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The Gut Microbiome and Multiple Sclerosis

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Abstract

The microbiome can be defined as the sum of the microbial and host's genome. Recent information regarding this complex organ suggests that in animal models of multiple sclerosis (MS), the composition of the gut microbiome can be altered, giving rise to both the effector and regulatory phases of central nervous system

(CNS) demyelination. Experimental findings during the past decade in animal models of MS have provided clear evidence for the significant role of gut microbes in both the effector and regulatory phase of this condition. There is mounting evidence in preliminary human studies suggesting that a dysbiotic MS gut microbiome could affect disease progression. We propose considering the gut microbiome as a key organ for the regulation of tolerance mechanisms and speculate that the gut microbiome is the major environmental risk factor for CNS demyelinating disease. Accordingly, we hypothesize that intervention of the gut microbiome could result in safer novel therapeutic strategies to treat MS.

Multiple sclerosis (MS) is a chronic autoimmune disease that affects the brain and spinal cord. Immune cells attack the myelin sheath that covers and protects neurons, resulting in a wide variety of neurological symptoms such as demyelination and axonal and neuronal loss ([Dendrou et al.](#)

2015). Most cases of relapsing MS are diagnosed in individuals between the ages of 20 and 50 with peak incidence at around 28. As noted in other autoimmune diseases, the gender ratio shows a higher frequency in females than in males. Some studies have shown evidence that suggests this ratio has shifted from 1:1, when MS was first described by Charcot at the end of the 19th century, to a current 3:1 female-to-male ratio (Fazekas et al. 2009). Furthermore, the gender ratio appears to be contingent on geographic location (Trojano et al. 2012). The etiological agent that may initiate disease remains unknown, and, although genetic susceptibility may be a factor, it cannot explain all instances (Compston and Coles 2008). The incomplete causative influence of genetic susceptibility suggests that other factors, such as environmental factors, might interact with the genetic susceptibility of the individual.

Whether MS is neurological or autoimmune by nature is still under debate (Dendrou et al. 2015),

although most genetic profiling shows a preponderance of immune-mediated molecules that are activated during the disease process. Regardless of pathology, it is now understood that inflammation and possibly immune dysregulation play a significant role during the progression of the disease. Both genetic and environmental factors have been proposed as putative factors that increase the risk for MS; some environmental risk factors include, but are not limited to, vitamin D deficiency, stress, smoking, viral or bacterial infections, and diet ([Lincoln and Cook 2009](#); [Mandia et al. 2014](#); [Ochoa-Repáraz and Kasper 2014](#)).

Within the last decade, a considerable effort has been made to ascertain a possible link between the functions of the human microbiome and human diseases. Public and private initiatives were launched to improve the technological tools needed for the study of the microbiome. It is possible now to evaluate the effects of genetic and environmental factors on the composition of

the human microbiota as well as perform functional studies focused on the understanding of the metabolic or immunological impact of gut microbes. The gut microbiome is a bidirectional organ in which changes in the gut microbiota drive both immune and physiologic alterations in the host that in turn are critical in maintaining homeostasis of the gut microbiota. The current understanding of the complexity in the gut environment suggests the relevance of dysbiosis, which can be defined as alterations of the microbiome composition serving as a potential mechanism to cause disease. Dysbiosis can affect immunological responses to the microbiota, but further affect the integrity of the epithelia that comprise cellular barriers essential to maintaining the integrity of both intestine and central nervous system (CNS) (Fig. 1). A number of factors that have been associated with MS as potential triggers of disease appear to have significant effect on the gut microbial composition (Ochoa-Repáraz and Kasper 2014). Here, we review

experimental evidence suggesting the vital importance of the gut microbiome in immune homeostasis as well as intestinal and blood–brain barrier (BBB) integrity. At present, there is both supporting and conflicting literature focused on the role of gut microbes in the regulation of MS and experimental autoimmune (or allergic) encephalomyelitis (EAE).



Figure 1.

The reciprocal association of the gut dysbiosis with central nervous system (CNS) inflammatory demyelination. Host's genetics and distinct environmental factors, including diet, sanitation, use of antibiotics, and lifestyle, have been linked to ...

THE GUT-ASSOCIATED LYMPHOID TISSUE IS A RESERVOIR FOR IMMUNE CELLS ASSOCIATED WITH MS

Because of the deadly effects that pathogenic

microbes have had in the history of human biology, we permanently link microbes with disease and illness. Nevertheless, among all gut microbes that have been identified thus far, only a minor fraction can be associated with disease.

The rest of the microorganisms, including bacteria, archaea, fungi, eukaryotic microbial organisms, and viruses, are not harmful, and, in many cases, they are beneficial. Humans harbor an extremely large population of these commensal microbes throughout life, from birth to death. We globally name these microbes as the microbiota, and recognize the sum of the genes and products that are secreted or excreted by the microbiota as the microbiome. We now know that different locations of our bodies exposed to the environment around us comprise different microbiomes, including the skin, oral, respiratory tract, stomach, urogenital tract, and the gut.

Furthermore, we are now beginning to understand how diverse these different microbiomes are and how different their functions might be. Of the

microbiomes, the gut microbiome is the most studied because of its relative abundance and biological importance.

Anatomically, the gastrointestinal (GI) tract is a large muscular tube covered with a mucosal epithelium. The surface area of the human intestine is $\sim 200 \text{ m}^2$. The gut of a human consists of the small intestine and the large intestine.

Although the mucosal epithelium is present in both small and large intestines, there is variability in both morphology and function. The main function of the small intestine is to digest food and absorb nutrients, whereas the large intestine reabsorbs water and ions. The biological geography of the gut appears to affect the composition of the microbiota. Physical and chemical factors, such as the differences in morphology, enzymes, and acids released in the stomach as components of pancreatic juice and bile, and larger quantities of antimicrobial peptides released by Paneth cells, affect the microbial load of the small intestine, which is

notably reduced when compared with the large intestine. Other factors, such as the lower nutrient retention times as well as oxygen and pH, also account for the longitudinal differences ([Donaldson et al. 2015](#)).

The gut microbiota is comprised of bacteria, archaea, viruses that infect bacteria (bacteriophages), fungi, and eukaryotic microbes. Of the microbiota, bacteria are most studied. The two most dominant phyla are Bacteroidetes and Firmicutes; these constitute some of the roughly 40 different species that the core gut microbiota is comprised of, resulting in 75% of the total abundance for at least 1 year in an individual ([Martinez 2013](#)). A second study argued that 60% of all bacterial species persisted for 5 years in individuals ([Faith 2013](#)). Taken together, it is evident that the gut microbiome is a highly complex ecosystem in which many factors merge and influence both the microbial communities and our immune, neuronal, and endocrine system ([Ochoa-Repáraz and Kasper 2014](#)). Because of its

complexity, animal models are used to determine the biological relevance of gut microbes in the immune system. Gnotobiotic (germ-free) mice, antibiotics, colonization with single or multiple microbial species, fecal transplantations, and also treatment with microbial antigens and metabolites are used to affect the microbiome and study the outcomes on immune populations.

Many of the observations that define the cellular and/or soluble mechanisms that contribute to MS development and progression are derived from experimental animal models, consistent with those currently being used to evaluate the effects of the gut microbiome on disease pathogenesis and regulation. The most widely used animal model is EAE. EAE is characterized by peripheral activation of $CD4^{+}$ T helper 1 (Th1) and T helper 17 (Th17) cells in draining lymph nodes and infiltration of activated and proliferative Th1 and Th17 into the CNS by crossing the BBB from circulation. Both T-cell populations have been associated with immunological tolerance

breakdown leading to autoimmunity. However, the sole involvement of Th1 cells in MS has been challenged with the identification of Th17 cells as drivers of CNS demyelination in EAE ([Hofstetter et al. 2005](#); [Komiyama et al. 2006](#); [Sutton et al. 2006](#)). Antibodies against interferon γ (IFN- γ) ([Billiau et al. 1988](#); [Duong et al. 1994](#)), IFN- γ deficiency ([Ferber et al. 1996](#); [Chu et al. 2000](#)), or IFN- γ receptor deficiency in mice ([Willenborg et al. 1996](#)) exacerbate EAE severity. In contrast, T cells expressing interleukin (IL)-17 were found in cerebrospinal fluid and plaques of MS patients ([Matusevicius et al. 1999](#)), as well as an enhanced expression of IL-17 receptor (IL-17R) and IL-22 receptor (IL-22R) in the vessels of MS lesions when compared to the vessels of healthy individuals ([Kebir et al. 2007](#)). In the EAE model, autoreactive CD4⁺ T cells cross the BBB and then are reactivated within the CNS by either resident tissue-fixed macrophages or peripherally antigen-presenting cells (APCs) that may be either dendritic cells or possibly B cells.

Multiple efforts are now being taken to target another lymphoid cell population, B cells, with critical importance in the pathology of MS (Hauser 2015; Longbrake and Cross 2016). B cells act both as an effector and as an APC and differentiate into plasma cells to produce antigen-specific antibodies. B cells recognize antigens with their B-cell receptor and mediate presentation in major histocompatibility complex (MHC) class II and costimulation. Major subsets of circulating B cells can produce proinflammatory cytokines, such as tumor necrosis factor α (TNF- α) and lymphotoxin. However, more restricted populations, such as B10 cells produce, anti-inflammatory cytokines such as IL-10 (Mauri and Menon 2015). B cells isolated from MS patients appear to be shifted toward the proinflammatory phenotype (Bar-Or et al. 2010). Furthermore, a recent published work has provided evidence to suggest that B cells from MS patients exacerbate neuronal antigen-specific Th17 cell responses in mechanisms

mediated by B cells as APCs ([Ireland et al. 2016](#)).

In recent years, experimental evidence gathered from the rodent EAE models suggest that the gut microbiome, particularly changes in the gut microbiome, affect the frequencies or the function of many of the immune cells described above.

Consequently, the involvement of the gut microbiota in immune homeostasis and its association with autoimmune diseases, including MS, has been proposed. Although for decades the study of the gut microbes in the context of immune homeostasis was limited, it is now increasingly understood that the gut and the gut-associated lymphoid tissues (GALTs) are the reservoir for many of the lymphoid cells in our bodies. The hypothesis that at least part of the 10^{13} microbes that our gut harbors functionally affects the local as well as the systemic immune balance is no longer just speculation. In the next section, we review different mechanisms by which gut microbes and, more specifically, changes in the gut microbial composition, affects

the numbers or the function of immune cells that are linked to MS pathology.

HOW CAN GUT MICROBES REGULATE MS?

The presence of gut microbes is required for the immune system's appropriate development and maturation. These properties are highlighted by the dramatic morphological, cellular, and functional deficiencies observed in germ-free animals when compared with conventionally housed animals ([Round and Mazmanian 2009b](#)). The immune dysfunction of germ-free mice is characterized by an unbalanced equilibrium between pro- and anti-inflammatory immune cells present systemically and, particularly, in the gut. These characteristics can be restored by the colonization of the gut with commensal microbes such as *Bacteroides fragilis* ([Mazmanian et al. 2005](#)). Thus, it is logical to postulate that gut microbes affect the proper maturation of the immune system as well as its function

immune system as well as its function.

Immunomodulation

EAE models of gut microbiome absence, alteration, or bacterial monocolonization provide substantial experimental evidence to suggest that the microbes of the gut influence immune homeostasis by affecting the balance of those immune responses elicited to promote inflammation and those directed to control the extent of the immune response. For instance, segmented filamentous bacterium (SFB), a Gram-positive resident of the rodent gut significantly affects the severity of EAE, among other experimental models of autoimmunity ([Lee et al. 2011](#)). The mechanism by which SFB appears to enhance disease severity appears to be mediated by the activation of gut-induced Th17 cells. The monocolonization of germ-free mice with SFB renders EAE disease-resistant germ-free mice susceptible by the induction of Th17 cells ([Lee et al. 2011](#)). Similar results have been observed in the germ-free mice model that is susceptible to

the germ-free mice model that is susceptible to rheumatoid arthritis (RA) ([Chappert 2014](#); [Wu et al. 2016](#)). Other commensal microbes such as *Porphyromonas gingivalis*, a constituent of the oral microbiome, increases glial activation and are shown to exacerbate proinflammation and EAE severity. The lipopolysaccharide (LPS) of *P. gingivalis* was shown to be recognized by Toll-like receptor 2 (TLR2) and promoted neuroinflammation ([Shapira et al. 2002](#); [Nichols et al. 2009](#)). Other bacteria taxa, such as specific clusters of *Clostridium* spp., have also been shown to promote enhanced Th17 cell responses in the gut ([Horai et al. 2015](#)). Interestingly, recent findings that will be discussed later in this work associate a significant increase in the relative abundances of specific clusters of *Clostridium* in stool samples obtained from relapsing remitting MS (RRMS) patients when compared to those from healthy donors ([Miyake et al. 2015](#)). The experimental evidence suggests Th17 cell induction by gut microbes, indicating that perhaps dysbiosis could influence the pool of

inflammatory cells with an autoreactive potential. No causative association has been found; yet, two recent published works suggest a functional impact of the MS gut microbiota determined by fecal transplantation studies using germ-free mice as recipients ([Berer et al. 2017](#); [Cekanaviciute et al. 2017](#)). Germ-free mice show reduced susceptibility to the disease when compared to conventionally housed mice, as previously published ([Berer et al. 2011](#); [Lee et al. 2011](#)). In [Cekanaviciute et al. \(2017\)](#), the fecal content from individual MS patients or healthy donors were transferred into recipient C57 germ-free mice. Mice that received MS fecal content suffered a significantly more severe form of EAE when compared with controls, whereas those that received fecal content from healthy individuals developed significant reduction in the EAE clinical scores when compared with controls. In [Berer et al. \(2017\)](#), transgenic mice that develop spontaneous EAE transferred with fecal samples of MS individuals had an increase in the

incidence of disease when compared with recipient mice that were transferred with fecal content isolated from their homozygotic siblings, not diagnosed with MS, and considered in their study healthy. Both studies highlighted the importance of IL-10-producing CD4⁺ T cells in the immunodulatory effects of the gut microbiota (Berer et al. 2017; Cekanaviciute et al. 2017).

The immune system has evolved to protect multicellular organisms from pathogens; therefore, the immune system must be able to distinguish between pathogenic signals and commensal signals. In autoimmune diseases, autoreactive cells target self-antigens and harm cells promoting tissue disruption, inflammation, and disease. The acquisition of the adaptive immune system by vertebrates also evolved in the generation of mechanisms to control the presence and effects of autoreactive cells. Central tolerance, which occurs in the thymus for developing T cells and in the bone marrow for maturing B cells, is a major mechanism for clonal

deletion of autoreactive T and B cells by the induction of apoptosis. The process of self-restriction in response to a high-affinity interaction between the T-cell receptor and B-cell receptor to exposed self-antigens in the thymus and the bone marrow, respectively, result in the destruction of the majority of T and B cells. This process ensures that only those T and B cells that do not respond to self-antigens will result in mature effector cells. The loss of central tolerance is caused by the escape of self-reactive cells that do not undergo clonal deletion. Similarly, peripheral tolerance mechanisms attempt to prevent the destructive effects of autoreactive cells. Anergy induced by nonresponsiveness and direct interaction with regulatory cell populations are proposed as the main mechanisms by which our immune system destroys these cells in the periphery. Among the cell compartments that play a protective role against autoimmunity, the regulatory T cells (Tregs) are the most studied.

The immune dysfunction in MS, characteristic of

The immune dysregulation in MS, characteristic of other autoimmune conditions, may be caused by the loss of immune homeostasis arising from varying patterns of microbial colonization. As the gut is the main reservoir of immune cells, immune homeostasis in both the GALT and the periphery may be dependent on other microbes, inducing the necessary anti-inflammatory responses.

Tregs are activated $CD4^{+}$ T cells with enhanced expression of the α -chain of the IL-2 receptor (IL-2R) or CD25, and the master regulator *Fork-head box 3* (*Foxp3*). Two main types of Tregs have been identified thus far: the naturally occurring Tregs (nTregs) that are generated in the thymus, and the inducible Tregs (iTregs), generated in the periphery ([Josefowicz and Rudensky 2009](#)). The nTregs were first identified as an immunosuppressive T-cell subset derived from the thymus ([Sakaguchi et al. 1982](#)) and later shown to express high levels of CD25 (IL-2R α -chain) and identified as immunosuppressive ([Sakaguchi et al. 1995](#)). Subsequently, the

(Sakaguchi et al. 1995). Subsequently, the transcription factor *Foxp3* was discovered in the early 2000s as a critical gene responsible for the tolerance induced by Tregs (Chatila et al. 2000; Bennett et al. 2001). *Foxp3* expression is required for Treg differentiation and suppressive activity, as well as for their proliferation and survival (Josefowicz and Rudensky 2009). Other important cell subsets have regulatory functions, including the already discussed regulatory B cells. Tr1 cells and Th3 cells have also been shown to be critical players in tolerance. Tr1 cells are IL-10-producing CD4⁺ T cells that do not express Foxp3 (Sundstedt et al. 2003). Th3 cells were described by Howard Weiner's laboratory as transforming growth factor β (TGF- β)-producing CD4⁺ CD25⁺ T cells (Weiner 2001; Faria and Weiner 2006). Furthermore, invariant natural killer T (iNKT) cells have shown regulatory phenotypes (Nowak and Stein-Streilein 2007).

Tregs suppress the proliferation of other cells, affect the function of dendritic cells, and promote

immune homeostasis. Immunosuppressive effects of Tregs are variable and include either direct cell-to-cell contact, the production of anti-inflammatory cytokines such as TGF- β and IL-10, or indirect by the modulation of APC function. Because of their importance in controlling the proliferation of immune cells, there has been great interest in understanding the role of Tregs in MS development and their potential protective function against MS, as well as many other autoimmune diseases ([Perdigoto et al. 2015](#)). There is some uncertainty as to whether the absolute number of Tregs is reduced in individuals with MS compared to healthy controls. However, insufficient suppressive function of Tregs in individuals with MS has been described. Early studies compared the in vitro function of Tregs by studying the suppressive capacity of Tregs isolated from 15 MS patients when cocultured with proliferating effector CD4⁺ CD25⁻ T cells ([Viglietta et al. 2004](#)). In MS patients, Tregs had a significantly reduced ability to suppress effector T-cell proliferation and

to suppress effector T-cell proliferation and reduced production of IFN- γ . Reduced immunosuppression was later confirmed by Haas and colleagues (Haas et al. 2005). Huan and collaborators (2005) detected reduced Foxp3 protein as well as reduced messenger RNA (mRNA) *FOXP3* expression levels in MS patients; however, although their study included RRMS, secondary progressive MS (SPMS), and primary progressive MS (PPMS) patients, all samples were pooled for their analysis. In contrast, Venken and colleagues (Venken et al. 2006) reported that the frequencies of circulating Tregs in RRMS and SPMS patients were not different from those detected in healthy donors. However, they documented a significant reduction in the ability of Tregs isolated from RRMS to suppress the proliferation of effector CD4⁺ T cells and their production of IFN- γ in vitro. Interestingly, no suppressive dysfunction was observed in Tregs isolated from circulating blood of SPMS patients. To date, the reasons why the regulatory effects of Tregs might not be

compromised in SPMS patients remain unknown (Fletcher et al. 2010). The differences may rely on the more pronounced inflammatory activity in relapsing forms of MS that would require the assistance from Tregs; however, this is a hypothesis that has yet to be tested. The suppressive ability of Tregs was associated with the levels of *FOXP3* mRNA expression (Venken et al. 2006). Feger and collaborators (Feger et al. 2007) compared the frequencies of nTregs in the cerebrospinal fluid (CSF) and circulating blood of MS patients. They showed that, in CSF of MS patients, the frequencies of nTregs were enhanced, but no differences were observed in circulation (Feger et al. 2007). In their studies, all MS patients, including RRMS, SPMS, and PPMS, were also pooled for analysis. As indicated earlier, the distinct function of Tregs in the different clinical types of MS has yet to be elucidated. It would be important to determine whether Tregs of MS patients respond differently to inflammation at different stages of the disease

process. Gut microbes are known to promote iTregs and provide an essential mechanism for the establishment of peripheral tolerance. Conversely, we are now beginning to appreciate that disease may affect the composition of the microbiota within the microbiome. Future studies will determine whether RRMS, PPMS, and SPMS patients have an identifiable influence on the microbiome. It would be important to evaluate whether the effects of those changes within the microbiota are the result of the regulatory function of Tregs. Another experimental barrier to overcome is the current inability to effectively discern the phenotype of nTregs and iTregs. Sorting this out would enhance our understanding of the role of natural versus microbiome-promoted iTregs in the context of the different forms of MS. As we will discuss in the next section, [Fletcher and colleagues \(2009\)](#) showed that both frequencies and suppressive function of circulating Tregs expressing the ectoenzyme CD39 are reduced in RRMS patients when compared with healthy individuals.

compared with healthy individuals.

The EAE models have provided evidence to suggest that specific gut microbes can affect EAE severity. Some of these findings suggest a role for Tregs in protective immunity promoted by gut bacteria. Treatment of Lewis rats with *Bifidobacterium animalis* (Ezendam et al. 2008) and mice with a mixture of *Lactobacillus* spp. (Lavasani et al. 2010) regulate the extent of CNS inflammation and clinical scores in EAE.

B. fragilis is a major constituent of the human gut microflora comprising between 1%–3% of the total bacteria. *B. fragilis* produces eight extracellular polysaccharides that are essential for their ability to colonize and survive within the gut (Mazmanian et al. 2005, 2008; Surana and Kasper 2012). One of these polysaccharides, the polysaccharide A (PSA), is a potent immunomodulatory symbiont factor that is recognized by APCs in a TLR2-dependent mechanism that potentiates CD4⁺ T-cell activation and induction of regulatory IL-10-

producing cells (Mazmanian et al. 2005; Wang et al. 2006; Round and Mazmanian 2009a; Ochoa-Repáraz et al. 2010b). PSA produced by *B. fragilis* is protective in models of experimental colitis (Mazmanian et al. 2008; Dasgupta et al. 2014) and asthma (Johnson et al. 2015). The induced IL-10 production that PSA stimulates in T cells is a common feature in the protective mechanism of action, although the phenotype of the CD4⁺ T-cell population appears to be disease specific (Johnson et al. 2015). In murine EAE, PSA is protective prophylactically and therapeutically by the induction of IL-10-producing Tregs (Ochoa-Repáraz et al. 2010b; Wang et al. 2014b). The protection that PSA confers is not observed in IL-10-deficient mice, suggesting that the anti-inflammatory cytokine is key in the immunomodulatory effects induced by PSA. Similarly, TLR2-deficient mice are unresponsive to the polysaccharide (Wang et al. 2014b). The recognition of PSA is mediated by both conventional (Mazmanian et al. 2005; Wang

et al. 2006) and plasmacytoid dendritic cells (Dasgupta et al. 2014). PSA is then presented to CD4⁺ T cell by MHC class II–mediated mechanisms (Cobb and Kasper 2008, 2005).

In EAE mice, PSA enhances a phenotypically distinct subpopulation characterized by the expression of an ectoenzyme, the ectonucleoside triphosphate diphosphohydrolase 1 (ENTPD1), also known as CD39, responsible for the catabolism of ATP into adenosine monophosphate (AMP) (CD39⁺ Tregs) (Wang et al. 2014b). In vitro, Fletcher’s findings showed the dysfunctional nature of the CD39⁺ Tregs isolated from MS patients when Th17 proliferation and IL-17 production were significantly reduced when compared with Tregs isolated from healthy donors. Of particular interest is the ability of this population of Tregs from both healthy and MS patients to suppress IFN- γ production. CD39 hydrolyzes ATP into 5'AMP. AMP is then catabolized to adenosine by another ectoenzyme, CD73 (Antonioli et al. 2013). ATP is released to

the extracellular media when cells lyse and through lysis-independent mechanisms by nucleotide-permeable channels and by mediation of lysosomes ([Antonioli et al. 2013](#)). During tissue damage, as occurs during inflammation, ATP is released to the media. The expression of CD39 in immune cells appears to be regulated by the inflammatory milieu through cytokines, hypoxic cells, and oxidative stress ([Antonioli et al. 2013](#)). Adenosine has anti-inflammatory effects on T cells, including Tregs, by binding to their adenosine receptors.

The acquisition of a CD39 phenotype independent of Foxp3 by Tregs promoted by the treatment with PSA enhanced the expression of regulatory markers measured by flow cytometry and the production of IL-10. Furthermore, the acquisition of the CD39⁺ phenotype by regulatory CD4⁺ T cells was associated with the up-regulation of several activation markers (PD-1, GITR, CTLA-4, and ICOS) when compared with CD39⁻ T cells. The CD39⁺ Foxp3-negative

population showed enhanced expression of surface-tracking molecules such as chemokine receptor-5 (CCR5), CCR6, and CXC chemokine receptor-3 (CXCR3), as well as the adhesion molecules CD49b and CD29 (Wang et al. 2014a). Most notable was the failure to protect CD39-deficient EAE mice by oral administration of PSA (Wang et al. 2014b). CNS levels of IL-17 of EAE mice are reduced when mice are treated orally with PSA. The adoptive transfer of PSA-CD39⁺ CD4⁺ T cells into RagKO recipient EAE mice (which lack mature B and T cells) induced protection, as opposed to CD39⁻ CD4 T cells (Wang et al. 2014b). The immunomodulatory and protective role of CD39⁺ Tregs that are induced in response to oral treatments with attenuated vaccine delivery vectors have also been shown in murine collagen-induced arthritis (CIA) (Kochetkova et al. 2010, 2011, 2014; Maddaloni et al. 2015). Ex vivo flow cytometry evaluation of the CNS of EAE mice confirmed that those animals treated with PSA had increased

frequencies and numbers of CD39⁺ CD4⁺ T cells, both Foxp3-positive and Foxp3-negative ([Wang et al. 2014a](#)). Taken together, the findings observed with PSA suggest that gut microbes and derived factors can promote a regulatory phenotype in T cells that augments their migratory and, perhaps, their protective effects against CNS inflammatory demyelination. Promoting a Treg phenotype that includes the expression of CD39 could be a novel strategy to promote immunoregulation in the gut, and also systemically in an autoimmune scenario. As such, fingolimod, an approved MS therapeutic that blocks lymphocyte egress from lymph nodes by antagonizing the sphingosin-1 phosphate-1 (S1P1) receptor enhances the levels of CD39 mRNA in circulating blood of MS-treated patients, as well as CD39⁺ Tregs measured by flow cytometry ([Muls et al. 2014](#)). We have observed that alemtuzumab, a humanized anti-CD52 monoclonal antibody (mAb), enhances the expression of Foxp3 and CD39 in the circulating

CD4⁺ T-cell population that recovers after treatment ([Mielcarz et al. 2013](#), presented at ECTRIMS 2013). In EAE mice treated with a murine anti-CD52 mAb, the effects observed are similar ([Pant et al. 2015](#), presented at ECTRIMS 2015). Remarkably, the CD39-positive phenotype is also exacerbated in GALT Tregs of mice treated orally with teriflunomide (ECTRIMS 2015) ([Kircher et al. 2015](#)), an approved oral therapy against relapsing forms of MS.

Teriflunomide's described mechanism of action (MOA) is the inhibition of the de novo synthesis of pyrimidine that stops the proliferation of autoreactive cells by the action targeted against the enzyme dihydroorotate dehydrogenase (DHODH). We observed an increase in the frequencies of GALT CD39⁺ Foxp3-negative CD4⁺ T cells and Tregs in naïve mice treated orally with daily doses of teriflunomide. Adoptive transfer of this isolated Treg population reduces significantly the severity of disease in recipient EAE mice. Our preliminary findings suggest an additional immunomodulatory MOA for the

additional immunomodulatory MCR for the therapeutic based on GALT CD39⁺-producing T cells. Based on our preliminary observations, we hypothesize that gut microbiome alterations lead to the long-term efficacy of some therapies. It appears that induction of a GALT-derived CD39⁺ population of regulatory T cells may offer a common pathway by which some and perhaps all of the Food and Drug Administration (FDA)-approved therapies for treating MS invoke their protective capacity. Accordingly, we speculate that the induction and the amplification of CD39⁺ T regulatory cells, which are dysfunctional in their ability to control Th17 cell proliferation and IL-17 production by Th17 cells in MS patients (Fletcher et al. 2009), may result in improved efficacy of therapies for treating relapsing MS.

Recent clinical observations on the effect of anti-CD20 indicate that B cells should be considered a primary target to treat MS (Hauser 2015; Longbrake and Cross 2016). The enhanced severity of EAE in B-cell-deficient mice (Wolf et

al. 2006) implies a direct and essential role

al. 1996) implies, as discussed previously, the existence of anti-inflammatory subsets of B cells, particularly those that produce IL-10 (Matsushita et al. 2008; Crampton et al. 2010). IL-10-producing B regulatory cells protect IL-10-deficient mice against EAE (Fillatreau et al. 2002). We have considered the impact of the gut microbiome on the phenotype and function of B cells during disease. In the gut, B cells can play a tolerogenic role against colitis (Wang et al. 2015). In colitis-induced mice, the disease is exacerbated in the absence of B cells and the number of GALT Tregs are reduced in B-cell-deficient mice. Based on their findings, it is suggested that Tregs and B cells are part of a tolerogenic loop wherein B cells promote the induction of Tregs and Tregs promote the maturation of immunoglobulin A (IgA)-producing plasma cells. Remarkably, the adoptive transfer of B cells obtained from IL-10-deficient mice also protected mice against colitis (Wang et al. 2015), suggesting an IL-10-independent immunomodulatory role for B cells (Ray et al. 2012). We have described that B cells

isolated from mesenteric lymph nodes from B6 mice treated orally with antibiotics that target the microbiome were protective when transferred into recipient EAE mice (Ochoa-Repáraz et al. 2010a). The transferred B cells expressed increased levels of Cd1d and CD5. Interestingly, treatment with antibiotics also affected the function of Tregs during EAE (Ochoa-Repáraz et al. 2009). The more recent findings (Wang et al. 2015) and our previous work suggest that both Tregs and B cells might have a cooperative function during autoimmune inflammation. The induction of a B regulatory population by the microbiome may represent another important novel target for the treatment of this CNS inflammatory condition.

From the data and experiments presented, it becomes clear that there is an intimate relationship established between the immune system and the microbiota of the human microbiome. This relationship is contingent on the presence of resident microbes as well as the

the presence of resident microbes as well as the modulations of the host immune system. In summation, it is the microbiome that is partially responsible for maintaining a healthy balance between pro- and anti-inflammatory responses that regulate and educate the host's immune system, particularly in instances of autoimmunity.

Gut-Derived Metabolites and the Control of Blood–Brain Barrier Permeability

Metabolically, the presence of the gut microbiota has beneficial implications to the host gut. Gut microbes process fibers from diet and produce metabolites with multiple diverse molecular and cellular functions in the metabolism and immune system of the host. Fermenting bacteria, such as members of the genera *Clostridium* and *Bacteroides*, of the colon are associated with these dietary fiber fermentation processes. Short-chain fatty acids (SCFAs), such as acetate, propionate, and butyrate, are products of the bacterial fermentation of nondigestible

carbohydrates that are derived from complex fibers; in the absence of a gut microbiota, fibers would traverse the GI tract unused. SCFAs also provide immunological benefits to the host by regulating gene expression in immune cells and shifting them toward a regulatory phenotype, decreasing oxidative stress and reinforcing the intestinal barrier as well as the BBB. The importance of gut-derived metabolites was recently highlighted in the IL-10 knockout model of spontaneous colitis. In mice that suffer from severe intestinal epithelium barrier disruption, the treatment with a fiber-rich diet significantly reduced the disruption. The beneficial effects of the diet were accompanied by increases in the levels of acetate, propionate, and butyrate in the gut, a reduced inflammatory cell infiltration into the lamina propria and enhanced frequencies of Tregs ([Wang et al. 2016](#)). Thus, gut microbes could affect key metabolites associated with gut intestinal integrity as well as promotion of immunomodulatory responses. We speculate that metabolites such as SCFA produced in the gut

metabolites such as SCFA produced in the gut affect the host systemically, as evidenced by their effects on BBB integrity. Although SCFA transport to the brain has been reported (Conn et al. 1983; Macfabe 2012), the migratory and effects mechanisms to and within the CNS require further investigation (Michel and Prat 2016).

During EAE, significant changes in metabolite patterns are found at the onset and at the peak of the disease of rats. In the blood of SJL EAE mice, ~15% of the metabolites detected (283) differed in their concentrations during disease (Mangalam et al. 2013). Some of the gut microbes that produce SCFA as a result of fibers fermentation, such as clusters of *Clostridium*, have been recently shown to be significantly reduced in fecal samples obtained from MS patients when compared with healthy donors (Miyake et al. 2015). Additionally, lipid 654, a bacteria-derived lipodipeptide produced by *Bacteroides* spp., is diminished in the serum of MS patients (Farrokhi et al. 2013). Other metabolites that may be

et al. 2015). Other metabolites that may be present in the gut microbiota might also be associated with pathways of Th17 cell differentiation, such as sterols that have been shown to be ligands for ROR γ t (Hu et al. 2015), for instance, oxysterols (Soroosh et al. 2014).

The importance of SCFA in the induction of regulatory responses mediated by Tregs has been extensively reviewed in recent years (Kinoshita and Takeda 2015). Recent findings suggest that these gut-derived metabolites could be implicated in processes with essential importance in inflammatory diseases of the CNS. Remarkably, the gut microbiome might be a key regulator of BBB integrity (Braniste et al. 2014). Germ-free mice show an increase in BBB permeability when compared to conventional specific pathogen-free (SPF) mice. Molecularly, the reduced integrity of the BBB was associated with an altered expression of occludin and claudin-5, which is significantly increased in the epithelium of germ-free mice. When fecal content obtained from SPF

mice was transferred to GF mice, the BBB integrity improved and enhanced the expression of the tight junction proteins. Further, when germ-free mice were monocolonized with SCFA-producing gut bacteria, the integrity of the BBB was also restored ([Braniste et al. 2014](#)). In light of the findings, it appears that the permeability of the BBB in germ-free mice is underdeveloped and “leaky.” As in MS, the integrity of BBB integrity is compromised; this is apparent in other CNS diseases as well ([de Vries et al. 2012](#)).

HOW ABOUT THE OTHER WAY AROUND—DOES DISEASE AFFECT THE GUT MICROBIOME?

As noted previously in this review, the gut microbiome is bidirectional. Microbiota drive a host immune or physiologic response and, in turn, the host maintains gut homeostasis and prevents the development of dysbiosis. During EAE, an increase in the frequencies of Th1, Th17, and IL-17⁺ $\gamma\delta$ T cells are observed in the GALT of mice

as early as 7 days postdisease induction (Nouri et al. 2014). APCs isolated from the GALT of EAE mice also produce enhanced quantities of proinflammatory cytokines. Furthermore, the integrity of the intestinal barrier appears to be disrupted (Nouri et al. 2014). The leaky gut observed in EAE mice is also observed in a murine model of autism spectrum disorders (ASDs) (Hsiao et al. 2013). In this model, mice show GI dysfunction, dysbiosis, and increased intestinal permeability. Importantly, the treatment with *B. fragilis* improves the integrity of the barrier (Hsiao et al. 2013). The absence of microbes in germ-free mice also affects the intestinal barrier integrity (Kozakova et al. 2015) restored by gut microbes. The colonization with a probiotic, consisting of *Lactobacillus rhamnosus* LOCK0900, *L. rhamnosus* LOCK0908, and *Lactobacillus casei* LOCK0919, improved the intestinal morphology and barrier integrity (Kozakova et al. 2015). Thus, the effects of dysbiosis on the intestinal integrity could affect significantly the levels of gut microbial

significantly the levels of gut microbial components, such as endotoxin (Cani et al. 2008), and exacerbate disease.

The findings obtained in the EAE model suggest that disease can affect intestinal integrity. Can MS also induce dysbiosis? The advances in the microbiological and population analytical tools, such as 16S rRNA and metagenomic approaches, are starting to provide evidence that dysbiosis can occur in those with MS. Recent findings support the hypothesis of dysbiosis in MS (Rumah et al. 2013; Cantarel et al. 2015; Miyake et al. 2015; Chen et al. 2016; Jangi et al. 2016; Tremlett et al. 2016a, 2016b; Berer et al. 2017; Cekanaviciute et al. 2017). The presence of *Clostridium perfringens* (Rumah et al. 2013), significant alterations in clusters of *Clostridia* (Miyake et al. 2015), reductions in *Bacteroidaceae*, and increases in *Ruminococcus* (Cantarel et al. 2015), uneven microbiota structures (Chen et al. 2016; Tremlett et al. 2016a, 2016b), and increases in *Akkermansia muciniphila*, among other bacterial

and archaeal taxa (Jangi et al. 2016; Berer et al. 2017; Cekanaviciute et al. 2017), have been described in MS gut microbiota when compared with healthy individuals. Using the EAE model that afflicts the nonobese diabetic (NOD) strain of mice, we studied whether different stages of the disease would affect the composition of the gut microbiota. In the NOD mice, induced EAE promotes an early mild peak of disease that is followed by a period of remission and a subsequent worsening stage, prolonged and progressive (Basso et al. 2008; Levy et al. 2010). Histologically, the NOD EAE mice have changes that are observed in human MS during secondary progressive disease, including a leaky BBB. Our preliminary data suggest a significant difference ($p < 0.017$) in the overall microbial relative abundances of the gut microbiome in healthy versus mice that developed severe EAE during early stages of disease, measured at day 14 post-EAE induction. However, the overall microbiota structure of mice that developed severe EAE was not different to control mice at chronic stages of

not differentiate control mice at chronic stages of disease (Colpitts et al. 2017).

CONCLUDING REMARKS

Here, we reviewed the experimental evidence associating disease with various EAE models of the gut microbiome, including germ-free mice, oral antibiotics, colonization with single and multiple strains of gut microbes, and oral treatment with products obtained from gut commensal symbionts. Together, these experimental findings show that alteration of the GALTs or the metabolic pathways triggered by gut microbes affects the severity of CNS inflammatory demyelination. The experimental nature of these findings limit their potential clinical relevance, and, although still speculative, the responses that gut microbes elicit in the host suggest that they form part of a master regulatory system of immune responses that could potentially impact inflammatory CNS demyelinating disease. Preliminary studies have shown that the gut microbiome appears to be

shown that the gut microbiome appears to be altered in those with relapsing MS. Recent preliminary evidence suggests that during disease the gut microbiome is at least affected quantitatively. Current efforts will determine whether the differences observed in the gut microbiome are translated into a functionally biological relevance. More importantly, in the coming years, we will determine whether these effects that are now observed in the gut microbial populations and their functions can be used as potential novel therapies against MS. If so, this would represent a major paradigm shift in both our understanding as well as the treatment of this devastating condition.

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