The role of the gut microbiota in multiple sclerosis

Jorge Correale[1], *Reinhard Hohlfeld*[2,3] *and Sergio E. Baranzini*[4] ✉

Abstract | During the past decade, research has revealed that the vast community of micro-organisms that inhabit the gut — known as the gut microbiota — is intricately linked to human health and disease, partly as a result of its influence on systemic immune responses. Accumulating evidence demonstrates that these effects on immune function are important in neuroinflammatory diseases, such as multiple sclerosis (MS), and that modulation of the microbiome could be therapeutically beneficial in these conditions. In this Review, we examine the influence that the gut microbiota have on immune function via modulation of serotonin production in the gut and through complex interactions with components of the immune system, such as T cells and B cells. We then present evidence from studies in mice and humans that these effects of the gut microbiota on the immune system are important in the development and course of MS. We also consider how strategies for manipulating the composition of the gut microbiota could be used to influence disease-related immune dysfunction and form the basis of a new class of therapeutics. The strategies discussed include the use of probiotics, supplementation with bacterial metabolites, transplantation of faecal matter or defined microbial communities, and dietary intervention. Carefully designed studies with large human cohorts will be required to gain a full understanding of the microbiome changes involved in MS and to develop therapeutic strategies that target these changes.

[1]*Department of Neurology, Fleni, Buenos Aires, Argentina.*

[2]*Institute of Clinical Neuroimmunology, University Hospital, Ludwig Maximilian University, Munich, Germany.*

[3]*Munich Cluster for Systems Neurology (SyNergy), Munich, Germany.*

[4]*Weill Institute for Neurosciences, Department of Neurology, University of California San Francisco, San Francisco, CA, USA.*

✉*e-mail: sergio.baranzini@ucsf.edu*

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During the past decade, research into the human gut microbiome has accelerated rapidly, revealing that the microbiota that inhabit multiple body niches — and particularly the gut — have an intricate relationship with human health[1,2]. The excitement around these microbiota and its genomic composition (known as the microbiome) and its influence on human health and disease has extended to every area of medicine, including neurology. Autoimmune and inflammatory conditions and their animal models — in particular, multiple sclerosis (MS) and its animal model experimental autoimmune encephalomyelitis (EAE) — were among the first diseases in which microbiome research demonstrated important basic concepts and began to suggest novel avenues for treatment[3,4].

In this Review, we summarize current knowledge of how the gut microbiome is involved in neuroimmunological disease, focusing on autoimmune demyelination and MS. We first outline how the microbiota influence immune function through complex interactions in the gut. We then discuss the evidence that dysbiosis is associated with MS and consider how the microbiota could be targeted therapeutically.

Microbiota and the immune system

Neuroinflammatory diseases such as MS involve immune dysfunction, and alterations in the microbiota can influence immune function in multiple ways. First, bacterial metabolites influence intestinal production of serotonin, which itself regulates the function of many immune cell types. In addition, through complex interactions with several components of the immune system, the gut microbiota make a major contribution to the regulation of host T cell and B cell maturation and activity[5]. In turn, these lymphocytes regulate the microbiota through maintenance of the intestinal barrier and low-grade microbial translocation to other body sites. These interactions begin at the intestinal epithelium, which commensal (and pathogenic) bacteria access by breaking through the mucus layer. This mucus layer is different in the small intestine (which has a single, tightly attached layer of mucus) and in the colon (where the mucus is organized into a loose outer layer and a denser, firmly attached inner layer)[6], with implications for the composition and function of immune cells that are resident in the gut-associated lymphoid tissue in these two regions — pro-inflammatory lymphocytes

Key points

- The colony of bacteria that inhabit the gut — known as the gut microbiota — varies in composition according to genetic factors and, more importantly, envorinmental influences, particularly diet.
- The composition of the gut microbiota influences the production of serotonin in the gut, which in turn influences serotonin-mediated regulation of systemic immune function.
- Gut microbiota are also involved in complex interactions with the gut and immune cells in the small intestine and the colon, thereby influencing immune responses in the periphery and the central nervous system.
- Abundant evidence indicates that the gut microbiota has a role in multiple sclerosis (MS) through its influence on immune function.
- Therapeutic strategies that target the microbiota — including dietary interventions, probiotics, short-chain fatty acids and faecal microbial transplantation — seem promising for the treatment of MS, but further work is needed to assess their effectiveness.

are predominantly produced in the small intestine, whereas anti-inflammatory lymphocytes predominate in the colon[4].

Signals from the microbiota create complex interactions between epithelial cells, dendritic cells, macrophages and innate lymphoid cells. Normally, these interactions are tightly controlled by innate and adaptive immune responses. However, a breakdown of intestinal homeostasis owing to dysbiosis can result in dysregulated systemic immune responses. Given that systemic immune processes can contribute to neuroinflammation in MS and other neuroinflammatory disorders, this dysregulation can contribute to and/or exacerbate neuroinflammation[7–10] (FIG. 1).

In the following sections, we discuss in detail how the microbiome influences immune function and how studies in mice and humans have demonstrated that these effects are important in neuroimmunological disease.

Serotonin

Serotonin — also known as 5-hydroxytryptamine (5-HT) — has well known, critical roles in the brain and gut. In the brain, it acts as a neurotransmitter involved in behaviour, cognition and locomotor activation, and in the gut, it influences intestinal peristalsis, motility, secretion of mucus, vasodilatation and absorption of nutrients[11]. However, serotonin and its metabolites (for example, *N*-acetylserotonin and melatonin) also affect immune regulation via receptors expressed on innate and adaptive immune cells[12–16]. Approximately 90% of all serotonin production occurs in the gut, and the microbiota strongly influence this production. Consequently, the microbiota can influence immune function indirectly via their effects on serotonin production.

Microbiota and serotonin production in the gut. Serotonin is produced by the metabolism of dietary tryptophan. This metabolism is mediated by the enzymes 5-hydroxytryptophan decarboxylase, tryptophan hydroxylase 1 (in the enterochromaffin cells of the gut[17,18]) and tryptophan hydroxylase 2 (in the brain[19]). Animal studies have demonstrated that the gut microbiota can influence the amount of serotonin synthesized by enterochromaffin cells by modulating expression of tryptophan hydroxylase 1.

Mice kept in a sterile environment (germ-free mice) have higher plasma levels of tryptophan and lower levels of serotonin than animals kept in specific-pathogen-free conditions, the conventional method for maintaining experimental mice colonies[20,21], and these levels normalize upon colonization after the weaning period. Normalization was associated with an increase in expression of tryptophan hydroxylase 1 with no change in the number of enterochromaffin cells[22]. Furthermore, administration of short-chain fatty acids (SCFAs; microbial metabolites derived from dietary fibre) to mice increased levels of tryptophan hydroxylase 1 mRNA in enterochromaffin cells and, subsequently, increased intestinal serotonin levels without changing levels of serotonin transporters[22,23]. This observation demonstrates that the microbiota modulate serotonin production via their metabolites. Reinforcing these findings, colonic administration of tryptophan hydroxylase inhibitors blocked the ability of microbiota to produce colonic and peripheral serotonin, suggesting that gut microbes require host tryptophan hydroxylase activity to upregulate serotonin production in the periphery[23].

Unlike eukaryotes, some bacteria, including *Corynebacterium* species, *Streptococcus* species and *Escherichia coli*, can synthesize tryptophan via tryptophan synthase[24]. Further, faecal metabolites produced by spore-forming bacteria, particularly *Clostridia* species, can increase serotonin levels in enterochromaffin cell cultures, as well as in the colon of germ-free mice[25]. These observations suggest that native members of the microbiota can also contribute directly to host serotonin levels through its de novo synthesis.

Serotonin and neuroinflammation. Serotonin receptors have been found on almost all types of immune cell and a growing body of data suggests that gut serotonin influences the innate and adaptive immune systems — via different mechanisms — during neuroinflammation[20–23,26,27]. For instance, increased levels of serotonin in the gut attenuate the severity of EAE in mice[28] by reducing IFNγ production and T cell proliferation, expression of 5-HT_{1A} receptors on $CD4^+$ T cells is increased in patients with MS[29], and serotonin suppresses the release of IL-17 and IFNγ, which are both neurotoxic in MS, by $CD8^+$ T cells in vitro.

In addition to its effects on T cells, serotonin can upregulate genes associated with M2 macrophage polarization and suppress lipopolysaccharide-induced pro-inflammatory cytokines through the activation of 5-HT_{2B} and 5-HT_7 receptors on monocytes[30]. In addition, in vitro activation of 5-HT_4 receptors in astrocytes inhibits IFNγ-mediated induction of major histocompatibility complex (MHC) class II and co-stimulatory molecules[31]. These effects may interfere with the immune response of astrocytes in the central nervous system (CNS). Serotonin can also activate autoreactive T cells through the 5-HT_3 receptor, thereby increasing the production of IL-6 and IL-17, which causes deleterious effects in EAE[32].

In addition to the direct effects of serotonin on various immune cells, serotonin metabolites can also have an immunosuppressive effect. Studies in EAE mice

have demonstrated that *N*-acetylserotonin has antioxidant, anti-inflammatory and neuroprotective properties mediated by its activation of the TrKB receptor[28,32]. Melatonin, another metabolite of serotonin, ameliorates EAE, and interferes with the differentiation of human and mouse T cells[28] by inducing expression of the repressor transcription factor Nfil3, thereby blocking the differentiation of T_H17 cells, and promoting differentiation of protective Tr1 cells by activating Erk1/2 signalling and transcription of the IL-10 promoter RORα[16].

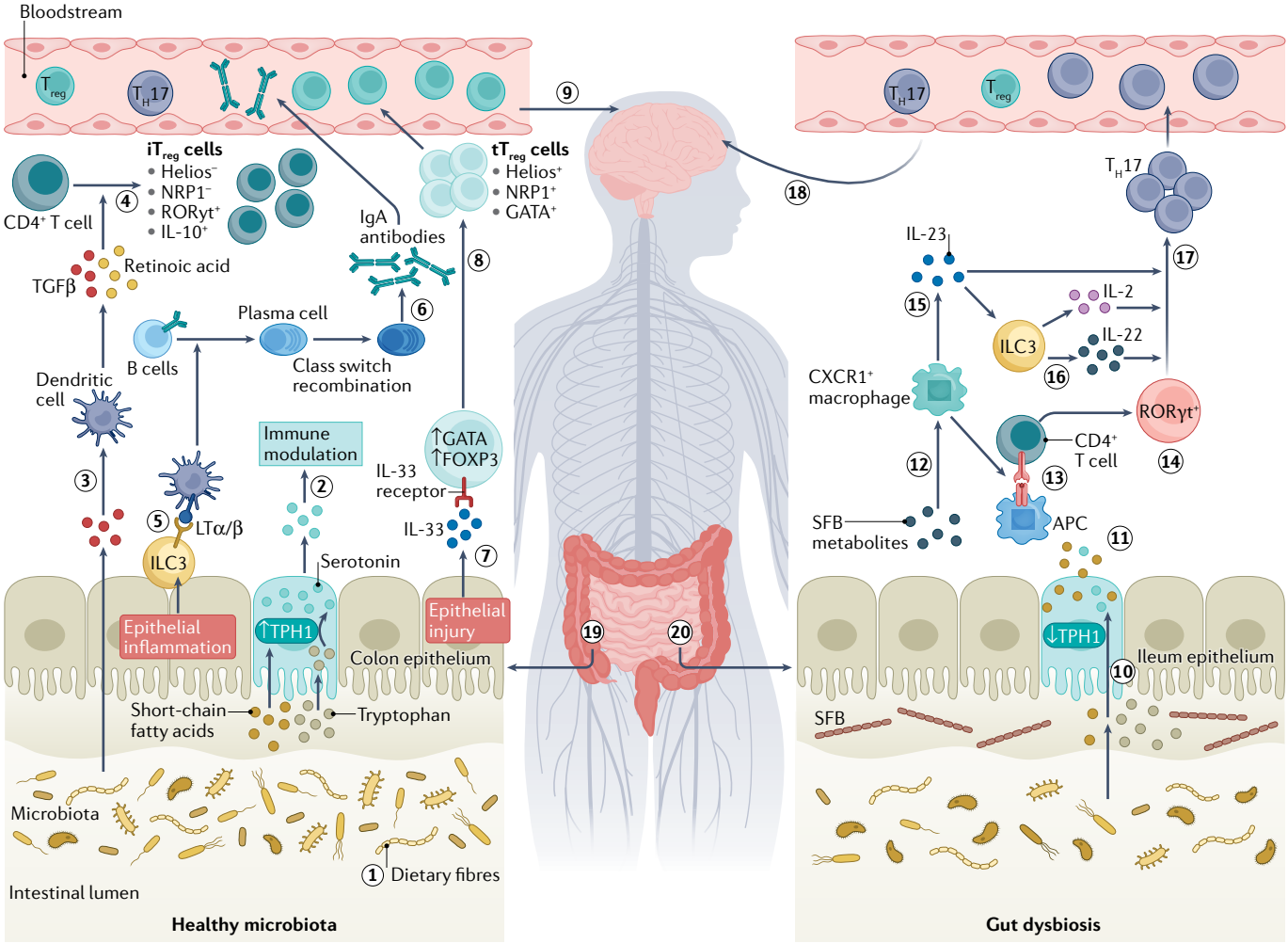

Fig. 1 | **The gut microbiota contributes to the modulation of neuroinflammation.** In healthy individuals (left), commensal and anti-inflammatory bacteria interact with the colonic mucosa to maintain homeostasis. Bacteria that are part of the commensal gut microbiota produce short-chain fatty acids through the fermentation of dietary fibre (1). These short-chain fatty acids increase the activity of tryptophan hydroxylase 1 (TPH1) in enterochromaffin cells of the gastrointestinal epithelium, which increases production of serotonin from dietary tryptophan (2). Serotonin regulates the secretion of cytokines (such as tumour necrosis factor (TNF), the interferon IFNγ and the interleukins IL-1β and IL-17) by immune cells, mediates recruitment of neutrophils and activation of T cells, and can activate autoreactive T cells to produce IL-6 and IL-17. Signals from the microbiota also have direct effects on immune processes. Short-chain fatty acids can activate dendritic cells (3) that release TGFβ and retinoic acid, which promote differentiation of CD4⁺ T cells into intestinal-induced T regulatory (iT_{reg}) cells (4). Inflammation of the epithelium induces production of lymphotoxin α and β (LTα/β) by type 3 innate lymphoid cells (ILC3s), which leads to activation of dendritic cells (5), thereby contributing to the development of plasma cells, which undergo class switch recombination to produce immunoglobulin A (IgA) antibodies (6). In addition, epithelial cell injury causes release of IL-33 (7), which leads to increased expression of GATA3 and FOXP3 in T cells, ultimately promoting the proliferation and maintenance of thymus-induced T_{reg} (tT_{reg}) cells (8). The T_{reg} cells and IgA antibodies generated through these processes reach the central nervous system via the circulation (9) and promote homeostasis. Disruption of intestinal homeostasis alters these processes and can result in dysregulated systemic immune responses that contribute to neuroinflammation. In gut dysbiosis (right), serotonin production in the gut is disrupted because a reduction in short-chain fatty acids reduces activity of TPH1 (10), leading to lower levels of serotonin and higher levels of tryptophan (11), with implications for immune regulation. Segmented filamentous bacteria (SFB) in the ileum also modulate the CD4⁺ T cell compartment in the gut-associated lymphoid tissue by promoting T_H17 cell differentiation and expansion. SFB metabolites activate CXCR1⁺ macrophages (12). These macrophages act as antigen-presenting cells (APCs), presenting microbial antigens to naive T cells (13) that consequently differentiate into RORγt⁺ cells (14) and, ultimately, T_H17 cells. Activated CXCR1⁺ macrophages also contribute to the synthesis of IL-23 (15) and stimulate ILC3s to produce IL-22 (16). In the presence of IL-2, which is also predominantly produced by ILCs, IL-22 and IL-23 promote the differentiation of RORγt⁺ T cells into T_H17 cells (17). T_H17 cells in the circulation can promote neuroinflammation (18). Gut bacteria can, therefore, modulate the balance between regulatory and pro-inflammatory cells that influences neuroinflammation. Gut microbiota differ in their functional potential, density and composition along different segments of the small and large intestine. Intestinal-induced T_{reg} cells are enriched in the colon (19), where they expand and differentiate in the presence of commensal bacteria. Conversely, during gut dysbiosis, SFB promote differentiation of T_H17 cells primarily in the small intestine (20).

Collectively, these findings suggest that the gut microbiome represents a plausible and tractable target for modulation of serotonin bioavailability. Evidence from animal and human studies that serotonin alters immune function and influences neuroimmunological disease suggests that targeting serotonin availability via the gut microbiota has potential in the treatment of CNS inflammation[33].

The microbiota and CNS serotonin. Whether the gut microbiota influences local effects of serotonin in the CNS is less clear. Tryptophan can cross the blood–brain barrier (BBB) via the cognate L-type amino acid transporter, which enables local serotonin production in the CNS. However, serotonin itself is a highly charged molecule and cannot passively diffuse across cell membranes, so a transport mechanism would be required for it to cross the BBB[26,34] and no such transport mechanism has yet been identified. Consequently, modulation of peripheral serotonin production by the microbiota is unlikely to influence serotonin levels in the CNS.

Nevertheless, some evidence suggests that the gut microbiota can influence levels of key central neurotransmitters, including serotonin, in other ways. For example, increased levels of tryptophan in the circulation, which can result from microbiota-mediated reduction of serotonin synthesis in the gut or tryptophan synthesis by some bacterial species, can lead to increased serotonin-mediated neurotransmission[35]. In addition, bacterial metabolites that derive from tryptophan can influence CNS inflammation through the transcription factor aryl hydrocarbon receptor (AHR), which has been shown to integrate environmental, dietary, microbial and metabolic cues to regulate microglial activation[36]. Finally, some studies in mice have demonstrated that extracellular vesicles released by *Akkermansia muciniphila* can increase serotonin levels in the hippocampus as well as the colon. The central effect could be related to the ability of these vesicles to cross the BBB or might be linked to specific signal transduction pathways[27]. Together, these observations indicate that the microbiota may influence CNS levels of serotonin with functional implications, and further work should be done to determine whether this is the case and whether these effects are important in neuroinflammation.

T cells

Effects on pro-inflammatory T cells. Specific commensal bacterial species can modulate the $CD4^+$ T cell compartment in the gut-associated lymphoid tissue and promote production of pro-inflammatory T cells (T_H1 or T_H17 cells)[37–39]. In germ-free mice that are resistant to EAE, T_H17 cells are undetectable, but monocolonization with segmented filamentous bacteria (SFB) promoted the differentiation and expansion of T_H17 cells, many of which were specific to SFB antigens[40], thereby restoring susceptibility to EAE[41]. Similarly, in patients with relapsing–remitting MS, levels of pro-inflammatory $IL\text{-}22^+IL17^+$ cells are increased in the small intestine mucosa in association with *Streptococcus* strains[42]. Furthermore, one study has shown that bacterial species that have been specifically associated with MS can promote inflammatory processes — in this study, *Akkermansia muciniphila* and *Acinetobacter calcoaceticus*, which are increased in patients with MS (see section 'Studies in people with MS') induced pro-inflammatory responses in human peripheral blood mononuclear cells[8]. Type 3 innate lymphoid cells (ILC3s) also contribute to production of T_H17 cells through their release of IL-22 in response to IL-23, which is secreted from macrophages after their activation by SFB metabolites[5].

Several other mucosa-associated bacteria in the microbiota can drive pro-inflammatory T_H17 cell responses, including *Escherichia coli*, *Bifidobacterium adolescentis*, *Staphylococcus aureus* and *Candida albicans*[43,44]. Each of these species induces a unique epithelial and T_H17 cell response, which depends on the taxonomic group of the colonizing microorganism and the activation state of the T cells, which is in turn determined by the surrounding cytokine environment and the genetic background of the host. Polarization of T_H17 cells can also be induced by a decrease in levels of microbes that limit their intestinal expansion, such as *Prevotella* species[45]. Following their priming in the mesenteric lymph nodes, T_H17 cells can reach the CNS directly via systemic circulation or through recirculation after trafficking to intestinal tissue.

Effects on T regulatory cells. T regulatory (T_{reg}) cells are anti-inflammatory, and the balance between these cells and pro-inflammatory T cells determines the strength of immune responses. The intestine contains intestinal-induced T_{reg} (iT_{reg}) cells and thymus-induced (tT_{reg}) cells (FIG. 1).

iT_{reg} cells are enriched in the colon, where they expand and differentiate in the presence of commensal bacteria and their metabolites. iT_{reg} cells are absent in germ-free mice and their production in the small intestine and the colonic lamina propria is induced by different components of the microbiota, including *Clostridium* clusters IV and XIVa and members of the *Escherichia*, *Bacteroides*, *Lactobacillus* and *Streptococcus* genera[46]. Many of these species are present in specific areas of the intestine, resulting in compartmentalized activation and induction of iT_{reg} cells that are essential for intestinal $CD4^+$ T cell homeostasis. Bacterial SCFAs are important metabolites that drive differentiation of $CD4^+$ T cells into iT_{reg} cells by acting on mucosal $CD103^+$ dendritic cells. These colonic dendritic cells consequently produce the polarizing factors TGFβ and retinoic acid[47–49] and suppress the expression of pro-inflammatory cytokines[50,51].

T cell receptors found on many T_{reg} cells in the intestine, including colonic T_{reg} cells, are also present on $CD4^+FOXP3^+$ thymocytes, suggesting that a substantial proportion of the T_{reg} cell repertoire in the intestine is of thymic origin[52]. Unlike iT_{reg} cells, the presence of these tT_{reg} cells is unaffected by the absence of microbiota[53]. Indeed, in germ-free mice, these cells can become activated upon colonization of the mice with standardized microbial flora[46] and can prevent colitis in mice that lack iT_{reg} cells[54]. tT_{reg} cells express the IL-33 receptor, so they respond to IL-33, which is produced by epithelial cells as a result of epithelial inflammation. IL-33 signalling leads to increased expression of GATA3 and upregulation of

FOXP3, ultimately promoting the proliferation and maintenance of tT_{reg} cells[55] (FIG. 1).

T_{reg} cells produced in the gut enter the circulation and influence systemic immune responses. In this way, the local microbiota influence systemic inflammatory processes, and therefore neuroinflammation, via their effects on T_{reg} cell levels. One specific pathway through which the commensal microbiota affect neuroinflammation is the regulation of T_{reg} cell activity via CD39, which increases the migratory capacity of T_{reg} cells (thereby facilitating the function of the CNS) and modulates the purinergic signal in immune cells and in the periphery[56,57]. Purinergic signalling in the astrocyte and migroglia is a particularly important factor in the development of pathological processes.

Studies in mice have demonstrated that these influences of the microbiota on T_{reg} cells are important in neuroinflammation. For example, administration of *Bacteroides fragilis*-derived polysaccharide A to mice induced production of tissue-specific $CD4^+FOXP3^+CD39^+$ T_{reg} cells, which protected the animals against CNS inflammation[58,59]. In another study, CD39 deficiency reduced accumulation of T_{reg} cells in the CNS in EAE, thereby increasing T_H1 and T_H17 signals and exacerbating neuroinflammation[60]. Furthermore, patients with MS have low levels of $FOXP3^+CD39^+$ T_{reg} cells, which suppress IL-17-producing pro-inflammatory T cells in vitro[61].

Mucosal-associated invariant T cells. Mucosal-associated invariant T (MAIT) cells are innate-like immune cells that respond to bacterial antigens and their dysregulation has been implicated in autoimmune diseases[62,63]. MAIT cells are absent in germ-free mice, suggesting that their function is linked to the intestinal microbiota[64]. The antigens that MAIT cells respond to are intermediates of microbial riboflavin synthesis and only bacteria that encode the riboflavin pathway — such as members of the *Bacteroidetes* and *Proteobacteria* phyla — can stimulate MAIT cells[65]. Once activated, MAIT cells produce cytokines related to inflammation and cell death, including TNF, IFNγ, IL-17A and granzyme[66]. In patients with MS, MAIT cells are recruited into the CNS at onset and persist for several years[67]. These cells express high levels of CD103, which is a defining marker of tissue residency, and produce IFNγ and IL-17 upon activation, suggesting that resident MAIT cells are pro-inflammatory and have deleterious effects in the CNS[62].

B cells

Immunoglobulin G is the main antibody isotype present in the serum but a substantial number of antibody-secreting cells in the blood secrete immunoglobulin A (IgA) antibodies, which are normally secreted into mucosal surfaces such as the gastrointestinal tract[68]. In the gut, plasma cells produce large quantities of IgA antibodies against commensal bacteria during homeostasis[69]. Development of these plasma cells is initiated by ILC3s, which respond to production of IL-1β from the epithelium that is driven by the microbiota[70]. Activated ILC3s secrete lymphotoxins α and β, and granulocyte-macrophage colony-stimulating factor (GM–CSF), which in turn act on dendritic cells and macrophages[71], initiating a downstream cascade that leads to B cell differentiation. However, some evidence indicates that cells that produce IgA antibodies specific for gut-encountered antigens are also present outside the gut. One study in mice has shown that gut-derived IgA^+ B cells are mobilized from the gut and subsequently attenuate inflammation in the CNS[72] (FIG. 2). This finding suggests that the role of these cells during autoimmune disease should be considered.

A follow-up investigation showed that IgA^+ B cells that are specific for gut microbiota traffic to the CNS in active MS[73]. In this study, the investigators characterized IgA-producing B cells and IgA antibodies in the gut, blood, cerebrospinal fluid and brain tissue of patients with MS and other neuroinflammatory diseases. In patients with MS, the IgA coating of gut bacteria differed from that in healthy controls, raising the possibility that MS-associated bacteria are potent IgA inducers. Interestingly, cerebrospinal fluid levels of IgA^+ B cells and IgA antibodies were higher during active relapses than during clinical remission; given that IgA^+ B cells have been shown to be anti-inflammatory in the brain[72], this observation suggests that they protect the brain during inflammatory disease activity. These findings are in agreement with the observation that in homeostasis, gut-educated IgA-producing plasma cells protect the meningeal venous sinuses upon fungal infection in mice[74].

These data have identifed crosstalk between B cells and the gut microbiota as a new avenue for exploration in MS and potentially other neuroinflammatory disorders. Given that B cell depletion is the most effective therapeutic option currently available for relapsing–remitting MS, the role of the microbiota in regulating B cell function is of immense interest.

Microbial translocation

Originally, interaction between the gut microbiota and the immune system was thought to occur mostly as a result of bacterial products and fragments crossing the gut epithelial barrier and reaching the neighbouring gut-associated lymphoid tissue. However, the use of modern techniques for the sensitive and specific detection of bacteria, including cultures, visualization and sequencing techniques, has produced evidence that translocated, viable bacteria are present in healthy non-mucosal tissues (reviewed in detail elsewhere[75]), challenging the traditional view that healthy mammals are sterile at non-mucosal sites. In addition, translocated commensal bacteria have been detected at extra-intestinal sites in several autoimmune diseases (including systemic lupus erythematosus and type 1 diabetes mellitus) and in tumours (including tumours of the pancreas, breast, bone and brain)[75,76]. Whether live commensal bacteria translocate to lymphoid tissues distal from the gut or to the CNS in MS is currently unknown.

In order for commensal bacteria or their products to translocate to the gut-associated lymphoid tissue or gut-distal sites, they first need to traverse the gut epithelial barrier, which is consequently of crucial importance for pathophysiology and therapy. In EAE mice, intestinal permeability is increased before disease onset, and this

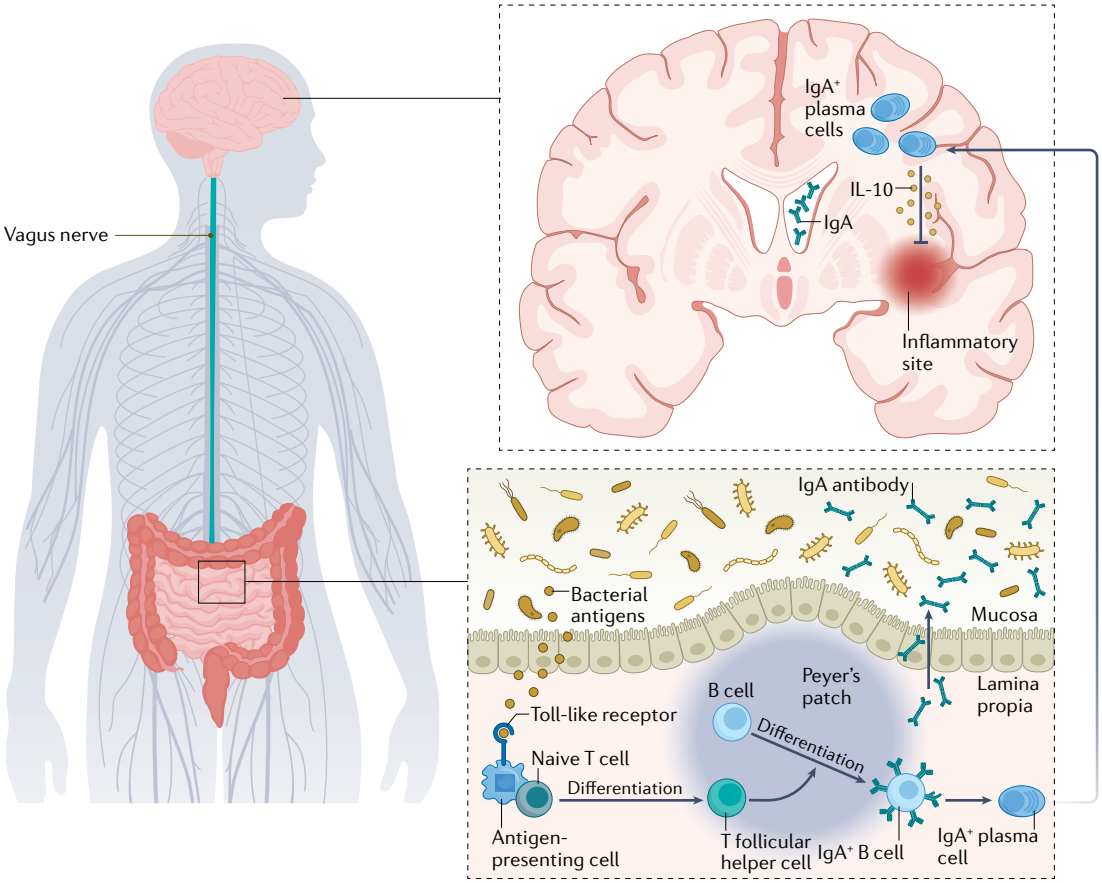

Fig. 2 | **Recirculation of IgA-producing-plasma cells in MS.** Bacterial antigens activate antigen-presenting cells, which lead to differentiation of naive T cells into T follicular helper cells. These cells in turn promote differentiation of B cells into IgA$^+$ B cells and plasma cells that produce IgA antibodies. The plasma cells and antibodies enter the circulation and reduce inflammation in the brain owing to IL10 release by the plasma cells.

permeability increases with disease progression[77]. This disrupted barrier function has been associated with alterations in mucosal structure, including increased crypt depth and the thickness of the jejunum and ileum mucosa, and overexpression of zonulin 1, changes that led to increased pro-inflammatory responses driven by T_H1 and T_H17 cells in the lamina propria, Peyer's patches and mesenteric lymph nodes. Besides the gut-associated lymphoid tissue, gut-distant lymphoid tissues such as lymph nodes and the spleen are sites where translocated live bacteria or bacterial products are likely to interact with the immune system.

Of particular relevance to MS is the question of whether the gut microbiota interacts with the BBB. A growing body of evidence indicates that direct and indirect interactions can occur. Some bacteria can cross the intact BBB through interactions of their cell wall components, such as lipoteichoic acid (LTA), with the brain endothelium[78]. Other bacteria require BBB disruption or engagement of peripheral immune cells that can facilitate their migration across the BBB to enter the CNS[79]. In addition, brain endothelial cells express Toll-like receptors, which means that they can directly respond to the presence of bacterial cell wall components such as lipopolysaccharides and LTA. Lipopolysaccharides and LTA can also induce other cell types to release pro-inflammatory mediators that modulate BBB function[80]. Interestingly, BBB permeability is greater in germ-free mice than in specific-pathogen-free mice. This permeability has been associated with reduced expression of the tight junction proteins occludin and claudin 5, which are known to regulate barrier function in endothelial tissues. Colonization of adult germ-free mice with gut microbiota decreased BBB permeability and upregulated the expression of these tight junction proteins[81]. Overall, these findings indicate that crosstalk between the gut microbiota and the immune system can affect the brain endothelium, with the potential to initiate and/or maintain pathological processes in the CNS.

On the basis of the evidence above, the BBB and the intestinal barrier could be therapeutic targets for MS therapy. Treatment of germ-free mice with SCFAs can suppress intestinal permeability[82] and restore BBB integrity[83], suggesting that SCFAs have potential as therapeutic agents. Butyrate, in particular, is known to have an important role in regulating the integrity of the epithelial barrier by influencing expression of tight junction proteins (increasing expression of claudin 5, claudin 7 and zonulin 1, and decreasing expression of claudin 2), increasing trans-epithelial electrical resistance[84] and altering cytokine and chemokine secretion[82].

The CNS immune system

Alterations to the microbiome in early life might have long-term implications for neuroimmunological disease through developmental effects in the brain. Specifically, the gut microbiota can influence microglial development and maturation[85,86]. Microglia perform canonical functions of myeloid cells, including phagocytosis, antigen presentation, and production of cytokines and reactive oxygen species[87], and constitute the main CNS cell population during a large part of the fetal stage. Experiments conducted in germ-free mice during early embryogenesis and at birth showed that the total count of microglial cells does not differ from that in conventionally colonized control mice, but the maturation of microglia is arrested[85]. Indeed, germ-free mice had higher numbers of immature microglia throughout the grey and white matter of the cortex, corpus callosum, hippocampus, olfactory bulb and cerebellum[85]. This phenotype was associated with impaired CNS immune responses[88]. Interestingly, morphological and gene expression abnormalities observed in microglia in germ-free animals were normalized by the administration of SCFAs, which are primary metabolites of some bacteria[85]. In accordance with these observations, similar alterations are observed in microglia from conventionally colonized mice that are deficient for the SCFA receptor GPR43[85]. Similarly, evidence suggests that alterations in the production of SCFAs by gut bacteria — which can result from insufficient dietary fibre or low levels of bacteria that produce SCFAs — can influence microglia during early postnatal development[89]. Given that microglia are long-lived, early-life influences on their development could lead to long-term modifications that have implications for neurological disease. Whether such effects are important in MS or other neuroinflammatory diseases has not yet been investigated.

The microbiota in MS

Given the involvement of the gut microbiota in immune regulation discussed above, alterations in the microbiota are likely to influence inflammatory disease, including neuroinflammatory disease. A considerable body of evidence from studies in mice and humans indicates that the microbiome is indeed important in the pathogenesis and progression of MS. Consequently, interventions to manipulate the gut microbiota and correct gut dysbiosis have therapeutic potential in MS.

Preclinical evidence

Early indications that bacteria might have a role in CNS autoimmunity came from observations that transgenic mice that expressed a myelin-specific T cell receptor[90] developed spontaneous EAE when housed in a non-sterile facility but not when housed in an specific-pathogen-free environment. At the time, these observations were interpreted as evidence that external pathogenic bacteria could trigger neurological autoimmune disease.

The first evidence that commensal bacteria have a role in neurological autoimmune disease came from studies in which antibiotic treatment was used to reduce the natural gut flora. Antibiotic treatment reduced levels of mesenteric T_H17 cells, resulting in reduced severity of EAE[91]. This effect depended on the presence of a subset of invariant natural killer cells, suggesting that innate immune mechanisms are involved in microbial regulation of CNS autoimmunity. In another pioneering study, oral antibiotic treatment protected against actively induced EAE, indicating that the treatment downregulated pro-inflammatory mechanisms or upregulated anti-inflammatory mechanisms[3]. Subsequent work identified commensal *Bacteroides fragilis* as protective bacteria that exert their beneficial effect by shedding capsular polysaccharide A[10], which activates T_{reg} cells via the Toll-like receptor 2 signalling pathway, thereby suppressing EAE[49,92].

The modulation of actively induced EAE by antibiotic treatment used in these early studies has several limitations. First, active induction of EAE involves immunization of experimental animals with autoantigen plus adjuvant, a procedure that affects the commensal gut flora in unpredictable and non-physiological ways. Second, antibiotic treatment reduces the microbiota incompletely and transiently. Third, antibiotics might have off-target effects that could confound the interpretation of data (reviewed in detail elsewhere[4]). Some of these limitations can be overcome by using germ-free mice, enabling studies of mice with no microbiota or with a defined microbiotic composition (gnotobiotic mice). However, the use of germ-free mice provides the greatest advantage when used in combination with spontaneous autoimmune disease models, which enables investigation of the role of the microbiota in triggering disease (reviewed in detail elsewhere[93]).

The role of the microbiota in triggering neurological autoimmune disease[7] was demonstrated in a pioneering study conducted in mice that expressed a myelin oligodendrocyte glycoprotein-specific T cell receptor on a large proportion of their $CD4^+$ T cells. These mice develop EAE with a very high incidence when housed in a specific-pathogen-free environment, and the symptoms and course of EAE in this model bear a striking resemblance to relapsing–remitting MS in humans. These mice were completely protected from EAE when housed under germ-free conditions. However, when the germ-free mice were colonized with faecal samples from their counterparts housed in specific-pathogen-free conditions, disease susceptibility returned[7]. These observations unequivocally demonstrated that the development of spontaneous autoimmune disease in this model was dependent on the presence of microbiota. Unexpectedly, the disease-triggering effect did not depend on the presence of segmented filamentous bacteria, which are known to promote differentiation of T_H17 cells[94,95] and exacerbate actively induced EAE[9].

Studies in people with MS

The evidence from animal models that the microbiome is involved in neurological autoimmune disease led to studies of the role of the gut microbiota in human MS. It is important to keep in mind when interpreting the results of microbiome studies in humans the limitation that faecal samples mostly include microbes from the intestinal lumen and relatively few from the mucus or epithelium-associated populations, so that some micro-organisms are under-represented or absent.

Table 1 | **Studies that have identified microbiome alterations in multiple sclerosis**

Authors	Number of patients (disease course)	Treatment	Controls	Ethnicity	OTUs or genera altered in MS	Ref.
Cantarel et al.	7 (RRMS)	5 treated (GA) 2 untreated	8	White	Increased: *Akkermansia*, *Faecalibacterium*, *Coprococcus*	167
Miyake et al.	20 (RRMS)	13 treated (IFNβ and/or PSL) 7 untreated	40 ancestry-matched and 10 others	Asian	Increased: *Eggerthella lenta*, *Streptococcus thermophiles/salivarius* Decreased: *Clostridium* spp., *Faecalibacterium prausnitzi*, *Anaerostipes hadrus*	168
Chen et al.	31 (RRMS)	20 treated (IFNβ, NTZ or GA) 11 untreated	36	Not reported	Increased: *Pseudomonas*, *Pedobacter*, *Mycoplana*, *Blautia*	169
Jangi et al.	60 (RRMS)	28 untreated	43	White, Black ($n = 2$)	Increased: *Akkermansia*, *Methanobrevibacter*, *Butyricimonas*, *Paraprevotella*, *Haemophilus*, *Slackia*	45
Tremlett et al.	18 (RRMS)	9 treated (IFNβ, NTZ or GA) 9 untreated	17	White (50%), not white (50%)	Increased: *Bilophila*, *Bifidobacterium*, *Desulfovibrio*, *Christensenellaceae*	170
Cekanaviciute et al.	71 (RRMS)	71 untreated	71 (household)	White	Increased: *Acinetobacter calcoaceticus*, *Akkermansia muciniphila*, *Eggerthella lenta*	8
Berer et al.	22 (RRMS) 7 (SPMS) 3 (CIS) 2 (PPMS)	19 treated (IFNβ, NTZ, GA, or AZT) 15 untreated	34 (monozygotic twins)	White	Increased: *Akkermansia muciniphila*	98
Ling et al.	22 (RRMS)	22 untreated	33	Asian	Decreased: *Faecalibacterium*	171
The iMSMS Consortium	128 (RRMS)	77% treated, 23% untreated	128 (household)	White	Not reported	115

CIS, clinically isolated syndrome; GA, glatiramer acetate; NTZ, natalizumab; OTU, operational taxonomic unit; PPMS, primary progressive MS; PSL, prednisolone; SPMS, secondary progressive MS; RRMS, relapsing-remitting MS.

In addition, the collection of samples can result in the selective elimination of obligatory anaerobic organisms[4,6]. Acquisition of duodenal or colonic samples by biopsy is an attractive strategy for adequate sampling of these populations but ethical concerns prevent widespread sample collection via colonoscopies unless patients strictly require the procedure for diagnostic reasons.

The first generation of studies in humans mostly described differences in microbiotic composition between patients and controls and did not provide mechanistic insight. Furthermore, they usually involved small, heterogeneous cohorts of patients with MS and healthy controls roughly matched for age and sex[96,97] (TABLE 1). These studies found that some bacterial genera, such as *Akkermansia*, *Prevotella* and *Methanobrevibacter* are altered in MS patients, providing the first evidence that components of the human gut microbiota might contribute to CNS-specific autoimmunity. However, no consistent picture emerged from these early studies.

A second generation of studies involved larger and/or better defined cohorts of patients with MS and controls and started to explore mechanistic questions. These studies are exemplified by two complementary studies in which germ-free mice were colonized with faecal microbiota from patients with MS and controls[8,98]. One of these studies involved 34 monozygotic twin pairs who were discordant for MS, meaning that influences of human genetics on the individual microbiome were controlled for. Analysis of the gut microbial composition demonstrated that the overall microbial profiles were similar but some bacterial genera, such as *Akkermansia*, were increased in untreated individuals with MS compared with their healthy twins. When faecal bacteria from participants were introduced into the germ-free transgenic spontaneous EAE model described above, the incidence of spontaneous EAE was significantly higher among mice that received bacteria from the participants with MS than among those that received bacteria from the healthy twins[98].

The other study involved 71 untreated patients with MS and 71 healthy controls[8]. Analysis of the microbiome identified no major shifts in the overall diversity of microbial communities but some bacterial taxonomic groups were significantly associated with MS. In vitro, the MS-associated bacteria *Akkermansia muciniphila* and *Acinetobacter calcoaceticus* induced pro-inflammatory responses in human peripheral blood mononuclear cells and in monocolonized mice. By contrast, *Parabacteroides distasonis* was reduced in people with MS and stimulated anti-inflammatory T cell responses. Finally, faecal transplantation from participants with MS into germ-free mice exacerbated actively induced EAE and reduced levels of IL-10$^+$ T_{reg} cells, whereas transplantation from healthy controls did not[8].

Together, these two studies provided the first functional evidence that the human gut microbiota contribute to CNS-specific autoimmunity. The precise mechanisms are still unknown, but MS-associated

alterations in the microbiome might include a lack of protective bacteria and an overabundance of bacteria that promote disease. Some evidence also suggests that specific microbes can have positive and negative immunomodulatory effects depending on the spatial and temporal context. For example, multiple studies have identified *Akkermansia* as a bacterial genus that is over-represented in the MS-associated microbiome[45,98] and these bacteria can induce pro-inflammatory responses in vitro and in vivo[8]. However, in a different EAE model, *Akkermansia* species seemed to contribute to a protective loop because its abundance was high at the peak of disease and it induced dendritic cells to produce cytokines that stimulated T_{reg} differentiation and thereby ameliorated disease[99]. This study suggested that the increase in *Akkermansia* species was mediated by an increase in levels of the microRNA miR-30 at the peak of clinical symptoms, and oral administration of miR-30 was also associated with the beneficial increase in T_{reg} cells. Furthermore, studies in ulcerative colitis[100], periodontal bone destruction[101], atherosclerosis[102] and liver injury[103] in mice and in obesity[104] in humans have suggested that *Akkermansia* bacteria are protective in these diseases. Together, these data suggest that *Akkermansia* species can interact with the host immune system in seemingly opposite fashions. A complex regulatory network of interactions between different taxonomic groups, their metabolites, the local cellular environment and its cytokine milieu might underlie these observations, but further study is needed to understand the precise mechanisms.

Adding to an already complex scenario, changes in the microbiome in MS can both precede and/or follow disease initiation. Therefore, different stages and/or phenotypes of MS are likely to be associated with different gut microbial communities. Accordingly, studies have also been done to investigate microbiome changes associated with progressive forms of MS, and several associations have emerged. Primary progressive MS has been associated with reductions in *Butyricococcus*, a spore-forming genus that is known to produce SCFAs and is therefore capable of mediating anti-inflammatory effects by inducing T_{reg} cells[105]. In another study, primary progressive MS was associated with increased levels of *Enterobacteriaceae* and *Clostridium*, and decreased levels of *Blautea* and *Agathobaculum*[106]. The same study showed that an increase in *Akkermansia*, which is increased in all forms of MS, was linked to lower clinical disability scores. Finally, metagenomic analyses have revealed that the presence of microbial genes involved in DNA mismatch repair was increased in secondary progressive MS compared with relapsing–remitting MS[107]. Sulphur metabolomics analysis in this study also revealed excessive faecal oxidation, and the combined evidence indicates that DNA oxidation is increased in the gut, which could contribute to chronic neuroinflammation and neurodegeneration in secondary progressive MS[107].

Given the extensive heterogeneity of MS and the enormous complexity of the commensal microbiota, a third generation of much larger and more comprehensive microbiome studies are clearly needed to obtain deeper insights into the role of microbiota in MS. These studies must be carefully controlled to ensure that findings are informative. For example, diet is the most important factor that affects the composition of the gut microbiota, so cases and controls need to be balanced with respect to diet to ensure that any differences in microbiota composition are associated with the disease rather than with differences in diet[108–110]. Besides diet, the gut microbiome composition can be altered by factors such as age[111], sex[112], ethnicity[113], geographical location[113] and exposure to environmental factors (for example, smoking and exercise[114]). The combination of these factors means that associations identified between components of the microbiota and disease are usually too small in magnitude to explain much of the variance observed in phenotype. Large, multi-centre controlled studies are needed to minimize the influence of these factors and identify associations with much more certainty.

In an effort to generate such data, the International Multiple Sclerosis Microbiome Study (iMSMS) was established in 2015 as a global effort to determine the role of gut bacteria and their genes in MS. The iMSMS amalgamates top-tier experts in clinical and translational aspects of MS and in microbiome research. The immediate goal of the iMSMS is the analysis of stool samples from thousands of patients with MS and healthy controls from their households[115].

Therapeutic implications

Given the influence of gut microbiota on immune function and the evidence that alterations to these bacterial communities exist in neuroinflammatory disease, targeted interventions to normalize the gut microbiota hold promise as therapeutic agents in MS. Various approaches are under investigation, as discussed in the following sections.

Diet

Notably, short-term, dramatic dietary interventions have demonstrated the ability to alter microbiota diversity quickly in humans. However, these alterations are transient and do not persist for more than a few days[116]. Long-term dietary patterns and habitual intake play a part in shaping each individual's microbiota profile. However, it is unclear how long a dietary intervention needs to be maintained to achieve a permanent alteration of the microbiota[117]. Various dietary protocols have been proposed to curb the progression of complex diseases, some beneficial effects of which can be related to their effects on the gut microbiota. In MS, several dietary interventions have been proposed that could reduce inflammation and promote clinical improvement; these interventions include a ketogenic diet, a palaeolithic diet (and modified versions) and intermittent fasting, among others.

A ketogenic diet primarily consists of high (55–60%) fats, moderate (30–35%) proteins and very low (0–10%) carbohydrates. Several studies of and three pilot clinical trials of ketogenic diet therapy for multiple sclerosis have suggested that this diet is safe and feasible and could be neuroprotective and disease-modifying[118].

The palaeolithic diet is a modern interpretation of the diet that humans ate during the Palaeolithic era (about 2.5 million years ago). This diet is mostly based on plants,

seafood and insects and excludes grains, dairy products, salt and refined sugar. One study has shown that this diet induced a high degree of biodiversity in the gut microbiota of healthy individuals[119]. The Wahls diet is a modification of the palaeolithic diet that involves high consumption of sulfur-rich and leafy green vegetables, and moderate intake of meat and fish, including organ meats (liver and kidney), and of seaweed, fermented food and nutritional yeast. Grains and dairy are excluded. This diet, together with the Swank protocol — a diet low in saturated fats — was tested as an intervention in relapsing–remitting MS in a randomized, parallel-arm clinical trial[120]. Both diets were associated with marginal but clinically meaningful within-group reductions in fatigue and improvements in quality of life. However, neither diet was associated with significant changes in mobility tests, such as the 6-minute walking test.

The anti-inflammatory diet is based on fruits, vegetables, lean protein, nuts, seeds and healthy fats. A modified version of the anti-inflammatory diet has been investigated in 100 patients with relapsing–remitting MS to investigate its effects on fatigue, quality of life and inflammatory markers[121]. Intervention with the diet was associated with a significant improvement in scores on a modified fatigue impact scale and on physical and mental components of the MS quality-of-life scale. The trial also produced evidence of a modest but statistically significant increase in serum levels of anti-inflammatory IL-4. No significant changes in levels of IL-17 and C reactive protein were detected.

Finally, intermittent fasting involves fasting periods that last longer than overnight and meals are restricted to specific time windows, with or without calorie restriction. Preclinical studies have demonstrated profound effects of intermittent fasting and time-restricted eating on the gut microbiota and on host metabolism, and a limited number of controlled trials in humans have produced similar results[122]. The effects of these microbiota changes in MS are yet to be investigated.

In summary, altering dietary habits and/or the frequency of food intake is a viable, feasible and low-cost intervention with potential benefits in MS. However, dietary interventions are notoriously difficult to enforce and few trials have been conducted; there is a need for larger, rigorous clinical studies.

Probiotics

Replenishing health-promoting bacteria in the gut microbiota by the use of probiotics has been proposed as an intervention to maintain gut integrity and prevent pathological alterations. According to the 2001 Expert Consultation of the Food and Agriculture Organization (FAO) of the United Nations and the World Health Organization, probiotics are "live organisms which when administered in adequate amounts confer a health benefit to the host"[123]. Studies in vitro and in animal models have suggested multiple mechanisms by which probiotics could mediate beneficial effects, including induction of host cell antimicrobial peptides, release of antimicrobial factors from the probiotic bacteria, suppression of immune cell proliferation and enhancement of gut barrier function[124–126].

Evidence from animal models suggests that probiotics can mitigate EAE by promoting IL-10 and TGFβ secretion from immune cells, expansion of T_{reg} cell populations in gut-related lymphoid organs and the CNS, and a concomitant reduction in levels of TNF, IFNγ and IL-17 (REFS.[92,127–130]). Despite these promising preclinical data, no strong evidence has demonstrated a benefit of probiotics in MS or any other disease[131]. In one study of patients with MS, administration of probiotics increased the abundance of several taxonomic groups that are known to be depleted in MS, such as *Lactobacillus* species, and decreased the abundance of others that have previously been associated with MS, including *Akkermansia* and *Blautia* species[132]. However, these findings need to be considered with caution because the number of patients included was small, the follow-up time was short, and the outcome measures were limited.

Lack of certainty about the use of probiotics as a treatment for systemic inflammatory conditions in humans stems from several issues. First, conclusions from many trials of probiotics are based on empirical clinical data but not functional analyses. Second, even though some of the outcomes assessed in these trials reached statistical significance (for example, changes in Expanded Disability Status Scale scores of 0.3), they are not necessarily clinically meaningful. Third, disparate bacterial strains are studied. Finally, unlike animal models, human diets, genetic backgrounds and microbiome compositions are highly heterogeneous, so the degree of colonization by probiotics varies considerably between individuals, leading to different responses to the same intervention[133]. Therefore, although the use of probiotics could have a synergistic effect with current disease-modifying therapies for MS, large-scale randomized and blinded clinical trials are required to determine whether their use is beneficial and feasible.

Short-chain fatty acids and other metabolites

SCFAs are saturated fatty acids with a chain length of one to six carbon atoms. They are the main metabolites produced by commensal gut microbiota from the fermentation of dietary fibre. Acetate, propionate and butyrate are the most abundant SCFAs in the human body and the most important in the colon[134]. In the human gut, *Bacteroidetes* bacteria secrete high levels of acetate and propionate, whereas *Firmicutes* bacteria primarily secrete butyrate[135]. SCFAs are important sources of energy not only for the gut microbiota itself but also for the intestinal epithelial cells. A growing body of evidence indicates that SCFAs have a key role in microbiota–gut–brain crosstalk[136,137].

SCFAs have diverse effects on the host immune system. One mechanism of these effects is inhibition of histone deacetylases (HDACs), which leads to anti-inflammatory effects in macrophages[138] and dendritic cells[139] and to increased production of T_{reg} cells[47]. These effects are critical for maintaining immune homeostasis. In EAE mice, administration of propionate ameliorated the disease course by increasing the numbers of T_{reg} cells[140]. Administration of butyrate to germ-free mice increased the number of T_{reg} cells in the colon lamina propria[50] and induced IL-10 secretion by

dendritic cells and macrophages[50,141]. Differentiation of T_{reg} cells was mediated by increased histone H3 acetylation in the promoter and conserved non-coding sequence regions of the *Foxp3* gene[50,141]. Inhibition of HDACs by SCFAs is concentration-dependent, so the dose–response relationship between SCFAs and HDAC inhibition needs to be established in humans. Besides, the anti-inflammatory effects of SCFAs by inhibiting activation of the nuclear factor kappa-B NFkB) have also been shown[142].

SCFAs can also affect host immunity by acting on G-protein-coupled receptors (GPCRs)[143], specifically GPCR43, GPCR41 and GPCR109A. SCFA-mediated activation of GPCR109A by butyrate and niacin increased the expression of anti-inflammatory molecules by colonic macrophages, and induced differentiation of T_{reg} cells[144]. GPCR-dependent effects of SCFAs have also been observed in the CNS. Studies of germ-free animals with compromised innate immunity owing to microglia-related defects have also demonstrated GPCR-dependent effects of SCFAs in the CNS. Recolonization of these animals with microbiota restored microglial homeostasis, maturation and function, and these effects depended on SCFAs and GPCR43 (REF.[85]). Microbiota depletion with antibiotics severely compromised microglial homeostasis in a similar way to that seen in the germ-free mice[85], reinforcing the finding that SCFAs produced by the microbiota are important for CNS immune function.

Most of the available evidence suggests that SCFAs have beneficial immunomodulatory effects, particularly in neurological disorders, but some evidence suggests that they can also have a detrimental effect. For example, systemically administered SCFAs caused altered T cell responses and tissue inflammation in the renal system in one study[145], although SCFA levels were higher than physiological levels. In another study, propionate increased GPCR43 expression during adipose differentiation and consequently upregulated PPARγ2, suggesting that SCFAs have important physiological roles in adipogenesis[146], which is detrimental because adipose tissue promotes inflammation. In addition, acetate is converted into acetyl-coenzyme A, so increases in acetate levels lead to increased availability of acetyl-coenzyme A in cellular metabolism, which can boost mTOR activation, leading to increased production of pro-inflammatory T_H1, T_H17 and IL-10$^+$ T cells[147,148].

SCFAs are not the only microbiota-derived products that influence systemic immunity. Bacterial metabolism of tryptophan, mediated by the enzyme tryptophanase, generates several metabolites, including tryptamine, indole-3 acetic acid, 3-methylindole, indole-3 aldehyde and indoxyl-3 sulphate[36,149]. These molecules can bind to the ligand-inducible transcription factor AHR, which is expressed by immune cells, epithelial cells and astrocytes[36,150], and trigger a transcriptional response with various immunological consequences[151]. Activation of AHR can promote generation of T_{reg} cells or T_H17 cells depending on the acting ligand and the immune cell microenvironment[152]. On the basis of this knowledge, dietary supplementation with tryptophan metabolites is a possible therapeutic approach. In EAE mice, such dietary supplementation ameliorated the symptoms of EAE[153]. This effect was associated with AHR-mediated repression of the cytotoxic enzyme nitric oxide synthase and the chemokine CCL2 in astrocytes[153]. Similarly, antibiotic suppression of the tryptophanase-positive bacteria *Lactobacillus reuteri* to reduce levels of tryptophan metabolites worsened EAE scores[153]. Future studies that focus on the regulation of the AHR by tryptophan metabolites are likely to shed more light on the potential for therapeutic intervention in this pathway.

One other metabolite that could have therapeutic potential is polysaccharide A derived from human commensal *Bacteroides fragilis*. This molecule promotes production of T_{reg} cells, and its administration ameliorated EAE in mice[92,154] (see subsection 'Effects on T regulatory cells'). Further studies are needed to determine whether its use could be beneficial in MS. Studies to test this compound in humans are ongoing.

Faecal microbiota transplantation

Faecal microbiota transplantation is a procedure in which faecal contents from a healthy donor are introduced into a patient (usually by colonoscopy) after they have received a high dose of broad-spectrum antibiotics. The principle is similar to that of an organ transplantation, with the goal of correcting a dysbiotic state induced by disease. This simple procedure gained traction on the basis of demonstrations that it is effective against *Clostridium difficile* infection — in a randomized clinical trial, faecal microbiota transplantation prevented relapses of *Clostridium difficile* infection in 90% of patients, whereas vancomycin prevented relapses in just 27%[155,156]. Subsequently, faecal microbiota transplantation was considered for use in diseases in which dysbiosis is thought to contribute to pathogenesis[157]. A limited number of studies in humans have been performed or are ongoing, and only animal experiments have been done for some diseases[158]. Large, double-blind, randomized, controlled trials are needed to further elucidate the effects of faecal microbiota transplantation in neurological disorders. Concerns about reproducibility, scalability and safety are likely to be limiting the development of this otherwise promising procedure. For example, heterogeneity in donor faecal material could lead to undesirable variability in the outcomes. In addition, donor material needs to be carefully tested for the presence of known pathogens, although the risk of transmitting a known (or unknown) pathogen cannot be completely eliminated.

A more recently developed approach is the delivery of rationally designed bacterial communities rather than undefined, bulk faecal contents. In principle, these communities would seed colonization of the recipient's gut and restart the 'dialogue' between the microbiota and the immune system. Rational design of such bacterial communities requires the identification of key functions that are missing and the development of a self-sustaining ecosystem of micro-organisms that reinstates these functions, primes further colonization and ultimately restores a healthy microbiome[159]. This approach has the advantage of being more amenable to standard manufacturing practices, thus overcoming the challenges of

reproducibility, safety and scalability posed by faecal microbiota transplantation.

Studies of the human microbiome

As the influence of the gut microbiota on human health becomes more evident, motivation increases to understand how the microbiome could be modified therapeutically and to develop interventions to implement these modifications. However, meticulous study design is needed for this kind of research to ensure that results are accurate and meaningful. First, the questions to be answered must be carefully considered and highly focused — trying to answer too many questions in one study could be a mistake. If the objectives are too broad in scope, the study design could become too complex and/or underpowered for analysis of subgroups. Once the research questions are defined, then the appropriate study design can be selected. The most frequently used study designs in medical microbiome research include cross-sectional studies, case–control studies, longitudinal studies and randomized controlled trials. The first three are observational studies, so no intervention wa used, whereas the last is the most widely used type of interventional study.

For studies of the microbiota, cross-sectional studies can be descriptive or analytical[160]. Descriptive studies are used to investigate the composition of the microbiota in one or more populations, whereas analytical studies explore the association between the microbiota and a specific pathology. Cross-sectional studies are generally used to explore the characteristics of the microbiome and serve as a preliminary step for future research.

Case–control studies and prospective longitudinal studies can be informative but their validity is highly dependent on the nature of the risk factors under investigation. Case–control studies are useful when the contrasts in exposure and relative risks between cases and controls are high, but this might not always be the case for microbiome studies in MS, so large sample sizes are required. Furthermore, the results of case–control studies can be misleading owing to combinations of selection bias, reporting bias and reverse causality[161]. These aspects need to be considered when planning and interpreting case–control studies of the microbiome in MS.

Finally, the purpose of randomized controlled trials is to determine the efficacy of a specific intervention. In this type of study, the control group must be strictly selected. Double-blind, placebo-controlled randomized controlled trials are considered the gold standard for interventional studies[162]. For human gut microbiota studies, parallel or crossover designs can be suitable[163]. Crossover studies have the advantage that each individual serves as their own control, eliminating the possibility of inherent differences in microbial composition or other parameters between treatment groups at baseline. However, exploratory studies might be needed to establish whether modification of a particular taxonomic group of the microbiome persists once intervention has ended and, if so, for how long, as such persistence could result in carry-over effects that need to be considered in crossover study designs[164]. Parallel design studies require larger sample sizes to overcome the effects of interindividual variation in the gut microbiota but they have the benefit of shorter study durations that require less commitment from participants[165].

Conclusions

Many studies in animal models and human disease have demonstrated that alterations in gut microbiome composition can affect CNS physiology and neuroinflammation, not only in MS but also in a wide spectrum of different conditions, including Alzheimer disease, Parkinson disease, stroke, brain injury and neuropsychiatric disorders, such as depression, autism and schizophrenia[166]. Neurological and immunological activity in the CNS can be influenced by microbiota-derived metabolites or by microbiota-derived systemic signals. However, despite growing evidence, considerable gaps remain in our understanding of the exact mechanisms involved in the communication between gut and brain during health and disease. Therefore, more translational and clinical studies are required to determine how alterations in these interactions begin and are sustained over time. Mouse models are essential for obtaining mechanistic insight into the role of the microbiome in CNS diseases, but studies in sufficiently large human cohorts are crucial for the identification of detrimental and beneficial bacteria. For translational and reverse-translational studies, the fundamental differences in physiology and anatomy between the species must be kept in mind, particularly differences in gastrointestinal and immunological physiology and in environmental factors, such as housing conditions and dietary habits.

Longitudinal studies in humans are also needed to determine whether targeting the microbiota is a viable therapeutic strategy. These studies should include characterization of the microbiota and a combination of genomic, proteomic and metabolomic analysis to identify products of the gut microbiota that are involved in disease, the signalling pathways that they affect to regulate host immune functions, and the bacterial metabolites that specifically affect the CNS. These insights will be critical not only for understanding the aetiology of neuroinflammation but also for identifying diagnostic biomarkers and developing novel treatment approaches in which the gut microbiota composition is modulated to restore immune cell homeostasis in immune-mediated CNS diseases.

Several possible therapeutic strategies aimed at altering the gut microbiota have been identified, including probiotics, dietary modifications, faecal microbiota transplantation and supplementation with bacterial metabolites, such as SCFAs. Controlled clinical trials of these approaches are needed to determine whether their effects on the gut microbiota are beneficial in MS. Regardless of the therapeutic strategy being tested, these studies should include large cohorts of carefully phenotyped patients — including characterization of host genetics, dietary habits, medication use and comorbid illness — who are compared with carefully matched individuals without the disease. If studies are designed and conducted well, the results should tell us whether modification of the gut microbiota could be added to the therapeutic toolbox for MS.

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