaceae* at the family level; and *Intestinimonas*, *Bilophila*, *Lactobacillus*, *Oscillibacter*, *Gemmiger*, and *Dialister* at the genus level. In contrast, *Clostridium XVIII* at genus level was significantly higher in the healthy control group compared to that in the HD group (**Figure 4**).

Relationships Between Fecal Microbiota and HD Clinical Characteristics

To determine the relationship between clinical characteristics and gut microbiota in HD patients, Spearman correlation analysis was performed to evaluate correlations among clinical characteristics and gut-microbiota genera obtained by LEfSe analysis. Our analysis revealed two correlations between fecal microbiota at the genus level with specific clinical scores, namely *Intestinimonas* with TFC scores and *Lactobacillus* with MMSE scores (**Figure 5**).

Cytokine Responses in HD Patients With Alterations in Fecal Microbiota

To evaluate plasma cytokine profiles from HD patients and healthy controls, we quantified the plasma concentrations of interferon gamma (IFN-γ), interleukin 1 beta (IL-1β), IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, and tumor necrosis factor alpha (TNF-α). IL-4 plasma concentrations, characteristic of responses from T-helper-2 cells, was significantly lower (p = 0.03) in plasma samples from HD patients (0.008 ± 0.001 pg/mL) than in samples from healthy controls (0.009 ± 0.001 pg/mL). In contrast, there were no significant differences ($p > 0.05$) in the plasma concentrations of IFN-γ, IL-1β, IL-2, IL-6, IL-8, IL-10, IL-12p70, IL-13, or TNF-α in HD patients (**Figure 6A**).

Finally, to identify correlations between components of fecal microbiota and cytokines, we examined correlations between systemic levels of cytokines and relative abundances of fecal

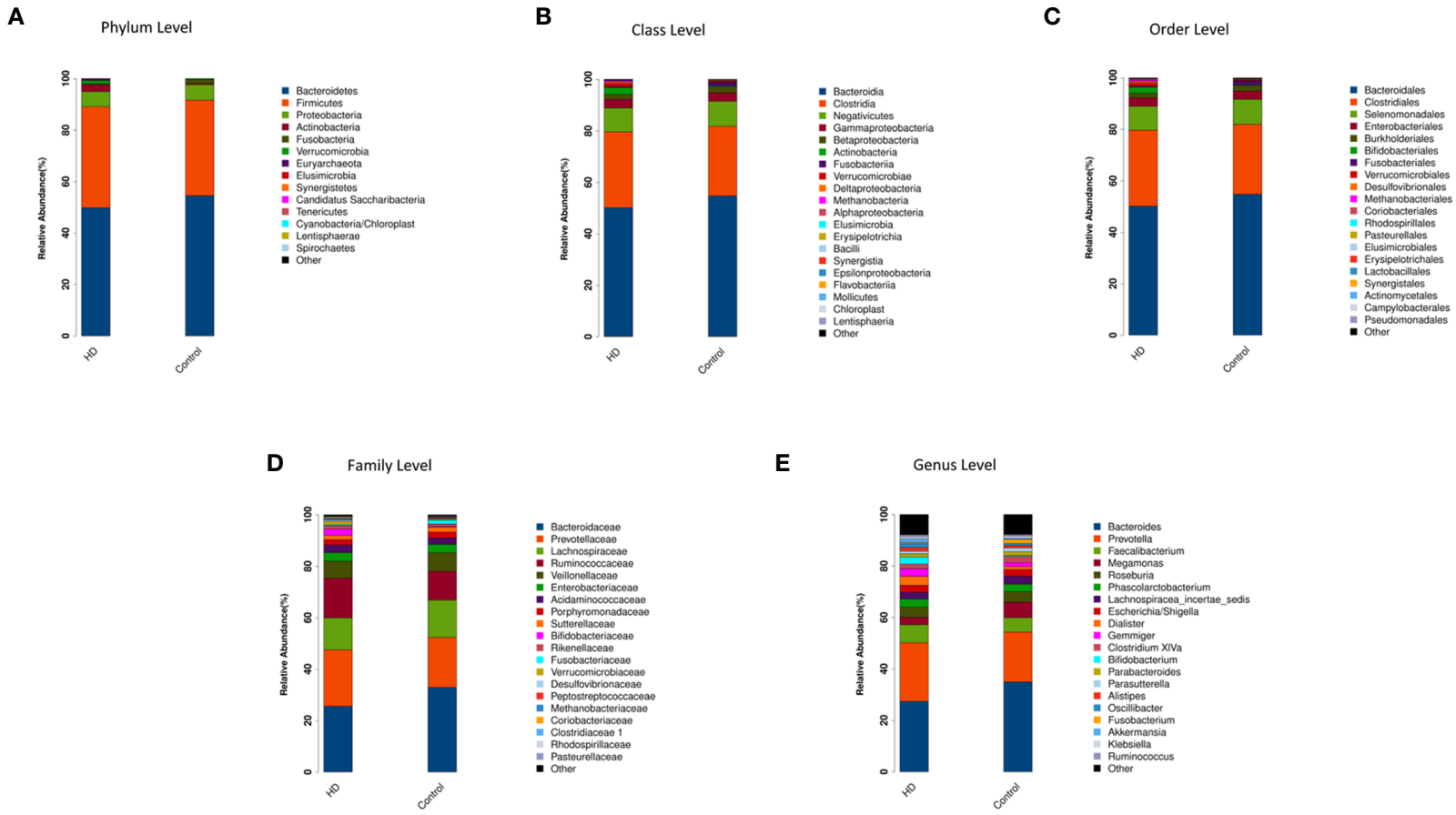


FIGURE 3 | Compositions and relative abundances of taxa at multiple phylogenetic ranks based on 16S rDNA sequences in HD patients and healthy controls. The compositions and relative abundances of major taxa in HD and controls are compared at level of phylum **(A)**, class **(B)**, order **(C)**, family **(D)** and genus **(E)**.

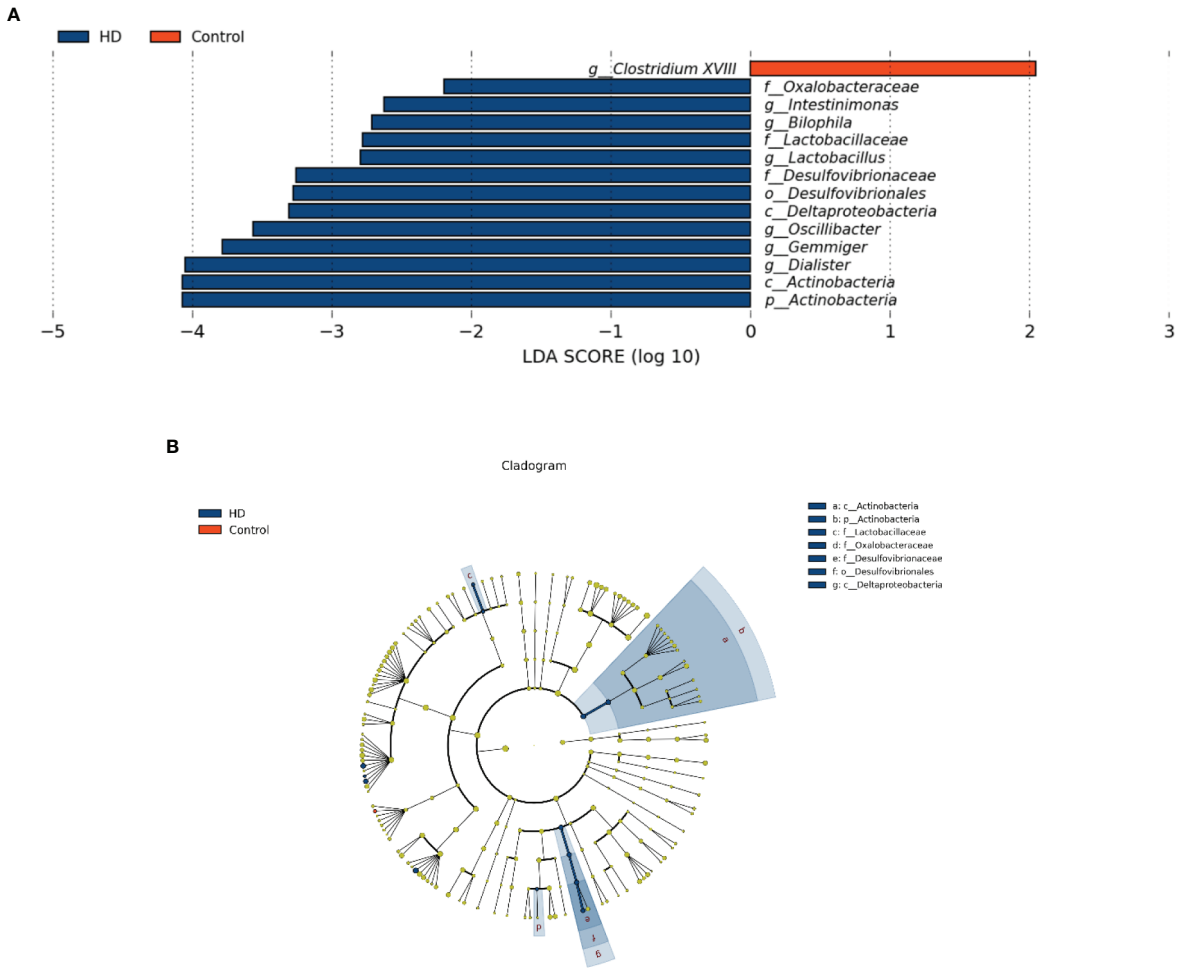


FIGURE 4 | Fecal microbiota differences between HD patients and healthy controls detected by LEfSe analysis. **(A)** Linear discriminant analysis (LDA) effect size (LEfSe) analysis showing significant bacterial differences in fecal microbiota between the HD patients and healthy controls. The LDA scores (log10) > 2 and $p < 0.05$ are listed. **(B)** A cladogram showing the taxonomic structure and relative abundances of the identified taxa. The size of each dot is proportional to the relative abundance of each taxon (p, phylum; c, class; o, order; f, family; g, genus).

microbiota in the HD group. We found correlations between fecal microbiota and cytokines (e.g., *Intestinimonas* with plasma IL-4 levels ($p = 0.028$, $\rho = 0.382$), *Bilophila* with plasma IL-6 levels ($p = 0.001$, $\rho = -0.544$) (**Figure 6B**).

DISCUSSION

The present study provides the evidence for gut dysbiosis in human patients with HD, providing clinical relevance to a previous study that reported gut dysbiosis in a transgenic mouse model of HD (11). We found that *Intestinimonas* and *Bilophila* correlated with concentrations of IL-4 and IL-6, respectively, in HD patients, suggesting the occurrence of a systemic chronic inflammatory status associated with altered gut microbiota.

In our present study, we used 16S rDNA gene sequencing on DNA isolated from fecal samples to systematically analyze characteristics of gut microbiota between HD patients and healthy controls. We observed that α-diversity (richness) in HD patients was significantly higher than that of healthy controls, as was β-diversity (structure), consistent with previous results from a mouse model of HD (11). However, in a recent study of the gut microbiota of HD patients (40), the α-diversity of HD patients was lower than that of healthy controls. The disparity is likely due to different ethnic origins, geography, host, genetics, age and other factors (41). Previous study showed an increase in gut microbiota richness was detected in autistic children (42), and it shared great similarities in the altered microbiota species with HD. Given HD was also considered as a neurodevelopmental disorder similar to autism (43), the greater bacterial diversity is potentially beneficial to the development of central nervous system (CNS) function, which remains subject to debate.

Furthermore, we analyzed the top-20 taxa at multiple phylogenetic ranks. At the genus level, the average ratios of

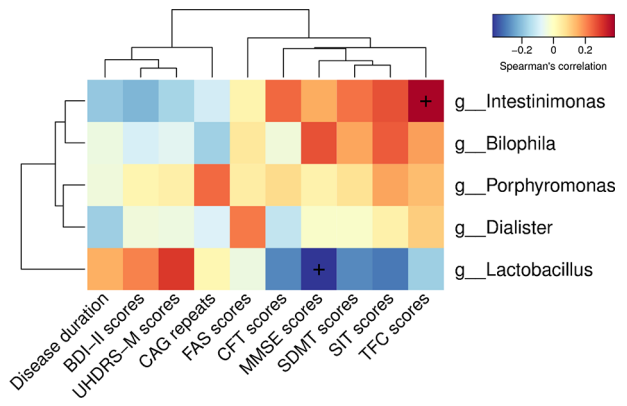


FIGURE 5 | Correlations among clinical characteristics and gut-microbiota genera obtained by LEfSe analysis. Correlation heatmaps showing the relationships among clinical characteristics and gut-microbiota genera. Each row in the heatmap represents a genus. Each column represents a clinical characteristic. The color temperature encodes the Spearman correlation coefficient (ρ). The sample size was n = 33. The "+" symbol within a cell indicates a $p < 0.05$. Abbreviations are as follows: BDI-II scores, Beck Depression Inventory II scores; UHDRS-M, motor section of the Unified Huntington's Disease Rating Scale scores; FAS scores, Functional Assessment Scale scores; CFT scores, Category Fluency Test scores; MMSE scores, Mini-Mental State Examination scores; SDMT scores, Symbol Digit Modalities Test scores; SIT scores, Stroop Interference Test scores; TFC scores, Total Functional Capacity scores.

Bacteroides and *Prevotella* between groups HD and healthy control were 0.78 and 1.18. The significant bacterial differences identified by LEfSe (LDA > 2.0) showed that *Clostridium XVIII* at the genus level was significantly higher in the healthy control group compared to that in the HD group, whereas the relative abundances of the following were higher in the HD group compared to those in the healthy control group: *Actinobacteria* at the phylum level; *Deltaproteobacteria* and *Actinobacteria* at the class level; *Desulfovibrionales* at the order level; *Oxalobacteraceae*, *Lactobacillaceae*, and *Desulfovibrionaceae* at the family level; and *Intestinimonas*, *Bilophila*, *Lactobacillus*, *Oscillibacter*, *Gemmiger*, and *Dialister* at the genus level. These results indicate that particular gut microbiota components are associated with HD. The increased abundance of *Actinobacteria* at the phylum level in the HD group compared to that in the healthy control group in our present study is similar to previous findings reported in patients with IBD (44), type-2 diabetes mellitus (T2DM) (45), AD (28), ASD (46), and major depressive disorder (47, 48). The increased abundance of *Actinobacteria* at the class level in the HD patients compared to that in the healthy controls in this study is consistent with previous studies in AD and ASD patients (28, 46). Family level analysis in our present study revealed significant increases in the abundance of *Desulfovibrionaceae* in the fecal samples of HD patients compared to those from healthy controls, whereas a previous study found that the abundance of *Desulfovibrionaceae* was increased in the feces of IBD patients (49), which is likely a breaker of the intestinal mucosal barrier that induces IBD (50).

The abundance of the *Intestinimonas* genus was higher in HD patients than in healthy controls and correlated with TFC scores. A previous study has shown that *Intestinimonas*, which is correlated with propionic/butyric acid, plays a key role in anti-inflammation (51, 52). In the present study, we observed a positive correlation between *Intestinimonas* and plasma concentrations of IL-4, an anti-inflammatory cytokine mainly produced by T-helper-2 cells (53). Since butyrate has the ability to regulate T cell differentiation (54), it suggests that *Intestinimonas* may confer efficacious anti-inflammatory effects on systemic inflammatory responses in HD patients. However, future studies are needed to reveal the interactions between changes in symbiotic gut microbiota and the immune reactions in HD pathogenesis.

Bilophila contains only species of *B. wadsworthia*, and a higher abundance of *B. wadsworthia* induces systemic inflammatory responses in specific pathogen-free (SPF) mice (55). In addition, *B. wadsworthia* plays an important role in the development of IBD-like colitis in *IL-10*$^{-/-}$ mice (56). Additionally, *B. wadsworthia* has been found to be positively associated with proinflammatory cytokines, such as IL-6 (57) and IL-1β (58). A previous study found that *Bilophila* was significantly negatively correlated ($p < 0.05$) with IL-6 mRNA levels in a T2DM rat model treated with stachyose (59). Interestingly, our present study also revealed that *Bilophila* were negatively correlated with proinflammatory IL-6 levels, suggesting that *Bilophila* may play an anti-inflammatory role in systemic inflammatory responses in HD patients. That said, further research is needed to determine the precise relationships between gut microbiota compositions and immune reactions in HD patients.

Lactobacillus has the ability to ferment a series of carbon sources, primarily to lactic acid, and is widely recognized as a source of probiotics (60). Several recent studies have found that *Lactobacillus* is more abundant in patients with T2DM (61), ASD (62, 63), IBD (64), and rheumatoid arthritis (65, 66), which is consistent with our present findings in HD patients. Interestingly, we found that the abundance of *Lactobacillus* was negatively correlated with MMSE scores, although we did not find any correlation between *Lactobacillus* and levels of inflammatory cytokines. These findings suggest that if the abundance of *Lactobacillus* adversely impacts MMSE scores, and consequently cognitive function, it likely does so *via* a non-cytokine-induced mechanism.

In the present study, we found that the abundance of *Clostridium XVIII* was significantly decreased in the HD group compared to that in healthy controls, indicating higher fecal water content in the HD group, similar to findings in HD mice (11). Strati et al. observed that *Clostridium XVIII* was significantly more abundant in constipated autistic subjects compared to that in non-constipated autistic subjects, and this increased abundance was associated with clinical manifestations of GI problems related to alterations in gut microbiota (62).

There are several limitations of this study. Although we managed to recruit HD and control pairs from the same family and at the same geographic location across China, the sample size is minimal and the nature of the present study is exploratory. Although this study was controlled for factors affecting the gut

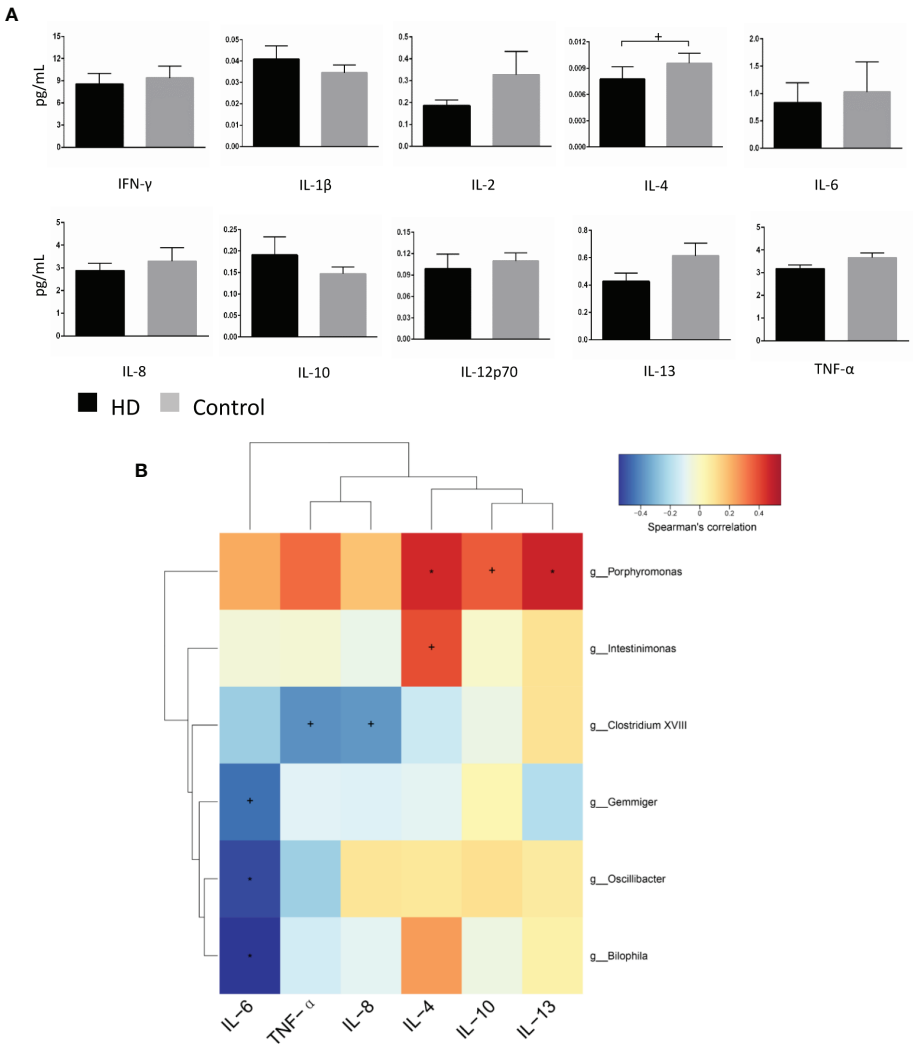


FIGURE 6 | Cytokine profiles in HD patients and healthy controls, as well as correlations between cytokines and gut-microbiota genera obtained by LEfSe analysis. **(A)** Plasma concentrations of interferon-gamma (IFN-γ), interleukin-1β (IL-1β), IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, and tumor necrosis factor alpha (TNF-α). **(B)** Correlation heatmaps showing the relationships among cytokines and gut-microbiota genera. Each row in the heatmap represents a genus. Each column represents a cytokine. The color temperature encodes the Spearman correlation coefficient (ρ). The sample size was n = 33. Statistical analyses were performed by Mann–Whitney tests **(A)** and Spearman's tests **(B)**. The error bars indicate the standard error of mean (SEM). HD denotes Huntington's disease. $^{+}p < 0.05$, $^{*}p < 0.01$.

microbiota, such as age, gender, diet, and region, physical activity, smoking, alcohol, drugs and other factors should be fully considered in the further study. Further studies with larger sample sizes comprised of independent cohorts originating from a different population are required to consolidate our findings of different microbial species between HD patients and healthy controls, as well as the microbial species associated with clinical symptoms. In addition, more advanced technology such as shotgun metagenome analysis, and more advanced association analysis platform such as HALLA (67) can be used in the future to provide more detailed information regarding any implicated microbiota in future studies to more comprehensively understand fecal microbiota composition in HD patients.

In summary, the present study first elucidates that gut microbiota are altered in HD patients and are correlated with specific clinical characteristics. Furthermore, we demonstrated that fecal microbiota was related to specific cytokine levels. Revealing the precise interactions between microbial and host immune responses may help to better understand the pathogenesis of HD.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and

accession number can be found below: National Center for Biotechnology Information (NCBI) BioProject database with project number PRJNA667318.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee, Beijing Tiantan Hospital, Capital Medical University, Beijing, China. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Clinical analyses and manuscript writing: GD. Participants recruitment: GD, WD, XY, WG, JM, and YH. Samples preparation: QY and WD. Experimental design and critical revision: YH. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by Beijing Tiantan Hospital, Capital Medical University for Neurogenetics Studies (YH) and Beijing Postdoctoral Research Foundation (GD).

ACKNOWLEDGMENTS

We thank the healthy individuals, the patients, and their close relatives who donated their fecal and blood samples.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.603594/full#supplementary-material

Supplementary Figure 1 | Dilution curves of α-diversity index. As the amount of sequencing increased, more species were found, and no new OTU was found by increasing the number of sampling strips until the species was saturated.

Supplementary Figure 2 | Species profiling histogram of the HD samples according to HD duration at phylum classification level.

Supplementary Figure 3 | Species profiling histogram of the HD samples according to HD duration at class classification level.

Supplementary Figure 4 | Species profiling histogram of the HD samples according to HD duration at order classification level.

Supplementary Figure 5 | Species profiling histogram of the HD samples according to HD duration at family classification level.

Supplementary Figure 6 | Species profiling histogram of the HD samples according to HD duration at genus classification level.

REFERENCES

1. Moffitt H, McPhail GD, Woodman B, Hobbs C, Bates GP. Formation of polyglutamine inclusions in a wide range of non-CNS tissues in the HdhQ150 knock-in mouse model of Huntington's disease. *PloS One* (2009) 4:e8025. doi: 10.1371/journal.pone.0008025
2. Sathasivam K, Hobbs C, Turmaine M, Mangiarini L, Mahal A, Bertaux F, et al. Formation of polyglutamine inclusions in non-CNS tissue. *Hum Mol Genet* (1999) 8:813–22. doi: 10.1093/hmg/8.5.813
3. Sharp AH, Loev SJ, Schilling G, Li SH, Li XJ, Bao J, et al. Widespread expression of Huntington's disease gene (IT15) protein product. *Neuron* (1995) 14:1065–74. doi: 10.1016/0896-6273(95)90345-3
4. Listed N. A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. *Cell* (1993) 72:971–83. doi: 10.1016/0092-8674(93)90585-E
5. van der Burg JM, Winqvist A, Aziz NA, Maat-Schieman ML, Roos RA, Bates GP, et al. Gastrointestinal dysfunction contributes to weight loss in Huntington's disease mice. *Neurobiol Dis* (2011) 44:1–8. doi: 10.1016/j.nbd.2011.05.006
6. Djoussé L, Knowlton B, Cupples LA, Marder K, Shoulson I, Myers RH. Weight loss in early stage of Huntington's disease. *Neurology* (2002) 59:1325–30. doi: 10.1212/01.wnl.0000031791.10922.cf
7. Farrer LA, Meaney FJ. An anthropometric assessment of Huntington's disease patients and families. *Am J Phys Anthropol* (1985) 67:185–94. doi: 10.1002/ajpa.1330670304
8. Sanberg PR, Fibiger HC, Mark RF. Body weight and dietary factors in Huntington's disease patients compared with matched controls. *Med J Aust* (1981) 1:407–9. doi: 10.5694/j.1326-5377.1981.tb135681.x
9. Trejo A, Tarrats RM, Alonso ME, Boll MC, Ochoa A, Velásquez L. Assessment of the nutrition status of patients with Huntington's disease. *Nutr (Burbank Los Angeles County Calif)* (2004) 20:192–6. doi: 10.1016/j.nut.2003.10.007
10. Menalled LB, Chesselet MF. Mouse models of Huntington's disease. *Trends Pharmacol Sci* (2002) 23:32–9. doi: 10.1016/s0165-6147(00)01884-8
11. Kong G, Cao KL, Judd LM, Li S, Renoir T, Hannan AJ. Microbiome profiling reveals gut dysbiosis in a transgenic mouse model of Huntington's disease. *Neurobiol Dis* (2020) 135:104268. doi: 10.1016/j.nbd.2018.09.001
12. Weiss A, Trager U, Wild EJ, Grueninger S, Farmer R, Landles C, et al. Mutant huntingtin fragmentation in immune cells tracks Huntington's disease progression. *J Clin Invest* (2012) 122:3731–6. doi: 10.1172/JCI64565
13. Bjorkqvist M, Wild EJ, Thiele J, Silvestroni A, Andre R, Lahiri N, et al. A novel pathogenic pathway of immune activation detectable before clinical onset in Huntington's disease. *J Exp Med* (2008) 205:1869–77. doi: 10.1084/jem.20080178
14. Trager U, Andre R, Lahiri N, Magnusson-Lind A, Weiss A, Grueninger S, et al. HTT-lowering reverses Huntington's disease immune dysfunction caused by NFkappaB pathway dysregulation. *Brain* (2014) 137:819–33. doi: 10.1093/brain/awt355
15. Wang Y, Kasper LH. The role of microbiome in central nervous system disorders. *Brain Behav Immun* (2014) 38:1–12. doi: 10.1016/j.bbi.2013.12.015
16. Clarke G, Grenham S, Scully P, Fitzgerald P, Moloney RD, Shanahan F, et al. The microbiome-gut-brain axis during early life regulates the hippocampal serotonergic system in a sex-dependent manner. *Mol Psychiatry* (2013) 18:666–73. doi: 10.1038/mp.2012.77
17. Hoban AE, Stilling RM, Moloney G, Shanahan F, Dinan TG, Clarke G, et al. The microbiome regulates amygdala-dependent fear recall. *Mol Psychiatry* (2018) 23:1134–44. doi: 10.1038/mp.2017.100
18. Ogbonnaya ES, Clarke G, Shanahan F, Dinan TG, Cryan JF, O'Leary OF. Adult Hippocampal Neurogenesis Is Regulated by the Microbiome. *Biol Psychiatry* (2015) 78:e7–9. doi: 10.1016/j.biopsych.2014.12.023
19. Stilling RM, Ryan FJ, Hoban AE, Shanahan F, Clarke G, Claesson MJ, et al. Microbes & neurodevelopment–Absence of microbiota during early life increases activity-related transcriptional pathways in the amygdala. *Brain Behav Immun* (2015) 50:209–20. doi: 10.1016/j.bbi.2015.07.009
20. Greenblum S, Turnbaugh PJ, Borenstein E. Metagenomic systems biology of the human gut microbiome reveals topological shifts associated with obesity and inflammatory bowel disease. *Proc Natl Acad Sci U States America* (2012) 109:594–9. doi: 10.1073/pnas.1116053109

21. Qin J, Li Y, Cai Z, Li S, Zhu J, Zhang F, et al. A metagenome-wide association study of gut microbiota in type 2 diabetes. *Nature* (2012) 490:55–60. doi: 10.1038/nature11450
22. Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* (2006) 444:1027–31. doi: 10.1038/nature05414
23. Ma B, Liang J, Dai M, Wang J, Luo J, Zhang Z, et al. Altered Gut Microbiota in Chinese Children With Autism Spectrum Disorders. *Front Cell Infect Microbiol* (2019) 9:40:40. doi: 10.3389/fcimb.2019.00040
24. Jiang H, Ling Z, Zhang Y, Mao H, Ma Z, Yin Y, et al. Altered fecal microbiota composition in patients with major depressive disorder. *Brain Behav Immun* (2015) 48:186–94. doi: 10.1016/j.bbi.2015.03.016
25. Brenner D, Hiergeist A, Adis C, Mayer B, Gessner A, Ludolph AC, et al. The fecal microbiome of ALS patients. *Neurobiol Aging* (2018) 61:132–7. doi: 10.1016/j.neurobiolaging.2017.09.023
26. Qian Y, Yang X, Xu S, Wu C, Song Y, Qin N, et al. Alteration of the fecal microbiota in Chinese patients with Parkinson's disease. *Brain Behav Immun* (2018) 70:194–202. doi: 10.1016/j.bbi.2018.02.016
27. Wallen ZD, Appah M, Dean MN, Sesler CL, Factor SA, Molho E, et al. Characterizing dysbiosis of gut microbiome in PD: evidence for overabundance of opportunistic pathogens. *NPJ Parkinson's Dis* (2020) 6:11. doi: 10.1038/s41531-020-0112-6
28. Zhuang ZQ, Shen LL, Li WW, Fu X, Zeng F, Gui L, et al. Gut Microbiota is Altered in Patients with Alzheimer's Disease. *J Alzheimer's Dis JAD* (2018) 63:1337–46. doi: 10.3233/JAD-180176
29. Beal MF, Matson WR, Swartz KJ, Gamache PH, Bird ED. Kynurenine pathway measurements in Huntington's disease striatum: evidence for reduced formation of kynurenic acid. *J Neurochem* (1990) 55:1327–39. doi: 10.1111/j.1471-4159.1990.tb03143.x
30. Verwaest KA, Vu TN, Laukens K, Clemens LE, Nguyen HP, Van Gasse B, et al. (1)H NMR based metabolomics of CSF and blood serum: a metabolic profile for a transgenic rat model of Huntington disease. *Biochim Biophys Acta* (2011) 1812:1371–9. doi: 10.1016/j.bbadis.2011.08.001
31. Landwehrmeyer GB. *Protocol CS. Enroll-HD Protocol Final Version 1.0 09 September 2011*. (2011). pp. 1–64. Available at: https://www.enroll-hd.org/enrollhd_documents/Enroll-HDProtocol-1.0.pdf.
32. Kieburtz K, Penney JB, Como P, Ranen N, Shoulson I, Feign A, et al. Unified Huntington's Disease Rating Scale: reliability and consistency. Huntington Study Group. *Mov Disord* (1996) 11:136–42. doi: 10.1002/mds.870110204
33. Shoulson I, Fahn S. Huntington disease: clinical care and evaluation. *Neurology* (1979) 29:1–3. doi: 10.1212/wnl.29.1.1
34. Lee JH, Lee KU, Lee DY, Kim KW, Jhoo JH, Kim JH, et al. Development of the Korean version of the Consortium to Establish a Registry for Alzheimer's Disease Assessment Packet (CERAD-K): clinical and neuropsychological assessment batteries. *J Gerontol Ser B psychol Sci Soc Sci* (2002) 57:P47–53. doi: 10.1093/geronb/57.1.p47
35. Wechsler D. *Manual for the Wechsler Adult Intelligence Scale - Revised*. New York: Psychological Corporation (1981).
36. Stroop RJ. Studies of interference in serial verbal reactions (Reprinted from Journal Experimental-Psychology, Vol 18, Pg 643-662, 1935). *J Exp Psychol Gen* (1992) 121:15–23. doi: 10.1037/0096-3445.121.1.15
37. Folstein MF, Folstein SE, McHugh PR. "Mini-mental state": A practical method for grading the cognitive state of patients for the clinician. *J Psychiatr Res* (1975). doi: 10.1016/0022-3956(75)90026-6
38. Beck AT, Steer RA, Brown GK. Beck Depression Inventory-second edition. Manual. *Psihologijski mjerni instrumenti* - (1996) 93:21. doi: 10.1007/978-3-642-70486-4_13
39. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* (2010) 7:335–6. doi: 10.1038/nmeth.f.303
40. Wasser CI, Mercieca EC, Kong G, Hannan AJ, McKeown SJ, Glikmann-Johnston Y, et al. Gut dysbiosis in Huntington's disease: associations among gut microbiota, cognitive performance and clinical outcomes. *Brain Commun* (2020) 2:fcaa110. doi: 10.1093/braincomms/fcaa110
41. Escobar JS, Klotz B, Valdes BE, Agudelo GM. The gut microbiota of Colombians differs from that of Americans, Europeans and Asians. *BMC Microbiol* (2014) 14:311. doi: 10.1186/s12866-014-0311-6
42. Finegold SM, Dowd SE, Gontcharova V, Liu C, Henley KE, Wolcott RD, et al. Pyrosequencing study of fecal microflora of autistic and control children. *Anaerobe* (2010) 16:444–53. doi: 10.1016/j.anaerobe.2010.06.008
43. Barnat M, Capizzi M, Aparicio E, Boluda S, Wennagel D, Kacher R, et al. Huntington's disease alters human neurodevelopment. *Science* (2020) 369:787–93. doi: 10.1126/science.aax3338
44. Alam MT, Amos GCA, Murphy ARJ, Murch S, Wellington EMH, Arasaradnam RP. Microbial imbalance in inflammatory bowel disease patients at different taxonomic levels. *Gut Pathog* (2020) 12:1. doi: 10.1186/s13099-019-0341-6
45. Hashimoto Y, Hamaguchi M, Kaji A, Sakai R, Osaka T, Inoue R, et al. Intake of sucrose affects gut dysbiosis in patients with type 2 diabetes. *J Diabetes Invest* (2020) 11:1623–34. doi: 10.1111/jdi.13293
46. Plaza-Diaz J, Gomez-Fernandez A, Chueca N, Torre-Aguilar MJ, Gil A, Perez-Navero JL, et al. Autism Spectrum Disorder (ASD) with and without Mental Regression is Associated with Changes in the Fecal Microbiota. *Nutrients* (2019) 11:337. doi: 10.3390/nu11020337
47. Lai WT, Deng WF, Xu SX, Zhao J, Xu D, Liu YH, et al. Shotgun metagenomics reveals both taxonomic and tryptophan pathway differences of gut microbiota in major depressive disorder patients. *Psychol Med* (2019) 51:90–101. doi: 10.1017/S0033291719003027
48. Yang C, Qu Y, Fujita Y, Ren Q, Ma M, Dong C, et al. Possible role of the gut microbiota-brain axis in the antidepressant effects of (R)-ketamine in a social defeat stress model. *Trans Psychiatry* (2017) 7:1294. doi: 10.1038/s41398-017-0031-4
49. Berry D, Reinisch W. Intestinal microbiota: a source of novel biomarkers in inflammatory bowel diseases? Best practice & research. *Clin Gastroenterol* (2013) 27:47–58. doi: 10.1016/j.bpg.2013.03.005
50. Ijssennagger N, van der Meer R, van Mil SWC. Sulfide as a Mucus Barrier-Breaker in Inflammatory Bowel Disease? *Trends Mol Med* (2016) 22:190–9. doi: 10.1016/j.molmed.2016.01.002
51. Zhuang P, Zhang Y, Shou Q, Li H, Zhu Y, He L, et al. Eicosapentaenoic and Docosahexaenoic Acids Differentially Alter Gut Microbiome and Reverse High-Fat Diet-Induced Insulin Resistance. *Mol Nutr Food Res* (2020) 64:e1900946. doi: 10.1002/mnfr.201900946
52. Cai W, Xu J, Li G, Liu T, Guo X, Wang H, et al. Ethanol extract of propolis prevents high-fat diet-induced insulin resistance and obesity in association with modulation of gut microbiota in mice. *Food Res Int* (2020) 130:108939. doi: 10.1016/j.foodres.2019.108939
53. Paul WE, Zhu J. How are T(H)2-type immune responses initiated and amplified? *Nat Rev Immunol* (2010) 10:225–35. doi: 10.1038/nri2735
54. Guilloteau P, Martin L, Eeckhaut V, Ducatelle R, Zabielski R, Van Immerseel F. From the gut to the peripheral tissues: the multiple effects of butyrate. *Nutr Res Rev* (2010) 23:366–84. doi: 10.1017/S0954422410000247
55. Feng Z, Long W, Hao B, Ding D, Ma X, Zhao L, et al. A human stool-derived Bilophila wadsworthia strain caused systemic inflammation in specific-pathogen-free mice. *Gut Pathog* (2017) 9:59. doi: 10.1186/s13099-017-0208-7
56. Devkota S, Wang Y, Musch MW, Leone V, Fehlner-Peach H, Nadimpalli A, et al. Dietary-fat-induced taurocholic acid promotes pathobiont expansion and colitis in Il10-/- mice. *Nature* (2012) 487:104–8. doi: 10.1038/nature11225
57. Lv LJ, Li SH, Li SC, Zhong ZC, Duan HL, Tian C, et al. Early-Onset Preeclampsia Is Associated With Gut Microbial Alterations in Antepartum and Postpartum Women. *Front Cell Infect Microbiol* (2019) 9:224:224. doi: 10.3389/fcimb.2019.00224
58. Li F, Han Y, Cai X, Gu M, Sun J, Qi C, et al. Dietary resveratrol attenuated colitis and modulated gut microbiota in dextran sulfate sodium-treated mice. *Food Funct* (2020) 11:1063–73. doi: 10.1039/c9fo01519a
59. Liu G, Bei J, Liang L, Yu G, Li L, Li Q. Stachyose Improves Inflammation through Modulating Gut Microbiota of High-Fat Diet/Streptozotocin-Induced Type 2 Diabetes in Rats. *Mol Nutr Food Res* (2018) 62:e1700954. doi: 10.1002/mnfr.201700954
60. Gibson GR, Roberfroid MB. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *J Nutr* (1995) 125:1401–12. doi: 10.1093/jn/125.6.1401
61. Karlsson FH, Tremaroli V, Nookaew I, Bergstrom G, Behre CJ, Fagerberg B, et al. Gut metagenome in European women with normal, impaired and diabetic glucose control. *Nature* (2013) 498:99–103. doi: 10.1038/nature12198

62. Strati F, Cavalieri D, Albanese D, De Felice C, Donati C, Hayek J, et al. New evidences on the altered gut microbiota in autism spectrum disorders. *Microbiome* (2017) 5:24. doi: 10.1186/s40168-017-0242-1
63. Tomova A, Husarova V, Lakatosova S, Bakos J, Vlkova B, Babinska K, et al. Gastrointestinal microbiota in children with autism in Slovakia. *Physiol Behav* (2015) 138:179–87. doi: 10.1016/j.physbeh.2014.10.033
64. Wang W, Chen L, Zhou R, Wang X, Song L, Huang S, et al. Increased proportions of Bifidobacterium and the Lactobacillus group and loss of butyrate-producing bacteria in inflammatory bowel disease. *J Clin Microbiol* (2014) 52:398–406. doi: 10.1128/JCM.01500-13
65. Breban M, Tap J, Leboime A, Said-Nahal R, Langella P, Chiocchia G, et al. Faecal microbiota study reveals specific dysbiosis in spondyloarthritis. *Ann Rheum Dis* (2017) 76:1614–22. doi: 10.1136/annrheumdis-2016-211064
66. Picchianti-Diamanti A, Panebianco C, Salemi S, Sorgi ML, Di Rosa R, Tropea A, et al. Analysis of Gut Microbiota in Rheumatoid Arthritis Patients: Disease-Related Dysbiosis and Modifications Induced by Etanercept. *Int J Mol Sci* (2018) 19:2938. doi: 10.3390/ijms19102938
67. Rahnavard G, Franzosa EA, McIver LJ, Schwager E, Lloyd-Price J, Weingart G, et al. *High-sensitivity pattern discovery in large multi"omic datasets*. Harvard T.H. Chan School of Public Health (2019). Available at: https://huttenhower.sph.harvard.edu/halla.

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

frontiers in Immunology

REVIEW
published: 05 May 2021
doi: 10.3389/fimmu.2021.662807

Exploring the Gut-Brain Axis for the Control of CNS Inflammatory Demyelination: Immunomodulation by *Bacteroides fragilis'* Polysaccharide A

Deniz Erturk-Hasdemir[1†], *Javier Ochoa-Repáraz*[2*†], *Dennis L. Kasper*[1] *and Lloyd H. Kasper*[3]

[1] Department of Immunology, Harvard Medical School, Boston, MA, United States, [2] Department of Biology, Eastern Washington University, Cheney, WA, United States, [3] Department of Microbiology and Immunology, Geisel School of Medicine at Dartmouth College, Hanover, NH, United States

OPEN ACCESS

Edited by:
Paulus Stefan Rommer,
Medical University of Vienna, Austria

Reviewed by:
Carlos Rodrigo Camara-Lemarroy,
University of Calgary, Canada
Anne-Katrin Pröbstel,
University Hospital of Basel, Switzerland

***Correspondence:**
Javier Ochoa-Repáraz
jochoareparaz@ewu.edu

[†]These authors have contributed equally to this work and share first authorship

Specialty section:
This article was submitted to Multiple Sclerosis and Neuroimmunology, a section of the journal Frontiers in Immunology

Received: 01 February 2021
Accepted: 16 April 2021
Published: 05 May 2021

Citation:
Erturk-Hasdemir D, Ochoa-Repáraz J, Kasper DL and Kasper LH (2021) Exploring the Gut-Brain Axis for the Control of CNS Inflammatory Demyelination: Immunomodulation by Bacteroides fragilis' Polysaccharide A. Front. Immunol. 12:662807. doi: 10.3389/fimmu.2021.662807

The symbiotic relationship between animals and their resident microorganisms has profound effects on host immunity. The human microbiota comprises bacteria that reside in the gastrointestinal tract and are involved in a range of inflammatory and autoimmune diseases. The gut microbiota's immunomodulatory effects extend to extraintestinal tissues, including the central nervous system (CNS). Specific symbiotic antigens responsible for inducing immunoregulation have been isolated from different bacterial species. Polysaccharide A (PSA) of *Bacteroides fragilis* is an archetypical molecule for host-microbiota interactions. Studies have shown that PSA has beneficial effects in experimental disease models, including experimental autoimmune encephalomyelitis (EAE), the most widely used animal model for multiple sclerosis (MS). Furthermore, *in vitro* stimulation with PSA promotes an immunomodulatory phenotype in human T cells isolated from healthy and MS donors. In this review, we discuss the current understanding of the interactions between gut microbiota and the host in the context of CNS inflammatory demyelination, the immunomodulatory roles of gut symbionts. More specifically, we also discuss the immunomodulatory effects of *B. fragilis* PSA in the gut-brain axis and its therapeutic potential in MS. Elucidation of the molecular mechanisms responsible for the microbiota's impact on host physiology offers tremendous promise for discovering new therapies.

Keywords: immunomodulation, microbiota, EAE (experimental autoimmune encephalomyelitis), multiple sclerosis, symbiotic molecules, *Bacteroides fragilis*, polysaccharide A (PSA)

INTRODUCTION

Mammals have co-evolved with eons of resident microorganisms that play an integral role in regulating the host immunity (1). These microorganisms live in a complex community called microbiota, which is dominated by bacteria and includes archaea, fungi, and viruses (2). Analysis of the composition and the human microbiota's diversity has significantly improved by culture-

independent methods and next-generation sequencing (3, 4). An updated catalog based on a metagenomic assembly of microbiomes across world populations shows that over 150,000 bacterial genomes can be found in the human body (5). The gastrointestinal tract harbors most of these microbes, some producing immunomodulatory molecules to educate the host immune system (6). The composition and gut microbiota function can affect the susceptibility to and progression of a wide range of diseases in the intestine and the extraintestinal tissues such as the central nervous system (CNS) (7, 8). While several microbial species have been identified concerning specific pathologies in humans, the mechanistic understanding of how the symbiotic molecules interact with the host is still limited. Determining the molecular mechanisms of the microbial molecules is crucial for the development of prophylactic and therapeutic interventions. *B. fragilis* polysaccharide A (PSA) is a prototypical symbiotic antigen that has been invaluable for understanding the mechanisms directing microbiota–host interactions (9). PSA mediates gut homeostasis by directing cellular and physical development of the immune system (10), stimulating Tregs (11) *via* plasmacytoid dendritic cells (PDCs) (12), and protecting animals from experimental diseases like colitis (11, 12), asthma (13), or pulmonary inflammation (14), and experimental autoimmune encephalomyelitis (EAE) (15–17). EAE is an animal model of multiple sclerosis (MS), an inflammatory demyelinating CNS disease (18). MS is characterized by inflammation and axonal damage resulting in progressive disability due to neurodegeneration. Although the disease's etiology is not entirely understood, multiple genetic and environmental factors have been implicated in MS's onset and progression. Mounting evidence suggests that microbiota plays an essential role in the development of the disease (19).

In this review, we cover some of the most recent literature on the gut-brain axis in the context of CNS inflammatory demyelination. Because of the impact of gut microbes regulating the immune system and the immune-mediated responses that characterize EAE/MS, we hypothesize that the large pool of microbes and microbial products present in the gut is an excellent source of novel therapeutics. Furthermore, we propose that PSA produced by *B. fragilis* is an identified symbiont factor model for immunomodulation. PSA could be one of the possibly numerous bacterial cellular components capable of promoting protective responses against neuroinflammation. Accordingly, our review will summarize how PSA regulates immune responses in MS/EAE and discusses PSA's therapeutic potential.

GUT MICROBIOTA

Although utero colonization is still debated (20), it is known that microbial colonization starts mainly after birth (21). It is suggested that establishing a diverse and balanced microbiota in early life is essential for developing a healthy immune system (22, 23). An imbalance in the microbiota's composition and function (dysbiosis) during this window of opportunity can have long-lasting consequences later in life, causing a wide range of immune diseases (24). The microbial composition of the infant's gut depends on many factors (25), including the type of delivery (26), gestational age (27), antibiotic use (28), and the mode of feeding (29). Human milk oligosaccharides (HMOs) in breast milk promote *beneficial* microbes like *Bifidobacterium* species in breast-fed infants (30). Cessation of breast-feeding and introducing solid foods drives the infant's gut microbiome's maturation (31), gradually reaching an adult-like composition after three years of life (32). Gut microbiota in adulthood is dominated by Bacteroidetes and Firmicutes and includes Actinobacteria, Proteobacteria, Verrucomicrobia, archaea, viruses, fungi, and protozoa (33, 34). While the gut microbiota in adults is more stable than in infants, the specific microbial species can vary interpersonally, creating a unique composition for every individual.

In homeostatic conditions, the host and the microbiota benefit each other and coexist in a mutualistic symbiosis (35). The host offers a nutrient-rich environment for the microbiota. In return, the microbiota provides metabolites (36), vitamins (37), and other micronutrients to the host by fermenting undigested dietary components in the large intestine (38). The microbiota's ability to produce energy by digesting complex carbohydrates in the gut has been an evolutionary driving force for establishing the host-microbiota symbiotic relationship (39, 40). In addition, the gut microbiota participates in the development of the host immune system and balances defense and tolerance to maintain homeostasis (41).

While the use of fecal microbiota transplantation (FMT) as a treatment for gastrointestinal problems in Chinese medicine dates back to the 4th century (42), the interconnectedness of gut microbiota, CNS, and neuropsychiatric health is a concept from the early 19th century (43). It is now known that the fundamental impact of the microbiota on host physiology reaches far outside the gastrointestinal tract and extends to CNS. Recently it is proposed that there is a bidirectional relationship between the gut microbiota and the CNS (44, 45). This reciprocal interaction occurs through different routes involving endocrine, immune, and neural mechanisms. The function of microbiota in the gut-brain axis is believed to affect the etiology of a wide range of neuropsychiatric disorders, including Parkinson's disease (46, 47), Alzheimer's disease (48, 49), depression (50, 51), anxiety (52, 53), autism (54, 55), amyotrophic lateral sclerosis (56) and multiple sclerosis (57–66). Increasing evidence points to alterations in the gut microbiota composition in patients with neuropsychiatric disorders. However, the molecular mechanisms by which the microbiota modulates these diseases are not fully understood. A mechanistic understanding of microbiota's role in the gut–brain axis will help develop prophylactic and therapeutic interventions for CNS diseases that are increasingly affecting large populations.

GUT MICROBIOTA AND CNS INFLAMMATORY DEMYELINATION

Multiple Sclerosis (MS) is an immune-mediated debilitating disease initiated by the immune system attacking the neuron

protecting myelin sheet, which results in inflammation, chronic demyelination, axonal degeneration, and loss of brain volume (67, 68). MS affects around 400,000 people in the United States (69) and 2.5 million worldwide, mainly living in higher latitudes (70). MS is divided into four clinical types: Relapsing-Remitting (RR-MS), Secondary Progressive (SP-MS), Progressive Relapsing (PR-MS), and Primary Progressive (PP-MS), with the majority of patients suffering from RR-MS type. There are various environmental risk factors associated with disease onset and progression (vitamin D, latitude, viral infections, smoking, diet) and genetic disposition (71). The immunopathology of MS is mainly driven by inflammatory $CD4^+$ T cell responses characterized by an increase in Th1 and Th17 cells (72) and decreased or impairment in Treg cells (73). Increasing importance is now appreciated for B cells' role in both the progression and modulation of this condition (74). $CD20^+$ B cells are targeted with monoclonal antibodies as approved MS therapies (75). Cerebrospinal fluid (CSF) of MS patients contains oligoclonal bands produced by plasmablasts and plasma cells (76), some of them auto-reactive against myelin self-peptides (77). B cells are present in CSF, CNS parenchyma, and meninges of MS patients, and germinal centers were identified in MS CNS (78). A recent study of single-cell RNA sequencing in CSF and blood suggest that in MS patients, B cells are clonally expanded and show an active inflammatory signature with memory plasma cell or plasmablast phenotype (79). More significant to this review's context, IgA produced by B cells that cross-react with gut microbes have been identified in the CNS of MS patients with active lesions (80) and regulate neuroinflammation through IL-10 production (81), highlighting the importance of B cells on the gut-microbiota-brain axis. In addition to T and B cells, dendritic cells and $CD8^+$ T cells also play a role in modifying MS's pathology (82).

Gut microbiota is considered an environmental factor that can promote protective roles in the development of multiple sclerosis. Other environmental risk factors for MS, such as diet, vitamin D, or geography, can directly affect the microbiota's composition. Gut microbiota can activate immune cells in the intestine or secrete immunomodulatory molecules and metabolites that orchestrate immune responses in the gut-brain axis. Previous studies suggest a link between changes in the composition of gut microbiota and MS pathogenesis. Limited but accumulating evidence points to an altered microbiota in MS patients compared to healthy individuals (57–66). The MS patients' microbiota shows a decreased abundance of *Bacteroides*, *Parabacteroides*, *Prevotella*, and *Lactobacillus* genera and increased *Akkermansia Blautia*, *Ruminococcus*, and *Bifidobacterium* (83). The relative abundances of members of the domain Archaea, such as *Methanobrevibacter*, are also increased in MS patients' gut and the bacteria such as *Akkermansia*, while *Butyricimonas* is decreased when compared to healthy controls (63). Recent findings link immunoglobulin (Ig) A (IgA)-coated gut microbiota with MS. IgA is the major neutralizing Ig in the human mucosa, including the gut, but is also found in circulation and periphery and a recent paper reports elevated IgA levels in cerebrospinal fluid of MS patients suffering active neuroinflammation (80). IgA^+ B cells capable of recognizing gut microbiota are present within active CNS lesions, where elevated *IL-10* transcripts are observed. Previous findings from the same group indicate that IL-10 was a principal component of gut-derived plasma cells' immunoregulatory role against neuroinflammation (81). There is now increasing evidence for the gut microbiota's role in the associated neuroinflammatory condition, neuromyelitis optica syndrome (84).

Despite the limitations of any experimental model of disease, EAE mice offer a practical approach to elucidate the clinical relevance of gut microbes in neuroinflammatory disorders, such as MS. The oral administration of broad-spectrum antibiotics reduces the severity of EAE (85–88). The treatment of C57BL/6 EAE mice with kanamycin, colistin, and vancomycin induced protection mediated by invariant natural killer cells T (iNKT) cells associated with reduced production of proinflammatory cytokines (IFN-γ, TNF-α, IL-6, and IL-17) in draining lymph nodes (LN) and a reduction in the percentages of mesenteric LN Th17 cells. In the mesenteric LNs of antibiotics-treated EAE mice, the levels of proinflammatory cytokines were reduced while IL-10 was increased (86). The treatment of SJL/J EAE mice with vancomycin, metronidazole, ampicillin, and neomycin induced protection against the disease that was associated with a reduced production of proinflammatory cytokines (IFN-γ, IL-17) in draining lymph nodes and increased frequencies of Foxp3 expressing $CD25^+CD4^+$T cells and increased levels of IL-10 and IL-13 (85). The ablation of CD25-expressing cells resulted in the lack of protection with antibiotics, while the adoptive transfer of $CD25^+CD4^+$ T cells with enhanced expression of Foxp3 isolated from antibiotics-treated SJL/J mice reduced the severity of EAE in recipient mice (85). The observation that antibiotics given orally but not intraperitoneally can protect the animals from disease emphasizes gut microbiota's importance in EAE pathophysiology. The results obtained after oral versus intraperitoneal administration of ampicillin supported these findings (87). A significant reduction in inflammatory antigen-presenting cells (peripheral macrophages and resident microglia) was also observed and associated with the protective effects promoted by antibiotics against EAE (88). Mechanistically, the protective effects induced by the oral treatment with antibiotics could be associated with alterations in microbial populations capable of triggering molecular mimicry pathways towards autoimmunity (87). Studies in GF mice confirmed the impact of the presence of gut microbiota in the severity of EAE. In GF conditions, mice show reduced EAE severity and reduced peripheral proinflammatory signals (89, 90). The causative association between the gut microbiota and EAE remains to be elucidated. EAE induction promotes alterations in the composition of the gut microbiota at early phases of disease (44), and disease results in alterations in the intestinal permeability and intestinal proinflammatory responses (91).

The use of gnotobiotic mice in EAE studies helped identify a few essential bacteria for positively or negatively modulating the disease outcome. When GF mice are monocolonized with *Segmented Filamentous Bacteria* (*SFB*), an increase in Th17 cell-mediated responses correlate with exacerbated EAE

severity (89). When the MS patients' microbiota was transferred to germ-free mice, it caused more severe symptoms in the EAE (57) and spontaneous brain autoimmunity (58). Furthermore, fecal microbiota transplantation (FMT) is reported to alleviate disease symptoms in EAE mice (92) and MS patients (93–95). Although it is still elusive if alterations in microbiota's composition and function are the cause or the result of the disease, microbiome-based therapeutics offer promise for MS treatment.

It has been shown that *Prevotella histicola* suppresses EAE through Tregs (96), while *Lactobacillus reuteri* exacerbates the disease through pathogenic $CD4^+$ and $CD8^+$ T cell responses (97). The prophylactic administration of individual lactobacilli strains reduces EAE severity through diminished myelin oligodendrocyte glycoprotein (MOG)- T cell reactivity (98). In contrast, the treatment with a mixture of three strains (*Lactobacillus paracasei* DSM 13434, *Lactobacillus plantarum* DSM 15312, and *Lactobacillus plantarum* DSM 15313) was able to reduce the progression of established severe EAE in a mechanism mediated by IL-10-producing Tregs (98). Single species of Enterococci, *Escherichia coli*, and others have also shown promising EAE study results (**Table 1**). The EAE model has successfully addressed the protective role of probiotic formulations with multiple species (105), some of which have already been assessed in MS patients (**Table 1**). A systematic review of the use of probiotic formulations in EAE and MS studies has been recently published (111).

As discussed above, gut microbiota modulates CNS inflammatory demyelination in murine models. In the following sections, we discuss *Bacteroides fragilis*, and its capsular polysaccharide A (PSA), identified as a member of the gut microbiota and microbial product with immunomodulatory effects that we hypothesize can regulate the extent of neuroinflammatory mechanisms associated with CNS demyelinating diseases.

THE GUT MICROBIOTA AS A SOURCE FOR IMMUNOMODULATORY FACTORS: POLYSACCHARIDE A (PSA) PRODUCED BY *BACTEROIDES FRAGILIS*

Bacteroides fragilis (B. fragilis), a prominent species of the genus *Bacteroides* within the Gram-negative Bacteroidetes phylum is part of the normal microbiota of the human colon. Bacteroides species are among the most abundant and adept colonizers of the human gut due to their ability to efficiently utilize complex host and dietary glycans (112) and express different surface structures by phase variation (113). *B. fragilis*, an obligate anaerobic Gram-negative bacillus, colonizes the majority of healthy individuals (114) and has profound effects on host physiology (115). *B. fragilis* was initially identified as the most common anaerobe in clinical isolates from abscesses caused by abdominal trauma and bacteremia (116). When contained in the gut, *B. fragilis* plays

TABLE 1 | Single probiotic species and probiotic multi-species mixes evaluated for protection in murine EAE [1] and MS clinical studies.

Model of EAE/MS study	Probiotic strains	Primary mechanisms of action proposed	Ref.
Prophylactic, in C57BL/6 EAE	*Escherichia coli Nissle*	Anti-inflammatory effects, reduction of Th1/Th17. Restored intestinal barrier disruption	(99)
C57BL/6 EAE, during disease	*Lactobacillus reuteri*	Reduction of Th1/Th17, reduced proliferation of autoreactive cells, restored dysbiosis	(100)
HLA-DR3.DQ8 double transgenic EAE	*Prevotella histicola*	Increased Treg, anti-inflammatory effects, and reduction of Th1/Th17	(96, 101)
Prophylactic and therapeutic in C57BL/6 EAE	*Lactobacillus paracasei* DSM 13434, *L. plantarum* DSM 15312, and DSM 15313	Increased Treg, anti-inflammatory effects, and reduction of Th1/Th17	(98)
Therapeutic, in C57BL/6 EAE	*Bifidobacterium animalis* and *Lactobacillus plantarum*	Increased Treg, anti-inflammatory effects, and reduction of Th1/Th17	(102)
Wistar rats EAE, during the disease	*Enterococcus faecium* L-3	Increased T cell function, with proposed involvement of IL-10	(103)
Prophylactic, in C57BL/6 and SJL/J EAE	*Pediococcus acidilactici*	IL-10-producing regulatory Tr1 cells	(104)
Therapeutic, in C57BL/6 EAE	Lactibiane iki [2]	Increased Treg	(105)
Therapeutic, in Theiler's murine encephalomyelitis virus	Vivomixx [3]	Anti-inflammatory responses, reduces astrogliosis, increased Bregs	(106)
EAE in Lewis rats	*Lactobacillus plantarum* NCIB 8826 and *L. murines* CNRZ	Reduce cumulative disease burden: Mechanism of action not evaluated	(107)
Prophylactic treatment in C57BL/6 EAE; ongoing EAE.	IRT5 [4]	Increased IL-10 producing $CD4^+$ T cells and IL-producing $CD11c^+$ monocytes	(108)
MS; RR-MS subjects on glatiramer acetate *vs*. untreated and healthy controls	Vivomixx	Reduced peripheral monocyte-mediated responses and APC function	(109)
MS; Randomized, double-blind, placebo-controlled trial	*L. acidophilus*, *L. casei*, *L. fermentum*, *Bifidobacterium bifidum*	Improved EDSS, anti-inflammatory effects	(110)

[1] *EAE protection studies performed with PSA and PSA-producing B. fragilis were not included since they are extensively discussed in the manuscript's body.*
[2] *Lactibiane iki: Bifidobacterium lactis LA 304, Lactobacillus acidophilus LA 201, and L. salivarius LA 302.*
[3] *Vivomixx: Lactobacillus acidophilus DSM 24735, L. plantarum DSM 24730, L. paracasei DSM 24733, L. delbrueckii subsp. Bulgaricus DSM 24734, Bifidobacterium longum DSM 24736, B. breve DSM 24732, B. infantis DSM 24737, and Streptococcus thermophilus DSM 24731.*
[4] *IRT5: Lactobacillus casei, L. acidophilus, L. reuteri, Bifidobacterium bifidum, and Streptococcus thermophilus.*

an intricate role in the colon and develops a beneficial relationship with the host (9). Monocolonization of germ-free mice with *B. fragilis* leads to the immune system's cellular and physical development (10, 117, 118). *B. fragilis* can also alleviate intestinal inflammation in animal models of colitis (12, 117, 119) and confer protection against infections (120–124). Recent studies show that *B. fragilis* exerts its beneficiary effects not only locally in the intestine but also systemically in extraintestinal tissues (13, 14, 125, 126) including CNS (15, 54, 127).

The capsular polysaccharide structure of *B. fragilis* plays an essential role in establishing a symbiotic relationship with its host (128). A large part of the *B. fragilis* genome is allocated to enzymes that degrade dietary polysaccharides and produce capsular polysaccharides of the organism (129–132). *B. fragilis* produces eight different distinct capsular polysaccharides, regulated by phase variation at the promoter region (133, 134). Variable expression of polysaccharides A through H creates a remarkable surface diversity which is vital for symbiosis (135, 136) and immunomodulation (112, 137). PSA is most abundantly expressed among those polysaccharides, and its immunomodulatory properties are most extensively studied (138).

PSA is a zwitterionic immunomodulatory polysaccharide consisting of a tetrasaccharide repeating unit (139, 140). The zwitterionic structure with a negative and a positive charge in each repeating unit is essential for the immunological potency of PSA (141, 142) and is required to activate T cells through the major histocompatibility complex II (MHCII) pathway (143, 144). High-resolution LC-MS/MS analysis shows that the terminal-reducing end of PSA contains a covalently attached lipid moiety required to activate antigen-presenting cells and protect against EAE (17). PSA is processed by antigen-presenting cells (APCs) through depolymerization in endocytic compartments in a nitric oxide-dependent manner (145, 146) and presented through the MHCII pathway to activate T cells (143, 147). The beneficial effects of PSA on the host immune system are manifested through multiple mechanisms. Microbial colonization in the gut is essential for host health. Host-specific microbiota is required for the full maturation of a functional immune system (148). Germ-free mice grown in sterile conditions develop physical and functional defects in their immune system, making them predisposed to infectious and inflammatory diseases (149). Recolonizing germ-free (GF) mice with PSA expressing *B. fragilis* mediates immune system development and can correct germ-free animals' deficiencies (10). PSA-dependent colonization of *B. fragilis* in a unique mucosal niche in the gut results in the induction of regulatory T cells and suppression of Th17 cells (150). WT *B. fragilis* but not the ΔPSA mutant induces anti-inflammatory $CD4^+$ $CD45Rb^{low}$ T cell population (119) and protects animals from the T cell transfer model of experimental colitis. In addition, animals orally treated with pure PSA (119) or PSA containing outer-membrane vesicles (OMVs) from WT *B. fragilis* (151) can protect animals from intestinal inflammation. PSA-mediated immunomodulation requires tolerogenic plasmacytoid dendritic cells (pDCs) (12), which activate a specific set of T cells defined as IL-10–producing $CD4^+CD25^+Foxp3^+$ Treg cells with an inducible phenotype (11). Innate and adaptive immune responses initiated by PSA require toll-like receptor 2 (TLR2). TLR2 is necessary for inducing the genes (e.g., iNOS, MHCII, and CD86) required for processing and presenting PSA by APCs (152). As a result, PSA exposure of APCs increases the antigen presentation capacity of the cells by increasing the expression of MHCII and costimulatory signals, including ICOSL (12). The enhanced production of IL-10 triggered by pDCs after PSA recognition is ablated in the absence of ICOSL/ICOS signal (12). In addition, TLR2 expression on APCs is necessary to induce IL-10 producing $CD4^+$ T cells (12) and protection against colitis (11, 12) and EAE (16, 17). PSA is recognized by the TLR2/TLR1 heterodimer in collaboration with Dectin-1 initiating a signaling cascade that involves the phosphoinositide 3-kinase (PI3K) pathway (17). Activation of PI3K pathway leads to phosphorylation and inactivation of glycogen synthase kinase 3β (GSK3β), promoting cAMP response element-binding protein (CREB)-dependent transcription of anti-inflammatory genes. Furthermore, TLR2 directs the expansion of $CD39^+CD4^+$ T cells in response to PSA, required for PSA-mediated protection against EAE. PSA's EAE protection is ablated in TLR2 (16, 17), TLR1, and Dectin-1 deficient mice (17).

The interactions between PSA and the host's intestinal dendritic cells are multifactorial. PSA recognition by colonic dendritic cells through TLR4-TRIF (TIR domain-containing adapter-inducing interferon-β) domain pathway, through the activation of interferon regulatory factors (IRFs), induces the production of IFN-β with anti-viral activity (153). The activation of the TLR4/TRIF pathway depends on the presence of a lipooligosaccharide (LOS) fraction linked covalently to the polysaccharide, anchoring the macromolecule to the outer membrane of *B. fragilis* (17). PSA produced by *B. fragilis* was identified as a symbiont factor promoting IFN-β-dependent protection against vesicular stomatitis virus infection. PSA's anti-viral effects were lost in the absence of both TLR4 and IFN-β (153). Thus, the production of IFN-β by colonic dendritic cells with $CD103^+CD11b^-$ and $CD103^-CD11b^+$ phenotypes is regulated by PSA and *B. fragilis* and likely by other commensal microbiota (153). **Figure 1** summarizes the recognition and cellular signaling pathways triggered by PSA in dendritic cells that result in the activation of immunomodulation dominated by IL-10-producing $CD4^+$ T cells and anti-viral responses.

PSA AGAINST NEUROINFLAMMATION

Studies demonstrating the impact of *Bacteroides fragilis* and PSA on EAE pathology (15, 127) were the first mechanistic examples of immunomodulation by gut microbiota in multiple sclerosis. *B. fragilis* and PSA's beneficial effects beyond the gastrointestinal tract are observed most strikingly in the gut-brain axis using EAE. When antibiotic-treated mice were colonized with WT *B. fragilis* but not the ΔPSA mutant, they were protected from EAE (127). Immunoprotection by PSA in EAE requires TLR2, TLR1, and Dectin-1 (17) and a specific tolerogenic DC subset called plasmacytoid dendritic cells (pDC), as shown in **Figure 1** (12). In the EAE model, WT *B. fragilis* protects by inducing $Foxp3^+$ Tregs and IL-10, whereas the ΔPSA mutant induces proinflammatory

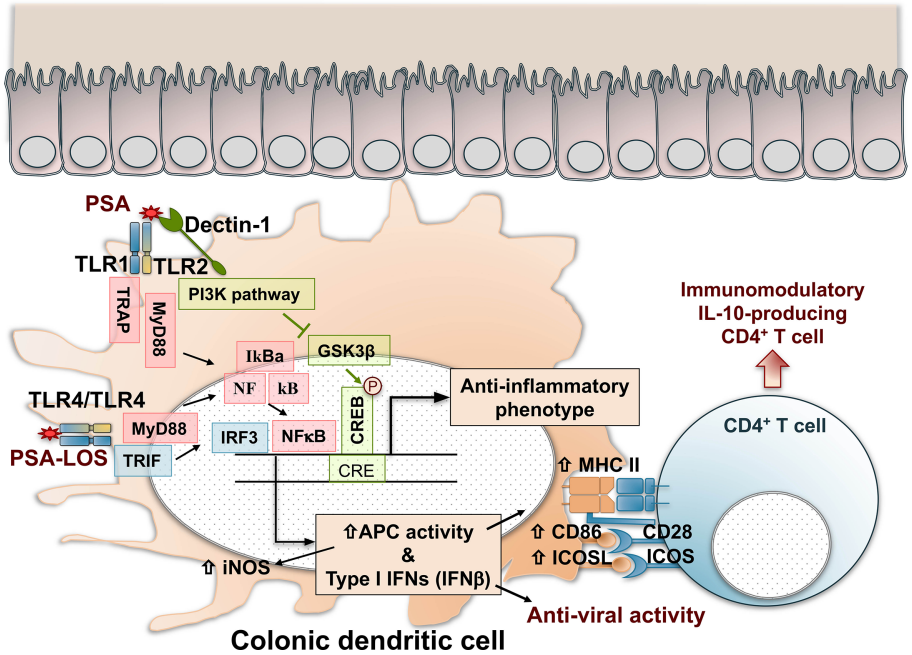


FIGURE 1 | Recognition, cell signaling, and immunomodulatory pathways triggered by PSA in colonic dendritic cells. PSA is recognized by TLR1/TLR2 dimers that result in NF-kB nuclear translocation and IRF-mediated activation of Type I IFN gene expression and enhanced antigen processing and presentation by increased expression of iNOS, MHC class II molecules, and costimulatory signals mediated by CD86 and ICOSL. In addition, Dectin-1, a C-Type Lectin pattern recognition receptor, contributes with TLR2 in the cell signal activation through the PI3K pathway, resulting in the nuclear phosphorylation and activation of CREB, triggering the expression of anti-inflammatory genes. As a result, naïve $CD4^+$ T cells are activated and differentiated in IL-10-producing immunomodulatory cells with Foxp3, CD39, Tr1 phenotypes that might depend on the inflammatory condition (IBD, asthma, EAE, or other). PSA recognition by TLR4 dimers induces the production of IFN-β with anti-viral activity through a MyD88 and TRIF-dependent pathway. The activation of TLR4/TRIF is dependent on the lipooligosaccharide (LOS) portion of the polysaccharide. CREB, cAMP response element–binding protein; GSK3β, glycogen synthase kinase 3β; IFN, interferon; IL-10, interleukin 10; iNOS, inducible nitric oxide synthase; IRF, interferon regulatory factors; MHC II, major histocompatibility complex class II; MyD88, myeloid differentiation primary response 88; NFkB, nuclear factor-kB; PSA, polysaccharide A; PI3K, phosphoinositide 3-kinase; TLR, toll-like receptor; TRAP, tumor necrosis factor receptor-associated protein. TRIF, TIR domain-containing adapter-inducing interferon-beta.

cytokines IL-17 IL-6 and causes pathology. In addition, $CD103^+CD11c^+$ DCs isolated from cervical lymph nodes of ΔPSA-colonized animals are unable to convert $Foxp3^-CD4^+$ T cells into $Foxp3^+$Treg cells (127). Furthermore, prophylactic or therapeutic treatment with pure PSA is sufficient to protect animals from EAE (15). PSA can induce accumulation of $CD11c^+CD103^+$ DCs in cervical lymph nodes, which convert naive $CD4^+$ T cells into $Foxp3^+$ Treg cells (15). IL-10–deficient mice are not protected from EAE, showing that PSA's immunoprotection in the EAE model requires IL-10–producing Treg cells induced by tolerogenic DCs (15). In addition, PSA-induced regulatory T cells express CD39 independent of their Foxp3 expression and require TLR2 for activation (16). CD39 [nucleoside triphosphate diphosphohydrolase-1 (NTPDase 1)] is an ectoenzyme that degrades ATP released from damaged cells to AMP and adenosine. Previous studies have shown that CD39 is expressed by regulatory T cells (145) and has an essential role in suppressing Th17 cells (154, 155). $CD39^+$ Tregs are reduced in MS patients (154), contributing to the Th17-driven pathology in this disease. CD39 deficiency increases IL-17 and decreases IL-10 production in mice and abrogates PSA-induced immunoprotection in EAE (16).

Furthermore, CD39 enhances the migratory capacity of $CD4^+$ T cells, which results in PSA-dependent accumulation of $CD39^+$ $CD4^+$ $Foxp3^+$ regulatory T cells in the CNS (156). In the DC-T cell coculture system, using cells from healthy human peripheral blood mononuclear cells (PBMCs), PSA induces IL-10 producing $CD39^+Foxp3^+$ cells *in vitro* (157). Furthermore, PSA increases the expression of CD39 and IL-10 and enhances the suppressive function of $Foxp3^+CD4^+$ cells (157). Similarly, PSA drives differentiation of regulatory T cells and IL-10 production using naïve T cells from MS patients (158). Notably, induced expression of Foxp3 in response to PSA was higher in MS patients than in healthy controls (158).

The protective effects of PSA against neuroinflammation were also addressed in a murine model of viral encephalitis. The induction of neuroinflammation with Herpes Virus was controlled by PSA's administration that promoted a protective mechanism by IL-10 (126). The phenotypes of IL-10-producing cells induced by the PSA treatment were heterogeneous, with inductions of $ICOS^+CD39^+CD37^+CD4^+$ T cells, $CD37^+CD8^+$ T cells, and IL-10-producing B cells. IL-10-producing Tregs were increased in draining LNs of PSA-treated mice compared to PBS-treated mice. The protection against neuroinflammation

triggered by the virus was also observed when mice were treated with PSA-producing *B. fragilis* (126). Thus, it appears that PSA is a potent modulator of neuroinflammation, in addition to the protective effects observed against infections (120–124), autoimmunity at the intestinal level (12, 117, 119), asthma (13), or pulmonary inflammation (14). More work is necessary to elucidate whether PSA's phenotypes depend on the inflammatory pathways triggered in specific target tissues of the different disorders. A recent paper showed that PSA promotes the activation of an interferon responsive gene (IRG) signature responsible for producing inflammatory cytokines and cellular signals resulting in PD1, Lag3, and Tim3 expression (159). PSA regulates Type I interferons' production by colonic dendritic cells, specifically IFN-β by TLR4-TRIF domain signaling mechanisms (153). The production of IFN-β directs the anti-viral protective responses induced by PSA (153).

CONCLUSIONS

The microbiome research field had expanded continuously since the early 2000s, which helped us understand the human microbiome's role in health and disease. In recent years, the field's scope shifted from the characterization of the microbiota composition and its association to diseases to mechanistic and causative understanding of microbes on human health.

The most prominent and most studied effect of gut microbiota is on the immune system, which can influence a wide range of infectious, inflammatory, metabolic, and autoimmune diseases (160). The immune system plays an essential role in the bidirectional communication within the gut-brain axis and modulates diseases in the CNS, including multiple sclerosis. Several different microbial species have been associated with susceptibility to and progression of multiple sclerosis. However, this is still an emerging field, and researchers must address numerous challenges in their research.

Separating correlation from causation in microbiome-disease association studies is one of the biggest challenges in the field. The literature has been dominated by associative studies comparing differences in microbiomes of MS patients with healthy controls. To translate these association studies into the clinic, researchers need to discover which of these differences are causing the disease. In addition, it is challenging to define "healthy microbiota" since there is tremendous intrapersonal and interpersonal variability in the composition of the human microbiota. Dysbiosis referring to disturbances in the microbiota structure is widely used without specific definitions of balanced and imbalanced microbial communities' compositions. Furthermore, while the GF and gnotobiotic animals are invaluable for microbiome research, animal studies' applicability to humans needs to be verified.

An accumulating body of research has proven the potential use of gut symbionts as microbial therapeutics. Following the success of FMT in treating *C. difficile* infections, several companies are testing groups of microbes or individual bacteria in clinical trials. However, understanding molecular interactions that shape host-bacterial interactions is crucial for the effective design of microbial therapeutics. This review highlights the microbiota's role in mediating immune responses to multiple sclerosis, focusing on an archetypical microbial molecule PSA. PSA is a hallmark of symbiotic molecules with immunoregulatory functions. Although further protection and toxicity studies are needed to address PSA's applicability as a therapeutic, we hypothesize that PSA, and likely other unidentified gut symbiont factors, is a safe and effective alternative for treating multiple sclerosis and other CNS diseases.

AUTHOR CONTRIBUTIONS

DE-H and JO-R contributed equally to the design and preparation of the manuscript, tables, and figures. DK and LK contributed equally to the idea, the design, and the review of the manuscript. All authors contributed to the article and approved the submitted version.

REFERENCES

1. Ferreiro A, Crook N, Gasparrini AJ, Dantas G. Multiscale Evolutionary Dynamics of Host-Associated Microbiomes. *Cell* (2018) 172:1216–27. doi: 10.1016/j.cell.2018.02.015
2. Erturk-Hasdemir D, Kasper DL. Resident Commensals Shaping Immunity. *Curr Opin Immunol* (2013) 25:450–5. doi: 10.1016/j.coi.2013.06.001
3. HMP. The Integrative Human Microbiome Project: Dynamic Analysis of Microbiome-Host Omics Profiles During Periods of Human Health and Disease. *Cell Host Microbe* (2014) 16:276–89. doi: 10.1016/j.chom.2014.08.014
4. Proctor LM, Creasy HH, Fettweis JM, Lloyd-Price J, Mahurkar A, Zhou W, et al. The Integrative Human Microbiome Project. *Nature* (2019) 569:641–8. doi: 10.1038/s41586-019-1238-8
5. Pasolli E, Asnicar F, Manara S, Quince C, Huttenhower C, Correspondence NS. Extensive Unexplored Human Microbiome Diversity Revealed by Over 150,000 Genomes From Metagenomes Spanning Age, Geography, and Lifestyle. *Cell* (2019) 176:649–62. doi: 10.1016/j.cell.2019.01.001
6. Zhao Q, Elson CO. Adaptive Immune Education by Gut Microbiota Antigens. *Immunology* (2018) 154:28–37. doi: 10.1111/imm.12896
7. Zheng D, Liwinski T, Elinav E. Interaction Between Microbiota and Immunity in Health and Disease. *Cell Res* (2020) 30:492–506. doi: 10.1038/s41422-020-0332-7
8. Riccio P, Rossano R. The Human Gut Microbiota is Neither an Organ Nor a Commensal. *FEBS Lett* (2020) 594:3262–71. doi: 10.1002/1873-3468.13946
9. Erturk-Hasdemir D, Kasper DL. Finding a Needle in a Haystack: Bacteroides Fragilis Polysaccharide A as the Archetypical Symbiosis Factor. *Ann N Y Acad Sci* (2018) 1417:116–29. doi: 10.1111/nyas.13660
10. Mazmanian SK, Liu CH, Tzianabos AO, Kasper DL. An Immunomodulatory Molecule of Symbiotic Bacteria Directs Maturation of the Host Immune System. *Cell* (2005) 122:107–18. doi: 10.1016/j.cell.2005.05.007
11. Round JL, Mazmanian SK. Inducible Foxp3+ Regulatory T-cell Development by a Commensal Bacterium of the Intestinal Microbiota. *Proc Natl Acad Sci U S A* (2010) 107:12204–9. doi: 10.1073/pnas.0909122107
12. Dasgupta S, Erturk-Hasdemir D, Ochoa-Reparaz J, Reinecker H-C, Kasper DL. Plasmacytoid Dendritic Cells Mediate Anti-Inflammatory Responses to a Gut Commensal Molecule Via Both Innate and Adaptive Mechanisms. *Cell Host Microbe* (2014) 15:413–23. doi: 10.1016/j.chom.2014.03.006

13. Johnson JL, Jones MB, Cobb BA. Bacterial Capsular Polysaccharide Prevents the Onset of Asthma Through T-cell Activation. *Glycobiology* (2015) 25:368–75. doi: 10.1093/glycob/cwu117
14. Johnson JL, Jones MB, Cobb BA. Polysaccharide-Experienced Effector T Cells Induce IL-10 in FoxP3+ Regulatory T Cells to Prevent Pulmonary Inflammation. *Glycobiology* (2018) 28:50–8. doi: 10.1093/glycob/cwx093
15. Ochoa-Repáraz J, Mielcarz DW, Wang Y, Begum-Haque S, Dasgupta S, Kasper DL, et al. A Polysaccharide From the Human Commensal Bacteroides Fragilis Protects Against CNS Demyelinating Disease. *Mucosal Immunol* (2010) 3:487–95. doi: 10.1038/mi.2010.29
16. Wang Y, Telesford KM, Ochoa-Reparaz J, Haque-Begum S, Christy M, Kasper EJ, et al. An Intestinal Commensal Symbiosis Factor Controls Neuroinflammation Via TLR2-mediated CD39 Signalling. *Nat Commun* (2014) 5:4432. doi: 10.1038/ncomms5432
17. Erturk-Hasdemir D, Oh SF, Okan NA, Stefanetti G, Gazzaniga FS, Seeberger PH, et al. Symbionts Exploit Complex Signaling to Educate the Immune System. *Proc Natl Acad Sci U S A* (2019) 116:26157–66. doi: 10.1073/PNAS.1915978116
18. Procaccini C, De Rosa V, Pucino V, Formisano L, Matarese G. Animal Models of Multiple Sclerosis. *Eur J Pharmacol* (2015) 759:182–91. doi: 10.1016/j.ejphar.2015.03.042
19. Ochoa-Repáraz J, Kirby TO, Kasper LH. The Gut Microbiome and Multiple Sclerosis. *Cold Spring Harbor Perspect Med* (2018) 1:a029017. doi: 10.1101/cshperspect.a029017
20. Rackaityte E, Halkias J, Fukui EM, Mendoza VF, Hayzelden C, Crawford ED, et al. Viable Bacterial Colonization is Highly Limited in the Human Intestine In Utero. *Nat Med* (2020) 26:599–607. doi: 10.1038/s41591-020-0761-3
21. Milani C, Duranti S, Bottacini F, Casey E, Turroni F, Mahony J, et al. The First Microbial Colonizers of the Human Gut: Composition, Activities, and Health Implications of the Infant Gut Microbiota. *Microbiol Mol Biol Rev* (2017) 81:e00036–17. doi: 10.1128/mmbr.00036-17
22. Olszak T, An D, Zeissig S, Vera MP, Richter J, Franke A, et al. Microbial Exposure During Early Life has Persistent Effects on Natural Killer T Cell Function. *Science* (2012) 336:489–93. doi: 10.1126/science.1219328
23. Ganal-Vonarburg SC, Hornef MW, Macpherson AJ. Microbial–Host Molecular Exchange and its Functional Consequences in Early Mammalian Life. *Science* (2020) 368:604–7. doi: 10.1126/science.aba0478
24. Gensollen T, Iyer SS, Kasper DL, Blumberg RS. How Colonization by Microbiota in Early Life Shapes the Immune System. *Science* (2016) 352:539–44. doi: 10.1126/science.aad9378
25. Chong CYL, Bloomfield FH, O'Sullivan JM. Factors Affecting Gastrointestinal Microbiome Development in Neonates. *Nutrients* (2018) 10:274. doi: 10.3390/nu10030274
26. Rutayisire E, Huang K, Liu Y, Tao F. The Mode of Delivery Affects the Diversity and Colonization Pattern of the Gut Microbiota During the First Year of Infants' Life: A Systematic Review. *BMC Gastroenterol* (2016) 16:86. doi: 10.1186/s12876-016-0498-0
27. Tirone C, Pezza L, Paladini A, Tana M, Aurilia C, Lio A, et al. Gut and Lung Microbiota in Preterm Infants: Immunological Modulation and Implication in Neonatal Outcomes. *Front Immunol* (2019) 10:2910. doi: 10.3389/fimmu.2019.02910
28. Bokulich NA, Chung J, Battaglia T, Henderson N, Jay M, Li H, et al. Antibiotics, Birth Mode, and Diet Shape Microbiome Maturation During Early Life. *Sci Translat Med* (2016) 8:343ra82. doi: 10.1126/scitranslmed.aad7121
29. Li Y, Faden HS, Zhu L. The Response of the Gut Microbiota to Dietary Changes in the First Two Years of Life. *Front Pharmacol* (2020) 11:334. doi: 10.3389/fphar.2020.00334
30. Zivkovic AM, German JB, Lebrilla CB, Mills DA. Human Milk Glycobiome and its Impact on the Infant Gastrointestinal Microbiota. *Proc Natl Acad Sci U S A* (2011) 108:4653–8. doi: 10.1073/pnas.1000083107
31. Bäckhed F, Roswall J, Peng Y, Feng Q, Jia H, Kovatcheva-Datchary P, et al. Dynamics and Stabilization of the Human Gut Microbiome During the First Year of Life. *Cell Host Microbe* (2015) 17:690–703. doi: 10.1016/j.chom.2015.04.004
32. Yatsunenko T, Rey FE, Manary MJ, Trehan I, Dominguez-Bello MG, Contreras M, et al. Human Gut Microbiome Viewed Across Age and Geography. *Nature* (2012) 486:222–7. doi: 10.1038/nature11053
33. Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, et al. Diversity of the Human Intestinal Microbial Flora. *Science* (2005) 308:1635–8. doi: 10.1126/science.1110591
34. Matijašić M, Meštrović T, Paljetak HČ, Perić M, Barešić A, Verbanac D. Gut Microbiota Beyond Bacteria-Mycobiome, Virome, Archaeome, and Eukaryotic Parasites in IBD. *Int J Mol Sci* (2020) 21:2668. doi: 10.3390/ijms21082668
35. Dethlefsen L, McFall-Ngai M, Relman DA. An Ecological and Evolutionary Perspective on Human-Microbe Mutualism and Disease. *Nature* (2007) 449:811–8. doi: 10.1038/nature06245
36. Postler TS, Ghosh S. Understanding the Holobiont: How Microbial Metabolites Affect Human Health and Shape the Immune System. *Cell Metab* (2017) 26:110–30. doi: 10.1016/j.cmet.2017.05.008
37. LeBlanc JG, Milani C, de Giori GS, Sesma F, van Sinderen D, Ventura M. Bacteria as Vitamin Suppliers to Their Host: A Gut Microbiota Perspective. *Curr Opin Biotechnol* (2013) 24:160–8. doi: 10.1016/j.copbio.2012.08.005
38. Flint HJ, Scott KP, Duncan SH, Louis P, Forano E. Microbial Degradation of Complex Carbohydrates in the Gut. *Gut Microbes* (2012) 3:289–306. doi: 10.4161/gmic.19897
39. Sonnenburg ED, Smits SA, Tikhonov M, Higginbottom SK, Wingreen NS, Sonnenburg JL. Diet-Induced Extinctions in the Gut Microbiota Compound Over Generations. *Nature* (2016) 529:212–5. doi: 10.1038/nature16504
40. Rook G, Bäckhed F, Levin BR, McFall-Ngai MJ, McLean AR. Evolution, Human-Microbe Interactions, and Life History Plasticity. *Lancet* (2017) 390:521–30. doi: 10.1016/S0140-6736(17)30566-4
41. Guzman-Bautista ER, Suzuki K, Asami S, Fagarasan S. Bacteria-Immune Cells Dialog and the Homeostasis of the Systems. *Curr Opin Immunol* (2020) 66:82–9. doi: 10.1016/j.coi.2020.05.010
42. Young VB. Therapeutic Manipulation of the Microbiota: Past, Present, and Considerations for the Future. *Clin Microb Infect* (2016) 22:905–9. doi: 10.1016/j.cmi.2016.09.001
43. Miller I. The Gut–Brain Axis: Historical Reflections. *Microb Ecol Health Dis* (2018) 29:1542921. doi: 10.1080/16512235.2018.1542921
44. Colpitts SL, Kasper EJ, Keever A, Liljenberg C, Kirby T, Magori K, et al. A Bidirectional Association Between the Gut Microbiota and CNS Disease in a Biphasic Murine Model of Multiple Sclerosis. *Gut Microbes* (2017) 8:561–73. doi: 10.1080/19490976.2017.1353843
45. Cox LM, Weiner HL. Microbiota Signaling Pathways That Influence Neurologic Disease. *Neurotherapeutics* (2018) 15:135–45. doi: 10.1007/s13311-017-0598-8
46. Sampson TR, Debelius JW, Thron T, Janssen S, Shastri GG, Ilhan ZE, et al. Gut Microbiota Regulate Motor Deficits and Neuroinflammation in a Model of Parkinson's Disease. *Cell* (2016) 167:1469–80. doi: 10.1016/j.cell.2016.11.018
47. Chao YX, Gulam MY, Chia NSJ, Feng L, Rotzschke O, Tan EK. Gut–Brain Axis: Potential Factors Involved in the Pathogenesis of Parkinson's Disease. *Front Neurol* (2020) 11:849. doi: 10.3389/fneur.2020.00849
48. Kim MS, Kim Y, Choi H, Kim W, Park S, Lee D, et al. Transfer of a Healthy Microbiota Reduces Amyloid and Tau Pathology in an Alzheimer's Disease Animal Model. *Gut* (2020) 69:283–94. doi: 10.1136/gutjnl-2018-317431
49. He Y, Li B, Sun D, Chen S. Gut Microbiota: Implications in Alzheimer's Disease. *J Clin Med* (2020) 9:2042. doi: 10.3390/jcm9072042
50. Kelly JR, Borre Y, O' Brien C, Patterson E, El Aidy S, Deane J, et al. Transferring the Blues: Depression-associated Gut Microbiota Induces Neurobehavioural Changes in the Rat. *J Psychiatr Res* (2016) 82:109–18. doi: 10.1016/j.jpsychires.2016.07.019
51. Capuco A, Urits I, Hasoon J, Chun R, Gerald B, Wang JK, et al. Current Perspectives on Gut Microbiome Dysbiosis and Depression. *Adv Ther* (2020) 37:1328–46. doi: 10.1007/s12325-020-01272-7
52. Jiang Hy, Zhang X, Yu Z, Zhang Z, Deng M, Zhao Jh, et al. Altered Gut Microbiota Profile in Patients With Generalized Anxiety Disorder. *J Psychiatr Res* (2018) 104:130–6. doi: 10.1016/j.jpsychires.2018.07.007
53. Peirce JM, Alviña K. The Role of Inflammation and the Gut Microbiome in Depression and Anxiety. *J Neurosci Res* (2019) 97:1223–41. doi: 10.1002/jnr.24476
54. Hsiao EY, McBride SW, Hsien S, Sharon G, Hyde ER, McCue T, et al. Microbiota Modulate Behavioral and Physiological Abnormalities Associated With Neurodevelopmental Disorders. *Cell* (2013) 155:1451–63. doi: 10.1016/j.cell.2013.11.024

55. Srikantha P, Hasan Mohajeri M. The Possible Role of the Microbiota-Gut-Brain-Axis in Autism Spectrum Disorder. *Int J Mol Sci* (2019) 20:2115. doi: 10.3390/ijms20092115
56. Burberry A, Wells MF, Limone F, Couto A, Smith KS, Keaney J, et al. C9orf72 Suppresses Systemic and Neural Inflammation Induced by Gut Bacteria. *Nature* (2020) 582:89–94. doi: 10.1038/s41586-020-2288-7
57. Berer K, Gerdes LA, Cekanaviciute E, Jia X, Xiao L, Xia Z, et al. Gut Microbiota From Multiple Sclerosis Patients Enables Spontaneous Autoimmune Encephalomyelitis in Mice. *Proc Natl Acad Sci U S A* (2017) 114:10719–24. doi: 10.1073/pnas.1711233114
58. Cekanaviciute E, Yoo BB, Runia TF, Debelius JW, Singh S, Nelson CA, et al. Gut Bacteria From Multiple Sclerosis Patients Modulate Human T Cells and Exacerbate Symptoms in Mouse Models. *Proc Natl Acad Sci U S A* (2017) 114:10713–8. doi: 10.1073/pnas.1711235114
59. Cignarella F, Cantoni C, Ghezzi L, Salter A, Dorsett Y, Chen L, et al. Intermittent Fasting Confers Protection in CNS Autoimmunity by Altering the Gut Microbiota. *Cell Metab* (2018) 27:1222–35.e6. doi: 10.1016/j.cmet.2018.05.006
60. Kohl HM, Castillo AR, Ochoa-Repáraz J. The Microbiome as a Therapeutic Target for Multiple Sclerosis: Can Genetically Engineered Probiotics Treat the Disease? *Diseases* (2020) 8:33. doi: 10.3390/diseases8030033
61. Miyake S, Kim S, Suda W, Oshima K, Nakamura M, Matsuoka T, et al. Dysbiosis in the Gut Microbiota of Patients With Multiple Sclerosis, With a Striking Depletion of Species Belonging to Clostridia Xiva and IV Clusters. *PLoS One* (2015) 10:e0137429. doi: 10.1371/journal.pone.0137429
62. Chen J, Chia N, Kalari KR, Yao JZ, Novotna M, Soldan MMP, et al. Multiple Sclerosis Patients Have a Distinct Gut Microbiota Compared to Healthy Controls. *Sci Rep* (2016) 6:28484. doi: 10.1038/srep28484
63. Jangi S, Gandhi R, Cox LM, Li N, von Glehn F, Yan R, et al. Alterations of the Human Gut Microbiome in Multiple Sclerosis. *Nat Commun* (2016) 7:12015. doi: 10.1038/ncomms12015
64. Tremlett H, Fadrosh DW, Faruqi AA, Hart J, Roalstad S, Graves J, et al. Associations Between the Gut Microbiota and Host Immune Markers in Pediatric Multiple Sclerosis and Controls. *BMC Neurol* (2016) 16:182. doi: 10.1186/s12883-016-0703-3
65. Tremlett H, Fadrosh DW, Faruqi AA, Zhu F, Hart J, Roalstad S, et al. Us Network of Pediatric Ms Centers. Gut Microbiota in Early Pediatric Multiple Sclerosis: A Case-Control Study. *Eur J Neurol* (2016) 23:1308–21. doi: 10.1111/ene.13026
66. Takewaki D, Suda W, Sato W, Takayasu L, Kumar N, Kimura K, et al. Alterations of the Gut Ecological and Functional Microenvironment in Different Stages of Multiple Sclerosis. *Proc Natl Acad Sci U S A* (2020) 117:22402–12. doi: 10.1073/pnas.2011703117
67. Dargahi N, Katsara M, Tselios T, Androutsou ME, De Courten M, Matsoukas J, et al. Multiple Sclerosis: Immunopathology and Treatment Update. *Brain Sci* (2017) 7:78. doi: 10.3390/brainsci7070078
68. Lemus HN, Warrington AE, Rodriguez M. Multiple Sclerosis: Mechanisms of Disease and Strategies for Myelin and Axonal Repair. *Neurol Clin* (2018) 36:1–11. doi: 10.1016/j.ncl.2017.08.002
69. Dilokthornsakul P, Valuck RJ, Nair KV, Corboy JR, Allen RR, Campbell JD. Multiple Sclerosis Prevalence in the United States Commercially Insured Population. *Neurology* (2016) 86:1014–21. doi: 10.1212/WNL.0000000000002469
70. Ghareghani M, Reiter RJ, Zibara K, Farhadi N. Latitude, Vitamin D, Melatonin, and Gut Microbiota Act in Concert to Initiate Multiple Sclerosis: A New Mechanistic Pathway. *Front Immunol* (2018) 9:2484. doi: 10.3389/fimmu.2018.02484
71. Baecher-Allan C, Kaskow BJ, Weiner HL. Multiple Sclerosis: Mechanisms and Immunotherapy. *Neuron* (2018) 97:742–68. doi: 10.1016/j.neuron.2018.01.021
72. Stromnes IM, Cerretti LM, Liggitt D, Harris RA, Goverman JM. Differential Regulation of Central Nervous System Autoimmunity by T H1 and TH17 Cells. *Nat Med* (2008) 14:337–42. doi: 10.1038/nm1715
73. Viglietta V, Baecher-Allan C, Weiner HL, Hafler DA. Loss of Functional Suppression by CD4+CD25+ Regulatory T Cells in Patients With Multiple Sclerosis. *J Exp Med* (2004) 199:971–9. doi: 10.1084/jem.20031579
74. Sabatino JJ, Zamvil SS, Hauser SL. B-Cell Therapies in Multiple Sclerosis. *Cold Spring Harb Perspect Med* (2019) 9:a032037. doi: 10.1101/cshperspect.a032037
75. Roach CA, Cross AH. Anti-CD20 B Cell Treatment for Relapsing Multiple Sclerosis. *Front Neurol* (2021) 11:595547. doi: 10.3389/fneur.2020.595547
76. Obermeier B, Mentele R, Malotka J, Kellermann J, Kümpfel T, Wekerle H, et al. Matching of Oligoclonal Immunoglobulin Transcriptomes and Proteomes of Cerebrospinal Fluid in Multiple Sclerosis. *Nat Med* (2008) 14:688–93. doi: 10.1038/nm1714
77. Warren KG, Catz I, Johnson E, Mielke B. Anti-Myelin Basic Protein and Anti-Proteolipid Protein Specific Forms of Multiple Sclerosis. *Ann Neurol* (1994) 35:280–9. doi: 10.1002/ana.410350307
78. Serafini B, Rosicarelli B, Magliozzi R, Stigliano E, Aloisi F. Detection of Ectopic B-Cell Follicles With Germinal Centers in the Meninges of Patients With Secondary Progressive Multiple Sclerosis. *Brain Pathol* (2004) 14:164–74. doi: 10.1111/j.1750-3639.2004.tb00049.x
79. Ramesh A, Schubert RD, Greenfield AL, Dandekar R, Loudermilk R, Sabatino JJ, et al. A Pathogenic and Clonally Expanded B Cell Transcriptome in Active Multiple Sclerosis. *Proc Natl Acad Sci U S A* (2020) 117:22932–43. doi: 10.1073/pnas.2008523117
80. Pröbstel A-K, Zhou X, Baumann R, Wischnewski S, Kutza M, Rojas OL, et al. Gut Microbiota-Specific Iga+ B Cells Traffic to the CNS in Active Multiple Sclerosis. *Sci Immunol* (2020) 5:eabc7191. doi: 10.1126/sciimmunol.abc7191
81. Rojas OL, Pröbstel A-K, Porfilio EA, Wang AA, Charabati M, Sun T, et al. Recirculating Intestinal Iga-Producing Cells Regulate Neuroinflammation Via IL-10. *Cell* (2019) 177:492–3. doi: 10.1016/j.cell.2019.03.037
82. Rangachari M, Kerfoot SM, Arbour N, Alvarez JI. Editorial: Lymphocytes in MS and EAE: More Than Just a CD4+ World. *Front Immunol* (2017) 8:133. doi: 10.3389/fimmu.2017.00133
83. Freedman SN, Shahi SK, Mangalam AK. The "Gut Feeling": Breaking Down the Role of Gut Microbiome in Multiple Sclerosis. *Neurotherapeutics* (2018) 15:109–25. doi: 10.1007/s13311-017-0588-x
84. Zamvil SS, Spencer CM, Baranzini SE, Cree BAC. The Gut Microbiome in Neuromyelitis Optica. *Neurotherapeutics* (2018) 15:92–101. doi: 10.1007/s13311-017-0594-z
85. Yokote H, Miyake S, Croxford JL, Oki S, Mizusawa H, Yamamura T. NKT Cell-Dependent Amelioration of a Mouse Model of Multiple Sclerosis by Altering Gut Flora. *Am J Pathol* (2008) 173:1714–23. doi: 10.2353/ajpath.2008.080622
86. Ochoa-Repáraz J, Mielcarz DW, Ditrio LE, Burroughs AR, Foureau DM, Haque-Begum S, et al. Role of Gut Commensal Microflora in the Development of Experimental Autoimmune Encephalomyelitis. *J Immunol* (2009) 183:6041–50. doi: 10.4049/jimmunol.0900747
87. Miyauchi E, Kim S-W, Suda W, Kawasumi M, Onawa S, Taguchi-Atarashi N, et al. Gut Microorganisms Act Together to Exacerbate Inflammation in Spinal Cords. *Nature* (2020) 585:102–6. doi: 10.1038/s41586-020-2634-9
88. Seifert HA, Benedek G, Nguyen H, Gerstner G, Zhang Y, Kent G, et al. Antibiotics Protect Against EAE by Increasing Regulatory and Anti-Inflammatory Cells. *Metab Brain Dis* (2018) 33:1599–607. doi: 10.1007/s11011-018-0266-7
89. Lee YK, Menezes JS, Umesaki Y, Mazmanian SK. Proinflammatory T-cell Responses to Gut Microbiota Promote Experimental Autoimmune Encephalomyelitis. *Proc Natl Acad Sci U S A* (2011) 108(Suppl 1):4615–22. doi: 10.1073/pnas.1000082107
90. Berer K, Mues M, Koutrolos M, Rasbi ZA, Boziki M, Johner C, et al. Commensal Microbiota and Myelin Autoantigen Cooperate to Trigger Autoimmune Demyelination. *Nature* (2011) 479:538–41. doi: 10.1038/nature10554
91. Nouri M, Bredberg A, Weström B, Lavasani S. Intestinal Barrier Dysfunction Develops At the Onset of Experimental Autoimmune Encephalomyelitis, and can be Induced by Adoptive Transfer of Auto-Reactive T Cells. *PLoS One* (2014) 9:e106335. doi: 10.1371/journal.pone.0106335
92. Li K, Wei S, Hu L, Yin X, Mai Y, Jiang C, et al. Protection of Fecal Microbiota Transplantation in a Mouse Model of Multiple Sclerosis. *Mediators Inflamm* (2020) 2020:2058272. doi: 10.1155/2020/2058272
93. Borody T, Leis S, Campbell J, Torres M, Nowak A. Fecal Microbiota Transplantation (FMT) in Multiple Sclerosis (Ms). *Am J Gastroenterol* (2011) 106:S352. doi: 10.14309/00000434-201110002-00942
94. Makkawi S, Camara-Lemarroy C, Metz L. Fecal Microbiota Transplantation Associated With 10 Years of Stability in a Patient With SPMS. *Neurol Neuroimmunol NeuroInflamm* (2018) 5:e459. doi: 10.1212/NXI.0000000000000459

95. Engen PA, Zaferiou A, Rasmussen H, Naqib A, Green SJ, Fogg LF, et al. Single-Arm, Non-randomized, Time Series, Single-Subject Study of Fecal Microbiota Transplantation in Multiple Sclerosis. *Front Neurol* (2020) 11:978. doi: 10.3389/fneur.2020.00978
96. Mangalam A, Shahi SK, Luckey D, Karau M, Marietta E, Luo N, et al. Human Gut-Derived Commensal Bacteria Suppress CNS Inflammatory and Demyelinating Disease. *Cell Rep* (2017) 20:1269–77. doi: 10.1016/j.celrep.2017.07.031
97. Montgomery TL, Künstner A, Kennedy JJ, Fang Q, Asarian L, Culp-Hill R, et al. Interactions Between Host Genetics and Gut Microbiota Determine Susceptibility to CNS Autoimmunity. *Proc Natl Acad Sci U S A* (2020) 117:27516–27. doi: 10.1073/pnas.2002817117
98. Lavasani S, Dzhambazov B, Nouri M, Fåk F, Buske S, Molin G, et al. A Novel Probiotic Mixture Exerts a Therapeutic Effect on Experimental Autoimmune Encephalomyelitis Mediated by IL-10 Producing Regulatory T Cells. *PLoS One* (2010) 5:e9009. doi: 10.1371/journal.pone.0009009
99. Secher T, Kassem S, Benamar M, Bernard I, Boury M, Barreau F, et al. Oral Administration of the Probiotic Strain Escherichia Coli Nissle 1917 Reduces Susceptibility to Neuroinflammation and Repairs Experimental Autoimmune Encephalomyelitis-Induced Intestinal Barrier Dysfunction. *Front Immunol* (2017) 8:1096. doi: 10.3389/fimmu.2017.01096
100. He B, Hoang TK, Tian X, Taylor CM, Blanchard E, Luo M, et al. Lactobacillus Reuteri Reduces the Severity of Experimental Autoimmune Encephalomyelitis in Mice by Modulating Gut Microbiota. *Front Immunol* (2019) 10:385. doi: 10.3389/fimmu.2019.00385
101. Shahi SK, Freedman SN, Murra AC, Zarei K, Sompallae R, Gibson-Corley KN, et al. Prevotella Histicola, A Human Gut Commensal, Is as Potent as COPAXONE® in an Animal Model of Multiple Sclerosis. *Front Immunol* (2019) 10:462. doi: 10.3389/fimmu.2019.00462
102. Salehipour Z, Haghmorad D, Sankian M, Rastin M, Nosratabadi R, Soltan Dallal MM, et al. Bifidobacterium Animalis in Combination With Human Origin of Lactobacillus Plantarum Ameliorate Neuroinflammation in Experimental Model of Multiple Sclerosis by Altering CD4+ T Cell Subset Balance. *Biomed Pharmacother* (2017) 95:1535–48. doi: 10.1016/j.biopha.2017.08.117
103. Abdurasulova IN, Ermolenko EI, Matsulevich AV, Abdurasulova KO, Tarasova EA, Kudryavtsev IV, et al. Effects of Probiotic Enterococci and Glatiramer Acetate on the Severity of Experimental Allergic Encephalomyelitis in Rats. *Neurosci Behav Phys* (2017) 47:866–76. doi: 10.1007/s11055-017-0484-1
104. Takata K, Kinoshita M, Okuno T, Moriya M, Kohda T, Honorat JA, et al. The Lactic Acid Bacterium Pediococcus Acidilactici Suppresses Autoimmune Encephalomyelitis by Inducing IL-10-producing Regulatory T Cells. *PLoS One* (2011) 6:e27644. doi: 10.1371/journal.pone.0027644
105. Calvo-Barreiro L, Eixarch H, Ponce-Alonso M, Castillo M, Lebrón-Galán R, Mestre L, et al. A Commercial Probiotic Induces Tolerogenic and Reduces Pathogenic Responses in Experimental Autoimmune Encephalomyelitis. *Cells* (2020) 9:906. doi: 10.3390/cells9040906
106. Mestre L, Carrillo-Salinas FJ, Feliú A, Mecha M, Alonso G, Espejo C, et al. How Oral Probiotics Affect the Severity of an Experimental Model of Progressive Multiple Sclerosis? Bringing Commensal Bacteria Into the Neurodegenerative Process. *Gut Microbes* (2020) 12:1813532. doi: 10.1080/19490976.2020.1813532
107. Maassen CBM, Claassen E. Strain-Dependent Effects of Probiotic Lactobacilli on EAE Autoimmunity. *Vaccine* (2008) 26:2056–7. doi: 10.1016/j.vaccine.2008.02.035
108. Kwon H-K, Kim G-C, Kim Y, Hwang W, Jash A, Sahoo A, et al. Amelioration of Experimental Autoimmune Encephalomyelitis by Probiotic Mixture is Mediated by a Shift in T Helper Cell Immune Response. *Clin Immunol* (2013) 146:217–27. doi: 10.1016/j.clim.2013.01.001
109. Tankou SK, Regev K, Healy BC, Cox LM, Tjon E, Kivisakk P, et al. Investigation of Probiotics in Multiple Sclerosis. *Mult Scler* (2018) 24:58–63. doi: 10.1177/1352458517737390
110. Kouchaki E, Tamtaji OR, Salami M, Bahmani F, Daneshvar Kakhaki R, Akbari E, et al. Clinical and Metabolic Response to Probiotic Supplementation in Patients With Multiple Sclerosis: A Randomized, Double-Blind, Placebo-Controlled Trial. *Clin Nutr* (2017) 36:1245–9. doi: 10.1016/j.clnu.2016.08.015
111. Morshedi M, Hashemi R, Moazzen S, Sahebkar A, Hosseinifard E-S. Immunomodulatory and Anti-Inflammatory Effects of Probiotics in Multiple Sclerosis: A Systematic Review. *J Neuroinflamm* (2019) 16:231. doi: 10.1186/s12974-019-1611-4
112. Comstock LE. Importance of Glycans to the Host-Bacteroides Mutualism in the Mammalian Intestine. *Cell Host Microbe* (2009) 5:522–6. doi: 10.1016/j.chom.2009.05.010
113. Troy EB, Carey VJ, Kasper DL, Comstock LE. Orientations of the Bacteroides Fragilis Capsular Polysaccharide Biosynthesis Locus Promoters During Symbiosis and Infection. *J Bacteriol* (2010) 192:5832–6. doi: 10.1128/JB.00555-10
114. Zitomersky NL, Coyne MJ, Comstock LE. Longitudinal Analysis of the Prevalence, Maintenance, and IgA Response to Species of the Order Bacteroidales in the Human Gut. *Infect Immun* (2011) 79:2012–20. doi: 10.1128/IAI.01348-10
115. Wexler AG, Goodman AL. An Insider's Perspective: Bacteroides as a Window Into the Microbiome. *Nat Microbiol* (2017) 2:17026. doi: 10.1038/nmicrobiol.2017.26
116. Polk F, Kasper D. Bacteroides Fragilis Subspecies in Clinical Isolates. *Ann Intern Med* (1977) 86:569–671. doi: 10.7326/0003-4819-86-5-569
117. An D, Oh SF, Olszak T, Neves JF, Avci FY, Erturk-Hasdemir D, et al. Sphingolipids From a Symbiotic Microbe Regulate Homeostasis of Host Intestinal Natural Killer T Cells. *Cell* (2014) 156:123–33. doi: 10.1016/j.cell.2013.11.042
118. Ennamorati M, Vasudevan C, Clerkin K, Halvorsen S, Verma S, Ibrahim S, et al. Intestinal Microbes Influence Development of Thymic Lymphocytes in Early Life. *Proc Natl Acad Sci U S A* (2020) 117:2570–8. doi: 10.1073/pnas.1915047117
119. Mazmanian SK, Round JL, Kasper DL. A Microbial Symbiosis Factor Prevents Intestinal Inflammatory Disease. *Nature* (2008) 453:620–5. doi: 10.1038/nature07008
120. Macfarlane GT, Hopkins MJ. Changes in Predominant Bacterial Populations in Human Faeces With Age and With Clostridium Difficile Infection. *J Med Microbiol* (2002) 51:448–54. doi: 10.1099/0022-1317-51-5-448
121. Pagliuca C, Cicatiello AG, Colicchio R, Greco A, Cerciello R, Auletta L, et al. Novel Approach for Evaluation of Bacteroides Fragilis Protective Role Against Bartonella Henselae Liver Damage in Immunocompromised Murine Model. *Front Microbiol* (2016) 7:1750. doi: 10.3389/fmicb.2016.01750
122. Li Z, Deng H, Zhou Y, Tan Y, Wang X, Han Y, et al. Bioluminescence Imaging to Track Bacteroides Fragilis Inhibition of Vibrio Parahaemolyticus Infection in Mice. *Front Cell Infect Microbiol* (2017) 7:170. doi: 10.3389/fcimb.2017.00170
123. Nagpal R, Tsuji H, Takahashi T, Nomoto K, Kawashima K, Nagata S, et al. Gut Dysbiosis Following C-section Instigates Higher Colonisation of Toxigenic *Clostridium Perfringens* in Infants. *Benef Microbes* (2017) 8:353–65. doi: 10.3920/BM2016.0216
124. Deng H, Yang S, Zhang Y, Qian K, Zhang Z, Liu Y, et al. Bacteroides Fragilis Prevents Clostridium Difficile Infection in a Mouse Model by Restoring Gut Barrier and Microbiome Regulation. *Front Microbiol* (2018) 9:2976. doi: 10.3389/fmicb.2018.02976
125. Li D, Pan Y, Xia X, Liang J, Liu F, Dou H, et al. Bacteroides Fragilis Alleviates the Symptoms of Lupus Nephritis Via Regulating CD1d and CD86 Expressions in B Cells. *Eur J Pharmacol* (2020) 884:173421. doi: 10.1016/j.ejphar.2020.173421
126. Ramakrishna C, Kujawski M, Chu H, Li L, Mazmanian SK, Cantin EM. Bacteroides Fragilis Polysaccharide A Induces IL-10 Secreting B and T Cells That Prevent Viral Encephalitis. *Nat Commun* (2019) 10:2153. doi: 10.1038/s41467-019-09884-6
127. Ochoa-Repáraz J, Mielcarz DW, Ditrio LE, Burroughs AR, Begum-Haque S, Dasgupta S, et al. Central Nervous System Demyelinating Disease Protection by the Human Commensal Bacteroides Fragilis Depends on Polysaccharide A Expression. *J Immunol* (2010) 185:4101–8. doi: 10.4049/jimmunol.1001443
128. Comstock LE, Kasper DL. Bacterial Glycans: Key Mediators of Diverse Host Immune Responses. *Cell* (2006) 126:847–50. doi: 10.1016/j.cell.2006.08.021
129. Cerdeno-Tarraga AM, Patrick S, Crossman LC, Blakely G, Abratt V, Lennard N, et al. Extensive DNA Inversions in the B. Fragilis Genome Control Variable Gene Expression. *Science* (2005) 307:1463–5. doi: 10.1126/science.1107008
130. Cao Y, Rocha ER, Smith CJ. Efficient Utilization of Complex N-linked Glycans is a Selective Advantage for Bacteroides Fragilis in Extraintestinal Infections. *Proc Natl Acad Sci U S A* (2014) 111:12901–6. doi: 10.1073/pnas.1407344111
131. Cuskin F, Lowe EC, Temple MJ, Zhu Y, Cameron EA, Pudlo NA, et al. Human Gut Bacteroidetes can Utilize Yeast Mannan Through a Selfish Mechanism. *Nature* (2015) 517:165–9. doi: 10.1038/nature13995

132. Pudlo NA, Urs K, Kumar SS, German JB, Mills DA, Martens EC. Symbiotic Human Gut Bacteria With Variable Metabolic Priorities for Host Mucosal Glycans. *mBio* (2015) 6:e01282–15. doi: 10.1128/mBio.01282-15
133. Pantosti A, Tzianabos A, Reinap BG, Onderdonk AB, Kasper DL. Bacteroides Fragilis Strains Express Multiple Capsular Polysaccharides. *J Clin Microbiol* (1993) 31:1850–5. doi: 10.1128/JCM.31.7.1850-1855.1993
134. Krinos CM, Coyne MJ, Weinacht KG, Tzianabos AO, Kasper DL, Comstock LE. Extensive Surface Diversity of a Commensal Microorganism by Multiple DNA Inversions. *Nature* (2001) 414:555–8. doi: 10.1038/35107092
135. Liu CH, Lee SM, Vanlare JM, Kasper DL, Mazmanian SK. Regulation of Surface Architecture by Symbiotic Bacteria Mediates Host Colonization. *Proc Natl Acad Sci U S A* (2008) 105:3951–6. doi: 10.1073/pnas.0709266105
136. Coyne MJ, Chatzidaki-Livanis M, Paoletti LC, Comstock LE. Role of Glycan Synthesis in Colonization of the Mammalian Gut by the Bacterial Symbiont Bacteroides Fragilis. *Proc Natl Acad Sci U S A* (2008) 105:13099–104. doi: 10.1073/pnas.0804220105
137. Blandford LE, Johnston EL, Sanderson JD, Wade WG, Lax AJ. Promoter Orientation of the Immunomodulatory Bacteroides Fragilis Capsular Polysaccharide A (PSA) is Off in Individuals With Inflammatory Bowel Disease (IBD). *Gut Microbes* (2019) 10:569–77. doi: 10.1080/19490976.2018.1560755
138. Surana NK, Kasper DL. The Yin Yang of Bacterial Polysaccharides: Lessons Learned From B. Fragilis PSA. *Immunol Rev* (2012) 245:13–26. doi: 10.1111/j.1600-065X.2011.01075.x
139. Baumann H, Tzianabos a O, Brisson JR, Kasper DL, Jennings HJ. Structural Elucidation of Two Capsular Polysaccharides From One Strain of Bacteroides Fragilis Using High-Resolution NMR Spectroscopy. *Biochemistry* (1992) 31:4081–9. doi: 10.1021/bi00131a026
140. Sharma S, Erickson KM, Troutman JM. Complete Tetrasaccharide Repeat Unit Biosynthesis of the Immunomodulatory *Bacteroides Fragilis* Capsular Polysaccharide a. *ACS Chem Biol* (2017) 12:92–101. doi: 10.1021/acschembio.6b00931
141. Tzianabos AO, Onderdonk AB, Rosner B, Cisneros RL, Kasper DL. Structural Features of Polysaccharides That Induce Intra-Abdominal Abscesses. *Science* (1993) 262:416–9. doi: 10.1126/science.8211161
142. Tzianabos AO, Kasper DL, Cisneros RL, Smith RS, Onderdonk AB. Polysaccharide-Mediated Protection Against Abscess Formation in Experimental Intra-Abdominal Sepsis. *J Clin Invest* (1995) 96:2727–31. doi: 10.1172/JCI118340
143. Cobb BA, Wang Q, Tzianabos AO, Kasper DL. Polysaccharide Processing and Presentation by the MHCII Pathway. *Cell* (2004) 117:677–87. doi: 10.016/j.cell.2004.05.001
144. Cobb BA, Kasper DL. Zwitterionic Capsular Polysaccharides: The New MHCII-dependent Antigens. *Cell Microbiol* (2005) 7:1398–403. doi: 10.1111/j.1462-5822.2005.00591.x
145. Duan J, Avci FY, Kasper DL. Microbial Carbohydrate Depolymerization by Antigen-Presenting Cells: Deamination Prior to Presentation by the MHCII Pathway. *Proc Natl Acad Sci U S A* (2008) 105:5183–8. doi: 10.1073/pnas.0800974105
146. Duan J, Kasper DL. Oxidative Depolymerization of Polysaccharides by Reactive Oxygen/Nitrogen Species. *Glycobiology* (2011) 21:401–9. doi: 10.1093/glycob/cwq171
147. Kalka-Moll WM, Tzianabos AO, Bryant PW, Niemeyer M, Ploegh HL, Kasper DL. Zwitterionic Polysaccharides Stimulate T Cells by MHC Class II-dependent Interactions. *J Immunol* (2002) 169:6149–53. doi: 10.4049/jimmunol.169.11.6149
148. Chung H, Pamp SJ, Hill JA, Surana NK, Edelman SM, Troy EB, et al. Gut Immune Maturation Depends on Colonization With a Host-Specific Microbiota. *Cell* (2012) 149:1578–93. doi: 10.1016/j.cell.2012.04.037
149. Fiebiger U, Bereswill S, Heimesaat MM. Dissecting the Interplay Between Intestinal Microbiota and Host Immunity in Health and Disease: Lessons Learned From Germfree and Gnotobiotic Animal Models. *Eur J Microbiol Immunol* (2016) 6:253–71. doi: 10.1556/1886.2016.00036
150. Round JL, Lee SM, Li J, Tran G, Jabri B, Chatila T a. Mazmanian SK. The Toll-like Receptor 2 Pathway Establishes Colonization by a Commensal of the Human Microbiota. *Science* (2011) 332:974–7. doi: 10.1126/science.1206095
151. Shen Y, Torchia MLG, Lawson GW, Karp CL, Ashwell JD, Mazmanian SK. Outer Membrane Vesicles of a Human Commensal Mediate Immune Regulation and Disease Protection. *Cell Host Microbe* (2012) 12:509–20. doi: 10.1016/j.chom.2012.08.004
152. Wang Q, McLoughlin RM, Cobb BA, Charrel-Dennis M, Zaleski KJ, Golenbock D, et al. A Bacterial Carbohydrate Links Innate and Adaptive Responses Through Toll-like Receptor 2. *J Exp Med* (2006) 203:2853–63. doi: 10.1084/jem.20062008
153. Stefan KL, Kim MV, Iwasaki A, Kasper DL. Commensal Microbiota Modulation of Natural Resistance to Virus Infection. *Cell* (2020) 183:1312–24.e10. doi: 10.1016/j.cell.2020.10.047
154. Fletcher JM, Lonergan R, Costelloe L, Kinsella K, Moran B, O'Farrelly C, et al. Cd39 + Foxp3 + Regulatory T Cells Suppress Pathogenic Th17 Cells and Are Impaired in Multiple Sclerosis. *J Immunol* (2009) 183:7602–10. doi: 10.4049/jimmunol.0901881
155. Borsellino G, Kleinewietfeld M, Di Mitri D, Sternjak A, Diamantini A, Giometto R, et al. Expression of Ectonucleotidase CD39 by Foxp3+ Treg Cells: Hydrolysis of Extracellular ATP and Immune Suppression. *Blood* (2007) 110:1225–32. doi: 10.1182/blood-2006-12-064527
156. Wang Y, Begum-Haque S, Telesford KM, Ochoa-Repáraz J, Christy M, Kasper EJ, et al. A Commensal Bacterial Product Elicits and Modulates Migratory Capacity of CD39+ Cd4 T Regulatory Subsets in the Suppression of Neuroinflammation. *Gut Microbes* (2014) 5:552–61. doi: 10.4161/gmic.29797
157. Telesford KM, Yan W, Ochoa-Reparaz J, Pant A, Kircher C, Christy MA, et al. A Commensal Symbiotic Factor Derived From Bacteroides Fragilis Promotes Human CD39+Foxp3+ T Cells and Treg Function. *Gut Microbes* (2015) 6:234–42. doi: 10.1080/19490976.2015.1056973
158. Burgess JN, Pant AB, Kasper LH, Colpitts Brass S. Cd4+ T Cells From Multiple Sclerosis Patients Respond to a Commensal-Derived Antigen. *Ann Clin Transl Neurol* (2017) 4:825–9. doi: 10.1002/acn3.465
159. Alvarez CA, Jones MB, Hambor J, Cobb BA. Characterization of Polysaccharide A Response Reveals Interferon Responsive Gene Signature and Immunomodulatory Marker Expression. *Front Immunol* (2020) 11:556813. doi: 10.3389/fimmu.2020.556813
160. Ruff WE, Greiling TM, Kriegel MA. Host–Microbiota Interactions in Immune-Mediated Diseases. *Nat Rev Microbiol* (2020) 18:521–38. doi: 10.1038/s41579-020-0367-2

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

frontiers in Immunology

REVIEW
published: 25 May 2021
doi: 10.3389/fimmu.2021.676255

Microbiota and Microglia Interactions in ASD

*Marcela Davoli-Ferreira, Carolyn A. Thomson and Kathy D. McCoy**

Department of Physiology and Pharmacology, Snyder Institute of Chronic Diseases, Cumming School of Medicine, University of Calgary, Calgary, AB, Canada

Autism spectrum disorders (ASD) are serious, highly variable neurodevelopmental disorders, commonly characterized by the manifestation of specific behavioral abnormalities, such as stereotypic behaviors and deficits in social skills, including communication. Although the neurobiological basis for ASD has attracted attention in recent decades, the role of microglial cells, which are the main resident myeloid cell population in the brain, is still controversial and underexplored. Microglia play several fundamental roles in orchestrating brain development and homeostasis. As such, alterations in the intrinsic functions of these cells could be one of the driving forces responsible for the development of various neurodevelopmental disorders, including ASD. Microglia are highly sensitive to environmental cues. Amongst the environmental factors known to influence their intrinsic functions, the gut microbiota has emerged as a central player, controlling both microglial maturation and activation. Strikingly, there is now compelling data suggesting that the intestinal microbiota can play a causative role in driving the behavioural changes associated with ASD. Not only is intestinal dysbiosis commonly reported in ASD patients, but therapies targeting the microbiome can markedly alleviate behavioral symptoms. Here we explore the emerging mechanisms by which altered microglial functions could contribute to several major etiological factors of ASD. We then demonstrate how pre- and postnatal environmental stimuli can modulate microglial cell phenotype and function, underpinning the notion that reciprocal interactions between microglia and intestinal microbes could play a crucial role in ASD aetiology.

Keywords: neurodevelopmental disorders, inflammation, dysbiosis, microbial metabolites, autism spectrum disorder (ASD), microglia, microbiome

OPEN ACCESS

Edited by:
Lloyd Kasper,
Dartmouth College, United States

Reviewed by:
Enrico Castroflorio,
The Institute of Photonic Sciences (ICFO), Spain
Manish Malviya,
Memorial Sloan Kettering Cancer Center, United States

***Correspondence:**
Kathy D. McCoy
kathy.mccoy@ucalgary.ca

Specialty section:
This article was submitted to Multiple Sclerosis and Neuroimmunology, a section of the journal Frontiers in Immunology

Received: *04 March 2021*
Accepted: *12 May 2021*
Published: *25 May 2021*

Citation:
Davoli-Ferreira M, Thomson CA and McCoy KD (2021) Microbiota and Microglia Interactions in ASD. Front. Immunol. 12:676255. doi: 10.3389/fimmu.2021.676255

BACKGROUND

Autism spectrum disorders (ASD) include a range of neurodevelopmental disorders, commonly characterized by repetitive behaviours, as well as impaired social skills, including verbal and nonverbal communication (1). These behavioral symptoms develop in early childhood and persist throughout life. In recent decades, there has been a major surge in ASD incidence globally (2). Although the precise aetiologies of ASD are complex, and remain to be fully understood, recent evidence points to abnormal synaptic development and function, and/or aberrant immune

responses, as potential drivers of ASD symptoms (3–6). Notably, microglial cells participate in these physiological processes and have been strongly associated with ASD development (7–10).

Microglia are the main resident immune cells of the central nervous system (CNS), providing the tissue with innate immune sensing, inflammatory effector functions and tissue repair. As such, they are the main producers of proinflammatory mediators in the context of neuroinflammation (11). Although immunomodulatory roles for microglia in neuroinflammatory and neurodegenerative diseases have been widely described, immune modulation is only one of an extensive array of discrete microglial functions. During CNS development, microglia regulate the number and strategic positioning of neurons and shape neuronal connectivity (10, 12). Moreover, they support gliogenesis and myelination (10, 12–15). Given both their immune and developmental functions, it would be attractive to propose that microglial dysfunction could contribute to neurodevelopmental disorders; either by influencing disease development or driving behavioral symptoms. However, the specific roles that microglial cells play in ASD pathophysiology are still controversial. Although several studies show that autistic individuals suffer from ongoing neuroinflammatory processes, characterized by microglial activation in several discrete regions of the brain (16–19), others dispute the significance of this and suggest that microglia may be intrinsically dysfunctional in their resting state, following a prenatal disruption to homeostatic brain development (20, 21). In this review, we explore both well-established and emerging literature and discuss perspectives on the role's microglia may play in the development of ASD; both in the context of abnormal immune signaling and altered neuronal connectivity. Given the vast array of peripheral factors that can modulate microglial maturation and function, we further discuss how perturbations in these extrinsic signals, particularly the gut microbiota, might promote microglial dysfunction in the context of the neurodevelopmental disorders.

MICROGLIA: ORIGIN AND PHYSIOLOGICAL FUNCTIONS IN THE BRAIN

Microglia are a highly specialised population of myeloid cells that inhabit the healthy CNS parenchyma, representing 5–12% of all cells in the CNS (22). Unlike the other cell types that coinhabit the CNS, microglia are not derived from the neuroectodermal germ layer. Rather, microglial ontogeny has been traced to erythromyeloid precursors, which differentiate into microglial progenitors in the yolk sac during embryogenesis (23, 24). At this stage, differentiation is critically controlled by the transcription factors Pu.1 and Irf8 with other transcription factors, such Runx1 and Jun, also providing a supporting role (21, 23, 24). On day 9.5 after conception (E9.5), microglial progenitors leave the yolk sac to seed the developing CNS in one single wave (22–24). Following an initial burst of proliferation and differentiation, mature microglia then colonize the parenchyma where they persist throughout the life of the host (**Figure 1**). There, within the healthy CNS, microglial numbers are maintained by gradual self-renewal, independently from the recruitment of any other hematopoietic myeloid cells or progenitors (11, 24, 25).

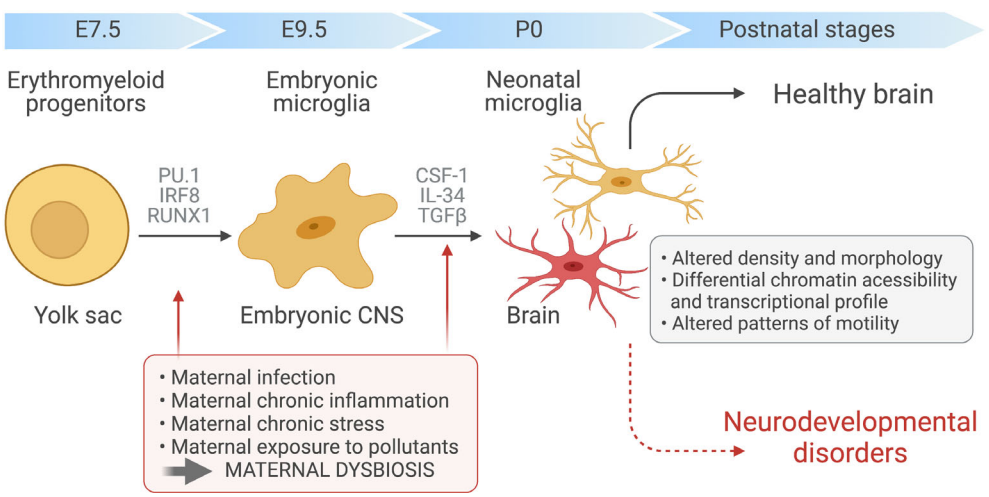


FIGURE 1 | Maternal immune activation and dysbiosis in microglial development. Yolk sac-derived erythroid progenitors differentiate into microglia progenitors, *via* Runx1, PU.1-and IRF8-dependent pathways, that then migrate and colonize the developing brain at around embryonic day 9.5. After microglial seeding of the embryonic CNS parenchyma and subsequent proliferation during prenatal and postnatal stages, factors such as CSF-1, IL-34 and TGF-β promote microglia terminal differentiation. Maternal chronic inflammatory diseases, maternal infection and exposure to environmental factors, such as pesticides and pollution, can induce immune activation during pregnancy and dramatic changes in maternal microbiota. These alterations can disrupt the normal prenatal microglia development, maturation and induce microglial epigenetics alterations, affecting the developing fetal brain and leading to ASD development.

Within the CNS parenchyma, microglia are imprinted by local environmental cues. Microglial differentiation and maintenance are strongly dependent on their expression of colony-stimulating factor 1 receptor (CSF1R), as well as the two main CSF1R ligands, CSF1 and IL-34 (**Figure 1**). Depleting either of these ligands reduces microglial cell abundance throughout the CNS. Moreover, the CNS of adult CSF1R-deficient mice are virtually devoid of all microglia (26, 27). By driving a microglia-specific gene signature, TGFβ signaling has recently also been shown to be indispensable for microglia maturation. The marker genes induced by TGFβ, which include *Tmem119, Sall1, Tgfbr1*, and *P2ry12*, can readily distinguish microglia from bone marrow-derived macrophages (28).

As tissue-resident macrophages, microglial cells are responsible for the continuous immunosurveillance of the CNS. Inflammatory insults induced by invading pathogens or local injuries trigger their production of immune mediators (29–31). These pathways also facilitate increased phagocytosis of cellular debris and/or pathogens (32). Previously, microglia were thought to be inactive during homeostasis and only activated in response to pathological insults. However, in addition to their "canonical" innate immune functions, recent findings suggest that microglia are intimately involved in CNS development through organising neuronal patterning and fine-tuning synaptic connections (13, 22).

During embryogenesis, microglia are the first glial cells to populate the developing CNS. In this early neurodevelopmental phase, they control neurogenesis by releasing neurotoxic or neurotrophic factors that orchestrate the survival, differentiation or apoptosis of neuronal progenitors (33–35). The survival-enhancing role of microglia is supported by findings showing that proliferation and survival of these progenitors is higher when they are co-cultured with microglia than when cultured alone (34). On the other hand, microglial respiratory bursts generate superoxide ions, which trigger the apoptosis of Purkinje cells in the postnatal cerebellum (33). Thus, through the selective release of neurotoxic or neurotrophic factors, microglia can shape the neuronal landscape.

In addition to modulating neurogenesis, microglia play important roles in the development and differentiation of neuronal circuits. From an early stage in postnatal neurodevelopment, microglia eliminate redundant neurons that do not establish functional circuits. Moreover, microglia modulate immature neuronal circuits by engulfing and eliminating dendritic spines at the synapse (10). This process, known as synaptic pruning, is critically important for the normal formation of synapses. Its disruption results in several neuronal abnormalities; including impaired functional connectivity, modifications to dopaminergic circuits, and an imbalance of the excitation-to-inhibition ratio in the cortex (9, 10, 36). Importantly, abnormal synaptic pruning in the CNS of the neonate, or even the developing fetus, could be important in the aetiology of ASD, as discussed later.

Finally, there is now cumulating evidence that microglial cells modulate synaptic plasticity, and subsequently, learning and memory (37–39). This is not only important during early developmental stages as depleting microglia from the CNS of adult mice also results in impaired synaptic plasticity and deficits in learning and memory (13, 40–42). Similar phenotypes are observed when microglia are unable to produce brain-derived neurotrophic factor (BDNF), as shown using conditional and inducible BDNF depletion under the CX_3CR1 promotor (42)

Thus, although microglia have several well-defined roles in neuroinflammation, it is becoming increasingly evident that they also shape neuronal survival and connectivity during development, interpret changes in the local milieu and modulate circuit formation accordingly (11, 43).

MICROGLIA IN ASD

To date, microglial cell participation in ASD and other neurodevelopmental disorders has been only speculated. While the causes of ASD are incompletely understood, some of the main symptoms, such as impairment in multisensory processing and integration, have been linked to defects in neurogenesis and the strategic positioning of neurons during CNS development, abnormal synaptic pruning and an altered neuronal excitation/inhibition ratio (44). Additionally, systemic and central inflammation may also be intrinsically involved in the pathogenesis of ASD and several other neurological disorders (45, 46). Considering both the physiological roles microglia play in regulating neurogenesis, neuronal migration and synaptic pruning, and their immunomodulatory roles in the CNS, it seems entirely plausible that aberrant microglial function may be a driving force in the pathogenesis of ASD.

Vargas et al. were the first to show an inflammatory phenotype in post-mortem brains from ASD individuals. In this pioneer work, neuropathologic analysis showed increased microglial activation, characterized by elevated expression of MHC class II, throughout the cerebral and cerebellar cortices in individuals with ASD. Moreover, increased expression of pro- and anti-inflammatory factors, such as such as CCL2, IL-6 and TGF-β, were observed in both the brain and cerebrospinal fluid (CSF) (16). Similar studies have reported increased expression of TNF-α, IL-6, IL-8, GM-CSF, and more recently, IL-18 and IL-37, in post-mortem brain tissue and CSF of children with ASD, suggesting a heightened immune response with associated localized brain inflammation (47–49). Consistent with this apparent microglial and astrocyte immune dysregulation, genome-wide analysis of brain tissue from ASD individuals showed enrichment of markers related to activated microglia and expression of genes associated with "immune and inflammatory" gene ontology categories, compared to neurotypical controls (50, 51). These changes in microglial activation markers in ASD brains were also accompanied by changes in microglial morphology, density and spatial localization (18, 52, 53). Not only do microglia have an increased density throughout the cerebral and cerebellar cortices of ASD patients, but they exhibit cell body enlargement, as well as process retraction and thickening. Filopodia also extend from the processes of ASD-associated microglia (18, 54). The putative microglial dysfunction detected in post-mortem samples has now been further

confirmed using *In Vivo* Positron Emission Tomography (PET). In this study, which focused on young adults with ASD, a radiotracer specific for activated microglia and astrocytes was used to show a marked activation of these cells in several discrete regions of the brain (17).

The combination of neuropathological analyses of post-mortem human brain samples and PET scanning of live human ASD patients has provided compelling evidence to suggest that aberrant microglia and astrocyte immune activation is a common hallmark of ASD. However, due to the small number of samples evaluated, variations in genetic backgrounds, lifestyle choices, medication use and socioeconomic status, more studies are required. In the case of post-mortem studies, the cause of death could also impact brain inflammation. Moreover, it remains to be established whether microglial activation is a secondary effect of aberrant brain development or whether microglia play a causative role in the initiation or manifestation of ASD. For that reason, environmental and genetic rodent models are widely employed to explore the range of contributions microglia make to ASD pathogenesis, including their effects on neuronal migration, neurotransmission, brain anatomy and inflammation.

In rodents, genetic manipulation of microglia can profoundly alter CNS function, culminating in behavioral abnormalities resembling those found in ASD. For example, mice lacking the gene encoding CX_3CR1 exhibit ASD-like behaviours, including social deficits (9, 10). Also known as the fractalkine receptor, CX_3CR1 is a chemokine receptor that facilitates direct contact between microglial cells and CX_3CL1 (fractalkine)-expressing neurons; an interaction known to suppress microglial cell activation and IL-1β production following peripheral immune stimulation (55). Signaling through the fractalkine/CX_3CR1 axis is required for the optimal recruitment of microglia to specific CNS locations during embryogenesis (56). As such, *Cx3cr1*-deficient mice have fewer microglia present in the CNS during early postnatal development, resulting in altered synaptic pruning and subsequent deficits in neuronal connectivity throughout life (9, 10). Similar to CX_3CR1, microglial expression of immunoglobulin superfamily-member, triggering receptor expressed on myeloid cells 2 (TREM2), is fundamental for synaptic pruning during prenatal neurodevelopment (57). TREM2 signalling transduction has a central role in promoting microglial activation (11) and variants in *TREM2* have been linked to different types of neurological diseases, including multiple sclerosis, Parkinson's and Alzheimer's diseases (58–62). Recent studies in mice show that the absence of this receptor results in defective remodelling of neuronal synapses, dysregulated excitatory/inhibitory neurotransmission, impaired neuronal connectivity and behavioral defects reminiscent of ASD (57). The expression of TREM2 was also significantly reduced in post-mortem brain tissue from individuals with ASD compared to neurotypical controls. This ASD-associated reduction in TREM2 expression was most prominent in samples collected from patients with severe symptoms, showing a negative correlation between TREM2 levels and ASD severity (57).

Recent studies have also shown that elevating protein synthesis, induced exclusively in microglia *via* overexpression of the translation initiation factor eIF4E, is sufficient to impair synaptic formation and drive the manifestation of ASD-like behaviors in young mice (63). Indeed, mutations that inactivate negative regulators of translation, such as in PTEN (phosphatase and tensin homolog), TSC1/2 (tuberous sclerosis complex 1/2), and FMR1 (fragile X mental retardation protein), are thought to cause ASD in a proportion of patients (64–68). Xu and collaborators suggested that defects in these ubiquitously expressed genes can alter microglial cell function sufficiently to drive ASD. In addition to an increased phagocytic potential, these microglia exhibit reduced mobility and impaired synaptic pruning, culminating in higher synapse density and higher excitatory neurotransmission compared to wild type mice, ultimately driving the development of ASD-like behaviors (63). Similar to the phenotype observed in mice that overexpressed eIF4E, the frequency, phenotype and function of microglia in the prefrontal cortex, hippocampus and striatum of *Pten*-deficient mice was substantially altered when compared with their wildtype littermates (63, 69, 70). Collectively, these studies add further weight to the hypothesis that aberrant microglial cell functions may help to drive the pathophysiology and behavioural symptoms associated with ASD.

Other models of autism in which risk genes are depleted in rodents to model symptomatic ASD variants, such as Rett syndrome and fragile X syndrome, are also related to microglial-dependent synaptic modulation. In *Fmr1*-deficient mice, a model of fragile X syndrome, microglia are increased in terms of size and abundance compared to those from wild-type littermates, and these physiological changes were associated with reduced microglial-mediated synaptic pruning (63, 71). As it is caused by loss-of-function mutations in the gene encoding methyl-CpG binding protein 2 (MECP2), Rett syndrome is modeled using variations of *Mecp2*-deficient mice. The specific deletion of *Mecp2* in murine microglial cells triggers an overproduction of glutamate, altering neuronal morphology and impeding the formation of synapses (72). Abnormal microglia–synapse interactions, and increased expression of inflammatory genes in macrophages and microglia, were also observed in mice lacking *Mecp2* (13, 73). These studies imply a pathological role for microglial cell dysfunction in ASD, apparently without the context of neuroinflammation. However, since both resting and activated microglia are able to secrete cytokines, neurotoxic and neurotrophic factors, as well as other soluble factors that have been implicated in ASD, it is possible that microglia use these mediators to influence a diverse range of neuronal functions and sculpt synaptic connections (19, 20, 74).

In addition to genetic factors, environmental factors also modify microglia function, affecting brain development, synaptic connectivity and CNS immune responses (75). Indeed, the behavioural abnormalities that are observed in mouse models in which environmental risk factors are the driving forces behind ASD development, such as the maternal immune activation (MIA) model, are similar to those induced by

genetic modification (75, 76). The MIA model, in which pregnant mice are challenged with polyinosinic-polycytidylic acid [Poly(I:C)] or lipopolysaccharide (LPS) during embryonic development (E9–12), was developed based on numerous epidemiological studies that have linked prenatal infection in humans to the development of several neurological disorders, including ASD, in the offspring (13, 77).

MIA remotely triggers the induction of multiple cytokines in the fetal brain of rodents, subsequently leading to abnormal neurodevelopment. Due to their rapid ability to respond to inflammatory signals and their role in modulating neuronal function and connectivity, microglial cells have been implicated in driving this disorder (78). However, contradicting results cloud the ability to draw clear conclusions on how and when MIA shapes microglial functions in the developing offspring. While some studies showed increased expression of microglial activation markers in adult offspring exposed to MIA *in utero*, others did not uncover any postnatal differences in microglial phenotype as a consequence of MIA. The most consistent microglial changes were found during pre- or perinatal developmental stages, suggesting that transient perturbations in microglial function might have life-long effects on neuronal patterning, functional connectivity and behaviour (79–82). Supporting this hypothesis, studies using genome-wide chromatin accessibility assays revealed a series of temporally distinct developmental stages, both pre- and perinatal, during which the susceptibility of microglia to immune mediators and other environmental cues was increased (21, 24). Microglia from newborns exposed to maternal immune activation showed an untimely downregulation of genes that are typically expressed during this early stage of development, such as *Spi1* (the gene encoding Pu.1) and *Irf8*, and instead exhibited a transcriptional phenotype more akin to that of adult microglia (21, 24). Although these alterations were transient, the authors suggested that accelerated microglial maturation could have sufficient detrimental consequences in the developing brain to induce and maintain neurological disorders that continue long after the microglia phenotype is restored (21).

Together, these data strengthen the notion that microglia can play a fundamental role in driving neurodevelopmental disorders, including ASD, *via* their effects on neuroimmune pathways, synaptic remodelling, neuronal survival and connectivity. Understanding the main factors that induce microglial dysfunction and identifying developmental timepoints when the CNS is most susceptible to the impacts of microglial dysregulation would help to identify novel therapeutic targets and prophylactic strategies to better treat or prevent ASD.

ENVIRONMENTAL FACTORS INFLUENCING ASD: FOCUS ON MICROBIOTA-MICROGLIA MODULATION

Although genetic factors can majorly influence the risk of ASD development, epidemiological and preclinical studies estimate that 50% of ASD pathophysiology is driven by non-heritable factors, suggesting that environmental factors may play an equally prominent role (83, 84). However, while progress has been made towards gaining an understanding of the genetic components that drive ASD, environmental risk factors are less understood. Several recent studies suggest that prenatal, perinatal and postnatal factors act synergistically to induce the development of ASD (85). Maternal diet and lifestyle, as well as exposure to infection, environmental chemicals and drugs during critical periods of CNS development, can induce various congenital malformations, culminating in a latent and long-term impact on brain function, and enhancing the risk of ASD development in the offspring (86–90). Many environmental components that are vertically transmitted *via* mother-to-child interactions can influence brain development during peri- and postnatal periods, whilst horizontally transferred external factors, i.e. those that are not dependent on the maternal interface, have the capacity to interfere with the maintenance and progression of ASD symptoms (91, 92).

The Role of the Gut Microbiota in ASD Development and Maintenance

Intestinal microbes are intimately involved in integrating the various environmental factors, such as diet, environment, sex, age and genetic background, which subsequently impact host immune responses (93). Remarkably, gastrointestinal (GI) dysfunction is one of the most prominent comorbidities in ASD patients, with 23-70% of the individuals developing symptoms associated with the GI tract, including abdominal discomfort, irritated bowel syndrome, chronic diarrhea and/or constipation (32, 94). Moreover, variations in the composition and richness (diversity) of the gut microbiota have been observed in children with ASD compared to neurotypical controls, with several reports of increased proportions of *Clostridium, Suterella, Ruminococcus* and *Lactobacillus* and lower abundances of *Bifidobacterium, Akkermansia, Blautia* and *Prevotella* (95–100).

Based on the apparent dysbiosis observed in ASD individuals, numerous cross-sectional studies have investigated whether exposure to antibiotics during different developmental stages could play a causative role in triggering the onset of ASD (101–106). Although current data are conflicting and inconclusive, the most consistent data obtained from the larger cohort studies indicate that the use of specific classes of antibiotics during early life may marginally increase the risk of ASD development (103, 104, 107).

The studies described above have been useful in identifying associations between ASD and the GI tract, particularly with dysbiosis of the gut microbiota. In an open-label trial, fecal microbiota transplant (FMT) therapy from neurotypical control donors to ASD patients significantly increased bacterial diversity and improved irritability, communication skills and sociability (108, 109). Thus, the gut microbiota may contribute to the behavioural symptoms associated with ASD.

In mice, the induction of dysbiosis – for example using dietary modulation, antibiotics or gnotobiotic models – can aggravate both genetic and environmental models of ASD (110). Germ-free

(GF) mice have significant social impairments compared to specific pathogen-free (SPF) mice, as do mice that receive antibiotics postnatally (111–113). Moreover, transferring dysbiotic gut microbiota from ASD donors was sufficient to induce further social deficits and increase repetitive behaviours in GF mice, compared to mice that received an FMT from neurotypical control donors (114). These data demonstrate that ASD-like behaviours can be transferred by the microbiota of ASD patients.

Dysbiosis of the commensal microbiota has also been observed in the offspring of mice exposed to the MIA model, apparently contributing to barrier permeability and behavioural changes. In particular, MIA-exposed offspring had reduced levels of *Bacteroides fragilis* in the gut compared to controls. Importantly, reintroducing *Bacteroides fragilis* to the GI tract of these mice was sufficient to restore GI function and improve the neurological symptoms related to ASD (115, 116). Together, these data demonstrate that the commensal microbiota may be crucial for the programming and presentation of neurotypical behaviours.

It is important to consider that the studies outlined above predominantly focus on how the microbiota of the individual impacts the progression of ASD. While ASD symptoms were alleviated following FMT from neurotypical donors, the effects were transient and do not constitute a cure. As ASD is established early in development, including during embryogenesis, it seems likely that environmental factors experienced by a mother during gestation may play an equally, if not more important role. It is therefore not surprising that in rodent models of ASD, the maternal gut microbiota has also been implicating in remotely conditioning neurodevelopment, subsequently leading to ASD-like behavioural changes in the offspring. In mice, the presence of bacteria that can drive Th17 cell induction, such as segmented filamentous bacteria (SFB), is required to induce ASD development in the offspring of dams exposed to MIA (117, 118). Moreover, while the maternal microbiota is essential for normal fetal neurodevelopment (119), dysbiosis induced in response to altered diet and stress during pregnancy has also been increasingly linked to aberrant brain development and behavioural abnormalities in murine offspring (110, 119, 120). Thus, the vertical transfer of microbial molecules or microbially-induced intermediates, may alter brain function in the developing offspring, ultimately triggering the development of ASD-like behaviours.

A conclusive link between the maternal microbiota and ASD development in human patients has yet to be established. However, mothers of children with ASD often present with compositional differences in their gut microbiota, including increased levels of *Proteobacteria*, *Alphaproteobacteria*, *Moraxellaceae*, and *Acinetobacter*, when compared to mothers of healthy, neurotypical children (121). Moreover, meta-analyses of large cohort studies suggest prenatal exposure to different classes of antibiotics could contribute to the development of ASD (122, 123). However, data linking prenatal antibiotic exposure to ASD development in the offspring are highly controversial, and these studies neglect to evaluate the impact that antibiotic treatments have on the composition of the maternal microbiota during pregnancy.

As further evidence that the maternal microbiota can impact neurodevelopment of human offspring, epidemiological studies show a clear association between maternal infections, particularly those occurring during the first trimester of pregnancy, and ASD development in the offspring. Prominent maternal infections associated with ASD development in children include viral pathogens, such as herpes simplex virus type 2, cytomegalovirus and rubella, as well as the *Toxoplasma gondii* parasite (124–127). The impact of these microbes on the developing fetus may by driven by the vertical transfer of pro-inflammatory cytokines, induced in response to infection. Indeed, children born to mothers with chronic inflammatory diseases, such as obesity, diabetes, autoimmune diseases and asthma, also have an increased risk of neurodevelopmental disorders (128). However, it should be noted that many of these chronic inflammatory diseases and infectious pathogens are also accompanied by shifts in the composition and diversity of the gut microbiota, suggesting that the vertical transfer of microbial molecules may also impact fetal development (129, 130). Thus, dysbiosis may be one of the driving forces by which inflammatory diseases can increase the risk of neurodevelopmental disorders.

Collectively, the correlative data linking maternal immune activation and/or dysbiosis to ASD development in humans, combined with the causative role of SFB in driving the murine MIA model, suggests that dysbiosis of the maternal microbiota during gestation may contribute the risk of ASD in children.

The Gut Microbiota Modulates Microglial Function

Microglia are highly sensitive to environmental changes, not just locally, but on a global scale. On the most basic level, microglia are readily activated in response to systemic inflammation or circulating LPS, specifically in CNS regions with fenestrated capillaries, including the choroid plexus and the circumventricular organs (131, 132). If the insult is great enough, systemic LPS challenge can trigger the activation of microglia that rapidly spreads from the circumventricular organs into the brain parenchyma, mediated by the autocrine and paracrine effects of microglial TNFα and IL-1β production (132–134). Whilst the gut microbiota will not induce systemic inflammation under homeostatic conditions, it has been well-documented that ASD is associated with barrier defects in the GI tract (135–137), often referred to as a "leaky gut", and impaired blood-brain barrier (BBB) integrity (115, 136, 138). Thus, it is possible that inappropriate trafficking of bacterial cell wall components through the intestinal barrier to the CNS, through a permissive BBB, could contribute to abnormal microglial activation and associated neurological symptoms.

Microglia can sense peripheral changes in more subtle ways, and it is becoming increasingly apparent that they respond to distal changes in the gut microbiota composition, in the absence of overt inflammation or endotoxemia. The absence of a microbiome certainly has profound and lasting effects on microglial cell phenotype and function. Microglia development can be modulated by the maternal microbiota in a sex- and time-dependent manner (**Figure 1**). Embryonic microglial cells

isolated from the offspring of GF dams exhibited marked and sex-specific differences in transcriptional profiles, increased density and ramification of embryonic microglia in different brain regions and altered chromatin accessibility compared to those from the offspring of SPF dams (139). Transcriptional differences were first apparent in the microglia of GF offspring at E14.5, with 19 differentially expressed genes differentiating GF and SPF microglia at this time. By E18.5, the transcriptional profile of microglia from male, but not female, GF embryos was profoundly distinct from their SPF counterparts, with a total of 1216 genes differentiating between male embryonic microglia from GF and SPF mice, compared to the 20 genes that differentiated between microglia from the two groups of females (139). Interestingly, most of these genes were upregulated in microglia from SPF compared to GF embryos. Not only does this work provide compelling evidence that the maternal microbiota can shape microglial cell development and maturation during pre- and perinatal stages, but the sex-specific differences highlighted in this study could account for the male bias associated with ASD development. However, it is still debated whether there is a biological mechanism accounting for the sex-specific differences associated with ASD prevalence.

The presence of an intact, complex microbiota is also required for normal microglial cell phenotype, morphology and functionality in adulthood. Microglia are more abundant in the brains of adult GF compared to SPF mice. Moreover, they are more proliferative, and exhibit altered cell morphology, characterised by longer dendrites with increased numbers of segments, branches and terminal points (140). Phenotypically, they are less mature, with increased expression of *Spi1*, CSF1R and F4/80, and are therefore less equipped to respond to immune challenges, as demonstrated by their tempered cytokine production following LPS stimulation *ex vivo* or lymphocytic choriomeningitis viral (LCMV) infection *in vivo*. Microglia from GF mice also have a reduced ability to expand in response to LCMV compared to microglia from adult SPF mice (140). Thus, microbial signals may be required for normal microglial maturation, priming them to respond to inflammatory insults later in life; see **Table 1** for a summary of some bacteria and associated metabolites shown to affect microglia.

Recolonizing mice with a complex microbiota or feeding them short-chain fatty acids (SCFA) can rescue the abnormal microglial maturation associated with GF mice (140). SCFA are bacterial metabolites derived from microbial fermentation. All the three main SCFAs, propionate, butyrate and acetate, are able to cross the blood brain barrier (BBB) during steady-state through monocarboxylate transporters, and are detectable in the CSF in humans (150). Altered concentrations of SCFA are observed in faecal samples from children with ASD, and ASD-associated bacteria, such as Clostridia and Bacteroidetes, are important producers of propionate and its derivates (151–154). Further supporting a role for propionate in neuropathology, the administration of high amounts of propionate, by different routes, can dramatically increase microglial cell activation, thus increasing the local production of inflammatory cytokines that induce bystander damage and the development of ASD-like behaviours in mice (148, 150). On the other hand, butyrate promotes the transcription of genes involved in neuronal inhibitory pathways, thus improving social behavior in the BTBR mouse strain, an idiopathic model of ASD (155). Considering that butyrate shows anti-inflammatory effects in microglia, and that microglia act as important modulators of neuronal inhibitory/excitatory pathways in ASD models, it seems entirely plausible that the beneficial role of butyrate is at least partially mediated through microglial cell-modulation (156–158).

Aberrant production of p-Cresol, a metabolite produced mainly by intestinal microbes, has been described in the fecal samples from children with ASD (142, 159). Interestingly, p-Cresol has recently been suggested induce the elevation of microglia-associated CD68 protein in the prefrontal cortex of mice with p-Cresol sulfate (PCS)-induced neuroinflammation (144). Although the specific mechanisms remain to be fully established, these data suggest that imbalances in the production of microbial metabolites might contribute to ASD pathogenesis *via* their effects on microglial cells (**Figure 2**). Notably, bacterial metabolites can be transferred from mother to fetus during gestation (160) and could thus account for the neurodevelopmental changes associated with maternal dysbiosis.

In addition to the direct effects that bacterial metabolites may have on the CNS, the immune system is a potential mediator of the gut-to-brain communication associated with ASD. Development, maturation and activation of the peripheral immune system is heavily influenced by the gut microbiota, particularly during early life (129, 161). Perturbation of the normal microbiota during this critical window, or even during pregnancy, can cause long-lasting immune alterations, conferring susceptibility to several disorders, including

TABLE 1 | Potential links between bacterial species and microglia development and function.

Gut microbiota Bacterial species	Metabolites	Potential effects on microglia	References
Bifidobacterium spp	SCFAs	Homeostatic expansion of ramified microglia	(141)
Blautia hydrogenotrophica, Clostridium spp.	p-cresol	Induce microglial activation and expression of microglia associated CD68 protein	(142–144)
Clostridium butyricum	Mainly butyrate	Attenuate microglia activation and microglia-mediated neuroinflammation	(145)
Lactobacillus spp.	unknown	Regulate microglial dystrophy and activation during prenatal periods	(146, 147)
Bacteroides spp, Clostridium spp.	propionate	Induce microglial activation and production of inflammatory mediators (in high concentrations)	(148, 149)

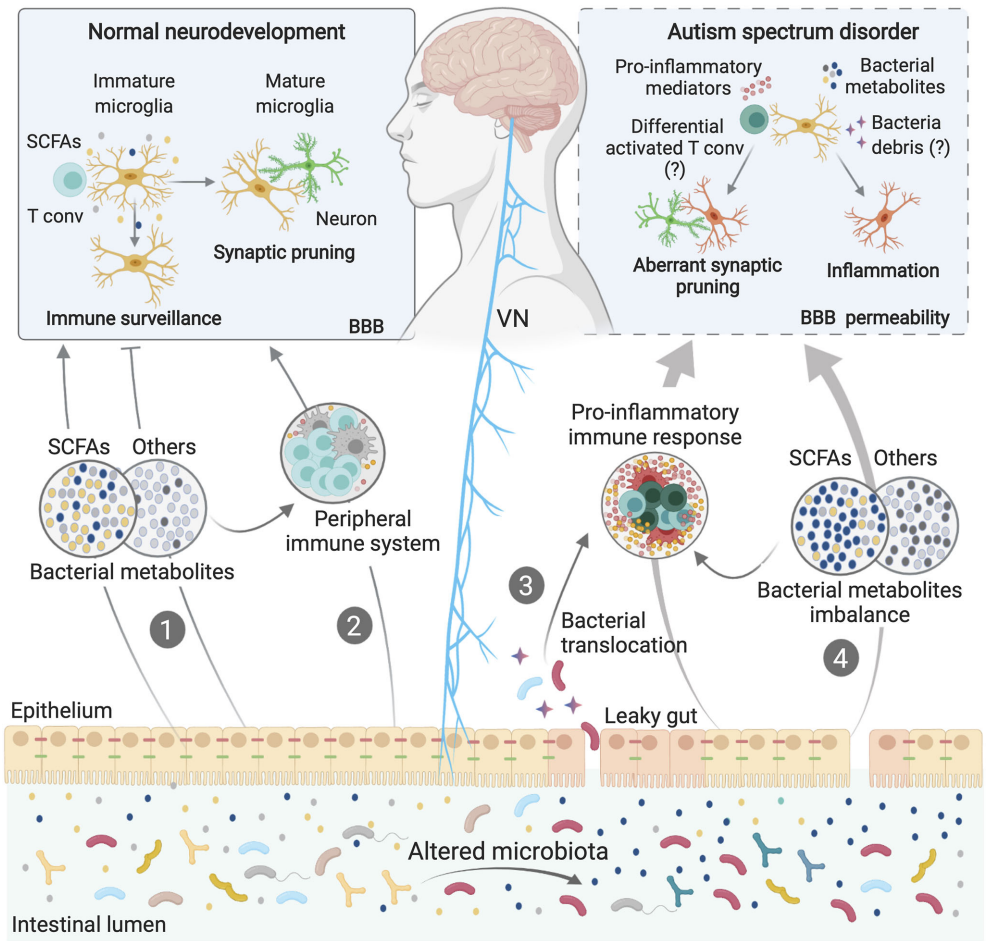


FIGURE 2 | Microbiota-Microglia modulation in ASD. Microbiota-microglia communication is mediated *via* multiple direct and indirect mechanisms, including the production of bacterial metabolites, such as SCFAs (1), direct modulation of the peripheral immune system and cytokine milieu (2), and direct activation of the vagus nerve (VN) by bacterial compounds and metabolites. During homeostasis, some bacterial metabolites and components of the immune system can activate the VN or reach the brain *via* the systemic circulation, directly affecting microglial maturation and functions (1; 2). In some neurodevelopmental disorders, including ASD, dysbiosis of the gut microbiota can induce loss of gut barrier integrity. Higher intestinal permeability may allow bacterial translocation (3), as well as an imbalance in circulating bacteria-derived components (4), thus activating immune signaling pathways, including the release of cytokines and other proinflammatory molecules. Both bacterial components and proinflammatory mediators can cross the blood brain barriers (BBB) or activate the VN, inducing aberrations in the normal homeostatic functions of microglia, such as surveillance, synaptic pruning and inflammatory states, contributing to ASD symptoms.

neurodevelopmental disorders (130, 162). Indeed, in addition to gut dysbiosis, children with ASD often present with abnormal activation of peripheral blood mononuclear cells and increased levels of systemic inflammatory mediators, including IL-1β, IL-6, CCL2, IFN-γ and IL-17 (163–166). Similarly, genetic and environmental ASD models show permanent systemic immune dysregulation and suggest a detrimental role of inflammation in the aetiology and/or maintenance of ASD (167, 168). For example, the behavioural deficits associated with the MIA model can be restored by a bone-marrow transplant from the offspring of PBS-injected control dams, thus highlighting the detrimental role the immune system can play in mediating the ASD-like symptoms associated with this model (167). It has been widely published that peripheral immune system activation can have a profound impact on brain function and behaviour (131, 169–171). Given that microglia can sense and respond to changes in circulating inflammatory mediators, it is possible that aberrant immune activation could contribute to the neuropsychiatric symptoms associated with ASD *via* effects on microglial cells (**Figure 2**).

Recent work has elucidated a novel pathway of immune-mediated microglial cell maturation whereby activated $CD4^+$ T helper cells migrate to the CNS, facilitating microglial fetal-to-adult transition (172). Crucially, peripheral activation of conventional $CD4^+$ T cells by the microbiome is essential to license their migration to the brain in steady state. An absence of $CD4^+$ T cells in the brain, as observed in MHC class II-deficient mice, induces altered neuronal synapses and abnormal behaviour

similar to those observed in some ASD models. In these mice, microglial differentiation was arrested between fetal- and adult-states. Although this study failed to address what direct effects, if any, microbial diversity might play on the phenotypes observed, it provided proof-of-principle that gut dysbiosis could impact microglial maturation in ASD patients *via* altered $CD4^+$ T cell peripheral activation (172).

Finally, both gut bacteria and their metabolites, as well cytokines and other immune mediators, can directly stimulate the vagus nerve (VN), which in turn, relays information to the CNS (173–177). The VN is one of the most prominent aspects of the parasympathetic nervous system and has been extensively studied for its involvement in digestion, satiety, stress response, and regulation of inflammation (178). It also constitutes one of the main pathways of neuroimmune communication, driving sickness behaviour in response to systemic LPS challenge (171). Vagal afferent fibers innervate all the layers of the intestinal wall (178). Although it does not extend into the lumen of the GI tract, the VN is exposed to bacterial components that diffuse across the GI barrier, such as neurotransmitters, metabolites and major components of bacterial cell walls. VN neurons express numerous pattern recognition receptors, as well as receptors for SCFA and serotonin, allowing them to interact with these molecules directly (177, 179–181). The gut microbiota may also interact with the VN indirectly, by altering the inflammatory milieu of the intestine (**Figure 2**). Afferent VN fibers also express numerous cytokine and chemokine receptors (182). As such, intestinal inflammation induced by dysbiosis can be sensed by the VN and transmitted to the brain; an effect known to influence microglial activation and neuroinflammation (183, 184). Thus, by stimulating the VN, either directly or indirectly, the gut microbiota may regulate behavior in patients with ASD (**Figure 2**). Indeed, experiments in mouse models have shown that by stimulating the VN, gut microbes, such as *L. reuteri*, can improve ASD symptoms (110). Moreover, *Lactobacillus* strains can regulate behavioral and physiological responses in a manner that requires VN stimulation (173).

Collectively, these data demonstrate some of the complex pathways by which the gut microbiota can remotely modulate microglial cell function and associated behavioural changes. They also provide proof of principle that microbiome-based therapies could alleviate ASD symptoms *via* their putative effect on microglia.

Dysbiosis, Immune Dysfunction and a Leaky Gut in ASD

Although it is yet to be fully established, a causal relationship between immune dysfunction, dysbiosis and the barrier defects associated with ASD patients seems likely, and we propose this as a major factor in the maintenance of neurological dysfunction in ASD (**Figure 2**). Dysbiosis of the intestinal microbiota could certainly induce both gut permeability and abnormal intestinal inflammation through interactions with local immune and mesenchymal cells (185). These interactions classically induce the production of a wide range of pro-inflammatory mediators, amplifying local inflammatory responses and possibly driving the GI-related co-morbidities that many ASD patients endure. As such, the intestine is a likely source of the chronic low-grade inflammation observed systemically in ASD patients (32, 186). Supporting these hypotheses, the dysbiosis observed in murine offspring exposed to the MIA model is accompanied by elevated levels of colonic IL-6 and a widespread defect in intestinal barrier integrity, all of which are restored following reconstitution with *B. fragilis* (115).

Microglia express numerous cytokine receptors, as well as toll-like receptors (TLR)-2, -4 and -7 (187–189), as does the BBB endothelium and the VN. Moreover, BBB permeability is known to increase in response to circulating cytokines. Thus, in addition to locally activating the VN, the putative release of inflammatory molecules and bacterial cell-wall components from the gut into the circulation of ASD patients could increase BBB permeability, resulting in widespread microglial cell activation and dysfunction (11, 190). In addition to the immune pathways described, dysbiosis and a leaky gut could create imbalances in circulating bacterial metabolites, which can cross the BBB to interact with microglia directly (**Figure 2**).

In summary, although a clear, causative role for the microbiota–microglia axis in ASD onset or development has yet to be fully described, the findings highlighted in this review suggest that progression of ASD may have microbial origins and thus paves the way for further research into whether therapeutic microbial manipulation could help stem the tide of increasing ASD incidence. We believe further study of this to be of fundamental importance for establishing novel prophylactic strategies that could prevent ASD.

CONCLUSIONS

We are still unravelling the complex tri-directional relationships linking the microbiota with microglial function and ASD development. Here we describe recent evidence implicating microglia in ASD development, and discuss how environmental risk factors, particularly gut dysbiosis, could compromise the immunological and neurological functions of microglia to drive permanent changes in the brain. We have also highlighted how perturbations in the gut microbiota during prenatal and neonatal periods, induced by antibiotics, dietary changes or infections, could compromise microglial function, thus altering brain function and increasing the risk of ASD. Recent studies have begun to clarify the significant influence the gut microbiota has on microglial phenotype during steady-state, and in numerous models of neurological disorders. However, more research is required to identify the precise mechanisms by which microglia and the gut microbiota collude to drive neurodevelopmental disorders, particularly in humans. We hope that future studies, using metabolomics assays and advanced next-generation sequencing platforms, will reveal specific microbial communities or molecules associated with ASD pathogenesis or alleviating symptoms, as well as the

precise molecular mechanisms involved. This could pave the way for the identification of novel treatment targets and/or the rational design of probiotics to treat or prevent ASD.

AUTHOR CONTRIBUTIONS

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

FUNDING

MD-F is supported by a Beverley Phillips Postdoctoral Fellowship and a Cumming School of Medicine Postdoctoral Fellowship.

ACKNOWLEDGMENTS

The figures were created with BioRender.com.

REFERENCES

1. Lord C, Bishop SL. Recent Advances in Autism Research as Reflected in DSM-5 Criteria for Autism Spectrum Disorder. *Annu Rev Clin Psychol* (2015) 11:53–70. doi: 10.1146/annurev-clinpsy-032814-112745
2. Hyman SL, Levy SE, Myers SM. Council ON Children WITH Disabilities SECTODABP. Identification, Evaluation, and Management of Children With Autism Spectrum Disorder. *Pediatrics* (2020) 145(1):e20193447. doi: 10.1542/peds.2019-3447
3. Matta SM, Hill-Yardin EL, Crack PJ. The Influence of Neuroinflammation in Autism Spectrum Disorder. *Brain Behav Immun* (2019) 79:75–90. doi: 10.1016/j.bbi.2019.04.037
4. Prata J, Machado AS, von Doellinger O, Almeida MI, Barbosa MA, Coelho R, et al. The Contribution of Inflammation to Autism Spectrum Disorders: Recent Clinical Evidence. *Methods Mol Biol* (2019) 2011:493–510. doi: 10.1007/978-1-4939-9554-7_29
5. Bourgeron T. From the Genetic Architecture to Synaptic Plasticity in Autism Spectrum Disorder. *Nat Rev Neurosci* (2015) 16(9):551–63. doi: 10.1038/nrn3992
6. Ebrahimi-Fakhari D, Sahin M. Autism and the Synapse: Emerging Mechanisms and Mechanism-Based Therapies. *Curr Opin Neurol* (2015) 28(2):91–102. doi: 10.1097/WCO.0000000000000186
7. Hong S, Dissing-Olesen L, Stevens B. New Insights on the Role of Microglia in Synaptic Pruning in Health and Disease. *Curr Opin Neurobiol* (2016) 36:128–34. doi: 10.1016/j.conb.2015.12.004
8. Butovsky O, Weiner HL. Microglial Signatures and Their Role in Health and Disease. *Nat Rev Neurosci* (2018) 19(10):622–35. doi: 10.1038/s41583-018-0057-5
9. Paolicelli RC, Bolasco G, Pagani F, Maggi L, Scianni M, Panzanelli P, et al. Synaptic Pruning by Microglia Is Necessary for Normal Brain Development. *Science* (2011) 333(6048):1456–8. doi: 10.1126/science.1202529
10. Zhan Y, Paolicelli RC, Sforazzini F, Weinhard L, Bolasco G, Pagani F, et al. Deficient Neuron-Microglia Signaling Results in Impaired Functional Brain Connectivity and Social Behavior. *Nat Neurosci* (2014) 17(3):400–6. doi: 10.1038/nn.3641
11. Colonna M, Butovsky O. Microglia Function in the Central Nervous System During Health and Neurodegeneration. *Annu Rev Immunol* (2017) 35:441–68. doi: 10.1146/annurev-immunol-051116-052358
12. Cheadle L, Rivera SA, Phelps JS, Ennis KA, Stevens B, Burkly LC, et al. Sensory Experience Engages Microglia to Shape Neural Connectivity Through a Non-Phagocytic Mechanism. *Neuron* (2020) 108(3):451–68.e459. doi: 10.1016/j.neuron.2020.08.002
13. Salter MW, Stevens B. Microglia Emerge as Central Players in Brain Disease. *Nat Med* (2017) 23(9):1018–27. doi: 10.1038/nm.4397
14. Hagemeyer N, Hanft KM, Akriditou MA, Unger N, Park ES, Stanley ER, et al. Microglia Contribute to Normal Myelinogenesis and to Oligodendrocyte Progenitor Maintenance During Adulthood. *Acta Neuropathol* (2017) 134(3):441–58. doi: 10.1007/s00401-017-1747-1
15. Miron VE, Boyd A, Zhao JW, Yuen TJ, Ruckh JM, Shadrach JL, et al. M2 Microglia and Macrophages Drive Oligodendrocyte Differentiation During CNS Remyelination. *Nat Neurosci* (2013) 16(9):1211–8. doi: 10.1038/nn.3469
16. Vargas DL, Nascimbene C, Krishnan C, Zimmerman AW, Pardo CA. Neuroglial Activation and Neuroinflammation in the Brain of Patients With Autism. *Ann Neurol* (2005) 57(1):67–81. doi: 10.1002/ana.20315
17. Suzuki K, Sugihara G, Ouchi Y, Nakamura K, Futatsubashi M, Takebayashi K, et al. Microglial Activation in Young Adults With Autism Spectrum Disorder. *JAMA Psychiatry* (2013) 70(1):49–58. doi: 10.1001/jamapsychiatry.2013.272
18. Morgan JT, Chana G, Pardo CA, Achim C, Semendeferi K, Buckwalter J, et al. Microglial Activation and Increased Microglial Density Observed in the Dorsolateral Prefrontal Cortex in Autism. *Biol Psychiatry* (2010) 68(4):368–76. doi: 10.1016/j.biopsych.2010.05.024
19. Liao X, Yang J, Wang H, Li Y. Microglia Mediated Neuroinflammation in Autism Spectrum Disorder. *J Psychiatr Res* (2020) 130:167–76. doi: 10.1016/j.jpsychires.2020.07.013
20. Maezawa I, Calafiore M, Wulff H, Jin LW. Does Microglial Dysfunction Play a Role in Autism and Rett Syndrome? *Neuron Glia Biol* (2011) 7(1):85–97. doi: 10.1017/S1740925X1200004X
21. Matcovitch-Natan O, Winter DR, Giladi A, Vargas Aguilar S, Spinrad A, Sarrazin S, et al. Microglia Development Follows a Stepwise Program to Regulate Brain Homeostasis. *Science* (2016) 353(6301):aad8670. doi: 10.1126/science.aad8670
22. Kierdorf K, Prinz M. Microglia in Steady State. *J Clin Invest* (2017) 127(9):3201–9. doi: 10.1172/JCI90602
23. Kierdorf K, Erny D, Goldmann T, Sander V, Schulz C, Perdiguero EG, et al. Microglia Emerge From Erythromyeloid Precursors Via Pu.1- and Irf8-dependent Pathways. *Nat Neurosci* (2013) 16(3):273–80. doi: 10.1038/nn.3318
24. Ginhoux F, Greter M, Leboeuf M, Nandi S, See P, Gokhan S, et al. Fate Mapping Analysis Reveals That Adult Microglia Derive From Primitive Macrophages. *Science* (2010) 330(6005):841–5. doi: 10.1126/science.1194637
25. Schulz C, Gomez Perdiguero E, Chorro L, Szabo-Rogers H, Cagnard N, Kierdorf K, et al. A Lineage of Myeloid Cells Independent of Myb and Hematopoietic Stem Cells. *Science* (2012) 336(6077):86–90. doi: 10.1126/science.1219179
26. Wegiel J, Wiśniewski HM, Dziewiatkowski J, Tarnawski M, Kozielski R, Trenkner E, et al. Reduced Number and Altered Morphology of Microglial Cells in Colony Stimulating Factor-1-Deficient Osteopetrotic Op/Op Mice. *Brain Res* (1998) 804(1):135–9. doi: 10.1016/S0006-8993(98)00618-0
27. Wang Y, Szretter KJ, Vermi W, Gilfillan S, Rossini C, Cella M, et al. IL-34 Is a Tissue-Restricted Ligand of CSF1R Required for the Development of Langerhans Cells and Microglia. *Nat Immunol* (2012) 13(8):753–60. doi: 10.1038/ni.2360
28. Spittau B, Dokalis N, Prinz M. The Role of Tgfβ Signaling in Microglia Maturation and Activation. *Trends Immunol* (2020) 41(9):836–48. doi: 10.1016/j.it.2020.07.003
29. Domínguez-Punaro MEL, Segura M, Contreras I, Lachance C, Houde M, Lecours MP, et al. In Vitro Characterization of the Microglial Inflammatory Response to Streptococcus Suis, an Important Emerging Zoonotic Agent of Meningitis. *Infect Immun* (2010) 78(12):5074–85. doi: 10.1128/IAI.00698-10
30. Han Q, Liu S, Li Z, Hu F, Zhang Q, Zhou M, et al. DCPIB, a Potent Volume-Regulated Anion Channel Antagonist, Attenuates Microglia-Mediated Inflammatory Response and Neuronal Injury Following Focal Cerebral Ischemia. *Brain Res* (2014) 1542:176–85. doi: 10.1016/j.brainres.2013.10.026
31. Chhatbar C, Detje CN, Grabski E, Borst K, Spanier J, Ghita L, et al. Type I Interferon Receptor Signaling of Neurons and Astrocytes Regulates Microglia Activation During Viral Encephalitis. *Cell Rep* (2018) 25(1):118–29.e114. doi: 10.1016/j.celrep.2018.09.003

32. Abdel-Haq R, Schlachetzki JCM, Glass CK, Mazmanian SK. Microbiome-Microglia Connections Via the Gut-Brain Axis. *J Exp Med* (2019) 216(1):41–59. doi: 10.1084/jem.20180794
33. Marín-Teva JL, Dusart I, Colin C, Gervais A, van Rooijen N, Mallat M. Microglia Promote the Death of Developing Purkinje Cells. *Neuron* (2004) 41(4):535–47. doi: 10.1016/S0896-6273(04)00069-8
34. Antony JM, Paquin A, Nutt SL, Kaplan DR, Miller FD. Endogenous Microglia Regulate Development of Embryonic Cortical Precursor Cells. *J Neurosci Res* (2011) 89(3):286–98. doi: 10.1002/jnr.22533
35. Sierra A, Encinas JM, Deudero JJ, Chancey JH, Enikolopov G, Overstreet-Wadiche LS, et al. Microglia Shape Adult Hippocampal Neurogenesis Through Apoptosis-Coupled Phagocytosis. *Cell Stem Cell* (2010) 7(4):483–95. doi: 10.1016/j.stem.2010.08.014
36. Squarzoni P, Oller G, Hoeffel G, Pont-Lezica L, Rostaing P, Low D, et al. Microglia Modulate Wiring of the Embryonic Forebrain. *Cell Rep* (2014) 8 (5):1271–9. doi: 10.1016/j.celrep.2014.07.042
37. Zaki Y, Cai DJ. Creating Space for Synaptic Formation-a New Role for Microglia in Synaptic Plasticity. *Cell* (2020) 182(2):265–7. doi: 10.1016/j.cell.2020.06.042
38. Wu Y, Dissing-Olesen L, MacVicar BA, Stevens B. Microglia: Dynamic Mediators of Synapse Development and Plasticity. *Trends Immunol* (2015) 36(10):605–13. doi: 10.1016/j.it.2015.08.008
39. Nguyen PT, Dorman LC, Pan S, Vainchtein ID, Han RT, Nakao-Inoue H, et al. Microglial Remodeling of the Extracellular Matrix Promotes Synapse Plasticity. *Cell* (2020) 182(2):388–403.e315. doi: 10.1016/j.cell.2020.05.050
40. Rogers JT, Morganti JM, Bachstetter AD, Hudson CE, Peters MM, Grimmig BA, et al. CX3CR1 Deficiency Leads to Impairment of Hippocampal Cognitive Function and Synaptic Plasticity. *J Neurosci* (2011) 31 (45):16241–50. doi: 10.1523/JNEUROSCI.3667-11.2011
41. Bachstetter AD, Morganti JM, Jernberg J, Schlunk A, Mitchell SH, Brewster KW, et al. Fractalkine and CX 3 CR1 Regulate Hippocampal Neurogenesis in Adult and Aged Rats. *Neurobiol Aging.* (2011) 32(11):2030–44. doi: 10.1016/j.neurobiolaging.2009.11.022
42. Parkhurst CN, Yang G, Ninan I, Savas JN, Yates JR, Lafaille JJ, et al. Microglia Promote Learning-Dependent Synapse Formation Through Brain-Derived Neurotrophic Factor. *Cell* (2013) 155(7):1596–609. doi: 10.1016/j.cell.2013.11.030
43. Zengeler KE, Lukens JR. Innate Immunity At the Crossroads of Healthy Brain Maturation and Neurodevelopmental Disorders. *Nat Rev Immunol* (2021). doi: 10.1038/s41577-020-00487-7
44. Lee E, Lee J, Kim E. Excitation/Inhibition Imbalance in Animal Models of Autism Spectrum Disorders. *Biol Psychiatry* (2017) 81(10):838–47. doi: 10.1016/j.biopsych.2016.05.011
45. Meltzer A, Van de Water J. The Role of the Immune System in Autism Spectrum Disorder. *Neuropsychopharmacology* (2017) 42(1):284–98. doi: 10.1038/npp.2016.158
46. Siniscalco D, Schultz S, Brigida AL, Antonucci N. Inflammation and Neuro-Immune Dysregulations in Autism Spectrum Disorders. *Pharmaceut (Basel)* (2018) 11(2):56. doi: 10.3390/ph11020056
47. Li X, Chauhan A, Sheikh AM, Patil S, Chauhan V, Li XM, et al. Elevated Immune Response in the Brain of Autistic Patients. *J Neuroimmunol* (2009) 207(1-2):111–6. doi: 10.1016/j.jneuroim.2008.12.002
48. Tsilioni I, Patel AB, Pantazopoulos H, Berretta S, Conti P, Leeman SE, et al. Il-37 Is Increased in Brains of Children With Autism Spectrum Disorder and Inhibits Human Microglia Stimulated by Neurotensin. *Proc Natl Acad Sci USA* (2019) 116(43):21659–65. doi: 10.1073/pnas.1906817116
49. Parker-Athill EC, Tan J. Maternal Immune Activation and Autism Spectrum Disorder: Interleukin-6 Signaling as a Key Mechanistic Pathway. *Neurosignals* (2010) 18(2):113–28. doi: 10.1159/000319828
50. Voineagu I, Wang X, Johnston P, Lowe JK, Tian Y, Horvath S, et al. Transcriptomic Analysis of Autistic Brain Reveals Convergent Molecular Pathology. *Nature* (2011) 474(7351):380–4. doi: 10.1038/nature10110
51. Gupta S, Ellis SE, Ashar FN, Moes A, Bader JS, Zhan J, et al. Transcriptome Analysis Reveals Dysregulation of Innate Immune Response Genes and Neuronal Activity-Dependent Genes in Autism. *Nat Commun* (2014) 5:5748. doi: 10.1038/ncomms6748
52. Morgan JT, Chana G, Abramson I, Semendeferi K, Courchesne E, Everall IP. Abnormal Microglial-Neuronal Spatial Organization in the Dorsolateral Prefrontal Cortex in Autism. *Brain Res* (2012) 1456:72–81. doi: 10.1016/j.brainres.2012.03.036
53. Lee AS, Azmitia EC, Whitaker-Azmitia PM. Developmental Microglial Priming in Postmortem Autism Spectrum Disorder Temporal Cortex. *Brain Behav Immun* (2017) 62:193–202. doi: 10.1016/j.bbi.2017.01.019
54. Tetreault NA, Hakeem AY, Jiang S, Williams BA, Allman E, Wold BJ, et al. Microglia in the Cerebral Cortex in Autism. *J Autism Dev Disord* (2012) 42 (12):2569–84. doi: 10.1007/s10803-012-1513-0
55. Corona AW, Huang Y, O'Connor JC, Dantzer R, Kelley KW, Popovich PG, et al. Fractalkine Receptor (CX3CR1) Deficiency Sensitizes Mice to the Behavioral Changes Induced by Lipopolysaccharide. *J Neuroinflamm* (2010) 7:93. doi: 10.1186/1742-2094-7-93
56. Arnoux I, Audinat E. Fractalkine Signaling and Microglia Functions in the Developing Brain. *Neural Plast* (2015) 2015:689404. doi: 10.1155/2015/689404
57. Filipello F, Morini R, Corradini I, Zerbi V, Canzi A, Michalski B, et al. The Microglial Innate Immune Receptor TREM2 Is Required for Synapse Elimination and Normal Brain Connectivity. *Immunity* (2018) 48(5):979–91.e978. doi: 10.1016/j.immuni.2018.04.016
58. Cady J, Koval ED, Benitez BA, Zaidman C, Jockel-Balsarotti J, Allred P, et al. TREM2 Variant p.R47H as a Risk Factor for Sporadic Amyotrophic Lateral Sclerosis. *JAMA Neurol* (2014) 71(4):449–53. doi: 10.1001/jamaneurol.2013.6237
59. Jonsson T, Stefansson H, Steinberg S, Jonsdottir I, Jonsson PV, Snaedal J, et al. Variant of TREM2 Associated With the Risk of Alzheimer's Disease. *N Engl J Med* (2013) 368(2):107–16. doi: 10.1056/NEJMoa1211103
60. Wang S, Mustafa M, Yuede CM, Salazar SV, Kong P, Long H, et al. Anti-Human TREM2 Induces Microglia Proliferation and Reduces Pathology in an Alzheimer's Disease Model. *J Exp Med* (2020) 217(9):e20200785. doi: 10.1084/jem.20200785
61. Gratuze M, Leyns CE, Sauerbeck AD, St-Pierre MK, Xiong M, Kim N, et al. Impact of TREM2R47H Variant on Tau Pathology-Induced Gliosis and Neurodegeneration. *J Clin Invest* (2020) 130(9):4954–68. doi: 10.1172/JCI138179
62. Zhou Y, Song WM, Andhey PS, Swain A, Levy T, Miller KR, et al. Human and Mouse Single-Nucleus Transcriptomics Reveal TREM2-Dependent and TREM2-Independent Cellular Responses in Alzheimer's Disease. *Nat Med* (2020) 26(1):131–42. doi: 10.1038/s41591-019-0695-9
63. Xu ZX, Kim GH, Tan JW, Riso AE, Sun Y, Xu EY, et al. Elevated Protein Synthesis in Microglia Causes Autism-Like Synaptic and Behavioral Aberrations. *Nat Commun* (2020) 11(1):1797. doi: 10.1038/s41467-020-15530-3
64. Lv JW, Cheng TL, Qiu ZL, Zhou WH. Role of the PTEN Signaling Pathway in Autism Spectrum Disorder. *Neurosci Bull* (2013) 29(6):773–8. doi: 10.1007/s12264-013-1382-3
65. Zhou J, Parada LF. PTEN Signaling in Autism Spectrum Disorders. *Curr Opin Neurobiol* (2012) 22(5):873–9. doi: 10.1016/j.conb.2012.05.004
66. Zalfa F, Giorgi M, Primerano B, Moro A, Di Penta A, Reis S, et al. The Fragile X Syndrome Protein FMRP Associates With BC1 RNA and Regulates the Translation of Specific mRNAs At Synapses. *Cell* (2003) 112(3):317–27. doi: 10.1016/S0092-8674(03)00079-5
67. Bagni C, Greenough WT. From mRNP Trafficking to Spine Dysmorphogenesis: The Roots of Fragile X Syndrome. *Nat Rev Neurosci* (2005) 6(5):376–87. doi: 10.1038/nrn1667
68. Jeste SS, Sahin M, Bolton P, Ploubidis GB, Humphrey A. Characterization of Autism in Young Children With Tuberous Sclerosis Complex. *J Child Neurol* (2008) 23(5):520–5. doi: 10.1177/0883073807309788
69. Kwon CH, Luikart BW, Powell CM, Zhou J, Matheny SA, Zhang W, et al. Pten Regulates Neuronal Arborization and Social Interaction in Mice. *Neuron* (2006) 50(3):377–88. doi: 10.1016/j.neuron.2006.03.023
70. Chen CJ, Sgritta M, Mays J, Zhou H, Lucero R, Park J, et al. Therapeutic Inhibition of mTORC2 Rescues the Behavioral and Neurophysiological Abnormalities Associated With Pten-Deficiency. *Nat Med* (2019) 25 (11):1684–90. doi: 10.1038/s41591-019-0608-y
71. Jawaid S, Kidd GJ, Wang J, Swetlik C, Dutta R, Trapp BD. Alterations in CA1 Hippocampal Synapses in a Mouse Model of Fragile X Syndrome. *Glia* (2018) 66(4):789–800. doi: 10.1002/glia.23284

72. Maezawa I, Jin LW. Rett Syndrome Microglia Damage Dendrites and Synapses by the Elevated Release of Glutamate. *J Neurosci* (2010) 30 (15):5346–56. doi: 10.1523/JNEUROSCI.5966-09.2010
73. Cronk JC, Derecki NC, Ji E, Xu Y, Lampano AE, Smirnov I, et al. Methyl-Cpg Binding Protein 2 Regulates Microglia and Macrophage Gene Expression in Response to Inflammatory Stimuli. *Immunity* (2015) 42 (4):679–91. doi: 10.1016/j.immuni.2015.03.013
74. Li L, Lu J, Tay SS, Moochhala SM, He BP. The Function of Microglia, Either Neuroprotection or Neurotoxicity, Is Determined by the Equilibrium Among Factors Released From Activated Microglia In Vitro. *Brain Res* (2007) 1159:8–17. doi: 10.1016/j.brainres.2007.04.066
75. Knuesel I, Chicha L, Britschgi M, Schobel SA, Bodmer M, Hellings JA, et al. Maternal Immune Activation and Abnormal Brain Development Across CNS Disorders. *Nat Rev Neurol* (2014) 10(11):643–60. doi: 10.1038/nrneurol.2014.187
76. Patterson PH. Maternal Infection and Immune Involvement in Autism. *Trends Mol Med* (2011) 17(7):389–94. doi: 10.1016/j.molmed.2011.03.001
77. Estes ML, McAllister AK. Maternal Immune Activation: Implications for Neuropsychiatric Disorders. *Science* (2016) 353(6301):772–7. doi: 10.1126/science.aag3194
78. Smolders S, Notter T, Smolders SMT, Rigo JM, Brône B. Controversies and Prospects About Microglia in Maternal Immune Activation Models for Neurodevelopmental Disorders. *Brain Behav Immun* (2018) 73:51–65. doi: 10.1016/j.bbi.2018.06.001
79. Giovanoli S, Weber-Stadlbauer U, Schedlowski M, Meyer U, Engler H. Prenatal Immune Activation Causes Hippocampal Synaptic Deficits in the Absence of Overt Microglia Anomalies. *Brain Behav Immun* (2016) 55:25–38. doi: 10.1016/j.bbi.2015.09.015
80. Manitz MP, Plümper J, Demir S, Ahrens M, Eßlinger M, Wachholz S, et al. Flow Cytometric Characterization of Microglia in the Offspring of PolyI:C Treated Mice. *Brain Res* (2016) 1636:172–82. doi: 10.1016/j.brainres.2016.02.004
81. Zhu F, Zheng Y, Liu Y, Zhang X, Zhao J. Minocycline Alleviates Behavioral Deficits and Inhibits Microglial Activation in the Offspring of Pregnant Mice After Administration of Polyriboinosinic-Polyribocytidilic Acid. *Psychiatry Res* (2014) 219(3):680–6. doi: 10.1016/j.psychres.2014.06.046
82. Missault S, Van den Eynde K, Vanden Berghe W, Fransen E, Weeren A, Timmermans JP, et al. The Risk for Behavioural Deficits Is Determined by the Maternal Immune Response to Prenatal Immune Challenge in a Neurodevelopmental Model. *Brain Behav Immun* (2014) 42:138–46. doi: 10.1016/j.bbi.2014.06.013
83. Hallmayer J, Cleveland S, Torres A, Phillips J, Cohen B, Torigoe T, et al. Genetic Heritability and Shared Environmental Factors Among Twin Pairs With Autism. *Arch Gen Psychiatry* (2011) 68(11):1095–102. doi: 10.1001/archgenpsychiatry.2011.76
84. Cryan JF, O'Riordan KJ, Sandhu K, Peterson V, Dinan TG. The Gut Microbiome in Neurological Disorders. *Lancet Neurol* (2020) 19(2):179–94. doi: 10.1016/S1474-4422(19)30356-4
85. Wang C, Geng H, Liu W, Zhang G. Prenatal, Perinatal, and Postnatal Factors Associated With Autism: A Meta-Analysis. *Med (Baltimore)* (2017) 96(18): e6696. doi: 10.1097/MD.0000000000006696
86. Lyall K, Schmidt RJ, Hertz-Picciotto I. Maternal Lifestyle and Environmental Risk Factors for Autism Spectrum Disorders. *Int J Epidemiol* (2014) 43 (2):443–64. doi: 10.1093/ije/dyt282
87. Hertz-Picciotto I, Schmidt RJ, Krakowiak P. Understanding Environmental Contributions to Autism: Causal Concepts and the State of Science. *Autism Res* (2018) 11(4):554–86. doi: 10.1002/aur.1938
88. Oudin A, Frondelius K, Haglund N, Källén K, Forsberg B, Gustafsson P, et al. Prenatal Exposure to Air Pollution as a Potential Risk Factor for Autism and ADHD. *Environ Int* (2019) 133(Pt A):105149. doi: 10.1016/j.envint.2019.105149
89. von Ehrenstein OS, Cui X, Yan Q, Aralis H, Ritz B. Maternal Prenatal Smoking and Autism Spectrum Disorder in Offspring: A California Statewide Cohort and Sibling Study. *Am J Epidemiol* (2021) 190(5):728–37. doi: 10.1093/aje/kwaa182
90. Guma E, Plitman E, Chakravarty MM. The Role of Maternal Immune Activation in Altering the Neurodevelopmental Trajectories of Offspring: A Translational Review of Neuroimaging Studies With Implications for Autism Spectrum Disorder and Schizophrenia. *Neurosci Biobehav Rev* (2019) 104:141–57. doi: 10.1016/j.neubiorev.2019.06.020
91. Bölte S, Girdler S, Marschik PB. The Contribution of Environmental Exposure to the Etiology of Autism Spectrum Disorder. *Cell Mol Life Sci* (2019) 76(7):1275–97. doi: 10.1007/s00018-018-2988-4
92. Karimi P, Kamali E, Mousavi SM, Karahmadi M. Environmental Factors Influencing the Risk of Autism. *J Res Med Sci* (2017) 22:27. doi: 10.4103/1735-1995.200272
93. Schmidt TSB, Raes J, Bork P. The Human Gut Microbiome: From Association to Modulation. *Cell* (2018) 172(6):1198–215. doi: 10.1016/j.cell.2018.02.044
94. Holingue C, Newill C, Lee LC, Pasricha PJ, Daniele Fallin M. Gastrointestinal Symptoms in Autism Spectrum Disorder: A Review of the Literature on Ascertainment and Prevalence. *Autism Res* (2018) 11 (1):24–36. doi: 10.1002/aur.1854
95. Wang L, Christophersen CT, Sorich MJ, Gerber JP, Angley MT, Conlon MA. Increased Abundance of Sutterella Spp. and Ruminococcus Torques in Feces of Children With Autism Spectrum Disorder. *Mol Autism* (2013) 4(1):42. doi: 10.1186/2040-2392-4-42
96. Strati F, Cavalieri D, Albanese D, De Felice C, Donati C, Hayek J, et al. New Evidences on the Altered Gut Microbiota in Autism Spectrum Disorders. *Microbiome* (2017) 5(1):24. doi: 10.1186/s40168-017-0242-1
97. Liu F, Li J, Wu F, Zheng H, Peng Q, Zhou H. Altered Composition and Function of Intestinal Microbiota in Autism Spectrum Disorders: A Systematic Review. *Transl Psychiatry* (2019) 9(1):43. doi: 10.1038/s41398-019-0389-6
98. Williams BL, Hornig M, Parekh T, Lipkin WI. Application of Novel PCR-based Methods for Detection, Quantitation, and Phylogenetic Characterization of Sutterella Species in Intestinal Biopsy Samples From Children With Autism and Gastrointestinal Disturbances. *mBio* (2012) 3(1): e00261–11. doi: 10.1128/mBio.00261-11
99. Wang L, Christophersen CT, Sorich MJ, Gerber JP, Angley MT, Conlon MA. Low Relative Abundances of the Mucolytic Bacterium Akkermansia Muciniphila and Bifidobacterium Spp. in Feces of Children With Autism. *Appl Environ Microbiol* (2011) 77(18):6718–21. doi: 10.1128/AEM.05212-11
100. Finegold SM, Molitoris D, Song Y, Liu C, Vaisanen ML, Bolte E, et al. Gastrointestinal Microflora Studies in Late-Onset Autism. *Clin Infect Dis* (2002) 35(Suppl 1):S6–16. doi: 10.1086/341914
101. Sandler RH, Finegold SM, Bolte ER, Buchanan CP, Maxwell AP, Väisänen ML, et al. Short-Term Benefit From Oral Vancomycin Treatment of Regressive-Onset Autism. *J Child Neurol* (2000) 15(7):429–35. doi: 10.1177/088307380001500701
102. Hamad AF, Alessi-Severini S, Mahmud SM, Brownell M, Kuo IF. Early Childhood Antibiotics Use and Autism Spectrum Disorders: A Population-Based Cohort Study. *Int J Epidemiol* (2018) 47(5):1497–506. doi: 10.1093/ije/dyy162
103. Wimberley T, Agerbo E, Pedersen CB, Dalsgaard S, Horsdal HT, Mortensen PB, et al. Otitis Media, Antibiotics, and Risk of Autism Spectrum Disorder. *Autism Res* (2018) 11(10):1432–40. doi: 10.1002/aur.2015
104. Axelsson PB, Clausen TD, Petersen AH, Hageman I, Pinborg A, Kessing LV, et al. Relation Between Infant Microbiota and Autism?: Results From a National Cohort Sibling Design Study. *Epidemiology* (2019) 30(1):52–60. doi: 10.1097/EDE.0000000000000928
105. Bittker SS, Bell KR. Acetaminophen, Antibiotics, Ear Infection, Breastfeeding, Vitamin D Drops, and Autism: An Epidemiological Study. *Neuropsychiatr Dis Treat* (2018) 14:1399–414. doi: 10.2147/NDT.S158811
106. Fallon J. Could One of the Most Widely Prescribed Antibiotics Amoxicillin/Clavulanate "Augmentin" be a Risk Factor for Autism? *Med Hypotheses* (2005) 64(2):312–5. doi: 10.1016/j.mehy.2004.06.023
107. Łukasik J, Patro-Gołąb B, Horvath A, Baron R, Szajewska H, Group SW. Early Life Exposure to Antibiotics and Autism Spectrum Disorders: A Systematic Review. *J Autism Dev Disord* (2019) 49(9):3866–76. doi: 10.1007/s10803-019-04093-y
108. Kang DW, Adams JB, Gregory AC, Borody T, Chittick L, Fasano A, et al. Microbiota Transfer Therapy Alters Gut Ecosystem and Improves Gastrointestinal and Autism Symptoms: An Open-Label Study. *Microbiome* (2017) 5(1):10. doi: 10.1186/s40168-016-0225-7

109. Kang DW, Adams JB, Coleman DM, Pollard EL, Maldonado J, McDonough-Means S, et al. Long-Term Benefit of Microbiota Transfer Therapy on Autism Symptoms and Gut Microbiota. *Sci Rep* (2019) 9(1):5821. doi: 10.1038/s41598-019-42183-0
110. Buffington SA, Di Prisco GV, Auchtung TA, Ajami NJ, Petrosino JF, Costa-Mattioli M. Microbial Reconstitution Reverses Maternal Diet-Induced Social and Synaptic Deficits in Offspring. *Cell* (2016) 165(7):1762–75. doi: 10.1016/j.cell.2016.06.001
111. Desbonnet L, Clarke G, Shanahan F, Dinan TG, Cryan JF. Microbiota Is Essential for Social Development in the Mouse. *Mol Psychiatry* (2014) 19 (2):146–8. doi: 10.1038/mp.2013.65
112. Diaz Heijtz R, Wang S, Anuar F, Qian Y, Björkholm B, Samuelsson A, et al. Normal Gut Microbiota Modulates Brain Development and Behavior. *Proc Natl Acad Sci USA* (2011) 108(7):3047–52. doi: 10.1073/pnas.1010529108
113. Leclercq S, Mian FM, Stanisz AM, Bindels LB, Cambier E, Ben-Amram H, et al. Low-Dose Penicillin in Early Life Induces Long-Term Changes in Murine Gut Microbiota, Brain Cytokines and Behavior. *Nat Commun* (2017) 8:15062. doi: 10.1038/ncomms15062
114. Sharon G, Cruz NJ, Kang DW, Gandal MJ, Wang B, Kim YM, et al. Human Gut Microbiota From Autism Spectrum Disorder Promote Behavioral Symptoms in Mice. *Cell* (2019) 177(6):1600–18.e1617. doi: 10.1016/j.cell.2019.05.004
115. Hsiao EY, McBride SW, Hsien S, Sharon G, Hyde ER, McCue T, et al. Microbiota Modulate Behavioral and Physiological Abnormalities Associated With Neurodevelopmental Disorders. *Cell* (2013) 155(7):1451–63. doi: 10.1016/j.cell.2013.11.024
116. Golubeva AV, Joyce SA, Moloney G, Burokas A, Sherwin E, Arboleya S, et al. Microbiota-Related Changes in Bile Acid & Tryptophan Metabolism Are Associated With Gastrointestinal Dysfunction in a Mouse Model of Autism. *EBioMedicine* (2017) 24:166–78. doi: 10.1016/j.ebiom.2017.09.020
117. Lammert CR, Frost EL, Bolte AC, Paysour MJ, Shaw ME, Bellinger CE, et al. Cutting Edge: Critical Roles for Microbiota-Mediated Regulation of the Immune System in a Prenatal Immune Activation Model of Autism. *J Immunol* (2018) 201(3):845–50. doi: 10.4049/jimmunol.1701755
118. Kim S, Kim H, Yim YS, Ha S, Atarashi K, Tan TG, et al. Maternal Gut Bacteria Promote Neurodevelopmental Abnormalities in Mouse Offspring. *Nature* (2017) 549(7673):528–32. doi: 10.1038/nature23910
119. Vuong HE, Pronovost GN, Williams DW, Coley EJL, Siegler EL, Qiu A, et al. The Maternal Microbiome Modulates Fetal Neurodevelopment in Mice. *Nature* (2020) 586(7828):281–6. doi: 10.1038/s41586-020-2745-3
120. Vuong HE, Yano JM, Fung TC, Hsiao EY. The Microbiome and Host Behavior. *Annu Rev Neurosci* (2017) 40:21–49. doi: 10.1146/annurev-neuro-072116-031347
121. Li N, Yang J, Zhang J, Liang C, Wang Y, Chen B, et al. Correlation of Gut Microbiome Between Asd Children and Mothers and Potential Biomarkers for Risk Assessment. *Genomics Proteomics Bioinf* (2019) 17(1):26–38. doi: 10.1016/j.gpb.2019.01.002
122. Lee E, Cho J, Kim KY. The Association Between Autism Spectrum Disorder and Pre- and Postnatal Antibiotic Exposure in Childhood-A Systematic Review With Meta-Analysis. *Int J Environ Res Public Health* (2019) 16 (20):4042. doi: 10.3390/ijerph16204042
123. Hamad AF, Alessi-Severini S, Mahmud SM, Brownell M, Kuo IF. Prenatal Antibiotics Exposure and the Risk of Autism Spectrum Disorders: A Population-Based Cohort Study. *PloS One* (2019) 14(8):e0221921. doi: 10.1371/journal.pone.0221921
124. Nayeri T, Sarvi S, Moosazadeh M, Hosseininejad Z, Sharif M, Amouei A, et al. Relationship Between Toxoplasmosis and Autism: A Systematic Review and Meta-Analysis. *Microb Pathog* (2020) 147:104434. doi: 10.1016/j.micpath.2020.104434
125. Mahic M, Mjaaland S, Bøvelstad HM, Gunnes N, Susser E, Bresnahan M, et al. Maternal Immunoreactivity to Herpes Simplex Virus 2 and Risk of Autism Spectrum Disorder in Male Offspring. *mSphere* (2017) 2(1):e00016–17. doi: 10.1128/mSphere.00016-17
126. Slawinski BL, Talge N, Ingersoll B, Smith A, Glazier A, Kerver J, et al. Maternal Cytomegalovirus Sero-Positivity and Autism Symptoms in Children. *Am J Reprod Immunol* (2018) 79(5):e12840. doi: 10.1111/aji.12840
127. Zerbo O, Qian Y, Yoshida C, Grether JK, Van de Water J, Croen LA. Maternal Infection During Pregnancy and Autism Spectrum Disorders. *J Autism Dev Disord* (2015) 45(12):4015–25. doi: 10.1007/s10803-013-2016-3
128. Han VX, Patel S, Jones HF, Nielsen TC, Mohammad SS, Hofer MJ, et al. Maternal Acute and Chronic Inflammation in Pregnancy Is Associated With Common Neurodevelopmental Disorders: A Systematic Review. *Transl Psychiatry* (2021) 11(1):71. doi: 10.1038/s41398-021-01198-w
129. Belkaid Y, Hand TW. Role of the Microbiota in Immunity and Inflammation. *Cell* (2014) 157(1):121–41. doi: 10.1016/j.cell.2014.03.011
130. McDonald B, McCoy KD. Maternal Microbiota in Pregnancy and Early Life. *Science* (2019) 365(6457):984–5. doi: 10.1126/science.aay0618
131. Thomson CA, McColl A, Graham GJ, Cavanagh J. Sustained Exposure to Systemic Endotoxin Triggers Chemokine Induction in the Brain Followed by a Rapid Influx of Leukocytes. *J Neuroinflamm* (2020) 17(1):94. doi: 10.1186/s12974-020-01759-8
132. Nadeau S, Rivest S. Role of Microglial-Derived Tumor Necrosis Factor in Mediating CD14 Transcription and Nuclear Factor Kappa B Activity in the Brain During Endotoxemia. *J Neurosci* (2000) 20(9):3456–68. doi: 10.1523/JNEUROSCI.20-09-03456.2000
133. Quan N, Whiteside M, Herkenham M. Time Course and Localization Patterns of interleukin-1beta Messenger RNA Expression in Brain and Pituitary After Peripheral Administration of Lipopolysaccharide. *Neuroscience* (1998) 83(1):281–93. doi: 10.1016/S0306-4522(97)00350-3
134. Laflamme N, Soucy G, Rivest S. Circulating Cell Wall Components Derived From Gram-Negative, Not Gram-Positive, Bacteria Cause a Profound Induction of the Gene-Encoding Toll-like Receptor 2 in the CNS. *J Neurochem* (2001) 79(3):648–57. doi: 10.1046/j.1471-4159.2001.00603.x
135. de Magistris L, Familiari V, Pascotto A, Sapone A, Frolli A, Iardino P, et al. Alterations of the Intestinal Barrier in Patients With Autism Spectrum Disorders and in Their First-Degree Relatives. *J Pediatr Gastroenterol Nutr* (2010) 51(4):418–24. doi: 10.1097/MPG.0b013e3181dcc4a5
136. Fiorentino M, Sapone A, Senger S, Camhi SS, Kadzielski SM, Buie TM, et al. Blood-Brain Barrier and Intestinal Epithelial Barrier Alterations in Autism Spectrum Disorders. *Mol Autism* (2016) 7:49. doi: 10.1186/s13229-016-0110-z
137. Fowlie G, Cohen N, Ming X. The Perturbance of Microbiome and Gut-Brain Axis in Autism Spectrum Disorders. *Int J Mol Sci* (2018) 19(8):2251. doi: 10.3390/ijms19082251
138. Runge K, Tebartz van Elst L, Maier S, Nickel K, Denzel D, Matysik M, et al. Cerebrospinal Fluid Findings of 36 Adult Patients With Autism Spectrum Disorder. *Brain Sci* (2020) 10(6):355. doi: 10.3390/brainsci10060355
139. Thion MS, Low D, Silvin A, Chen J, Grisel P, Schulte-Schrepping J, et al. Microbiome Influences Prenatal and Adult Microglia in a Sex-Specific Manner. *Cell* (2018) 172(3):500–16.e516. doi: 10.1016/j.cell.2017.11.042
140. Erny D, Hrabě de Angelis AL, Jaitin D, Wieghofer P, Staszewski O, David E, et al. Host Microbiota Constantly Control Maturation and Function of Microglia in the CNS. *Nat Neurosci* (2015) 18(7):965–77. doi: 10.1038/nn.4030
141. Luck B, Engevik MA, Ganesh BP, Lackey EP, Lin T, Balderas M, et al. Bifidobacteria Shape Host Neural Circuits During Postnatal Development by Promoting Synapse Formation and Microglial Function. *Sci Rep* (2020) 10 (1):7737. doi: 10.1038/s41598-020-64173-3
142. Gabriele S, Sacco R, Altieri L, Neri C, Urbani A, Bravaccio C, et al. Slow Intestinal Transit Contributes to Elevate Urinary P-Cresol Level in Italian Autistic Children. *Autism Res* (2016) 9(7):752–9. doi: 10.1002/aur.1571
143. Saito Y, Sato T, Nomoto K, Tsuji H. Identification of Phenol- and P-Cresol-Producing Intestinal Bacteria by Using Media Supplemented With Tyrosine and Its Metabolites. *FEMS Microbiol Ecol* (2018) 94(9):fiy125. doi: 10.1093/femsec/fiy125
144. Sun CY, Li JR, Wang YY, Lin SY, Ou YC, Lin CJ, et al. P-Cresol Sulfate Caused Behavior Disorders and Neurodegeneration in Mice With Unilateral Nephrectomy Involving Oxidative Stress and Neuroinflammation. *Int J Mol Sci* (2020) 21(18):6687. doi: 10.3390/ijms21186687
145. Sun J, Xu J, Yang B, Chen K, Kong Y, Fang N, et al. Effect of Clostridium Butyricum Against Microglia-Mediated Neuroinflammation in Alzheimer's Disease Via Regulating Gut Microbiota and Metabolites Butyrate. *Mol Nutr Food Res* (2020) 64(2):e1900636. doi: 10.1002/mnfr.201900636

146. Lebovitz Y, Kowalski EA, Wang X, Kelly C, Lee M, McDonald V, et al. Lactobacillus Rescues Postnatal Neurobehavioral and Microglial Dysfunction in a Model of Maternal Microbiome Dysbiosis. *Brain Behav Immun* (2019) 81:617–29. doi: 10.1016/j.bbi.2019.07.025
147. Lee HJ, Hwang YH, Kim DH. Lactobacillus Plantarum C29-Fermented Soybean (Dw2009) Alleviates Memory Impairment in 5XFAD Transgenic Mice by Regulating Microglia Activation and Gut Microbiota Composition. *Mol Nutr Food Res* (2018) 62(20):e1800359. doi: 10.1002/mnfr.201800359
148. Choi J, Lee S, Won J, Jin Y, Hong Y, Hur TY, et al. Pathophysiological and Neurobehavioral Characteristics of a Propionic Acid-Mediated Autism-Like Rat Model. *PloS One* (2018) 13(2):e0192925. doi: 10.1371/journal.pone.0192925
149. Koh A, De Vadder F, Kovatcheva-Datchary P, Bäckhed F. From Dietary Fiber to Host Physiology: Short-Chain Fatty Acids as Key Bacterial Metabolites. *Cell* (2016) 165(6):1332–45. doi: 10.1016/j.cell.2016.05.041
150. Silva YP, Bernardi A, Frozza RL. The Role of Short-Chain Fatty Acids From Gut Microbiota in Gut-Brain Communication. *Front Endocrinol (Lausanne)* (2020) 11:25. doi: 10.3389/fendo.2020.00025
151. Wang L, Christophersen CT, Sorich MJ, Gerber JP, Angley MT, Conlon MA. Elevated Fecal Short Chain Fatty Acid and Ammonia Concentrations in Children With Autism Spectrum Disorder. *Dig Dis Sci* (2012) 57(8):2096–102. doi: 10.1007/s10620-012-2167-7
152. Cryan JF, Dinan TG. Mind-Altering Microorganisms: The Impact of the Gut Microbiota on Brain and Behaviour. *Nat Rev Neurosci* (2012) 13(10):701–12. doi: 10.1038/nrn3346
153. Bojović K, Ignjatović Đ, Soković Bajić S, Vojnović Milutinović D, Tomić M, Golić N, et al. Gut Microbiota Dysbiosis Associated With Altered Production of Short Chain Fatty Acids in Children With Neurodevelopmental Disorders. *Front Cell Infect Microbiol* (2020) 10:223. doi: 10.3389/fcimb.2020.00223
154. Yu R, Wu Z, Wang S, Zhang M, Zhou G, Li B. Isolation, Identification and Characterization of Propionic Acid Bacteria Associated With Autistic Spectrum Disorder. *Microb Pathog* (2020) 147:104371. doi: 10.1016/j.micpath.2020.104371
155. Kratsman N, Getselter D, Elliott E. Sodium Butyrate Attenuates Social Behavior Deficits and Modifies the Transcription of Inhibitory/Excitatory Genes in the Frontal Cortex of an Autism Model. *Neuropharmacology* (2016) 102:136–45. doi: 10.1016/j.neuropharm.2015.11.003
156. Huuskonen J, Suuronen T, Nuutinen T, Kyrylenko S, Salminen A. Regulation of Microglial Inflammatory Response by Sodium Butyrate and Short-Chain Fatty Acids. *Br J Pharmacol* (2004) 141(5):874–80. doi: 10.1038/sj.bjp.0705682
157. Wenzel TJ, Gates EJ, Ranger AL, Klegeris A. Short-Chain Fatty Acids (Scfas) Alone or in Combination Regulate Select Immune Functions of Microglia-Like Cells. *Mol Cell Neurosci* (2020) 105:103493. doi: 10.1016/j.mcn.2020.103493
158. Koyama R, Ikegaya Y. Microglia in the Pathogenesis of Autism Spectrum Disorders. *Neurosci Res* (2015) 100:1–5. doi: 10.1016/j.neures.2015.06.005
159. Gabriele S, Sacco R, Cerullo S, Neri C, Urbani A, Tripi G, et al. Urinary P-Cresol Is Elevated in Young French Children With Autism Spectrum Disorder: A Replication Study. *Biomarkers* (2014) 19(6):463–70. doi: 10.3109/1354750X.2014.936911
160. Gomez de Aguero M, Ganal-Vonarburg SC, Fuhrer T, Rupp S, Uchimura Y, Li H, et al. The Maternal Microbiota Drives Early Postnatal Innate Immune Development. *Science* (2016) 351(6279):1296–302. doi: 10.1126/science.aad2571
161. McCoy KD, Thomson CA. The Impact of Maternal Microbes and Microbial Colonization in Early Life on Hematopoiesis. *J Immunol* (2018) 200(8):2519–26. doi: 10.4049/jimmunol.1701776
162. Al Nabhani Z, Dulauroy S, Marques R, Cousu C, Al Bounny S, Déjardin F, et al. A Weaning Reaction to Microbiota Is Required for Resistance to Immunopathologies in the Adult. *Immunity* (2019) 50(5):1276–88.e1275. doi: 10.1016/j.immuni.2019.02.014
163. Masi A, Quintana DS, Glozier N, Lloyd AR, Hickie IB, Guastella AJ. Cytokine Aberrations in Autism Spectrum Disorder: A Systematic Review and Meta-Analysis. *Mol Psychiatry* (2015) 20(4):440–6. doi: 10.1038/mp.2014.59
164. Moaaz M, Youssry S, Elfatatry A, El Rahman MA. Th17/Treg Cells Imbalance and Their Related Cytokines (IL-17, IL-10 and TGF-β) in Children With Autism Spectrum Disorder. *J Neuroimmunol* (2019) 337:577071. doi: 10.1016/j.jneuroim.2019.577071
165. Molloy CA, Morrow AL, Meinzen-Derr J, Schleifer K, Dienger K, Manning-Courtney P, et al. Elevated Cytokine Levels in Children With Autism Spectrum Disorder. *J Neuroimmunol* (2006) 172(1-2):198–205. doi: 10.1016/j.jneuroim.2005.11.007
166. Al-Ayadhi LY, Mostafa GA. Elevated Serum Levels of Interleukin-17A in Children With Autism. *J Neuroinflamm* (2012) 9:158. doi: 10.1186/1742-2094-9-158
167. Hsiao EY, McBride SW, Chow J, Mazmanian SK, Patterson PH. Modeling an Autism Risk Factor in Mice Leads to Permanent Immune Dysregulation. *Proc Natl Acad Sci USA* (2012) 109(31):12776–81. doi: 10.1073/pnas.1202556109
168. Careaga M, Schwartzer J, Ashwood P. Inflammatory Profiles in the BTBR Mouse: How Relevant Are They to Autism Spectrum Disorders? *Brain Behav Immun* (2015) 43:11–6. doi: 10.1016/j.bbi.2014.06.006
169. McColl A, Thomson CA, Nerurkar L, Graham GJ, Cavanagh J. TLR7-Mediated Skin Inflammation Remotely Triggers Chemokine Expression and Leukocyte Accumulation in the Brain. *J Neuroinflamm* (2016) 13(1):102. doi: 10.1186/s12974-016-0562-2
170. Thomson CA, McColl A, Cavanagh J, Graham GJ. Peripheral Inflammation Is Associated With Remote Global Gene Expression Changes in the Brain. *J Neuroinflamm* (2014) 11:73. doi: 10.1186/1742-2094-11-73
171. Dantzer R, O'Connor JC, Freund GG, Johnson RW, Kelley KW. From Inflammation to Sickness and Depression: When the Immune System Subjugates the Brain. *Nat Rev Neurosci* (2008) 9(1):46–56. doi: 10.1038/nrn2297
172. Pasciuto E, Burton OT, Roca CP, Lagou V, Rajan WD, Theys T, et al. Microglia Require Cd4 T Cells to Complete the Fetal-to-Adult Transition. *Cell* (2020) 182(3):625–40.e624. doi: 10.1016/j.cell.2020.06.026
173. Bravo JA, Forsythe P, Chew MV, Escaravage E, Savignac HM, Dinan TG, et al. Ingestion of Lactobacillus Strain Regulates Emotional Behavior and Central GABA Receptor Expression in a Mouse Via the Vagus Nerve. *Proc Natl Acad Sci USA* (2011) 108(38):16050–5. doi: 10.1073/pnas.1102999108
174. Sgritta M, Dooling SW, Buffington SA, Momin EN, Francis MB, Britton RA, et al. Mechanisms Underlying Microbial-Mediated Changes in Social Behavior in Mouse Models of Autism Spectrum Disorder. *Neuron* (2019) 101(2):246–59.e246. doi: 10.1016/j.neuron.2018.11.018
175. Zanos TP, Silverman HA, Levy T, Tsaava T, Battinelli E, Lorraine PW, et al. Identification of Cytokine-Specific Sensory Neural Signals by Decoding Murine Vagus Nerve Activity. *Proc Natl Acad Sci USA* (2018) 115(21):E4843–52. doi: 10.1073/pnas.1719083115
176. Goehler LE, Gaykema RP, Opitz N, Reddaway R, Badr N, Lyte M. Activation in Vagal Afferents and Central Autonomic Pathways: Early Responses to Intestinal Infection With Campylobacter Jejuni. *Brain Behav Immun* (2005) 19(4):334–44. doi: 10.1016/j.bbi.2004.09.002
177. Johnston GR, Webster NR. Cytokines and the Immunomodulatory Function of the Vagus Nerve. *Br J Anaesth* (2009) 102(4):453–62. doi: 10.1093/bja/aep037
178. Bonaz B, Bazin T, Pellissier S. The Vagus Nerve At the Interface of the Microbiota-Gut-Brain Axis. *Front Neurosci* (2018) 12:49. doi: 10.3389/fnins.2018.00049
179. Hosoi T, Okuma Y, Matsuda T, Nomura Y. Novel Pathway for LPS-induced Afferent Vagus Nerve Activation: Possible Role of Nodose Ganglion. *Auton Neurosci* (2005) 120(1-2):104–7. doi: 10.1016/j.autneu.2004.11.012
180. Lacolley P, Owen JR, Sandock K, Lewis TH, Bates JN, Robertson TP, et al. 5-HT Activates Vagal Afferent Cell Bodies In Vivo: Role of 5-HT2 and 5-HT3 Receptors. *Neuroscience* (2006) 143(1):273–87. doi: 10.1016/j.neuroscience.2006.07.032
181. Lal S, Kirkup AJ, Brunsden AM, Thompson DG, Grundy D. Vagal Afferent Responses to Fatty Acids of Different Chain Length in the Rat. *Am J Physiol Gastrointest Liver Physiol* (2001) 281(4):G907–15. doi: 10.1152/ajpgi.2001.281.4.G907
182. Pavlov VA, Tracey KJ. The Vagus Nerve and the Inflammatory Reflex–Linking Immunity and Metabolism. *Nat Rev Endocrinol* (2012) 8(12):743–54. doi: 10.1038/nrendo.2012.189

183. Forsythe P, Bienenstock J, Kunze WA. Vagal Pathways for Microbiome-Brain-Gut Axis Communication. *Adv Exp Med Biol* (2014) 817:115–33. doi: 10.1007/978-1-4939-0897-4_5
184. Madore C, Yin Z, Leibowitz J, Butovsky O. Microglia, Lifestyle Stress, and Neurodegeneration. *Immunity* (2020) 52(2):222–40. doi: 10.1016/j.immuni.2019.12.003
185. Thomson CA, Nibbs RJ, McCoy KD, Mowat AM. Immunological Roles of Intestinal Mesenchymal Cells. *Immunology* (2020) 160(4):313–24. doi: 10.1111/imm.13191
186. Kelly JR, Kennedy PJ, Cryan JF, Dinan TG, Clarke G, Hyland NP. Breaking Down the Barriers: The Gut Microbiome, Intestinal Permeability and Stress-Related Psychiatric Disorders. *Front Cell Neurosci* (2015) 9:392. doi: 10.3389/fncel.2015.00392
187. Hickman SE, Kingery ND, Ohsumi TK, Borowsky ML, Wang LC, Means TK, et al. The Microglial Sensome Revealed by Direct RNA Sequencing. *Nat Neurosci* (2013) 16(12):1896–905. doi: 10.1038/nn.3554
188. Lee YB, Nagai A, Kim SU. Cytokines, Chemokines, and Cytokine Receptors in Human Microglia. *J Neurosci Res* (2002) 69(1):94–103. doi: 10.1002/jnr.10253
189. Olson JK, Miller SD. Microglia Initiate Central Nervous System Innate and Adaptive Immune Responses Through Multiple Tlrs. *J Immunol* (2004) 173 (6):3916–24. doi: 10.4049/jimmunol.173.6.3916
190. Rochfort KD, Collins LE, Murphy RP, Cummins PM. Downregulation of Blood-Brain Barrier Phenotype by Proinflammatory Cytokines Involves NADPH Oxidase-Dependent ROS Generation: Consequences for Interendothelial Adherens and Tight Junctions. *PloS One* (2014) 9(7): e101815. doi: 10.1371/journal.pone.0101815

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

frontiers in Immunology

ORIGINAL RESEARCH
published: 24 June 2021
doi: 10.3389/fimmu.2021.670500

Chronic Stress-Induced Depression and Anxiety Priming Modulated by Gut-Brain-Axis Immunity

*Susan Westfall[1], Francesca Caracci[1], Molly Estill[2], Tal Frolinger[1], Li Shen[2] and Giulio M. Pasinetti[1,3]**

[1] Department of Neurology, Icahn School of Medicine at Mount Sinai, New York, NY, United States, [2] Department of Neuroscience, Icahn School of Medicine at Mount Sinai, New York, NY, United States, [3] Geriatric Research, Education and Clinical Center, James J. Peters Veterans Affairs Medical Center, Bronx, NY, United States

Chronic stress manifests as depressive- and anxiety-like behavior while recurrent stress elicits disproportionate behavioral impairments linked to stress-induced immunological priming. The gut-brain-microbiota-axis is a promising therapeutic target for stress-induced behavioral impairments as it simultaneously modulates peripheral and brain immunological landscapes. In this study, a combination of probiotics and prebiotics, known as a synbiotic, promoted behavioral resilience to chronic and recurrent stress by normalizing gut microbiota populations and promoting regulatory T cell (Treg) expansion through modulation of ileal innate lymphoid cell (ILC)3 activity, an impact reflecting behavioral responses better than limbic brain region neuroinflammation. Supporting this conclusion, a multivariate machine learning model correlatively predicted a cross-tissue immunological signature of stress-induced behavioral impairment where the ileal Treg/T helper17 cell ratio associated to hippocampal chemotactic chemokine and prefrontal cortex IL-1β production in the context of stress-induced behavioral deficits. In conclusion, stress-induced behavioral impairments depend on the gut-brain-microbiota-axis and through ileal immune regulation, synbiotics attenuate the associated depressive- and anxiety-like behavior.

Keywords: probiotic, nutraceutical, innate lymphocyte cells, psychiatry, microbiota

OPEN ACCESS

Edited by:
Francesca Ronchi, University of Bern, Switzerland

Reviewed by:
Kenjiro Ono, Showa University, Japan
Gerard M. Moloney, University College Cork, Ireland

***Correspondence:**
Giulio M. Pasinetti
giulio.pasinetti@mssm.edu

Specialty section:
This article was submitted to Multiple Sclerosis and Neuroimmunology, a section of the journal Frontiers in Immunology

Received: *21 February 2021*
Accepted: *08 June 2021*
Published: *24 June 2021*

Citation:
Westfall S, Caracci F, Estill M, Frolinger T, Shen L and Pasinetti GM (2021) Chronic Stress-Induced Depression and Anxiety Priming Modulated by Gut-Brain-Axis Immunity. Front. Immunol. 12:670500. doi: 10.3389/fimmu.2021.670500

INTRODUCTION

Chronic stress imposes persistent immunological changes to the periphery and brain priming the host to disproportionally respond to recurrent subthreshold stresses (1). The most common psychiatric responses to chronic stress include mood disorders such as major depressive disorder (MDD) and anxiety, while future sensitivity to recurring stressors can manifest as post-traumatic stress disorder (PTSD). Gut microbiota modifying agents including probiotics and prebiotics attenuate symptoms of stress-induced behavioral impairment (2, 3), yet the mechanisms remain in their infancy. Depression and anxiety associated with chronic stress have complex etiologies linked to maladaptive responses in neurovascular architecture, neuroendocrine signaling, and immune signaling (4, 5). Chronic stress has been causally linked to inflammation in the periphery (6, 7) and the brain (8) and this chronic low-grade inflammation resulting from chronic stress has become a major risk factor for the evolution of stress-induced psychiatric impairment (9). Inflammation

could act as a therapeutic target for depression and anxiety; however, the unique disparate immune regulation of the periphery and the brain provides pharmacological challenges (10). Notably, the microbiome-gut-brain-axis can indiscriminately regulate maladaptive immune responses in both the periphery and the brain (11) making it an attractive therapeutic target. This multifaceted strategy could reform the reductionist approaches of current therapeutic regimes for psychiatric impairment, which classically target only one protein or receptor leading to inconsistent and poor clinical outcomes (12).

Chronic stress priming to recurrent psychological impairment has been attributed to microglia activation (13, 14), associated to sterile inflammation in the hippocampus (15), peripheral IL-6 production (6), activation of major histocompatibility complex (MHC)II$^+$ CD11c$^+$ dendritic cells and Ly6C^{hi} monocytes (10), proinflammatory leukocytes (16), a migratory phenotype in myeloid cells (17) and trafficking of myeloid cells from the spleen into limbic brain regions (18). Expansion of circulating inflammatory monocytes may also confer susceptibility to recurrent stressful episodes (19) while infiltration of chemokine receptor 2 (CCR2$^+$) Ly6C^{hi} monocytes into the brain and their subsequent differentiation to IL-1β producing macrophages may exasperate neuroinflammatory phenotypes to recurrent challenges (20). It has also been proposed that the adaptive immune system may store a stressor's immunological memory facilitating protection against or sensitivity to future stress exposures (21).

The composition of the gut microbiota is an important influence for managing psychiatric health (22, 23). Anxiety-like behavior can be transferred by the gut microbiota (24) and attenuated by supplementation with certain probiotics (i.e. psychobiotics) (25–27). In general, reconstitution of germ-free mice with commensal microbiota or treatment with psychobiotics promotes IL-10 production and expansion of Tregs (27, 28). Communication between the gut microbiota and the immune system occurs through both cognate interactions and *via* its secreted metabolome including tryptophan metabolites, polyphenolic metabolites and short chain fatty acid production, which can modulate immune cells' responsiveness (29). Tryptophan catabolism is controlled by the gut microbiota producing metabolites that interact with the aryl hydrocarbon receptor (AHR). These interactions transcriptionally regulate innate immune cells and intestinal epithelial cells influencing the balance of pro- and anti-inflammatory cell types in the gastrointestinal associated lymphoid tissue (GALT) (30). We also identified that synbiotic-specific polyphenolic metabolites alter the Th17/Treg ratio, possibly through mechanisms involving the AHR (28). Although many pathways have been identified, there remains an innate complexity to the relationship between microbial metabolites and the immune system. Likewise, direct interactions have been identified between microbial metabolites and innate lymphoid cells (ILCs), dendritic cells, macrophages, monocytes, neutrophils and naïve lymphocytes [reviewed in (29)] diluting the discovery of direct gut microbiome-gut-brain axis interactions in the context of chronic or recurrent stress.

In a previous study, we showed that a synbiotic composed of *Lactobacillus fermentum, Bifidobacterium longum* and a polyphenol-rich prebiotic attenuated behavioral deficits to chronic stress by altering the Treg/Th17 ratio in the ileum through mechanisms potentially implicating the AHR (28). The prebiotic used was a Botanical Derived Polyphenolic Preparation (BDPP) composed of grape seed extract, concord grape juice and resveratrol, a cocktail we previously shown to be neuroprotective (31, 32). Our previous studies also showed that the plasma and brain bioavailability of BDPP's bioactive polyphenolic metabolites is greatly enhanced when administered in conjunction with probiotics as a synbiotic (28). In the current study, correlative gut-brain-axis associations in the context of recurrent stress-induced anxiety and depression were formulated using an unbiased machine learning algorithm. This technique was employed to build generalized associations between chronic and recurrent stress, gut microbiota composition and inflammatory markers in a manner that considers the variation between individuals within groups and the synergistic activities between the periphery and brain. This analysis created a set of putative mechanisms to be addressed in future work demonstrating that the gut-brain-axis cannot be considered as isolated tissues, but a synergistic system of gut, periphery and brain that can ultimately change the behavioral signature of stress-induced depression and anxiety.

MATERIALS AND METHODS

Bacteria and Fermentation Conditions

The bacterial cell lines *Lactobacillus plantarum* ATCC 793 (Lp793) and *Bifidobacterium longum* ATCC 15707 (Bl15707) were cultivated from frozen stock in Man-Rogosa-Sharpe (MRS) media and MRS with 0.05% cysteine, respectively, in an anaerobic incubator at 37°C as previously described (28).

Animal Husbandry

C57BL/6J male mice were purchased from The Jackson Laboratory (age 8 weeks; Bar Harbor, ME, USA) and group housed in the centralized animal care facility of the Center for Comparative Medicine and Surgery at the Icahn School of Medicine at Mount Sinai. Male mice were exclusively used in this study to normalize the behavioral effects due to stress; further studies will be conducted to directly compare sex in stress-induced behavioral deficits. All animals were maintained on a 12-h light/dark cycle in a temperature-controlled (20 ± 2°C) vivarium with access to food and water *ad libitum*. All procedures, protocols and behavioral experiments were approved by the Mount Sinai Institutional Animal Care and Use Committee (IACUC).

Animals were fed a polyphenol-free diet for the duration of all experiments (**Table S1**). Following the 2-week stabilization and acclimatization period for the mice, animals were placed on their respective treatment for 2 weeks prior to starting the stress protocol. The Bioactive Dietary Polyphenol Preparation (BDPP) was comprised of 1% *w/v* grape seed polyphenol

extract (GSPE; Healthy Origins), 1% *w*/*v* resveratrol (BulkSupplements.com) and a 5% *w*/*v* concord grape extract (AA Pharmachem, San Diego, USA) made in sterile water. All tested compounds were analyzed by liquid chromatography–mass spectrometry and archived as previously reported (28, 31) in compliance with National Institutes of Health, National Center for Complementary and Integrative Health (Bethesda, MD, USA) product integrity guidelines. Probiotic doses for mice were prepared by growing cultures in bulk as previously described (28). The bacteria were incorporated into the animals' drinking water at a final dosage of 1.0x10 (9) CFU/day per bacterium, calculated based on the average daily water consumption per cage, which was recalculated and replaced daily. The synbiotic was composed of BDPP and the probiotics and was replaced daily. Bacteria cultures were prepared in bulk, aliquoted at a known density and frozen at -80ºC until needed. This viability of this preparation was tested by freezing bacterial stocks as indicated and replating from thawed samples and preforming colony counts.

Chronic Unpredictable Stress Protocol

Mice were randomly subdivided into 8 treatment groups including untreated control (Control), BDPP-only (BDPP), probiotic or synbiotic and each treatment group was treated with one condition, either stressed or non-stressed. During the stress protocol, mice were submitted to 28 days of random mild stressors (CUS), allowed to rest for 28 days (CUS+Rest) followed by re-stimulation to a subthreshold mild unpredictable stress (US) for 7 days (CUS+US) to model recurrent stress. The CUS+US timepoint was controlled to a 7 day US without prior stress exposure as demonstrated in previous studied (**Figure 1A**) (15). For each treatment, condition (stressed or non-stressed) and timepoint, the group size included *n*=16 animals, combined over three independent experiments where tissues were allocated towards the biochemical and imaging studies equally. This group size was determined based on power analyses from previous data conducted by us (28) and others (15). During the stress periods, animals in all study groups, including the mice for the immunophenotyping, were subjected twice daily to random mild stressors (**Table S2**). Stressors included 45° cage tilt for 12 h, wet bedding for 10-12 h, no bedding for 10-12 h, food and/or water deprivation for 12 h, 4°C cold exposure for 1 h, cold water swim for 5 min, cage shaking for 20 min, reversed light schedule, restraint stress for 1 h, predator scent exposure for 8 h, or crowding with 12 animals/cage for 1 h. Consistent with our ongoing and published (28) experiments, no significant changes between groups were observed for weight, water or food consumption throughout the testing period. Behavioral assessment on all animals (n =16 per group) was conducted following each timepoint. From the 16 animals per group, 6 mice were perfused and used for immunofluorescent analysis, 6 mice were used for tissue RNA, protein (brain and peripheral tissues), blood and feces collection and the remaining 4 for other purposes not relevant to this manuscript. Animals were sacrificed immediately following behavior, blood was collected by cardiac puncture into heparinized tubes while tissues were frozen on dry ice and stored at -80°C until analysis.

Behavioral Experiments

Behavioral experiments were performed with a NIR camera and measured with ANY-maze™ tracking software (Stoelting Co., IL, USA. Version 5.1 Beta). All animals were handled for 5 min per day for 3 days prior to behavioral testing and were habituated to the testing room for 1 h at the beginning of the test day. Details for anxiety-like behavior with the open field test and depressive-like behavior with the forced swim test can be found as previously described (28).

16S DNA Extraction and Gut Microbiome Profiling

Fecal pellets from six mice were isolated from the distal colon from 6 mice per group. The Qiagen PowerFecal Pro DNA kit (Cat No. 12830-50) was used for DNA extraction with the Omni International BeadRupter 24 bead mill homogenizer. DNA from extracted samples was amplified using Invitrogen's AccuPrime High Fidelity kit using primers containing adapters for MiSeq sequencing and single-end barcodes allowing pooling and direct sequencing of PCR products (515F/806R) (33). The 16S rRNA gene sequencing was conducted by Diversigen (Houston, TX) (34). The 16S rRNA V4 gene region was amplified from the extracted community DNA by PCR and sequenced with the MiSeq platform (Illumina) using the 2x250 bp paired-end protocol. This generated paired-end reads that overlapped almost completely. Sequences that passed chimera slaying post sequencing were merged and blasted against the 16S specific curated Silva database (v132).

RNA Extraction and Real Time PCR

Previous studies showed promising changes in immunological gene regulation in the ileum, spleen and multiple brain regions (28). To confirm these changes in the current study for multiple time points, RNA from the spleen and ileal tissues were extracted using the Trizol reagent (ThermoFisher) while RNA from brain regions was extracted using the RNeasy Mini kit (Qiagen), with the same n = 6 animals per group as used for the 16S sequencing. cDNA synthesis was conducted with the High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (ThermoFisher). qPCR was conducted in collaboration with the Quantitative PCR CoRE at the Icahn School of Medicine at Mount Sinai using an ABI 7900HT Real-Time instrument and SDS software. Relative gene expression was assessed using the delta-delta CT method (35) and all primer pairs and annealing temperatures are provided in **Table S3**. All data was expressed in relative terms to the housekeeping gene *gapdh* following its demonstration of stability across tissue and treatment (36).

Protein Extraction and ELISA Assays

Protein from brain regions, spleen and ileum from the same mice used for RNA and fecal collection (n = 6) were homogenized in RIPA buffer (Sigma) with an added protease inhibitor cocktail (Sigma) and phenylmethylsulfonyl fluoride (PMSF) phosphatase inhibitor (ThermoFisher). ELISA assays for the cytokines IL-1β (limit of detection, LOD 15.6 pg/ml, R&D Biosystems), IL-10 (31.2 LOD, R&D Biosystems), IL-6 (LOD 4.0 pg/ml ThermoFisher) and

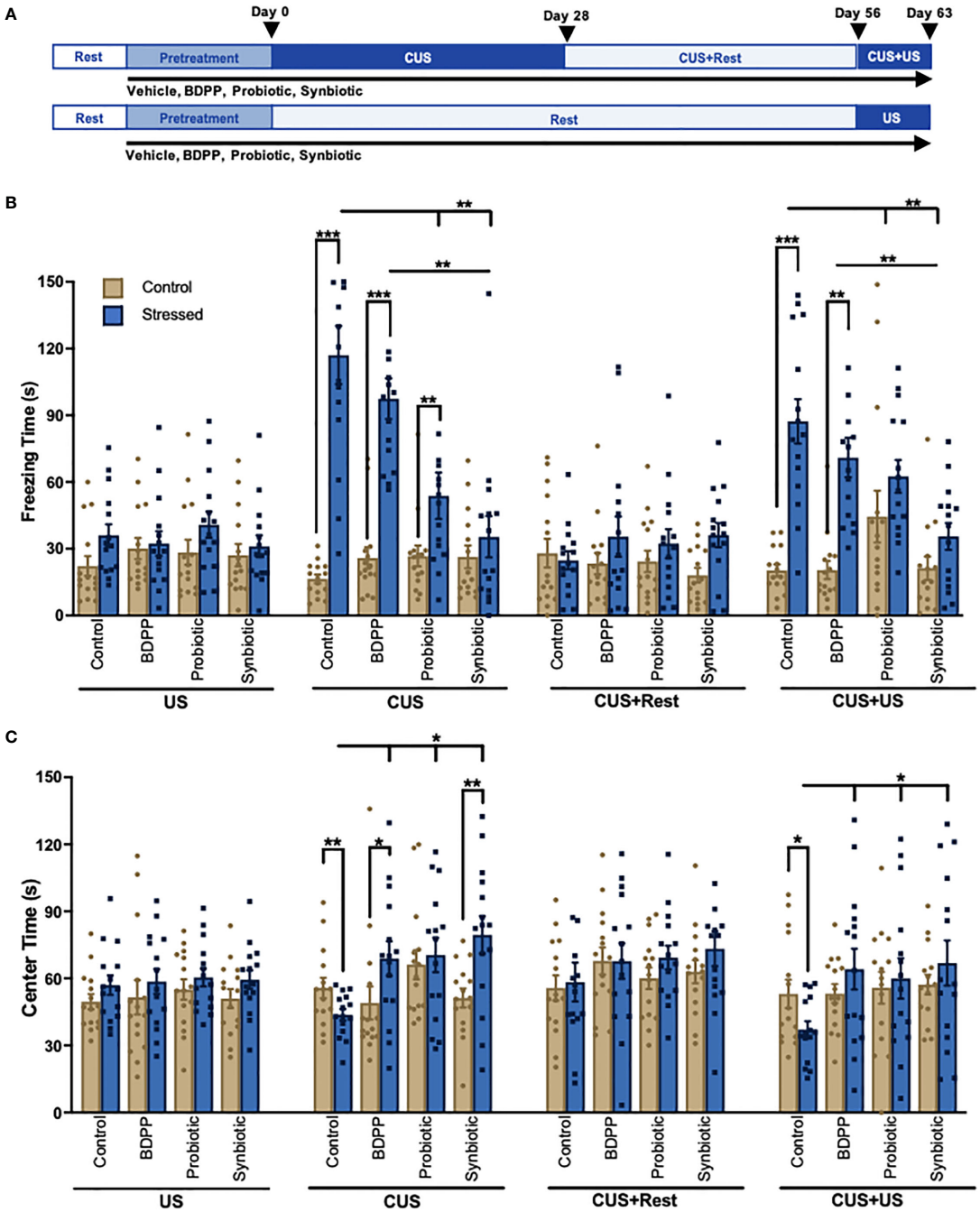


FIGURE 1 | A Synbiotic Attenuates Chronic-Stress Induced Psychological Deficits. **(A)** Chronic and recurrent stress are modelled using the chronic unpredictable stress (CUS) protocol. Following 2 weeks of rest and 2 weeks of pretreatment with the BDPP, probiotics or synbiotics, mice are exposed to 28 days of random mild unpredictable stressors (CUS) following by 28 days of rest (CUS+Rest) and a re-stimulation of 7 days of unpredictable stress (US) modelling recurrent stress (CUS+US). The final CUS+US timepoint can be compared to the 7-day subthreshold US. Each group contained $n = 16$ animals. Behavior phenotypes were assessed using the **(B)** forced swim test for depressive-like behavior and **(C)** open field test for anxiety-like behavior where for each group there are $n = 16$ mice +/- SEM and significance is determined with a two-way ANOVA and Tukey's post-hoc analysis. DC, dendritic cell; ILC, innate lymphoid cell; BC, B cell; Treg, regulatory T cell; Th17, T helper 17 cell; TNFβ, tumor necrosis factor β; TLR4, toll-like receptor 4; NFκB, nuclear factor kappa light chain enhancer of B cells; Casp1, caspase1; IL-1β, interleukin 1 beta; CCL2(MCP1), monocyte chemoattractant protein 1; CCL5(RANTES), C-C motif 5; ICAM, intercellular adhesion molecule; VCAM, vascular cell adhesion molecule. In all cases, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

IL-17A (ThermoFisher, LOD 4 pg/ml) were conducted as per company instructions.

Immunohistochemistry

At the time of sacrifice, mice (n = 6 per group, separate from the RNA, protein and fecal analysis) were cardiac perfused first with saline and then with 4% paraformaldehyde (PFA) and the brain including the brain stem was removed. Brain tissue was preserved in 4% PFA overnight and subsequently transferred to a saline solution containing sodium azide (0.02%). Sagittal sections were cut to 50 μm thickness using a vibratome (Leica VT1000S) and stored in saline with sodium azide. For immunostaining, slices were washed with PBST (PBS + 0.1% Triton X-100) and blocked in 5% normal goal serum. Slices were incubated with primary antibody (rabbit anti-mouse Iba1, clone EPR16588, abcam and rat anti-mouse CD68, clone FA-11, BioRad) and then secondary antibody (goat anti-rabbit AF568 and goat anti-rat AF488, ThermoFisher) before mounting with ProLong Diamond Antifade mounting media (ThermoFisher). Images were acquired on a Zeiss LSM880 Airyscan confocal microscope under an X20/0.8 NA air immersion 20 X objective. Image analysis was conducted with Image J. Microscopy image analysis was performed at the Microscopy CoRE at the Icahn School of Medicine at Mount Sinai.

Single Cell Suspensions

Samples processed for CyTOF were derived from a different cohort of mice than the reported behavioral and biochemical analyses with n = 3 animals per group. Blood was drawn by cardiac puncture and placed in EDTA blood collection tubes on ice. PBMCs were extracted by serially lysing red blood cells (RBC) in RBC lysis buffer. The spleen and ileum (distal 5 cm of the small intestine) were removed and placed in ice-cold RPMI supplemented with FBS (2%) and HEPES (15 mM). The spleen was washed, macerated and placed in a digestion buffer containing RPMI supplemented with FBS (5%), DNase I (0.5 mg/mL) and collagenase IV (0.4 mg/mL) at 37°C for 30 min with agitation. After 30 min, the spleen was pulverized with a 18G needle and filtered through 100 μM mesh on ice. Ileum samples, with Peyer's Patches removed, were washed in HBSS to remove fecal matter and cut longitudinally so mucus and remaining fecal matter could be removed. The whole ileum was placed in a dissociation buffer of RPMI supplemented with FBS (5%), EDTA (5 mM) and HEPES (15mM) for 20 min at 37°C with agitation. Following the 20 min, the dissociation buffer and ileum was filtered through a 100 μM filter the lamina propria was transferred to a digestion buffer of RPMI supplemented with FBS (5%), DNase I (0.5 mg/mL) and collagenase VIII (0.4 mg/mL) and incubated 25 min at 37°C with agitation. Like the spleen, the ileum was pulverized with a 18G needle and filtered through a 100 μM mesh on ice.

GIPA02 CyTOF Sample Processing and Data Acquisition

Samples were processed by the Mount Sinai Human Immune Monitoring Center. Cell counts were performed on the Nexcelom Cellaca Automated Cell Counter (Nexcelom Biosciences, Lawrence, MA, USA) and cell viability was measured utilizing Acridine Orange/Propidium Iodide viability staining reagent (Nexcelom). Cell counts were normalized such that four million cells were taken for downstream processing. After washing cells, live-cell barcoding was performed. Live-cell barcoding allows for the pooling of samples prior to CyTOF antibody labeling, thus eliminating batch staining variability between replicates (37). In this study, cadmium conjugated CD45 and MHCI (H-2) antibodies were utilized for live-cell barcoding, and replicates of each condition were live-cell barcoded together. Fc receptor blocking (Biolegend Inc., San Diego, CA, USA) and Rhodium-103 viability staining (Fluidigm) were performed simultaneously with live-cell barcoding. Next, surface staining (antibody list **Table S4**) was performed by resuspending each pooled sample in a scaled amount of CyTOF antibody cocktail. Next, the eBioscience Foxp3/Transcription Factor Staining Buffer Set (Thermo Fisher Scientific, Waltham, MA, USA) was used to perform intranuclear staining. Samples were then washed and palladium barcoding of each condition was performed utilizing the Fluidigm Cell-ID 20-Plex Pd Barcoding Kit (Fluidigm) following manufacturer's instructions. Conditions with unique combinatorial palladium labels were pooled such that only three samples remained (PBMC pool, spleen pool, ileum pool). This second tier of barcoding allowed intracellular staining to be performed on all of the conditions/replicates per tissue type in bulk. Heparin blocking (100 units/mL) was utilized to prevent non-specific binding of intracellular antibodies to eosinophils (38). Samples were fixed in 2.4% PFA. 125nM Iridium-193 (Fluidigm) and 2nM Osmium tetroxide (EMS) cell labeling was performed simultaneously with sample fixation (39). Samples were washed twice with CSB and stored in FBS + 10% DMSO at -80°C until acquisition (40).

Prior to data acquisition, samples were washed and resuspended at a concentration of 1 million cells per ml in Cell Acquisition Solution containing a 1:20 dilution of EQ Normalization beads (Fluidigm). The samples were acquired on a Helios Mass Cytometer equipped with a wide bore sample injector at an event rate of <400 events per second (41). After acquisition, repeat acquisitions of the same sample were concatenated and normalized using the Fluidigm software, and palladium-based debarcoding was performed utilizing the CyTOF debarcoding software made available from the Eli Zunder lab (42). These debarcoded files were uploaded to Cytobank for manual data clean-up/live-cell debarcoding.

For manual cleaning, immune cells were first identified based on Ir-193 DNA intensity and CD45 expression; Ce140+ normalization beads, CD45-low/Ir-193-low debris and cross-sample and Gaussian ion-cloud multiplets were excluded from downstream analysis. After this data cleanup, manual gating was utilized to debarcode the live-cell cadmium barcoded replicates. After this final debarcoding, the files were split by population such that each FCS file contained fully cleaned and debarcoded data from its corresponding sample. Lastly, for unbiased analysis of cell-subsets within each sample, the debarcoded files were run through the Astrolabe Diagnostics Data Processing pipeline (43).

viSNE plots and cell type gating were preformed using Cytobank (44). viSNE clustering was performed on 22 parameters (**Table S5**) where equal event sampling was selected using 6666 events in the spleen, ileum and PBMCs, the lowest common denominator in all samples.

MARS Algorithm and Correlation Analysis

All linear regression and Multivariate Adaptive Regression Splines (MARS) analyses were performed in R (version 4.0.2). The samples included were all derived from biological material from the same mice used for the 16S sequencing, RNA, protein and behavioral tests with $n = 6$ per group. Basic linear regression was performed in R using the lm function ("stats" R package, version 4.0.2). The association of individual bacterial species, gene expression and cytokines with subject behavior was examined with the following formula: log10(response) ~ Behavior + Day + Condition, where Day and Condition served as control variables. MARS analysis was performed in R using the "earth" package (version 5.1.2). The MARS approach allowed for the systematic identification of pertinent primary, secondary or higher levels of interactions among predictor variables. Briefly, MARS constructs a linear model with all possible basis terms (defined as hinge functions of predictors and the products of them), then by performing a stepwise term deletion in the full model, identifies the set of model terms that yield the best Generalized Coefficient of Variation (GCV). To assess the influence of microbiome on behavior, the MARS algorithm was run with the behavior as the response variable, while all available microbiome genera and conditions (i.e. time, stress or treatment) as predictors. To assess the influence of measured genes and cytokines on behavior, the MARS algorithm was run with the behavior as the response variable, while all available genes, cytokines, and conditions (i.e. time, stress or treatment) were predictors. The optimal parameters for MARS analysis, including the maximum degree of interactions, was determined using the generalized R-squared metric, which is the estimated model performance on unseen datasets. To systematically dissect which minor cell population was varying with respect to treatment and/or time, the MARS algorithm was repeated with the cell frequencies jointly as the predictor variable, and treating the timepoint, treatment and stress conditions as the response variable.

Statistics

All statistical analyses were completed in Graphpad Prism version 8.0. All within group comparisons for the behavior, gene and cytokine data were conducted with 2-way ANOVAs with Tukey's posthoc test while one-way ANOVAs with Tukey's post-hoc analysis for the microglia activation marks and immune cell frequency data. For the gut microbiome analysis, between group statistics of the phyla and genera were conducted with the Mann-Whitney U test (due to the non-parametric nature of gut microbiome data) with false discovery rate (FDR) corrections using the Benjamini-Hochberg method. Alpha diversity, representing microbiome variance within a sample, was calculated with the Fisher diversity index and Observed OTU abundances. Beta diversity, between sample variance, was estimated quantitatively with weighted UniFrac analysis distance matrices. Variation in community structure was assessed with permutational multivariate analyses of variance (PERMANOVA) between groups with visualization of the data using Principal Coordinates Analysis (PCoA).

RESULTS

A Synbiotic Attenuates Chronic and Recurrent Stress-Induced Behavioral Impairment

Chronic and recurrent stress were modelled using the CUS paradigm (**Figure 1A**). This paradigm was used to elucidate the gut microbiome-peripheral-brain-inflammation connection in driving chronic- and recurrent-stress induced behaviors. As previously shown, behavioral changes were not observed following US (15); however, significant depressive- (**Figure 1B**) and anxiety-like (**Figure 1C**) behaviors were observed in the stressed vehicle controls following CUS. Both behavioral impairments recovered following the rest period (CUS+Rest) and were re-observed with CUS+US re-stimulation. The probiotic and synbiotic attenuated depressive-like behavior compared to stressed vehicle controls following CUS, while the synbiotic rescued the phenotype following CUS and CUS+US (**Figure 1B**). All treatment groups elicited a beneficial effect on anxiety-like behavior following CUS and CUS+US compared to the stressed vehicle control (**Figure 1C**). Notably, only BDPP and the synbiotic, however, improved anxiety-like behavior compared to their respective unstressed controls at the CUS timepoint. Taken together, the synbiotic promoted more consistent and robust resilience to stress-induced behavioral impairments compared to its components.

Neuroinflammation in Limbic Brain Regions Do Not Associate With Synbiotic-Induced Behavioral Phenotypes in Chronic and Recurrent Stress

Studies have shown that microglia activation in limbic brain regions mirror behavioral responses of chronic and recurrent stress (15). In this study, microglia activation was determined by the activated CD68 positive surface area. There were no variations in microglia activation or number in the prefrontal cortex (PFC) with US, but following CUS, microglial activation was elevated in the stressed vehicles and attenuated by all treatments (**Figure 2A**, **Figure S2**, **Table S7**). Notably, microglia activation remained elevated at the CUS+Rest and CUS+US timepoints in discordance with behavioral observations while the synbiotic ubiquitously attenuated activated microglia at all time points. A similar analysis was done in the amygdala, hippocampus and nucleus of the solitary tract (NTS) of the cerebellum. In the amygdala, a similar trend was observed with respect to timepoint and treatment, while there were few inconsistent changes in both the hippocampus and the NTS (**Figures S1**, **S2**, **Tables S8–13**). To test if microglial activation could be reflected by transcriptional control of immune genes,

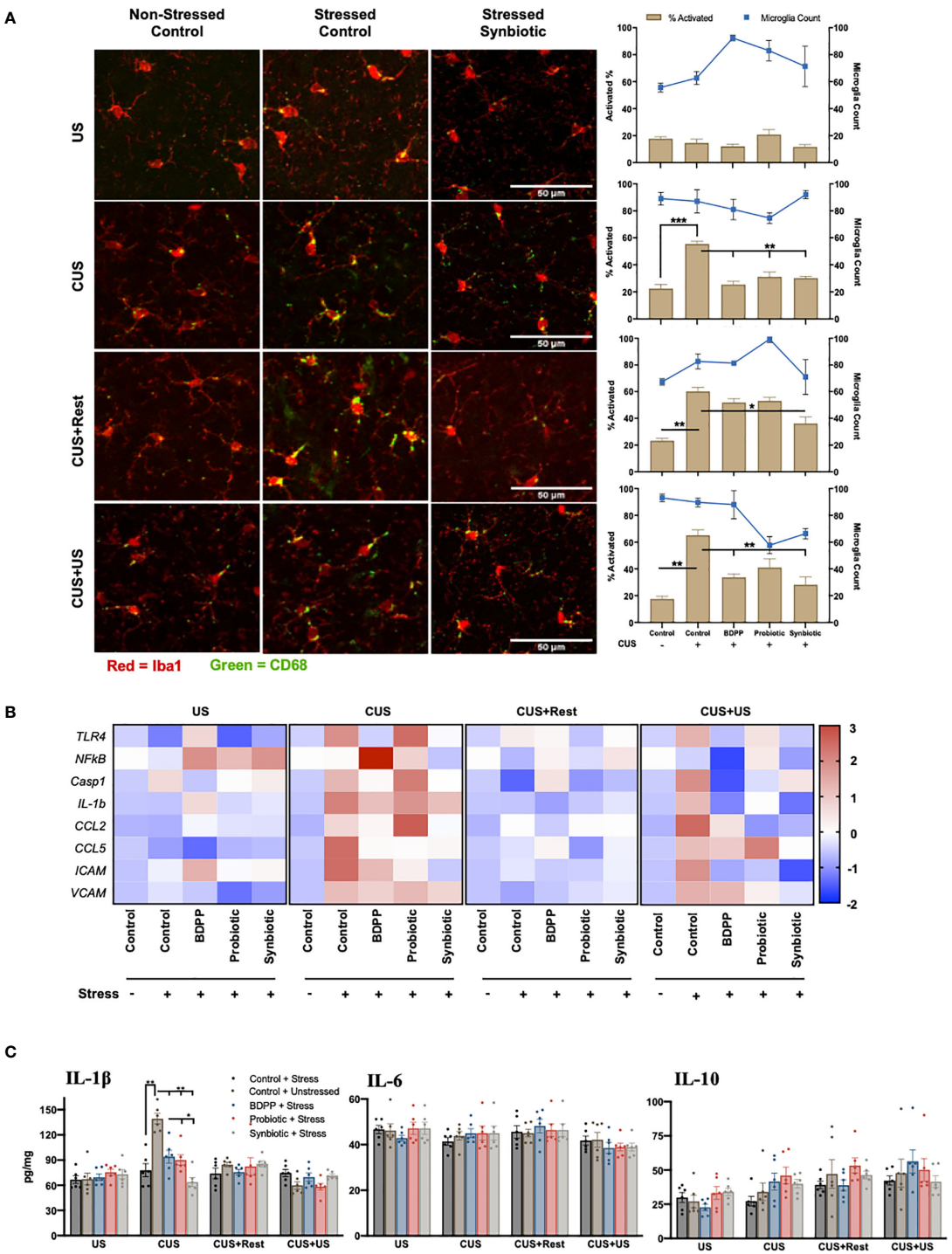


FIGURE 2 | Dissimilarity between recurrent stress behavioral phenotype with neuroinflammatory correlates. The behavioral phenotype was aligned with microglia activation in the prefrontal cortex as determined by **(A)** immunohistochemical analysis of Iba1 (red) and CD68 (green) for area and activation, respectively (n = 6 +/- SEM). Quantification was determined as the percentage of surface area covered by CD68 *vs.* Iba1 (brown bars, left axis) and displayed with the microglia count per frame (blue line, right axis) with significance determined with one-way ANOVA and Tukey's post-hoc analysis. **(B)** Immunological markers of microglia activation were validated with region-specific gene expression (n = 6, different mice than used for the IHC) and represented as the ratio of stressed *vs.* non-stressed for the respective group normalized with the z-score across all timepoints for a single gene. A positive z-score is represented with red and a negative z-score with blue. Absolute quantification of key cytokine expression **(C)** of IL-1β (left), IL-6 (middle) and IL-10 (right). Statistical markers are delineated by *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

quantification of key sterile immune factors, canonical activators and immune cell recruitment genes in the respective brain regions was conducted. Following CUS and CUS+US, there was an upregulation of almost all immune-associated genes including toll-like receptor (*Tlr*)4, caspase 1 (*Casp1*), *Il1β*, *Ccl2*, *Ccl5*, intercellular adhesion molecular (*Icam*) and vascular adhesion molecule (*Vcam*) in the PFC (**Figure 2B**, **Table S4**). At the gene level, the synbiotic, but not the probiotic or BDPP, robustly attenuated inflammatory gene expression profiles. Importantly, the most consistent and drastically impacted factors by both stress and the synbiotic, paralleling the behavioral response, were the immune cell recruitment genes, *Ccl2*, *Ccl5* and *Icam*. In the PFC, their expression in response to stress and treatment mirrored the behavioral response with the synbiotic attenuating their stress-induced elevation. This was similarly true for the gene and cytokine expression in the PFC, hippocampus, cortex and cerebellum, however less defined compared to the PFC (**Figure 2C**). This suggests that the recruitment of peripheral immune cells into the brain may be more important than microglia activation for driving the gut-microbiota stress-induced behavioral responses.

Gut Microbiota Variations Under Chronic and Recurrent Stress Normalized by Synbiotic Treatment

The gut microbiome is sensitive to both stress and treatment and plays a critical role in directing the immune response along the gut-brain-axis (2). To determine how the synbiotic modulates the gut microbiome's response to the stress protocol, 16S metagenomic sequencing was conducted. The stress protocol caused a significant shift in both the Fisher alpha (within sample variance) and beta diversities (between sample variance) in the gut microbiome of stressed vehicle controls (**Figure 3A**). The stress-induced variations in alpha and beta diversities were normalized by BDPP (**Figure 3B**) and the synbiotic (**Figure 3D**), but not probiotic treatment (**Figure 3C**). Based on these results, the chronic stress-induced variation following CUS can be interpreted as a loss of microbial diversity, which partially recovered after a period of rest. Note, that the alpha diversity was unchanged in unstressed treated controls; however all treatments elicited a similar shift in the beta diversity (**Figure S3A**). Likewise, treatment affected beta diversity following US and CUS (**Figures S3B, C**), while alpha diversity varied only following CUS due to an increase in diversity observed with probiotic and synbiotic treatment (**Figure S3C**). Interestingly, treatment had no effect on the alpha or beta diversities following CUS+Rest or CUS+US (**Figures S3D, E**). Taken together, the synbiotic was the most effective at normalizing the changes in gut microbiota diversity due to chronic and recurrent stress.

Quantification of the individual gut microbiome populations confirmed the consistent beneficial role of the synbiotic. There were no variations in either the phyla (**Figure S3F**) or genera (**Figure 3E**) of non-stressed treated controls due to either stress or treatment; however, both treatment and time elicited variations in the genera of stressed mice (**Figures 3F–I**, **S5**). The most drastic variation following CUS in the stressed vehicles was an increase in *Dubosiella* spp., which recovered after the rest period but failed to increase upon CUS+US (**Figure S4**). Following CUS, both probiotic and synbiotic treatments effectively reduced *Dubosiella* spp. levels (**Figure S5**), while the synbiotic attenuated *Dubosiella* levels at all time points (**Figure S4**). *Faecalibacterium* spp. is related to *Dubosiella* spp. yet there were no variations in *Faecalibacterium* spp. in stressed vehicle controls at US or CUS in contrast to the increase observed following CUS+Rest and CUS+US (**Figure S4**). This trend was paralleled by all treatment groups (**Figure S4**); however, the synbiotic elicited a proportionally larger increase in *Faecalibacterium* spp. than the other treatments (**Figures 3F**, **S5**). *Akkermansia* spp. is another important genera in terms of metabolite production, but there were no variations due to stress in vehicle controls at any timepoint (**Figure S4**). Importantly, the synbiotic alone elicited a positive increase in *Akkermansia* spp. following CUS compared to stressed vehicle controls (**Figures 3F**, **S5**). Finally, the probiotic and synbiotic elicited a stark increase (and BDPP a decrease) in *Lactococcus* spp. at all time points, compared to the non-stressed vehicle controls (**Figure S4**), yet there were no variations in *Lactococcus* spp. due to stress or timepoint in vehicle controls. Overall, the probiotic and synbiotic treatments normalized the major genera variations induced by stress in vehicle controls while the synbiotic elicited some unique changes in genera populations that play important roles in managing barrier integrity.

To understand how variations in the gut microbiota, and their interactions, may relate to the behavioral response in the context of chronic and recurrent stress, a MARS model was performed with behavior as the response and all available microbiome genera and conditions (i.e. time, stress or treatment) as predictors. In this context, the MARS algorithm takes into account the variations of all the gut microbiota populations, group and timepoint effect and the combined behavioral data to unbiasedly predict which groups of bacteria influencing the overall phenotype of chronic-stress induced behavioral impairment. The use of the MARS approach allows for the systematic identification of primary and secondary levels of interactions between gut microbiome populations, which optimize the prediction of stress-induced behavioral responses. It also controls the individual covariates' influence (i.e. time, stress or treatment) on the behavioral response based on the microbiome data. Depressive-like behavior associated with variations in *Lactobacillus* spp., *Ruminoclostridium* spp., and secondary interactions of *Lactobacillus* spp. with *Faecalibaculum, Blautia*, or *Bifidobacterium* spp. (**Figure 3J**). While the log10 transformed MARS coefficients appear very small, these interactions remain significant considering the extensive size of the model with multiple covariate influences. With a larger coefficient, the interaction of stress with either probiotic or synbiotic imposed a positive effect on the depressive-like behavior (**Figure 3J**). In contrast, anxiety-like behavior did not associate to any specific genera (**Figure 3K**), but synbiotic and probiotic treatment did associate to anxiety-like behavior. Additionally, the interaction of the synbiotic with stress or the CUS timepoint both associated to lower anxiety demonstrating the beneficial effect of the synbiotic.

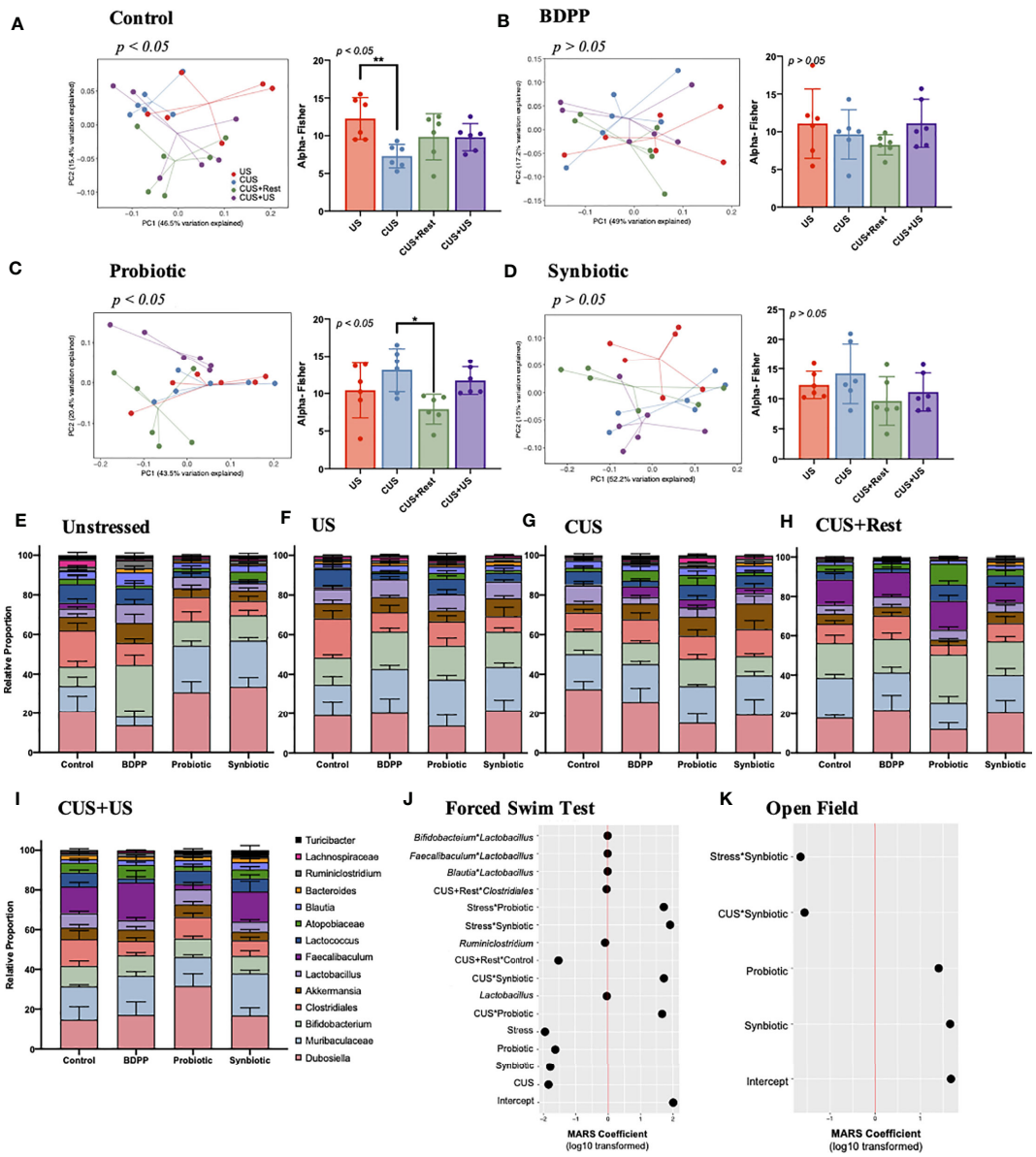


FIGURE 3 | Chronic and recurrent stress induced variations in the gut microbiota in response to synbiotic treatment. 16S V4 metagenomic sequencing of the gut microbiota following chronic and recurrent stress and treatment with variations in genera across all timepoints in the CUS protocol: US, CUS, CUS+Rest and CUS+US. Variation in community structure was assessed with permutational multivariate analyses of variance (PERMANOVA) between groups (beta diversity) with visualization of the data using Principal Coordinates Analysis (PCoA) with the lines representing the distance of each individual to the centroid. The alpha diversity calculated as the Fisher's Alpha test following **(A)** stressed vehicle control, and stress with **(B)** BDPP, **(C)** Probiotic and **(D)** Synbiotic. Statistical significance is marked by *p < 0.05 and **p < 0.01. Variations in the gut microbiota over time, within treatment group can be found in **Figure S3**. Variations in the 14 most abundant genera with respect to treatment is shown in **(E)** unstressed vehicle controls, **(F)** US, **(G)** CUS, **(H)** CUS+Rest and **(I)** CUS +US with statistical differences outlined in **Figure S4**. Results from the MARS algorithm show the interactions of gut microbiome and study covariates predicting **(J)** forced swim test and **(K)** open field test. The x-axis depicts the signed log10 transformed absolute value of the term coefficient in the MARS model. Note that the hinge function was removed for simplicity of visualization. Each group contains $n = 6$ mice (the same mice used for RNA/protein studies) +/- SEM individuals while significance determined with one-way ANOVA with Tukey's posthoc analysis. Statistics for the β-diversity is as outlined in material and methods section.

These results indicate that probiotics and synbiotics, but not BDPP, alter the gut microbiota in a manner correlating to attenuated stress-induced anxiety- and depressive-like behavior, while depressive-like behavior is more sensitive to specific gut microbiota genera including *Lactobacillus* spp. Further studies will be required to understand the direct relationship between genera specially altered by the synbiotic and the direct psychological consequences.

A Synbiotic Protects Against Intestinal Inflammation

Chronic stress and variations in the gut microbiota are intimately related to barrier immunity and programming of peripheral cellular immune responses. Further, the behavioral responses to stress and synbiotic treatment were more sensitive to the recruitment of peripheral immune cells to the brain than neuroinflammation. To characterize the peripheral innate and adaptive immune cell variations in response to stress and treatment, immunophenotyping was conducted with CyTOF. In the ileum, viSNE analysis gated by the major cell populations (**Figure 4A**) and the corresponding quantification of the major cell types (**Figure 4B**) revealed that neither treatment nor time elicited significant variations in the major ileal immune cell populations. Note that the viSNE plots were calculated for each timepoint so direct comparisons of the phenotypic islands cannot be compared between timepoints. Continuing broad characterization of the ileal immunological response, the gene and cytokine expression of key inflammatory components were determined. Following CUS and CUS+US, but not US or CUS+Rest, there was a strong upregulation of kynurenine and the proinflammatory cytokines in the ileum including IL-17A, IL-1β and IL-6, an effect rescued most strongly by the synbiotic (**Figure 4C**, **Table S5**). There was a correlating downregulation of the anti-inflammatory cytokine IL-10 in control mice in response to stress, while the synbiotic ubiquitously up-regulated IL-10 release. Notably, unlike the major cell populations, cytokine expression mirrored the stress-induced behavioral responses. A similar trend was observed for gene expression. US invoked few variations in immunological gene expression, but CUS and CUS+US strongly upregulated *Rorγt* and *Cyp1a1* in stressed vehicle controls, which were both downregulated exclusively by the synbiotic following CUS, but not CUS+US (**Figure 4D**, **Table S6**). The gene expression ratio of *Rorγt* to *Foxp3*, reflecting broadly the Th17 to Treg ratio, was upregulated by CUS and CUS+US in the stressed vehicle controls, and rescued by the synbiotic following CUS and all treatments following CUS+US (**Figure S6A**). This trend was similar to the ratio of IL-17A to IL-10 cytokine release (**Figure S6B**) indicating that the variations in proinflammatory gene expression elicited by stress and beneficially affected by the synbiotic may be due to the regulation of Th17 and Treg cell differentiation.

Enumeration of Tregs and Th17 cells and their activated states was determined using the CyTOF data. In stressed vehicle controls, ileal Tregs were reduced following CUS and CUS+US, an effect ubiquitously upregulated by the synbiotic, CUS+US by BDPP and CUS+Rest and CUS+US by probiotics (**Figure 4E**). Interestingly, $CTLA4^+$ Tregs (i.e. activated Tregs) were correspondingly decreased following CUS and CUS+US in stressed vehicle controls and elevated at all timepoints exclusively by the synbiotic (**Figure 4E**). A different trend was observed for the Th17 cells. Th17 cell numbers were elevated following CUS and CUS+US in the stressed vehicle controls, but only downregulated by the synbiotic in the CUS+US group (**Figure 4F**). Likewise, there were no significant changes in the $IL\text{-}17A^+ICOS^+$ Th17 cells (i.e. activated Th17) with respect to time or treatment. Concatenating this, the ratio of inactivated Th17/Treg cells in the ileum was significantly elevated in stressed vehicle controls following CUS and CUS+US, compared to unstressed vehicle controls. This stress-induced elevation was ubiquitously rescued only by the synbiotic at all time points (Fig S6c). A similar trend was observed for the ratio of "activated" Th17/Treg, except the synbiotic exclusively reduced the activated Th17/Treg ratio except at the CUS+US timepoint where probiotics also elicited a beneficial effect (**Figure S6C**). These trends were unique to the Treg and Th17 cells, as the other effector lymphocytes in the ileum including CD8 T cells (IFNγ+ and TNFα+), Th1 and Th2 cells showed inconsistent variations with respect to time and treatment (**Figure S7**, panels within).

To understand which innate immune cells may be activating the lymphocytes in the context of gut microbiota-inflammatory-stress interactions, several APCs were also assessed in the ileum. The quantities of monocytes, macrophages, dendritic cells (DC1, DC2, pDC), natural killer (NK) cells and neutrophils showed little variation with respect to either time or treatment (**Figure S7**, panels within). As reported in other studies, there was an increase in $MHCII^+$ monocytes in the ileum of stressed vehicle controls following CUS and CUS+Rest, which was reduced by all treatments (**Figure S7C**); however, there was no effect following CUS+US. Interestingly, ILC, ILC1, ILC2 (**Figure S7**) and ILC3 (**Figure 4G**) populations elicited few variations over time and treatment; however, the $IL\text{-}22^+$ (NCR^+) and $IL\text{-}17A^+$ (NCR^-) producing ILC3 cells showed trends paralleling the behavioral responses. IL-22 producing ILC3s were significantly downregulated in response to stress in vehicle controls, while synbiotic treatment exclusively upregulated their expression (**Figure S7J**). Likewise, synbiotic treatment downregulated the stress-induced increase in IL-17A producing ILC3 cells (**Figure S7K**). Overall, the ratio of IL-22/IL-17A producing ILC3s was reduced in response to stress and rescued exclusively by the synbiotic at both the CUS and CUS+US timepoints (**Figure 4H**). This is a significant result as IL-22 and IL-17A producing ILC3s are, in part, responsible for the differentiation of either Treg or Th17, respectively (45). It also suggests that manipulation of the gut microbiota with a synbiotic may reprogram the barrier immune architecture towards an NCR+ ILC3-Treg dominating phenotype that consequently prevents the stress-induced inflammatory milieu associated with chronic and recurrent stress.

Crosstalk Between Peripheral Tissues Predict Stress-Induced Behavioral Responses

To expand on the immunophenotyping of the ileum and gather a full understanding of the microbiota-peripheral-brain immune crosstalk, innate and adaptive immune cells in both the spleen (**Figure 5A**, **Figure S8A**, **Tables S14**, **15**) and PBMCs (**Figure 5B**, **Figure S8D**, **Tables S16**, **17**) were also immunophenotyped. In the spleen, stress-induced alterations in the Th17/Treg activated cell ratio was similar to the ileum, except the synbiotic elicited a less drastic impact (**Figure 5C**), which can be attributed to variations in the Treg population (**Figure S9G**). Variations in the Th17/Treg

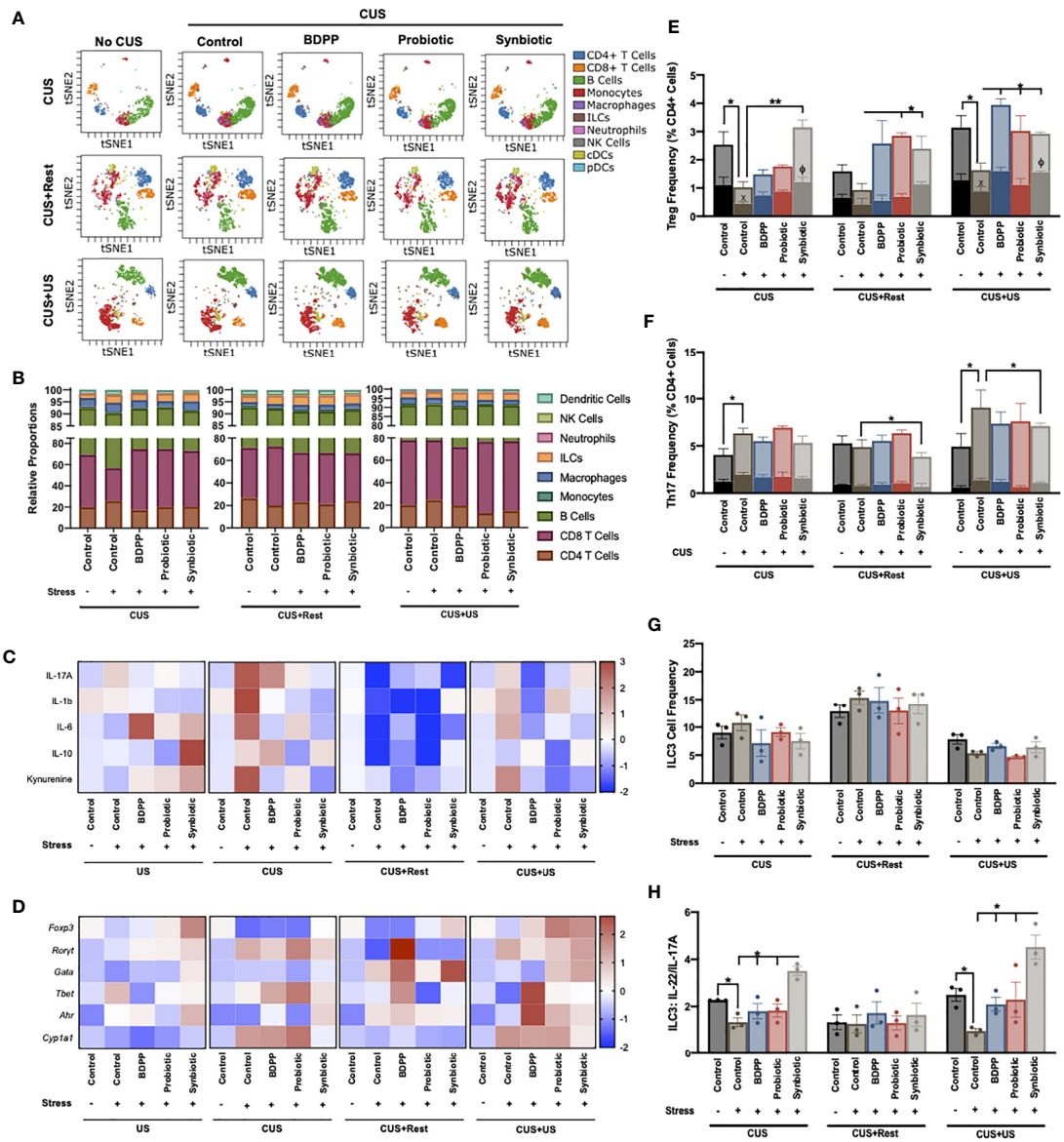


FIGURE 4 | Synbiotic alleviates ileal immune variations in response to chronic and recurrent stress. Immunophenotyping of the ileum was determined with a CyTOF panel and **(A)** overall visualization of the similarity of major cell populations in a single representative sample is depicted as a viSNE plot. The viSNE plot is two-dimensional figure with the axes tSNE1 and tSNE2, with cells plotted on a continuum of expression with phenotypically related cells clustered together based on gating of major cell populations and colored based on cell type called a "phenotypic island". Quantification of each of these cell populations (n =3) is shown in **(B)**. Overall immunological response is depicted as a heatmap of the **(C)** cytokine and **(D)** transcriptional profiles (n = 6) and represented as the ratio of relative values of stressed *vs.* non-stressed for the respective group normalized with the z-score across all timepoints for a single gene or cytokine. A positive z-score is represented with red and a negative z-score with blue. Individual cell frequencies of **(E)** regulatory T cells (Tregs) and **(F)** T helper (Th) 17 cells relative to total $CD4^+$ cell populations are shown with a solid color inset of activated Treg ($CTLA^+$) and Th17 ($IL17A^+ICOS^+$) cells, respectively. Frequency of **(G)** innate lymphoid cell (ILC)3 relative to total cell populations and **(H)** the ratio of IL-22 to IL-17A producing ILC3 cells are shown. All cell frequencies represent n = 3 mice +/- SEM with significance determined with a one-way ANOVA and Tukey's post-hoc analysis where *$p < 0.05$, **$p < 0.01$.

ratio were not observed among the PBMCs (**Figure 5F**). There was also an increase in $MHCII^+$ monocytes in the spleen following CUS in the stressed vehicle controls (**Figure 5D**) and a trending decrease of migratory $CD103^+$ DC1 cells, where the latter was rescued by the synbiotic following CUS and CUS+Rest (**Figure 5E**). There was a similar increase in activated monocytes among PBMCs following CUS in the stressed vehicle control, an effect rescued by synbiotics alone (**Figure 5G**); but no alterations due to stress or treatment were observed in the migratory DC1 population (**Figure 5H**).

Gene and cytokine expression previously shown to be altered by stress and synbiotic treatment in the periphery (28), showed

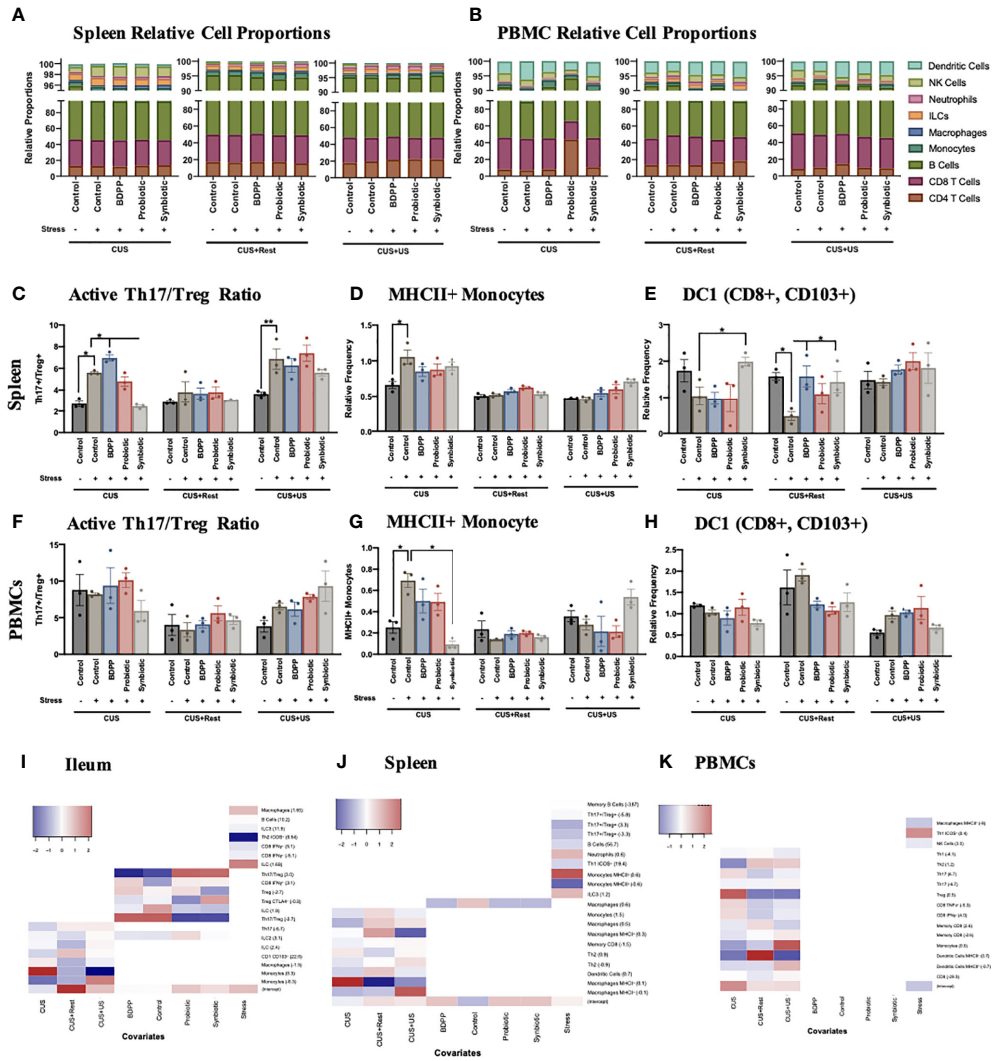


FIGURE 5 | Interaction between peripheral and central immune cell profiles determine stress-induced behaviors. Immunophenotyping of the major cell types in the **(A)** spleen and **(B)** PBMCs show little variation across time during the CUS protocol, which is also reflected in the viSNE plots (**Figure S9**). Splenic cell frequencies reflect the activated **(C)** Th17/Treg ratio, **(D)** MHCII+ monocytes and **(E)** migratory CD103+ dendritic cells (DC)1 relative to total cell populations. PBMC cell frequencies of **(F)** Th17/Treg ratio, **(G)** MHCII+ monocytes and **(H)** migratory CD103+ dendritic cells (DC)1 relative to total cell populations are also shown. All cell frequencies have $n = 3$ mice +/- SEM with significance calculated with a one-way ANOVA and Tukey's post-hoc analysis where *$p < 0.05$ and **$p < 0.01$. The cellular frequencies were associated to the individual study predictors (stress, timepoint and treatment) using a MARS additive model in the **(I)** ileum, **(J)** spleen and **(K)** PBMCs with the model hinge function in parentheses. The MARS term coefficient is represented as a heatmap with the higher coefficient represented with red color and the lower coefficient with blue.

less consistent variations in the context of chronic and recurrent stress. There was a stark increase in *Rorγt, Cyp1a1* and *Tlr4* in the splenic stressed vehicle controls following CUS, all rescued by synbiotic treatment (**Figure S8B**). Importantly, these variations were not observed in the CUS+US group uncoupling the inflammatory response in the spleen from the behavioral response. A similar trend was observed for splenic cytokine expression (**Figure S8C**). In the serum, the proinflammatory cytokines IL-1β and IL-17A were upregulated following both CUS and CUS+US in the stressed vehicle controls, and downregulated by the synbiotic (**Figure S8E**). Notably, IL-17A expression remained elevated following rest (CUS+Rest) in the stressed vehicle controls, also uncoupling it from the behavioral phenotype. Importantly, unlike in the ileum, the variations in immune cells observed in the PBMCs and spleen generally did not associate with the chronic and recurrent stress-induced behavioral impairment, yet a trend towards a chronic-stress induced peripheral immune cell phenotype may be apparent. As such, in both the spleen (**Figure S9**) and PBMCs (**Figure S10**), variations in minor cell populations were observed, yet not obviously correlated to the behavioral phenotypes.

To systematically dissect which minor cell populations were varying with respect to treatment and/or time, the MARS model was repeated, using an additive model, with the cell frequencies

as the predictors to the timepoint, treatment and stress as responses. As these data were collected from a separate cohort of animals not used for the above behavioral analyses, exploration of a link between cell frequencies and behavior was not attempted. This analysis will determine which of the cell frequencies are associated to the study conditions, i.e. day, stress or treatment. In the ileum, the Th17/Treg ratio was positively associated to the control and BDPP treatments, while negatively associated to probiotic and synbiotic, defining the beneficial effect specifically of the probiotic and synbiotic on this lymphocyte ratio within the context of chronic and recurrent stress (**Figure 5I**). A similar trend was observed for the activated CTLA4+ activated Tregs. Regarding the variation associated to timepoint (CUS, CUS+Rest or CUS+US), the monocytes, macrophages and dendritic cells all displayed significant associations. This apparent dichotomy between cell types responsive to timepoint (mostly APCs), cell types associated to treatment (lymphocytes and ILCs) and cell types varying with stress demonstrate a clear separation of regulatory mechanisms to be considered in the ileum of the current stress and treatment model.

A similar separation was observed in the spleen and PBMCS. In the spleen, there was a strong association of the immune cell subsets with the timepoint, while only macrophages weakly associated with treatment (**Figure 5J**). In addition, and in support of the cell frequency quantification, $MHCII^+$ monocytes were highly associated with the overall stress condition, whereas $MHCII^+$ macrophages more strongly associated to the individual timepoints. Finally, cell subsets in stressed animals significantly varied with respect to timepoint in the PBMCs, not treatment and only weakly with stress (**Figure 5K**). The strongest association in PBMCs was the activated $MHCII^+$ dendritic cells and the Tregs, which were both responsive to timepoints associated with stress. This analysis revealed that there is an immune cell tissue-dependency in the context of chronic and recurrent stress, with the ileum having a considerable association to both time and treatment and in the spleen and PBMCs, the associations are primarily with the timepoint.

A Peripheral-Central Immune Crosstalk Drives Chronic- and Recurrent-Stress Behavioral Deficits

The peripheral, especially ileal, immune response is an important predictor of chronic and recurrent-stress induced behavioral impairment; however, neuroinflammatory markers in limbic brain regions are common signatures of stress-induced depressive- and anxiety-like behaviors. To dissect which peripheral and/or brain-derived immune factors are associated to the chronic and recurrent stress-induced behavioral responses, linear and multivariate interactions between immune factors and the behavioral response were made. Data was only included which was collected from the same animal, allowing only the 16S sequencing, behavioral, RNA and protein enumerations to be included. Linear associations between the gene and cytokine factors with the behavior response were conducted, with the gene and cytokines acting as the response variable and the behavior as the main predictor. In this model, the stress, timepoint and treatment were all treated as covariates giving an overall indication of the how the behavioral response associates with single tissue-specific immune genes and cytokines. Note, that the effect size represents the strength of the association while correlation represents the overall fit of the model in the context of all the covariates. In both cases where the forced swim test (**Figure 6A**) or the open field test (**Figure 6B**) were used as the predictor, one striking observation was that there were more peripheral immune associations compared to brain-derived ones. Predicting depressive-like behavior, only *Il1β* gene expression in the PFC and cerebellar *Ccl5* elicited a positive effect size, with the PFC response having a higher correlation than the cerebellum, indicating that the entire model fit well for the behavioral prediction. Splenic IL-6 (gene and cytokine) and *Cyp1a1* also displayed positive effect sizes with splenic *Cyp1a1* having a strong correlation demonstrating the importance of AHR pathway signaling. The remaining seven associations occurred in the ileum with the proinflammatory markers kynurenine, IL-17, IL-1β and IL-6 having positive and *Foxp3* and *Gata3* gene expression negative effect sizes (**Figure 6A**). Predicting anxiety-like behavior, which has an opposite direction of beneficial effect compared to the forced swim test (i.e. "lower" value in the open field test means less anxiety-like behavior), cerebellar IL-6 had a positive effect size and good correlation while *Icam, Il10* and *Il1β* all had negative effect sizes and weak correlations (**Figure 6B**). Similar to the forced swim test data, splenic *Cyp1a1* had a negative effect size and *Gata3* gene expression a positive effect size and strong correlation to the model. Also similar to the depressive-like behavior, was the dominant representation of ileal immune associations. Again, *Foxp3* had a strong response to anxiety-like behavior in the ileum with an opposing effect size compared to the other proinflammatory markers such as kynurenine, *Nfkb, Tlr2, Il1β* and *Il10* (**Figure 6B**). Overall, the linear model confirms that the peripheral, namely ileal, immune responses are better associated than the limbic brain-derived immune factors to the chronic and recurrent stress-induced behavioral response within the context of gut microbiota modifying prebiotics, probiotics and synbiotic suggesting that a direct relationship may exist.

Linear associations give valuable insights to the impact of the behavioral response on a single gene or cytokine predictor; however, this model is limited as the genes and cytokines are assumed to be independent, which could contribute to the positive association or explain the low associative coefficient. To detangle these covariates and predict which individual factors across tissue, treatment and time may be synergizing in response to the behavioral output, the MARS approach was implemented. Similar to the linear model, behavior [either depressive-like (**Figure 6C**) or anxiety-like (**Figure 6D**)] was used as a response variable, while all available genes, cytokines and sample characteristics (timepoint, stress, and treatment) were used as predictors. Using the generalized R-squared as criterion, allowing a maximum of three interactions in a single term provided a better model fit than when a maximum of two

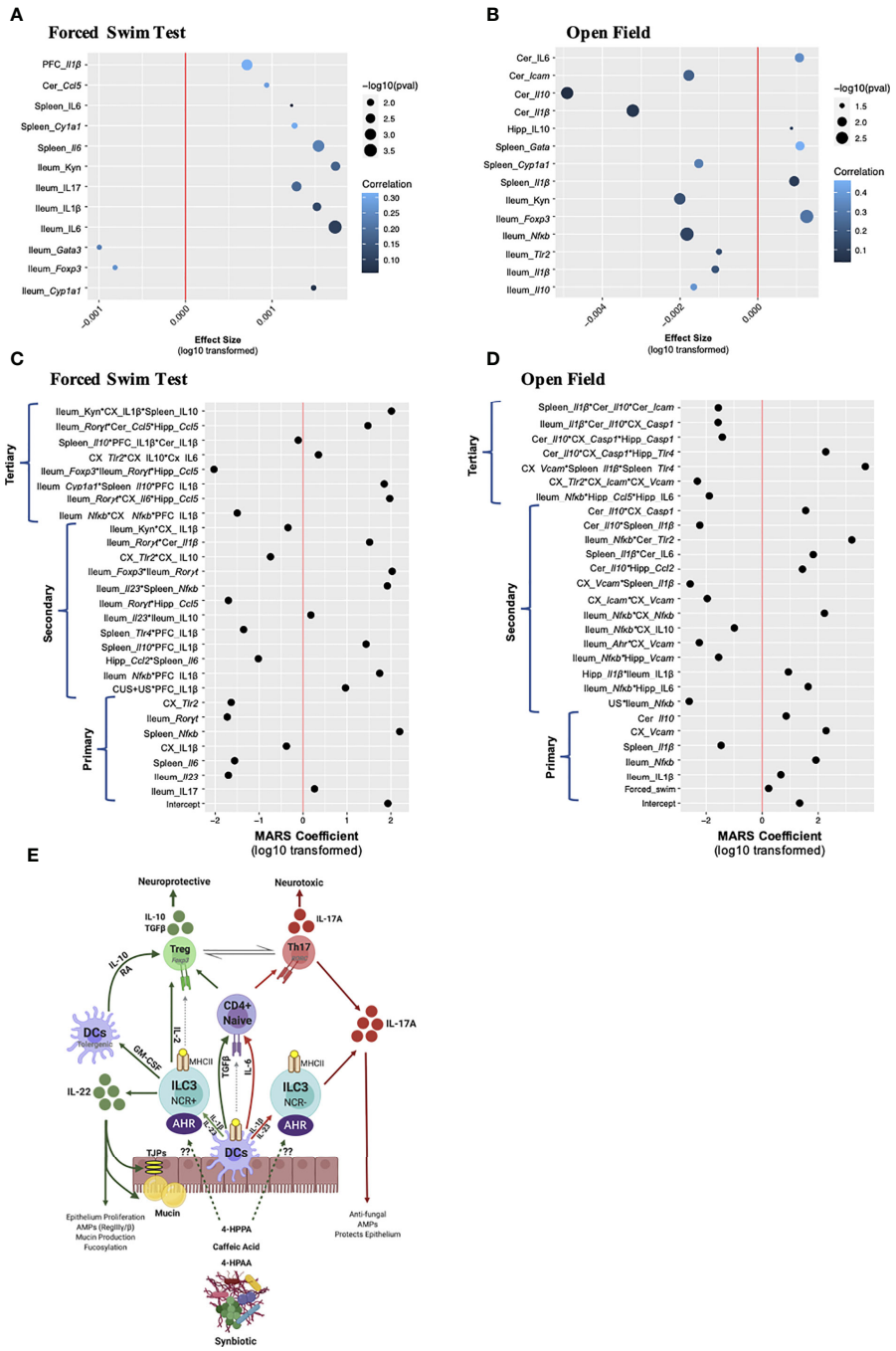


FIGURE 6 | Multivariate analysis of gut-brain-axis coordination towards chronic- and recurrent-stress behavioral predictions. Linear regression modeling in which the individual gene, cytokine, and microbiota were used as response variables, and the behavior was used as the main predictor of interest, while condition, treatment and timepoint were considered covariates. Association to **(A)** depressive-like behavior and **(B)** anxiety-like behavior are shown with the x-axis representing the effect size, the y-axis the term and the significance shown as dot size and correlation (i.e. how well the model fits the response variable) as a heatmap. The x-axis represents the absolute value of the response variable log10 transformed, and multiplied by the sign, to establish a normal distribution: i.e. log10(effectsize+1) when the effect size was positive, and log10(abs(effectsize-1))*(-1) when the effect size was negative. A multivariate adaptive regression splines (MARS) model was designed to test the cross-tissue association of gene and cytokine factors (up to tertiary interactions) in the context of predicting **(C)** depressive-like behaviors and **(D)** anxiety-like behaviors. The x-axis shows the log10 of the absolute value of the coefficient, multiplied by the coefficient sign. For both the linear and MARS models, factor names in italics represent mRNA gene expression while normal text is cytokine expression. **(E)** A working scheme of barrier immunity affected by chronic and recurrent stress and the role of the gut microbiota and its associated metabolites. For all MARS associations, data from the same $n = 6$ mice were included from the 16S sequencing, RNA, protein and behavioral analyses. DC, dendritic cell; ILC, innate lymphoid cell; NCR, natural cytotoxicity receptor; AHR, aryl hydrocarbon receptor; RA, retinoic acid.

interactions were allowed (see details in materials and methods section). Using this analysis, primary, secondary and tertiary interactions can be identified. Similar to the linear associations, the ileum had the highest predictive power, with genes (*Rorγt, IL-23R* and *Nfκb*) and cytokines (IL-17A and IL-1β) having significant primary associations in both behaviors tested. For the spleen, gene expression of *Il6, Il1β* and *Nfκb* likewise associated with behavior. In the brain, IL-1β in the PFC associated with depressive-like behavior whereas *Vcam, Tlr2* and IL-1β in the cortex and *Il-10* in the cerebellum also had significant primary effects.

The tertiary interactions bring a novel understanding to the capacity of the entire gut-brain-axis to drive chronic and recurrent stress-induced depressive-like behaviors synergizing across the tissues investigated. Interestingly, the triple association between ileal *Foxp3, Rorγt* and hippocampal *Ccl5* strongly associated with depressive-like behavior (**Figure 6C**) linking the Th17/Treg ratio to the recruitment of peripheral immune cells to the hippocampus. Likewise, ileal *Rorγt*, cerebellar *Ccl5* and hippocampal *Ccl5* were a strong predictor of depressive-like behavior as was ileal *Cyp1a1*, splenic *Il10* and PFC IL-1β. Ileal *Nfkb* expression also coordinated with hippocampal *Ccl5* and IL-6 production to predict anxiety-like behavior (**Figure 6D**). A splenic increase in *Il1β* and *Tlr4* with cortical *Vcam* expression strongly associated with the anxiety-like behavior while splenic IL-10 associated with IL-1β from both the PFC and cortex, predicted depressive-like behavior. These and the other associations illustrate how the gut-brain-axis may functionally coordinate with the limbic brain regions to predict stress-induced depressive- and anxiety-like behavior outlining the importance of the gut microbiota and barrier immunity in driving these responses. These correlations set the foundation for further studies to directly validate the impact of these singular and synergistic associations using transgenic mice and specifically immuno-compromised mice.

DISCUSSION

Chronic and recurrent psychological impairment are causally associated with inflammation (9, 46); however, the role of barrier immunity and the impact of gut microbiota on the peripheral and neuroinflammatory biological signatures of chronic and recurrent stress remain undefined. This is surprising as gut-brain associations were previously shown with studies outlining comorbidities between irritable bowel syndrome and psychiatric disorders including depression (47) and the benefit of probiotics for managing both the gastrointestinal and psychiatric symptoms (48). The gap in understanding for the role of barrier and peripheral immunity on the biological signature of chronic- and recurrent-stress induced psychiatric disorders formed the motivation of this study: to characterize the peripheral immunological changes elicited by a synbiotic that prophylactically prevents chronic and recurrent psychiatric symptoms associated with stress.

Chronic stress alters the commensal microbiota in mice (49) and humans (50). A dysbiotic microbiota has also been associated with MDD (51) and PTSD (52); however, neither functional nor causal links have been made between the gut microbiota and the symptoms associated with chronic or recurrent stress. We show that the diversity and complexity of the gut microbiota is reduced with chronic stress, which recovers following a period of rest yet does not recur following subthreshold recurrent stress. Despite this behavioral discordance, synbiotic treatment prevented the loss of diversity and complexity due to the stress protocol. Although there were no significant changes in phyla due to stress or treatment, *Dubosiella* spp. were highly upregulated following chronic, but not recurrent, stress, which was rescued by both probiotic and synbiotic treatment. *Dubosiella* spp. is composed largely of the species *Dubosiella newyorkensis*, which is closely related to *Faecalibaculum rodentium* (53). This novel genera was identified a part of the family *Erysipelotrichaceae* known to be important for host metabolism and inflammatory conditions associated with diet (54). *Erysipelotrichaceae* family members have been identified as "colitogenic strains", which are heavily coated in IgA antibodies, can transfer colitis-like symptoms through cohousing and associate with inflammasome-mediated intestinal dysbiosis (55, 56). These are significant associations as they confirm how the *Dubosiella* spp. may influence the CUS-induced behavioral phenotypes by driving intestinal inflammation. Since the synbiotic at all timepoints reduced *Dubosiella* spp. populations, it demonstrates how a synbiotic proves superior to its probiotic or prebiotic constituents to regulate the commensal gut microbiome populations, protecting it against an inflammatory microbiome.

Chronic and recurrent stress invoked several minor gut microbiome alterations with respect to treatment and timepoint and a machine learning MARS algorithm was used to dissect which species were associated with chronic and recurrent stress-induced psychiatric impairment, in the context of time and treatment covariates. The MARS algorithm is a powerful approach as it considers all the variations occurring simultaneously between multiple data, groups, treatments and timepoints taking into the consideration the variations of each of the individual animals tested. The current study produces a large dataset across tissue, treatment, time and modality to gather enough information to make accurate predictions. This unbiased approach allows educated associations to be built facilitating mechanistic conclusions to be made. Primary associations between the gut microbiome and behavior included *Lactobacillus* and *Ruminoclostridium* spp., while the interaction of *Lactobacillus* with either *Faecalibaculum, Blautia* or *Bifidobacterium* spp. elicited significant secondary associations. The association of *Lactobacillus* spp. with depression has been shown before as exogenous *Lactobacillus* supplementation attenuates symptoms of depression in humans (57) while reduced abundance of *Lactobacillus* and *Bifidobacterium* showed possible clinical associations with MDD (58). Nevertheless, based on the present comprehensive sampling and characterization of the chronic and recurrent-stress induced microbiome in the context of gut microbiota manipulation with prebiotics, probiotics and synbiotics, it can

be confirmed that these species, especially *Lactobacillus* spp., are the most significant at predicting stress-induced depressive like behavior and could serve as a biomarker of disease severity with therapeutic implications. As such, *Lactobacillus* spp. have been shown to improve barrier immunity by strengthening the gut epithelium in the context of endotoxin challenge and stimulating the increase in butyrate-producing *Faecalibacterium* and *Anaerotruncus* spp (59). while *Lactobacillus* and *Bifidobacterium* supplementation in aging mice improved barrier function, attenuated peripheral inflammation and simultaneously improved cognitive performance (60).

The impact of the gut microbiota on epithelial integrity can be attributed, in part, to its reprogramming of barrier immunity under homeostatic and challenged conditions. The GALT is the largest secondary lymphoid organ composed of a myriad of immune cells of the innate and adaptive immune systems that are in direct communication with the gut microbiota. The gut microbiota maintains barrier immune homeostasis by modulating the reactivity of resident APCs, facilitating the recruitment of immune cells under times of immune challenge and influencing differentiation of effector T cells (61). These actions alter the barrier and peripheral immune milieu subsequently influencing the inflammatory state of the periphery and the brain. For example, *Lactobacillus* spp. have been shown to influence dendritic cell maturation in mice and consequently bias the effector lymphocyte populations to either Treg (immature dendritic cells) or Th1/Th2 (mature DCs) responses (62) whereas *Bacteriodes fragilis* secretes bacterial polysaccharides (PSA) that alter CD4 T cell reactivity (63). Despite these observations, little is known about the gut microbiota-derived molecular meditators that facilitate this effect or how the microbiota can modulate the interaction of innate immune cells with the naïve effector T cells in the context of chronic and recurrent stress. The comprehensive immunophenotyping with CyTOF in multiple peripheral tissues revealed that variations in the ileal NCR^+ ILC3 population and correlating upregulation of Treg/Th17 ratio best associated to chronic- and recurrent-stress induced behavioral deficits. The ILCs are a group of innate immune cells expressing interleukin-7 receptor (IL-7Ra/CD127) and are found enriched at mucosal sites in the gut where they contribute to the maintenance of tissue homeostasis and host defense (64). The ILCs form cognate interactions with naïve CD4+ T cells directing their maturation in an activity- and microbiota-dependent manner (65). Murine ILC3 cells, characterized by *Rorγt* and IL-22 expression (66) lack TLRs (67) and are responsive to IL-1β production by macrophages, which stimulates IL-2 production facilitating differentiation of naïve CD4 cells into Tregs. IL-1β production by macrophages is dependent on MYD88- and NOD2-dependent sensing of the microbiota giving the microbiota a key role in determining the functional activity of ILC3 (68). These previously identified mechanisms are in line with the immunological data collected in this study. IL-1β and IL-6 production were primarily associated with the chronic and recurrent behavioral phenotypes in the ileum and PFC. In addition, based on the MARS association of the individual terms within the study covariates, ileal IL-1β production was associated with synbiotic treatment and the stress condition showing ileal IL-1β's contribution to recurrent stress-induced behavior. Microbiota-driven IL-1β production by macrophages also promotes GM-CSF (colony-stimulating factor 2 (Csf2)) by ILC3, which in turn stimulates production of retinoic acid and IL-10 driving Treg differentiation (45, 69). Csf2 production by ILC3s additionally promotes the generation of proinflammatory dendritic cells in the spleen (45), while migratory $CD103^+$ $CD11b^+$ dendritic cells have been implicated in the differentiation of Tregs (70). This putative downstream association was also observed in the current study as migratory splenic $CD103^+$ dendritic cells were downregulated due to chronic stress, and uniquely rescued by synbiotic treatment. This also demonstrates how multiple tissues are cooperating to elicit an adaptive response to chronic and recurrent stress following gut microbiota manipulation.

The AHR is another important component of ILC3 activation and the crosstalk of the gut microbiota with barrier immunity (71). Gut microbiota metabolites, especially those derived from tryptophan (i.e. kynurenine) or dietary polyphenols, activate the AHR (72). For example, *Lactobacillus* spp. ferment tryptophan producing indole-3-aldehyde augmenting IL-22 production by ILC3s (30), the stimulator of Treg differentiation. We previously showed that the AHR could be a putative communicator between the gut microbiota and the host in the context of stress-induced psychological impairment due to the variations in tryptophan metabolism (28), and the current study supports this conclusion. From the MARS analysis, ileal *Cyp1a1* transcription, a factor immediately downstream of the AHR, interacted with splenic IL-10 and PFC IL-1β to drive depressive-like behavior. This suggests that AHR signaling in the ileum could be contributing to the neuroinflammatory phenotype in the brain characteristic of chronic and recurrent stress-induced depression. Examining in depth the association of ileal *Cyp1a1* with the study covariates, ileal *Cyp1a1* variations were associated primarily with the behavioral response, and less with the timepoint or treatment conditions suggesting that microbial-AHR-inflammatory signaling could be a fundamental mechanism driving this gut-brain-axis communication.

An important distinction observed in the current study is that ILC3 polarization and subsequent lymphocyte activation is more critical than monocyte or dendritic cell activation for recurrent stress-induced psychological impairment. Previously, groups had postulated that variations in the activation of the innate immune response in $MHCII^+$ monocytes (19), $CD11c^+$ dendritic cells (10) and/or the infiltration of $Ly6C^{hi}$ monocytes into the brain (20) could be driving sensitivity to recurrent stress. We (28) and other groups (73) also identified that Tregs could drive the sensitivity to chronic and recurrent stress. In this study, comprehensive immunophenotyping of the peripheral and central, innate and adaptive immune responses demonstrated that the microbiome-ILC3-Treg-Th17 axis aligns best with the behavioral phenotypes of chronic and recurrent stress following supplementation with gut microbiota modulating factors (**Figure 6E**). Importantly, we also identified that a synbiotic can indiscriminately attenuate

stress-induced alterations in barrier and peripheral immunity and consequently, neuroinflammation. Activated monocytes and dendritic cells remained important for the chronic-stress associated behaviors; however, did not respond to synbiotic treatment nor correspond to the recurrent stress phenotype. As the nature of the machine learning algorithm is correlative, further studies should investigate how removing AHR signaling in Tregs or compromising ILC3 activity causally impacts the behavioral responses to chronic and recurrent stress. Future studies will directly address the question of whether the gut microbiota can alter the recruitment of immune cells into the brain to bring an additional level of understanding to the direct gut-brain-interactions. An important caveat to note is that different mouse strains from different vendors may express correspondingly different immune cell types, mostly due to the presence and activity of the gut microbiota. One group describes how the T:APC cell ratio may determine the functional activity of the immune system, another function of gut microbiota, dependent on strain background and diet (74). Based on this, we can draw these conclusions exclusively for the C57/Bl6 mouse and similar backgrounds on the polyphenol-free diet described in this study.

Epigenetic mechanisms are emerging as an important factor in immunological memory and depression (75, 76). Stress invokes stable epigenetic variations in the innate and adaptive immune cells termed trained adaptive/innate immunity with implications in the development of stress-induced neuropsychiatric disorders (77). In a six-year clinical study of 581 MDD patients, methylation profiles in the blood were found to be highly correlated to disease status with the major themes being immune cell migration and inflammation (78). Importantly, gut microbiota derived metabolites, including the histone deacetylase inhibitor butyrate, can reprogram the cellular epigenome, both proximally and distally (79). Indeed, we previously showed that epigenetic modifications driven by gut microbiota derived metabolites, especially methylation patterns in the IL-6 promoter, promoted resilience to stress-induced depression (80). Specific to immune cell function, ILCs activity is controlled, in part, by their epigenetic landscape. Each ILC subtype contains uniquely regulated enhancer elements enriched with H3K4me3 creating unique motif signatures. Interestingly, H3K4me3 enhancer regions were extensively altered following depletion of the gut microbiota with broad-spectrum antibiotic treatment. This depletion facilitated a shift in the ILC1 and ILC2 populations towards the RORγt-driven ILC3-like transcriptional profile confirming the dependence of ILC3 on microbial regulation (81). This substantiates the possibility that the synbiotic-specific metabolites may promote resilience to chronic and recurrent stress by invoking epigenetic modifications in the ILC3 cells promoting the differentiation of Tregs over Th17 cells. This, and other specific epigenetic programs should be explored in future studies to understand how cellular imprinting may be contributing to the exaggerated recurrent-stress induced psychiatric phenotypes.

In conclusion, this study describes how the gut-brain-axis can prime the barrier immune response to promote resilience to chronic and recurrent stress associated depressive- and anxiety-like psychological impairment. In particular, synbiotic-specific metabolites can shift ILC3 activity towards a NCR^+ IL-22 producing phenotype driving an increase in the beneficial Treg/Th17 ratio. Compared to the expression and activation of monocytes, macrophages, dendritic cells and microglia, only the microbiome-ILC3-Th17-Treg axis paralleled the behavioral phenotype in response to chronic and recurrent stress. In addition, these gut microbiota induced variations associated to the release of immune cell recruitment chemokines in the PFC and hippocampus causally linking the different tissues of the gut-brain-axis for chronic and recurrent stress management. Future experiments should explore possible epigenetic mechanisms involving the AHR and associated pathways to causally link specific synbiotic-derived metabolites with immune regulation of stress.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Mount Sinai Institutional Animal Care and Use Committee (IACUC).

AUTHOR CONTRIBUTIONS

SW conceived, conducted all *in vivo* work, analyzed data and wrote the manuscript. FC assisted SW in all aspects of conducting of the study. TF was instrumental in developing the CUS protocol and assisted in analyzing behavioral data. ME under the supervision of LS developed the MARS algorithm, data representation and interpretation, based on the data provided by SW and GP oversaw all aspects of the study and provided funding. All authors contributed to the article and approved the submitted version.

FUNDING

The study was supported by grant number P50 AT008661-01 and U19 AT010835 from the NCCIH and the ODS.

ACKNOWLEDGMENTS

The authors would also like to thank Daniel Geanon and Brian Lee at the Human Immunophenotyping Monitoring Core (HIMC) at Mount Sinai and Steven Chen for their support for the CyTOF experiments. The authors would also like to acknowledge the Mount Sinai QPCR core and Microscopy Cores for the support of the facility and staff.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.670500/full#supplementary-material

REFERENCES

1. Charney DS, Manji HK. Life Stress, Genes, and Depression: Multiple Pathways Lead to Increased Risk and New Opportunities for Intervention. *Sci STKE* (2004) 2004(225):re5. doi: 10.1126/stke.2252004re5
2. Burokas A, Arboleya S, Moloney RD, Peterson VL, Murphy K, Clarke G, et al. Targeting the Microbiota-Gut-Brain Axis: Prebiotics Have Anxiolytic and Antidepressant-Like Effects and Reverse the Impact of Chronic Stress in Mice. *Biol Psychiatry* (2017) 82(7):472–87. doi: 10.1016/j.biopsych.2016.12.031
3. Petra AI, Panagiotidou S, Hatziagelaki E, Stewart JM, Conti P, Theoharides TC. Gut-Microbiota-Brain Axis and Its Effect on Neuropsychiatric Disorders With Suspected Immune Dysregulation. *Clin Ther* (2015) 37(5):984–95. doi: 10.1016/j.clinthera.2015.04.002
4. Franklin TB, Saab BJ, Mansuy IM. Neural Mechanisms of Stress Resilience and Vulnerability. *Neuron* (2012) 75(5):747–61. doi: 10.1016/j.neuron.2012.08.016
5. Yang L, Zhao Y, Wang Y, Liu L, Zhang X, Li B, et al. The Effects of Psychological Stress on Depression. *Curr Neuropharmacol* (2015) 13 (4):494–504. doi: 10.2174/1570159x1304150831150507
6. Hodes GE, Pfau ML, Leboeuf M, Golden SA, Christoffel DJ, Bregman D, et al. Individual Differences in the Peripheral Immune System Promote Resilience Versus Susceptibility to Social Stress. *Proc Natl Acad Sci U S A* (2014) 111 (45):16136–41. doi: 10.1073/pnas.1415191111
7. Grippo AJ, Francis J, Beltz TG, Felder RB, Johnson AK. Neuroendocrine and Cytokine Profile of Chronic Mild Stress-Induced Anhedonia. *Physiol Behav* (2005) 84(5):697–706. doi: 10.1016/j.physbeh.2005.02.011
8. Wang YL, Han QQ, Gong WQ, Pan DH, Wang LZ, Hu W, et al. Microglial Activation Mediates Chronic Mild Stress-Induced Depressive- and Anxiety-Like Behavior in Adult Rats. *J Neuroinflamm* (2018) 15(1):21. doi: 10.1186/s12974-018-1054-3
9. Ménard C, Pfau ML, Hodes GE, Russo SJ. Immune and Neuroendocrine Mechanisms of Stress Vulnerability and Resilience. *Neuropsychopharmacology* (2017) 42(1):62–80. doi: 10.1038/npp.2016.90
10. Ambrée O, Ruland C, Scheu S, Arolt V, Alferink J. Alterations of the Innate Immune System in Susceptibility and Resilience After Social Defeat Stress. *Front Behav Neurosci* (2018) 12:141. doi: 10.3389/fnbeh.2018.00141
11. van de Wouw M, Boehme M, Dinan TG, Cryan JF. Monocyte Mobilisation, Microbiota & Mental Illness. *Brain Behav Immun* (2019) 81:74–91. doi: 10.1016/j.bbi.2019.07.019
12. Swinney DC, Anthony J. How Were New Medicines Discovered? *Nat Rev Drug Discov* (2011) 10(7):507–19. doi: 10.1038/nrd3480
13. Wohleb ES, Hanke ML, Corona AW, Powell ND, Stiner LM, Bailey MT, et al. β-Adrenergic Receptor Antagonism Prevents Anxiety-Like Behavior and Microglial Reactivity Induced by Repeated Social Defeat. *J Neurosci* (2011) 31(17):6277–88. doi: 10.1523/jneurosci.0450-11.2011
14. Iwata M, Ota KT, Li XY, Sakaue F, Li N, Dutheil S, et al. Psychological Stress Activates the Inflammasome *Via* Release of Adenosine Triphosphate and Stimulation of the Purinergic Type 2x7 Receptor. *Biol Psychiatry* (2016) 80 (1):12–22. doi: 10.1016/j.biopsych.2015.11.026
15. Franklin TC, Wohleb ES, Zhang Y, Fogaça M, Hare B, Duman RS. Persistent Increase in Microglial Rage Contributes to Chronic Stress-Induced Priming of Depressive-Like Behavior. *Biol Psychiatry* (2018) 83(1):50–60. doi: 10.1016/j.biopsych.2017.06.034
16. Cohen S, Janicki-Deverts D, Doyle WJ, Miller GE, Frank E, Rabin BS, et al. Chronic Stress, Glucocorticoid Receptor Resistance, Inflammation, and Disease Risk. *Proc Natl Acad Sci U S A* (2012) 109(16):5995–9. doi: 10.1073/pnas.1118355109
17. Powell ND, Sloan EK, Bailey MT, Arevalo JM, Miller GE, Chen E, et al. Social Stress Up-Regulates Inflammatory Gene Expression in the Leukocyte Transcriptome *Via* β-Adrenergic Induction of Myelopoiesis. *Proc Natl Acad Sci U S A* (2013) 110(41):16574–9. doi: 10.1073/pnas.1310655110
18. Wohleb ES, McKim DB, Shea DT, Powell ND, Tarr AJ, Sheridan JF, et al. Re-Establishment of Anxiety in Stress-Sensitized Mice is Caused by Monocyte Trafficking From the Spleen to the Brain. *Biol Psychiatry* (2014) 75(12):970–81. doi: 10.1016/j.biopsych.2013.11.029
19. Gururajan A, van de Wouw M, Boehme M, Becker T, O'Connor R, Bastiaanssen TFS, et al. Resilience to Chronic Stress Is Associated With Specific Neurobiological, Neuroendocrine and Immune Responses. *Brain Behav Immun* (2019) 80:583–94. doi: 10.1016/j.bbi.2019.05.004
20. Wohleb ES, Powell ND, Godbout JP, Sheridan JF. Stress-Induced Recruitment of Bone Marrow-Derived Monocytes to the Brain Promotes Anxiety-Like Behavior. *J Neurosci* (2013) 33(34):13820–33. doi: 10.1523/jneurosci.1671-13.2013
21. Lewitus GM, Schwartz M. Behavioral Immunization: Immunity to Self-Antigens Contributes to Psychological Stress Resilience. *Mol Psychiatry* (2009) 14(5):532–6. doi: 10.1038/mp.2008.103
22. Luczynski P, McVey Neufeld KA, Oriach CS, Clarke G, Dinan TG, Cryan JF. Growing Up in a Bubble: Using Germ-Free Animals to Assess the Influence of the Gut Microbiota on Brain and Behavior. *Int J Neuropsychopharmacol* (2016) 19(8):pyw020. doi: 10.1093/ijnp/pyw020
23. Neufeld KM, Kang N, Bienenstock J, Foster JA. Reduced Anxiety-Like Behavior and Central Neurochemical Change in Germ-Free Mice. *Neurogastroenterol Motil* (2011) 23(3):255–64, e119. doi: 10.1111/j.1365-2982.2010.01620.x
24. Bercik P, Denou E, Collins J, Jackson W, Lu J, Jury J, et al. The Intestinal Microbiota Affect Central Levels of Brain-Derived Neurotropic Factor and Behavior in Mice. *Gastroenterology* (2011) 141(2):599–609, 609.e1-3. doi: 10.1053/j.gastro.2011.04.052
25. Sudo N, Chida Y, Aiba Y, Sonoda J, Oyama N, Yu XN, et al. Postnatal Microbial Colonization Programs the Hypothalamic-Pituitary-Adrenal System for Stress Response in Mice. *J Physiol* (2004) 558(Pt 1):263–75. doi: 10.1113/jphysiol.2004.063388
26. Ait-Belgnaoui A, Durand H, Cartier C, Chaumaz G, Eutamene H, Ferrier L, et al. Prevention of Gut Leakiness by a Probiotic Treatment Leads to Attenuated HPA Response to An Acute Psychological Stress in Rats. *Psychoneuroendocrinology* (2012) 37(11):1885–95. doi: 10.1016/j.psyneuen.2012.03.024
27. Dinan TG, Stanton C, Cryan JF. Psychobiotics: A Novel Class of Psychotropic. *Biol Psychiatry* (2013) 74(10):720–6. doi: 10.1016/j.biopsych.2013.05.001
28. Westfall S, Caracci F, Zhao D, Wu QL, Frolinger T, Simon J, et al. Microbiota Metabolites Modulate the T Helper 17 to Regulatory T Cell (Th17/Treg) Imbalance Promoting Resilience to Stress-Induced Anxiety- and Depressive-Like Behaviors. *Brain Behav Immun* (2021) 91:350–68. doi: 10.1016/j.bbi.2020.10.013
29. Wang G, Huang S, Wang Y, Cai S, Yu H, Liu H, et al. Bridging Intestinal Immunity and Gut Microbiota by Metabolites. *Cell Mol Life Sci* (2019) 76 (20):3917–37. doi: 10.1007/s00018-019-03190-6
30. Zelante T, Iannitti RG, Cunha C, De Luca A, Giovannini G, Pieraccini G, et al. Tryptophan Catabolites From Microbiota Engage Aryl Hydrocarbon Receptor and Balance Mucosal Reactivity *Via* Interleukin-22. *Immunity* (2013) 39 (2):372–85. doi: 10.1016/j.immuni.2013.08.003
31. Frolinger T, Sims S, Smith C, Wang J, Cheng H, Faith J, et al. The Gut Microbiota Composition Affects Dietary Polyphenols-Mediated Cognitive Resilience in Mice by Modulating the Bioavailability of Phenolic Acids. *Sci Rep* (2019) 9(1):3546. doi: 10.1038/s41598-019-39994-6
32. Jiang C, Sakakibara E, Lin WJ, Wang J, Pasinetti GM, Salton SR. Grape-Derived Polyphenols Produce Antidepressant Effects *Via* VGF- and BDNF-dependent Mechanisms. *Ann N Y Acad Sci* (2019) 1455(1):196–205. doi: 10.1111/nyas.14098
33. Caporaso JG, Lauber CL, Walters WA, Berg–Lyons D, Huntley J, Fierer N, et al. Ultra-High-Throughput Microbial Community Analysis on the Illumina HiSeq and MiSeq Platforms. *Isme J* (2012) 6(8):1621–4. doi: 10.1038/ismej.2012.8
34. Structure, Function and Diversity of the Healthy Human Microbiome. *Nature* (2012) 486(7402):207–14. doi: 10.1038/nature11234
35. Livak KJ, Schmittgen TD. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* (2001) 25(4):402–8. doi: 10.1006/meth.2001.1262
36. Barber RD, Harmer DW, Coleman RA, Clark BJ. GAPDH as a Housekeeping Gene: Analysis of GAPDH mRNA Expression in a Panel of 72 Human Tissues. *Physiol Genomics* (2005) 21(3):389–95. doi: 10.1152/physiolgenomics.00025.2005
37. Hartmann FJ, Simonds EF, Bendall SC. A Universal Live Cell Barcoding-Platform for Multiplexed Human Single Cell Analysis. *Sci Rep* (2018) 8 (1):10770. doi: 10.1038/s41598-018-28791-2

38. Rahman AH, Tordesillas L, Berin MC. Heparin Reduces Nonspecific Eosinophil Staining Artifacts in Mass Cytometry Experiments. *Cytom A* (2016) 89(6):601–7. doi: 10.1002/cyto.a.22826
39. Stern AD, Rahman AH, Birtwistle MR. Cell Size Assays for Mass Cytometry. *Cytom A* (2017) 91(1):14–24. doi: 10.1002/cyto.a.23000
40. Amir ED, Guo XV, Mayovska O, Rahman AH. Average Overlap Frequency: A Simple Metric to Evaluate Staining Quality and Community Identification in High Dimensional Mass Cytometry Experiments. *J Immunol Methods* (2018) 453:20–9. doi: 10.1016/j.jim.2017.08.011
41. Lee BH, Kelly G, Bradford S, Davila M, Guo XV, Amir ED, et al. A Modified Injector and Sample Acquisition Protocol can Improve Data Quality and Reduce Inter-Instrument Variability of the Helios Mass Cytometer. *Cytom A* (2019) 95(9):1019–30. doi: 10.1002/cyto.a.23866
42. Zunder ER, Finck R, Behbehani GK, Amir el AD, Krishnaswamy S, Gonzalez VD, et al. Palladium-Based Mass Tag Cell Barcoding With a Doublet-Filtering Scheme and Single-Cell Deconvolution Algorithm. *Nat Protoc* (2015) 10 (2):316–33. doi: 10.1038/nprot.2015.020
43. Amir ED, Lee B, Badoual P, Gordon M, Guo XV, Merad M, et al. Development of a Comprehensive Antibody Staining Database Using a Standardized Analytics Pipeline. *Front Immunol* (2019) 10:1315. doi: 10.3389/fimmu.2019.01315
44. Kotecha N, Krutzik PO, Irish JM. Web-Based Analysis and Publication of Flow Cytometry Experiments. *Curr Protoc Cytom* (2010) Chapter 10: Unit10.17. doi: 10.1002/0471142956.cy1017s53
45. Mortha A, Chudnovskiy A, Hashimoto D, Bogunovic M, Spencer SP, Belkaid Y, et al. Microbiota-Dependent Crosstalk Between Macrophages and ILC3 Promotes Intestinal Homeostasis. *Science* (2014) 343(6178):1249288. doi: 10.1126/science.1249288
46. Pfau ML, Ménard C, Russo SJ. Inflammatory Mediators in Mood Disorders: Therapeutic Opportunities. *Annu Rev Pharmacol Toxicol* (2018) 58:411–28. doi: 10.1146/annurev-pharmtox-010617-052823
47. O'Mahony SM, Clarke G, Dinan TG, Cryan JF. Irritable Bowel Syndrome and Stress-Related Psychiatric Co-morbidities: Focus on Early Life Stress. *Handb Exp Pharmacol* (2017) 239:219–46. doi: 10.1007/164_2016_128
48. Pinto-Sanchez MI, Hall GB, Ghajar K, Nardelli A, Bolino C, Lau JT, et al. Probiotic Bifidobacterium Longum NCC3001 Reduces Depression Scores and Alters Brain Activity: A Pilot Study in Patients With Irritable Bowel Syndrome. *Gastroenterology* (2017) 153(2):448–59.e8. doi: 10.1053/j.gastro.2017.05.003
49. Gao X, Cao Q, Cheng Y, Zhao D, Wang Z, Yang H, et al. Chronic Stress Promotes Colitis by Disturbing the Gut Microbiota and Triggering Immune System Response. *Proc Natl Acad Sci U S A* (2018) 115(13):E2960–e2969. doi: 10.1073/pnas.1720696115
50. Nishida K, Sawada D, Kuwano Y, Tanaka H, Rokutan K. Health Benefits of Lactobacillus Gasseri CP2305 Tablets in Young Adults Exposed to Chronic Stress: A Randomized, Double-Blind, Placebo-Controlled Study. *Nutrients* (2019) 11(8):1859. doi: 10.3390/nu11081859
51. Jiang H, Ling Z, Zhang Y, Mao H, Ma Z, Yin Y, et al. Altered Fecal Microbiota Composition in Patients With Major Depressive Disorder. *Brain Behav Immun* (2015) 48:186–94. doi: 10.1016/j.bbi.2015.03.016
52. Bajaj JS, Sikaroodi M, Fagan A, Heuman D, Gilles H, Gavis EA, et al. Posttraumatic Stress Disorder Is Associated With Altered Gut Microbiota That Modulates Cognitive Performance in Veterans With Cirrhosis. *Am J Physiol Gastrointest Liver Physiol* (2019) 317(5):G661–g669. doi: 10.1152/ajpgi.00194.2019
53. Cox LM, Sohn J, Tyrrell KL, Citron DM, Lawson PA, Patel NB, et al. Description of Two Novel Members of the Family Erysipelotrichaceae: Ileibacterium Valens Gen. Nov., Sp. Nov. And Dubosiella Newyorkensis, Gen. Nov., Sp. Nov., From the Murine Intestine, and Emendation to the Description of Faecalibaculum Rodentium. *Int J Syst Evol Microbiol* (2017) 67 (5):1247–54. doi: 10.1099/ijsem.0.001793
54. Turnbaugh PJ, Ridaura VK, Faith JJ, Rey FE, Knight R, Gordon JI. The Effect of Diet on the Human Gut Microbiome: A Metagenomic Analysis in Humanized Gnotobiotic Mice. *Sci Transl Med* (2009) 1(6):6ra14. doi: 10.1126/scitranslmed.3000322
55. Palm NW, de Zoete MR, Cullen TW, Barry NA, Stefanowski J, Hao L, et al. Immunoglobulin A Coating Identifies Colitogenic Bacteria in Inflammatory Bowel Disease. *Cell* (2014) 158(5):1000–10. doi: 10.1016/j.cell.2014.08.006
56. Chen L, Wilson JE, Koenigsknecht MJ, Chou WC, Montgomery SA, Truax AD, et al. NLRP12 Attenuates Colon Inflammation by Maintaining Colonic Microbial Diversity and Promoting Protective Commensal Bacterial Growth. *Nat Immunol* (2017) 18(5):541–51. doi: 10.1038/ni.3690
57. Romijn AR, Rucklidge JJ, Kuijer RG, Frampton C. A Double-BlindRandomized, Placebo-Controlled Trial of Lactobacillus Helveticus and Bifidobacterium Longum for the Symptoms of Depression. *Aust N Z J Psychiatry* (2017) 51(8):810–21. doi: 10.1177/0004867416686694
58. Aizawa E, Tsuji H, Asahara T, Takahashi T, Teraishi T, Yoshida S, et al. Possible Association of Bifidobacterium and Lactobacillus in the Gut Microbiota of Patients With Major Depressive Disorder. *J Affect Disord* (2016) 202:254–7. doi: 10.1016/j.jad.2016.05.038
59. Wang J, Ji H, Wang S, Liu H, Zhang W, Zhang D, et al. Probiotic Lactobacillus Plantarum Promotes Intestinal Barrier Function by Strengthening the Epithelium and Modulating Gut Microbiota. *Front Microbiol* (2018) 9:1953. doi: 10.3389/fmicb.2018.01953
60. Ni Y, Yang X, Zheng L, Wang Z, Wu L, Jiang J, et al. Lactobacillus and Bifidobacterium Improves Physiological Function and Cognitive Ability in Aged Mice by the Regulation of Gut Microbiota. *Mol Nutr Food Res* (2019) 63 (22):e1900603. doi: 10.1002/mnfr.201900603
61. Zhao Q, Elson CO. Adaptive Immune Education by Gut Microbiota Antigens. *Immunology* (2018) 154(1):28–37. doi: 10.1111/imm.12896
62. Christensen HR, Frøkiaer H, Pestka JJ. Lactobacilli Differentially Modulate Expression of Cytokines and Maturation Surface Markers in Murine Dendritic Cells. *J Immunol* (2002) 168(1):171–8. doi: 10.4049/jimmunol.168.1.171
63. Mazmanian SK, Liu CH, Tzianabos AO, Kasper DL. An Immunomodulatory Molecule of Symbiotic Bacteria Directs Maturation of the Host Immune System. *Cell* (2005) 122(1):107–18. doi: 10.1016/j.cell.2005.05.007
64. Artis D, Spits H. The Biology of Innate Lymphoid Cells. *Nature* (2015) 517 (7534):293–301. doi: 10.1038/nature14189
65. Buela KA, Omenetti S, Pizarro TT. Cross-Talk Between Type 3 Innate Lymphoid Cells and the Gut Microbiota in Inflammatory Bowel Disease. *Curr Opin Gastroenterol* (2015) 31(6):449–55. doi: 10.1097/mog.0000000000000217
66. Cording S, Medvedovic J, Cherrier M, Eberl G. Development and Regulation of Rorγt(+) Innate Lymphoid Cells. *FEBS Lett* (2014) 588(22):4176–81. doi: 10.1016/j.febslet.2014.03.034
67. Crellin NK, Trifari S, Kaplan CD, Satoh-Takayama N, Di Santo JP, Spits H. Regulation of Cytokine Secretion in Human CD127(+) Lti-Like Innate Lymphoid Cells by Toll-like Receptor 2. *Immunity* (2010) 33(5):752–64. doi: 10.1016/j.immuni.2010.10.012
68. Zhou L, Chu C, Teng F, Bessman NJ, Goc J, Santosa EK, et al. Innate Lymphoid Cells Support Regulatory T Cells in the Intestine Through Interleukin-2. *Nature* (2019) 568(7752):405–9. doi: 10.1038/s41586-019-1082-x
69. Reynders A, Yessaad N, Vu Manh TP, Dalod M, Fenis A, Aubry C, et al. Identity, Regulation and *In Vivo* Function of Gut Nkp46+Rorγt+ and Nkp46+Rorγt- Lymphoid Cells. *EMBO J* (2011) 30(14):2934–47. doi: 10.1038/emboj.2011.201
70. Idoyaga J, Fiorese C, Zbytnuik L, Lubkin A, Miller J, Malissen B, et al. Specialized Role of Migratory Dendritic Cells in Peripheral Tolerance Induction. *J Clin Invest* (2013) 123(2):844–54. doi: 10.1172/jci65260
71. Li S, Bostick JW, Zhou L. Regulation of Innate Lymphoid Cells by Aryl Hydrocarbon Receptor. *Front Immunol* (2017) 8:1909. doi: 10.3389/fimmu.2017.01909
72. Koper JEB, Loonen LMP, Wells JM, Troise AD, Capuano E, Fogliano V. Polyphenols and Tryptophan Metabolites Activate the Aryl Hydrocarbon Receptor in an *In Vitro* Model of Colonic Fermentation. *Mol Nutr Food Res* (2019) 63(3):e1800722. doi: 10.1002/mnfr.201800722
73. Cohen H, Ziv Y, Cardon M, Kaplan Z, Matar MA, Gidron Y, et al. Maladaptation to Mental Stress Mitigated by the Adaptive Immune System *Via* Depletion of Naturally Occurring Regulatory CD4+CD25+ Cells. *J Neurobiol* (2006) 66(6):552–63. doi: 10.1002/neu.20249
74. Denning TL, Norris BA, Medina-Contreras O, Manicassamy S, Geem D, Madan R, et al. Functional Specializations of Intestinal Dendritic Cell and Macrophage Subsets That Control Th17 and Regulatory T Cell Responses are Dependent on the T Cell/APC Ratio, Source of Mouse Strain, and Regional Localization. *J Immunol* (2011) 187(2):733–47. doi: 10.4049/jimmunol.1002701

75. Penner-Goeke S, Binder EB. Epigenetics and Depression. *Dialogues Clin Neurosci* (2019) 21(4):397–405. doi: 10.31887/DCNS.2019.21.4/ebinder
76. Lau CM, Adams NM, Geary CD, Weizman OE, Rapp M, Pritykin Y, et al. Epigenetic Control of Innate and Adaptive Immune Memory. *Nat Immunol* (2018) 19(9):963–72. doi: 10.1038/s41590-018-0176-1
77. Salam AP, Borsini A, Zunszain PA. Trained Innate Immunity: A Salient Factor in the Pathogenesis of Neuroimmune Psychiatric Disorders. *Mol Psychiatry* (2018) 23(2):170–6. doi: 10.1038/mp.2017.186
78. Clark SL, Hattab MW, Chan RF, Shabalin AA, Han LKM, Zhao M, et al. A Methylation Study of Long-Term Depression Risk. *Mol Psychiatry* (2020) 25 (6):1334–43. doi: 10.1038/s41380-019-0516-z
79. Woo V, Alenghat T. Host-Microbiota Interactions: Epigenomic Regulation. *Curr Opin Immunol* (2017) 44:52–60. doi: 10.1016/j.coi.2016.12.001
80. Wang J, Hodes GE, Zhang H, Zhang S, Zhao W, Golden SA, et al. Epigenetic Modulation of Inflammation and Synaptic Plasticity Promotes Resilience Against Stress in Mice. *Nat Commun* (2018) 9(1):477. doi: 10.1038/s41467-017-02794-5
81. Gury-BenAri M, Thaiss CA, Serafini N, Winter DR, Giladi A, Lara–Astiaso D, et al. The Spectrum and Regulatory Landscape of Intestinal Innate Lymphoid Cells Are Shaped by the Microbiome. *Cell* (2016) 166(5):1231–46.e13. doi: 10.1016/j.cell.2016.07.043

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

frontiers in Immunology

REVIEW
published: 12 July 2021
doi: 10.3389/fimmu.2021.679055

An Integrated View on Neuronal Subsets in the Peripheral Nervous System and Their Role in Immunoregulation

Manuel O. Jakob[1], *Michael Kofoed-Branzk*[1], *Divija Deshpande*[1], *Shaira Murugan*[2] *and Christoph S. N. Klose*[1]*

[1] Department of Microbiology, Infectious Diseases and Immunology, Charité – Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin and Humboldt-Universität zu Berlin, Berlin, Germany, [2] Department of BioMedical Research, Group of Visceral Surgery and Medicine, University of Bern, Bern, Switzerland

OPEN ACCESS

Edited by:
Francesca Ronchi, University of Bern, Switzerland

Reviewed by:
Sebastien Talbot, Université de Montréal, Canada
Warren G. Tourtellotte, Cedars-Sinai Medical Center, United States

***Correspondence:**
Christoph S. N. Klose
christoph.klose@charite.de

Specialty section:
This article was submitted to Multiple Sclerosis and Neuroimmunology, a section of the journal Frontiers in Immunology

Received: *10 March 2021*
Accepted: *15 June 2021*
Published: *12 July 2021*

Citation:
Jakob MO, Kofoed-Branzk M, Deshpande D, Murugan S and Klose CSN (2021) An Integrated View on Neuronal Subsets in the Peripheral Nervous System and Their Role in Immunoregulation. Front. Immunol. 12:679055. doi: 10.3389/fimmu.2021.679055

The peripheral nervous system consists of sensory circuits that respond to external and internal stimuli and effector circuits that adapt physiologic functions to environmental challenges. Identifying neurotransmitters and neuropeptides and the corresponding receptors on immune cells implies an essential role for the nervous system in regulating immune reactions. Vice versa, neurons express functional cytokine receptors to respond to inflammatory signals directly. Recent advances in single-cell and single-nuclei sequencing have provided an unprecedented depth in neuronal analysis and allowed to refine the classification of distinct neuronal subsets of the peripheral nervous system. Delineating the sensory and immunoregulatory capacity of different neuronal subsets could inform a better understanding of the response happening in tissues that coordinate physiologic functions, tissue homeostasis and immunity. Here, we summarize current subsets of peripheral neurons and discuss neuronal regulation of immune responses, focusing on neuro-immune interactions in the gastrointestinal tract. The nervous system as a central coordinator of immune reactions and tissue homeostasis may predispose for novel promising therapeutic approaches for a large variety of diseases including but not limited to chronic inflammation.

Keywords: neuro-immune interactions, neuronal classification, peripheral nervous system, enteric nervous system, dorsal root ganglia (DRG), function of neurons

INTRODUCTION

The nervous system in multi-cellular organisms consists of a complex network of neurons, which can rapidly and precisely transmit signals. Signal transmission within the nervous system is mediated by the release of neurotransmitters or neuropeptides from the presynaptic neuron into the synaptic cleft, the engagement of the cognate receptor on the postsynaptic neuron, and the elicitation of a signaling cascade within the postsynaptic neuron. The nervous system is organized in circuits linking afferent sensory input with a broad array of reactions at effector sites. In this way, the nervous system coordinates physiological functions, such as behavior, motor functions, blood pressure and hormone release (1–4).

The nervous system is classified into the central nervous system (CNS) and the peripheral nervous system (PNS). The CNS, which includes the brain and the spinal cord, is enclosed by the dura mater. The PNS, placed outside of the dura mater, is sub-classified into the somatic and autonomic nervous systems. The somatic nervous system consists of peripheral somatosensory nerves, which convey afferent signals and efferent nerves controlling motor functions, e.g. regulating movements of extremities. The autonomic nervous system can be functionally distinguished into the sympathetic, the parasympathetic, and the enteric nervous system (5). The parasympathetic and sympathetic nervous system are typical functional counter players for opposing physiological functions: the rest-and-digest (parasympathetic nervous system) and the fight-or-flight reaction (sympathetic nervous system). These functions are highly conserved across species and build the basis for survival during external threats (6). The third component of the autonomic nervous system is the gut *intrinsic* enteric nervous system (ENS), which controls intestinal movement, mixing of ingested food and the secretion of fluids. In the gastrointestinal tract, *intrinsic* neurons are those whose cell bodies lie within the organ, whereas *extrinsic* nerves (e.g. sensory nerve fibers) have their cell bodies outside the innervated organ. Typically, the soma of extrinsic sensory afferents is located within dorsal root ganglia, celiac ganglia, superior or inferior mesenteric ganglia or the nodose/jugular ganglia. The intrinsic and independent coordinator ability of the ENS has been underlined in experiments following extrinsic denervation, where a lack of extrinsic signals only impaired physiologic functions of the intestine to a minor degree. Conversely, loss of the intrinsic ENS can be disastrous, as shown in Hirschsprung or Chagas disease, where intestinal motor functions are significantly reduced or absent (7, 8).

The ENS is composed of different neuronal populations, which fulfil specific physiologic functions. The traditional classification of neurons solely according to their chemical signature is not entirely sufficient to define a functional type of neuron because similar neurotransmitters seem to exert different physiologic functions. In the same line, anatomical/morphological classifications do not adhere to specific neuronal functions because of similar shapes of enteric neurons in distinct functional classes. Thus, current neuronal classifications need to be extended by a broader array of markers to better understand the peripheral nervous system in detail. Single-cell RNA-sequencing is a powerful tool, which provides several vital transcripts per cell and can zoom in at the potential correct resolution.

Here we review the current understanding of the function of different neuronal subsets that relay the signals to subsets of immune cells in the peripheral nervous system, which regulate intestinal physiology as a response to environmental challenges and physiologic perceptions.

THE ENTERIC NERVOUS SYSTEM

The intestine has its own nervous system, the ENS, which operates to a large degree autonomously and outside of voluntary control, despite being innervated by extrinsic nerve fibers. The ENS is the largest collection of neurons outside the CNS comprising hundreds of millions of neurons. It is the major coordinator of physiologic bowel functions, including but not limited to peristaltic movement. The importance of the autonomous function of the ENS has been demonstrated by experiments using extrinsic denervation of the intestine, in which the bowel function was only mildly affected by the cessation of extrinsic signals (9).

The ENS has a characteristic spider web structure, is embedded in the intestinal wall and closely associated with the muscle layers. The somas of enteric neurons mainly cluster in two anatomically distinct but strongly interconnected ganglionated plexuses (10). The outer, myenteric plexus (Auerbach Plexus) lies within the longitudinal and the circular muscle layer, and the inner, submucosal plexus (Meissner Plexus) is located below the muscle layers (11).

While a classification based on morphological aspects has been proposed, this classification does not take into account that functionally similar neurons can vary in their morphology. Using open-end single-cell or single-nuclei sequencing approaches, several studies have revealed the transcriptomic landscape of the ENS and thus provide a classification, which is more functionally substantiated. A classification based on a transcriptional code, as discussed in the following paragraph, includes marker genes encoding for receptors, ion channels and neuropeptides and can thus broaden our understanding of physiologic and immunologic functions.

Populations of Neurons

The intrinsic micro-circuitry regulation of intestinal function consists of five neuronal classes with distinct functional specializations. Sensory neurons, also referred to as intrinsic primary afferent neurons (IPANs), detect chemical and physical alterations in the intestine and transmit the signal *via* interneurons to excitatory motor neurons, inhibitory motor neurons or secretomotor/vasodilator neurons (**Figure 1**). Enteric neurons are replenished from neuronal stem cells of the intestine. While the traditional sub-classification was based on the neuronal type, the anatomic location and axonal projection as well as neurochemical signature, recent advances in single-cell sequencing has enabled a comprehensive clustering based on gene expression.

Single-cell sequencing of the ENS using fluorescence-activated sorting of Wnt1-Cre; R26Tomato mice revealed 1105 enteric neurons (with ~ 3066 genes detected on average) and eventually found nine clusters of neurons in the muscular sheet of the intestine (12). Based on neuro-transmitters nitric oxide synthase 1 (*NOS1*) and choline acetyltransferase (*ChAT*), two main groups can be classified, $NOS1^+$ neurons and $ChAT^+$ neurons. The authors assigned three distinct neuronal clusters of nitrergic neurons, which comprise inhibitory motor neurons and secretomotor/vasodilator neurons (**Table 1**). Further, 6 clusters of cholinergic neurons, which express *ChAT* and the solute carrier family 5 member 7 (*Slc5a7*, gene encodes for an ion transporter) could be distinguished (12). $ChAT^+$ neurons are further subdivided into excitatory motor neurons, sensory neurons and interneurons according to their gene expression (**Table 1**).

Using the pan-neuronal Baf53b-cre deleter mice crossed to R26R-tomato to enable sort-purification of enteric neurons from the myenteric plexus of the small intestine combined with single-cell RNA-sequencing, 4,892 high-quality enteric neurons have been analyzed and 12 classes of neurons could be identified based

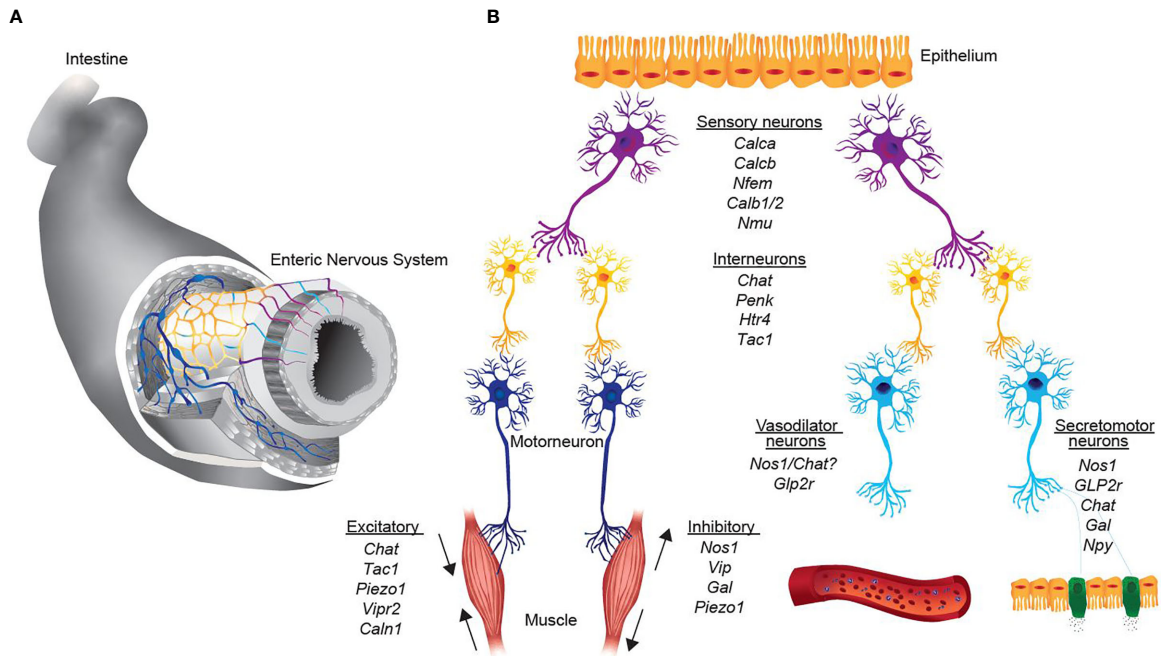


FIGURE 1 | Representation of neurons, key transcripts and functional assignments in the intrinsic enteric nervous system. **(A)** 2-Dimensional scheme of the enteric nervous system. Colors represent the schematic representation of distinct functional neuronal groups. **(B)** Simplified representation from sensing of the environment of different stimuli (sensory neurons, purple), signaling transmission to interneurons (yellow) and excitation of effector neurons (motor neurons, dark blue; vasodilator and secretomotor, light blue). Italics are the key transcripts of the respective neuronal group.

TABLE 1 | Reference genes for the identification of neuronal subsets based on unbiased single-cell RNA-sequencing (12–14).

Secretomotor, vasodilator	IMN		EMN		IN		SN	
NOS1			**ChAT**					
ENT1	ENT2	ENT3	ENT4	ENT5	ENT6	ENT7	ENT8	ENT9
VIP low	**VIP**	**VIP**	**Tac1**	**Tac1**	**Penk**	**Penk**	CCK	NMU
Gal high	**Gal**	**Gal**	**Piezo1**	**Piezo1**	**Htr4**	**Htr4**	Ucn3	Calcb
NeuroD6	**Piezo1**	**Piezo1**	**VIPr2**	**VIPr2**	**Tac1 high**	Calcb		Nog
Glp2r	NPY high	NPY low	**Caln1**	**Caln1**		Sst		

Bold genes represent key transcripts. IMN, inhibitory motor neurons; EMN, excitatory motor neurons; IN, Interneurons; SN, sensory neurons.
VIP, vasoactive intestinal peptide; Tac1, gene encoding Substance P; PENK, gene encoding proenkephalin (Opioid); Gal, galanin (neuroendocrine peptide); Piezo1, piezo type mechanosensitive ion channel component 1; Htr4, 5-hydroxytryptamine receptor 4; Caln1, Calneuron 1.

on their gene expression (13). Matching these results to known entities of enteric neurons based on functional properties, the authors could propose the following classification: Populations 1-4 can be classified as excitatory motor neurons predominantly defined *via* the expression of *Tac1* and *Calb2*. Class 8 and 9 have been assigned to inhibitory motor neurons and are characterized by the expression of *Nos1/Gal/VIP/Npy*. Different classes (Populations 6, 7, 11) of sensory intrinsic primary afferent neurons have been identified based on the known markers *Calca/Calcb/Nfem/Calb1/Calb2* and the selective expression for *NMU*, *Ucn-3/Cck* or *Nxph2*. Interneurons are represented with a mixed neurochemical signature, such as *Nos1/ChAT* for Interneurons group 1, *Sst/Calcb/Calb2* for interneurons group 2. The co-expression of *Nos1* and *ChAT* was also found in the dataset of Zeisel and, thus, may reflect the profile of interneurons, which connect different neuronal groups (12). While all the single-cell RNA-sequencing studies nicely delineate distinct populations of enteric neurons based on specific transcripts, dissociation and sort-purification of entire neurons from the muscular sheet of the intestine might not equally represent all neurons in the ENS, some of which might be sensitive to the isolation or sorting procedure. It should be noted that even for tissue-resident immune cells, which are presumably easier to release from the fabric of the tissue after digestion, a discrepancy between cells recovered after digestion and those detected in situ was reported (15).

To overcome this potential limitation, a study by the Regev lab performed single-nuclei sequencing by using sort-purification of labelled nuclei with a nuclear-tagged fluorescent protein (14). This approach is expected to result in an equal representation of neuronal nuclei present in the tissue. The authors sequenced 1´187´535 colonic and ileal nuclei and eventually profiled 2´657 neuronal nuclei (with 7´369 genes detected per nucleus). By using nuclear isolation and sequencing, Drokhlyansky and colleagues identified 21 neuronal populations based on known marker genes. These 21 identified neuronal classes could be further broadly sub-classified into 5 populations of *ChAT*$^+$*Nos1*$^+$ double-expressing putative excitatory motor neurons, 7 populations of *Nos1*$^+$ inhibitory motor neurons (4 subsets are *Nos1*$^+$*Vip*$^+$), 2 populations of *Glp2r*$^+$ secretomotor and vasodilator neurons, 4 populations of *CGRP*$^+$ sensory neurons, 3 populations of *Penk*$^+$ Interneurons (**Table 1**). Apart from the transcripts at the single-cell level, there is limited data available that reports a deeper characterization of neuronal subsets after sort purification. Thus, the detection limit of single-cell RNA-sequencing can miss important transcripts in certain subpopulations of cells. Purification of neuronal subpopulations may allow to assign defined functions and to delineate the neuronal subclasses in more detail in the near future. In-between the above-mentioned studies, minor discrepancies in the representation of neuronal subclasses were reported, which may be due to different reporter mouse strains used for sort purification, different isolation/sequencing techniques or differences in bioinformatic analyses. However, even though transcripts may differ in between different single-cell RNA-sequencing datasets, the known functional subsets of enteric neurons are uniformly present in all studies. Consequently, each study concluded putative functional roles of identified neuronal subclasses by associating gene expression to a certain known function, even though without experimental proof. Thus, future studies need to experimentally confirm functional coherence of the identified transcripts.

Within the enteric nervous system, only one study performed sequencing of human neurons (14). By using MIRACL-sequencing, a total of 436'202 human nuclei were profiled and 1'445 neurons clustered into 14 neuronal subsets (with ~ 4302 genes detected on average). By comparing human and mouse colon neuronal nuclei, the authors found strong congruence between species but also differences in ENS composition, such as a higher abundance of motor neurons and a lower diversity of sensory neurons, interneurons and secretomotor/vasodilator neurons in humans. The fact that the abundance of neurons is relatively low, future studies should aim to address to enrich neurons in specimens to gain deeper insights in regulated neuronal gene expression.

EXPRESSION OF GENES MEDIATING NEURO-IMMUNE INTERACTION IN THE ENS

Understanding which genes enteric neurons have adopted for sensing the immune system is one of the burning questions in the field today. We aimed to provide an overview by re-examining published single-cell and single-nuclei datasets deposited in publicly available databases in silico for expression of cytokine receptors, chemokine receptors, NOD-like receptors (NLRs) and toll-like receptors (TLRs) (**Figures 2–4**) (12, 14). With regard to cytokine receptors *Il11ra1*, *Il4Ra*, *Il13ra1*, and *Il6st* (**Figure 2**) were detectable in enteric neurons.

The expression of the type 2 cytokine receptors *Il4Ra* and *IL13ra1* in sensory ganglia and their functional role has been studied in the context of chronic itch in mice and humans (17). Functionally, type 2 cytokines can directly activate sensory neurons, activate itch-sensory pathways and are critical players in the development of chronic itch sensations. Furthermore, type 2 cytokines induce the JAK signaling pathway in neurons and JAK inhibitors have been successfully tested in chronic itch diseases in humans (17). In the same line, the important initiator of type 2 responses thymic stromal lymphopetin (TSLP) activates TRPA1$^+$ sensory neurons and promotes itch responses in mice (18). However, their role in the ENS needs to be delineated in future studies.

Recent studies suggest a role for pattern recognition receptors (PRR) in DRG neurons and pain sensation (19). Given its location, the ENS is constantly exposed to microbial factors. A role for the ENS in sensing microbial metabolites was found but the importance of PRR on enteric neurons is not well defined. Reanalysis of the datasets shows expression of *Nlrp6* and *TLR3* and is consistently found in enteric neurons among several studies (**Figures 3** and **4**).

The role of the inflammasome components Nlrp6 and caspase 11 has recently been highlighted to control enteric neuronal cell death and glucose metabolism in a microbiota-dependent manner (20). In fact, microbiota-depletion with antibiotics leads to loss of enteric associated CART$^+$ neurons in a Nlrp6 and Caspase 11 dependent manner. Analysis of SPF-colonized and GF mice revealed reduction in blood glucose levels, which was linked to CART$^+$ neurons suggesting the regulation of blood glucose level independent from CNS control (20). The role of TLRs in neurons is intriguing because it may suggest direct microbial sensing of enteric neurons (11). However, the role of TLR3 in the ENS remains elusive.

The expression pattern of cytokine receptors, NLRs and TLRs, and chemokine receptors can guide the readers to design their research projects accordingly (**Figures 2–4**, **Supplementary Figure 1**).

REGULATION OF NEURONAL POPULATIONS

Excitatory and Inhibitory Motor Neurons

Within the group of motor neurons, a sub-classification proposed by Furness and colleagues (21) distinguishes five main classes of motor neurons present in the intestine including excitatory motor neurons, inhibitory motor neurons, secretomotor/vasodilator neurons, secretomotor neurons that are not vasodilator and neurons to enteroendocrine cells (**Figure 1**).

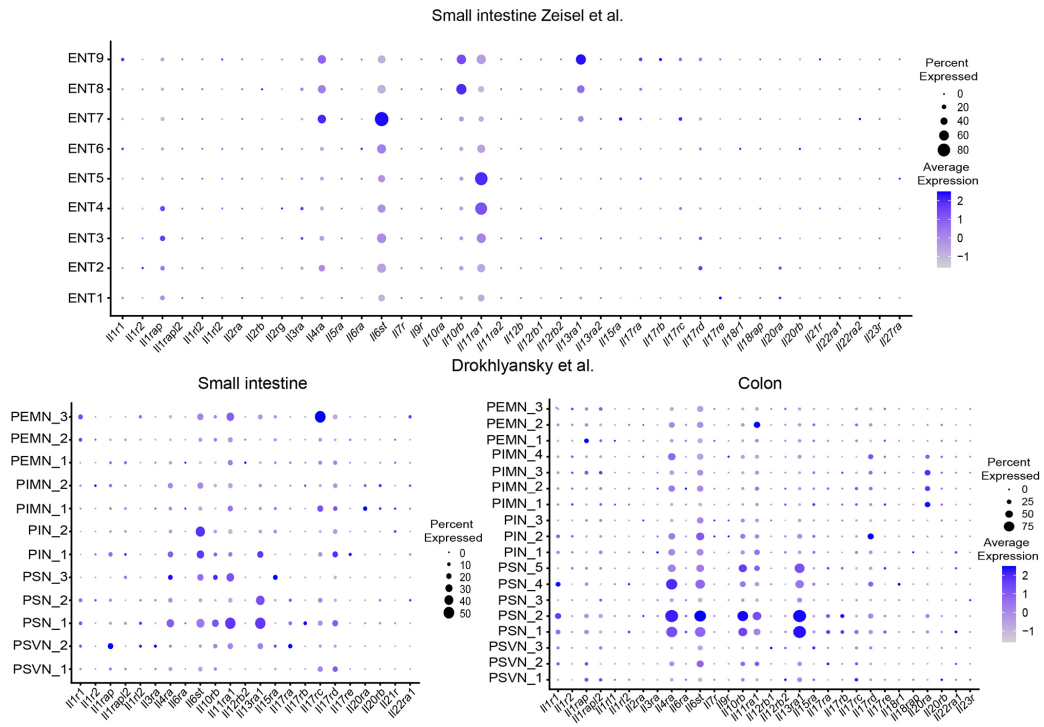


FIGURE 2 | Expression of interleukin receptors in different ENS subsets. Dotplots showing the percentage of expressing cells as well as average expression within the indicated identified neuronal clusters for selected murine interleukin receptor genes. Data was downloaded from http://mousebrain.org/ (12) or from https://singlecell.broadinstitute.org/ (14). Genes not present in the plot showing the Drokhlyansky data have been filtered. All dataset were normalized and transformed before the plots were created using the Seurat package (16).

Excitatory motor neurons use the main neurotransmitter acetylcholine (Ach) for signal transmission and are thus distinguishable from inhibitory motor neurons, which use nitric oxide (NO) as the main neurotransmitter (5). This difference in signal transmission of excitatory versus inhibitory motor neurons becomes evident in anticholinergic medications, such as atropine or antidepressants, that typically lead to constipation due to the lack of signals from excitatory motor neurons (22). Furthermore, conditional deletion of ChAT in neural-crest derived neurons by using the Wnt1-cre driver in mice resulted in gastrointestinal dysmotility, dysbiosis and eventually death of the mice at post-natal day 30. This phenotype highlights the extraordinary role of *ChAT* for host physiology and its role in the coordination of motor functions (23).

Even though Ach is the dominant neurotransmitter used by excitatory motor neurons, these neurons show a residual excitation after muscarinic block suggesting that other neurotransmitters are involved (24, 25). The residual excitation is assumed to be mediated by tachykinins, in particular Substance P (gene *Tac1*), which engage on NK1 and NK2 receptors on muscle cells (26). Altogether, these data indicate that excitation of motor neurons is mediated by cholinergic and non-cholinergic neurotransmitters and each excitation may be fine-tuned dependent on the transmitter involved.

On the contrary, experiments in mice with the deletion of *NOS1* revealed that neurons normally expressing NOS remain intact (apart from grossly enlarged stomach due to pyloric stenosis) and respective mice do not show evident histopathological abnormalities (27). These results suggest that inhibitory neurons are also co-regulated by other neurotransmitters to terminate the excitatory signal. Several transmitters/neuropeptides have been described to regulate inhibitory motor neurons including adenosine triphosphate (ATP) (28), vasoactive intestinal peptide (VIP) (29), pituitary adenylyl cyclase activating peptide (PACAP) and carbon monoxide (30) in addition to NO as neuromuscular transmitters (31).

In summary, excitatory and inhibitory motor neurons in the ENS control muscle contraction and relaxation in an autonomous manner (**Figure 1**). In this way, ingested food is squirted, mixed with digestive enzymes and eventually transported aborally. Based on gene profiles detected in single-cell RNA-sequencing studies, neurons can be associated to either excitatory or inhibitory motor neurons. However, the exact functional role of the subpopulations within excitatory or inhibitory motor neurons remains elusive.

In this context, putative intrinsic but also extrinsic neurons are equipped with mechanosensory ion channels. Different cell types have been described to use mechanosensitive ion channels

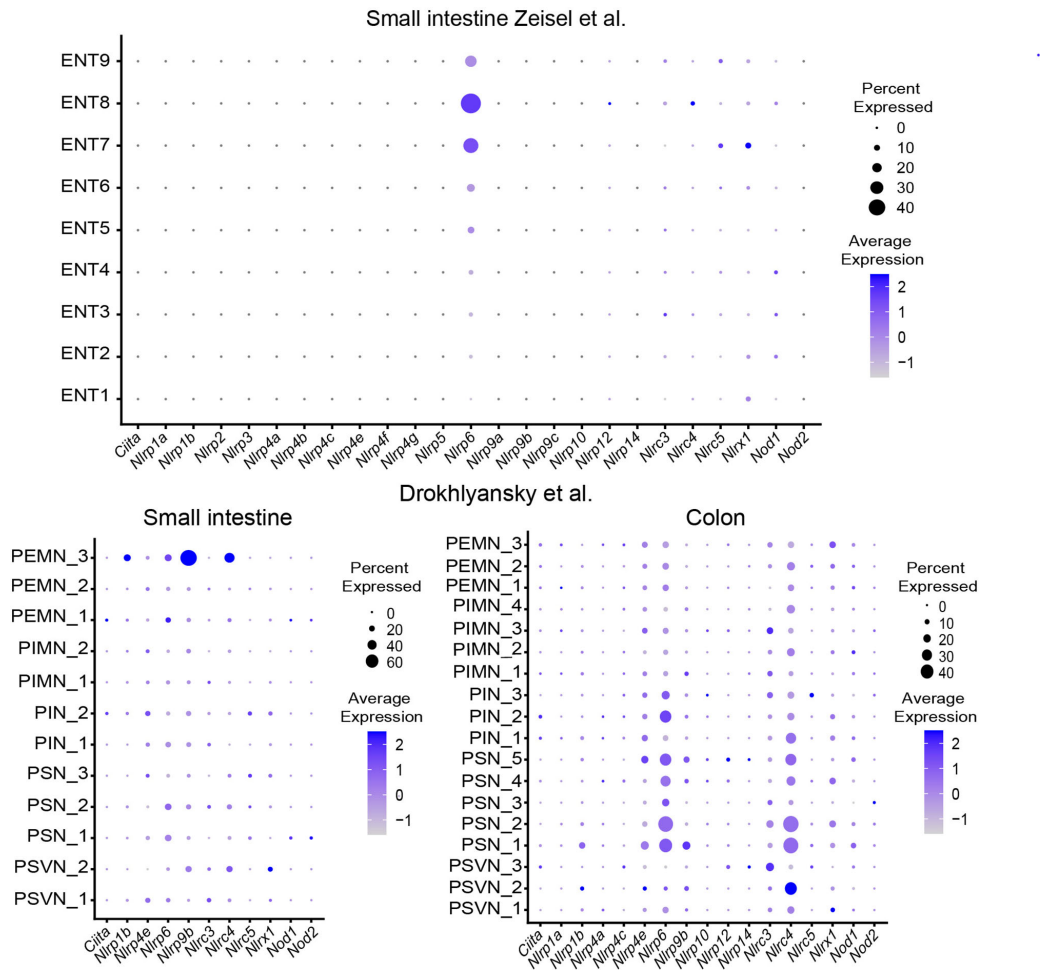


FIGURE 3 | Expression of NOD-like receptors in different ENS subsets. Dotplots showing the percentage of expressing cells as well as average expression within the indicated identified neuronal clusters for selected murine NOD-like receptor genes. Data was downloaded from http://mousebrain.org/ (12) or from https://singlecell.broadinstitute.org/ (14). Genes not present in the plot showing the Drokhlyansky data have been filtered. All dataset were normalized and transformed before the plots were created using the Seurat package (16).

to detect alterations in mechanical forces. In this way, neurons can adapt their functions dependent on mechanical perturbations. Piezo ion channels are important signaling cationic ion channels for mechanosensation and their role in the extrinsic nervous system and the enteric nervous system are emerging (32). Functionally in the peripheral nervous system, PIEZO ion channels have been linked to the sensation of nociceptive signals, proprioception and touch. An interesting finding from an immunologic perspective is that mechanical processes can also regulate immune cell activity (33). The authors found that the mechanosensory ion channel PIEZO1 is able to mount a proinflammatory reprogramming in macrophages. Upon conditional deletion of PIEZO1 in myeloid cells in the context of *P. aeruginosa* infection in the lung, the PIEZO1-mediated mechanosensation protected against bacterial infection (33). This data argues for similar mechanisms used by the nervous and the immune system to adapt physiological functions to mechanical forces. Thus, mechanosensory ion channels may be important for neuro-immune disturbances in ileus and other gastrointestinal diseases. However, their exact role, in particular the role of PIEZO1 in the gastrointestinal tract, needs further investigation.

Secretomotor and Vasodilator Neurons

The intestine is constantly exposed to potential external threats, such as the commensal microbiota, in addition to the needs to control bowel absorption and to avoid electrolyte disturbances. Intrinsic secretomotor neurons have their cell bodies predominantly in the submucosa and play an important role in the regulation of these functions (**Figure 1**). In the guinea-pig small intestine, three different *ChAT* expressing secretomotor/vasodilator neurons have been described. Co-expression of *ChAT* and Calretinin, both of which innervate glands and arterioles, has been linked to vasomotor function. Functionally,

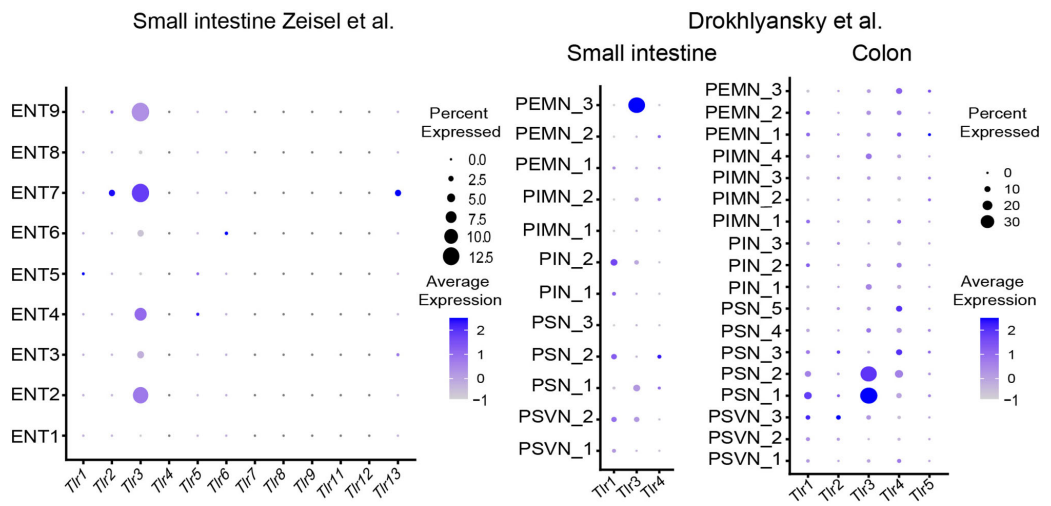


FIGURE 4 | Expression of Toll-like receptors in different ENS subsets. Dotplots showing the percentage of expressing cells as well as average expression within the indicated identified neuronal clusters for selected murine Toll-like receptor genes. Data was downloaded from http://mousebrain.org/ (12) or from https://singlecell.broadinstitute.org/ (14). Genes not present in the plot showing the Drokhlyansky data have been filtered. All dataset were normalized and transformed before the plots were created using the Seurat package (16).

excitation of vasomotor neurons lead to vasodilation and, thus, increase local blood flow (34). This is interesting because the topical application of Ach on vascular smooth muscle normally causes vasoconstriction and thus Ach seems to exert context-dependent effects. The two other neuronal classes described in the guinea-pig can be functionally assigned to different secretomotor neuronal populations. These neurons and their associated regulatory mechanisms are of great importance in host physiology because interference with secretory mechanisms may lead to constipation or diarrhea. Secretomotor neurons are activated *via* intrinsic primary afferent neurons following chemical or physical interactions with luminal contents (34). Such stimuli can activate cAMP or Ca^{2+} activated chloride ion channels, which regulate the movements of chloride towards the intestinal lumen or through epithelial cells into the lamina propria and cause water to diffuse along (35, 36). Based on expressed neuro-transmitters, two types of secretomotor neurons can be distinguished; the first group co-expresses *ChAT* and neuropeptide Y, whereas the second group is characterized by the expression of *ChAT* only (37). However, future studies need to delineate the functional role of each neuro-transmitter in detail, which will allow to correctly assign the transcriptional profiles to neuronal classes.

Sensory Neurons

The ENS needs to monitor mechanical and chemical perturbations in order to react to incoming physiologic or pathologic signals and to tune local cellular components (**Figure 1**). Extrinsic sensory innervation includes spinal and vagal afferent neurons, whose cell bodies lie outside the intestine, e.g. in the Dorsal Root Ganglia or in the nodose/jugular ganglia. While anatomically defined, distinct afferent neurons originating from the intestine project into dorsal root ganglia, the vagus nerve innervates the entire intestine with a denser innervation in the small intestine as compared to the large intestine. Both types of extrinsic axons, DRG-derived or vagal, project into the inner and outer layers of the intestinal wall and perceive signals that are important to guide homeostatic functions. The respective neuronal population and its functional role are discussed in the next paragraph (Dorsal Root Ganglia section of the review).

Intrinsic innervation is characterized by cell bodies lying inside the gut wall. Neurons that perceive and integrate sensory information in the ENS are called intrinsic primary afferent neurons (IPANs). IPANs are integrated in the neuronal architecture to act in concert with motor neurons, interneurons and secretomotor neurons to direct homeostatic functions depending on external stimuli and the needs of digestive functions (38). Similar to the ENS, sensory neurons can be classified according to their neurochemical signature. Sensory IPANs have been identified based on the known markers *Calca*/*Calcb*/*Nfem*/*Calb1*/*Calb2* and the selective expression for *NMU*, *Ucn-3*/*Cck* or *Nxph2*.

Depending on the function of the neurons, three types of IPANs are identified using small intestine of the guinea pig as a model organism. First, chemosensitive IPANs respond to chemicals present on the surface of the small intestine (39). Because nerve fibers do not reach the surface of the intestine, and thus, do not come into direct contact with luminal contents, chemical changes in the intestinal lumen have to be sensed indirectly *via* signals from epithelial cells (40). Enteroendocrine cells are specialized epithelial cells, which for instance release 5-Hydroxytryptamine (5-HT) upon mucosal chemical or mechanical stimulation, which is a potent stimulator of IPANs and act as a signal transducer (41). However, knowledge concerning how endothelial cells communicate with the

ENS remains a black box. Analysis of single-cell RNA-sequencing data from epithelial cells suggest the presence of potential stimulating peptides, such as Substance P, cholecystokinin, ghrelin, and synthesizing enzymes of signaling amines (5-HT) in these cells (42). In line with these observations, receptors for the respective stimulators, such as the 5-HT_3 receptor and Substance P receptor 1, is expressed in IPANs suggesting an enteroendocrine to ENS signaling hub. Thus, sensory neurons functionally assigned to chemosensitive IPANs could be identified by a distinct gene profile for receptors of stimulating peptides and amines. However, the stimulation of other classes of IPANs, the mucosal mechanoreceptors, is also mostly indirect *via* 5-HT released from enterochromaffine cells (43). The second functional class described are stretch-sensitive IPANs that react to mechanical tension/distortion (38). Interestingly, these neurons seem to be not only mechanosensitive, but can also directly act as inhibitory motor neurons (44). Thus, their gene profile may be mixed and complicated for a functional assignment. The third group of IPANs are mucosal mechanoreceptors, which may be identified by putative mechanosensitive ion channels (e.g. Piezo2). However, as stated above, the stimulation of mucosal mechanoreceptors is mostly indirect and further studies need to unravel their profiles in more detail.

Interneurons

The interneurons, as the name suggests, are neurons, which connect functionally diverse neuronal populations in order to complete a neuronal circuit of varying complexities. For instance, interneurons receive signal from sensory neurons (which sense environmental perturbations) and relay the signal to either inhibitory or excitatory motor neurons to trigger an effector function to the sensed stimulus (**Figure 1**). The interneurons, as a type of 'bridging neurons', are multipolar and can be excitatory or inhibitory. They are primarily located within the myenteric plexus forming uniaxonal chains along the length of the gut with the ascending interneurons projecting orally and the descending interneurons projecting anally. There are different interneurons within the myenteric plexus with distinct neurochemical signatures, which can differ between gut regions. Using the guinea pig as a model organism, one class of excitatory ascending interneurons and three classes of descending interneurons have been described in the small intestine (45). In the colon on the other hand, three neurochemical classes of ascending interneurons and four classes of descending interneurons have been identified (46). These anatomic differences may come from the distinct local environment (e.g. microbiota) and the functional role it has to execute in different anatomic regions. The method applied by the authors was to morphologically distinguish interneuronal subpopulations. Whereas, upon distinguishing the subpopulations of interneurons based on their transcriptional signature, Morarach et al. was able to identify two subpopulations: one expressing motor-neuron-like inhibitory and excitatory neuropeptides *Nos1*/*ChAT* and the other expressing sensory-neuron-like neuropeptides *Sst*/*Calcb*/*Calb2* (13). The discrepancy in the findings/characterization of these studies might be a result of difference in either their method of evaluation (morphological vs transcriptomic) or the anatomic locations within the gut studied. However, several studies demonstrate that neurochemical markers of the interneurons overlap with other neuronal populations and, as such, do not have their unique neurochemical signature.

INTEGRATION OF SENSORY NEURONAL SIGNALS ORIGINATING FROM THE LUMINAL CONTENT OF THE INTESTINE

Since nerve fibers do not reach the lumen of the intestine under homeostatic conditions, microbiota and metabolites could directly stimulate neurons by penetrating the epithelial barrier or indirectly through epithelial cells or other cell types. Epithelial enteroendocrine cells were shown to be directly innervated by neurons and transduce signals to the CNS within milliseconds after being exposed to sugar (47). Although the finding that epithelial cells are innervated is still controversial (48), sensing of secondary signals released from epithelial cells by enteric neurons is likely to contribute to the regulation of intestinal homeostasis.

Using AAV particles for neuronal-specific deletion of aryl hydrocarbon receptor (Ahr) in enteric neurons, Obata et al. could demonstrate a pivotal functional for enteric neurons in metabolite sensing (49). Genetic ablation of Ahr resulted in reduced peristalsis and increased intestinal transit times. Further, Ahr expression was altered in germ-free mice suggesting a role for commensal microbiota in regulating Ahr expression in neurons. Several studies have reported that the absence of an intact microbiota resulted in activation of neurons and alterations of neuronal composition in ENS (48–50) as well as effects on CNS function were reported (51, 52). This leads to the question if and how neurons sense microorganisms. In vitro data show that neurons are able to respond to PAMPs, for example LPS *via* an TLR4-independent, or excretory secretory product of helminth in an Myd88-dependent manner (53, 54). Analysis of enteric neuronal populations in germ-free mice has revealed alterations in the enteric nervous system, in particular of $NOS1^+$ neurons, although there is some discrepancy between the studies whether $NOS1^+$ neurons are over- or underrepresented (50, 55, 56). Similar findings were reported in Myd88-deficient mice arguing for a role of TLRs in microbial sensing and development of the ENS. However, it remains unclear if the phenotype is explained by direct sensing of neurons *via* TLRs or indirect mechanisms *via* secondary messengers. Conditional gene targeting of TLR4 using WNT1-Cre resulted in altered ENS development. However, it should be mentioned that this targeting strategy does not exclude a major role for glial cells in TLR4 microbial sensing instead of or in addition to enteric neurons (13, 50).

Investigating neuronal-specific gene expression in germ-free animals by using the Ribotag system activated by SNP25Cre, Muller et al. could detect cell death of $CART^+$ neurons, which are involved in regulation of blood glucose levels, mediated by NLRP6 and Casape-11-dependent pathway (20). Further, the absence of an intact commensal microbiota is detected in parts

via SCFA and GPR41 resulted in an increased sympathetic neuronal activity, as measured by c-FOS expression in the coeliac-superior mesenteric ganglia, activation of glutamatergic sensory neurons in the brainstem and decreased intestinal transit time (48). Similarly, cell death controlled by a NLRP6 and Casape-11-dependent pathway as well as sympathetic activation have been reported following *Salmonella enterica* infection resulting in decreased intestinal motility. In this context, activation of the sympathetic nervous system and release of norepinephrine can instruct a tissue-protective program in muscularis macrophages characterized by Arginase 1 and BMP-2 expression and responsiveness to enteric neuron-derived CSF-1 (48, 57).

Altogether, the signaling circuits sensing mucosal homeostasis are emerging and the integration of these signals in the CNS can provide on more holistic view on how intestinal homeostasis is regulated.

DORSAL ROOT GANGLIA

Dorsal root ganglia (DRGs) comprise of neuronal cell bodies of sensory neurons, whose nerve fibers innervate different anatomic regions (e.g. thorax, small intestine, colon and extremities). The cell bodies represent the somas of the first-order sensory neurons and constitute an integral part of the somatosensory system. DRGs process sensory information transduced from afferent nerves, which may include nociceptive information, in addition to signals generated in steady-state conditions such as the feeling of discomfort and satiety (**Figure 5**). The afferent neurons of DRGs integrate sensory information from distinct body regions and transmit the signal to the CNS (58). Sensory afferent signals from the thoracolumbar region predominantly originate from the small intestine and lumbosacral DRGs mainly project the lower extremities, but also to the large intestine (59–61). Different types of sensory neurons within DRGs allow sensing of distinct stimuli. Initially, sensory neurons were categorized based on their degree of myelination and the associated conduction velocity. This classification led to four main classes of neurons, namely thinly myelinated Aδ fibers, unmyelinated C fibers, heavily and moderately A myelinated fibers. Because of a significant heterogeneity in the degree of myelination and conduction velocity in functionally similar classes, this classification insufficiently reflects function. To gain further insight into different populations and to functionally discern the neurons within DRGs, there are now many studies available, which have performed single-cell RNA-sequencing and associated gene expression with a function in mice, primates and humans (12, 62–68). The respective datasets can be accessed *via* online tools and screened for genes of interest (as outlined in **Table 2**).

Single-cell RNA-sequencing, if not used with any selection, is an untargeted and unbiased approach that allows identification of neuronal cell types based on transcripts expressed by the cells. However, there is a certain noise in between studies that complicates the interpretation of the results. Furthermore, a clear limitation of current techniques is the rather superficial sequencing depth at a single-cell level and the required dissociation of cells, which may omit large and long axonal neurons during sort-purification. In future studies, such limitation may be overcome by the single-nuclei sequencing approach as described above (14).

However, with the available data, neurons can be classified into neurofilamentous, peptidergic and non-peptidergic neurons, which

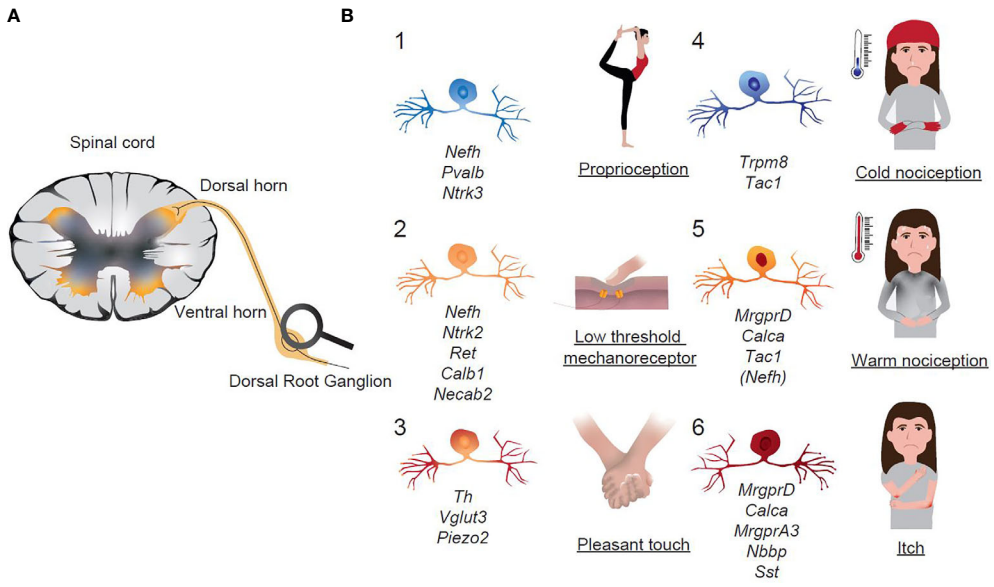


FIGURE 5 | Functional classification of sensory neurons in Dorsal Root Ganglia. **(A)** Schematic representation of extrinsic sensory afferent neurons that transmit signals from the periphery to the CNS. **(B)** Schematic representation of sensory neuronal groups in DRGs with assigned functions. Italics are the key transcripts of the respective neuronal group.

are uniformly detected in all the above-mentioned studies. A recent study used a machine-learning approach for three available nomenclatures/classification of DRG neurons (12, 63, 68) in finding the corresponding cell types in different datasets (**Table 3**) (64). The Usoskin classification showed the least 'noise' and highest prediction score when comparing different datasets and may therefore be the classification of choice (64). However, it has to be mentioned that the Usoskin study detected fewer neurons (622 neurons) compared to the work of Zeisel (1'580 neurons) or Sharma (10´922 neurons). The Usoskin-classification proposed a classification of DRG neurons into a total of 11 groups: 5 subgroups within the neurofilamentous group (NF1-5), 4 non-peptidergic neurons including TH^+ neurons (NP1-3, TH), 2 peptidergic neurons (PEP) including $Nav1.8^+$ neurons (**Table 3**) (63). Another study by Zeisel et al. was consistent with the results obtained by Usoskin, but due to more sequenced neurons, sub-clusters within peptidergic neurons (PEP1, 2, Trpm8), non-peptidergic neurons (NP 1, 2) were identified (12). One has to consider that available single-cell RNA-sequencing studies may compare DRGs that receive afferents from different anatomic locations and the subtype composition may vary across axial levels, which therefore could explain minor discrepancies when comparing neuronal population datasets (68, 70). Thus, sequencing of neurons isolated from either thoracic, lumbar or sacral regions may unravel anatomic fingerprints of gene expression in future studies.

Another recent study identified a total of 12 classes of DRG neurons, which consists of Aβ-rapidly adapting (RA) low threshold mechanoreceptors (LTMR), Aδ-LTMR, C-LTMR, 6 groups of Calcitonin Gene-related Peptide (CGRP) neurons, $Mrgrd^+$ polymodal nociceptors, $Somatostatin^+$ and cold thermosensors (68). Interestingly, a direct comparison of the three main classifications (Usoskin et al. *vs* Zeisel et al. vs Sharma et al.) by the before mentioned machine-learning approach found a high probability of finding a distinct cell across different datasets (64). Thus, the same cell can be found in different datasets and the difference between studies relies on the annotation and is not based on biologic differences. All the above-mentioned DRG-related studies have been performed in naïve animals and no specific disease model was profiled in detail. Therefore, it is of great interest to decipher transcriptional regulators in disease states such as nerve injury and concomitant neuronal regeneration at a single-cell resolution. A recent single-cell sequencing paper describes interesting features associated with different nerve injury models (69). First, a reduced expression of neuronal-subtype marker genes, such as *Tac1*, *Mrgprd*, and *Nefh* is described. Second, but not unexpected, genes involved in axon guidance, axogenesis and cell migration have overall increased. Of particular interest are specific transcripts found in nociceptive neurons because of their role in the development of neuropathic pain (69). Genes of interest can be browsed in the available online tool (see **Table 2**).

A large set of publications has studied different neuronal molecules, which help to propose a relation of the molecular profile of the respective neuron with modality-specific function within DRG neurons. These studies allow to functionally predict the different neuronal populations found in single-cell RNA-sequencing studies (**Figure 5**). Generally, neurofilamentous and TH populations are proprioceptors (control of body positioning and balance) and LTMRs (touch sensations), whereas non-peptidergic and peptidergic neurons are nociceptors (damage/potential damage signals), which represent the majority of all DRG neurons. Within the group of nociceptors and based on the molecular signature, a broad functional sub-classification is made: TRP cation channel subfamily member 8 (TRPM8) neurons are cold-sensory neurons, peptidergic neurons detect heat and pain, isolectin4-binding non-pepdidergic neurons can sense noxious touch, itch and chemical signals (**Figure 5**).

Because of available top gene transcripts, the populations can be identified and targeted, either *via* immunohistochemistry, potentially flow cytometry or used for a conditional deletion by using specific Cre-drivers in mice that allow deleting genes within a neuronal subpopulation.

Colonic Afferent DRGs

A recent single-cell RNA-sequencing study on retrogradely traced colonic sensory neurons in the mouse identified seven neuronal subtypes (70). The authors identified 5 specific subtypes in the thoracolumbar region and seven subtypes in the lumbosacral region, two of them being exclusively found in the lumbosacral region. The populations identified in both anatomic regions included Neurofilament-a and Neurofilament-b, which express genes typically associated with myelinated DRG neurons, such as neurofilament heavy chain (*Nefh*) and lactate dehydrogenase B (*Ldhb*). The third subtype was classified as non-peptidergic neurons and showed an expression pattern of non-peptidergic nociceptors, such as the purinergic receptors P2X3 or glial-cell line derived neurotrophic factor family receptor alpha2 (*Gfrα2*). The last two subtypes express *Calca*, *Tac1* and *TrkA* and have been termed as peptidergic nociceptors. The subtypes of neurons exclusively found in the lumbosacral region included a neurofilament and a

TABLE 2 | links to access the respective datasets.

Study	Sequencing site	Links
Zeisel et al. (12)	ENS	http://loom.linnarssonlab.org/dataset/cellmetadata/Mousebrain.org.level6/L6_Enteric_neurons.loom
Usoskin et al. (63)	DRG naive	http://linnarssonlab.org/drg/
Sharma et al. (68)	DRG naive	https://kleintools.hms.harvard.edu/tools/springViewer_1_6_dev.html?datasets/Sharma2019/all
Zeisel et al. (12)	DRG naive	http://loom.linnarssonlab.org/dataset/cellmetadata/Mousebrain.org.level6/L6_Peripheral_sensory_neurons.loom
Renthal et al. (69)	DRG after axonal injury	http://www.painseq.com
Hockley et al. (70)	DRG colon	http://hockley.shinyapps.io/ColonicRNAseq.
Häring et al. (71)	Dorsal horn of spinal cord	https://linnarssonlab.org/dorsalhorn/

TABLE 3 | Reference genes for the identification of neuronal subsets in DRGs based on unbiased single-cell RNA sequencing (adapted from (12, 58, 63, 64, 68).

	Proprioceptive/touch sensation neurons				Nociceptive neurons															
					Non-peptidergic					Peptidergic neurons										
Usoskin	NF1	NF2	NF3	NF4	TH	NP1	NP1	NP2	NP2	NP3	Pep2	Pep1	Pep1	Pep1	Pep1	TRPM8	TRPM8	TRPM8
Zeisel	NF1	NF2	NF2	NF3	NP1	NP2	NP3	NP4	NP5	NP6	Pep1	Pep2	Pep3	Pep4	Pep5	Pep6	Pep7	Pep8
Shama	Adelta-LTMR	Abeta-RA-LTMR	Abeta-Field	Proprioceptors	C-LTMR	NP-nociceptors	NP-nociceptors	CGRP-theta	CGRP-theta	Sst	CGRP-zeta	CGRP-ata	CGRP-gamma	CGRP-epsilon	CGRP-alpha	CGRP-beta	TRPM8	TRPM8	TRPM8
Signature genes	Nefh	Nefh	Nefh	Nefh	TH	Mrgprd	Mrgprd	Calca	Calca	Tac1	Calca	Calca	Calca	Calca	Calca	Calca	Trpm8	Trpm8	Trpm8
	Ntrk2	Ntrk2low	Ntrk3high	Pvalb	Vglut3	Prkcq	Prkcq	MgprA3	MgprA3	Nppb	Nefh	Nefh	Tac1	Tac1	Tac1	Tac1	Tac1	Tac1	Tac1
	Necab2	Ret	Fam19a1	Ntrk3	Piezo2	Agtr1a	Agtr1a	Gfra1	Gfra1	Nts	Ntrk1	Ntrk1	Sertm1	Ltk	Sstr2	Dcn	Angpt4	Ntm	Pnoc
	Cacna1h	Calb1	Ret	Runx3	Zfp521	Lpar3	Cyp26b1	Mlc1	Mlc1	Il31ra	Smr2	Smr2	Mrap2	Traf3ip3	Dcdc2a				Penk
						Barx2				Osmr	Creg2	Creg2	Slc5a7						

NF 1-4, neurofilamentous neurons; TH, thyroxin hydroxylase; NP1-6, non-peptidergic neurons; Pep, peptidergic neurons; TRPM8, transient receptor potential cation channel subfamily M member 8 neurons; LTMR, low threshold mechanoreceptor.

peptidergic subtype, both of which show a relatively similar basic expression pattern (neurofilament subgroup: *Piezo2*, *Nefh*, *Ldhb*; peptidergic subgroup: *Calca*, *Tac1*) but are furthermore characterized by a specific gene set suggesting that neurons have an anatomic fingerprint.

DRGs Originating From Iliac/Skin-Innervating Lymph Nodes

Apart from sensory neurons directly originating from organs such as the intestine, lymph nodes show a spatial distribution of sensory and sympathetic neurons (72). A more comprehensive view by using tracing experiments *via* the injection of Cre-expressing viruses into iliac lymph nodes and single-cell RNA-sequencing of labelled DRG neurons reveals 4 transcriptionally distinct neuronal subtypes (72). The vast majority of detected neurons express *Nav1.8* and only few co-expressed *TH*. By comparing the detected neurons to publicly available datasets (63, 68), the study revealed that lymph nodes are equipped with transcriptionally heterogenous, predominant peptidergic nociceptors. Of great interest in inguinal-lymph nodes are particularly enriched genes involved in inflammation and neuro-immune-communication, such as *Il33*, *TLRs* or *Ptgir*. The same authors also observed sensory neuronal remodeling after LPS application suggesting modular sensing of danger signals by the nervous system (72). Future studies should focus on the sensory DRGs of mesenteric lymph nodes to delineate transcriptional profiles associated with the primary hubs exposed to constant PRR signals.

Vagal Ganglia

Apart from DRGs located in close proximity to the spinal cord, vagal ganglia receive afferent signal input and mediate host protection against a broad variety of sensations. The upper airways are densely innervated by the vagus nerve with the fused primary order neurons located in the nodose/jugular/petrosal superganglia clustering near the jugular foramen. These sensory neurons play a pivotal role in swallowing reflex pathways and coordinate the upper airways to prevent aspiration pneumonia or dysphagia (73). Single-cell RNA-sequencing of nodose/jugular/petrosal ganglia with a coverage of 25'117 sensory neurons (spanning 2293 genes) revealed a total of 37 classes of neurons. *P2RY1*$^{+}$ neurons seem to be particularly relevant with respect to swallow reflexes because conditional ablation of P2RY1 neurons led to impaired swallowing responses (73). Given the wide variety of neurons located in vagal ganglia, the role of other subclasses remains elusive and needs to be studied in future projects.

FUNCTIONAL ROLE OF NEURONAL SUBPOPULATION IN DRGs

Neurofilaments-Expressing Neurons (Groups NF1-4)

Neurofilaments (NF, gene *Nefh*) are 10nm bundles of fibrils within neurons (74). In contrast to the perikarya, NF can be highly abundant in axons (75). In concert with microtubule and microfilaments, NF form the neuronal cytoskeleton and are

aligned in parallel along the axonal axis. The filaments primarily support neuronal structure and regulate the axonal diameter, which is a critical determinant of neuronal conduction velocity (76, 77). Based on the marker NF200, two distinct neuronal subtypes have been identified that allow the discrimination between large myelinated or thinly myelinated neurons with fast conduction velocities (NF200 positive) and small unmyelinated neurons with low conduction velocities (NF200 negative) (78). Because of their high conduction velocities, NF neurons are involved in signals that require fast signal transmission, such as touch sensations and proprioception. Apart from NF, spinal proprioceptive neurons, which innervate muscle spindles and Golgi tendon organs, are further characterized by the expression of neurotrophin-3 (*Ntf3)* and parvalbumin (*Pvalb*) (79). The expression of other marker genes, such as Tropomyosin receptor kinase A and B (*TrkA*, *TrkB*), Ret, calbindin (*Calb*), functional predictions can be made of NF neurons and allow for a certain functional classification (58).

According to the Usoskin-classification, gene expression profiles of NF group 1-3 is characterized by a pattern of *Nefh*, *Ntrk2*, *Ret*, *Calb1* and *Ntrk3* (63). These groups can be functionally assigned to low threshold mechanoreceptors (LTMRs). The expression of *Ntrk3* and *Pvalb* in NF groups 4/5 suggests that these neurons have a proprioceptive role (**Table 3**) (63). In general, proprioceptors are located within the musculoskeletal system and relay the information to the CNS concerning body position and movements (**Figure 5**).

A more comprehensive view into the subgroups and the associated gene expression suggests that NF group 1 are lightly myelinated Aδ LTMRs and very sensitive velocities detectors tuned to the deflection of body hairs (80). NF group 2 are rapid adapting LTMRs that end in Meissner corpuscle and longitudinal lanceolate endings and are critical for the perception of skin movement and vibration (81). Because of the high expression of *Ntrk3*, NF group 3 can be assigned to slowly adapting LTMRs, which control the perception of stretch and indentation (81).

TH Neurons

TH-expressing DRG neurons have been shown to be C-LTMRs (82). One of the top genes expressed in this population, *Vglut3*, has been identified to be uniquely found in C-LTMRs. Functionally, absence of Vglut3 in *Vglut3*$^{-/-}$ mice has been linked to defects in acute mechanical pain sensations upon intense noxious stimuli (83). An additional feature of these neurons is the high expression of the mechanosensitive ion channel, *Piezo2*. Apart of mechanical pain, C-LTMRs perceive low mechanical forces such as pleasant touch of the skin.

Peptidergic and Non-Peptidergic Neurons

Slow conducting DRG neurons are classified into peptidergic and non-peptidergic neurons. Peptidergic neurons are defined based on the expression of the neuropeptides substance P, calcitonin gene-related peptide (CGRP), and somatostatin, whereas non-peptidergic neurons bind the plant lectin IB4, express the Mrg family of G-protein coupled receptors and P2X3 (84, 85). However, one must take into account that all of these markers are only partially selective and some overlap can exist (86). Basically, peptidergic and non-peptidergic neurons are unmyelinated, primary afferent neurons assigned to nociceptors and thus respond to damage and potential damage stimuli (**Figure 5**).

From an immunologic perspective, peptidergic neurons and their secreted neuropeptides, including Substance P, CGRP and NMU, have a marked immunoregulatory potential, which has been shown by many studies (54, 87–91) and will be discussed in detail in a later paragraph.

Non-Peptidergic Neurons

The expression of the Mas-related G-protein coupled receptor member D (*MrgprD*) in non-peptidergic neurons group 1 (NP1) suggests its role in the perception of noxious mechanical and thermal stimuli as well as in the perception of itch (92, 93). NP2, expressing *MrgprA3* and *Calca*, are polymodal nociceptors and evoke itch responses (94). Both groups, NP1 and NP2, have been linked to the development of neuropathic pain (63, 95, 96). Even though characterized by a different set of key genes, NP3 have similar functions than NP2 and are also itch-sensing neurons. Relevant genes in this neuronal subset include the natriuretic polypeptide B (*Nbbp*) and Somatostatin (*Sst*). Injection of Nbbp intrathecal triggered itch responses in mice whereas itch-responses were blocked upon ablation of Nbbp-receptor-expressing cells (97). In the same line, Sst co-expressed with Nbbp neurons and Sst^{+} neurons triggered itch behavior (98).

Peptidergic Neurons

Pep1 neurons can be largely grouped by the expression of *Tac1*, which represents the gene for substance P. In terms of neuronal function, this neuronal population is involved in thermosensation and thus reacts to noxious heat and cold stimuli (63, 99, 100). However, substance P has also a major role in neuro-immune interactions, which will be discussed in a later paragraph. Similarly, these neurons express CGRP (*Calca*), which has also been implicated in regulating immune functions. Pep2 neurons express neurofilament heavy chain (*Nefh*) as well as *Ntrk1*, suggesting that these neurons are lightly myelinated Aδ nociceptors, which have a higher conduction velocity than unmyelinated neurons (63). Thus, these neurons respond to dangerously intense mechanical or mechanothermal stimuli. TRPM8 neurons are involved in thermal perception of temperatures <20°C and cold-triggered nociception (101).

THE DORSAL HORN OF THE SPINAL CORD

Somatosensory sensations, as described above, can be activated by a large variety of stimuli. Excitatory and inhibitory afferent neurons transmit the signal to DRGs, where a first processing of the signal input occurs. Afferents from DRGs relay the information to the CNS *via* interneurons located in the dorsal horn of the spinal cord. The incoming information is further processed within the dorsal spinal cord and ultimately relayed to different brain areas (102). A recent study using single-cell RNA-sequencing of the dorsal spinal cord identified that all neurons

either express the vesicular glutamate receptor 2 (Vglut2) or the vesicular GABA transporter (Vgat), thus, representing glutamatergic excitatory or GABAergic inhibitory neurons. To enrich neurons, the authors used the reporter mouse lines $Vgat^{tdTom}$ and $Vglut2^{tdTom}$ and FACS-enriched the samples for the respective neuronal subsets (71). The final dataset of 1'545 neurons revealed 15 glutamatergic and 15 GABAergic neuronal subsets and based on literature, the expressed transcripts have been linked to certain physiologic functions (71). For detailed information, we kindly refer to the original articles since the complexity of spinal cord neuronal population is beyond the scope of this review (71, 103). A very interesting novel feature that allows to monitor activation of cell types involved in sensory signal transmission is the sensory-transcription coupling. By using triple in-situ hybridization and the combination of the immediate-early gene expression of *Arc* with markers for distinct neuronal cell types revealed that noxious heat and cold activate different sets of excitatory and inhibitory neurons (71). This finding underlines the complexity of neuronal networks and argues for a broad activation of neurons in response to one distinct modality.

NEURO-IMMUNE SIGNALING IN HOST PHYSIOLOGY AND DISEASE

Enteric and DRG neurons express the neuro-peptides VIP, CGRP, Substance P, and NMU, and the neuro-transmitters norepinephrine and acetylcholine. Many studies identified the respective receptor expression on a large set of immune cells, which implies the regulation of immune responses by the nervous system. Furthermore, the close co-localization of hematopoietic cells with neuronal fibers suggests a bidirectional signaling exchange. These neuro-immune modules constantly interact in order to adapt physiologic processes. The following paragraph reviews neuro-immune interactions based on neuro-peptides or transmitters.

Vasoactive Intestinal Peptide (Gene: *VIP*)

VIP engages on two subsets of receptors, Vipr1 and Vipr2. Thus, solely studying the experimental effects of VIP may be difficult to interpret because of receptor-dependent responses. Furthermore, mechanisms behind such studies do not allow to conclude specific effects on a cellular level. This fact has been observed in the following experimental designs: On one hand and in the context of gastrointestinal inflammation, VIP knock-out mice showed a more severe phenotype in DNBS- and DSS-induced colitis in mice and VIP has been linked to the maintenance of intestinal integrity (104). On the other hand, models of DSS-induced colitis in $Vipr1^{-/-}$ and $Vipr2^{-/-}$ mice displayed opposite results. In fact, $Vipr1^{-/-}$ mice showed a milder disease score compared to wild type mice, whereas $Vipr2^{-/-}$ developed a more severe colitis (105, 106). Thus, studies addressing the role of VIP need to take into account the distinct affinity of VIP onto its receptors and the distinct receptor expression on a broad array of immune cells. *Vipr2* is expressed by a large set of immune cells including innate lymphoid cells type 3 (ILC3), which play a crucial role in host defense mechanism against bacteria. Recent studies have now described *Vipr2* expression by ILC3s and binding of the ligand VIP regulates the release of IL-22 (106, 107). Because IL-22 controls the antimicrobial peptide production and regulates lipid absorption, the VIP-IL-22 axis has been proposed to shift the balance in host defense mechanisms and lipid uptake (107). Furthermore, VIP and IL-22 is released in a feeding-dependent manner and, thus, underlines the role of neuro-immune signaling for adaptations of barrier mechanisms dependent on food-uptake. However, the role played by VIP on ILC3s seems to be context dependent acknowledged by controversial results published by different authors (106, 107). VIP has also been shown to regulate type 2 immune reactions in the lung (108). By using ovalbumin-induced lung inflammation, the authors showed an increase of VIP transcripts in nodose ganglia of inflamed lungs, which was dependent on nociceptive neuronal firing. Upon release, VIP engages to Vipr2 and activated the release of type 2 cytokines from ILC2 and $CD4^+$ T cells (108). In the same context, nociceptors express FcεR1 and directly sense IgE-OVA complexes to initiate type 2 immune reactions (109). In summary, VIP appears as an important regulator of type 2 and type 3 immune responses.

Calcitonin Gene-Related Peptide (Gene: *Calca* and *Calcb*)

By using Capsaicin-induced denervation in neonatal mice and immunoassays, early studies already suggested that the vast majority of CGRP neurons have an extrinsic source in the intestine (110). According to current knowledge, the main source of CGRP are the unmyelinated sensory C fibers in DRGs (111). As reviewed above, CGRP-expressing neurons are assigned to nociceptive neurons and, thus, sense damage and potential damage stimuli and are involved in the perception of pain. Because CGRP expressing nerve fibers have been found in many immunologic organs including bone marrow, spleen, lymph nodes, skin and the intestine, and the receptor is expressed on many different immune cells, the immunoregulatory potential of CGRP has been repeatedly shown (54, 88, 89, 111–113). Following the release, CGRP engages the calcitonin receptor-like receptor (CALCRL) and the receptor-modifying protein 1 (RAMP1). It is important to note that immune cells have also been shown to express CGRP, which suggests a bi-directional crosstalk between immune cells and the nervous system (114, 115). In general, CGRP seems to have anti-inflammatory effects in the GI-tract. This has been highlighted in gain-of function experiments by modelling inflammatory bowel disease and the systemic administration of CGRP in rats, which resulted in an amelioration of TNBS-induced colitis (116, 117). In line with these results, loss-of-function experiments by using CGRP antagonists in rats or CGRP knock-out mice revealed increased susceptibility to colitis (118, 119). These results suggest that CGRP agonists may be potential therapeutic targets for the treatment of inflammatory diseases. The anti-inflammatory role of CGRP has also been highlighted in the context of *Streptococcus pyogenes* infection in the skin, which is the leading cause of the life-threatening necrotizing fasciitis. The

authors found that S. pyogenes directly secretes streptolysin S and promotes the release of CGRP from nociceptors, which inhibits neutrophil recruitment and phagocytic killing (120). In the same line and in the context of *Salmonella enterica* infection, TRPV1$^+$ nociceptors release CGRP and modulate M cells to eventually control host defense against Salmonella (121). In terms of type 2 immune reactions, innate lymphoid cells type 2 (ILC2) express the receptors CALCRL/Ramp1 and binding of CGRP modulate ILC2 activation, whereas deletion of this signaling cascade elevated ILC2 responsiveness and type 2 immunity (88, 89, 113). Thus, the prevailing view regarding the role of the neuropeptide CGRP is its overall anti-inflammatory properties. With regard to many overwhelming immune reactions, topic or systemic application of CGRP may be a valuable treatment strategy. However, further studies need to investigate the related mechanisms in more detail.

Substance P (Gene: *Tac1*)

Substance P (SP) is a member of the tachykinin family of neuropeptides and is encoded by the Tachykinin 1 (*Tac1*) gene. SP exerts its function *via* the engagement on G-protein coupled neurokinin receptors but predominantly binds on the neurokinin 1 receptor (NK1R) (122). SP is expressed in the central and peripheral nervous system and intrinsic neurons seem to be the major source in the intestine (123, 124). Once synthesized, SP is transported in large dense-core vesicle and released *via* exocytosis where it exerts its function on the same cell or the adjacent cell (125). Apart from neurons, immune cells have been implicated to express both, SP and its receptor NK1R (126). The relative broad expression of the receptor on immune cells in the lamina propria, such as mast cells, eosinophils, neutrophils, macrophages, dendritic cells and natural killer cells implies its tight regulatory immune function. In general, SP has a pro-inflammatory role *via* the induction of pro-inflammatory cytokines in immune cells (127). In particular, it has been highlighted that SP and its receptor NK1R increase the susceptibility to DSS- and TNBS-induced colitis (128, 129). A newly developed intestinal organ culture system observed an anti-correlation of Tac1 and its receptor TacR1 after the application of different microbiota strains into the in vitro system. This negative correlation has then been linked to alterations of Rorgt$^+$Tregs. Such observations suggest the potential of microbiota-sensing by Tac1$^+$ neurons and the consecutive modulation of immunologic reactions (130). In the allergic setting, nociceptors release SP after allergen exposure and promote migration and activation of adaptive type 2 immune responses (131). However, because of its broad expression in several tissues, the exact cellular role of SP/NK1R has to be further studied in conditional deletion models in order to decipher the mechanism behind the observed phenotypes.

Neuromedin U (Gene: *NMU*)

NMU is a short neuropeptide with highly conserved amidated C-terminus required for receptor binding. Neurons of the CNS, the pituitary gland and the ENS express NMU (132). Within the ENS, NMU labels together with CGRP a subset of ChAT$^+$ sensory neurons (12, 53, 132). Further, NMU expression is regulated by the commensal microbiota and modulated by secretory excretory products of helminths (48, 53, 133). To mediate its biological function, NMU binds to two large G-protein coupled receptors Nmur1 and Nmur2. Nmur2 is expressed in neurons in particular in the CNS and regulates feeding-behavior, circadian rhythm as well as pain perception and bone formation (132). The immunomodulatory functions of NMU were recognized years ago (134–136), however, before ILCs were emerging. Thus, the mechanism remained elusive until several publications demonstrated that NMU acts specifically *via* ILC2s in different organs (53, 133, 137). By binding to Nmur1, NMU triggers a signal cascade *via* Gαq - PLC, activation of NFAT, resulting in activation of ILC2, proliferation and cytokine production. Upon NMU stimulation, ILC2 promote the type 2 immune response characterized by eosinophil recruitment, goblet cell hyperplasia and mucus production, resulting in increased worm expulsion during *N. brasiliensis* infection or enhanced airway inflammation following papain challenge. Altogether, these data indicate that sensory neurons regulate ILC2 activation *via* NMU and CGRP with downstream effects on various immune cell types participating in type 2 inflammation at mucosal barriers.

Norepinephrine (Synthesizing Gene: Thyroxine Hydroxylase)

Norepinephrine has a dual function in mammalian hosts. On one hand, it is a stress or danger hormone, which is released during a fight-or-flight reaction and results in a concomitant increase in blood pressure, heart rate, glucose mobilization and other stress reactions. On the other hand, norepinephrine acts as a neurotransmitter released from sympathetic nerves located in sympathetic ganglia. Once released, norepinephrine binds and activates α- and β-adrenergic receptors, which are G protein-coupled receptors and thus exert their effect *via* a second messenger system. In general, β-adrenergic receptors seem to be immunologically more important given their anti-inflammatory effects in neutrophils, macrophages and ILCs (138–140). More specifically, β-adrenergic stimulation mediates the polarization of intestinal macrophages, which reside in close proximity to sympathetic neurons (138). After the activation of the β2-receptor, macrophages upregulate tissue protective-programs (138). Reciprocally, macrophages upregulate neuro-protective programs through an arginase1-polyamine axis, and, thus limit neuronal damage (57).

β2-adrenergic receptors are expressed in ILC2s and argue for a regulatory role of the sympathetic nervous system in controlling type 2 immune reactions. Indeed, norepinephrine has been shown to inhibit ILC2s, whereas ILC2 specific ablation of the β2 receptor magnified type 2 immune reactions and improved worm clearance in the context of *N. brasiliensis* infection (139). In summary, sympathetic neurons have an anti-inflammatory role in a broad range of immune cells.

Acetylcholine (Synthesizing Gene: Choline Acetyltransferase)

Acetylcholine (Ach) is a neurotransmitter predominantly used in the autonomous nervous system and the major neurotransmitter of the parasympathetic nervous system. In addition, excitatory motor neurons, sensory neurons and interneurons within the

ENS are capable of producing Ach. Ach mediates biological effects by binding to two different families of cholinergic receptors, the nicotinic and the muscarinic Ach receptors. Among immune cells, the nicotinic α7-Ach receptor is expressed on macrophages and ILC subsets and was investigated in detail due to its anti-inflammatory effects in various disease settings including but not limited to sepsis, IBD and arthritis. This intriguing finding, termed 'cholinergic anti-inflammatory pathway', uncovered a vagal regulated release of Ach, which binds on the nicotinic α7-Ach receptor on macrophages to suppress release of pro-inflammatory cytokines and in particular TNF-α, which is mediating immunopathology during sepsis or intestinal inflammation (141, 142). The cholinergic anti-inflammatory pathway has profound and rapid anti-inflammatory properties. The potent effects are illustrated by the prevention of septic shock in mice mediated by this pathway (143). Furthermore, stimulation of the vagal nerves using a medical device was successfully used in patients suffering from chronic arthritis to mitigate disease symptoms, which have been proven resistant to glucocorticoid treatment (144).

α7-nicotinic Ach receptor agonists were also shown to suppress ILC2 activation during allergic asthma (145). These data suggest a broad role of α7-Ach receptor signaling in damping chronic inflammation in various settings. On the contrary, vagotomy was shown to delay the resolution of inflammation following peritoneal *E. coli* infection. In this disease model, vagal stimulation promoted biosynthesis of resolvins in ILC3s (146). In addition, ILC3 might also serve as a source of Ach as described during allergic airway inflammation (147). In summary, Ach emerges as an important checkpoint for immune activation but the multifaceted actions as mucosal barriers require further investigation.

SUMMARY AND FUTURE PERSPECTIVE

The identification of different neuronal subpopulations within the intrinsic ENS, as well as the extrinsic sensory compartment, provides the basis for studying the peripheral nervous system in detail. We noticed that certain genes are more ubiquitously expressed by different neuronal subpopulations and may therefore be involved in different functions depending on the neuronal subtype. However, our review combines several datasets and allows for the identification of neuronal subtypes based on genes expressed, morphology and function. The identification of key transcripts of neuronal populations now allows to design specific Cre-driver mouse lines to study neuronal programs in health and disease. There are three technical strategies available to study gene expression in neuronal populations: The desired Cre-driver mouse line can be crossed to a mouse with conditional expression of a fluorescent protein. Fluorescent cells can then be FACS-sorted und analyzed for gene expression (12). The other option is the nuclear dissociation and FACS-enrichment of neuronal nuclei as described recently (14). And the third option is the use of Ribotag mice, which have a hemagglutinin-tagged ribosomal protein specifically active activated following Cre-recombination. Such an approach allows to study ribosomal transcripts specifically active for the desired cre-driver (148). To gain more fundamental insights into expressed genes, their regulation in health and disease as well as more definite view into neuronal subpopulations, the readers are kindly encouraged to upload their sequencing datasets into public available bioportals. This will allow the scientific community to browse for their genes of interest. Future studies should especially focus on comparing steady state gene expression to different diseases to unravel important transcripts in certain neuronal subpopulations.

If we consider the central role of the nervous system in regulating immune functions, the potential is remarkable. The work of Tracey and colleagues has underlined its potential by the discovery of the 'cholinergic anti-inflammatory' pathway (143, 149). Such profound anti-inflammatory properties are of need for overwhelming inflammatory diseases, such as inflammatory bowel disease, sepsis, rheumatoid arthritis, and many more. Of note, the therapeutic potential of the anti-inflammatory properties of the nervous system has been successfully tested in clinical trials in humans (144, 150, 151). Even more impressive is the notion that patients in one clinical trial did no longer respond to conventional anti-inflammatory treatments, whereas vagal nerve stimulation improved clinical symptoms of rheumatoid arthritis patients (144). However, there is an emerging need to identify neuronal mechanisms that construct anti- as well as pro-inflammatory reactions in more detail. Such insights may uncover key neuro-immune cues that can potentially be harnessed by drugs or biologics in the near future.

AUTHOR CONTRIBUTIONS

MJ: writing of manuscript. MK-B: bioinformatic analysis and critical revision of the manuscript. DD: writing of the manuscript. SM: design of figures and critical revision of the manuscript. CK: writing of manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by grants from the Swiss National Science foundation (Grant-ID: 184425 to MJ), the German Research Foundation (DFG; Neuromac SFB TR project B17, FOR2599 project 5 - KL 2963/5-2, KL 2963/2-1 and KL 2963/3-1 to CK), (DFG; SPP1937-DI764/9 to MK-B), and the European Research Council Starting Grant (ERCEA; 803087 to CK).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.679055/full#supplementary-material

Supplementary Figure 1 | Expression of chemokine receptors, C-type leptin receptors and other genes of interest in different ENS subsets. Dotplots showing the percentage of expressing cells as well as average expression within the indicated identified neuronal clusters for selected murine chemokine receptor, C-type leptin receptor and other genes of interest. Data was downloaded from http://mousebrain.org/ (12) or from https://singlecell.broadinstitute.org/ (14). Genes not present in the plot showing the Drokhlyanski data have been filtered. All dataset were normalized and transformed before the plots were created using the Seurat package (16).

REFERENCES

1. Morton GJ, Cummings DE, Baskin DG, Barsh GS, Schwartz MW. Central Nervous System Control of Food Intake and Body Weight. *Nature* (2006) 443(7109):289–95. doi: 10.1038/nature05026
2. Stifani N. Motor Neurons and the Generation of Spinal Motor Neuron Diversity. *Front Cell Neurosci* (2014) 8:293. doi: 10.3389/fncel.2014.00293
3. Guyenet PG. The Sympathetic Control of Blood Pressure. *Nat Rev Neurosci* (2006) 7(5):335–46. doi: 10.1038/nrn1902
4. Ulrich-Lai YM, Herman JP. Neural Regulation of Endocrine and Autonomic Stress Responses. *Nat Rev Neurosci* (2009) 10(6):397–409. doi: 10.1038/nrn2647
5. Rao M, Gershon MD. Enteric Nervous System Development: What Could Possibly Go Wrong? *Nat Rev Neurosci* (2018) 19(9):552–65. doi: 10.1038/s41583-018-0041-0
6. Jansen AS, Nguyen XV, Karpitskiy V, Mettenleiter TC, Loewy AD. Central Command Neurons of the Sympathetic Nervous System: Basis of the Fight-or-Flight Response. *Science* (1995) 270(5236):644–6. doi: 10.1126/science.270.5236.644
7. Rao M, Gershon MD. The Bowel and Beyond: The Enteric Nervous System in Neurological Disorders. *Nat Rev Gastroenterol Hepatol* (2016) 13(9):517–28. doi: 10.1038/nrgastro.2016.107
8. Furness JB, Callaghan BP, Rivera LR, Cho HJ. The Enteric Nervous System and Gastrointestinal Innervation: Integrated Local and Central Control. *Adv Exp Med Biol* (2014) 817:39–71. doi: 10.1007/978-1-4939-0897-4_3
9. Furness JB. The Enteric Nervous System: Normal Functions and Enteric Neuropathies. *Neurogastroenterol Motil* (2008) 20 Suppl 1:32–8. doi: 10.1111/j.1365-2982.2008.01094.x
10. Furness JB, Kunze WA, Clerc N. Nutrient Tasting and Signaling Mechanisms in the Gut. II. The Intestine as a Sensory Organ: Neural, Endocrine, and Immune Responses. *Am J Physiol* (1999) 277(5):G922–8. doi: 10.1152/ajpgi.1999.277.5.G922
11. Jakob MO, Murugan S, Klose CSN. Neuro-Immune Circuits Regulate Immune Responses in Tissues and Organ Homeostasis. *Front Immunol* (2020) 11:308. doi: 10.3389/fimmu.2020.00308
12. Zeisel A, Hochgerner H, Lonnerberg P, Johnsson A, Memic F, van der Zwan J, et al. Molecular Architecture of the Mouse Nervous System. *Cell* (2018) 174(4):999–1014 e22. doi: 10.1016/j.cell.2018.06.021
13. Morarach K, Mikhailova A, Knoflach V, Memic F, Kumar R, Li W, et al. Diversification of Molecularly Defined Myenteric Neuron Classes Revealed by Single-Cell RNA Sequencing. *Nat Neurosci* (2021) 24(1):34–46. doi: 10.1038/s41593-020-00736-x
14. Drokhlyansky E, Smillie CS, Van Wittenberghe N, Ericsson M, Griffin GK, Eraslan G, et al. The Human and Mouse Enteric Nervous System at Single-Cell Resolution. *Cell* (2020) 182(6):1606–22.e23. doi: 10.1016/j.cell.2020.08.003
15. Steinert EM, Schenkel JM, Fraser KA, Beura LK, Manlove LS, Igyarto BZ, et al. Quantifying Memory CD8 T Cells Reveals Regionalization of Immunosurveillance. *Cell* (2015) 161(4):737–49. doi: 10.1016/j.cell.2015.03.031
16. Hao Y, Hao S, Andersen-Nissen E, Mauck WM, Zheng S, Butler A, et al. Integrated Analysis of Multimodal Single-Cell Data. *Cell* (2020) 184 (13):3573–87.e29. doi: 10.1101/2020.10.12.335331
17. Oetjen LK, Mack MR, Feng J, Whelan TM, Niu H, Guo CJ, et al. Sensory Neurons Co-Opt Classical Immune Signaling Pathways to Mediate Chronic Itch. *Cell* (2017) 171(1):217–28 e13. doi: 10.1016/j.cell.2017.08.006
18. Wilson SR, The L, Batia LM, Beattie K, Katibah GE, McClain SP, et al. The Epithelial Cell-Derived Atopic Dermatitis Cytokine TSLP Activates Neurons to Induce Itch. *Cell* (2013) 155(2):285–95. doi: 10.1016/j.cell.2013.08.057
19. Kato J, Agalave NM, Svensson CI. Pattern Recognition Receptors in Chronic Pain: Mechanisms and Therapeutic Implications. *Eur J Pharmacol* (2016) 788:261–73. doi: 10.1016/j.ejphar.2016.06.039
20. Muller PA, Matheis F, Schneeberger M, Kerner Z, Jove V, Mucida D. Microbiota-Modulated CART(+) Enteric Neurons Autonomously Regulate Blood Glucose. *Science* (2020) 370(6514):314–21. doi: 10.1126/science.abd6176
21. Furness JB. Types of Neurons in the Enteric Nervous System. *J Auton Nerv Syst* (2000) 81(1-3):87–96. doi: 10.1016/S0165-1838(00)00127-2
22. Collamati A, Martone AM, Poscia A, Brandi V, Celi M, Marzetti E, et al. Anticholinergic Drugs and Negative Outcomes in the Older Population: From Biological Plausibility to Clinical Evidence. *Aging Clin Exp Res* (2016) 28(1):25–35. doi: 10.1007/s40520-015-0359-7
23. Johnson CD, Barlow-Anacker AJ, Pierre JF, Touw K, Erickson CS, Furness JB, et al. Deletion of Choline Acetyltransferase in Enteric Neurons Results in Postnatal Intestinal Dysmotility and Dysbiosis. *FASEB J* (2018) 32(9):4744–52. doi: 10.1096/fj.201701474RR
24. Taylor GS, Bywater RA. Antagonism of non-Cholinergic Excitatory Junction Potentials in the Guinea-Pig Ileum by a Substance P Analogue Antagonist. *Neurosci Lett* (1986) 63(1):23–6. doi: 10.1016/0304-3940(86)90006-6
25. Holzer P, Schluet W, Maggi CA. Ascending Enteric Reflex Contraction: Roles of Acetylcholine and Tachykinins in Relation to Distension and Propagation of Excitation. *J Pharmacol Exp Ther* (1993) 264(1):391–6.
26. Qu ZD, Thacker M, Castelucci P, Bagyanszki M, Epstein ML, Furness JB. Immunohistochemical Analysis of Neuron Types in the Mouse Small Intestine. *Cell Tissue Res* (2008) 334(2):147–61. doi: 10.1007/s00441-008-0684-7
27. Huang PL, Dawson TM, Bredt DS, Snyder SH, Fishman MC. Targeted Disruption of the Neuronal Nitric Oxide Synthase Gene. *Cell* (1993) 75 (7):1273–86. doi: 10.1016/0092-8674(93)90615-W
28. Burnstock G. Historical Review: ATP as a Neurotransmitter. *Trends Pharmacol Sci* (2006) 27(3):166–76. doi: 10.1016/j.tips.2006.01.005
29. Keef KD, Shuttleworth CW, Xue C, Bayguinov O, Publicover NG, Sanders KM. Relationship Between Nitric Oxide and Vasoactive Intestinal Polypeptide in Enteric Inhibitory Neurotransmission. *Neuropharmacology* (1994) 33(11):1303–14. doi: 10.1016/0028-3908(94)90030-2
30. Xue L, Farrugia G, Miller SM, Ferris CD, Snyder SH, Szurszewski JH. Carbon Monoxide and Nitric Oxide as Coneurotransmitters in the Enteric Nervous System: Evidence From Genomic Deletion of Biosynthetic Enzymes. *Proc Natl Acad Sci U S A* (2000) 97(4):1851–5. doi: 10.1073/pnas.97.4.1851
31. Lecci A, Santicioli P, Maggi CA. Pharmacology of Transmission to Gastrointestinal Muscle. *Curr Opin Pharmacol* (2002) 2(6):630–41. doi: 10.1016/S1471-4892(02)00225-4
32. Alcaino C, Farrugia G, Beyder A. Mechanosensitive Piezo Channels in the Gastrointestinal Tract. *Curr Top Membr* (2017) 79:219–44. doi: 10.1016/bs.ctm.2016.11.003
33. Solis AG, Bielecki P, Steach HR, Sharma L, Harman CCD, Yun S, et al. Mechanosensation of Cyclical Force by PIEZO1 Is Essential for Innate Immunity. *Nature* (2019) 573(7772):69–74. doi: 10.1038/s41586-019-1485-8
34. Hansen MB. The Enteric Nervous System II: Gastrointestinal Functions. *Pharmacol Toxicol* (2003) 92(6):249–57. doi: 10.1034/j.1600-0773.2003.920601.x
35. Cooke HJ, Xue J, Yu JG, Wunderlich J, Wang YZ, Guzman J, et al. Mechanical Stimulation Releases Nucleotides That Activate P2Y1 Receptors to Trigger Neural Reflex Chloride Secretion in Guinea Pig Distal Colon. *J Comp Neurol* (2004) 469(1):1–15. doi: 10.1002/cne.10960
36. Bornstein JC. *Physiology of the Gastrointestinal Tract, Sixth Edition*. (2018). pp. 429–51, JPPF. Elsevier Inc. (2018)
37. Clerc N, Furness JB. Intrinsic Primary Afferent Neurones of the Digestive Tract. *Neurogastroenterol Motil* (2004) 16 Suppl 1:24–7. doi: 10.1111/j.1743-3150.2004.00470.x
38. Furness JB, Jones C, Nurgali K, Clerc N. Intrinsic Primary Afferent Neurons and Nerve Circuits Within the Intestine. *Prog Neurobiol* (2004) 72(2):143–64. doi: 10.1016/j.pneurobio.2003.12.004
39. Bertrand PP, Kunze WA, Bornstein JC, Furness JB, Smith ML. Analysis of the Responses of Myenteric Neurons in the Small Intestine to Chemical Stimulation of the Mucosa. *Am J Physiol* (1997) 273(2 Pt 1):G422–35. doi: 10.1152/ajpgi.1997.273.2.G422

40. Bellono NW, Bayrer JR, Leitch DB, Castro J, Zhang C, O'Donnell TA, et al. Enterochromaffin Cells Are Gut Chemosensors That Couple to Sensory Neural Pathways. *Cell* (2017) 170(1):185–98.e16. doi: 10.1016/j.cell.2017.05.034
41. Kirchgessner AL, Tamir H, Gershon MD. Identification and Stimulation by Serotonin of Intrinsic Sensory Neurons of the Submucosal Plexus of the Guinea Pig Gut: Activity-Induced Expression of Fos Immunoreactivity. *J Neurosci* (1992) 12(1):235–48. doi: 10.1523/JNEUROSCI.12-01-00235.1992
42. Fung C, Vanden Berghe P. Functional Circuits and Signal Processing in the Enteric Nervous System. *Cell Mol Life Sci* (2020) 77(22):4505–22. doi: 10.1007/s00018-020-03543-6
43. Pan H, Gershon MD. Activation of Intrinsic Afferent Pathways in Submucosal Ganglia of the Guinea Pig Small Intestine. *J Neurosci* (2000) 20(9):3295–309. doi: 10.1523/JNEUROSCI.20-09-03295.2000
44. Mazzuoli-Weber G, Schemann M. Mechanosensitivity in the Enteric Nervous System. *Front Cell Neurosci* (2015) 9:408. doi: 10.3389/fncel.2015.00408
45. Neal KB, Bornstein JC. Targets of Myenteric Interneurons in the Guinea-Pig Small Intestine. *Neurogastroenterol Motil* (2008) 20(5):566–75. doi: 10.1111/j.1365-2982.2007.01052.x
46. Lomax AE, Furness JB. Neurochemical Classification of Enteric Neurons in the Guinea-Pig Distal Colon. *Cell Tissue Res* (2000) 302(1):59–72. doi: 10.1007/s004410000260
47. Kaelberer MM, Buchanan KL, Klein ME, Barth BB, Montoya MM, Shen X, et al. A Gut-Brain Neural Circuit for Nutrient Sensory Transduction. *Science* (2018) 361(6408):eaat5236. doi: 10.1126/science.aat5236
48. Muller PA, Schneeberger M, Matheis F, Wang P, Kerner Z, Ilanges A, et al. Microbiota Modulate Sympathetic Neurons *Via* a Gut-Brain Circuit. *Nature* (2020) 583(7816):441–6. doi: 10.1038/s41586-020-2474-7
49. Obata Y, Castano A, Boeing S, Bon-Frauches AC, Fung C, Fallesen T, et al. Neuronal Programming by Microbiota Regulates Intestinal Physiology. *Nature* (2020) 578(7794):284–9. doi: 10.1038/s41586-020-1975-8
50. Anitha M, Vijay-Kumar M, Sitaraman SV, Gewirtz AT, Srinivasan S. Gut Microbial Products Regulate Murine Gastrointestinal Motility *Via* Toll-Like Receptor 4 Signaling. *Gastroenterology* (2012) 143(4):1006–16.e4. doi: 10.1053/j.gastro.2012.06.034
51. Olson CA, Vuong HE, Yano JM, Liang QY, Nusbaum DJ, Hsiao EY. The Gut Microbiota Mediates the Anti-Seizure Effects of the Ketogenic Diet. *Cell* (2018) 173(7):1728–41.e13. doi: 10.1016/j.cell.2018.04.027
52. Chu C, Murdock MH, Jing D, Won TH, Chung H, Kressel AM, et al. The Microbiota Regulate Neuronal Function and Fear Extinction Learning. *Nature* (2019) 574(7779):543–8. doi: 10.1038/s41586-019-1644-y
53. Cardoso V, Chesne J, Ribeiro H, Garcia-Cassani B, Carvalho T, Bouchery T, et al. Neuronal Regulation of Type 2 Innate Lymphoid Cells *Via* Neuromedin U. *Nature* (2017) 549(7671):277–81. doi: 10.1038/nature23469
54. Meseguer V, Alpizar YA, Luis E, Tajada S, Denlinger B, Fajardo O, et al. TRPA1 Channels Mediate Acute Neurogenic Inflammation and Pain Produced by Bacterial Endotoxins. *Nat Commun* (2014) 5:3125. doi: 10.1038/ncomms4125
55. McVey Neufeld KA, Mao YK, Bienenstock J, Foster JA, Kunze WA. The Microbiome Is Essential for Normal Gut Intrinsic Primary Afferent Neuron Excitability in the Mouse. *Neurogastroenterol Motil* (2013) 25(2):183–e88. doi: 10.1111/nmo.12049
56. Collins J, Borojevic R, Verdu EF, Huizinga JD, Ratcliffe EM. Intestinal Microbiota Influence the Early Postnatal Development of the Enteric Nervous System. *Neurogastroenterol Motil* (2014) 26(1):98–107. doi: 10.1111/nmo.12236
57. Matheis F, Muller PA, Graves CL, Gabanyi I, Kerner ZJ, Costa-Borges D, et al. Adrenergic Signaling in Muscularis Macrophages Limits Infection-Induced Neuronal Loss. *Cell* (2020) 180(1):64–78.e16. doi: 10.1016/j.cell.2019.12.002
58. Emery EC, Ernfors P. Dorsal Root Ganglion Neuron Types and Their Functional Specialization. In: JN Wood, editor. *The Oxford Handbook of the Neurobiology of Pain Oxford Handbook Online*. Oxford University Press (2018).
59. Robinson DR, McNaughton PA, Evans ML, Hicks GA. Characterization of the Primary Spinal Afferent Innervation of the Mouse Colon Using Retrograde Labelling. *Neurogastroenterol Motil* (2004) 16(1):113–24. doi: 10.1046/j.1365-2982.2003.00456.x
60. Tan LL, Bornstein JC, Anderson CR. Distinct Chemical Classes of Medium-Sized Transient Receptor Potential Channel Vanilloid 1-Immunoreactive Dorsal Root Ganglion Neurons Innervate the Adult Mouse Jejunum and Colon. *Neuroscience* (2008) 156(2):334–43. doi: 10.1016/j.neuroscience.2008.06.071
61. Deshpande D, Agarwal N, Fleming T, Gaveriaux-Ruff C, Klose CSN, Tappe-Theodor A, et al. Loss of POMC-Mediated Antinociception Contributes to Painful Diabetic Neuropathy. *Nat Commun* (2021) 12(1):426. doi: 10.1038/s41467-020-20677-0
62. Li CL, Li KC, Wu D, Chen Y, Luo H, Zhao JR, et al. Somatosensory Neuron Types Identified by High-Coverage Single-Cell RNA-Sequencing and Functional Heterogeneity. *Cell Res* (2016) 26(8):967. doi: 10.1038/cr.2016.90
63. Usoskin D, Furlan A, Islam S, Abdo H, Lonnerberg P, Lou D, et al. Unbiased Classification of Sensory Neuron Types by Large-Scale Single-Cell RNA Sequencing. *Nat Neurosci* (2015) 18(1):145–53. doi: 10.1038/nn.3881
64. Kupari J, Usoskin D, Lou D, Parisien M, Hu Y, Fatt M, et al. Single Cell Transcriptomics of Primate Sensory Neurons Identifies Cell Types Associated With Human Chronic Pain. *Nat Commun* (2020) 12:1510. doi: 10.1038/s41467-021-21725-z
65. Finno CJ, Peterson J, Kang M, Park S, Bordbari MH, Durbin-Johnson B, et al. Single-Cell RNA-Seq Reveals Profound Alterations in Mechanosensitive Dorsal Root Ganglion Neurons With Vitamin E Deficiency. *iScience* (2019) 21:720–35. doi: 10.1016/j.isci.2019.10.064
66. Faure L, Wang Y, Kastriti ME, Fontanet P, Cheung KKY, Petitpre C, et al. Single Cell RNA Sequencing Identifies Early Diversity of Sensory Neurons Forming *Via* Bi-Potential Intermediates. *Nat Commun* (2020) 11(1):4175. doi: 10.1038/s41467-020-17929-4
67. Hu G, Huang K, Hu Y, Du G, Xue Z, Zhu X, et al. Single-Cell RNA-seq Reveals Distinct Injury Responses in Different Types of DRG Sensory Neurons. *Sci Rep* (2016) 6:31851. doi: 10.1038/srep31851
68. Sharma N, Flaherty K, Lezgiyeva K, Wagner DE, Klein AM, Ginty DD. The Emergence of Transcriptional Identity in Somatosensory Neurons. *Nature* (2020) 577(7790):392–8. doi: 10.1038/s41586-019-1900-1
69. Renthal W, Tochitsky I, Yang L, Cheng YC, Li E, Kawaguchi R, et al. Transcriptional Reprogramming of Distinct Peripheral Sensory Neuron Subtypes After Axonal Injury. *Neuron* (2020) 108(1):128–44.e9. doi: 10.1016/j.neuron.2020.07.026
70. Hockley JRF, Taylor TS, Callejo G, Wilbrey AL, Gutteridge A, Bach K, et al. Single-Cell RNAseq Reveals Seven Classes of Colonic Sensory Neuron. *Gut* (2019) 68(4):633–44. doi: 10.1136/gutjnl-2017-315631
71. Haring M, Zeisel A, Hochgerner H, Rinwa P, Jakobsson JET, Lonnerberg P, et al. Neuronal Atlas of the Dorsal Horn Defines Its Architecture and Links Sensory Input to Transcriptional Cell Types. *Nat Neurosci* (2018) 21(6):869–80. doi: 10.1038/s41593-018-0141-1
72. Huang S, Ziegler CGK, Austin J, Mannoun N, Vukovic M, Ordovas-Montanes J, et al. Lymph Nodes are Innervated by a Unique Population of Sensory Neurons With Immunomodulatory Potential. *Cell* (2021) 184(2):441–59 e25. doi: 10.1016/j.cell.2020.11.028
73. Prescott SL, Umans BD, Williams EK, Brust RD, Liberles SD. An Airway Protection Program Revealed by Sweeping Genetic Control of Vagal Afferents. *Cell* (2020) 181(3):574–89 e14. doi: 10.1016/j.cell.2020.03.004
74. Yuan A, Rao MV, Veeranna, Nixon RA. Neurofilaments and Neurofilament Proteins in Health and Disease. *Cold Spring Harb Perspect Biol* (2017) 9(4). doi: 10.1101/cshperspect.a018309
75. Burton PR, Wentz MA. Neurofilaments Are Prominent in Bullfrog Olfactory Axons But Are Rarely Seen in Those of the Tiger Salamander, Ambystoma Tigrinum. *J Comp Neurol* (1992) 317(4):396–406. doi: 10.1002/cne.903170406
76. Hoffman PN, Cleveland DW, Griffin JW, Landes PW, Cowan NJ, Price DL. Neurofilament Gene Expression: A Major Determinant of Axonal Caliber. *Proc Natl Acad Sci U S A* (1987) 84(10):3472–6. doi: 10.1073/pnas.84.10.3472
77. Yuan A, Rao MV, Veeranna, Nixon RA. Neurofilaments at a Glance. *J Cell Sci* (2012) 125(Pt 14):3257–63. doi: 10.1242/jcs.104729
78. Lawson SN, Waddell PJ. Soma Neurofilament Immunoreactivity is Related to Cell Size and Fibre Conduction Velocity in Rat Primary Sensory Neurons. *J Physiol* (1991) 435:41–63. doi: 10.1113/jphysiol.1991.sp018497
79. Ernfors P, Lee KF, Kucera J, Jaenisch R. Lack of Neurotrophin-3 Leads to Deficiencies in the Peripheral Nervous System and Loss of Limb

Proprioceptive Afferents. *Cell* (1994) 77(4):503–12. doi: 10.1016/0092-8674(94)90213-5
80. Rutlin M, Ho CY, Abraira VE, Cassidy C, Bai L, Woodbury CJ, et al. The Cellular and Molecular Basis of Direction Selectivity of Adelta-LTMRs. *Cell* (2014) 159(7):1640–51. doi: 10.1016/j.cell.2014.11.038
81. Abraira VE, Ginty DD. The Sensory Neurons of Touch. *Neuron* (2013) 79(4):618–39. doi: 10.1016/j.neuron.2013.07.051
82. Li L, Rutlin M, Abraira VE, Cassidy C, Kus L, Gong S, et al. The Functional Organization of Cutaneous Low-Threshold Mechanosensory Neurons. *Cell* (2011) 147(7):1615–27. doi: 10.1016/j.cell.2011.11.027
83. Seal RP, Wang X, Guan Y, Raja SN, Woodbury CJ, Basbaum AI, et al. Injury-Induced Mechanical Hypersensitivity Requires C-Low Threshold Mechanoreceptors. *Nature* (2009) 462(7273):651–5. doi: 10.1038/nature08505
84. Averill S, McMahon SB, Clary DO, Reichardt LF, Priestley JV. Immunocytochemical Localization of trkA Receptors in Chemically Identified Subgroups of Adult Rat Sensory Neurons. *Eur J Neurosci* (1995) 7(7):1484–94. doi: 10.1111/j.1460-9568.1995.tb01143.x
85. Basbaum AI, Bautista DM, Scherrer G, Julius D. Cellular and Molecular Mechanisms of Pain. *Cell* (2009) 139(2):267–84. doi: 10.1016/j.cell.2009.09.028
86. Le Pichon CE, Chesler AT. The Functional and Anatomical Dissection of Somatosensory Subpopulations Using Mouse Genetics. *Front Neuroanat* (2014) 8:21. doi: 10.3389/fnana.2014.00021
87. Kashem SW, Riedl MS, Yao C, Honda CN, Vulchanova L, Kaplan DH. Nociceptive Sensory Fibers Drive Interleukin-23 Production From CD301b+ Dermal Dendritic Cells and Drive Protective Cutaneous Immunity. *Immunity* (2015) 43(3):515–26. doi: 10.1016/j.immuni.2015.08.016
88. Wallrapp A, Burkett PR, Riesenfeld SJ, Kim SJ, Christian E, Abdulnour RE, et al. Calcitonin Gene-Related Peptide Negatively Regulates Alarmin-Driven Type 2 Innate Lymphoid Cell Responses. *Immunity* (2019) 51(4):709–23 e6. doi: 10.1016/j.immuni.2019.09.005
89. Nagashima H, Mahlakoiv T, Shih HY, Davis FP, Meylan F, Huang Y, et al. Neuropeptide CGRP Limits Group 2 Innate Lymphoid Cell Responses and Constrains Type 2 Inflammation. *Immunity* (2019) 51(4):682–95.e6. doi: 10.1016/j.immuni.2019.06.009
90. Serra MC, Bazzoni F, Della Bianca V, Greskowiak M, Rossi F. Activation of Human Neutrophils by Substance PEffect on Oxidative Metabolism, Exocytosis, Cytosolic Ca2+ Concentration and Inositol Phosphate Formation. *J Immunol* (1988) 141(6):2118–24.
91. Theoharides TC, Zhang B, Kempuraj D, Tagen M, Vasiadi M, Angelidou A, et al. IL-33 Augments Substance P-Induced VEGF Secretion From Human Mast Cells and Is Increased in Psoriatic Skin. *Proc Natl Acad Sci U S A* (2010) 107(9):4448–53. doi: 10.1073/pnas.1000803107
92. Rau KK, McIlwrath SL, Wang H, Lawson JJ, Jankowski MP, Zylka MJ, et al. Mrgprd Enhances Excitability in Specific Populations of Cutaneous Murine Polymodal Nociceptors. *J Neurosci* (2009) 29(26):8612–9. doi: 10.1523/JNEUROSCI.1057-09.2009
93. Liu Q, Sikand P, Ma C, Tang Z, Han L, Li Z, et al. Mechanisms of Itch Evoked by Beta-Alanine. *J Neurosci* (2012) 32(42):14532–7. doi: 10.1523/JNEUROSCI.3509-12.2012
94. Han L, Ma C, Liu Q, Weng HJ, Cui Y, Tang Z, et al. A Subpopulation of Nociceptors Specifically Linked to Itch. *Nat Neurosci* (2013) 16(2):174–82. doi: 10.1038/nn.3289
95. Kang SA, Govindarajan R. Anti-Calcitonin Gene-Related Peptide Monoclonal Antibodies for Neuropathic Pain in Patients With Migraine Headache. *Muscle Nerve* (2020) 63(4):563–7. doi: 10.1002/mus.27153
96. Iyengar S, Ossipov MH, Johnson KW. The Role of Calcitonin Gene-Related Peptide in Peripheral and Central Pain Mechanisms Including Migraine. *Pain* (2017) 158(4):543–59. doi: 10.1097/j.pain.0000000000000831
97. Mishra SK, Hoon MA. The Cells and Circuitry for Itch Responses in Mice. *Science* (2013) 340(6135):968–71. doi: 10.1126/science.1233765
98. Huang J, Polgar E, Solinski HJ, Mishra SK, Tseng PY, Iwagaki N, et al. Circuit Dissection of the Role of Somatostatin in Itch and Pain. *Nat Neurosci* (2018) 21(5):707–16. doi: 10.1038/s41593-018-0119-z
99. McKemy DD, Neuhausser WM, Julius D. Identification of a Cold Receptor Reveals a General Role for TRP Channels in Thermosensation. *Nature* (2002) 416(6876):52–8. doi: 10.1038/nature719
100. Caterina MJ, Rosen TA, Tominaga M, Brake AJ, Julius D. A Capsaicin-Receptor Homologue With a High Threshold for Noxious Heat. *Nature* (1999) 398(6726):436–41. doi: 10.1038/18906
101. Lolignier S, Gkika D, Andersson D, Leipold E, Vetter I, Viana F, et al. New Insight in Cold Pain: Role of Ion Channels, Modulation, and Clinical Perspectives. *J Neurosci* (2016) 36(45):11435–9. doi: 10.1523/JNEUROSCI.2327-16.2016
102. Peirs C, Seal RP. Neural Circuits for Pain: Recent Advances and Current Views. *Science* (2016) 354(6312):578–84. doi: 10.1126/science.aaf8933
103. Sathyamurthy A, Johnson KR, Matson KJE, Dobrott CI, Li L, Ryba AR, et al. Massively Parallel Single Nucleus Transcriptional Profiling Defines Spinal Cord Neurons and Their Activity During Behavior. *Cell Rep* (2018) 22(8):2216–25. doi: 10.1016/j.celrep.2018.02.003
104. Wu X, Conlin VS, Morampudi V, Ryz NR, Nasser Y, Bhinder G, et al. Vasoactive Intestinal Polypeptide Promotes Intestinal Barrier Homeostasis and Protection Against Colitis in Mice. *PloS One* (2015) 10(5):e0125225. doi: 10.1371/journal.pone.0125225
105. Yadav M, Huang MC, Goetzl EJ. VPAC1 (Vasoactive Intestinal Peptide (VIP) Receptor Type 1) G Protein-Coupled Receptor Mediation of VIP Enhancement of Murine Experimental Colitis. *Cell Immunol* (2011) 267(2):124–32. doi: 10.1016/j.cellimm.2011.01.001
106. Seillet C, Luong K, Tellier J, Jacquelot N, Shen RD, Hickey P, et al. The Neuropeptide VIP Confers Anticipatory Mucosal Immunity by Regulating ILC3 Activity. *Nat Immunol* (2020) 21(2):168–77. doi: 10.1038/s41590-019-0567-y
107. Talbot J, Hahn P, Kroehling L, Nguyen H, Li D, Littman DR. Feeding-Dependent VIP neuron-ILC3 Circuit Regulates the Intestinal Barrier. *Nature* (2020) 579(7800):575–80. doi: 10.1038/s41586-020-2039-9
108. Talbot S, Abdulnour RE, Burkett PR, Lee S, Cronin SJ, Pascal MA, et al. Silencing Nociceptor Neurons Reduces Allergic Airway Inflammation. *Neuron* (2015) 87(2):341–54. doi: 10.1016/j.neuron.2015.06.007
109. Crosson T, Wang JC, Doyle B, Merrison H, Balood M, Parrin A, et al. FcepsilonR1-Expressing Nociceptors Trigger Allergic Airway Inflammation. *J Allergy Clin Immunol* (2021) 147(4):2330–42. doi: 10.1016/j.jaci.2020.12.644
110. Sternini C, Reeve JRJr., Brecha N. Distribution and Characterization of Calcitonin Gene-Related Peptide Immunoreactivity in the Digestive System of Normal and Capsaicin-Treated Rats. *Gastroenterology* (1987) 93(4):852–62. doi: 10.1016/0016-5085(87)90450-1
111. Assas BM, Pennock JI, Miyan JA. Calcitonin Gene-Related Peptide Is a Key Neurotransmitter in the Neuro-Immune Axis. *Front Neurosci* (2014) 8:23. doi: 10.3389/fnins.2014.00023
112. Hosoi J, Murphy GF, Egan CL, Lerner EA, Grabbe S, Asahina A, et al. Regulation of Langerhans Cell Function by Nerves Containing Calcitonin Gene-Related Peptide. *Nature* (1993) 363(6425):159–63. doi: 10.1038/363159a0
113. Xu H, Ding J, Porter CBM, Wallrapp A, Tabaka M, Ma S, et al. Transcriptional Atlas of Intestinal Immune Cells Reveals That Neuropeptide Alpha-CGRP Modulates Group 2 Innate Lymphoid Cell Responses. *Immunity* (2019) 51(4):696–708.e9. doi: 10.1016/j.immuni.2019.09.004
114. Wang X, Xing L, Xing Y, Tang Y, Han C. Identification and Characterization of Immunoreactive Calcitonin Gene-Related Peptide From Lymphocytes of the Rat. *J Neuroimmunol* (1999) 94(1-2):95–102. doi: 10.1016/S0165-5728(98)00230-6
115. Bracci-Laudiero L, Aloe L, Caroleo MC, Buanne P, Costa N, Starace G, et al. Endogenous NGF Regulates CGRP Expression in Human Monocytes, and Affects HLA-DR and CD86 Expression and IL-10 Production. *Blood* (2005) 106(10):3507–14. doi: 10.1182/blood-2004-10-4055
116. Mazelin L, Theodorou V, Fioramonti J, Bueno L. Vagally Dependent Protective Action of Calcitonin Gene-Related Peptide on Colitis. *Peptides* (1999) 20(11):1367–74. doi: 10.1016/S0196-9781(99)00144-8
117. Evangelista S, Tramontana M. Involvement of Calcitonin Gene-Related Peptide in Rat Experimental Colitis. *J Physiol Paris* (1993) 87(4):277–80. doi: 10.1016/0928-4257(93)90017-N
118. Engel MA, Khalil M, Siklosi N, Mueller-Tribbensee SM, Neuhuber WL, Neurath MF, et al. Opposite Effects of Substance P and Calcitonin Gene-Related Peptide in Oxazolone Colitis. *Dig Liver Dis* (2012) 44(1):24–9. doi: 10.1016/j.dld.2011.08.030

119. Reinshagen M, Flamig G, Ernst S, Geerling I, Wong H, Walsh JH, et al. Calcitonin Gene-Related Peptide Mediates the Protective Effect of Sensory Nerves in a Model of Colonic Injury. *J Pharmacol Exp Ther* (1998) 286 (2):657–61.
120. Pinho-Ribeiro FA, Baddal B, Haarsma R, O'Seaghdha M, Yang NJ, Blake KJ, et al. Blocking Neuronal Signaling to Immune Cells Treats Streptococcal Invasive Infection. *Cell* (2018) 173(5):1083–97 e22. doi: 10.1016/j.cell.2018.04.006
121. Lai NY, Musser MA, Pinho-Ribeiro FA, Baral P, Jacobson A, Ma P, et al. Gut-Innervating Nociceptor Neurons Regulate Peyer's Patch Microfold Cells and SFB Levels to Mediate Salmonella Host Defense. *Cell* (2020) 180(1):33–49 e22. doi: 10.1016/j.cell.2019.11.014
122. Mantyh PW. Neurobiology of Substance P and the NK1 Receptor. *J Clin Psychiatry* (2002) 63 Suppl 11:6–10.
123. Hokfelt T, Vincent S, Dalsgaard CJ, Skirboll L, Johansson O, Schultzberg M, et al. Distribution of Substance P in Brain and Periphery and Its Possible Role as a Co-Transmitter. *Ciba Found Symp* (1982) 1982(91):84–106. doi: 10.1002/9780470720738.ch6
124. Lai NY, Mills K, Chiu IM. Sensory Neuron Regulation of Gastrointestinal Inflammation and Bacterial Host Defence. *J Intern Med* (2017) 282(1):5–23. doi: 10.1111/joim.12591
125. De Camilli P, Jahn R. Pathways to Regulated Exocytosis in Neurons. *Annu Rev Physiol* (1990) 52:625–45. doi: 10.1146/annurev.ph.52.030190.003205
126. Suvas S. Role of Substance P Neuropeptide in Inflammation, Wound Healing, and Tissue Homeostasis. *J Immunol* (2017) 199(5):1543–52. doi: 10.4049/jimmunol.1601751
127. Shimizu Y, Matsuyama H, Shiina T, Takewaki T, Furness JB. Tachykinins and Their Functions in the Gastrointestinal Tract. *Cell Mol Life Sci* (2008) 65 (2):295–311. doi: 10.1007/s00018-007-7148-1
128. Engel MA, Khalil M, Mueller-Tribbensee SM, Becker C, Neuhuber WL, Neurath MF, et al. The Proximodistal Aggravation of Colitis Depends on Substance P Released From TRPV1-Expressing Sensory Neurons. *J Gastroenterol* (2012) 47(3):256–65. doi: 10.1007/s00535-011-0495-6
129. Engel MA, Leffler A, Niedermirtl F, Babes A, Zimmermann K, Filipovic MR, et al. TRPA1 and Substance P Mediate Colitis in Mice. *Gastroenterology* (2011) 141(4):1346–58. doi: 10.1053/j.gastro.2011.07.002
130. Yissachar N, Zhou Y, Ung L, Lai NY, Mohan JF, Ehrlicher A, et al. An Intestinal Organ Culture System Uncovers a Role for the Nervous System in Microbe-Immune Crosstalk. *Cell* (2017) 168(6):1135–48 e12. doi: 10.1016/j.cell.2017.02.009
131. Perner C, Flayer CH, Zhu X, Aderhold PA, Dewan ZNA, Voisin T, et al. Substance P Release by Sensory Neurons Triggers Dendritic Cell Migration and Initiates the Type-2 Immune Response to Allergens. *Immunity* (2020) 53 (5):1063–77 e7. doi: 10.1016/j.immuni.2020.10.001
132. Brighton PJ, Szekeres PG, Willars GB. Neuromedin U and its Receptors: Structure, Function, and Physiological Roles. *Pharmacol Rev* (2004) 56 (2):231–48. doi: 10.1124/pr.56.2.3
133. Klose CSN, Mahlakoiv T, Moeller JB, Rankin LC, Flamar AL, Kabata H, et al. The Neuropeptide Neuromedin U Stimulates Innate Lymphoid Cells and Type 2 Inflammation. *Nature* (2017) 549(7671):282–6. doi: 10.1038/nature23676
134. Moriyama M, Sato T, Inoue H, Fukuyama S, Teranishi H, Kangawa K, et al. The Neuropeptide Neuromedin U Promotes Inflammation by Direct Activation of Mast Cells. *J Exp Med* (2005) 202(2):217–24. doi: 10.1084/jem.20050248
135. Moriyama M, Fukuyama S, Inoue H, Matsumoto T, Sato T, Tanaka K, et al. The Neuropeptide Neuromedin U Activates Eosinophils and Is Involved in Allergen-Induced Eosinophilia. *Am J Physiol Lung Cell Mol Physiol* (2006) 290(5):L971–7. doi: 10.1152/ajplung.00345.2005
136. Moriyama M, Matsukawa A, Kudoh S, Takahashi T, Sato T, Kano T, et al. The Neuropeptide Neuromedin U Promotes IL-6 Production From Macrophages and Endotoxin Shock. *Biochem Biophys Res Commun* (2006) 341(4):1149–54. doi: 10.1016/j.bbrc.2006.01.075
137. Wallrapp A, Riesenfeld SJ, Burkett PR, Abdulnour RE, Nyman J, Dionne D, et al. The Neuropeptide NMU Amplifies ILC2-Driven Allergic Lung Inflammation. *Nature* (2017) 549(7672):351–6. doi: 10.1038/nature24029
138. Gabanyi I, Muller PA, Feighery L, Oliveira TY, Costa-Pinto FA, Mucida D. Neuro-Immune Interactions Drive Tissue Programming in Intestinal Macrophages. *Cell* (2016) 164(3):378–91. doi: 10.1016/j.cell.2015.12.023
139. Moriyama S, Brestoff JR, Flamar AL, Moeller JB, Klose CSN, Rankin LC, et al. beta2-adrenergic Receptor-Mediated Negative Regulation of Group 2 Innate Lymphoid Cell Responses. *Science* (2018) 359(6379):1056–61. doi: 10.1126/science.aan4829
140. Nicholls AJ, Wen SW, Hall P, Hickey MJ, Wong CHY. Activation of the Sympathetic Nervous System Modulates Neutrophil Function. *J Leukoc Biol* (2018) 103(2):295–309. doi: 10.1002/JLB.3MA0517-194RR
141. Wang H, Yu M, Ochani M, Amella CA, Tanovic M, Susarla S, et al. Nicotinic Acetylcholine Receptor Alpha7 Subunit Is an Essential Regulator of Inflammation. *Nature* (2003) 421(6921):384–8. doi: 10.1038/nature01339
142. Tracey KJ. Physiology and Immunology of the Cholinergic Antiinflammatory Pathway. *J Clin Invest* (2007) 117(2):289–96. doi: 10.1172/JCI30555
143. Borovikova LV, Ivanova S, Zhang M, Yang H, Botchkina GI, Watkins LR, et al. Vagus Nerve Stimulation Attenuates the Systemic Inflammatory Response to Endotoxin. *Nature* (2000) 405(6785):458–62. doi: 10.1038/35013070
144. Koopman FA, Chavan SS, Miljko S, Grazio S, Sokolovic S, Schuurman PR, et al. Vagus Nerve Stimulation Inhibits Cytokine Production and Attenuates Disease Severity in Rheumatoid Arthritis. *Proc Natl Acad Sci U S A* (2016) 113(29):8284–9. doi: 10.1073/pnas.1605635113
145. Galle-Treger L, Suzuki Y, Patel N, Sankaranarayanan I, Aron JL, Maazi H, et al. Nicotinic Acetylcholine Receptor Agonist Attenuates ILC2-Dependent Airway Hyperreactivity. *Nat Commun* (2016) 7:13202. doi: 10.1038/ncomms13202
146. Dalli J, Colas RA, Arnardottir H, Serhan CN. Vagal Regulation of Group 3 Innate Lymphoid Cells and the Immunoresolvent PCTR1 Controls Infection Resolution. *Immunity* (2017) 46(1):92–105. doi: 10.1016/j.immuni.2016.12.009
147. Darby M, Roberts LB, Mackowiak C, Chetty A, Tinelli S, Schnoeller C, et al. ILC3-Derived Acetylcholine Promotes Protease-Driven Allergic Lung Pathology. *J Allergy Clin Immunol* (2020) 147(4):1513–6.e4. doi: 10.1016/j.jaci.2020.10.038
148. Sanz E, Bean JC, Carey DP, Quintana A, McKnight GS. Ribotag: Ribosomal Tagging Strategy to Analyze Cell-Type-Specific mRNA Expression *In Vivo*. *Curr Protoc Neurosci* (2019) 88(1):e77. doi: 10.1002/cpns.77
149. Tracey KJ. Reflex Control of Immunity. *Nat Rev Immunol* (2009) 9(6):418–28. doi: 10.1038/nri2566
150. Bonaz B, Sinniger V, Hoffmann D, Clarencon D, Mathieu N, Dantzer C, et al. Chronic Vagus Nerve Stimulation in Crohn's Disease: A 6-Month Follow-Up Pilot Study. *Neurogastroenterol Motil* (2016) 28(6):948–53. doi: 10.1111/nmo.12792
151. Steyn E, Mohamed Z, Husselman C. Non-Invasive Vagus Nerve Stimulation for the Treatment of Acute Asthma Exacerbations-Results From an Initial Case Series. *Int J Emerg Med* (2013) 6(1):7. doi: 10.1186/1865-1380-6-7

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

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: frontiersin.org/about/contact

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership