Published in final edited form as:

Transl Res. 2017 January ; 179: 126–138. doi:10.1016/j.trsl.2016.07.016.

The influence of gut derived CD39 regulatory T cells in CNS demyelinating disease

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Abstract

There is considerable interest in trying to understand the importance of the gut microbiome in human diseases. The association between dysbiosis, an altered microbial composition, as related to human disease is being explored in the context of different autoimmune conditions, including multiple sclerosis (MS). Recent studies suggest that MS affects the composition of the gut microbiota by altering the relative abundances of specific bacteria and archaea species. Remarkably, some of the bacterial species shown reduced in the gut of MS patients are known to promote immunosuppressive regulatory T cells (Tregs). In MS, the function of a phenotype of Tregs that express CD39, an ectoenzyme involved in the catabolism of ATP as immunomodulatory cells, appears to be reduced. In this review we discuss the involvement of the gut microbiota in the regulation of experimental models of CNS inflammatory demyelination and review the evidence that link the gut microbiome with multiple sclerosis. Further, we hypothesize that the gut microbiome is an essential organ for the control of tolerance in multiple sclerosis patients and a potential source for safer novel therapeutics.

Keywords

CD39; gut microbiota; dysbiosis; gut-brain axis; Multiple Sclerosis

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Conflict of Interest:

All authors have read the journal's policy on conflicts of interest. Javier Ochoa-Repáraz Ph.D, reports grants from Genzyme, personal remuneration from Symbiotix Biotherapies, during the conduct of the study. Dr. Lloyd H Kasper MD, reports grants from Genzyme, Teva and Symbiotix Biotherapies, personal remuneration from TEVA Neuroscience and Genzyme that are outside the submitted work.

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Introduction

Multiple sclerosis

Multiple sclerosis (MS) is a chronic autoimmune disease that affects the brain and spinal cord in the central nervous system (CNS). During MS, immune cells attack the myelin sheet that covers and protects neurons causing demyelination and eventually axonal loss leading to neurodegeneration and loss of brain volume.¹ Due to the essential role of myelin as an insulating cover that allows electric impulses to travel through the neurons its destruction may result in relapses that include paralysis, sensory changes, and a variety of associated neurologic abnormalities. MS is characterized by the formation of plaques of demyelination in the white and grey matter of the brain or spinal cord as a result of inflammation and subsequent axonal loss.¹ Several clinical forms of MS have been described. The overlap during transition from relapsing-remitting MS to secondary progress MS is not distinct. However, for clinical purposes, the major forms of MS include early MS (formerly referred to as clinically isolated syndrome), relapsing remitting, secondary progressive, progressive relapsing and primary progressive. 85% of all MS patients suffer from relapsing remitting MS (RR-MS). RR-MS is diagnosed initially as a clinically isolated syndrome that is followed by a series of relapses and remissions over decades. During the relapses, inflammatory lesions are observed in the CNS forming pathological plaques in the white matter that characterize the disease. Approximately 70% of RR-MS patients develop secondary-progressive MS (SP-MS). SP-MS is characterized by lesions in the grey matter, by axonal loss, and brain atrophy that causes a progressive neurological impairment. A third form of MS affects the remaining 15% of patients, the primary progressive (PP-MS). A chronic and progressive increase in disability is observed in PP-MS due to axonal loss and brain atrophy and no remission periods are present. During MS, peripheral inflammatory cells enter the CNS parenchyma by crossing the blood-brain barrier (BBB). Activated CNS resident cells contribute to inflammation and plaque formation; however, their involvement in the disease is less understood.

Approximately 2.5 million human suffer from the disease worldwide. Both genetic²⁻⁸ and environmental factors have been associated with the disease.⁹⁻¹¹ Whether MS is a neurological disease that triggers an autoimmune inflammatory reaction or an autoimmune disease that, when triggered, attacks neurons is still under debate. As reviewed by Dendrou and colleagues recently, MS could be triggered within the CNS as a neurodegenerative process or even triggered by an external agent such as a viral infection. The lesion would then promote an uncontrollable inflammatory cascade of reactions and infiltration of peripheral immune cells within the CNS. More likely, MS could be triggered in the periphery by the activation of autoreactive T cells that then would cross the BBB and target the myelin sheet of neurons.¹ Current opinion based on GWAS and other genetic testing indicates that the principal molecules upregulated in MS are immunologically based. This scenario requires that T cells as well as B cells acquire the pathological function of targeting self-antigens, which could result by bystander activation or by molecular mimicry. Infiltration of T cells would then promote the infiltration of macrophages, B cells, and perhaps other immune cell types such as dendritic cells. The involvement of autoreactive T cells in MS has been extensively studied using experimental animal models. For MS, the

most widely utilized animal model is experimental autoimmune (or allergic) encephalomyelitis (EAE), characterized by the peripheral activation of CD4⁺ T helper 1 (Th1) and Th17 cells in draining lymph nodes. After activation, Th1 and Th17 proliferate in the periphery and enter the CNS by crossing the BBB from the circulation. In the EAE model, autoreactive CD4⁺ T cells cross the BBB and then are re-activated within the CNS by either resident or peripherally infiltrated APCs.

The autoimmune nature of MS implies that deficiencies in one or more mechanisms of immune tolerance occurs. As a mechanism to control the presence of circulating autoreactive T and B cells that can cause autoimmune reactions, our bodies have evolved methods of cellular anergy in both the thymus and bone marrow, which are primary lymphoid organs responsible for the maturation of T and B cells respectively. These mechanisms are defined as central tolerance. In central tolerance the T-cell receptors (TCR) in T cells and B-cell receptors (BCR) in B cells, both members of the immunoglobulin superfamily, are exposed and restricted to self-antigens. High-affinity interaction to the self-antigen results in anergy and depletion of the cells, a process that reduces a large percentage of those cells that leave the thymus and bone-marrow. Although the mechanisms of central tolerance are highly effective, breaches occur and auto-reactive cells escape the primary lymphoid tissues through circulation. These errors occur and it is the role of the peripheral tolerance to detect and destroy the auto-reactive cells that escaped the process. Regulatory cells are important in the maintenance of peripheral tolerance.

Peripheral tolerance is mostly accomplished by T cell subsets with regulatory function: the regulatory T cells (Tregs). Tregs are activated CD4⁺ T cells with enhanced expression of CD25 and the transcription regulator *Fork-head box 3 (Foxp3)*. *Foxp3* expression is required for Treg differentiation and suppressive activity as well as for their proliferation and survival.¹² Other important cell subsets have regulatory functions, including regulatory B cells. B cells have been also shown to be relevant in MS pathology. Experimental evidence obtained in the EAE model suggest that distinct subpopulations of B cells with regulatory phenotypes could be involved in the protection to the disease¹³ by the production of increased levels of anti-inflammatory IL-10.¹⁴ Because of their importance in disease control, novel therapeutics have been designed to target them.^{8,15} Tr1 cells and Th3 cells have been also shown to be critical players in tolerance. Tr1 cells are IL-10 producing CD4⁺ T cells that do not express Foxp3.¹⁶ Th3 were described by Howard Weiner's laboratory as TGF- β producing CD4⁺CD25⁺ T cells.^{17,18} Furthermore, invariant natural killer (iNKT) cells, have shown regulatory phenotypes.¹⁹

Regulatory cells in multiple sclerosis

The main function of Tregs is to control the proliferation of inflammatory cell subsets and promote immune homeostasis. This anti-inflammatory effect is promoted by a variety of regulatory mechanisms including direct cell-to-cell contact, the production of anti-inflammatory cytokines, mainly TGF- β and IL-10, and also by effects modulating the function of antigen presenting cells (APCs). Because of their importance in controlling the proliferation of immune cells, great interest has been put in the understanding of Tregs in MS development and their potential protective function against MS as well as many other

autoimmune diseases.²⁰ Early studies by Viglietta and colleagues compared the *in vitro* function of Tregs by studying the suppressive capacity of Tregs isolated from 15 RR-MS patients when co-cultured with proliferating effector CD4⁺CD25⁻ T cells.²¹ Their studies were first to demonstrate that, when compared with Tregs isolated from healthy donors, the MS Tregs had reduced capacity to suppress the proliferation and IFN- γ production by effector T cells. The reduced immunosuppression of MS Tregs was later confirmed by Haas and colleagues in blood samples isolated from RR-MS patients.²² Huan and collaborators detected reduced Foxp3 protein and mRNA *FOXP3* expression levels in MS patients; however, although their study included RR-MS, SP-MS, and PP-MS patients, all samples were pooled for analysis. Pooled analysis of blood serves as an experimental factor that could limit the appreciation of the potential effects that the different forms of MS has in the circulating Treg frequencies.²³ By contrast, Venken and colleagues reported that the frequencies of circulating Tregs in RR-MS and SP-MS patients were not different to those detected in healthy donors. They, however, documented a significant reduction in the ability of Tregs isolated from relapsing remitting MS to suppress the proliferation of effector CD4⁺ T cells and their production of IFN- γ *in vitro*. Remarkably, no suppressive dysfunction was observed in Tregs isolated from circulating blood of SP-MS patients. The suppressive ability of Tregs was associated with the levels of *FOXP3* mRNA expression.²⁴ Feger and collaborators compared the frequencies of nTregs in the cerebrospinal fluid (CSF) and circulating blood from MS patients suffering from RR-MS, SP-MS, and PP-MS and demonstrated that when samples of CSF obtained from all forms of MS patients were pooled for analysis the frequencies of nTregs were enhanced but no differences were observed in circulation.²⁵ Fletcher and colleagues showed that both frequencies and suppressive function of circulating Tregs that express the ectoenzyme CD39 are reduced in RR-MS patients and not reduced in SP-MS patients when compared to healthy individuals.²⁶ The conclusion that can be reached based on these studies is that MS patients appear to have a near normal level of circulating Tregs but they appear to be dysfunctional in that they are unable to adequately suppress the proliferation of pro-inflammatory cells and the secretion of pro-inflammatory cytokines such as IFN- γ and IL-17. Furthermore, the different forms of the disease appear to affect the frequencies and immunosuppressive function of circulating Tregs of MS patients. These observations might be of interest considering the potential role of the microbiome as inducers of peripheral tolerance and immunomodulation, which we review below.

Immunomodulatory effects of the gut microbiota

The reservoir for both the IL-17 pro-inflammatory secreting Th17 cells and Foxp3 expressing Tregs is within the gut-associated lymphoid tissues (GALT). The gut is the largest immune organ in our body containing upwards to 80% of the immune compartment. The role of the GALT in the microbiome may be critical in understanding both immunopathogenesis and regulation in CNS demyelinating disease.

In 1683, Antonie van Leuwenhoek wrote to the Royal Society that “the dental plaque is an unbelievably great company of living animalcules”. That is perhaps the first description of the human oral microbiota. We now define the oral microbiota as all the microorganisms that are found on or in the human oral cavity and its contiguous extensions such as the tonsils

and pharynx. We also have a better understanding of other microbiota present in our bodies, such as the skin microbiota, the nasal microbiota, the urogenital microbiota, or the gut microbiota. We can consider that the microbiota is the microbial populations that inhabit a particular location of the body. As noted above, of those studied, the gut microbiota comprises the largest and most diverse. As in any other ecosystem, living organisms need to adapt to environmental factors, such as oxygen availability and tolerance, temperature, pH, humidity, water activity and osmolarity, nutrients, and also factors including enzymes and immunological soluble and cellular triggers that are produced by the host in response to them. The combination of the genomes that belong to the microbiota, our genes and products of the microbiota, and the host environment is now defined as the microbiome. The gut microbiome is then the sum of the genomes of bacteria, archaea, viruses, and also eukaryotic microbes such as fungi, their products, our genes, and their products. Overall, we carry with us around 3 pounds of microbial weight making this organ comprised of alien genetic material the largest organ in our body other than the skin. Some studies suggest that for each human cell there are 10 microbial cells. Furthermore, instead of the 20,000 genes of the human genome the total microbial genome comprises 20 million genes. Recent studies have lowered those numbers. Despite the discrepancies in the numbers, the importance of the microbiota for human health is now beginning to be fully understood.

In terms of composition, eukaryotic parasites, fungi, bacteriophages, and several archaea species have been found in the gut, such as *Methanobrevibacter smithii* (97.5% of 700 stool samples analyzed), and *Methanosphaera stadtmanae*. But the most studied constituents of the gut microbiota are bacteria. The two most dominant phyla are *Bacteroidetes* and *Firmicutes*. One study suggest that the gut has a core stable bacterial population with 40 species (75% of the gut microbiota in terms of abundance) that persisted one year in the individuals, whereas a second study showed that 60% of all bacterial species persisted for 5 years in individuals. Colonization by microbes starts in the moment when we are born²⁷, if not earlier.⁵⁵ In this context, different studies have associated the delivery method, natural through birth canal or cesarean, with differences in the established microbiota. Furthermore, diet during early days of life and abrupt interventions such as the use of antibiotics influence and shape the microbiome. Among all factors studied, diet is perhaps the most influential in the microbiome composition; changes can occur in just two days. David and colleagues administered mice with either a plant or animal-based diet and observed rapid changes in the composition of the gut microbiome and metabolites produced, such as short-chain fatty acids.²⁸

With the launch of the Human Microbiome Project and other similar approaches we have now started to comprehend the extent of the microbial distribution and variability of the different human microbiomes. Using the 16S rRNA gene as a marker to define operational taxonomic units (OTUs), we are now able to evaluate the effects of diet, infection, vitamin deficiencies, and other factors in the distribution, relative abundances, and population diversities of the gut microbiota. Furthermore, metagenomic approaches allow us to understand functionally the relative importance of the microbiome constituents. Currently, multiple efforts are being made in order to determine a correlation between diseases and changes in the microbiome. In the context of human diseases, dysbiosis has gained increased attention over the last years. In dysbiosis, alterations in the composition of the microbiome can lead to disease. The

effects of dysbiosis could be associated with significant changes in the metabolic, neuroendocrine, or immunological responses to the changes in the microbiota. Here we will discuss the effects that gut microbes have shaping, educating, and modulating immunological pathways as well as cells associated with pro/anti-inflammatory responses and immune homeostasis.

The role of gut microbes controlling the epithelial barriers integrity

As earlier discussed, diets enriched in fat content induce significant changes in the gut microbiota as well as an increased intestinal permeability, inflammation, and oxidative stress.²⁹ Altered intestinal permeability could then promote a stage of chronic inflammation that might exacerbate CNS inflammatory diseases, such as multiple sclerosis. Remarkably, recent studies demonstrate that CNS demyelination directly enhanced small intestine epithelial barrier permeability. As it is discussed later in this review, the induction of EAE, both active and passively, promoted the increase in intestinal barrier permeability as soon as seven days after induction.³⁰ More evidence is needed, however, in order to conclude that a demyelinating disease that occurs within the CNS is responsible for intestinal dysfunction. Nevertheless, it has been shown that the human commensal gut microbe *Bacteroides fragilis* regulates the intestinal barrier permeability in the maternal immune activation (MIA) mouse model, in which mothers are administered with the viral mimic poly(I:C). Resulting offspring shows social and behavioral impairments that relate to autism spectrum disorders (ASD).³¹ ASD mice also exhibit GI dysfunction, dysbiosis, and increased intestinal permeability. Treatment with *B. fragilis* restored gut integrity and resulted in improved behavior. The effects promoted by *B. fragilis* treatment were associated with a reduction in the levels of 4-ethylphenylsulfate (4EPS) observed in ASD mice, levels that were found significantly enhanced in untreated ASD mice.³¹

The absence of microbes in germ-free mice also affects morphology³², angiogenesis³³ and intestinal barrier integrity⁶¹. A recent study has shown that the colonization with a probiotic mixture of three different Lactobacilli, including *Lactobacillus rhamnosus* LOCK0900, *L. rhamnosus* LOCK0908, and *L. casei* LOCK0919, reduced permeability and improved intestinal morphology.³² Effects of dysbiosis on the intestinal integrity could affect significantly the levels of gut microbial components, such as endotoxin²⁹, and exacerbate disease. However, other mechanisms yet to be described could be responsible for the gut-brain association during CNS inflammation. As it will be covered later in this review, we have shown that a gut symbiont factor, the polysaccharide A (PSA) produced by *B. fragilis*, is protective against murine models of MS by the induction of tolerogenic dendritic cells and Tregs.^{34–36} The treatment of EAE mice with PSA promoted an accumulation of gut-derived regulatory T cells and dendritic cells in cervical lymph nodes that was not observed in healthy mice treated with PSA.^{34–36} These results could potentially indicate the involvement of an unknown inflammatory signal released during disease that would promote the migration of these cells.

Remarkably, Nouri and colleagues recently showed that during both active and passive EAE induction the GALT of diseased mice had increased infiltration of IFN- γ ⁺ producing Th1 cells, IL-17⁺ producing Th17 cells, and IL-17⁺ $\gamma\delta$ T cells, and pro-inflammatory levels

produced by gut-derived APCs of EAE mice were enhanced when compared to healthy mice. More importantly, the intestinal morphology and function of EAE mice was significantly affected seven days after disease induction with increased expression of the tight junction regulator zonulin in duodenum, jejunum and ileum and resulted in enhanced intestinal permeability.³⁰ These findings are even more intriguing when considering the evidence that suggests gut microbes are key in regulating the integrity of the blood-brain barrier (BBB) and that the integrity is significantly compromised during CNS diseases.³⁷ Previously considered to be isolated from the immune system by the BBB, the blood-cerebrospinal fluid, and blood-leptomeningeal barrier, it is understood that the CNS interacts more actively with its surrounding tissues and circulating vessels, and memory T cells survey the brain and spinal cord of healthy individuals³⁸. Furthermore, recent evidence demonstrates in mice the existence of a CNS lymphatic system³⁹. Although the findings require the validation in human CNS, these early studies suggest again that the separation between the CNS and the periphery is not as defined as initially speculated or as recently reviewed.¹

Could gut microbes or gut-derived metabolites regulate the integrity of these barriers? According to recent studies the hypothesis that metabolites produced by gut microbes control the permeability of the CNS barriers is plausible. Short-chain fatty acids (SCFAs), such as acetate, propionate, and butyrate, are products of the bacterial fermentation of non-digestible carbohydrates that are derived from fibers. These carbohydrates would pass through the GI tract if they were not processed by fermenting bacteria of the gut, particularly of the colon, such as *Clostridium* and *Bacteroides* species. Some animal species such as gorillas derive over a 50% of their energy from short chain fatty acids, as opposed to a 5–10% in humans.⁵⁷ SCFAs are more than energy sources and provide immunological benefits to the host by regulating gene expression in immune cells shifting them towards a regulatory phenotype, decreasing oxidative stress, and reinforcing intestinal barrier as well as the blood-brain barrier (BBB), as it will be discussed later. Gut microbes are also essential for the maintenance of intestinal morphology³², angiogenesis³³ and intestinal barrier integrity⁶¹.

In germ-free mice the integrity of the BBB is reduced when compared to conventional laboratory mice, with altered expression of tight junction proteins.⁴⁰ In this work, authors explored the importance of the BBB during early stages of life when the barrier is relevant protecting the CNS from gut microbes during brain development and from the changes in the metabolites pool that occur due to the switch from the nutritional dependence of the fetus to nutrition after birth.⁴⁰ Using germ-free mice they observed that embryos from mothers devoid of gut microbiota had increased blood-brain barrier permeability and reduced expression of the tight junction modulator occludin. In adult germ-free mice, the integrity of the BBB was also compromised with reduced occludin and claudin-5 expression in brain. SCFAs had been previously associated with improved intestinal barrier integrity, using in vitro Caco-2 cell models⁴¹ and butyrate in specifically.⁴² The administration of a high fiber diet was also shown to increase the intestinal barrier integrity in rats.⁴³ The effects of the gut microbiota in occludin expression has been also documented.⁴⁴ Dietary-derived SCFAs effects in intestinal barrier integrity may be benefited from close association between the gut microbiome and the intestinal epithelium, but how can SCFAs affect the integrity of the

BBB? In their work, Braniste and colleagues compared the ability of gut microbes that are known to produce different SCFAs in response to diet. They compared the disruption in the BBB permeability in germ-free mice that were monocolonized with either *Clostridium tyrobutyricum*, a butyrate producer, or with *Bacteroides thetaiotaomicron*, known to produce acetate and propionate, and observed that both gut microbes that produce SCFAs by fermentation of dietary fibers also restored BBB integrity. Furthermore, fecal transplants from conventional mice to germ-free mice restored the BBB integrity.⁴⁰ The transport of SCFAs, such as propionate, from the gut to distal organs, such as the CNS, through blood-stream has been previously documented.^{45,46} However, as recently reviewed by Michel and Prat, although these intriguing experimental findings suggest a remarkable role for SCFAs controlling the integrity of the BBB, an evident therapeutic target for CNS diseases, such as MS, and their mechanisms behind the effects are yet to be elucidated.⁴⁷

Gut microbiome effects on homeostasis and autoimmunity

The presence of commensal and mutualistic microbes in the gut also help us by directly competing for space and nutrients with pathogens and by directly responding against them⁴⁸. Additionally, germ-free studies and microbial mono-colonization experiments have demonstrated that the gut microbiome helps to develop and allow for the maturation of the immune system as well as the neuronal system that can affect behavior and mental health.⁴⁹ The effects of gut microbes in the immune system are required for its appropriate development and maturation, as noted by the dramatic morphological, cellular, and functional deficiencies observed in germ-free animals when compared with conventionally housed animals.⁵⁰ The immune dysfunction of germ-free mice are associated with a disequilibrium between pro- and anti-inflammatory immune cells both in the GALT as well as systemically that can be restored by the colonization of the gut with commensal microbes such as *Bacteroides fragilis*.⁵¹ Thus, gut microbes affect both the proper maturation and function of the immune system. Our body has evolved mechanism of central and peripheral tolerance by which self-antigens are discriminated from non-self-components, such as those from microbes or other external insults. The role of peripheral tolerance is to destroy those autoreactive clones that escape from the thymus and bone marrow, by apoptosis, anergy, or by the direct effects of Tregs, as described previously. The gut microbiome is perhaps the most important factor when considering the induction of peripheral Tregs by immunomodulatory effects promoted by either direct effect of microbial components or metabolites that are produced during microbial metabolism of diet.⁵²

Immune development and modulation are the basis for the hygiene hypothesis. According to this hypothesis, the increased use of antibiotics and other hygienic measures to reduce exposure to infectious diseases provokes significant alterations in immune function that can be associated with an increased incidence of developing an autoimmune disorder later in life.^{53,54} As strides have been made to make ourselves cleaner a corresponding increase in autoimmune diseases has become evident. Assuming the validity of the hypothesis, children raised in environments with more exposure to contaminated animal and plant debris, such as farms, would develop an immune system that would reduce the incidence of autoimmune diseases. Two different population studies that when combined included more than 15,000 children among ages between 6 and 13 showed that children raised on farms from Germany,

Austria, and Switzerland had a significantly lower asthma rates and tendency to develop atopy than control children, those not living on farms.⁵⁵ It is reasonable to propose that gut microbes and the effects of self-exposure to these microbes have a fundamental effect on the development and maturation of an effective immune system. Gut microbes appear to educate the immune system during early stages of life. However, it remains unclear how the influence of the gut microbe-host interactions would affect later autoreactive reactions, whether it be by a molecular mimicry mechanism, bystander activation, superantigen responses or combination of these effects.

The immunomodulatory role of gut microbes in experimental CNS inflammatory demyelination

Gut microbes have been shown to affect the balance between pro- and anti-inflammatory responses in the gut and regulate CNS inflammatory demyelinating diseases experimentally. The best described pro-inflammatory gut microbe that affects EAE severity is the gram-positive *Segmented filamentous bacterium* (SFB). SFB promotes gut-induced Th17 cell responses that could enhance inflammatory autoreactive responses in the CNS. The monocolonization of germ-free mice with SFB restores the lost susceptibility of the mice to EAE by the induction of Th17 cells.⁵⁶ Similar results have been observed in germ free mice susceptible to rheumatoid arthritis (RA).^{57,58} Other commensal microbes such as *Porphyromonas gingivalis*, a constituent of the oral microbiome, increase glial activation and shown to exacerbate pro-inflammation and EAE severity in a mechanism dependent on the immune recognition of its lipopolysaccharide (LPS) by TLR2.^{59,60}

If dysbiosis, defined as the alterations in the composition of the microbiome can lead to disease, affects the frequencies and function of pro- and anti-inflammatory reactions in response to the change in the microbiome, other microbes must be responsible for potentiating peripheral tolerance by promoting the induction of regulatory cells, such as Tregs and Tr-1 cells. EAE studies show that different gut microbes shift the immune balance towards a regulatory stage and can control the severity of EAE in mice or rats. Treatment of Lewis rats with *Bifidobacterium animalis*,⁶¹ and mice with a mixture of *Lactobacillus* spp.⁶² regulate the extend of CNS inflammation and clinical scores in EAE. Table 1 shows a list of microbes present in the gut microbiome that have been shown to control EAE. Among the gut microbes known to promote regulatory responses in mice, *Bacteroides fragilis* and specifically the human commensal *B. fragilis* that produces the extracellular capsular polysaccharide A (PSA) is best characterized. PSA produced by *B. fragilis* is a potent immunomodulatory symbiont factor that is recognized by antigen presenting cells in a TLR2-dependent mechanism and that potentiates the CD4⁺ T cell activation and induction of regulatory IL-10 producing cells.^{34,51,63,64} PSA produced by *B. fragilis* is protective in models of experimental colitis^{65,66} and asthma.⁶⁷ The induced IL-10 production that PSA stimulates in T cells is a common feature in the protective mechanism of action, although the phenotype the CD4⁺ T cell population producing it might actually be disease-specific.⁶⁷ Although there are many aspects of the mechanism by which PSA promotes immunomodulation, the polysaccharide is recognized by APCs of the gut in a TLR2 dependent mechanism. Early studies by Kasper's laboratory showed that PSA is internalized by dendritic cells that are found next in the mesenteric lymph nodes.⁵¹ The recognition of

PSA is mediated by conventional^{51,63} and plasmacytoid dendritic cells.⁶⁶ In the mesenteric lymph nodes, PSA promotes the proliferation of CD4⁺ T cells, their differentiation, and cytokine production after its presentation by APCs in a MCH class II dependent mechanism to naïve T cells.^{68,69} These immunomodulatory effects of *B. fragilis* appear to be independent of the intestinal barrier permeability and more associated with its recognition by APCs of the gut that traffic to the mesenteric lymph nodes, sites for antigen presentation. As earlier discussed, treatment with *B. fragilis* enhances intestinal barrier integrity in a process that is linked with 4EPS levels, which are significantly increased in untreated ASD mice.³¹ Our work focused on the potential protective role of PSA in murine models of EAE. PSA promoted prophylactic and therapeutic protection in EAE mice by the induction of IL-10-producing Tregs.^{34,36} In IL-10 deficient mice, PSA is not protective. Similarly, TLR2 deficient mice are unresponsive to the protective and regulatory effect of polysaccharide.³⁶ We will discuss next the effects of PSA promoting an enhanced regulatory phenotype in Tregs.

Murine CD39 expressing T cells as enhanced immunomodulation mediators

In EAE mice, PSA also affects the phenotype of the regulatory cells induced by favoring a subclass of regulatory T cells characterized by the expression of an ectoenzyme, the ectonucleoside triphosphate diphosphohydrolase 1 (ENTPD1), also known as CD39, responsible for the catabolism of ATP into AMP (CD39⁺Tregs).³⁶ The CD4⁺ T cells expressing CD39, that are induced by PSA, have an extended regulatory phenotype and produce increased levels of anti-inflammatory IL-10. PSA fails to protect CD39 deficient mice subjected to suboptimal EAE.³⁶ The levels of IL-17 in brains and CNS-draining cervical lymph nodes of PSA-treated mice were significantly reduced when compared with control EAE mice. Adoptively transferred 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.³⁶ Further, the acquisition of a CD39⁺ phenotype by regulatory CD4⁺ T cells, independent of Foxp3 expression (PD-1, GITR, CTLA-4 and ICOS) when compared with CD39⁻ T cells, enhanced the expression of surface molecules associated with cellular trafficking, such as Chemokine receptor-5 (CCR5), CCR6, and CXC chemokine receptor-3 (CXCR3) as well as the adhesion molecules CD49b and CD29.³⁵ The enhanced migratory capacity of the CD39⁺ T cells from EAE mice treated with PSA was confirmed in *in vitro* transwell studies. When compared to CD39⁺ T cells isolated from lymph nodes of PBS-treated EAE mice, PSA-Treated CD39⁺ T cells migrated more efficiently towards homogenates of EAE CNS. *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 negative.⁷⁰ Thus, these studies revealed how a gut symbiont factor produced by a human commensal has the ability to promote a regulatory phenotype in T cells that augments their migratory and protective effects against CNS inflammatory demyelination.

Why is CD39 expression important in the immunomodulation induced by T cells? CD39 increases the immunosuppressive activity of the cells expressing it by mediating the hydrolysis of ATP into adenosine monophosphate (5'AMP). AMP is then catabolized to adenosine by another ectoenzyme, CD73.⁷¹ ATP is released to the extracellular media when

cells lyse and by lysis-independent mechanisms, in vesicles, by nucleotide-permeable channels and by mediation of lysosomes.⁷¹ During tissue damage, which occurs during inflammation, ATP is released into the media. The expression of CD39 in immune cells appears to be regulated by the inflammatory milieu: through cytokines, hypoxic cells, and oxidative stress.⁷¹ By metabolizing ATP through the action of CD39 and then CD73, the adenosine formed increases the anti-inflammatory effects in Tregs and effector cells. P1 receptors expressed in immune cells, G protein-coupled P1 or adenosine receptors (A₁, A_{2A}, A_{2B} and A₃), respond to adenosine. In Tregs, CD39 increase their immunosuppressive activity and expansion in inflamed tissues and reduces the effects of ATP-mediated Treg cell death and pro-inflammation immune cells.

CD39-expressing CD4⁺ T cells have been shown to be important modulators of other experimental autoimmune diseases. In colitis-mediated experimental rheumatoid arthritis, the oral administration of a *Salmonella enterica* serovar Thyphimurium life attenuated vaccine that expresses in the surface the colonization factor antigen 1 (CFA/I) of enterotoxigenic *Escherichia coli* (ETEC) induced protection mediated by both Foxp3 positive and negative CD39⁺ CD4⁺ T cells.^{72–74} The same research group of Pascual and collaborators have recently demonstrated similar regulatory effects mediated by CD39⁺ CD4⁺ T cells in the RA model when mice were treated orally with the Gram-positive probiotic *Lactococcus lactis* expressing CFA/I.⁷⁵ It was shown that the adapted probiotic induced the expansion of CD39⁺ Tregs that produce both TGF- β and IL-10, that suppress the production of tumor necrosis factor alpha (TNF- α) and neutrophil infiltration into the joints of collagen-induced arthritis (CIA) model of RA.⁷⁵ It is known that CFA/I promotes a cytokine profile shift in disease mice, towards an anti-inflammatory profile⁷⁶. Furthermore, CFA/I enhanced the ability of IL-35 to exacerbate the regulatory function of CD39⁺Tregs^{72,73}. Although the protection induced by CFA/I fimbriae expressed in bacterial vectors are observed in both EAE and CIA, and also purified CFA/I in the RA model⁷³ have been demonstrated, the molecular mechanisms by which CD39⁺ Tregs are induced remain unknown. The regulatory effects of CD39⁺ Tregs on experimental RA and RA patients subjected to anti-IL-6 receptor (IL-6R) treatment were also been shown by flow cytometric analysis of peripheral blood.⁷⁷ Low peripheral expression levels of CD39 have also been proposed as biomarkers for RA in patients⁷⁸, and CD39-expressing Tr1 cells proposed as potential therapy against arthritis.⁷⁹ In patients suffering from inflammatory bowel disease, peripheral Th17 cells expressing CD39 had enhanced *in vitro* immunosuppressive effects.^{80,81} In a toxin-induced diabetes model, overexpression of CD39 rendered the transgenic mice protected against the disease.⁸² In summary, experimental evidence using animal models suggest that the acquisition of a CD39 phenotype by T cells, Tregs and even Th17 cells exacerbate their protective effects. We will now discuss the potential role of CD39 expressing cells in immunomodulation during multiple sclerosis, and propose the importance of the gut microbiota.

The potential beneficial effects of gut microbes or their products in multiple sclerosis patients

As discussed above, results obtained in experimental models of CNS inflammatory demyelinating diseases suggest a regulatory role of the gut microbiota. Efforts are underway in order to determine whether similar effects occur in patients suffering from multiple sclerosis. Studies are focused on comparing the gut microbiota relative abundances and diversity in MS patients with healthy individuals. A study conducted by Weiner's lab showed that species of *Methanobrevibacteriaceae* were found to be more abundant gut symbiont in of MS patients vs. healthy donors.⁵³ A more recent study found that specific clusters of *Clostridia* and species of *Bacteroidetes* were significantly depleted in the gut of MS patients. The relative abundances of a total of 21 different species were affected.⁸³ Further, the levels of lipid 654, a bacteria-derived lipopeptide produced by *Bacteroides* species was found to be reduced in serum of MS patients when compared to healthy individuals.⁸⁴ In addition to bacteria, archaea, fungi, and viruses (mainly bacteriophages) have been found in the human gut, with high intra-individual spatial and temporal variability. Further, diet and other environmental factors significantly affect the composition of the microbiome. Among those, glatiramer acetate, an approved immunomodulatory drug used by MS patients over the last two decades has been also shown to affect the composition of the gut microbiota.⁸⁵ Despite the descriptive relevance of this study and others, no causative microbial species has been identified. Metagenomic functional analysis as well as other approaches, such as metabolomics, currently being used might potentially be beneficial in order to understand the biological relevance of the observed dysbiosis.

To our knowledge, all studies reporting the potential dysbiosis that occurs in MS is compared in samples from those suffering from RR-MS. Further studies are necessary to determine whether dysbiosis occurs in all forms of MS. More importantly, it would be of great interest to determine whether different stages of disease affect differently the composition and function of the gut microbiome. In this context, would it be possible to predict the shift in the MS stage by better understanding the microbial composition of the gut? We have obtained preliminary data that suggest, in a biphasic model of EAE in non-obese diabetic (NOD) mice, that early stages of disease, but not the chronic phase, affects significantly the relative abundances and overall diversity of the microbiome⁸⁶. More studies are needed in order to understand the biological significance of these early findings.

The recent study by Miyake and colleagues described that specific *Clostridia* clusters and species of *Bacteroidetes* are among those affected during the dysbiosis observed in the intestine of MS patients. Remarkably, colonic clusters of *Clostridia* and also *Bacteroides fragilis* have been identified as strong Treg inducers.^{87,88} As we previously discussed, Tregs in MS patients might be dysfunctional in their ability to suppress the proliferation of autoreactive cells. Remarkably, it was shown that the ATP hydrolytic function of CD39⁺ Tregs is impaired in MS patients.⁸⁹ Similar dysfunction was observed in patients with autoimmune hepatitis.⁹⁰ Fletcher and colleagues showed that CD39⁺ Tregs isolated from MS patients respond less efficiently than CD39⁺ Tregs from healthy individuals when controlling the proliferation of Th17 cells and IL-17 production in vitro.⁸⁹ Due to the

observed dysfunction it is possible that gut microbes that promote peripheral tolerance mechanisms, such as the induction of Tregs, could regulate disease severity. We recently showed that a purified form of PSA produced by *B. fragilis* also modulates the regulatory phenotype of human Tregs. Using circulating peripheral mononuclear cells (PBMC) from healthy individuals, we identified PSA as a strong CD39⁺ Treg inducer. Exposure of human dendritic cells to PSA promoted the conversion of naïve T cells into CD39⁺ Tregs. The Treg induction was seen to be dependent of HLA class II presentation and costimulatory signals. Further, the exposure of circulating Tregs to PSA enhanced the expression of CD39 and IL-10 production by these cells, as well as their ability to reduce the production of TNF- α by pro-inflammatory monocytes stimulated with LPS.⁹¹ It will be relevant to determine whether PSA or other commensal derived antigens exacerbate the regulatory function of Tregs and perhaps control their tolerogenic dysfunction.

The potential role for GALT derived regulatory CD39⁺ T cells is not limited to specific products derived from the microbiota that colonize the intestine. As noted previously, the gut microbiome is a bidirectional organ in that microbial products secreted by the microbiota transit the gut lumen providing critical biologic information that controls host physiology and immunity. In turn, these shifts in the host influences the growth and metabolism of the microbiota. Thus, therapies directed at altering the peripheral immune system may result in changes in both the GALT as perhaps the microbiota itself. Recent studies show that approved MS therapeutics, such as fingolimod, increases CD39-expressing regulatory T cells in MS patients, as measured by mRNA expression levels of CD39 and by flow cytometry.⁹² These striking observations has been supported by recent studies in our laboratory where it was observed that treatment with alemtuzumab, a humanized anti-CD52 monoclonal antibody (mAb), reduced the frequencies of circulating CD4⁺ T cells; however, the remaining population had an enhanced expression of Foxp3 and CD39 (Mielcarz, *et al.* presented atECTRIMS 2013⁹³). Similar findings have been observed in EAE mice treated with a murine anti-CD52 mAb (Pant *et. al.*, manuscript under review, and presented atECTRIMS 2015⁹⁴). Further, preliminary data obtained in our laboratory and presented atECTRIMS 2015⁹⁵ provides new evidence that teriflunomide, an approved oral therapy against relapsing forms of MS, promotes the induction of GALT regulatory CD39⁺ T cells that when adoptively transferred into EAE recipient mice are sufficient to reduce the severity of the disease. The protective effects are observed when CD39⁺ T cells isolated from the GALT of teriflunomide-treated mice are transferred both prophylactic and therapeutically. Remarkably, the main mechanism of action proposed for the immunodulatory effects of teriflunomide is the inhibition of the *de novo* synthesis of pyrimidine, required for the proliferation of autoreactive cells, by the action targeted against the enzyme dihydroorotate dehydrogenase (DHODH). Our findings, (under review for publication) would provide an additional immunomodulatory effect for the therapeutic based on the induction of CD39⁺ producing T cells. Taking together, the experimental and clinical data suggest that gut microbial factors could be used to augment the regulatory function in MS patients while avoiding the deleterious side effects that characterize the current therapies. We further speculate that alterations of the gut microbiota may lead to the long term efficacy of some therapies by the induction and amplification of CD39⁺ T regulatory cells that have been shown to be insufficient in their capacity to reduce pro-inflammatory response such as IL-17

in those with multiple sclerosis. Amplification and restoration of the GALT derived CD39⁺ T regulatory pathway may represent the critical mechanism by which the currently available therapies for treating relapsing MS demonstrated efficacy.

Concluding remarks

The current effort directed toward understanding the complexity of the gut microbiome and its effects in human diseases is providing critical information that could benefit how we interpret disease pathogenesis and design novel therapies. Autoimmune diseases require the effective function of peripheral tolerance mechanism to control exacerbated autoreactive responses. In multiple sclerosis patients, the function of regulatory cells may be insufficient to control the proliferation and inflammatory effects of autoreactive cells leading to a break in peripheral tolerance. Because of the importance of the gut microbiota regulating the balance between anti- and pro-inflammatory immune reactions efforts are now focusing on the ability of specific microbial species to promote the regulatory function of the cells that respond to them. Future studies will determine whether controlling dysbiosis or treating MS patients with immunomodulatory factors derived from gut microbes become new therapeutic approaches.

Acknowledgments

The authors acknowledge the support of the National MS Society grant CA 1027A1/3 and the National Institutes of Health grants R41 AI110170/AI/NIAID and R56 AI098282/AI/NIAID, and Sanofi-Genzyme Corporate (Genzyme/IST Concept Award# GF-2014-11153). All authors have read the journal's authorship agreement and that the manuscript has been reviewed by and approved by all named authors.

Abbreviations

APC	antigen presenting cell
ATP	adenosine triphosphate
ASD	autism spectrum disorders
BBB	blood brain barrier
CNS	central nervous system
CSF	cerebrospinal fluid
DHODH	dihydroorotate dehydrogenase
EAE	experimental autoimmune or allergic encephalomyelitis
Foxp3	Fork-head box 3
GALT	gut-associated lymphoid tissues
IFN-γ	Interferon gamma
IL	Interleukin

LPS	lipopolysaccharide
MS	multiple sclerosis
PBMC	peripheral mononuclear cell
PSA	polysaccharide A
RR-MS	relapsing-remitting multiple sclerosis
SCFA	short-chain fatty acids
TGF-β	tumor growth factor beta
Th cell	T helper cell
TLR	toll-like receptor
TNF-α	tumor necrosis factor alpha
Tregs	regulatory T cells

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Table 1

Interventions to the gut microbiome that affect the severity of animal models of CNS inflammatory demyelination

Experimental model	Microbe	Species	Major effects observed	Refs
Germ-free (GF)	None	Mouse	Reduced severity. Reduced peripheral Th17 cell and increased Treg frequencies and anti-inflammatory cytokines	56
Germ-free (GF)	None	Mouse	Reduced severity. Deficit in Th17 cell in lamina propria and peyer's patches. Lack of autoimmune T cells and B cell recruitment and autoantibody production reduced	96
GF + mono-colonization	<i>Segmented filamentous bacteria</i>	Mouse	Disease susceptibility restored	56
GF + colonization	Specific pathogen free microbiota	Mouse	Disease susceptibility restored	96
Antibiotics	None	Mouse	Reduced severity, decreased IL-17 and IFN- γ , increased Tregs	97
Antibiotics	None	Mouse	Reduced severity, invariant NKT cell-mediated effects	98
Colonization	PSA-producing <i>B. fragilis</i>	Mouse	PSA-deficient, but not PSA-producing <i>B. fragilis</i> restores EAE susceptibility in mice treated with antibiotics	99
Colonization	<i>Lactococcus</i> spp.	Mouse	EAE protection by induction of IL-10-producing Tregs	62
Colonization	<i>Bifidobacterium animalis</i>	Rat	Reduces EAE severity	61
Colonization	<i>Pediococcus acidilactici</i> R037	Mouse	Reduces the severity of EAE by induction of IL-10-producing Tr1 cells	100
Colonization	<i>Lactococcus lactis</i> Hsp65	Mouse	Reduces the severity of EAE by induction of Tregs cells and LAP ⁺ CD4 ⁺ Tregs	101
Treatment with purified symbiont factor	Purified PSA produced by <i>B. fragilis</i>	Mouse	Prophylactic and therapeutic protection by IL-10-producing CD39 ⁺ and Tregs	34–36