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INVITED REVIEW

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Multiple sclerosis and the intestine: Chasing the microbial offender

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Summary

Multiple sclerosis (MS) affects more than 2.8 million people worldwide but the distribution is not even. Although over 200 gene variants have been associated with susceptibility, studies of genetically identical monozygotic twin pairs suggest that the genetic make-up is responsible for only about 20%–30% of the risk to develop disease, while the rest is contributed by milieu factors. Recently, a new, unexpected player has entered the ranks of MS-triggering or facilitating elements: the human gut microbiota. In this review, we summarize the present knowledge of microbial effects on formation of a pathogenic autoreactive immune response targeting the distant central nervous system and delineate the approaches, both in people with MS and in MS animal models, which have led to this concept. Finally, we propose that a tight combination of investigations of human patients with studies of suitable animal models is the best strategy to functionally characterize disease-associated microbiota and thereby contribute to deciphering pathogenesis of a complex human disease.

KEYWORDS
CNS autoimmunity, FMT, gnotobiotic mice, gut-brain-axis, microbiota, MS/EAE

1 | INTRODUCTION

The number of people with MS is currently estimated to amount to more than 2.8 million worldwide,[1] an incidence, which per se may not rank MS among the major medical problems of our time. But the importance of the disease rather derives from the immense burden of individual disability, its enormous socioeconomic impact, and the unmet need of curative treatments.[2] The risk of developing MS is unevenly distributed. MS preferentially affects women, develops mostly in young adults, and it displays a marked geographical preference of "Caucasian" ethnicities living in temperate climatic zones. This distribution pattern remains to be fully understood, but twin and family studies provide compelling evidence that both genetic as well as environmental factors together have a causative role.[3]

Anneli Peters and Lisa Ann Gerdes contributed equally to this work.

1.1 | Disease risk

Susceptibility to MS is governed by a composite of more than 200 risk genes, as identified by large genome-wide association studies (GWAS).[4] Most of these gene variants, for example, the most relevant HLADRB1*15:01, are related to the immune system and immune reactivity, but they are by no means exclusive to MS patients. A most recent study revealed that a MS predisposing genetic set-up emerged in a steppe pastoral population due to positive selection driven by pathogenic challenges coinciding with changes in diet, lifestyle and population density, and was distributed predominantly over northern Europe via migration more than 5000 years ago.[5] To date only one risk gene (homozygous carriers of rs10191329 in the DYSF–ZNF638 locus) has been proposed to associate with higher disease activity and progression, with a shortening in the median time to requiring a walking aid, with increased brainstem and cortical pathology in brain tissue, and with brain atrophy on MRI.[4,6]

As a matter of fact, among the many people who share a genetic disposition and live in environments favoring MS, only few actually come down with clinical disease. It is known from studies of monozygotic twin pairs that the genetic predisposition contributes just about 20%–30% to overall disease risk, while the majority of the risk, including the actual trigger, comes from milieu factors, including contributions from life style, previous infections and the surrounding environment.[7] But which then are the actual triggers that spark MS in susceptible individuals?

1.2 | Triggers of MS?

Over time, numerous microbial organisms have been suspected as sparking factors, but an MS-specific infectious organism has never been identified with certainty.[8] This holds true for the strongest candidate, the Epstein–Barr virus (EBV), an ubiquitous herpesvirus: although infection with EBV on its own does not necessarily lead to disease development in all infected people, it seems to be an obligatory prerequisite for those people that do develop MS.[9] Furthermore, in many MS patients EBV triggers a virus-specific T cell response within the central nervous system (CNS),[10] and an expansion of EBV-specific B cells which secrete antibodies that cross-react with a cell adhesion molecule, GlialCAM.[11] Nonetheless, at present it remains open whether these activities trigger or just facilitate the disease.

Much evidence supports an autoimmune pathogenesis of MS.[12] Genetic, neuropathological, therapeutic and experimental model observations seem to harmonize with autoimmunity, but so far, a universal pathognomonic target autoantigen, such as the acetylcholine receptor in myasthenia gravis, has escaped identification. In fact, a number of potential autoantigens have been listed, but most of these labeled only minor subgroups, or turned out to be biomarkers for disorders related to but different from MS.[13] This is the case for aquaporin-4 and myelin oligodendrocyte glycoprotein (MOG), as autoantibodies against these antigens are now used to differentiate neuromyelitis optica spectrum disorder (NMOSD) and MOG-antibody associated disease (MOGAD) from classical MS.[14]

Over the past years, fresh evidence points to an unexpected player in the field: the intestinal microbiota, the world of microbes occupying the digestive tract in health and disease. This review will discuss evidence supporting a role of gut microbes in the pathogenesis of MS, and present approaches to study the underlying mechanisms in clinics and experimental models.

1.3 | MS, gut and gut microbiota

Obviously, MS affects the brain with pathogenic lesions that are distributed throughout the CNS, preferentially in the myelinated white matter, but also in the less myelinated gray matter. These changes are diverse, by their distribution, as well as in their state of inflammatory activity, and they change over the course of disease, from the relapsing–remitting stage to the chronic phase.[15] These lesions unroll within the tightly shielded CNS tissue, inaccessible to most extrinsic signaling. So, how could a remote organ system like the bowel and its associated immune system, the gut associated lymphoid tissue (GALT) (Figure 1), control onset and course of MS?

1.3.1 | MS and the intestine

Conventional microbiology relied on methods like differential culturing of microbial samples, morphology and metabolic markers, which provided limited insights into the complexity of the intestinal microbiomes. This has been radically changed with the advent of molecular genetics, combination of DNA/RNA sequencing and neuroinformatics. Advanced 16S ribosomal RNA (rRNA) sequencing and metagenomic approaches for the first time allowed detailed insights into the stunning diversity and dynamics of human-associated microbial communities.[16] Implicitly it has become clear that the intestine, and especially the intestinal microbiota profoundly impact the pathogenesis of MS. These insights are based both on clinical as well as experimental model studies.

For ages, changes of intestinal function have been familiar both to people with MS as well as to their doctors. Thus, many, if not most patients report problems of their digestive tract, such as constipation or fecal incontinence,[17] which may be the result of changes resident in the CNS, affecting the regulation of the enteric nervous system[18] (Box 1). Conversely, the intestinal tube and its contents have been known to contribute to the risk of developing the disease, or modulating its course and severity. However, most reports suffered from incomplete study designs, hampering unambiguous interpretation.[19]

1.3.2 | Bowel dysfunction

Neurogenic bowel dysfunction manifesting as constipation and fecal incontinence are common especially in MS patients with lesions

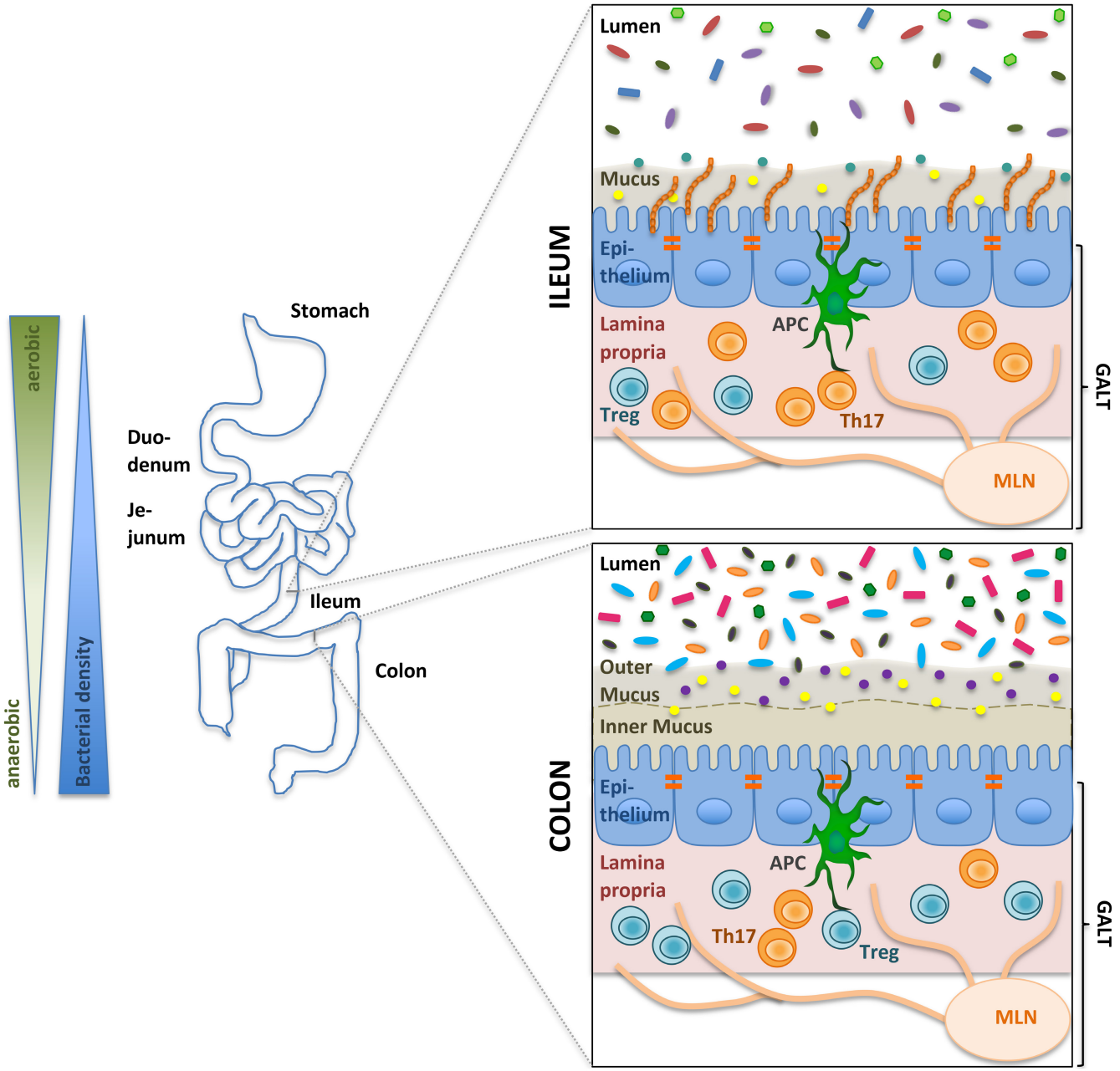

FIGURE 1 Microbiota in different intestinal segments. Composition and density of microbiota change over the entire length of the intestine. The ileum is covered by a relatively thin mucus layer allowing for close contact of the microbiota and the GALT in the underlying Lamina propria and certain microbiota in the ileum have been associated with induction of pro-inflammatory Th17 responses. In contrast, the colon has a much thicker two-layered mucus preventing close contact of the microbiota and the GALT. Instead, the dense and mostly anaerobic microbiota in the colon induce higher frequencies of Tregs for example via production of SCFA. APCs, antigen presenting cells; GALT, gut associated lymphoid tissue; MLN, mesenteric lymph node; Tregs, regulatory T cells.

located in the spinal cord. Bowel symptoms can occur early in the disease course and significantly interfere with the patient's quality of life, social integrity, and daily activities.[23] Two larger studies reported overall a range of 88% (minor) to 12% (moderate–severe) bowel symptoms, and with tools defining presence, absence and severity they reported constipation in 38% and fecal incontinence in 18% of patients.[24–26] Bowel symptoms may wax and wane with an acute MS relapse and may respond to relapse treatment, but are less responding in chronical phases (Box 1).

1.3.3 | Diet

A key report underlining this connection was from Swank et al., who associated MS incidences to different dietary traditions. He noted high disease incidence in areas with high consumption of milk products and lower incidence in fish eating communities.[27] Subsequently, countless dietary protocols were listed for treatment of MS.[28,29]

Indeed, there are direct and indirect ways how diet may affect onset and course of MS disease. Alimentary components may

BOX 1 CNS and its bidirectional connection to the intestine

CNS and gut are distant in the body, but they are tightly interconnected by a diversity of signaling mechanism. The connections are bidirectional. Neurons control the gut function via a local neuronal network, the enteric nervous system (ENS). This is organized in two plexus: one located in the muscularis layer (Auerbach's plexus) controlling intestinal motility, the other one in the submucosal layer (Meissner's plexus) regulating secretion, endocrine functions and vascular activity. These connect to the CNS via the vagus nerve and autonomous networks. In addition, the intestine secretes a broad range of neurotransmitters, most prominently acetylcholine, serotonin, and GABA.[20]

Contrariwise, the gut has several ways to connect to the CNS. It releases hormones by endocrine cells plus microbial metabolic products.[21] Finally, and debatably most directly relevant in our context, the intestine hosts immune cells that pick up local information and then travel to the CNS with these signals to modulate local inflammatory reactivity, and behavior.[22]

stimulate local inflammation and permeability by affecting the diversity of microbiota. They may impact CNS function either directly via soluble metabolites transported via the blood stream, or indirectly by modulating immune cells within the GALT, which reach the CNS through the vascular blood-CNS barriers.[30] Diet impacts onset and progression of MS disease by shaping composition and dynamics of the intestinal microbiome.[31] Diet components trigger the production of bile acids as well as products toxic for fiber fermenting bacteria resulting in reduced diversity of microbiota and dysbiosis, which sets up the stage for intestinal inflammation and permeability. In addition, diet also affects the rate of synthesis of gut microbiome metabolites with potentially immunomodulatory or neuroprotective properties, such as short-chain fatty acids (SCFA), secondary bile acids and neurotransmitters.[32]

1.3.4 | Obesity

Obesity, especially in adolescence, constitutes a major lifetime risk for MS[33,34] and at the same time associates with changes in the gut microbial profile.[35] A worldwide increasing incidence of autoimmune diseases including MS has been recorded in the last decade and might relate to a profound shift from traditional diet habits (vegetables, wheat, rice, high fiber content and legumes, "mediterranean" diet) to modern formulas ("western diet") with heavily processed food with high salt, saturated fats and sugar contents, and a lot of meat. This is well supported by the rising number of MS cases in Japan which parallels with an increase in the prevalence of obesity in the population, which again associates with a replacement of traditional Asian foods with a western diet.[36] In addition, obesity combined with low diet quality appears to worsen clinical MS disability and disease activity on MRI, either through pro-inflammatory properties of fat tissue, a decline in gray matter, or as a consequence of additional comorbidities which themselves impact MS progression.[37]

2 | CLINICAL STUDIES OF MICROBIOTA AND MS

2.1 | Classical population trials

One early report by Miyake et al.[38] used 16S ribosomal RNA (rRNA) sequencing to compare the microbial profiles of fecal samples from people with MS with samples from matched healthy control donors. As a most remarkable observation, spore-forming organisms were reduced in MS-derived samples. These were related to regulatory T cell (Treg) control of allergy and autoimmune disorders.[39,40] In a subsequent trial the group could not observe striking differences related to MS stages, other than increase of DNA repair activity displayed by metagenomic sequencing.[41] Trials in 2016 pointed to discrepant microbial profiles, such as increased *Akkermansia* and archaea in one study,[42] increased *Pseudomonas*, *Mycoplana*, *Haemophilus*, *Blautia*, and *Dorea* genera[43] in a second one, while, thirdly, a trial of pediatric MS showed a trend of increased *Desulfovibrionaceae* versus decreased *Lachnospiraceae*.[44]

As a whole, these harbinger studies yielded perplexing, if not frustrating results, with little consensus between the groups, and with no overriding link between disease and any particular bacterial organism. Many of these studies suffered from various limitations including small sample sizes and analysis of populations with heterogeneous genetic background, different diet and environmental and lifestyle factors.[45] Clearly, this called for fresh trials with refined study designs and technologies to settle the dispute. These should examine cohorts with stronger statistical power and stricter selected and matched MS versus healthy control cohorts.

2.2 | Refined trials-shared household

The field has been spearheaded by a recent trial by the *international Multiple Sclerosis Microbiome Study* (iMSMS) consortium, which screened the microbiomes from 576 people with MS (pwMS) using a household control design to optimally control for various confounding factors and strengthening statistical power.[46] This approach exposed clear associations of the gut microbiome with MS risk, disease course and progression and identified treatment effects. In pwMS major SCFA-producing genera such as *Faecalibacterium prausnitzii* and *Bacteroides* were found to be reduced, in addition, *Prevotella* spp. known to inhibit Th17 activation showed lower levels. On the other hand, the study observed increased proportions of *Eisenbergiella and Akkermansia muciniphilia*,

a bacterium dwelling in the mucin layer and promoting luminal SCFA production. Intriguingly, functional analyses pointed to a double-edged mode of action of *Akkermansia*. Against expectation, the disease associated organism mitigated EAE in mice,[47] a finding that emphasizes the need of corroborating association studies with functional experimentation (vide infra).

2.3 | Refined trials-twin studies

The composition of the mature, healthy gut microbiota is profoundly influenced by the host's genotype independent of specific disease risk.[48] Such potentially confounding effects can be largely eliminated by pairwise comparison of monozygotic twins discordant for MS. Of note however, although monozygotic twins share most of their "hardwired" DNA, they are not absolutely *identical* individuals. They may be distinguished by substantial epigenetic modifications, including methylation of DNA and acetylation of histones, which could affect MS risk,[49] similar to milieu factors like sunlight, infections etc. With these caveats,[50] twin studies have been instructive in various investigations, including for appreciating the relative contributions of genetic vs. environmental effects.[51]

Early work, which explored the role of immune responses in MS twins found a decreased T cell response to measles virus,[52] although anti-viral antibodies were elevated by trend.[52,53] In contrast, T cell reactivity against the putative myelin autoantigen MBP were not elevated in twins with MS.[52] Twin studies of CNS changes using MRI were more consequential. Against intuition, a fore-running small-scale imaging screening of mono- and dizygotic MS discordant twins detected lesions in "some of the unaffected monozygotic twins",[54] a very early description of Radiologically Isolated Syndrome (RIS). RIS turned out to be a fairly common phenomenon, as it has been observed in about 14% of healthy people with familial risk.[55]

2.4 | The Munich MS Twin Study

This German consortium currently oversees a cohort of 101 monozygotic pairs with discordance for MS and serves primarily as a discovery cohort. As an obvious advantage, each twin with MS is matched with a control person who shares the same age, gender and genetic background, and—as household partners up to early adulthood/throughout adolescence—the participants also share a broad range of (early) environmental factors. The MS Twin Study also presents a high-risk cohort since the healthy co-twins carry the highest familial risk and therefore offer the chance to monitor disease evolution from the very beginning, starting with preclinical disease stages up to the clinical disease manifestation. Indeed, within this cohort a sizable proportion (20%) of the clinically healthy co-twins display subclinical evidence of CNS inflammation (subclinical neuroinflammation: SCNI) diagnosed via cerebral MRI and/or CSF analysis (vide supra). This observation opens up new opportunities for addressing important unsolved questions related to the prodromal (clinically non-apparent) stage of MS.

2.4.1 | MS Twin Study and epigenetics

Beyond genetic risk factors, epigenetic modifications co-determine susceptibility to MS. However, epigenome-wide-association studies for MS are potentially influenced by a heterogeneous genetic background noise by genetically unmatched cases and controls. Following up on an earlier report,[56] which detected no epigenomic differences between MS and HD twins, we investigated the epigenome in the MS Twin Study.[57] Ultra-deep sequencing of blood-derived mitochondrial DNA (mtDNA) from 34 twin pairs revealed 25 heteroplasmic variants with potentially pathogenic features in 18 pairs. Our analysis excluded mtDNA variation as a major driver of MS discordance in monozygotic twins, but provided valuable insights into the occurrence of heteroplasmic variants within monozygotic twins and across different tissues. PBMC-based methylomes in 45 MS-discordant monozygotic twins were overall very similar; nevertheless, we identified MS-associated differentially methylated positions including a region in the TMEM232 promoter and ZBTB16 enhancer. Furthermore, we identified epigenetic biomarkers for current interferon-beta treatment, and extensive validation showed that the ZBTB16 differentially methylated position is a signature for prior glucocorticoid treatment. Using the twin design we established an important reference for epigenomic MS studies, identified new candidate epigenetic markers, and highlighted the until then unknown treatment effects and genetic background as major confounders.[57]

2.4.2 | MS Twin Study and lipidomics

The search for a blood biomarker of MS has always been the goal of many researchers since it might provide a better understanding of the pathophysiology and enable disease monitoring. For this aim, we performed quantitative shotgun lipidomics on the plasma of 73 pairs of our MS Twin Study. We analyzed 243 lipid species, evaluated lipid features, and detected phospholipids that were significantly altered in the plasma of co-twins with MS compared to their non-affected siblings. Strikingly, changes were most prominent in ether phosphatidylethanolamines and ether phosphatidylcholines, pointing to the possibility that lipid signaling may be altered in MS. However, whether differences in lipid metabolism affect pathogenesis or are rather a consequence of the disease needs to be further studied.[58]

2.4.3 | MS Twin Study and immunological studies in prodromal MS

The current concepts of MS immune pathogenesis have been shaped by knowledge from EAE models with a strong emphasis on

autoreactive CD4$^+$ T effector cells. Previous studies of the human immune response have often produced ambiguous results. In particular, studying the early phase of the disease process has been challenging in humans, because the diagnosis of definite MS requires the occurrence of a first clinical episode, which is known to be preceded by an undetermined period of subclinical neuroinflammation. It has therefore been very difficult to distinguish between early, potentially primary, and later, potentially secondary, immunological mechanisms. To address the immunological changes during earliest stages of neuroinflammation we compared gene expression patterns of CSF cells from MS-discordant monozygotic twin pairs with signs of subclinical neuroinflammation (SCNI). By single-cell RNA sequencing of 2752 CSF cells, we identified clonally expanded CD8$^+$ T cells, plasmablasts, and, to a lesser extent, CD4$^+$ T cells not only from MS patients, but also from co-twins with SCNI. Clonally expanded T cells showed characteristics of activated tissue-resident memory T (TRM) cells. The TRM-like phenotype was detectable already in cells from SCNI subjects but more pronounced in cells from patients with definite MS. Our data provided evidence for very early concomitant activation of three components of the adaptive immune system in MS, with a notable contribution of clonally expanded TRM-like CD8$^+$ cells.[59]

An independent study aimed to assess MS related peripheral immune signatures in 43 twins of the MS Twin Study and used a systems biology approach covering a broad range of adaptive and innate immune populations on the protein level. Despite disease discordance, the immune signatures of MS-affected and unaffected co-twins were remarkably similar. Twinship alone contributed 55% of the immune variation, whereas MS explained 1%–2% of the immune variance. Notably, distinct traits in CD4$^+$ effector T cell subsets emerged when we focused on a subgroup of twins with SCNI in the clinically healthy co-twin. Early involvement of effector T cell subsets thus points to a key role of T cells in MS disease initiation.[60]

Finally, the influence of genetic predisposition and environmental triggers was explored in a detailed study combining multimodal high-throughput and high-dimensional single-cell technologies in conjunction with data-driven computational tools. This approach allowed identification of an inflammatory shift in a monocyte cluster of twins with MS, coupled with the emergence of a population of IL-2 hyper-responsive transitional naive helper T cells.[61]

2.4.4 | MS Twin Study and microbiome

As discussed above, the complex genetic set-up of humans profoundly complicates attempts to correlate microbial variation with genomic profiles. Thus, in a first study, we compared fecal samples from 34 pairs from the MS Twin Study and analyzed the microbiome composition by 16S rRNA sequencing as well as metagenomics shotgun sequencing. While there were no major differences in the overall microbial profiles, confirming the influence of the host's genetics in the composition of the gut microbiome, there were significant expansions in some taxa such as *Akkermansia* in untreated MS twins ($n=17$), which was also detected in another independent cohort of MS patients.[42,62] However, as described in detail below, transplantation of fecal samples from MS twins into germfree transgenic mice induced a significantly higher incidence of experimental autoimmunity than fecal samples from the healthy twin. These findings provided evidence that MS-derived microbiota contain factors that precipitate an MS-like autoimmune disease in a transgenic mouse model, encouraging further searches for protective and pathogenic microbial components in human MS.[63]

3 | GUT MICROBIOTA AND EAE

So far, most studies have analyzed the microbiome in different patient cohorts, but these datasets remain largely descriptive and offer little mechanistic insight. However, approaches that are more mechanistic are severely limited by ethical and practical constraints. To be able to study the impact of the microbiota on the immune system and identify which changes in the microbial composition may be pathogenic or protective, we need to simulate the human disease in animal models. Unfortunately, complex clinical patterns are rarely represented to completeness by any single experimental paradigm. In the case of human diseases, models commonly replicate isolated segments but not the entire pathological spectrum.[64] This is strikingly the case with MS and its model experimental autoimmune encephalomyelitis (EAE). In fact, the term EAE embraces a set of experimental systems, that all create inflammatory, and sometimes demyelinating changes in the CNS, which go along with specific neurological deficiencies.[65] As a commonality, most EAE variants involve the activation of CNS-specific autoimmune CD4$^+$ T cells, which ignite and drive an inflammatory reaction that culminates in MS-like CNS lesions.[66]

Consequently, the key event in EAE (and in MS as well) involves the activation of CNS-specific autoimmune T cell clones, a process that takes place outside of the CNS target tissue. Importantly, autoreactive T cell clones are components of the healthy mammalian immune system, where they sit in a dormant state, innocuous throughout lifetime.[67] The existing EAE variants differ by their mode of activation, by their target autoantigens, and by their genetic set-up. Classical EAE depends on active immunization with a strong immune adjuvant along with protein autoantigens. This procedure activates previously dormant T cells within the naïve immune repertoire. In transfer EAE models, previously selected CNS-specific autoimmune T cells are activated in vitro and subsequently transferred into experimental hosts. Spontaneous EAE develops in transgenic rodents most of which express a transgenic TCR for CNS autoantigens with or without a second transgene encoding autoreactive B cells. When choosing a model, it is important to remember that each EAE variant has advantages and disadvantages and can only model specific aspects of such a heterogeneous human disease as MS (summarized in Table 1).

Actively induced, passively transferred and spontaneous EAE variants all have contributed to the understanding of the connections between CNS autoimmunity and our microbial commensals.

3.1 | Actively induced EAE (aEAE)

Jules Freund invented the eponymous immune adjuvant (Complete Freund's Adjuvant, CFA), a water–oil immersion combining tissue specific autoantigens with mycobacterial products.[95] He showed that microbial components acting via innate immune responses are required to overwhelm the tolerogenic mechanisms that keep autoreactive T cell clones in dormancy and activate their auto-aggressive potential. This protocol provided the basis of actively induced autoimmune responses against a broad spectrum of tissues and organs, including, besides the CNS, testis, eye, muscle etc.[64] In fact, aEAE variants have been remarkably productive in mapping the autoimmune epitope landscape of the CNS. Apart from the classical CNS autoantigens, myelin basic protein (MBP) and proteolipid protein (PLP), aEAE studies identified MOG, which became a most popular target antigen in experimental autoimmune research,[96] along with a host of additional encephalitogenic proteins including non-myelin components.[65] The CNS-specific T cells are primed in the lymph nodes draining the immunization site and then travel to the CNS to initialize the inflammatory cascade and induce disease. Importantly, aEAE models were the first to link autoimmune CNS responses to the intestine. Microbiota depletion by treatment with a cocktail of broad-spectrum antibiotics ameliorates disease.[68,69] Likewise, also germfree animals immunized with MOG/CFA develop attenuated disease with reduced incidence and milder scores.[71] This indicates that even when the autoantigen/adjuvant is externally provided, the microbiota or microbiota-derived metabolites contribute to activation of autoreactive T cells. A recent study described that administration of vancomycin, which targets gram-positive bacteria, is protective in aEAE, whereas administration of neomycin, which targets primarily gram-negative bacteria, does not attenuate development of aEAE.[70] In contrast, another study described ampicillin, but not vancomycin nor neomycin to be protective in aEAE,[74] suggesting that the effectiveness of specific antibiotics is dependent on the hygienic status and composition of the microbiota prevalent in the individual animal facilities.

Later studies identified bacterial components that affected responsiveness to aEAE. Ileal colonization of germfree C57BL/6 mice with segmented filamentous bacteria (SFB) restored induction of aEAE by activating pro-inflammatory Th17 effector cells.[71] Contrariwise, a colonic organism, *Bacteroides fragilis*, substantially protected PLP-immunized SJL mice from aEAE, via activation of regulatory T cells by its capsular polysaccharide A, which in turn inhibited Th17 cell differentiation in the gut.[77,97] Similarly, Clostridia cluster IV and XIVa were shown to increase the frequency of Tregs in the colon[98] and administration of Clostridia cluster IV and XIVa strains during aEAE ameliorated disease, and was associated with enhanced Treg responses and increased levels of butyrate.[72] Furthermore, the butyrate producing bacterium *Anaerotruncus colihominis* also ameliorated EAE when continuously administered over the course of disease.[70] Another intriguing species is *Akkermansia muciniphila*, which is one of the very few organisms that has consistently been observed to be increased in MS patients across different cohorts. *A. muciniphila* is a mucus-degrading species and therefore has often been suspected to weaken intestinal barrier function. Further, it was shown to promote differentiation of pro-inflammatory Th1 cells.[62] On the other hand, it was shown that the micro RNA miR-30d, which is protective in aEAE, increases *A. muciniphila*,[47] and similarly, protective antibiotics like vancomycin often lead to a bloom of *A. muciniphila*.[70,99] Finally, Cox and colleagues demonstrated that administration of *A. muciniphila* strains derived from MS patients were able to attenuate aEAE with varying potency,[73] suggesting that the increase of *A. muciniphila* in MS patients is part of a counterregulatory rather than a pathogenic mechanism. Lactobacillus species have been suggested to be beneficial in MS, and their therapeutic use as probiotics is being assessed in clinical trials. For example, administration of *Lactobacillus reuteri* in aEAE was associated with milder EAE.[100] In contrast, Montgomery and colleagues showed that *L. reuteri* exacerbates EAE via tryptophane metabolites,[101,102] while another study suggests that *L. reuteri* can activate MOG-specific T cells via mimicry in the presence of a newly identified strain of the *Erysipelotrichaceae* family.[74] This underlines the importance to assess the effect of single microbial species in the context of a complex microbial community.

3.2 | T cell transfer EAE (tEAE)

The actively induced EAE variants provided limited information on the nature of induced CNS inflammation. Indeed, their original designation as Experimental *Allergic* Encephalomyelitis illustrates this. This changed radically, when it turned out that EAE could be transferred by infusing spleen cells sensitized against CNS matter.[103] The encephalitogenic potential of sensitized immune cells was substantially enhanced by in vitro pre-activation with mitogen[104] or the myelin autoantigen MBP.[105] Transfers of subset depleted populations *in vivo*[106] or *in vitro*[107,108] narrowed encephalitogenic effector cells to the T cell compartment. Ultimate proof of autoimmune T cells driving CNS autoimmunity came from Ben-Nun who transferred EAE in rats via in vitro selected MBP-specific T cell lines and clones.[109] This paradigm has become invaluable for several central issues of CNS autoimmunity.

It offers the possibility to characterize and manipulate effector T cells before transfer. This includes culture conditions driving T cell subsets towards Th1 versus Th17 lineages,[110] or genetically introduced markers for in vivo imaging of migratory pathways and in situ activation events. Encephalitogenic T cell lines expressing activation-dependent fluorochromes have been especially valuable revealing the migration patterns and activation events of encephalitogenic T cells from peripheral immune milieus through the blood–brain barrier into the CNS parenchyma.[111] This body of work

TABLE 1 How different EAE models contributed to our understanding of the connection between intestinal microbiota and CNS autoimmunity.

Model	Characteristics	Disadvantages	What has been shown in context with intestinal microbiota (selected studies)
aEAE in C57Bl/6 mice immunized with MOG_{35-55} in CFA + PTX	• Chronic progressive disease with demyelination, axonal damage • Autoreactive T cells are activated from a wild type repertoire • CD4 T cell (Th1, Th17) driven inflammation • Inflammatory macrophages	• No relapses • No spontaneous disease • Provision of exogenous autoantigen with adjuvant precludes spontaneous activation of autoreactive T cells • No involvement of B cells/antibodies	• Abx treatment attenuates disease[68–70] • GF mice develop attenuated disease[71] • Colonization of GF mice with SFB induces ileal Th17 cells and restores susceptibility to EAE[71] • Administration of butyrate-producing bacteria Clostridia cluster IV and XIVa[72] or *A. colihominis*[70] enhances Treg responses and ameliorates EAE • Administration of *A. muciniphila* ameliorates EAE[47,73] • *L. reuteri* can activate MOG-specific T cells via molecular mimicry and in conjunction with *Erysipelotrichaceae* exacerbates EAE in GF mice[74] • Colonization of GF mice with fecal microbiota from MS patients leads to exacerbated EAE compared to recipients of healthy donor microbiota[62] • Part of the CNS-infiltrating T cells were shown to originate from the intestine[75,76]
aEAE in SJL mice immunized with $PLP_{139-151}$ in CFA + PTX	• Relapsing–remitting disease with demyelination, axonal damage • Autoreactive T cells are activated from a wild type repertoire • CD4 T cell (Th1, Th17) driven inflammation • Inflammatory macrophages	• No spontaneous disease • Provision of exogenous autoantigen with adjuvant precludes spontaneous activation of autoreactive T cells • No involvement of B cells/antibodies	• Abx treatment attenuates disease[69] • Colonization of Abx-treated mice with *B. fragilis* induces Tregs/inhibits Th17 cells and protects from EAE[77]
aEAE in human MHC II-transgenic mice immunized with PLP_{91-110} in CFA + PTX	• Autoreactive T cells are selected/activated from a wild type repertoire via human MS-associated MHC II molecules • Chronic progressive disease with demyelination • CD4 T cell (Th1, Th17) driven inflammation	• No spontaneous disease • No relapses • Provision of exogenous autoantigen with adjuvant precludes spontaneous activation of autoreactive T cells • No involvement of B cells/antibodies	• Administration of *P. histicola* ameliorates EAE via induction of Tregs and tolerogenic APCs[94]
sEAE in MBP-specific TCR transgenic mice on B10.PL background	• Spontaneous disease (low incidence)	• No WT repertoire but all T cells have the same TCR	• First observation that hygenic conditions influence incidence of sEAE[81]
sEAE in OSE mice[87,88]: MOG-specific TCR transgenic mice crossed with MOG-specific BCR KI mice on C57Bl/6	• Spontaneous disease (high incidence, early onset) • Chronic progressive disease with demyelination • Lesions primarily in spinal cord and optic nerve • CD4 T cell (Th1, Th17) driven inflammation • MOG-specific B cells act as APCs • High titers of MOG-specific antibodies • Formation of eLFs in the meninges	• No WT repertoire but all T cells have the same TCR and all B cells have the same heavy chain • Lesion pattern and high MOG-specific antibody titers are not typical for MS but rather for MOGAD and NMOSD	• Abx treatment before onset is protective[89]

TABLE 1 (Continued)

Model	Characteristics	Disadvantages	What has been shown in context with intestinal microbiota (selected studies)
sEAE in RR mice[91]: MOG-specific transgenic TCR on SJL background	• Spontaneous disease (high incidence, late onset) • Relapsing–remitting disease course with demyelination • CD4 T cell (Th1, Th17) driven inflammation • MOG-specific B cells are recruited from WT repertoire • High titers of MOG-specific antibodies	• No WT repertoire but all T cells have the same TCR	• GF RR mice are protected from sEAE but develop disease quickly after colonization with microbiota from SPF mice[92] • GF RR mice colonized with fecal microbiota from MS twins develop EAE with higher incidence than recipients of microbiota from healthy twins[63]

Abbreviations: Abx, antibiotics; CFA, complete Freund's adjuvant; eLF, ectopic lymphoid follicles; GF, germfree; PTX, pertussis toxin; SFB, segmented filamentous bacteria; WT, wild type.

revealed that activated T cells do not home directly to the CNS, but first travel through peripheral immune organs, where they undergo profound transcriptional rearrangement, which endows them with a set of adhesion molecules and chemokine receptors required to pass through the layers of the cerebrovascular barriers and to communicate with local antigen presenting cells. The latter interactions take place in the leptomeningeal compartment providing cues that guide the T cells to enter the parenchyma, their ultimate destination.

In both aEAE and tEAE in mice, there is much evidence that encephalitogenic T cells travel through the intestine where they may become reactivated in a microbiota-dependent manner before traveling from there to the CNS. Thus, it was shown that in aEAE part of the MOG-specific T cells in the CNS have their origins in the intestine, as shown by tissue-specific gene expression patterns, and require IL-23 to convert into pathogenic effector T cells.[75,76] Similarly, in Th17 transfer EAE MOG-specific Th17 cells were detected in the colonic lamina propria prior to disease onset, where they proliferated suggesting that they received stimulatory signals.[78] Furthermore, treatment of recipient mice with antibiotics prior to Th17 cell transfer significantly delayed disease onset, indicating that microbiota contribute to activation of encephalitogenic T cells also in murine tEAE.

Very recently, a sophisticated functional imaging approach allowed to follow the interaction of fluorochrome-tagged encephalitogenic T cells with components of the intestinal tube in situ. This approach demonstrated that MOG-specific T cells receive stimulation signals as measured with a Ca^{++}-sensitive activation reporter directly in the ileal lamina propria in a microbiota-dependent and partially also in a MHC II-dependent manner providing formal proof of intestinal activation of encephalitogenic effector T cells.[79]

3.3 | TCR transgenic spontaneous EAE (spEAE)

Actively induced and T cell transfer models of EAE were of limited value in studying the initial stages of unfolding CNS autoimmunity in vivo. To avoid the artificial procedure of immunization with myelin antigen and adjuvant or of activating CNS-specific T cells in vitro, genetic models were developed that carry transgenic autoreactive T cell and sometimes also B cell receptors, which spontaneously led to development of EAE. The factors and mechanisms underlying initiation of CNS autoimmunity were much better represented in these spontaneous EAE variants. In fact, there is now a considerable panel of spontaneous EAE models available, all of them expressing transgenic, CNS-specific TCRs in the immune repertoire. In amazing contrast, transgenic mice with generalized autoimmune responses due to defective regulatory circuits seem to be spared from EAE-like CNS disease.[80]

The first transgenic mouse strain with spontaneous EAE was created by Goverman.[81] These mice were of the B10.PL strain and expressed a TCR specific for an MBP epitope. While injection of pertussis toxin triggered a high rate of EAE, a few animals also developed disease without experimental manipulation. Furthermore, transgenic mice bred on an immunodeficient background showed an EAE incidence of 100%.[82] Remarkably, it was already described in this model that different hygiene conditions affect spontaneous EAE incidence[81] and thus, without knowing this model provided the first hint that environmental factors such as the microbiota may be somehow involved in activation of encephalitogenic T cells. Of interest, not only mouse-derived anti-MBP TCR transgenes mediated spontaneous EAE, but also MBP-specific TCRs from a CD4 T cell isolated from peripheral blood of a person with MS induced development of spontaneous disease when expressed in an immunodeficient mouse.[83,84]

Especially useful are models carrying a transgenic TCR specific for MOG_{35-55} on the C57BL/6 background (2D2 strain),[85] which as a single-transgenic primarily leads to development of optic neuritis but exhibits spontaneous EAE only at very low rates. However, in the opticospinal EAE (OSE) model expressing both the MOG_{35-55}-specific TCR, as well as a MOG-specific BCR derived from the heavy chain of an anti-MOG antibody,[86] the cooperation of both cell types induces a very early onset of spontaneous disease around 4 weeks of age with high incidences of 40%–60%.[87,88] In OSE mice, as well as in another spontaneous EAE model featuring both a human MS-associated MHC-II gene and an MBP-specific TCR, treatment with antibiotics was protective if started before disease onset.[89,90] However, once disease has begun antibiotics did not have a beneficial effect anymore suggesting that the microbiota primarily influences the initial activation of autoreactive lymphocytes in the periphery.

The disease-triggering effect of the intestinal microbiota was even earlier demonstrated in the relapsing remitting (RR) model: this line carries a MOG_{96-105} specific transgenic TCR on the SJL/J background and additionally features recruitment of MOG-specific B cells from the endogenous repertoire. Animals kept under normal (SPF) housing conditions develop spontaneous disease with high incidence at the age of 3–6 months, and some show a relapsing–remitting disease course reminiscent of human disease.[91] Under germ-free housing conditions, RR mice are almost completely protected from spontaneous disease; however, upon colonization with fecal material from SPF mice they start to develop EAE within a few weeks.[92] This line of experiments demonstrated the importance of the intestinal microbiota for the activation of encephalitogenic T cells required for initiating pathogenesis.

4 | A COMPOSITE CLINICAL-EXPERIMENTAL STRATEGY TO IDENTIFY INTESTINAL MICROBES FACILITATING MS

The identification of intestinal microbes causally related to MS is challenging in several respects. Ideally, the human donors should be from optimally recruited cohorts (such as MS discordant twins), then the organisms should be sampled from segments suspected to harbor immune activation, rather than from the feces. Third, the brain autoimmune potential of the samples should be monitored in vivo by a suitable biological system, such as transfers into germfree RR mice (Figure 2).

While the spontaneous EAE models are well suited to study the dynamics of microbiome composition associated with disease development, the ultimate goal should be to identify pathogenic changes in microbial composition occurring in pwMS. To begin to approach this problem, either germfree mice or mice treated with antibiotics can be colonized with human fecal material to generate "humanized" mice. Here, it is important to understand that due to the inherent differences of the hosts only part of the microbial species are transferrable from human to mouse. In addition, different diets also have a huge impact on which species will thrive and which will not within the new murine host. Thus, the humanized microbiome in the mouse does not fully represent the microbiome of the human donor. Nonetheless, this approach enables us to study the influence of the successfully transferred species on the development of disease. With this approach, it was shown that colonization of germfree RR mice with fecal material from MS twins leads to development of EAE with a much higher incidence compared to RR mice colonized with fecal material from healthy twins.[63] Analysis of the microbial composition of the recipient mice showed a significant reduction in *Sutterella* in recipients of MS-twin derived fecal material concomitant with a decrease in IL-10 producing $CD4^+$ T helper cells. A parallel study demonstrated that in aEAE colonization of germfree C57Bl/6 mice with fecal material from pwMS also leads to exacerbated disease compared to mice colonized with fecal material from healthy controls[62] and also showed decreased *Sutterella* and reduced IL-10 producing regulatory T cells in recipients of pwMS-derived fecal material. These studies suggested that the loss of protective species within the microbiome of pwMS may be associated with disease development. One disadvantage of colonization with fecal material is that luminal colonic species are overrepresented. However, especially the mucosa-associated bacteria in the small intestine seem to have the biggest influence and the closest contact to the gut-associated immune system. Thus, it was recently shown that specifically during active disease pwMS show an increase in intestinal Th17 cell frequency as determined via intestinal biopsies and that this is associated with an altered Firmicutes/Bacteroidetes ratio in the mucosa of the small intestine.[93] Therefore, colonization experiments with segment-specific microbial consortia would be advantageous and may help to identify both pathogenic and protective species and thereby enable development of therapeutic approaches in the future. In this context, it was recently shown that administration of *Prevotella histicola* isolated from human small intestine can ameliorate disease in PLP-immunized mice via induction of Tregs and tolerogenic antigen presenting cells.[94]

To obtain representative microbial samples from the small intestine is technically challenging, as it requires invasive procedures and bowel cleansing, which significantly influences the number and composition of microbiota, and also because especially mucosa-associated species are difficult to propagate in vitro. Currently, we are following up on this with a focus on the microbiome of the small intestine since multiple studies point to the microbiome in the terminal ileum as the most important activator of proinflammatory autoreactive immune responses (Figure 1). To study site-specific properties of human microbiota, we enteroscopically obtained microbial samples from different locations (ileum and colon) and niches (luminal and mucosa-associated). To determine the disease-triggering potential of microbial samples and identify pathogenic species we employ our spontaneous EAE mouse model, and colonized germ-free RR mice with ileal microbial material derived from monozygotic twins discordant for MS (Figure 2). Using this experimental set-up our preliminary results show that ileal microbiota from MS twins, but not from healthy twins, trigger EAE development and identified the disease-triggering species as members of the *Lachnospiraceae* family. Although additional experiments are needed to confirm and expand these findings, they support the validity of our experimental approach. We thus believe that continued efforts will finally help us understand the impact of the microbiota in different segments on the immune response driving inflammation in the distant CNS.

5 | CONCLUSIONS

The intestinal microbiota and the gut-associated lymphoid tissue are closely connected, and these complex systems can influence each other directly and indirectly via both contact-dependent

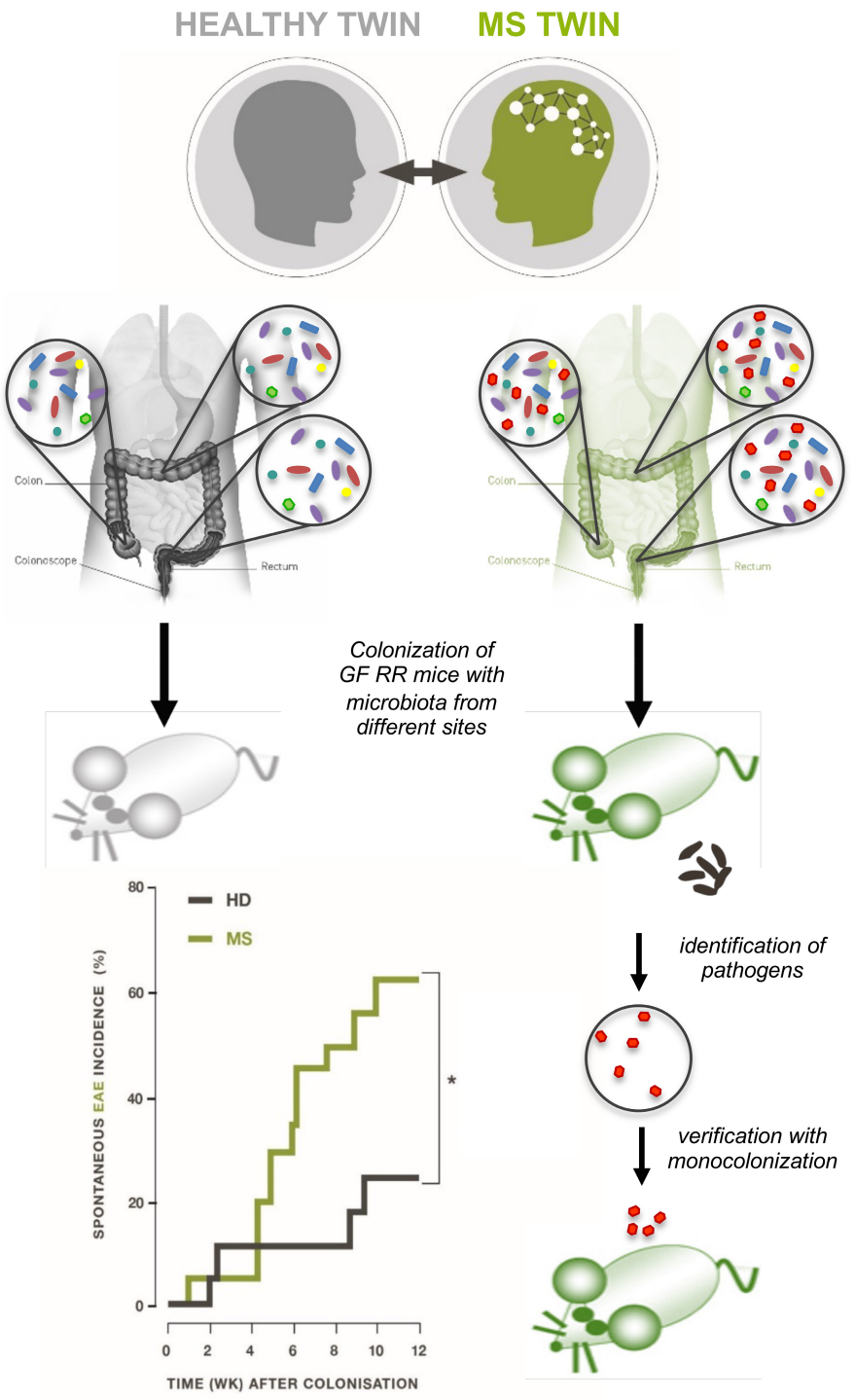

FIGURE 2 Functional characterization of MS-derived microbiota via transfer into spontaneous EAE model. Monozygotic twins with discordance for MS were selected for enteroscopy. After a routine cleansing procedure microbial samples are collected via enteroscopy from different niches, such as luminal (lavage) and mucosa-associated (biopsy) samples, as well as from different gut segments namely terminal ileum and colon as well as fecal samples. Site-specific microbiota are then transferred into transgenic GF RR mice for development of EAE. Microbiota are profiled using 16S rRNA sequencing to assess diversity and richness and to identify potentially pathogenic dominant bacterial species. Additional mono-colonization experiments with isolated bacteria might serve to identify disease-triggering bacteria and to gain insight into the underlying cellular and molecular pathogenic mechanisms induced by these specific bacteria.

mechanisms and soluble factors. Microbiota have emerged as a risk factor for the development of MS and many studies have attempted to identify the "disease-causing bug" by comparing microbiota composition between pwMS and controls. With the exception of *A. muciniphila*, which seems increased in pwMS across cohorts, these studies have not yielded many consistent reproducible results, highlighting the need for noise reduction by using either household controls (reduction of "environmental noise") or monozygotic healthy twin controls (reduction of "genetic noise"). Furthermore, in order to not only describe disease-associated changes, but progress to functional characterization of the altered species, it is required to test their properties and dissect their mechanism of action on the autoreactive immune response via transfer into a suitable animal model such as the RR mouse, where one can study the disease-triggering effect of microbiota. While this approach also has shortcomings due to inherent host differences and should be further refined in future efforts, it is in our opinion currently the best approach to gain mechanistic

insight into the disease-triggering process driven by the intestinal microbiota.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

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