Immunology
The Journal of cells, molecules, systems and technologies

British Society for immunology

IMMUNOLOGY REVIEW ARTICLE

The role of gut microbiome and associated metabolome in the regulation of neuroinflammation in multiple sclerosis and its implications in attenuating chronic inflammation in other inflammatory and autoimmune disorders

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doi:10.1111/imm.12903
Received 29 November 2017; revised 23 January 2018; accepted 24 January 2018.
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Summary

The importance of the gut microbiome in the regulation of non-infectious diseases has earned unprecedented interest from biomedical researchers. Widespread use of next-generation sequencing techniques has prepared a foundation for further research by correlating the presence of specific bacterial species with the onset or severity of a disease state, heralding paradigm-shifting results. This review covers the mechanisms through which a dysbiotic gut microbiota contributes to the pathological symptoms in an autoimmune neurodegenerative disorder, multiple sclerosis (MS). Although the central nervous system (CNS) is protected by the blood–brain barrier (BBB), it is unclear how gut dysbiosis can trigger potential immunological changes in the CNS in the presence of the BBB. This review focuses on the immunoregulatory functionality of microbial metabolites, which can cross the BBB and mediate their effects directly on immune cells within the CNS and/or indirectly through modulating the response of peripheral T cells to stimulate or inhibit pro-inflammatory chemokines and cytokines, which in turn regulate the autoimmune response in the CNS. Although more research is clearly needed to directly link the changes in gut microbiome with neuroinflammation, focusing research on microbiota that produce beneficial metabolites with the ability to attenuate chronic inflammation systemically as well as in the CNS, can offer novel preventive and therapeutic modalities against a wide array of inflammatory and autoimmune diseases.

Keywords: inflammation; microbiome; microbiota; neurodegeneration; neuroinflammation.

Introduction

Multiple sclerosis (MS) is a neurodegenerative autoimmune disorder in which the host immune system recognizes the myelin sheath surrounding axon terminals to be immunogenic, resulting in breakdown of tolerance. This triggers chronic inflammation within the central nervous system (CNS), lesion formation, demyelination of axons, along with breakdown in the blood–brain barrier (BBB), and gradual paralysis stemming from an inability to perpetuate action potentials over time. Multiple sclerosis has various forms with varying degrees of severity possessing distinct phenotypic characteristics; however, most cases share a common theme of varying symptoms at early stages followed by unrelenting neurodegeneration over time.[1] The inflammation in the CNS in patients with MS is mediated primarily by the T helper type 1 (Th1) and Th17 T-cell subsets producing pro-inflammatory cytokines, which leads to infiltrating monocytes and macrophages in the CNS following damage to the BBB. These infiltrating mononuclear cells carry out these inadvertent effector functions resulting in neurodegeneration.[2]

Experimental autoimmune encephalomyelitis (EAE) is a $CD4^+$ T-cell-mediated autoimmune disease induced in model organisms (primarily mouse and rat) in the research setting.[3,4] EAE is widely accepted as a reference standard

disease model for *in vivo* laboratory research geared towards studying the physiology and potential treatments of MS. EAE is inducible in mice through administration of antigens from the myelin sheath along with an adjuvant, which leads to induction of inflammatory T helper cells that infiltrate the CNS. These cells typically include Th1 and Th17 subsets that produce cytokines and chemokines, including interleukin-17 (IL-17), tumour necrosis factor-α (TNF-α), interferon-γ and CCL5, observed both locally in the CNS as well as systemically in circulation.[2,5–7] Along with the up-regulation of pro-inflammatory markers following induction of EAE, there is suppression of functionality of an anti-inflammatory T-cell subset known as T regulatory (Treg) cells.[8] Treg cells are $CD4^+$ cells characterized primarily by their expression of the transcription factor Foxp3 and production of the inhibitory cytokines such as IL-10 and transforming growth factor-β.[3] The production of these suppressive cytokines inhibiting T helper cell proliferation and function is the key mechanism by which Treg cells accomplish their effector function of inducing immune tolerance.[9] In addition to producing inhibitory cytokines, Treg cells accomplish their functions through cell–cell contact.[9] Treg cells have garnered great interest as a potential focal point for MS treatment due to their induction of tolerance displaying protective effects against demyelination.[10]

The microbiota is a microbial collective composed of commensal organisms and opportunistic pathogens alike that reside along barrier sites of the host organism. The microbial diversity along these barrier sites plays a regulatory role in digestion, immune system activation, protection from development of opportunistic pathogens, and various other physiological processes.[11–13] The importance of a tightly regulated microbiota on host physiology has gained a greater appreciation over recent years due largely to novel technologies giving a previously unavailable ability to culture fastidious organisms, rapidly sequence various macromolecules, and characterize microbe–host interactions with greater detail. The commensal relationship between host and microbial community possesses a highly complicated web of modulatory cross-talk among organisms and has been recognized as an undeniable factor affecting host response in disease states.[14] The count of living microbial cells, as well as genes expressed by those cells, possesses a towering presence over the host it resides within. The relative parity between these numbers is disputed due to difficulties in calculating definitive quantities in an ever-changing microbiota; however, the ratio is believed to be at the very least a 10-fold difference favouring the microbial flora.[15]

Gut–brain axis in MS and EAE

Interactions between the host microbiota and the gut-associated lymphoid tissue (GALT) help shape the immune system both locally and systemically throughout the host.[16] The GALT has become a focal point in research targeting autoimmune disorders because of Treg activation by bacterial strains inducing immune tolerance within host organisms.[17] The gut–brain axis is a term used to describe the interactions between host and microbiota residing within the gut and the lingering effects between these interactions that take effect within the CNS. The biodiversity present within the gastrointestinal tract has a collective metabolome very different from that of the human host that regulates the availability of nutrients for the host.[18] The result of bacterial metabolism under dysbiotic conditions can either prevent nutrients from reaching the host through bacterial sequestering or provide an excess of unwanted xenobiotic metabolites with detrimental effects. The study of outward products of bacterial metabolism in the gut–brain axis is relatively new but rapidly expanding. In combination with microbial metabolism, the interactions between bacteria and the immune cells are an important aspect of the gut–brain axis and are often dependent on the products of bacterial metabolism. Interventional therapies focusing on regulation of microbial diversity as well as the metabolic traits of the microbiome have started showing progress in the treatment of MS, including the use of established non-pathogenic probiotic regimens in the treatment of EAE.[19]

Although there are numerous reviews on the immune mechanisms that regulate the pathogenesis of EAE and the role of the microbiome in shaping the immune response in the gut, there is a paucity of reviews that help to understand the effects of microbiota in the gut–brain axis. It was therefore the primary objective of the current review to elucidate the complex interactions between the microbial metabolome found in the gut and how it can regulate immune response in the CNS in EAE and MS.

Amino acid metabolism

Cysteine metabolism

Desulfovibrionaceae are strictly anaerobic bacteria that desulfinate the amino acid 'cysteine' for downstream purposes as a primary carbon source.[20] The presence of *Desulfovibrionaceae* within the human gut has been shown in healthy colons; however, intestinal dysbiosis resulting from elevated levels of *Desulfovibrionaceae* has been shown in studies by sequencing the intestinal microbiota in both patients with MS and EAE-induced mice.[21,22] An overabundance of *Desulfovibrionaceae* plays a vital role in the host metabolism due to its sequestering of the amino acid cysteine within the gastrointestinal tract. Abnormal availability of the amino acid cysteine can have widespread effects throughout the host body because it is a building block required for host production of sulphur-containing

bioactive compounds, such as glutathione. The rate-limiting step of glutathione production, which is a tripeptide antioxidant, is the condensation reaction of cysteine with glutamate to form γ-glutamylcysteine.[23] Glutathione levels in particular are garnering focus due to their role as the primary countermeasure against reactive oxygen species (ROS) -related damage within the CNS. Currently, clinical studies are being performed to better understand the pathogenic role of underlying glutathione deficiencies in MS patients at different stages of the disease.[24] Damage related to ROS in glutathione-deficient patients has been shown to be reversible by the dietary supplementation of cysteine and glycine.[25] Supplementation of sulphur-containing precursor molecules was also shown to reduce oxidative stress in studies focusing on EAE-induced mice.[7] These studies suggest that the link between the bioavailability of these precursor molecules and the effector function of glutathione to eliminate elevated levels of ROS are directly related in both mice and humans.

Elevated levels of reactive oxygen species within the CNS arise from residential microglia, infiltrating macrophages and general mitochondrial activity in combination with faulty countermeasures to rid the tissues of free radicals. The multifaceted role of ROS in MS pathogenesis has been gaining a greater appreciation within the research community in recent years[26] due to elevated concentrations of ROS in CNS tissues playing potential roles in the initiation of lesions, recruitment of lymphocytes and the phagocytosis of myelin. High levels of superoxide in the CNS initiate lesions along the BBB due to disruption of the tight cell–cell junctions within cerebral endothelial cells as well as instigating a chemotaxis of monocytes towards the BBB.[27] The phagocytosis of myelin results in the loss of action potentials being perpetuated throughout the nervous system (causing the symptoms of paralysis) and is carried out by infiltrating macrophages. This phagocytosis of myelin sheathing has been shown to be dependent on the concentration of ROS present in the surrounding microenvironment.[28] In addition to an elevated ROS production, there is an increased expression of genes involved in the detoxification of ROS in an effort to alleviate self-inflicted damage caused by inflammation targeting the CNS.[29] This increased demand for antioxidant molecules locally within the CNS exposes and further exacerbates a potential susceptibility, if substantial levels of antioxidant precursor molecules are not readily available.

Collectively, the above studies suggest that pathological symptoms directly caused by accumulation of ROS during an inflammatory response in the CNS can be attributed at least in part to a lack of sulphur-containing precursor molecules related to either diet or bacterial dysbiosis. Dysbiosis arising from an overabundance of *Desulfovibrionaceae* within the host gut, provides a linkable mechanism of cysteine sequestering inducing a host deficiency of the sulphur-containing bioactive antioxidant glutathione. This glutathione deficiency aids in an abundance of ROS, which degrade the BBB, aid in the recruitment of immune cells to the CNS and are required for myelin phagocytosis.

Tryptophan metabolism

Bacterial metabolism of tryptophan can be linked to the modulation of inflammation through the production of indole-based compounds.[30] The enzyme tryptophanase, unique to bacteria, breaks down the amino acid residues into indole, ammonia and pyruvate for further bacterial use as an energy source or building block.[31] The outward indole by-products of this reaction can come with a variety of side chains attached to the third carbon. Some of the known side-chain functional groups include, but are not limited to, acetate, aldehyde and propionate.[30] These indole-based by-products have the capability to bind as well as activate the aryl hydrocarbon receptor (AHR) present in host lymphocytes, similar to plant-derived indoles.[32] The AHR is a cytosolic receptor that upon activation undergoes conformational changes, binds a carrier protein to import it into the nucleus, and carries out an effector function within the nucleus as a transcription factor.[33] The ligands for AHR are primarily planar hydrophobic molecules. Each of the mentioned indole by-products containing unique side chains possesses a unique binding efficiency to the AHR ligand-binding site. AHR ligands come from both endogenous and exogenous sources. Endogenous molecules produced by the host add a layer of complexity to the biological activity of ligands as environmental products and can either work as agonists activating the receptor, or as antagonists occupying the binding site with low efficacy to defer the basal levels of endogenous ligand activation.[34] This contribution of various indole-based compounds from the microbiome, which then compete with endogenous ligands produced by the host, results in disruption of balance between commensal organisms and host immune tissue.

Modulation of AHR activity through ligand binding by environmental pollutants such as 2,3,7,8-tetrachlorodibenzodioxin to dietary indoles has been shown primarily to lead to induction of Treg cells.[35–37] However, some endogenous ligands such as 6-formylindolo[3,2-b]carbazole have also been shown to induce Th17 cells.[32,38,39] The ability of AHR ligands to induce Treg cells versus Th17 cells may depend on their capacity to cause epigenetic changes including microRNA,[37] and clearly more research is needed to understand the complexity of the receptor. Studies focusing on AHR activation and inhibition in EAE have resulted in polarized effects of either amelioration or perpetuation of symptoms, through induction of Treg cells and Th17 cells, respectively.[40–42]

It has become apparent that AHR activation via bacterial derivatives of tryptophan metabolism has a net positive effect on symptomatic outcomes of EAE. It was shown in one study that using a tryptophan-depleted diet worsened the overall disease state of EAE in mice, whereas tryptophan supplementation resulted in an amelioration of symptoms.[41] This alleviation of symptoms within the CNS was found to be mediated in part by repressing the expression of Nos2 and Ccl2 in astrocytes.[41] An up-regulation of Nos2 and Ccl2 expression within astrocytes aggravated EAE symptoms due to a localized pro-inflammatory phenotype. Ccl2 is a cytokine involved in chemotaxis of memory T cells and monocytes to peripheral tissue sites, helping to initiate local inflammatory responses.[43] Nos2, commonly known as nitric oxide synthase, produces the reactive molecule nitric oxide (NO), which contributes to vasodilatation and is used as a ROS in targeted killing.[44] There does appear to be promise in the protective nature of tryptophan by-products against autoimmune inflammation in the CNS through the gut–brain axis. However, this area of research is relatively new and will require much more focus in the coming years to pinpoint the exact roles of these dynamic processes in EAE.

Carbohydrate metabolism

Short-chain fatty acid production from dietary fibres

The gut microbiota also plays a key role in the production of short chain fatty acids (SCFAs) through the fermentation of carbohydrates that are indigestible to the host organism. These SCFAs direct inflammatory responses throughout the body by modulating the chromatin structure within the nuclei of lymphocytes favouring gene products that result in the proliferation of anti-inflammatory $Foxp3^+$ Treg cells.[45,46] SCFAs regulate T-cell populations through the inhibition and activation of histone-modifying enzymes that either up-regulate (through acetylation) or down-regulate (through deacetylation) transcription at proximal promoter regions. Treg induction by SCFA is accomplished through the inhibition of histone deacetylase activity near the Foxp3 promoter region while simultaneously promoting acetylation of histone 3 at the Foxp3 promoter region.[47] The actual SCFA binding sites among $CD4^+$ T cells for inhibition of histone deacteylase enzymes are currently disputed and could occur through either intracellular binding affecting the mTOR pathway,[48] or through the cell surface G-protein-coupled receptors such as GPR41 and GPR43, specific for SCFAs.[49] The SCFA ligand binding site on $CD4^+$ T cells is currently debated due to GPR41 and GPR43 double knockouts having seemingly no effect on Treg cell proliferation as well as SCFAs being entirely permeable through the plasma membrane requiring no need for active transport.[50]

Butyrate production stems from prokaryotic fermentation of acetyl CoA precursors originating from dietary fibre catalysis. A diverse collection of species within the gastrointestinal tract have shown capabilities to ferment acetyl CoA into butyrate within the colon, however there is a primary focus on the *Clostridium* clusters XIVa and IV and the phylum Bacteroidetes, which both produce large quantities of butyrate.[51,52] Clinical studies focusing on sequencing the microbiome have shown a relative depletion of Bacteroidetes as well as the *Clostridium* clusters XIVa and IV in human samples from patients with MS when compared with healthy individuals.[53] The direct effect of butyrate induction within the colon has been tested through both high-fibre diet supplementation as well as direct oral butyrate treatments. Observed in both of these routes of butyrate supplementation was an amelioration of clinical symptoms of EAE using *in vivo* scoring algorithms combined with an increase in the numbers of $Foxp3^+$ $CD4^+$ T cells.[45]

Propionic acid is another SCFA produced from bacterial fermentation of indigestible fibres that could play a role in the prevention and relief of symptoms in inflammatory disorders. Propionic acid is produced by fermentation within the succinate pathway and modulates histone modifications at the Foxp3 promoter region in a manner similar to butyrate.[46,52] A large portion of the intestinal flora has been linked to the production of propionic acid, including the Gram-negative phylum Bacteroidetes and the Gram-positive phylum Firmicutes, which contains the genus *Clostridium*. Studies have shown that faecal transplants high in propionic acid in combination with propionic-acid-producing bacterial species have been beneficial in ameliorating the clinical symptoms of EAE in mice.[54]

Short-chain fatty acids are found within the body in staggering concentrations for biologically active compounds, relatively ~150 mM when pooled as a collective within the large intestine of healthy individuals, making changes in compositional and collective abundance more than sufficient to have changes in the phenotypic properties of lymphocytes within the GALT.[52]

Lipid metabolism

Polyunsaturated fatty acids

Another mechanism modulating the inflammatory response throughout the host is perpetuated by the metabolism of polyunsaturated fatty acids, namely omega-6 and omega-3 fatty acids, from host cells as well as bacterial cells within the gut. These long hydrocarbon chains are directly involved in the regulation of metabolic endotoxaemia, a disorder in which an overabundance of lipopolysaccharide (LPS) -producing gut bacteria leads to activation of Toll-like receptor 4 (TLR4), producing widespread chronic low-grade inflammation.[55] Omega-3 fatty

acids display an anti-inflammatory phenotype when ingested in higher proportions than omega-6 fatty acids due to the activation of intestinal alkaline phosphatase, an antimicrobial peptide that targets Gram-negative organisms while simultaneously promoting the growth of Gram-positive organisms.[56] In addition to reducing Gram-negative abundance, intestinal alkaline phosphatase exhibits anti-toxic effects by hydrolysing phosphate groups coupled to Lipid-A present on degraded LPS, reducing its binding efficiency to the TLR4 100-fold.[56] TLR4 activation leads to cryopyrin inflammasome activation releasing subsequent amounts of pro-inflammatory cytokines, TNF-α, IL-1 and IL-6 circulating in host organisms.[57] This chronic low-grade systemic inflammation connects the effects of microbial dysbiosis instigated by host diet to perpetual worsening of autoimmune disorders. This potential explanation coincides with the geographic distribution of diets high or low in omega-3 fatty acids. Regions of the world with diets rich in omega-3 fatty acids tend to have fewer instances of MS in comparison with those of the world that have a lower ratio of omega-3 fatty acids to omega-6 fatty acids,[58] adding a potential geographical proof to polyunsaturated fatty acids as a contributing factor. With focus on polyunsaturated fatty acids as a non-invasive intervention in the treatment of MS, clinical trials have tested the effects of supplementation of unsaturated fatty acids and found that there was a positive trend in the attenuation of symptoms, although it did not produce statistically significant results.[59]

Saturated long-chain fatty acids

Saturated long-chain fatty acids, such as lauric acid, have been shown to have antimicrobial properties in high concentrations.[60] These lipid molecules have a tendency to primarily target Gram-positive microbes. They have even been implicated recently as a potential topical therapy for inflammatory skin conditions caused by Gram-positive organisms such as *Propionibacterium acnes*.[60] Because saturated fatty acids form an increasingly substantial portion of energy-supplying molecules in westernized diets, there has been growing interest in the role of lauric acid in EAE due to shaping the microbiota with antimicrobial properties as well as shaping the GALT with TLR4 agonist properties similar to that of LPS.[61]

In vitro experiments supplementing media with saturated long-chain fatty acids, namely lauric acid, showed an up-regulation of Th17 cells and Th1 cells alongside a down-regulation of Treg cells.[62] In depth analysis of the transcriptome from these experiments showed an increase in pro-inflammatory markers (TNF-α, interferon-γ and Csf2) as well as a decrease in the anti-inflammatory marker Foxp3. Interestingly, these differential cytokine levels coincided with an up-regulation of the AHR, which shows a potential increased susceptibility to both endogenous and microbiota-produced compounds to compete as antagonists/agonists to drive further T-cell proliferation towards either an inflammatory or anti-inflammatory phenotype.

To focus on the effects of microbial by-products, researchers included faecal filtrates from saturated long-chain fatty acid-fed mice to cell cultures in Th17 cell polarizing conditions, which resulted in higher proportions of activated Th17 cells than that of control groups when compared with media that were supplemented with the faecal filtrates of mice fed a normal diet.[62] Sequencing analysis from these experiments showed decreased abundance of Bacteroidetes families as well as of *Prevotellaceae*. Both of these bacterial populations have previously been implicated in having protective roles against the progression of EAE symptoms through production of the SCFA butyrate,[51,52] which in turn up-regulates Treg cell proliferation.

Passage through the BBB

Passage of compounds from the peripheral blood supply into the CNS through the vascular endothelium composing the BBB is dependent on characteristics such as lipid solubility, tertiary structure, concentration, molecular weight and charge of the compound.[63] Generally compounds that can freely pass through the endothelium are low-molecular-weight lipid-soluble molecules with little to no charge. Majority of the bioactive compounds previously covered in this review are either theorized to be BBB permeable due to having all of the key characteristics of BBB-positive compounds, or have been proven to be BBB permeable through previous experimentation. Cytokines, generally hydrophilic in nature, present in the peripheral blood can modulate immunological functions within the CNS due to transporter-protein-mediated passage through the BBB and deposition into the CNS.[64–66] The passage of cytokines produced by peripheral lymphatic tissue through the BBB endothelium presents a bridge for BBB-negative compounds to have lasting effects on the physiology of the CNS through the modulation of peripheral lymphocytes. Intravenously injected indole compounds similar in structure to those produced by bacterial tryptophan metabolism have been shown to be able to freely pass through the BBB when present in the peripheral blood in high enough concentrations.[67] While structurally similar to many BBB-positive compounds, the indole-based microbial by-products of tryptophan metabolism have not been characterized as BBB-negative or -positive *in vivo*. The antioxidant glutathione is known to be BBB-positive due to well-defined transport proteins along the BBB that permit the deposition of glutathione in the non-oxidized state into the CNS.[68] LPS has been shown to be minimally invasive through the BBB endothelium of mice *in vivo*.[69] The neuroinflammatory effects of LPS can be accomplished by TLR activation in peripheral tissues inducing secondary effects within the

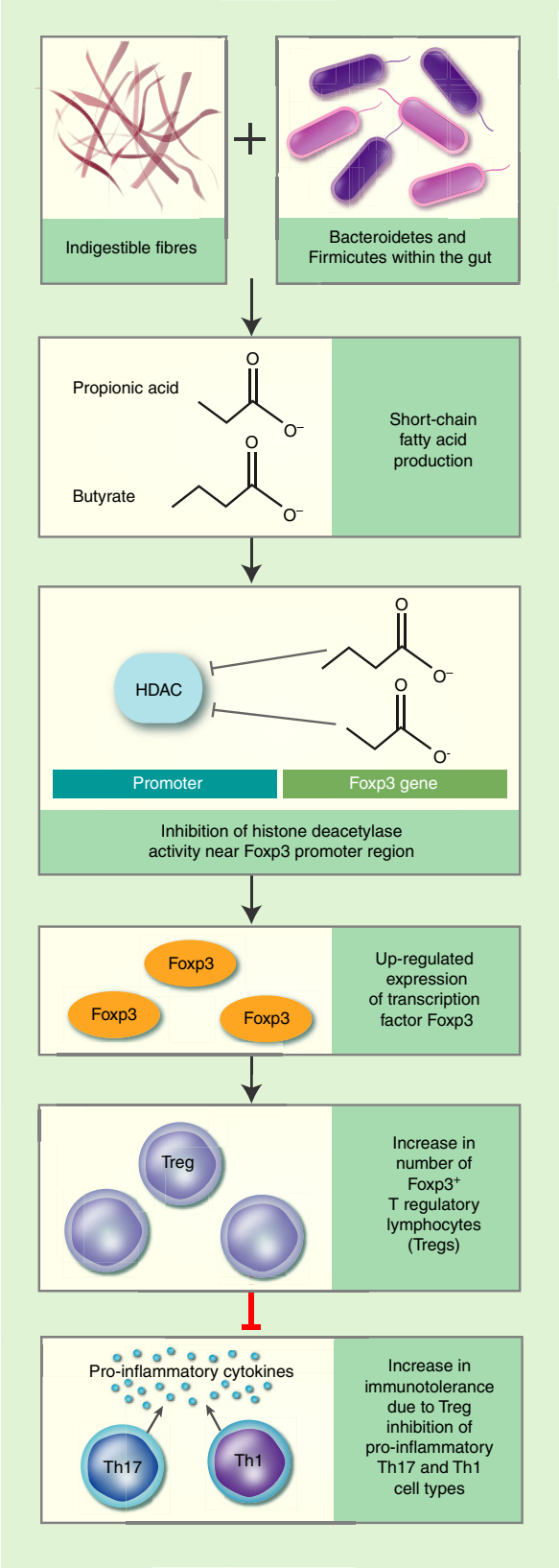

Figure 1. Graphical summary of immune tolerance induction through the production of short chain fatty acids by gut microbes.

CNS through BBB-positive mediators such as pro-inflammatory cytokines. SCFAs are BBB-positive, used by the CNS, and are integral in the development of proper permeability within the BBB.[70,71] It is currently unknown how much of the protective effects displayed by SCFAs in EAE are the result of direct interaction with the CNS versus interactions in the periphery.

Summary and conclusion

Multiple sclerosis is a debilitating autoimmune disease and, similar to 80 other autoimmune disorders, has no cure and limited treatment options aimed at reducing the clinical symptoms. Although there is increasing evidence suggesting that gut microbiota may play a critical role in the development or progression of MS, the precise mechanisms through which the gut microbiome can influence immune functions in the CNS despite the presence of BBB is unclear. This review systematically analysed the role of microbial metabolites and how they may regulate the immune functions of the host both within and outside the CNS, thereby providing evidence to support the hypothesis on the gut–brain axis (Fig 1). So far, research on the role of microbiota in MS has been fruitful in developing a better understanding of the pathogenesis of MS, but much more research is clearly needed in this field. Specifically, whether MS results from alterations in the microbiota caused by environmental factors and whether the microbiota influence the severity of the disease through regulation of immunological tolerance, needs additional research. Focusing research on probiotic organisms that produce beneficial metabolites or introducing changes in diet or other environmental factors to induce dysbiosis that is associated with induction of Treg cells or other immunosuppressive cytokines to attenuate chronic inflammation, can offer novel preventive and therapeutic modalities against a wide array of inflammatory and autoimmune diseases. Additionally, identification of specific deleterious microbiota triggering or promoting MS, and approaches to replace them with those that dampen chronic inflammation, can yield microbially based therapies that can help patients with minimally invasive procedures as well as cutting down on the costs of expensive long-term medications and their associated toxicity.

Disclosures

The authors declare that there are no conflicts of interest.

Funding

NIH grants R01ES019313, R01MH094755, R01AI123947, R01AI129788, P01AT003961, P20GM103641, and R01AT006888.

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