NEURO-IMMUNE CONNECTIONS TO ENABLE REPAIR IN CNS DISORDERS

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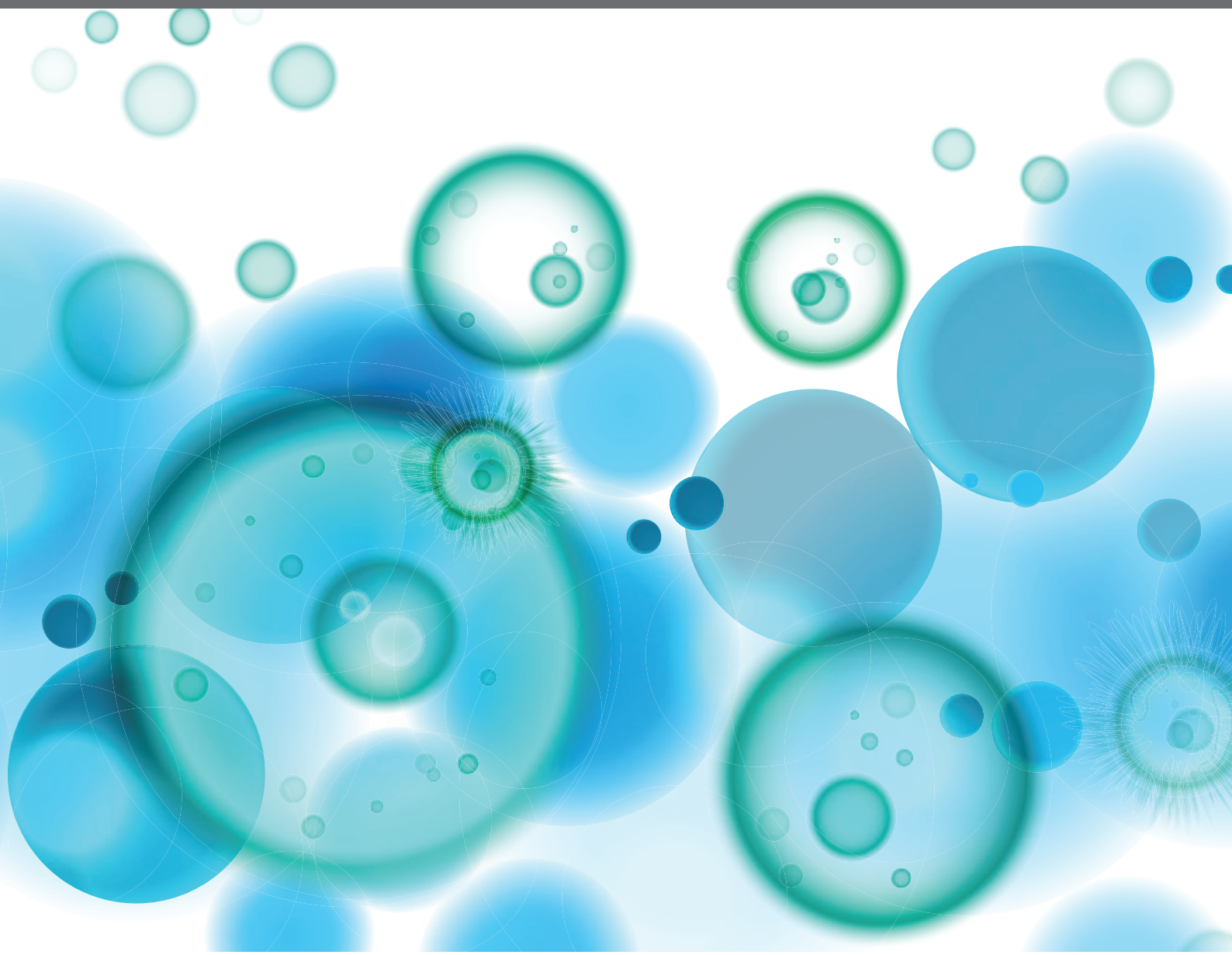

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NEURO-IMMUNE CONNECTIONS TO ENABLE REPAIR IN CNS DISORDERS

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EDITORIAL
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Editorial: Neuro-Immune Connections to Enable Repair in CNS Disorders

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Editorial on the Research Topic

Neuro-Immune Connections to Enable Repair in CNS Disorders

The pathology of neurological disorders, such as multiple sclerosis (MS), Alzheimer's disease (AD), spinal cord injury, and stroke is characterized by immune responses located within the central nervous system (CNS). This close neuro-immune interaction is not only involved in the onset, progression, and clinical manifestation of neurological diseases, but in some cases also plays a protective or even regenerative role. A complex interplay between peripheral immune cells, cells of the neurovascular unit, and CNS resident immune and glial cells steers the destructive, as well as the regenerative capacity of the CNS. An additional layer of immunological complexity is provided by the interaction between the adaptive and innate immune system in both inflammation and repair. Elucidating the underlying mechanisms involved in the neuro-immune interplay, may provide deep insight into novel therapeutic leads for future treatment strategies.

This Research Topic provides a comprehensive overview of neuro-immunological connections that enable vs. hamper CNS repair. High-quality research papers, as well as detailed overview papers have been collected in five different sections.

First, molecular mechanisms involved in peripheral and/or central immune cell functioning related to CNS disorders and repair are addressed. Evans et al. and Yeola et al. describe peripheral T cell-mediated mechanisms in CNS disorders. Evans et al. provide an extensive overview of T cell subsets and mechanisms that are beneficial for neuro-inflammatory and neurodegenerative disorders. They describe recently discovered pro-regenerative T cell-mediated mechanisms in amyotrophic lateral sclerosis, AD, Parkinson's disease, MS, and CNS trauma/injury. Yeola et al. investigated cellular mechanisms in the pathogenesis of experimental autoimmune encephalomyelitis (EAE), the animal model for MS. They studied EAE in the 1C6 NOD model, where T cells have a transgenic T cell receptor (TCR) which is specific for MOG_{35-55}. They found that regulatory T cells (Tregs) are essential for suppression of CNS autoimmunity in this model, and that endogenous TCR rearrangements, *via* recombination-activating gene (RAG) enzymes, are necessary for the development of these Tregs. With regard to central immune mechanisms,

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both Galloway et al. and Gervois and Lambrichts describe microglial functions, with a focus on phagocytosis, in health and disease. Gervois and Lambrichts provide an overview of TREM2- mediated microglial functions, including phagocytosis, migration, survival, and a pro-regenerative phenotype switch, which could be harnessed in the resolution of ischemic stroke. Galloway et al. provide an overview of the mechanisms of phagocytosis in the brain and how it is involved in brain injury and repair. They describe the role of phagocytosis in brain development, homeostasis and aging, and its function in disease states, including acute injury, MS, and AD.

Second, the blood brain barrier (BBB), which is the gateway to the brain, was addressed in this Research Topic. Wouters et al. found that liver X receptor (LXR) alpha, and not LXR-beta, is crucial for maintaining BBB integrity and immune quiescence, in a mouse model for MS. LXRs are ligand-activating transcription factors with important roles in cholesterol and lipid metabolism, but as this report now shows, they are also involved in neuro-inflammatory processes.

Third, the identification of novel molecular leads in the prevention and regeneration of neuro-immunological disorders is an important section of this Research Topic. Not only the identification and classification, but in particular the preclinical validation of potential targets provide key leads for future treatment strategies. In a first study, Sisa et al. show that properdin, a positive regulator of alternative complement activation, is crucially involved in neonatal hypoxia-ischemia induced brain damage. The results indicate that global properdin deletion in two independent mouse models for hypoxic ischemia (HI), reduced forebrain cell death, microglial activation, and tissue loss. The identification of properdin as a mediator of HI, renders properdin an interesting target to prevent HI-induced CNS damage. Next, Schepers et al. provide an elaborative overview on the involvement of second messengers in neuroinflammation and CNS repair. Intracellular second messengers are tightly regulated by phosphodiesterases (PDEs). The unique cell type-specific fingerprint of different PDE isoforms allows a tailor-made treatment strategy. Inhibition of selected PDEs in MS limits inflammation, while inhibition of others stimulates regenerative processes. Kolahdouzan et al. review novel therapies currently in clinical trial, and that are likely to appear in clinical practice in the near future. They focus on compounds that target the immune system and/or enhance endogenous repair mechanisms in the CNS. Although the authors primarily discussed the upcoming treatments in the context of MS, they indicate that most of the strategies can be extrapolated to the treatment of other neuro-inflammatory disorders.

Fourth, the contribution of glia to CNS repair was addressed, with a specific focus on the impact of immune mediators on glial function. Houben et al. provide a detailed overview of the known functions of oncostatin M (OSM), a neuropoietic cytokine, in CNS homeostasis and pathology. Here, they focus on the effects of OSM on neurons, astrocytes, microglia/macrophages and BBB endothelial cells, and discuss the current insights of OSM's involvement in reparative processes seen in murine models of CNS pathology. Lee et al. provide an overview of TNF superfamily reverse signaling in phagocytes of the CNS, both in physiological and pathological circumstances. In addition, they discuss the possibility of targeting these pathways for clinical application. Two reports from Kamermans, Rijnsburger et al. and Kamermans, Verhoeven et al. describe novel pathways showing how astrocytes are involved in MS pathogenesis. First, Kamermans, Verhoeven et al. showed that melanocortin receptor 4 (MC4R) is expressed on astrocytes in active MS lesions, and that activation of astrocytic MC4R ameliorates their reactive phenotype. These data suggest that targeting MC4R on astrocytes might be a novel therapeutic strategy to halt inflammation-associated neurodegeneration in MS. Second, Kamermans, Rijnsburger et al. showed that the expression of angiopoietin-like protein 4 (ANGPTL4), which is an inhibitor of lipoprotein lipase (LPL), is reduced on astrocytes in active MS lesions. ANGPTL4 inhibits uptake of myelin-derived lipids by LPL-expressing phagocytes. These data suggest that the strong reduction in astrocytic ANGPTL4 expression in active demyelinating MS lesions enables phagocytes to adequately clear myelin debris, setting the stage for remyelination.

Fifth and finally, other immunopathogenic mechanisms involved in neurodegeneration were addressed. For this part, four review papers discuss a diverse collection of CNS disorders and immunopathogenic mechanisms. Salani et al. summarize evidence evoking innate immune memory mechanisms in AD, and interpret their potential function, either protective or harmful, in disease progression. Mazón-Cabrera et al. provide an extensive overview of the antibodies described in autism spectrum disorders according to their target antigens, their different origins, and timing of exposure during neurodevelopment. Jin et al. review recent progress in understanding how BDNF influences mood disorders, by participating in alterations of the neuro-immune axis. Wang et al. highlight and discuss how the host microbiome, as a crucial extrinsic factor, influences microglia within the CNS. In addition, they summarize which CNS diseases are associated with host microbiome and microglia alterations and explore potential pathways by which gut bacteria can influence the pathogenesis. He et al. show that microglia mediate the remodeling of rod bipolar cells by phagocytosing postsynaptic materials and inhibiting ectopic neuritogenesis, thus reducing the deterioration of vision in a rat model of retinitis pigmentosa. Wetzels et al. show that advanced glycation end products (AGEs) are increased in MS brain lesions, and specifically expressed in astrocytes. Their receptors, RAGEs, are expressed on brain phagocytes, and together, this system could contribute to MS pathology.

In conclusion, this Research Topic emphasizes the importance of exploiting immunological mechanisms to boost repair in CNS disorders. In depth knowledge of these complex neuro-immune interactions will feed the

pipeline of novel treatment paradigms to efficiently treat a variety of diseases, for which currently no optimal treatment options exist.

AUTHOR CONTRIBUTIONS

TV, JH, NH, and BB edited the Research Topic and wrote the Editorial. All authors contributed to the article and approved the submitted version.

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REVIEW
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The Gut-Microglia Connection: Implications for Central Nervous System Diseases

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The importance of the gut microbiome in central nervous system (CNS) diseases has long been recognized; however, research into this connection is limited, in part, owing to a lack of convincing mechanisms because the brain is a distant target of the gut. Previous studies on the brain revealed that most of the CNS diseases affected by the gut microbiome are closely associated with microglial dysfunction. Microglia, the major CNS-resident macrophages, are crucial for the immune response of the CNS against infection and injury, as well as for brain development and function. However, the current understanding of the mechanisms controlling the maturation and function of microglia is obscure, especially regarding the extrinsic factors affecting microglial function during the developmental process. The gut microflora has been shown to significantly influence microglia from before birth until adulthood, and the metabolites generated by the microbiota regulate the inflammation response mediated by microglia in the CNS; this inspired our hypothesis that microglia act as a critical mediator between the gut microbiome and CNS diseases. Herein, we highlight and discuss current findings that show the influence of host microbiome, as a crucial extrinsic factor, on microglia within the CNS. In addition, we summarize the CNS diseases associated with both the host microbiome and microglia and explore the potential pathways by which the gut bacteria influence the pathogenesis of CNS diseases. Our work is thus a comprehensive theoretical foundation for studies on the gut-microglia connection in the development of CNS diseases; and provides great potential for researchers to target pathways associated with the gut-microglia connection and overcome CNS diseases.

Keywords: brain, central nervous system diseases, gut microbiome, microglia, gut-microglia connection

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BACKGROUND

Microglia—the major brain-resident macrophages—are involved in a myriad of processes as the first line of defense against injury and infections, including brain development, brain function, and immune response, in the central nervous system (CNS) (1). Consistent with these diverse roles, microglial dysfunction has been shown to be involved in the initiation or progression of multiple CNS diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), and even autism spectrum disorder (ASD) and depression (2–7). Despite their critical role, their regulatory dynamics during brain development are poorly understood. Several intrinsic factors, such as the fractalkine receptor CX3C chemokine receptor (CX3CR1), MAF BZIP transcription factor B (MafB), and molecules of the complement system, have been found to control the function of microglia (1, 5, 8, 9). However, our understanding of the mechanisms that regulate the maturation and function of microglia *in vivo* is limited, especially those involving extrinsic signals, such as those from the gut microbiome. Indeed, the mammalian microbiome is a signal that integrates environmental cues.

The gut microbiome has been showed to be a crucial signal for multiple biological processes, especially in maintaining the function of CNS, including brain circuitry, neurophysiology, and behavior (10–12). Indeed, the gut-brain axis is a crucial connection associated with multiple CNS diseases (11, 13, 14). The most comprehensive and typical example of this is the generation of host serotonin. In detail, the gut microbiota has been suggested to regulate the biosynthesis of host serotonin, affecting multiple aspects of host behavior in mice and humans (15–17). Furthermore, the gut microbiota has been shown to regulate the permeability of the blood-brain barrier (BBB) (18, 19). Moreover, a recent study has identified a lifelong close correlation between the brain metabolome and the intestinal microbiome in rats (20). Nevertheless, the detailed mechanisms linking the host microbiome and specific CNS diseases are poorly understood. Gut dysbiosis was identified as a crucial factor in animal models of AD and ASD (11, 13); furthermore, germ-free (GF) mice exhibited an excessive stress response (10, 21). These results suggested that several factors significant for CNS dysfunction may be controlled or generated by the gut microbiome. Indeed, in addition to acting as the regulators of peripheral immune function, studies have revealed that the microbiome controls the maturation and immune response of microglia (1, 12, 22, 23). Given that microglia are crucial for CNS immune response, the prior notion that the brain is "immune-privileged" is losing traction (24).

Notably, most microbiome-associated CNS diseases are closely related to the dysfunction of microglia, including AD, PD, MS, depression, and ASD (11, 23), suggesting that microglia may be a potential mediator that link the host microbiome with CNS diseases; however, confirming this hypothesis requires further studies to be conducted. Depression is a microglia-associated disorder because microglial dysfunction is often observed in the brains of patients suffering from depression (25). In addition, the gut microbiome has been suggested to be associated with depression in rodent models (26–29). Moreover, several specific metabolites, generated by the gut microbiome, control the inflammatory response of astrocytes in the experimental autoimmune encephalomyelitis (EAE) mouse model of multiple sclerosis (MS) by activating microglia (23). Collectively, microglia may be crucial mediators in the interaction between CNS diseases and the gut microbiota.

However, the detailed mechanisms controlling the maturation of microglia *in vitro* and *in vivo* remain uncertain; in particular, the mechanisms through which the microbiome affects microglia are only partially understood. Therefore, a comprehensive investigation of the regulation of signaling between microglia and the microbiome may uncover information with tremendous clinical implications for the treatment of several CNS diseases. In this review, we highlight and discuss the recent findings, showing how the host microbiome acts as a crucial environmental factor to control the function and maturation of microglia from before birth until adulthood. In addition, we describe several CNS diseases associated with both the microbiome and microglia in animal (mainly focused on mice) and humans. Our work, thus, provides a comprehensive theoretical foundation for the role of the gut microbiome in the function of microglia; this will provide a more comprehensive understanding of the role of gut flora, as extrinsic signals, in regulating CNS diseases via modulating the maturation and responses of microglia within the CNS.

MICROGLIA STRONGLY AFFECT CNS ARCHITECTURE AND FUNCTION

Microglia are major players in maintaining the health of the CNS, controlling embryonic wiring, and regulating cell death and survival (5, 30–33). During prenatal development, microglia are the first glial cells to migrate to the CNS, where they participate in the formation of CNS architecture, including neurogenesis, synapse shaping, and excitotoxicity prevention (4), as summarized in **Table 1**.

Macrophages reprogram their functions in response to pathogens, tissue damage, and lymphocyte interactions through polarization (49). As the major brain-resident macrophages, microglia also have the capacity to be polarized to M1-like or M2-like monocytes (4). Notably, phagocytic M2 phenotype microglia are sub-classified into M2a, M2b or M2c in the absence of inflammation (50) and induce a Th2-like response, M1 and M2 represent a spectrum of activation patterns and not separate cell subtypes. In general, M1-polarized microglia exert cytotoxic effects on specific cells *in vitro*, such as neurons and oligodendrocytes, whereas M2-polarized cells promote neurite outgrowth, exhibit phagocytic capacity (51–53), and support CNS remyelination by driving oligodendrocyte differentiation (54). However, overlapping phenotypes that co-expressed M1 and M2 markers were identified in most human inflammatory and neurodegenerative diseases; therefore, there is limited evidence on microglia polarization in these diseases (55, 56). Nevertheless, substantial evidence has demonstrated that microglia play multiple roles in homeostatic and pathological conditions of the CNS, similar

TABLE 1 | Major functions of microglia in maintaining health in the CNS.

Function	Microglia related process	Mediators	References
Neurogenesis	Guiding neurons and axons during the formation of neural circuits in prenatal development	-	(34)
	Integral components in neurogenic niches	CXCL12; CXCR4; ATP	(35, 36)
	Elimination of apoptotic neural stem cells and excess newborn progenitor cells	TAM; Gas6; Protein S	(37, 38)
	Age-associated reduction in neural stem cell proliferation	Proinflammatory cytokines secreted by microglia	(39)
Shaping Synapses	Synaptic pruning or connectivity	C1q; C3; CR3 (Microglia); CX3CR1 (Microglia); CX3CL1	(40, 41) (42, 43)
	Synaptic plasticity	Proinflammatory cytokines; ROS; NO; Neurotrophic factors; BDNF; IL-1β	(44, 45)
	Synaptic transmission	Glutamate; NADPH oxidase; NADPH receptor; PP2A; AMPA receptor;	(46)
	Synapse activity during neuropathic pain transmission	ATP; BDNF; Trk; KCC2 (Cation-chloride cotransporter KCC2)	(47)
Excitotoxicity prevention	Protection against NMDA-induced toxicity	ATP; P2X7; TNF-α	(48)

CXCL12, C-X-C motif chemokine 12; CXCR4, C-X-C chemokine receptor type 4; TAM, Tyro3, Axl, and Mertk receptors; Gas6, Growth arrest–specific 6; C1q, Complement component 1q; C3, Complement component 3; CR3, Complement receptor 3; CX3CR1, CX3C chemokine receptor 1; CX3CL1, C-X3-C motif chemokine ligand 1; PP2A, Protein phosphatase 2A; AMPA,α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; BDNF, Brain-derived neurotrophic factor; Trk, Neurotrophic receptor tyrosine kinase; P2X7, P2X purinoceptor 7; TNF-α, Tumor necrosis factor alpha.

to other tissue-resident macrophages (4). Besides, microglia are the first line of defense against extrinsic microbial infection and injury in the nervous system (22, 57). To perform this role, microglia continuously probe the peripheral environment using a series of receptors and signaling molecules collectively known as sensome genes, which are primarily expressed by the so-called "resting" microglia with high motility (58, 59). Once injury signals are sensed, resting microglia are activated and quickly move toward the indicated sites (60, 61). The phenotypes of these reactive microglia are different, depending on the specific signal and action of the modulators that activated the microglia (62). For instance, when the CNS was infected with the herpes simplex virus (HSV)-1, microglia were identified as the major source of HSV-1-induced chemokine production, including that of CXC ligand 10 (CXCL10), CC ligand 2 (CCL2), and CXCL9, all of which led to peripheral immune cell infiltration into the brain (63). In contrast, HSV-1 infection in CNS led to prolonged activation of microglial cells and retention of T lymphocyte, suggesting that microglia activation contributes to the neuropathological sequelae observed in herpes encephalitis patients (64). However, this does not mean that microglial activation is always beneficial for maintaining the health of the host CNS (65). Indeed, mounting evidence indicated that microglial overactivation contributes to neuronal damage in neurodegenerative diseases. Particularly, reactive oxygen species (ROS), generated from overactivated microglia in response to certain environmental toxins and endogenous proteins, causes neurotoxicity (65, 66).

Given the dual and critical roles of microglia in the CNS, the regulatory mechanisms of microglia are now a major research focus. Microglia originate from hematopoietic stem cells in the yolk sac, and are the first neurons generated at approximately embryonic day 9.5 (E9.5) in mice (9); they then expand and self-renew during adulthood and differentiate to play multiple crucial functions in the CNS (4, 9, 32, 67). The detailed transcriptional program controlling the differentiation process remains unclear, although several factors showed a potential role in their differentiation. *Spi1* (encoding PU.1), *Csf1r*, and interferon regulatory factor 8 (*IRF8*) are the primary microglial transcription and survival genes, all of which are essential for the development of microglia from erythromyeloid progenitors in the yolk sac in mice (8, 9, 68).

GUT MICROBIOTA AFFECTS PRENATAL AND ADULT MICROGLIA

Our current *in vitro* and *in vivo* knowledge of the mechanisms controlling microglia is limited. There is a critical gap in our understanding of the environmental factors that control the maturation and function of microglia *in vivo*. The gut microbiota is most associated with extrinsic signals, such as life style, regional strain pools, and has gained considerable attention from researchers, because the human intestine contains a wide range of microbial cells with major roles in multiple host biological processes, including food digestion and pathogen invasion blocking, to maintain general good health (10–12). Moreover, the gut flora influences multiple peripheral immune cells (69) and modulates hematopoietic stem cells and myeloid precursors in the bone marrow to maintain systemic populations of neutrophils in circulation (70). Bone marrow-derived splenic macrophage and monocyte populations were also found to be reduced in GF mice compared with controls (71). Of note, the microbiome appears to play a role in CNS diseases, along with microglia, as discussed in the following section, suggesting a

potential association between microbiota and microglia in the development of CNS diseases.

Indeed, the host gut microbiota has been suggested to control the maturation and function of microglia (1, 12, 22, 72). An experimental demonstration of the association between microglia and the microbiome was first performed by Erny et al. (22), who were inspired by numerous studies on the interactions between the CNS and the gastrointestinal system. In their study, GF mice displayed significant microglial defects, with altered cell proportions and an immature phenotype, including more segments, longer processes, and greater numbers of branching and terminal points. This defective microglia phenotype was also observed in other mice with altered microbiota, including the altered Schaedler flora (ASF) mice, which harbored only three bacterial strains (73), and the acute microbiome-depleted mice, following treatment with short-term broad-spectrum antibiotics. Collectively, microglia in conventionally colonized mice would require continuous input from the gut microbiome; this input appeared to be associated with bacterial complexity. In that study (22), treatment with antibiotics failed to increase the number of microglia, in contrast with the effect observed in adult GF mice. Moreover, the expression of *Ddit4*, a regulator of cell growth, proliferation, and survival, was markedly elevated in the microglia of adult GF mice; in contrast, *Ddit4* expression was unaffected in antibiotic-treated mice **(Table 2)**. Several reasons may contribute to the different effect of altering host microbiota between antibiotics-treated mice and GF mice. Firstly, microglia originate from the yolk sac, and their proliferation in adult mice was observed solely in brain-resident microglia (9). The GF mice lacked gut microbiota since birth; it is possible that the host microbiome influences prenatal microglia formation, thus contributing to the emergence of defective microglia in adult mice, which has already been confirmed in a later study (12). Secondly, the antibiotics utilized in the study (22) may have directly targeted microglia in the brain in a microbiota-independent manner. Indeed, several antibiotics, such as minocycline, have been shown to cross the BBB and regulate the activity of microglia, independent of the abundance of the microbiota (75). Furthermore, topical application of aminoglycoside antibiotics has been shown to enhance the host's innate immune response against viral infections in a microbiota-independent manner (76). Of the antibiotics used in that study (22), only metronidazole was able to cross the BBB (77). Therefore, further research is required to test whether treatment with metronidazole only can affect microglial function independent of the abundance of the gut microbiome. Thirdly, Iba1 was selected as a specific indicator of microglia in their study (22); however, it is not an ideal indicator for microglia because multiple myeloid cells are known to express Iba1, including perivascular cells, choroid plexus macrophages, and meningeal macrophages (78), thus, a more specific marker, such as P2Y12, is required to label microglia to analyze their abundance and morphology (79). Therefore, the observed reduction in Iba-1^+ parenchymal microglia in the CNS of GF mice, which was not observed in the antibiotic-treated mice, may have been due to the label of Iba1 in other myeloid cells within the brain.

TABLE 2 | Dominant microglial factors in GF and ABX-treated mice at 6–10 weeks of age.

Microglia-associated factors	Function of indicated factor (References)	GF	ABX
Spi1 (encodes PU.1)	Central microglial transcription and survival factor (68)	↑	-
Ddit4	Activation of cell proliferation (74)	↑	→
Csf1r	Central microglial transcription and survival factor (8)	↑	-
CSF1R	Surface factors of microglia downregulated during maturation (22)	↑	-
F4/80	As above (22)	↑	↑
CD31	As above (22)	↑	→

Ddit4, DNA damage-inducible transcript 4; Spi1, Spleen focus forming virus (SFFV) proviral integration oncogene; Csf1r, Colony stimulating factor 1 receptor; F4/80, adhesion G protein-coupled receptor E1 (Aliases); CD31, Platelet/endothelial cell adhesion molecule 1 (Aliases); GF, germ-free; ABX, antibiotics; ↑ means upregulation, → means unaltered, and – means uncertain.

Moreover, the consequence of defective microglia on viral genes expression and virus loading in the CNS of GF mice or antibiotic-treated mice was not determined as the researchers only assessed the immune response of microglia under GF conditions by analyzing the levels of several immune response genes after exposing the animals to either bacteria or lymphocytic viruses (22). However, microglia are the first line of defense against extrinsic microbial infection in the CNS; thus, the final functional consequences of microglial malformation and immaturity under GF conditions or after antibiotic treatment require further study.

Furthermore, the detailed mechanism underlying the regulation of microglia by the gut microbiota requires further research. Although microglia express many pattern-recognition receptors that recognize microbial-associated molecular patterns (MAMPs), no changes in microglia morphology and maturation were found in the absence of several MAMPs, which were recognized by toll-like receptor 3 (TLR3), TLR7, and TLR9 (22), suggesting that commensal microbes may regulate the function of microglia in a MAMP-independent manner. Finally, it has been indicated that short-chain fatty acids (SCFAs) produced by the microbiota through the catabolism of complex carbohydrates (22, 80), are involved in the regulation of microglia maturation (12). Given that GF mice exhibited an increased permeability of the BBB (18) and that SCFAs have been shown to cross the BBB (81, 82), it was not surprising that SCFA administration largely restored microglial activity under GF conditions (**Figure 1**). Current receptors known to recognize SCFAs, including FFAR2 [also called G protein-coupled receptor (GPR) 43], FFAR3 (also called GPR 41), and GPR109A, are expressed by multiple immune cells and intestinal epithelial cells (80). However, FFAR2 is not expressed in any adult brain cell, although the microglia of mice lacking FFAR2 exhibited defects and the receptors were not essential for SCFA to enter the CNS (83–85). Of note, microglia activity can be modulated by receptors of proinflammatory and anti-inflammatory cytokines in the CNS

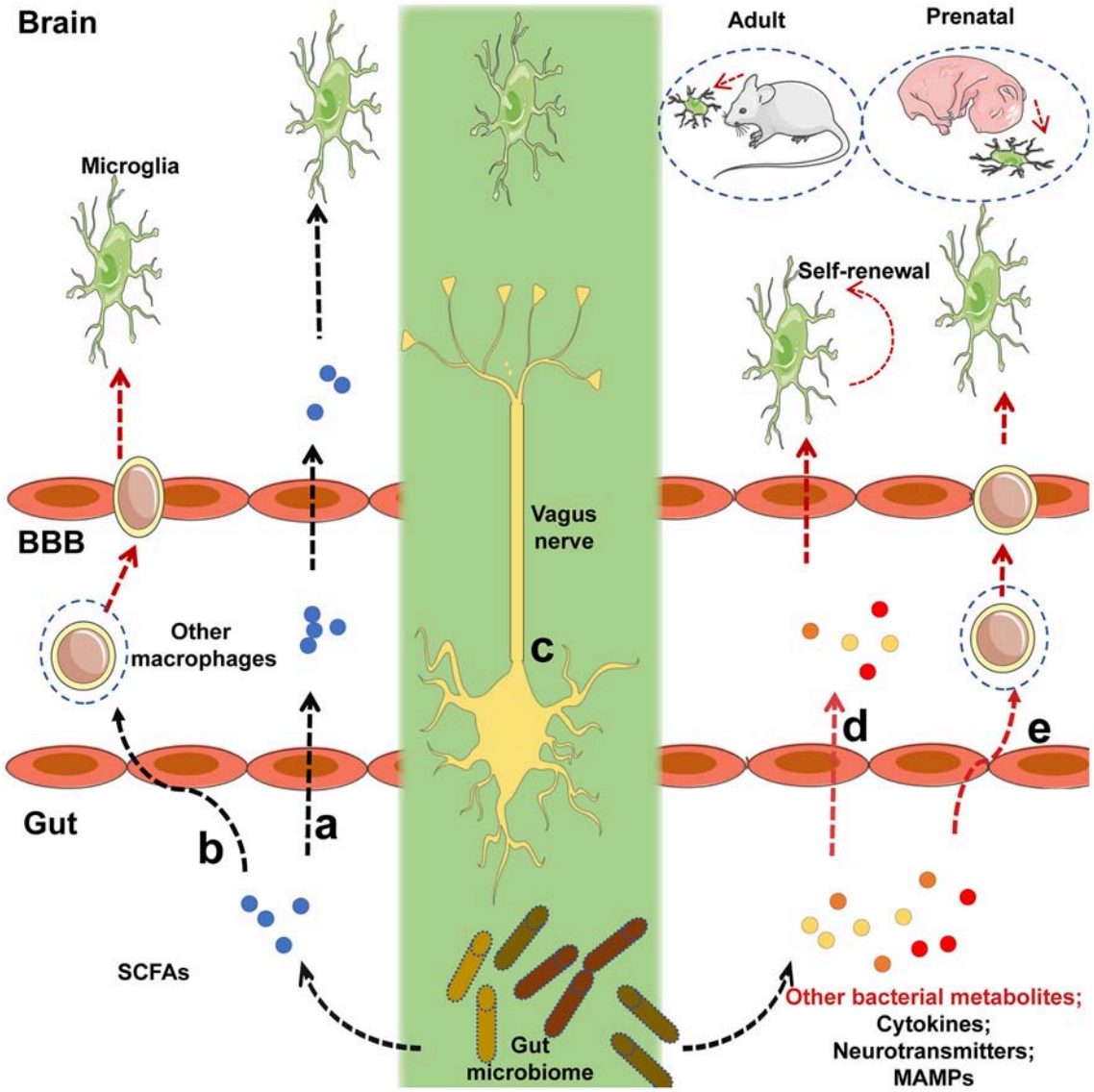

FIGURE 1 | Potential mechanisms by which intestinal microbiota regulate the maturation and function of microglia. **(a)** Short-chain fatty acids (SCFAs) generated by the gut microbiota cross the blood-brain barrier (BBB) via the circulatory system of the host, and target microglia to regulate their function or maturation. **(b)** Immune cells expressing receptors that recognize SCFAs can migrate to the brain via the BBB after signaling by SCFAs that originate from the gut flora. **(c)** The gut microbiota may communicate directly with brain-resident microglia via the vagus nerve. **(d)** Before receptors recognizing SCFAs are expressed, other bacterial metabolites or microbe-associated molecular patterns (MAMPs) generated by the gut microbiota can cross the BBB and target microglia to regulate their function or maturation. **(e)** Peripheral macrophages that can recognize the relevant metabolites or MAMPs can migrate to the brain via the BBB after receiving signals from bacterial metabolites or MAMPs released by the gut flora. Black lines indicated that the corresponding pathways were recognized in a prior study, and red lines represented uncertain pathway. Black lines represent known pathways and red lines indicated uncertain pathways.

and in circulation; SCFAs are crucial regulators of nuclear factor-κB (NF-κB) activity and proinflammatory innate immune responses (80, 86). Therefore, in the absence of a receptor expressed on the microglia, that can recognize SCFAs, the possibility that other metabolites from the gut microbiota directly regulate microglia cannot be excluded. SCFAs may control the activity of microglia through other peripheral myeloid cells that express FFAR2, recognize signals from SCFAs, and migrate to the brain or secrete specific factors that can cross the BBB to regulate microglia within the CNS (**Figure 1**). Propionate, a major SCFA, exhibited protective effects on the BBB by recognizing FFAR3 on the surface of endothelial cells in a recent study (19), indirectly supporting the notion that SCFAs, as gut-derived microbial metabolites, may be crucial mediators in the gut-brain connection. Other bacterial metabolites or MAMPs generated by the gut microbiota may cross the BBB and directly regulate microglial function (**Figure 1**), especially when the receptors that recognize SCFAs are not yet expressed, given

that microbiota may control the microglia before birth (12). Peripheral macrophages that can recognize target metabolites or MAMPs can cross the BBB and migrate to the brain after receiving signals such as bacterial metabolites or MAMPs generated by gut flora (**Figure 1**). The gut can connect directly to the CNS through the vagus nerve, and the modulation of microglia through external vagus nerve stimulation was found in a murine model of AD (87, 88); the role this link plays in the gut-microglia connection remains uncertain (**Figure 1**). Further study is warranted to determine the signaling pathways of the gut microbiome that ensure the maturation and function of microglia. Despite these unresolved questions, this study provided evidence that the gut microbiome is closely associated with the maturation of microglia, and that SCFAs are crucial mediators of the association between the gut microbiome and microglia.

In addition to affecting the microglia of adult mice, the maternal microbiome has emerged as a significant regulator of prenatal microglia in a sex-specific manner (12); this is not surprising as the male and female microglia behave differently during the embryonic phase. Under normal conditions, for pain perception, microglia in rodents exhibited sexually dimorphic properties and showed differences in colonization rates in males and females (89–92). Based on data from RNA-sequencing (RNA-seq), the increased expression of genes in microglia from E18.5, and in adult female mice, was associated with the apoptotic process, response to lipopolysaccharides (LPS), and inflammatory response (12, 92). This suggests that microglia were in a more immune-activated state in females; this is in line with a previous study, illustrating that females showed stronger innate and adaptive immune responses (93). In that study (12), the microglia from GF mice exhibited increased ramification and production during the embryonic stages, which is based on the fact that no other differences were observed in the development of embryos from GF mice and specific-pathogen-free (SPF) controls. Under GF conditions, the microglia of male mice were affected more strongly *in utero*; in the adult phase, the host microbiota exhibited a more profound effect on the microglia of female mice. Transcriptome data indicated that 1,216 differentially expressed genes (DEGs) were observed in E18.5 male microglia between control and GF embryos, while only 20 DEGs were found in female microglia at the same phase. These DEGs included immune response-associated genes, such as *Ly86* and *Aoah*, which are involved in the response to LPS, raising the possibility that maternal microbiota primes the microglia for their response to postnatal challenges. In contrast, the variation in DEGs was reversed between the sexes in adults; the number of DEGs in adult female microglia between GF and control mice was 433, but only 26 DEGs were observed in adult male microglia. Acute microbiome perturbation with antibiotic administration produced a reversed effect between the sexes in the microglia of adult mice; this effect was distinct from that in the control and GF mice (12). More DEGs were observed between the microglia of SPF and antibiotic-treated mice in males than in females. In addition, there was no significant difference in the density and morphology of microglia from antibiotic-treated and control adult mice (P60, where P0 is the day of birth). Confronted with the different effects of treatment with antibiotics and GF conditions on the microglia, Thion et al. thought that the primary effect of gut flora on microglia may have been caused by the long-term absence of the microbiota, i.e., under GF conditions. Of note, specific antibiotics utilized to clear commensal microbiota affected the microglia directly in a microbiota-independent manner; for example, minocycline is known to directly regulate microglia (75). However, the study (12) did not include detailed information regarding the antibiotics used; thus, we cannot exclude the possibility that this direct function of the antibiotics on microglia probably diminished the effect mediated by the absence of microbiota. Additionally, given that their RNA-seq data displayed several DEGs between different sexes that were dominantly present on the X and Y chromosomes, sex chromosome-associated genes may have interfered with the effects of the antibiotics on microglia, contributing to the differences between the antibiotic-treated mice and GF mice. Of note, consistent with the differences between GF and control adult mice, the DEGs in antibiotic-treated female adult mice were primarily associated with the regulation of transcription. However, DEGs in antibiotic-treated male adult mice were mainly associated with the immune response, which was distinct from GF adult male mice. Moreover, that study (12) failed to determine the specific bacterial cluster or clusters that affect microglia in the brain. It can be difficult to screen one bacterial species, given that seeding a specific species of bacteria into the host may greatly alter the composition of the host microbiota but not sole change the number of specific bacteria. However, it seems that the microbiome is unlikely to directly affect prenatal microglia, as most evidence has indicated that fetuses live in an environment devoid of any bacteria before birth, although there is a hypothesis that the first bacteria in the microbiota take root before birth (94). This indicated that indirect signaling may have occurred from intrauterine microorganisms or microbiota-generated factors from the mother via the umbilical cord during the later phases of embryonic development.

Taken together, the gut microbiota does regulate the function of microglia although the comprehensive mechanism remains uncertain. Of note, several studies revealed the potential mechanism by which the gut microbiome controls the function of microglia. Serotonin (5-hydroxytryptamine, 5-HT) is known as an important neurotransmitter in the brain and a vital factor in neurogenesis; it is primarily derived (about 90%) from the brain and the gastrointestinal system (95). Indigenous bacteria within the gut produce metabolites that signal enterochromaffin cells, which are a major producer of 5-HT in the digestive tract (16, 17). Until recently, the BBB was generally thought to be impermeable to 5-HT; however, new evidence has shown that 5-HT might cross the endothelial cells of the BBB using a serotonin transporter (15, 96). Moreover, 5-HT regulates microglial development via 5-HT2B receptors (97); therefore, it is possible that the gut microbiota affects the brain microglia via 5-HT. The gut microbiome is closely related to the maturation of microglia; several bacterial metabolites such as SCFAs may act as crucial mediators. These findings reinforce the view that the gut flora has a wide range of effects, especially on the immune

response mediated by microglia in the CNS; this is supported by a study showing that differences in the microbiota composition of wild and laboratory mice modified their immune responses (98).

THE GUT MICROBIOME AND MICROGLIA AFFECT SEVERAL CNS DISEASES

Given the importance of the microglia in the CNS, it is not surprising that microglial dysfunction results in neurodevelopmental, neurodegenerative, and neuroinflammatory diseases such as AD, PD, and MS (2–5, 88). A potential linker in this association is nitric oxide (NO) that can be generated by activated microglia (99). Indeed, during neuropathogenesis, microglia enter a hyperactive state in which they exacerbate the progression of neuropathogenesis by generating a series of factors including induced nitric oxide synthase (iNOS), chemokines, and cytokines (6). Notably, aberrant NO pathways have been implicated in several neurological disorders, including AD and PD (6). Furthermore, several microglia-associated genes are related to neurological and neuropsychiatric disorders in humans and mice, including CD33 and CXC3R1 (100–102). Commensal bacteria have emerged as a crucial factor in controlling the function and maturation of microglia; this role is mediated by the generation of SCFAs to a certain extent, as mentioned above (12, 22, 57). The host microbiota has also been found to be a critical regulator of neurogenesis, neurophysiology, myelination, and exploratory behavior (11, 81, 103, 104). The microbiome or its disruption has been shown to contribute to CNS diseases such as ASD, AD, MS, and PD, all of which are also affected by host microglia, as described below (11). Therefore, microglia that lie at the interface between environmental signals and the brain may be the critical link between the microbiome and CNS diseases.

AD

AD is a common cognitive degenerative disease characterized by the accumulation of phosphorylated tau/tangles and amyloid plaques consisting of amyloid β (Aβ), a cleavage product of amyloid precursor protein (APP) (105, 106). Microglia are known to be related with AD and are majorly involved in the alteration of the levels of many AD-associated factors. Specifically, the phagocytic ability of microglia against Aβ aggregates can be enhanced by apolipoprotein E (ApoE), whose polymorphism has been suggested to be closely related to sporadic AD (107, 108). ApoE can be produced by microglia to some extent through regulation by peroxisome proliferator-activated receptor γ (PPARγ) and liver X receptor (LXR) (108). Both PPARγ and LXR can form heterodimers with PXR, and agonists of PPARγ and PXR can reverse some neuropathological changes and restore cognitive ability, as well as shrink the Aβ plaque burden, which depends upon ApoE and microglia (4, 108–110). Although soluble Aβ peptides and small amounts of seeded Aβ plaques are cleared by microglia under normal conditions (as mentioned above), microglia are incapable of removing excessive Aβ aggregates that chronically activate and damage both neurons and microglia during the progression of pathological Aβ accumulation (4). Triggering receptor expressed on myeloid cells 2 (TREM2), a promoter for the activation of microglia (M2-like) and phagocytosis, is also a risk factor for late-onset of AD (111). In a study identifying new susceptibility loci for AD, the microglia-associated genes *CD33* and *ABCA7* were identified as two novel susceptibility genes (100, 101).

The gut microbiome is also closely related to AD in mice model and humans, although the detailed regulatory mechanisms surrounding this association require further research (6, 112–115). Different gut bacteria, such as *Escherichia coli*, *Salmonella enterica*, *S. typhimurium*, *Bacillus subtilis*, *Mycobacterium tuberculosis*, and *Staphylococcus aureus* (6), generate a significant concentration of amyloids and LPS (116). *E. coli* endotoxin potentiates the formation of Aβ *in vitro*, and as Aβ exceeds a certain threshold, it self-propagates, both of which may further compromise CNS function (6). As a result of aging, the gastrointestinal epithelium and BBB are more permeable to small molecules, which is exacerbated by alterations in tight junctions that result from changes in the gut microbiota when *Bacteroidetes* outnumbers *Firmicutes* and *Bifidobacterium* (6). From such perspective, it is reasonable that the migration of amyloids and leukocytes from the gut to the brain is more prominent in aging individuals. Notably, amyloids can activate the microglia to cause prolonged inflammation (116, 117). Of note, activated microglia can produce APP in response to exhausted neuronal excitation by NO and APP from the gut, which further contributes to AD pathogenesis (116) (**Figure 2**). APP overexpression is associated with the upregulation of peroxynitrite, which can transform into NO, and activated microglia also produce high concentrations of inducible NO synthase (**Figure 2**); this supports the hypothesis that NO generated by activated microglia is closely related to Aβ deposition (99). Importantly, the gut is the main site of NO generation, and the generation of high concentrations of NO is mediated by several bacterial species (**Figure 2**). For example, gut *lactobacteria*, *bifidobacteria*, and *E.coli Nissle 1917* can generate NO through the conversion of nitrite and nitrate (6). Moreover, NO produced in the gastrointestinal system can be scavenged by hemoglobin in red blood cells, thereby entering the peripheral vasculature and crossing the BBB into the cerebral vasculature (118), initiating a vicious cycle, as demonstrated in **Figure 2**.

Given that the gut flora controls the maturation and function of microglia (12, 22), it is likely that microglial activation controlled by gut microbiota could be strongly associated with AD. Indeed, the proportion of *Allobaculum* and *Akkermansia* in the gut was found to be reduced in an APP/PS1 mouse model of AD, along with an increase in the proportion of *Rikenellaceae* (112). Of note, colonization of GF APP transgenic mice by the microbiota from conventionally-raised APP transgenic mice through the fecal route increased cerebral Aβ pathology (113). AD occurs in the elderly and aged individuals exhibit a different microbiome, which supports the possibility that altered microbiome mediated by aging is associated with the initiation and development of AD. Additionally, another study confirmed the modulation of microglia through external vagus nerve stimulation in a murine model of AD (88) **(Figure 2)**. However, whether

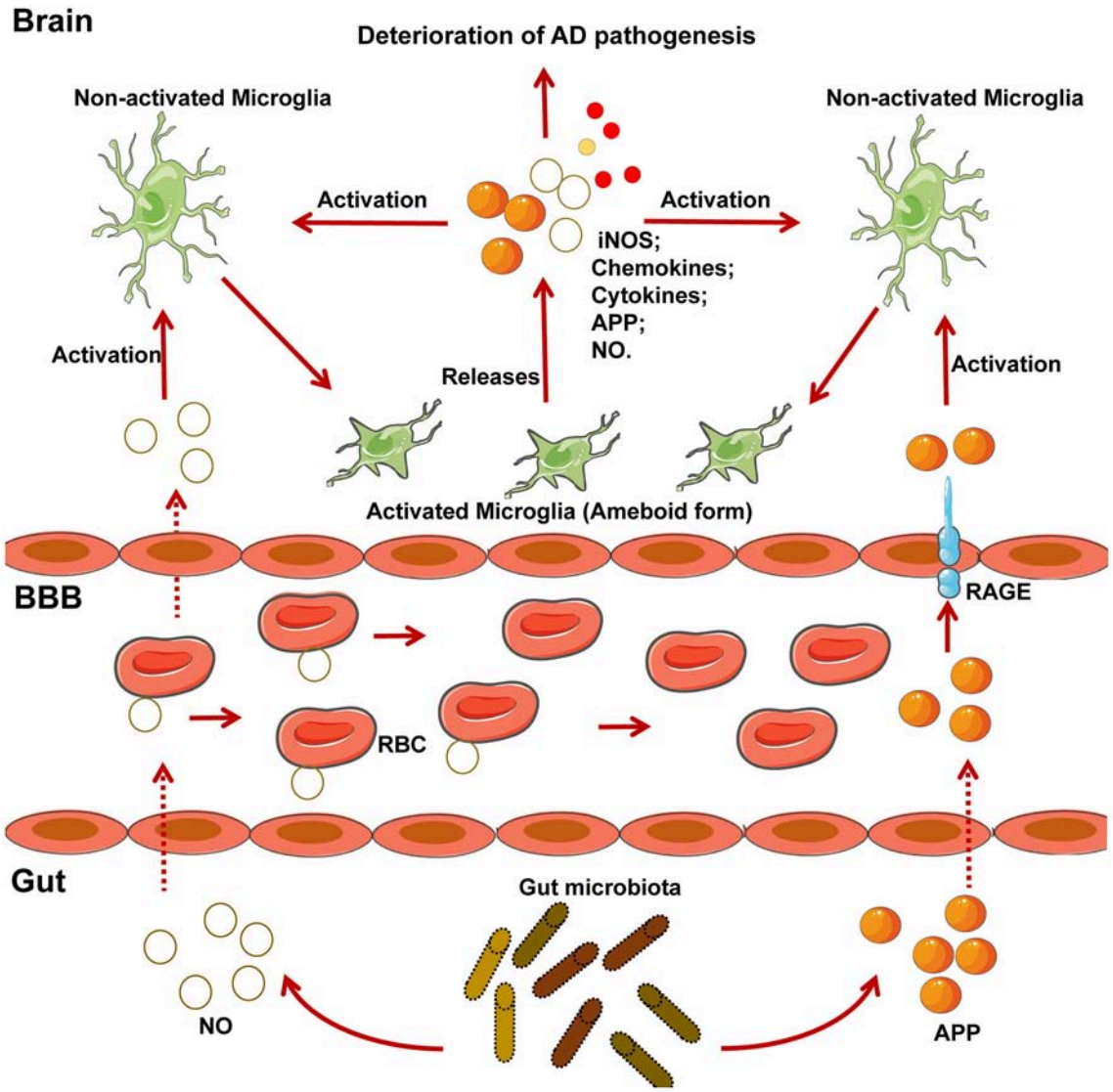

FIGURE 2 | NO and APP generated by microglia and several gut-specific microbes accelerate the development of AD pathogenesis**.** Chronically activated microglia contribute to the progression of neurodegenerative diseases such as AD. Several gut-specific microbes are able to generate NO and APP, which activate microglia and further exacerbate the development of AD. Specifically, NO generated by gut flora, can be carried to the brain by RBC. APP secreted by gut flora can cross the BBB via the RAGE receptor. After reaching the CNS, both NO and APP activate the microglia, which exhibit an ameboid form. Activated microglia can then secrete several risk factors for AD, including iNOS, chemokines, cytokines, APP, and NO, which further accelerate the pathogenesis of AD. iNOS, inducible nitric oxide synthase; NO, nitric oxide; RBC, red blood cell; RAGE, receptor for advanced glycosylation products.

the microbiota affects the progression of AD through several bacterial species or their associated factors remains unresolved.

MS

MS is a chronic inflammatory demyelinating disease of the CNS with heterogeneous histopathological features (119–121). In an EAE rat model of MS, relapse was characterized by an imbalance of monocyte activation profiles toward the M1 phenotype and the suppression of immunomodulatory M2 macrophages and/or microglia at lesion sites (122, 123). In addition, several microglia-associated genes, such as *TNFRSF1A* and *IRF8*, were also found to be associated with MS (122, 124). Moreover, TGFα and VEGF-B produced by microglia modulate the pathogenic activities of astrocytes in the EAE mouse model of MS and humans (23). In detail, TGFα acts via the Erbb1 receptor and suppresses the pathogenic activities of astrocytes to limit EAE development, whereas VEGF-B activates FLT-1 signaling in astrocytes and worsens EAE **(Figure 3)**. More specifically, metabolites of dietary tryptophan, generated by gut microbiota, modulate the production of TGFα and VEGF-B by microglia through an aryl hydrocarbon(AhR) receptor-mediated mechanism to further regulate CNS inflammation (23).

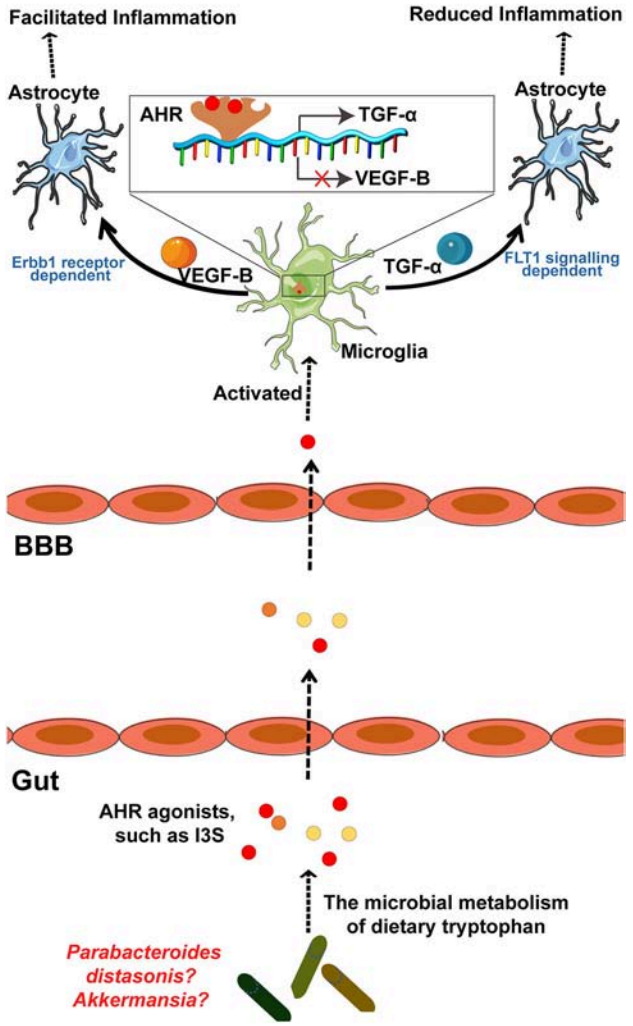

FIGURE 3 | The microbial metabolism of dietary Trp regulates the inflammatory response of astrocytes by microglia in EAE mouse model of MS through the AHR generated within microglia. Metabolism of dietary Trp by microbiota generates AHR agonists, which cross the BBB into the brain to activate microglia through an AHR-mediated mechanism within the microglia. In detail, these AHR agonists function as ligands for the AHR expressed in the microglia to bind the genes encoding VEGF-B and TGF-α, as indicated, to facilitate TGF-α transcription and to inhibit VEGF-B expression. Notably, VEGF-B increases the inflammatory activation of astrocytes to control the development of EAE. In contrast, TGF-α weakens the inflammatory activation of astrocytes to worsen EAE. Of note, both of *Akkermansia* and *Parabacteroides* distasonis, MS associated microbiota, may be the producer of AHR agonist, which required to be further researched. AHR, aryl hydrocarbon receptor; Trp, tryptophan; MS, multiple sclerosis; EAE, experimental autoimmune encephalomyelitis.

Commensal bacteria can also affect the autoreactivity of peripheral immune cells to CNS self-antigens, and thus, great attention has been focused on the contribution of the microbiota to MS pathogenesis (69, 120, 121, 125). Moreover, there is a significant increase in some taxa such as *Akkermansia* and reduction in other taxa such as *Parabacteroides* distasonis in untreated MS patients, while whether these bacteria regulate the microglia mediated inflammatory response through a mechanism by which the metabolites of tryptophan AhR ligands remains uncertain (**Figure 3**). However, AhR ligands are solely produced by a few bacteria, such as *Peptostreptococcus* russellii and *Lactobacillus* spp (126). Moreover, microbiota transplants from MS patients into GF mice resulted in worsening the symptoms of EAE compared with those in mice transplanted with microbiota from healthy controls (121). Additionally, on transplanting the microbiota to a transgenic mouse model of spontaneous brain autoimmunity, MS twin-derived microbiota induced a significantly higher incidence of autoimmunity than the healthy twin-derived microbiota (120). A majority of MS patients harbor antibodies against certain gastrointestinal antigens that are not found in healthy individuals, which further supports the possibility that alterations in the immune status of the brain against gut microbiota may be associated with MS (127). In an experimental model of MS, GF mice exhibited complete protection, which may have resulted from attenuated Th17 and B cell responses (128). The short-term effect of microbiota removal by treatment with antibiotics also attenuated EAE progression by inducing T cell, B cell, and immature natural killer (iNK), and regulatory T cell (Treg) responses (125, 129).

The close relationship between the gut microbiota and MS was also found in Theiler's murine encephalomyelitis virus (TMEV)-induced demyelinating disease (IDD), another relevant MS model (57). Unlike the effect showed by the gut microbiome exhibited in the previously mentioned MS model, antibiotic administration exaggerated the progression and severity of TMEV-IDD. A study into the mechanisms of this effect revealed that antibiotic-treated mice displayed lower levels of $CD4^+$ and $CD8^+$ T cells in their cervical and mesenteric lymph nodes. Furthermore, the activation of microglia was enhanced in TMEV-infected mice following treatment with antibiotics. Collectively, microglia act as crucial mediators in the connection between the microbiome and MS. However, the detailed mechanism underlying gut microbiota regulation of MS remains uncertain.

Depression

Despite the abundance of research on the mechanisms of depression as a psychiatric disease, our current understanding of depression is limited. One hypothesis claims that depression is a microglial disorder because microglial dysfunction has been commonly observed in the brain at the onset of depression (25). Minocycline, an antibacterial agent known to inhibit the activation of microglia after crossing the BBB, has been shown to significantly diminish depressive behaviors in rodents and humans (130, 131). Given that the gut microbiome affects the maturation of microglia, it is not clear whether the effect of minocycline is due to its antimicrobial properties by destroying gut microbiota or due to the direct inhibition of microglia. Microglia exhibit varying degrees of activation in patients with schizophrenia and depression, especially those who are suicidal (132). This was also confirmed in animal models of depression, although the pathophysiological role of microglial activation has not been clearly confirmed (25, 132–134). Studies of rodent models showed that the gut microbiome is also involved in the

development of depression (26–29), and beta diversity of the gut microbiome was significantly different between depressed and control patients. Of note, administration of *Bifidobacterium infantis* reversed experimentally created anxiety and depression in GF mice (26), and *Bifidobacterium infantis* is defined as a "psychobiotic" because of its antidepressant effect (135). GF mice transplanted with fecal microbiota from depressed mice also exhibited depressive-like phenotypes, which was confirmed in a rat depression model (29, 136). Treatment with antibiotics during the first year of life is also closely correlated to depression later in the life of humans (137). Probiotic supplementation was shown to improve the symptoms of anxiety and depression, especially *Lactobacillus helveticus Ns8*, which improved the behavioral, cognitive, and biochemical aberrations caused by chronic restraint stress (138, 139). However, the detailed mechanisms behind this association remain unclear, especially whether these results were mediated by the known interaction between the gut microbiome and microglia, direct effect of other unknown factors generated by gut bacteria, or a combination of the two.

PD

PD, a progressive neurodegenerative disease with motor and non-motor symptoms, is associated with misfolded α-synuclein seeds that assemble into fibrillary inclusions and with the loss of dopaminergic neurons in the substantia nigra (4, 140). α-synuclein, generated from neuron-derived exosomes, triggers the activation of microglia, which may accelerate lethality by the TAM-dependent phagocytosis of distressed spinal motor neurons (9). The mice lacking CX3CR1, a microglial-associated inflammation factor, exhibited a higher loss of neuron cells than control mice in a PD model established by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (141).

From the perspective of gut microbiology, PD patients exhibited significantly different microbial populations compared with healthy controls (142–144). PD patients displaying the tremor-dominant phenotype had significantly lower *Enterobacteriaceae* than those with more severe postural and gait instability, suggesting that the relative abundance of specific bacteria is sufficient to distinguish between the different forms of PD (143). Several microbial molecules that mimic host structures promote an immune response during the development of AD and PD (145–147). Of note, gut microbiome regulates movement disorders in mice and alterations in the human microbiome represent a risk factor for PD (140). In detail, antibiotic treatment ameliorates while microbial re-colonization promotes the pathophysiology of PD in adult animals. Administration of specific microbial metabolites to GF mice promotes neuroinflammation, and colonization of α-Syn-overexpressing mice with microbiota from PD-affected patients enhances physical impairments compared to microbiota transplants from healthy humans (140). However, given that there is no definite association among microglia, microbiota, and PD, the detailed underlying mechanisms remain uncertain.

ASD

ASD, a disorder associated with neurodevelopmental difficulties and mood disorders, is also associated with microglial dysfunction. Current understanding of the role of microglia in ASD is focused on the excessive microglia-mediated loss of synaptic tissue, which has been shown to contribute to patients suffering from Rett syndrome, an X-linked form of ASD (148). The gut microbiome in ASD patients exhibited differences in species variety and complexity compared with neurotypical controls (149–156). Generally, the gut microbiome of ASD patients exhibited more diversity and an abundance of *Clostridia* species (149–151, 155), but lacked several beneficial bacterial populations, such as *Prevotella* (154). In addition, *Sutterella* was found in intestinal biopsies of children with ASD presenting with gastrointestinal symptoms, while it was absent in control samples (152). Moreover, microbiota transfer therapy (MTT) alters the gut ecosystem and significantly improves GI and behavioral symptoms of ASD (157). Overall bacterial diversity and the abundance of *Bifidobacterium*, *Prevotella*, and *Desulfovibrio* among other taxa increased following MTT. Collectively, these studies indicate a potential relationship between the microbiome (or specific microbes) and the brain in ASD patients; as to whether microglia function as a mediator in this relationship remains uncertain. However, augmenting the microbiome with a specific microbe may be beneficial for ASD patients.

Abnormal Behavior

Microglial dysfunction is also closely associated with host abnormal behavior(13, 102, 158). Indeed, microglia interact with the nervous and endocrine systems during development, in which prostaglandin E2 (PGE2), generated through the aromatization of estradiol, plays a role (158). Additionally, mice lacking microglia-specific genes, such as CX3CR1, a chemokine receptor, exhibited impaired brain connectivity and abnormal social behavior because of defective neuron-microglia signaling (102). Of note, modifying the host microbiome with antibiotic or probiotic treatment also has a profound effect on the development of individuals, with long-lasting effects on host behavior in humans and animals (137, 159–161). Antibiotic administration during the first year of life may result in negative neurocognitive outcomes in later phases of life, including behavioral difficulties (137). Additionally, another study indicated that 7-day administration of nonabsorbable antibiotics was adequate to decrease anxiety-like behavior in mice (21). In contrast, short-term treatment with vancomycin failed to lead to anxiety- and depressive-like behaviors in neonate rats (16, 162), suggesting that the effect of gut dysbiosis on host behavior varies in different animals and depleting the microbiome in the short-term may not always impact behavior (162–164). In addition, compared to SPF mice, GF mice displayed several phenotypes associated with behavior (10, 21, 165–169). Moreover, several bacterial species improved the symptoms of stress, anxiety, and depressive-like behavior, as well as facilitating improvements in social behavior, communication, and cognitive function in animal models (13, 27, 163, 165, 170–174). Notably, several probiotic strains in rodents produced behavioral effects in a vagus nerve activation-dependent manner (175). Given that the vagus nerve also regulates the function of microglia at specific disease states, it may play a crucial role in the gut-microglia connection in abnormal behavior (**Figure 2**).

Collectively, the gut-microglia connection is clearly implicated in many CNS diseases, although the detailed

mechanisms controlling many of these associations remain to be determined. Of note, CNS disease-associated microRNAs have been shown to be closely related to the gut microbiome (176). MicroRNAs act as translational repressors to regulate gene translation and have also been implicated in anxiety-like behaviors (176). A previous study revealed that the gut microbiome regulates microRNA expression in the amygdala, affecting factors such as miR-183-5p and miR-206-3p, both of which are implicated in influencing anxiety levels and the expression of neurotrophins such as brain-derived neurotrophic factor (176). Moreover, in a study identifying region-specific miRNA-mRNA networks in the dorsal raphe nucleus and amygdala of high-responder/low-responder rats, miR-206-3p was shown to be associated with microglia and the immune response (177). Collectively, microRNAs may be potential mediators in the function of the gut-microglia connection in CNS diseases. In addition, in relation to the gut-microglia connection, HSV has clinically been associated with several neurodegenerative diseases, such as AD and MS, with uncertain mechanisms (106, 178). Of note, as a neurotropic virus, HSV-1 was also found to infect enteric neurons in addition to affecting the CNS, leading to gut dysbiosis (179). This may increase the abundance of several NO- and APP-generating bacteria, which remains uncertain. Consequently, NO and APP cross the BBB to activate microglia, thereby accelerating the progression of CNS diseases (**Figure 2**); HSV-1 may, therefore, be considered as a chronic risk factor for the development of several neurodegenerative diseases, especially AD (178).

CONCLUSIONS AND FUTURE DIRECTIONS

Research over the past few years has revealed that the development of a healthy brain requires crucial pre- and post-natal events that integrate environmental cues, especially the gut microbiome (11). The gut microbiome has been shown to influence various aspects of CNS biology through multiple mechanisms, including the alteration of both neurotransmitter levels (17) and BBB permeability (18). Furthermore, gut microbiome is closely associated with CNS diseases such as AD, depression, PD, and even ASD (11). However, most research to date has only demonstrated that different proportions of bacteria genera are associated with several CNS diseases; our understanding of the detailed signaling pathways through which the microbiome modulates CNS diseases remains poor. Importantly, microglia may be the crucial mediators linking gut microbiome and CNS diseases, given that microglia are crucial immune cells in the CNS, and their dysfunction has been shown to be related to most CNS-associated diseases. Moreover, emerging studies have revealed that the gut microbiome controls the maturation and function of microglia (12, 22) although the detailed mechanisms are yet to be elucidated. Indeed, as summarized by this study, multiple CNS diseases, such as AD, PD, and ASD, that are closely associated with microglia are also related to the gut flora. Additionally, indirect evidence supports the notion that the microbiota may affect the brain by modulating the microglia. For example, prostaglandin D2, which is produced by microglia, acts on the DP1 receptor in astrocytes to induce astrogliosis and demyelination (180). Microglia are known to participate in the demyelination process and the gut microbiome is also involved in myelination because myelin-related transcripts were found to be increased in the prefrontal cortex of the brains of GF mice and antibiotic-treated mice as compared to control mice (181, 182). However, the possibility that the gut microbiome-mediated effect on myelination is associated with microglia cannot be excluded and requires further research. Furthermore, just as several CNS diseases have shown significant sexual bias—such as ASD, which affects males at a higher rate (183, 184)—the composition of the gut microbiome and microglia also exhibits sexually dimorphic properties. Thus, it is essential to determine whether the gut-microglia connection contributes to the progression of CNS diseases with a sex bias. It is possible that the microbiome affects CNS diseases by modulating microglia; however, extensive research is required to confirm this. Therefore, it is critical to determine the importance of the gut-microglia connection in CNS diseases and to clearly define the specific mechanisms involved. Furthermore, understanding the interactions between specific microbial species and microglia would be significant for developing a novel therapeutic strategy for the treatment of neurological disorders in humans. Recently, the Kallyope company was allowed to continue its work on harnessing the communication pathways between the gut and the brain to develop novel therapies for various illnesses of the CNS. However, further studies are required to determine whether probiotics can be exploited to improve microglial function and, ultimately, alleviate CNS diseases.

AUTHOR CONTRIBUTIONS

YilW conception, design, collection and/or assembly of references, discussion, interpretation, and manuscript writing. ZW and YuW collection and/or assembly of references, interpretation, and manuscript writing. FL, XS, JJ, SQ, and RW collection and/or assembly of references. FJ, KK, and YifW conception, design, interpretation, and final approval of manuscript. YilW, ZW, and YuW contributed equally to this article.

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REVIEW
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Reverse Signaling of Tumor Necrosis Factor Superfamily Proteins in Macrophages and Microglia: Superfamily Portrait in the Neuroimmune Interface

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The tumor necrosis factor (TNF) superfamily (TNFSF) is a protein superfamily of type II transmembrane proteins commonly containing the TNF homology domain. The superfamily contains more than 20 protein members, which can be released from the cell membrane by proteolytic cleavage. Members of the TNFSF function as cytokines and regulate diverse biological processes, including immune responses, proliferation, differentiation, apoptosis, and embryogenesis, by binding to TNFSF receptors. Many TNFSF proteins are also known to be responsible for the regulation of innate immunity and inflammation. Both receptor-mediated forward signaling and ligand-mediated reverse signaling play important roles in these processes. In this review, we discuss the functional expression and roles of various reverse signaling molecules and pathways of TNFSF members in macrophages and microglia in the central nervous system (CNS). A thorough understanding of the roles of TNFSF ligands and receptors in the activation of macrophages and microglia may improve the treatment of inflammatory diseases in the brain and periphery. In particular, TNFSF reverse signaling in microglia can be exploited to gain further insights into the functions of the neuroimmune interface in physiological and pathological processes in the CNS.

Keywords: tumor necrosis factor superfamily, immunity, inflammation, macrophage, microglia, neuroinflammation, neuroimmune interface

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INTRODUCTION

Cell-to-cell communication, particularly for immune cells, occurs through either soluble mediators or direct contact. In cases of communication through soluble mediators, molecules, such as cytokines, chemokines, and hormones act in an autocrine, paracrine, or endocrine manner to stimulate cell surface receptors. In contrast, direct contact requires the interaction between cell surface molecules, such as cell adhesion molecules. Members of the tumor necrosis factor (TNF) superfamily (TNFSF) constitute a special class of molecules that are involved in both types of cell communication.

TNFSF members are type II membrane proteins that are present on the cell surface or in intracellular compartments. In their membrane-bound form, TNFSF members can interact with their cognate TNF receptor superfamily (TNFRSF) members present on the cell surface or on adjacent cells. Cellular activation increases cell surface expression and secretion of homotrimeric forms of TNFSF members released from the cell surface. For TNF-α, this proteolytic cleavage is carried out by TNF-α-converting enzyme, a member of the ADAM family of metalloproteases. Released trimeric forms of these ligands then act as cytokines by interacting with their cognate receptors.

Increasing evidence has demonstrated that during direct contact within or among cells expressing TNFSF and TNFRSF members, signals are generated from the receptor part (forward signaling) as well as the ligand part (reverse signaling). Another interesting feature of the interactions between TNFSF/TNFRSF members is that there is substantial crosstalk among cognate ligand-receptor pairs, and some receptors can also be solubilized and released into the surrounding tissues, thereby serving as competitive inhibitors of ligand action on receptor-bearing cells (1, 2).

Macrophages are immune cells that express most members of the TNFSF and TNFRSF before and/or after activation. Macrophages perform immunoregulatory functions at sites of acute and chronic inflammation, pathogenesis, and tumorigenesis. Many of the functions of macrophages are mediated by TNFSF and TNFRSF members. Moreover, TNFSF and TNFRSF members are expressed in brain glial cells and mediate diverse biological effects, including neuroinflammation and cell death. Neuroinflammation is closely associated with diverse neuropathologies, such as CNS injury and neurodegenerative diseases (3). Recent evidence indicates that neuroinflammation is one of the major components of the disease mechanisms. Under pathological conditions, neuroinflammation and brain injury constitute a positive feedback loop that perpetuates damages in the nervous system. Brain glial cells, particularly microglia, play a pivotal role in these processes. Inflammatory and neurotoxic mediators produced from excessively activated microglia contribute to neurodegeneration. Intracellular and intercellular signaling of microglia has been proposed as a therapeutic target to dampen deleterious microglial activation and to protect neurons from microglial neurotoxicity (4–6). In that vein, TNFSF and TNFRSF members expressed in brain microglia may provide insights into the intercellular signaling of microglia, and shed light on the regulatory mechanisms of microglia-mediated neuroinflammation.

To date, many reviews have summarized the roles of forward signaling in various processes associated with normal immunity and the pathogenesis of cancer and other conditions. Therefore, in this review, we will focus on reverse signaling initiated from membrane-bound form of TNFSF with an emphasis on recent developments. Especially, members of TNFSF that are expressed in macrophage/microglial lineage cells, such as BAFF/APRIL, LIGHT, GITRL, FasL, TWEAK, and CD137L (4-1BBL), will be the main topic of this review.

B-CALL ACTIVATION FACTOR OF THE TNF FAMILY (BAFF)/A PROLIFERATION-INDUCING LIGAND (APRIL)

BAFF (also known as TALL-1, THANK, and TNFSF13B), a B-cell survival factor, and its close relative APRIL (also known as TNFSF13) are expressed in both membrane-bound and soluble forms in various cells lineages, including myeloid cells (monocytes, macrophages/microglia, neutrophils, and dendritic cells [DCs]), stromal cells within lymphoid organs, and osteoclasts (7–10). APRIL shares ∼30% sequence identity with BAFF in the TNF domain (11). BAFF interacts with three types of receptors: transmembrane activator and a calcium-modulating cyclophilin ligand interactor (TACI), B-cell maturation antigen (BCMA), and BAFF receptor (BAFFR and BR3; **Figure 1**). These receptors can be found in lymphoid cells (i.e., B cells and plasma cells, but also in some subsets of T cells) and myeloid cells (8, 10). Although BAFF-R interacts with only BAFF, TACI, and BCMA interact with both BAFF and APRIL. Studies of APRIL and BAFF transgenic/knockout mice have revealed that these molecules are essential for B-cell survival, T-cell costimulation, autoimmune diseases, and cancer (8–11). Moreover, ligation of BAFFR activates B-cell survival through activation of the nuclear factor (NF)-κB pathway and downstream anti-apoptotic genes (12, 13). Although both BAFF and APRIL are required for B-cell maturation and survival, BAFF has major effects on pre-immune B cells, whereas APRIL acts on antigen-experienced B cells (14).

Both BAFF and APRIL contain a short cytoplasmic region of ∼30 amino acids, a transmembrane domain (TMD), and a 200-residue extracellular domain consisting of a stalk and a TNF domain (11, 15–17). In macrophages, both are capable of inducing reverse signaling, which triggers inflammatory changes for the induction of various inflammatory mediators, including matrix-degrading enzymes and pro-inflammatory cytokines (18, 19). Treatment of either primary mouse macrophages or human macrophage-like cell lines with the TACI:Fc fusion protein or anti-BAFF/APRIL-specific monoclonal antibodies (mAbs) stimulates the cells to express various pro-inflammatory markers while suppressing cytoskeletal rearrangement associated with phagocytosis and transmigration (18–21). Furthermore, co-incubation with Ramos cells, which express both TACI and BCMA, results in pro-inflammatory activation of THP-1 cells in a BAFF- or APRIL-dependent manner, indicating that cell-to-cell interactions can stimulate BAFF- or APRIL-mediated reverse signaling (18, 21). These pro-inflammatory responses initiated by BAFF are mediated by the mitogen-activated protein kinase (MAPK) extracellular signal-regulated kinase (ERK) and NF-κB, and suppression of cytoskeletal rearrangement is mediated by phosphatidylinositol 3-kinase (PI3K)/AKT and Rac-1, a Rho-family GTPase. Interestingly, BAFF-mediated signaling shows significant crosstalk with Toll-like receptor (TLR) 4-mediated signaling such that simultaneous treatment with anti-BAFF mAbs and lipopolysaccharide (LPS) results in a synergistic response with respect to pro-inflammatory

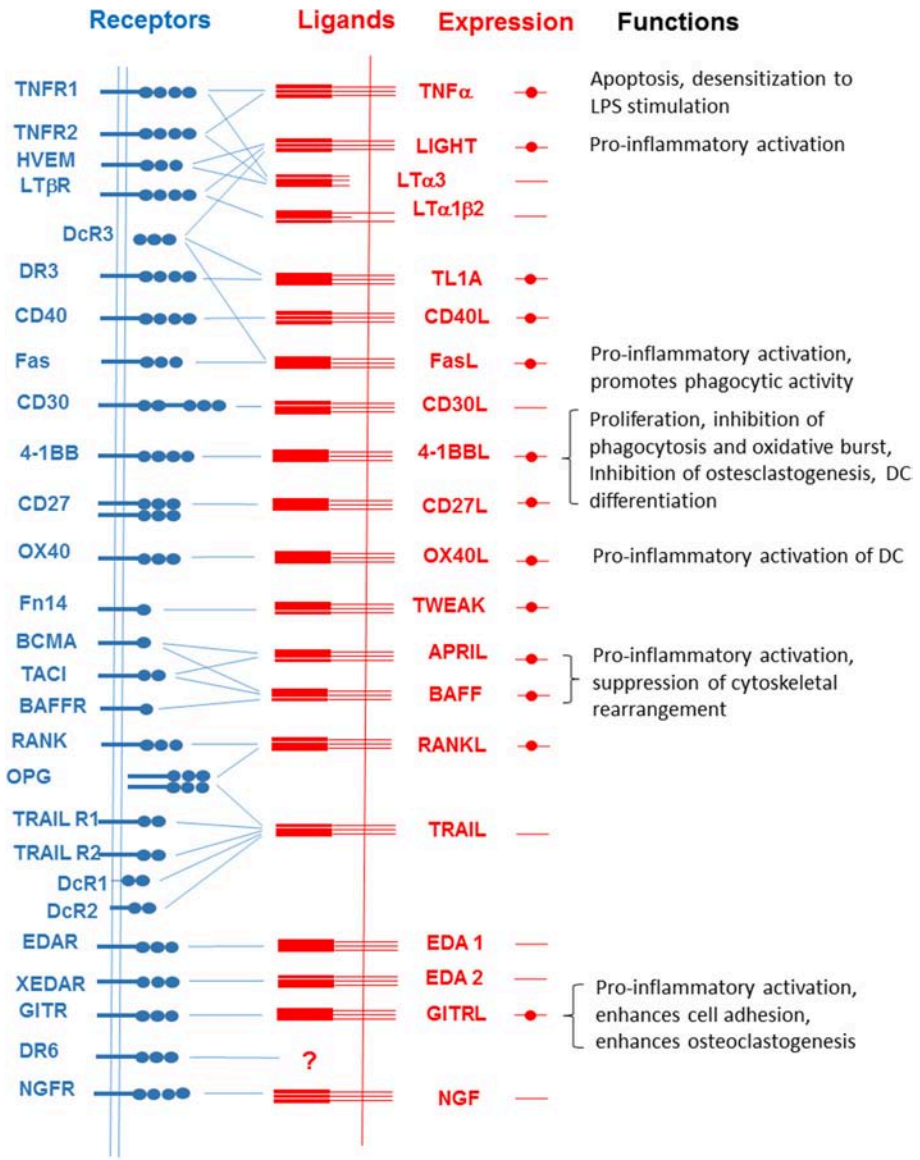

FIGURE 1 | Interactions among members of the TNFSF and TNFRSF. Filled circles represent the expression of each member of the TNFSF in macrophages and microglial cells. Functions of reverse signaling in the activities of macrophages and microglial cells are listed on the right.

activation. The cellular response is mediated by PI3K/AKT and MAPK/Mitogen- and stress-activated protein kinase 1 (MSK1) pathways, which culminate at the formation of a trimeric complex containing NF-κB, cyclic AMP-response element binding protein (CREB), and CREB binding protein. This trimeric complex is responsible for the synergistic activation of NF-κB and, consequently, pro-inflammatory responses of the cell (22). The involvement of ERK in pro-inflammatory activation has been further confirmed in studies showing the existence of crosstalk between BAFF-mediated signaling and signals initiated from immune receptor expressed on myeloid cells 1 (IREM-1, CD300F) (23–25). IREM-1 is an immunoreceptor tyrosine-based inhibition motif (ITIM)-containing cell surface molecule that exerts its inhibitory effects through interaction of its ITIMs with SH2-containing tyrosine phosphatase (SHP)-1. Via its phosphatase activity, SHP-1 suppresses cellular signals associated with PI3K, Janus kinase 2, MAPKs, signal transducers and activators of transcription, and NF-κB (26, 27). Simultaneous stimulation of BAFF and IREM-1 results in suppression of BAFF-mediated ERK activation owing to IREM-1-mediated activation of SHP-1 (22). These findings indicate that it is necessity to re-evaluate the role of BAFF in diseases in which BAFF is overexpressed in macrophages.

Despite the similarities in their extracellular domains and receptors, BAFF and APRIL have quite different intracellular domains (ICDs; **Figure 2**). On the other hand, the ICD in

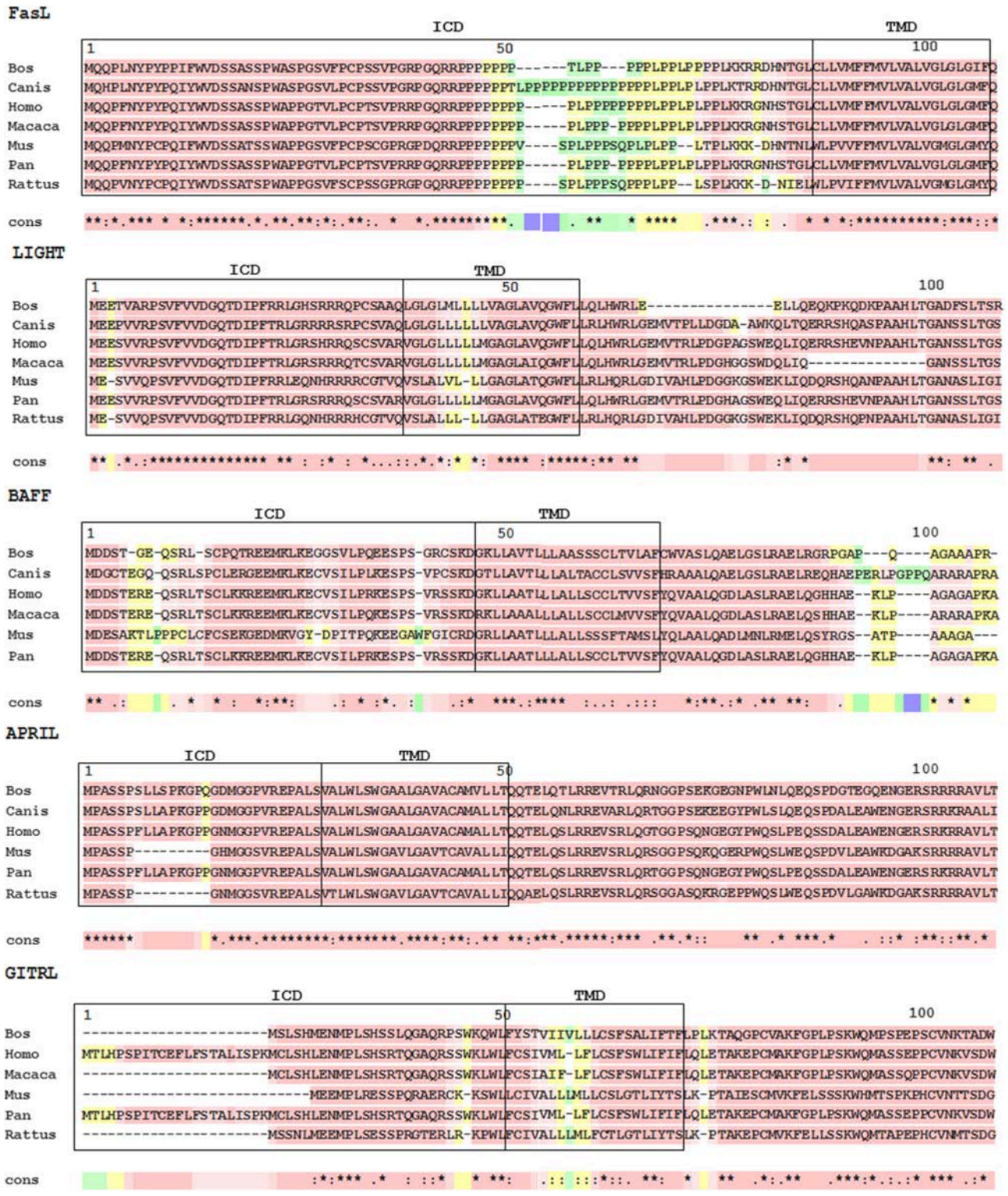

FIGURE 2 | Multiple sequence alignments of TNFSF ligands. Protein sequences were aligned using NCBI homoloGene website (https://www.ncbi.nlm.nih.gov/homologene). Alignments of N-terminal sequences were constructed with T-coffee (The European Bioinformatics Institute [EMBL-EBI], https://www.ebi.ac.uk/). The transmembrane domain of APRIL was computationally predicted with Phobius (EMBL-EBI, https://www.ebi.ac.uk/). The pink area indicates amino acid conservation. ICD, intracellular domain; TMD, transmembrane domain.

each member is highly conserved in among different species, supporting the importance of their intracellular domains for the generation of reverse signaling.

BAFF/APRIL and their receptor systems are believed to be involved in the pathogenesis of various autoimmune diseases. Accordingly, serum BAFF levels have been shown to be abnormally upregulated in patients with systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and Sjögren's syndrome (28–31). The mAb-based therapeutic belimumab (LymphoStat-B) was approved by the US Food and Drug Administration for the treatment of SLE in 2011 (32). Belimumab was developed by screening of a phage-display library and consists of two heavy chains and two light chains with specificity against BAFF; this antibody blocks BAFF-mediated activation of its receptors and subsequent cellular activation (33). Although belimumab interacts with soluble BAFF and not with the membrane-bound form of BAFF (34), the antibody may crosslink with and thus activate the membrane bound-form of BAFF on cells of monocyte lineage. Another mAb-based therapeutic currently being evaluated in clinical trials is tabalumab (35), an anti-BAFF human mAb that has been reported to neutralize both membrane-bound and soluble forms of BAFF (36). Because tabalumab binds the membrane-bound form of BAFF, exploring whether this antibody induces reverse signaling from BAFF in various cell types could be beneficial for the future development of agents targeting BAFF or APRIL.

BAFF and its receptors are widely expressed in brain glial cells (**Figure 3**). Microglia express BAFF, BAFFR, and TACI (37). In contrast, astrocytes and neurons only express BAFF and BAFFR, respectively (38, 39). Microglial expression and release of BAFF is increased by ganglioside mixture treatment (37) and brain injury (38). In particular, cerebral ischemia and reperfusion injury enhance microglial BAFF and neuronal BAFFR expression, suggesting important roles of the BAFF/BAFFR interaction in brain injury conditions (38). Neuronal survival was promoted by BAFF/BAFFR ligation under ischemic stress conditions *in vitro* as well as middle cerebral artery occlusion *in vivo*. Interactions between microglial BAFF and neuronal BAFFR seem to exert neuroprotective effects in brain ischemia injury and may represent a promising therapeutic target for patients with stroke. BAFF released from microglia has been proposed to act on microglia themselves or B cells infiltrated into the brain to regulate central nervous system (CNS) inflammation (37). A previous study by Krumbholz et al. identified astrocytes as a main cellular source of BAFF in multiple sclerosis plaques, suggesting that BAFF produced by brain astrocytes may be involved in B-cell survival under inflammatory conditions (39). BAFF has also been reported to have a different functional role in experimental autoimmune encephalomyelitis (EAE); specifically, *BAFFR* gene-deficient mice show increased peripheral inflammatory cytokines and higher disease severity compared with wild-type animals, suggesting alteration of macrophage activation and immune responses in the absence of BAFFR (40).

Reverse signaling of BAFF has not been specifically investigated in microglia or other glial cell types. Nevertheless, the wide distribution of BAFF and its receptors in various neural cell types indicates that BAFF/BAFFR signaling may be important for interglial crosstalk or neuron/glia interactions.

APRIL has been shown to be expressed by astrocytes in areas of gliosis and by several glioblastoma cell lines (**Figure 3**) (41). Under inflammatory conditions, astrocytes act like microglia, producing pro-inflammatory cytokines, chemokines, and nitric oxide. Astrocytic expression of APRIL has been shown to be increased in the brains of patients with multiple sclerosis (41). Thus, APRIL expressed in reactive astrocytes may participate in the regulation of neuro-inflammatory responses and gliotic scar formation in multiple sclerosis and other pathological conditions. Notably, in this previous study, microglia were negative for APRIL expression. However, the role of APRIL in glioblastoma cells is still not clear.

Further evidence of the role of BAFF and APRIL in CNS inflammation was obtained from a marmoset monkey model of multiple sclerosis (42). Indeed, administration of antibodies against either human BAFF or APRIL delayed EAE development via different mechanisms.

LIGHT

The expression of LIGHT (also known as TNFSF14 or CD258) has been observed in activated T and B lymphocytes, monocyte/macrophages, granulocytes, natural killer (NK) cells, and DCs (43–46). LIGHT can interact with three types of receptors, i.e., herpes virus entry mediator (HVEM), lymphotoxin β receptor (LTβR), and decoy receptor (DcR3) (43, 47). HVEM or LTβR mediates LIGHT-induced T-cell costimulation and/or subsequent cytokine production (48–52), whereas DcR3, which is a soluble receptor without a TMD, works as a competitive inhibitor of LIGHT-induced cellular responses (43, 47, 53). HVEM (also known as TNFRSF14, LIGHTR, or TR2), which was initially identified as a cellular coreceptor for herpes simplex virus (HSV) entry (54), has a wide tissue distribution, including lymphoid tissues, and is expressed on peripheral blood leukocytes, such as T and B lymphocytes and monocytes (55, 56). Similar to other members of this receptor superfamily, HVEM stimulation leads to the activation of transcription factors, including NF-κB and activator protein (AP-1) (56). The expression of LTβR has been detected on endothelial, epithelial, and myeloid cells (57). LTβR functions as a mediator of cancer-associated inflammation (58, 59), regulator of lymphoid organ development (60, 61) and homeostatic stimulator of DC expansion (62, 63). LTβR-mediated signaling induces the classical NF-κB pathway via TNF receptor-associated factor 2/5 (TRAF2/5) (64, 65) or the non-canonical NF-κB pathway via TRAF3 (66, 67). LTβR can also interact with and be stimulated by LTα1β2, which is expressed on the surface of the cell. Because HVEM also interacts with the homotrimer of LTα (LTα3) (57, 64), there seems to be extensive crosstalk between LIGHT/HVEM and LT/LT receptor systems (**Figure 1**).

The possibility of LIGHT-mediated reverse signaling has been reported in T cells, in which stimulation of LIGHT has costimulatory effects; indeed, treatment with anti-LIGHT mAbs enhances responses induced by T-cell receptor ligation. These

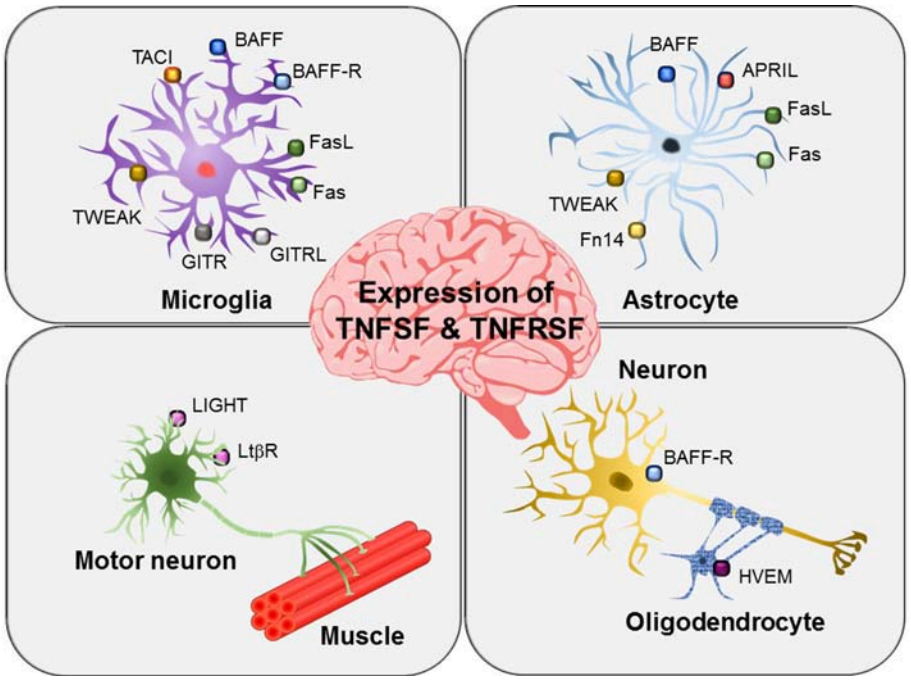

FIGURE 3 | Expression of TNFSF and TNFSRSF members in brain glial cells and neurons. Different members of the TNFSF and TNFSRSF are expressed on microglia, astrocytes, oligodendrocytes, and neurons as indicated. In particular, motor neurons have been shown to express LIGHT and LTβR.

responses include cell proliferation, cytokine production, and cytotoxic activity via MAPK activation. Although treatment of mice with DcR3-Fc downregulates graft-vs.-host responses and ameliorates the rejection of mouse heart allografts, it is not clear whether these effects are mediated by direct stimulation of membrane-bound LIGHT or perturbation of LIGHT-induced activation events (68, 69). Reverse signaling in macrophage lineage cells was demonstrated when the human macrophage-like cell line THP-1 was treated with a LIGHT-specific agonistic mAb. Cells responded by inducing the expression of pro-inflammatory mediators, such as interleukin (IL)-8 and matrix metalloproteinase (MMP)-9 while suppressing phagocytic activity. The signaling pathway initiated by LIGHT is mediated by the MAPK ERK and by PI3K, leading to activation of the major inflammatory transcription factor NF-κB (70).

Sequence analysis of the LIGHT ICD indicated a high level of conservation among different species. However, there were no similarities with currently known protein motifs (**Figure 2**).

LIGHT/HVEM/LTβR expression has not been thoroughly investigated in brain microglia or astrocytes. Instead, oligodendrocytes have been shown to express HVEM (71), and motoneurons express LIGHT and LTβR (**Figure 3**) (72). In an amyotrophic lateral sclerosis animal model, interferon (IFN)-γ secreted from astrocytes was found to induce LIGHT/LTβR signaling in motoneurons, thereby stimulating non-cell autonomous neurotoxic pathways (72). Moreover, researchers found that astrocyte/neuron crosstalk contributed to the elimination of motoneurons expressing both LIGHT and LTβR under pathological conditions. Microglia may also participate in the non-cell autonomous motoneuron selective death pathways by communicating with astrocytes or motoneurons. The study further suggested that IFN-γ/LIGHT/LTβR pathways may be useful therapeutic targets in motoneuron disease.

Although less is known about the glial expression of LIGHT/HVEM/LTβR, a previous study by Mana et al. investigated the role of LIGHT in CNS inflammation (73). In the EAE model, LIGHT was found to be involved in restraining macrophages and microglia, thereby limiting disease progression and nerve damage. However, further studies are required to elucidate the cell type-specific roles of LIGHT in autoimmune CNS inflammation because only conventional LIGHT-deficient mice have been evaluated.

HVEM expression has been found in oligodendrocytes (71). This study, however, focused on the role of HVEM as a receptor for viral entry during HSV-1 infection in a human oligodendrocytes. They observed the colocalization of HVEM and nectin-1 with HSV-1 particles, implying that HVEM may be a major viral receptor functioning in these cells.

GLUCOCORTICOID-INDUCED TNFR-RELATED PROTEIN (GITR) LIGAND (GITRL)

GITR (also known as AITR or TNFRSF18) was originally identified in activated T-lymphocytes, functioning as a regulator of T-cell receptor-mediated cell death (74). Later, its expression was detected in regulatory T cells (Tregs), effector T cells,

macrophages, and microglia (75–79). The ligand of GITR (GITRL) is mainly expressed in immature and mature DCs, B cells, endothelial cells, macrophages, and microglia (75, 80, 81). Forward signaling initiated from GITR acts to costimulate CD25-effector T cells, which respond through proliferation and cytokine production. This GITRL-induced forward signaling is mediated TRAFs and NF-κB (82–85). In human macrophage-like THP-1 cells, ligation of GITR results in the expression of pro-inflammatory mediators via activation of MAPK and PI3K (77). The GITRL/GITR system has also been implicated in various processes, including suppression of $CD4^+CD25^+$ Tregs, antiviral and antitumoral responses, leukocyte extravasation, RA development, and chronic lung inflammation (79, 86–88).

Reverse signaling through GITRL has been the subject of intense investigations. $GITR^{-/-}$ mice show decreased numbers of leukocytes in inflamed areas (89, 90), and treatment of experimental animals with GITR-Fc fusion protein ameliorates the symptoms of autoimmune or chronic inflammatory diseases (91). These effects may be due to induction of GITRL-mediated reverse signaling or blockage of GITR signaling (neutralizing GITRL). Additional studies have indicated that both of these mechanisms are possible. Some reports have favored the reverse signaling mechanism. For example, adherence of $GITR^{-/-}$ murine splenocytes or HL60 human monocytic cells to endothelial cells was found to be enhanced when the cells were treated with the GITR-Fc fusion protein. Moreover, stimulation with GITRL triggers the upregulation of intracellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule-1 (80). In contrast, other reports have favored the neutralizing effect of the fusion protein. Indeed, analysis of a spinal cord injury model in $GITR^{-/-}$ mice indicated that the GITR-Fc fusion protein failed to alter the disease severity in the knockout mice but decreased disease severity in wild-type mice (92).

Stimulation of murine primary macrophages or human macrophage-like THP-1 cells with GITR-Fc fusion protein or anti-GITRL mAbs induces pro-inflammatory mediators (e.g., MMP-9, IL-8, monocyte chemotactic protein-1, TNFα, and IL-β) and upregulates ICAM-1 expression. GITRL-mediated activation signals were found to be mediated by ERK and NF-κB (93). Treatment of murine monocytic cells with recombinant soluble GITR (rsGITR) in combination with IFN-γ results in synergistic induction of inducible nitric oxide (NO) synthase (iNOS), cyclooxygenase (COX)-2, and MMP-9. Analysis of the signaling mechanisms indicated the involvement of tyrosine phosphorylation and NF-κB (94–96).

Both GITR and GITRL are expressed at the same time in some cell types, particularly macrophages and microglial cells (75–77). Clusters of macrophages/microglia can be easily observed in lesion areas in atherosclerotic plaques, synovium of patients with RA, and amyloid plaques in Alzheimer's brain. Activation signals initiated from both the receptor and ligand, which can occur within one cell or among adjacent cells, may cause synergistic pro-inflammatory activation.

GITRL-mediated reverse signaling is also involved in osteoclastogenesis. When osteoclast precursors are treated with receptor activator of NF-κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF), the expression of both GITR and GITRL is induced. Additional treatment with rsGITR enhances osteoclastogenesis, which is blocked by neutralizing anti-GITRL antibodies. This effect is related to rsGITR-induced production of prostaglandin E_2 via COX-2. Prostaglandin E_2 then downregulates the steady-state level of osteoprotegerin (OPG), which has anti-osteoclastogenic effects (97).

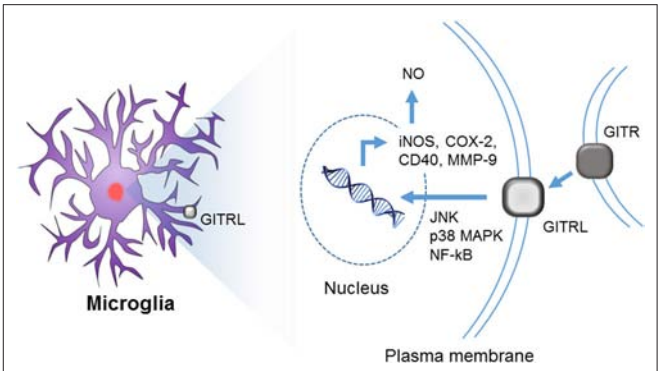

FIGURE 4 | Reverse signaling through GITRL in brain microglia. Although both GITR and GITRL are expressed on microglia, only GITRL participates in the inflammatory activation of microglia. Upon ligation of GITRL, MAPKs (such as JNK and p38) and NF-κB are activated, and consequently, the expression of *iNOS*, *COX-2*, *CD40*, and *MMP-9* genes is induced with concurrent NO production.

Reverse signaling through GITRL has been well-characterized in microglia (**Figures 3**, **4**). Hwang et al. first reported the expression of both GITR and GITRL in brain microglia and showed that reverse signaling through GITRL in microglia induces inflammatory activation, as determined by NO production and pro-inflammatory gene expression, such as iNOS, MMP-9, COX-2, and CD40 (**Figure 4**) (75). Furthermore, they demonstrated that GITRL-mediated microglial activation is executed by canonical inflammatory signaling, such as NF-κB and MAPK pathways. These results indicate that the GITR/GITRL system, particularly GITRL reverse signaling, may play a regulatory role in microglia-mediated neuroinflammation.

FAS LIGAND (FasL)

Fas (CD95), a type I transmembrane protein with characteristic cysteine-rich domains, works as a receptor for FasL (CD95L/CD178) (98, 99). Ligation of Fas induces caspase-dependent apoptotic cell death through its death domain, which is found in the ICD (100–103). Constitutive expression of Fas has been detected in many cell types, although FasL expression is restricted to $CD4^+$ T helper cells, activated $CD8^+$ cells, NK cells, and macrophages (104). Developmental stage-dependent expression of Fas during hematopoiesis has also been reported (105). Alternative splicing of *Fas* mRNA generates seven isoforms, which include soluble forms that can serve as DcRs (106). Activation of T cells leads to upregulation of cell surface expression of FasL, which then interacts with Fas on the same or adjacent cells. This interaction triggers apoptotic cell death, called activation-induced cell death (107). NK cells and $CD8^+$

cytotoxic T lymphocytes use Fas/FasL interaction as one of the two main pathways that are responsible for their cytotoxic effector functions (108). Cells in immune-privileged sites and some tumor cells constitutively express FasL for the suppression of immune responses against them (109, 110).

In addition to its apoptosis-inducing properties during tissue injury and organ dysfunction (111–113), Fas also works as an enhancer of pro-inflammatory responses (114, 115) though caspase-independent and myeloid differentiation factor 88 (MyD88)-dependent signaling pathways (116–118). MyD88 serves as cross point for the crosstalk between the Fas-mediated signaling pathway and IL-1R1 and/or TLR4-mediated signaling pathways (119). Fas also enhances the proliferation of fibroblasts and T cells (120–122), which are involved in caspase activation without cell death (123–126). In THP-1 cells, treatment with anti-Fas mAbs or incubation with FasL-expressing cells results in pro-inflammatory activation of these cells through activation of ERK and NF-κB (127). These reports indicate that Fas-mediated signaling pathways are under complex regulation and provoke various responses in different cell types.

Unlike other members of the TNFSF, FasL has a long ICD containing around 80 amino acids with a high level of conservation across different mammalian species. The ICD of Fas contains two casein kinase I (CKI) binding sites and a proline-rich region that contains multiple binding sites for the SH3 domain (2). The CKI phosphorylation motif can be found in five other members of the TNFSF and is required for the FasL-mediated activation of nuclear factor of activated T cells (NFAT) and costimulation of T cells (104) (128). Possible interactions of this ICD with SH3-containing signaling adapters, such as Grb2, Fyn, and PI3K, have been reported (129, 130). The proline-rich sequence is also required for the storage of FasL in specialized secretory vesicles and the translocation of FasL to the plasma membrane upon activation (131). This intracellular localization appears to be regulated by the interaction of the proline-rich region with the SH3-containing adapter protein PSTPIP, which further interacts with the tyrosine phosphatase PTP-PEST (132).

Reverse signaling initiated from FasL, particularly via its proline-rich sequence, is involved in costimulation of $CD8^+$ T cells, optimal thymocyte maturation, and antigen-driven proliferation of mature T cells (133–138). Upon activation, increasing fractions of FasL have been reported to be localized in lipid rafts, sphingolipid- and cholesterol-enriched dynamic membrane microdomains required for some of signaling and trafficking processes (133, 139). Along with increased localization in lipid rafts, activated FasL associates with SH3-containing proteins. The proline-rich domain is required for phosphorylation of FasL itself and other signaling molecules, including AKT, ERK, and c-Jun N-terminal kinase (JNK). These signaling events then activate transcription factors (NFAT and AP-1) and enhance IFN-γ production (1, 128, 133). Interestingly, FasL with an alteration in the proline-rich region (deletion of amino acids 45–54) abolishes its costimulation activity without affecting its death-inducing activity, indicating the separation of functional domains for different functions (128).

Although various functions of FasL have been well-documented in T cells, its roles in other cell types have not been extensively investigated. In breast cancer cell lines, IFN-γ treatment induces the translocation of pre-existing FasL to the cell membrane, and treatment with Fas-Fc fusion protein induces apoptotic cell death (140). In the human macrophage-like cell line THP-1, treatment with either anti-FasL mAbs or Fas-Fc fusion protein induces the production of pro-inflammatory mediators (e.g., MMP-9, TNF-α, and IL-8) and promotes phagocytic activity. This pro-inflammatory activation is mediated by MAPKs and NF-κB. In addition, FasL-mediated inflammatory activation is blocked by triggering of IREM-1 (141).

Fas and FasL are expressed in both microglia and astrocytes (**Figure 3**). As recently reviewed by Jha et al., microglia/astrocyte crosstalk constitutes an important component of neural cell communication, orchestrating a range of physiological and pathological processes in the CNS, such as brain development and neurological dysfunction (142). The bi-directional communication between microglia and astrocytes is mediated by either secreted or cell surface proteins. Fas/FasL expressed in microglia and astrocytes may mediate such crosstalk. Moreover, a previous study by Badie et al. suggested a role of the Fas/FasL interaction in microglia/glioma crosstalk (143). The authors reported that expression of the membrane-bound form of FasL is increased in the glioma environment and that FasL-expressing microglia may contribute to the local immunosuppressive environment of malignant glioma. However, the precise role of microglia in glioma biology remains unclear.

Fas is expressed at low levels and is upregulated upon TNF-α or IFN-γ treatment in primary mouse microglia cultures (144, 145). Moreover, Fas is expressed constitutively on astrocytes and is upregulated by treatment with IL-1, IL-6, or TNF-α. FasL is expressed on fetal and adult astrocytes and on microglia (144, 145). Thus, glial Fas/FasL may have a role in the induction of apoptosis in the CNS (144). Interestingly, however, the Fas/FasL interaction results in different signals in microglia vs. astrocytes. For example, Fas mediates cell death signaling in microglia, but transmits an inflammatory signal in astrocytes (145). Wang et al. have reported that astrocytic FasL mediates the elimination of autoimmune T cells in the CNS, contributing to recovery from EAE (146). This regulatory role of FasL expressed in astrocytes was demonstrated using glial fibrillary acidic protein/Cre FasL (fl/fl) mice in which the *FasL* gene was selectively deleted in astrocytes. In contrast, a study by Okuda et al. showed a tissue destructive role of FasL in the acute phase of EAE (147). When neutralizing antibodies against FasL were injected intrathecally, the disease severity was attenuated, and neuroinflammation and myelin damage were reduced in the CNS.

TNF-LIKE WEAK INDUCER OF APOPTOSIS (TWEAK)

As a member of TNFRSF, fibroblast growth factor-inducible 14 (FN14) is expressed in various cell types, including lymphocytes, macrophages, endothelial cells, fibroblasts, and keratinocytes, particularly under conditions, such as inflammation and malignancy. Its ligand, TWEAK, is expressed in lymphocytes,

macrophages, NK cells, renal tubular epithelial cells, and glomerular mesangial cells (148–150). Interestingly, both TWEAK and FN14 are widely distributed among many tissue types after exposure to inflammation (151). As a result, the TWEAK/FN14 system has been shown to be involved in inflammation, angiogenesis, cell proliferation, and apoptosis and in various diseases, including SLE, renal damage, RA, cancer, and conditions associated with cutaneous inflammation (152–154). FN14-mediated forward signaling leads to NF-κB activation (155, 156).

TWEAK-mediated reverse signaling has not been reported. However, a naturally occurring fusion protein between the ICD of TWEAK and the receptor binding domain of APRIL, called TWE-PRIL (157–159), has been reported. Analysis of APRIL$^{-/-}$ mice (which lack APRIL and TWE-PRIL) demonstrated the involvement of TWE-PRIL reverse signaling in suppression of sympathetic axon growth and tissue innervation (160).

TWEAK mRNA expression has been detected in both microglia and astrocytes (161). FN14 expression has been reported in astrocytes; however, its expression has not been detected in brain microglia. The TWEAK/FN14 interaction has been implicated in CNS inflammation. Proliferation of FN14-expressing astrocytes is increased upon exposure to recombinant TWEAK protein. Moreover, *TWEAK* mRNA expression is enhanced in spinal cords during EAE, and disease severity is increased in transgenic mice overexpressing TWEAK. These results indicate that the TWEAK/Fn14 interaction in spinal glia is involved in CNS autoimmune inflammatory responses and can be targeted for EAE and MS therapy. Consistent with this, treatment of cultured human astrocytes with TWEAK increases ICAM expression and IL-6/IL-8 secretion, inducing reactive astrocyte-like characteristics (162). TWEAK also induces C-C motif chemokine ligand 2 (CCL2) release from astrocytes and endothelial cells in culture. Blockade of TWEAK/FN14 signaling inhibits TWEAK-induced CCL2 production and ameliorates EAE (163). Furthermore, administration of anti-TWEAK neutralizing antibodies reduces leukocyte infiltration and disease severity in EAE animals (164). These results are consistent with the pro-inflammatory and disease-promoting effects of TWEAK in CNS inflammation.

CD137L (4-1BBL)

CD137 (4-1BB), originally identified as a T-cell costimulatory molecule, is expressed in activated T cells, B cells, NK cells, neutrophils, macrophages, and DCs and functions to promote their effector functions (165–167). The ligand of CD137 (CD137L, 4-1BBL) was found to be expressed in B cells, macrophages, and DCs (168, 169). Although CD137- or CD137L-knockout mice show no severe defects, they have a higher sensitivity to viral infection (170). Treatment with agonistic anti-CD137 antibodies or CD137L-Fc fusion protein results in expansion of tumor-specific T cells and ameliorates experimental autoimmune encephalomyelitis through modulation of the balance between Th17 and Tregs (171, 172).

For CD137L-mediated reverse signaling, most studies have been conducted using monocytes/macrophages. Stimulation of peripheral blood monocytes or bone marrow-derived macrophages with anti-CD137L mAbs or CD137-Fc fusion protein triggers a robust proliferative response, enhances cell adhesion, and/or stimulates pro-inflammatory activation associated with phosphotyrosine-mediated signaling (173, 174). This proliferation-inducing effect of CD137L has been reported to be mediated by the AKT/mammalian target of rapamycin (mTOR) pathway, resulting in reprogramming of glucose metabolism in a way that supports energy demand and biomass production. CD137L stimulation increases glucose uptake and upregulates enzymes involved in glucose transport/lysis and lactate production. Expression of genes involved in the pentose phosphate pathway and lipogenesis is also enhanced (175).

Bone marrow macrophages can be differentiated into osteoclasts by M-CSF and RANKL treatment. This osteoclastogenic process is inhibited by additional treatment with immobilized CD137L-Fc fusion protein or recombinant CD137 (176). Various experiments investigating this reverse signaling pathway have indicated that the signaling pathway is mediated by MAPKs, AKT, mTOR, PI3K, PKA, C/EBP, and CREB, resulting in induction of IL-6 and TNF expression (174, 177–179). Recombinant CD137 treatment also inhibits phagocytosis and oxidative burst (180). Interestingly, the extracellular domain of CD137L has been reported to directly interact with TNFR1, and this interaction appears to be required for CD137L-mediated reverse signaling. As a consequence, treatment of monocytes with TNF augments CD137-induced IL-8 expression, and inhibition of TNFR1 using TNFR1-neutralizing antibodies results in inhibition of CD137L-mediated responses, such as cell adhesion, apoptosis, CD14 expression, and IL-8 production (181). Using a two-hybrid system in a mouse macrophages, a novel transmembrane protein TMEM126A was found to interact with CD137L, and knockdown of TMEM126A was shown to abolish CD137L-mediated induction of tyrosine phosphorylation and pro-inflammatory cytokines (182). These results suggested the complex nature of CD137L-mediated reverse signaling. Further studies are needed to fully elucidate these mechanisms.

CD137L-mediated reverse signaling enhances DC maturation and potentiates the ability of DCs to stimulate T cells (180, 183). In contrast, a recent report showed that blockade of CD137L-mediated reverse signaling resulted in promotion of intratumoral differentiation of IL-12-producing CD103^{+} DCs and type 1 tumor-associated macrophages, which are required for the generation of IFN-γ-producing CD8^{+} T cells (184).

Most recently, CD137-CD137L signaling has been implicated in the hypothalamic interglial crosstalk under obese conditions (185). Mice fed with high-fat diet (HFD) showed an enhanced expression of CD137 and CD137L in the brain hypothalamus (186). Treatment of cultured glial cells with obesity-related molecules including free fatty acid and glucose promoted the expression of CD137 in astrocytes and CD137L in microglia, respectively (186). While forward signaling through CD137

in astrocytes increased their reactivity, reverse signaling through CD137L in microglia augmented the secretion of proinflammatory mediators, such as MCP-1. These recent findings suggest that CD137-CD137L signaling mediates microglia-astrocyte crosstalk in hypothalamic inflammatory responses under obese conditions, and CD137L reverse signaling in microglia might be a potential therapeutic target for the suppression of obesity-induced hypothalamic inflammation and related metabolic diseases.

REVERSE SIGNALING INITIATED FROM OTHER TNFSF MEMBERS

The list of TNSF members that can induce reverse signaling is increasing as more studies focus on this aspect of the TNFSF/TNFRSF system. In the case of TNF, its ICD contains a nuclear localization signal sequence that can be liberated upon stimulation of the membrane-bound form of TNF (mTNF) with anti-TNF antibodies. This cleaved 10-kD fragment containing the ICD and TMD were found to be localized to internal membranes and nuclear fractions (187). Stimulation of mTNF with soluble TNFR increases intracellular calcium levels in RAW264.7 mouse monocytes (188). Additionally, soluble TNFR treatment causes changes in mTNF phosphorylation status, and casein kinase, which can phosphorylate the serine residues in the ICD of mTNF, has been implicated in this reverse signaling mechanism (188–190).

In monocyte/macrophage lineage cells, stimulation of mTNF with mAbs or soluble receptors leads to activation of MAPKs, particularly ERK, and the cells have been shown to be resistant to subsequent stimulation with LPS (191, 192). Other investigators have shown that stimulation of mTNF with anti-TNF antibodies results in internalization of the mTNF/anti-TNF complex into early endosomes and then lysosomes in macrophages and DCs (193). In addition, stimulation of synovial macrophages in RA joints with chimeric anti-TNF mAbs (infliximab) or soluble TNFR (etanercept) results in the induction of caspase-independent apoptotic cell death (194–196).

Some members of the TNFSF/TNFRSF show major crosstalk among ligand/receptor pairs. For example, DcR3, which is the counterpart of LIGHT, TNF-like ligand 1A (TL1A), and FasL, contains three conserved cysteine-rich domains characteristic of a TNFR (47, 197–199). Originally, DcR3 was thought to neutralize these members of the TNFSF through competition with their receptors. However, treatment of DCs with DcR3 modulates the differentiation and activation of DCs, which then directs naïve T cells to differentiate into a Th2 phenotype (200). In addition, monocytes and THP-1 cells respond to DcR3 treatment with induction of actin reorganization and enhancement of adhesion. Analysis of this reverse signaling revealed the involvement of protein kinase C (PKC), PI3K, focal adhesion kinase, and Src kinases (69). Additionally, the involvement of DcR3 in osteoclast development was also reported. When cells of monocyte/macrophage lineage were treated with DcR3, osteoclastogenesis was induced through MAPK signaling and TNF-α expression. These responses enhanced the development of osteoclast phenotypes, such as polynuclear giant morphology, bone resorption, and expression of tartrate-resistant acid phosphatase, CD51/61, and MMP-9 (201).

Of the three counterparts (LIGHT, TL1A, and FasL) of DcR3, reverse signaling has been reported for LIGHT and FasL, but not in TL1A. To date, there is no direct evidence supporting the generation of reverse signaling from TL1A. However, the expression of full-length TL1A has been shown to be correlated with the senescence of endothelial cells, and knockdown of TL1A expression has been shown to reverse the senescence phenotype (202). In a murine colitis model, cell surface expression levels of TL1A were found to be related to the suppressive activity of Tregs in a DR3-dependent manner, suggesting that the strength of signaling initiated from TL1A closely regulates Treg activity (203).

OX40 (CD134) is mainly expressed in activated T cells and acts as a costimulatory molecule for receiving activation and survival signals (204–206). The ligand of OX40 (OX40L) is mainly expressed in T and B cells, activated macrophages and DCs, and endothelial cells (207–209). The OX40/OX40L system has been implicated in T-cell costimulation, Treg generation, cell adhesion, and extravasation of T cells (210–214).

When B cells are co-incubated with OX40-expressing T cells or stimulated with soluble OX40, OX40L-mediated reverse signaling is induced, and the B cells undergo terminal differentiation into plasma cells. Because T cells are also activated through this interaction, the OX40/OX40L interaction appears to induce bidirectional signaling events (215, 216). A recent analysis of OX40L expression levels in B cells from patients with allergic rhinitis indicated that OX40L expression is positively correlated with allergic markers, such as serum levels of IgE and IL-4 (217).

In freshly isolated human blood DCs, mAb-mediated crosslinking of OX40L enhances CD40L-mediated expression of IL-12. In DCs derived from monocytes with IL-4 and granulocyte-macrophage CSF treatment, ligation of OX40L enhances the production of pro-inflammatory cytokines (e.g., TNF-α, IL-12 p40, IL-1β, and IL-6) and the expression of DC activation markers (e.g., CD83, CD80, CD86, CD54, and CD40) (218). Although the signaling pathway has not been elucidated, these data clearly support the role of OX40L-mediated reverse signaling in DC activation and maturation.

As a ligand of CD40, CD40L (CD154, gp39) is expressed in and activates T cells, B cells, DCs, macrophages, smooth muscle cells, endothelial cells, and platelets. CD40 expression has been detected in B cells, monocyte/macrophages, DCs, mast cells, fibroblasts, and endothelial cells (219–221). The CD40/CD40L system is important for activation of B cells and subsequent differentiation of these cells into plasma cells and stimulation of immunoglobulin class switching. In addition, this system is also involved in T-cell priming, T cell-mediated effector functions, macrophage/NK cell/endothelial cell activation, organ-specific autoimmune diseases, graft rejection, and atherosclerosis (222–225).

CD40L-mediated reverse signaling has been studied in CD40-knockout mice, in which defective germinal center development

and antibody production were restored by soluble CD40 treatment. Additionally, reverse signaling was also found to be required for acquisition of B-cell activating potential (226). Although its ICD is only 22 amino acids, this region is highly conserved across various species and generates signaling through Lck, Rac1, MAPKs, and PKC in T cells (227–229). The presence of CD40 and CD40L in lipid rafts has also been reported and could explain the ability of these proteins to generate signaling (230, 231).

The expression of RANK has been detected mainly in osteoclasts and their precursors, DCs, and activated T and B cells. In addition, RANK expression has also been detected in a wide variety of tissues. The ligand of RANK (RANKL, also known as TRANCE) has been detected at osteoblasts, T cells, and stromal cells (232). The interactions between RANK and RANKL can be regulated by the decoy receptor OPG, which has affinity for both RANKL and TNF-related apoptosis-inducing ligand (233, 234). The RANK/RANKL/OPG system is involved in osteoclast differentiation/activation, bone remodeling, immune cell function, lymph node development, thermal regulation, and mammary gland development (235–238).

A few reports have provided evidence of RANKL-mediated reverse signaling. The expression of both RANK and RANKL has been detected in B chronic lymphocytic leukemia cells. Treatment of these cells with RANK-Fc fusion protein, but not with RANKL-Fc fusion protein, results in potent enhancement of IL-8 expression (239). Additionally, immobilization of RANK-Fc fusion protein augments IFN-γ secretion by Th1 cells in a p38 MAPK-dependent manner. Addition of RANK-Fc fusion protein during coculture of Th1 cells with antigen-presenting cells results in suppression of IFN-γ expression from Th1 cells, probably by blocking the interaction between RANK and RANKL (240). Moreover, osteoblasts, which express RANKL, regulate the differentiation and activation of osteoclasts and their precursors through the interactions of RANK and RANKL. Recent reports, however, have shown that reverse signaling initiated from RANKL is also possible. Soluble RANK treatment enhances p38-mediated mineralization of osteoblasts, which is abolished by knockdown of RANKL. When co-incubated with osteoclasts, osteoblasts respond by increasing p38 MAPK phosphorylation levels, and this response is blocked by abundant soluble RANKL (241).

CD30 is expressed in activated T and B cells and is a clinical marker for Hodgkin's lymphoma and related malignancies (242, 243). Interestingly, crosslinking of surface CD30 can activate latent human immunodeficiency virus in T cells (244, 245). CD30-mediated signaling has costimulatory effects in T and B cells, and serum levels of soluble CD30 serve as a prognostic marker of Hodgkin's disease and acquired immunodeficiency syndrome (246–248). The ligand of CD30 (CD30L, CD153) is expressed in activated T cells, B cells, and neutrophils. When peripheral blood neutrophils were stimulated with CD30L-specific mAbs or CD30-Fc fusion protein, cells responded by IL-8 production and oxidative burst. Peripheral blood T cells also responded to anti-CD3 and anti-CD30L antibody co-treatment by increasing metabolic activity, proliferation, and IL-6 production (249). According to a recent report, IgD^+ IgM^+ B cells express CD30L after activation with CD40L, IL-4, and specific antigen. Additional treatment with anti-CD30L antibodies or CD30-Fc fusion protein inhibits CD40-mediated signaling through TRAF2 and NF-κB, which results in reductions in class switch DNA recombination and subsequent production of IgG, IgA, and IgE (250).

The expression of CD27L (the ligand of CD27, CD70) has been detected in T cells, B cells, and NK cells. CD27 serves as a T-cell costimulatory molecule that enhances T-cell receptor-mediated signaling, proliferation, differentiation, and effector functions. The ligand of CD27 (CD27L, CD70) can be detected in lymphocytes, NK cells, and subsets of DCs (251–255). There have been numerous reports on the role of CD27-mediated forward signaling in the activation of T cells, B cells, and NK cells; however, few reports have demonstrated the existence of CD27L-mediated reverse signaling. In a study that explored the immune surveillance function of NK cells in cancer, B cells expressing cytoplasmic deletion mutant of CD27 were implanted in a B-cell acute lymphoblastic leukemia xenotransplant model. Expression of a truncation mutant in malignant cells increased the number of tumor-infiltrating IFN-γ-producing NK cells. Further analysis indicated that signaling mediated by CD70 on NK cells was transduced by AKT signaling and enhanced the survival and effector function of NK cells (256). In an earlier investigation, a subset of leukemic B cells was found to express CD27L, and stimulation with this ligand using specific mAbs resulted in enhanced cell proliferation. Furthermore, the proliferative response was synergistically enhanced by CD40 ligation (257).

FUTURE PERSPECTIVES

Although many studies have demonstrated the existence of reverse signaling initiated from TNFSF, it is still unclear how such signals are actually generated. The main reason for this is the short ICDs of these molecules, which usually lack any known signaling motifs. One exception is FasL, which contains several known protein motifs that can interact with multiple signaling adaptors. Although most members of TNFSF has short ICDs with lack of known signaling motifs, the high level conservation of ICD among various mammalian species and its uniqueness in each members of the TNFSF supports that these ICDs are involved in signal generation through yet unidentified mechanism. Bidirectional activation and possible crosstalk among signaling generated from these molecules are expected to generate a complex signaling network that regulates macrophage activity. Further studies are needed to explore this aspect of macrophage regulation.

Many antibody-based therapeutic approaches target the ligand part of TNFSF/TNFRSF system and aim for the blocking of receptor-mediated forward signaling. Some of these agents were proven to be effective for blocking the interaction between cognate ligand and receptor and thus the induction of forward signaling, which manifested in the alleviation of the severity of target disease(s). However, agents targeting the ligand itself or mimicking soluble receptor have the risk of activating membrane-bound form of ligands and, subsequently, generate

the reverse signaling. These unwanted effects may degrade the therapeutic potential of the agents and may be able to explain some of the side effects observed during clinical trials. Additionally, it is also possible to develop agents that aim for the blockage of reverse signaling in the future.

Finally, the roles of the TNFSF/TNFRSF in CNS inflammation are complex and can be pro-inflammatory or anti-inflammatory depending on the context. Different members of the TNFSF and their receptors are expressed in distinct types of brain glial cells and neurons and exert context-dependent effects on neuroinflammation. Because the expression of these TNFSF members is dynamically regulated under a diverse CNS milieu, their functional roles may be modulated accordingly, with spatiotemporal regulation of the crosstalk of different TNFSF/TNFRSF members. Given the critical role of these TNFSF/TNFRSF members in regulating neuroinflammation, TNFSF/TNFRSF members and related signaling pathways can be potential drug targets for the control of neuroinflammation and the treatment of related diseases in the CNS. However, the benefits and challenges of such an approach must be weighed carefully given the multiple cell-cell interactions that might be affected. Compared with forward signaling of the TNFRSF, little is known about the reverse signaling through the TNFSF. Thus, further studies are needed to better understand reverse signaling pathways in brain glial cells and to determine the therapeutic applications of these pathways in the field of CNS inflammation. Finally, targeting forward and reverse signaling may have its own advantages and disadvantages depending on specific TNFSF and TNFRSF members; therefore, a combination of both is likely to be useful in the clinical settings.

AUTHOR CONTRIBUTIONS

All authors have made a substantial intellectual contribution to this work and approved submission of the manuscript. W-HL and KS formulated the focus of the review. DS and S-GL conducted the literature review and participated in the discussion. W-HL and KS wrote the manuscript.

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REVIEW
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Phagocytosis in the Brain: Homeostasis and Disease

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Microglia are resident macrophages of the central nervous system and significantly contribute to overall brain function by participating in phagocytosis during development, homeostasis, and diseased states. Phagocytosis is a highly complex process that is specialized for the uptake and removal of opsonized and non-opsonized targets, such as pathogens, apoptotic cells, and cellular debris. While the role of phagocytosis in mediating classical innate and adaptive immune responses has been known for decades, it is now appreciated that phagocytosis is also critical throughout early neural development, homeostasis, and initiating repair mechanisms. As such, modulating phagocytic processes has provided unexplored avenues with the intent of developing novel therapeutics that promote repair and regeneration in the CNS. Here, we review the functional consequences that phagocytosis plays in both the healthy and diseased CNS, and summarize how phagocytosis contributes to overall pathophysiological mechanisms involved in brain injury and repair.

Keywords: phagocytosis, microglia, macrophage, neurodegeneration, neuroinflammation

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INTRODUCTION

Phagocytosis is the process through which cells recognize, engulf, and digest large particles (>0.5 microns), including, but not limited to, bacteria, apoptotic cells, and cell debris. Phagocytosis is a receptor-mediated process involving three major steps: "find me," "eat me," and "digest me," with each of these steps being regulated by multiple receptors, unique molecules, and signaling pathways. Specific receptors involved in phagocytosis can be either opsonic (i.e., Fc receptors, complement receptors) or non-opsonic (i.e., C-type lectin receptors, phosphatidylserine receptors). Following recognition by phagocytic receptors, the plasma membrane extends around the phagocytic target in an actin-dependent manner, with particles ultimately being enclosed within a vesicular phagosome. Following formation, this nascent phagosome proceeds through a series of maturation steps, culminating in fusion with lysosomes (phagolysosome) for the eventual destruction of the phagocytosed particles. Importantly, following destruction, byproducts must be effectively dealt with by the phagocytic cell, either through storage, recycling or efflux mechanisms. The basic cell biology of phagocytosis has been extensively reviewed elsewhere (1).

Adding additional complexity to phagocytosis is the requirement for specific outcomes in the context of different phagocytic targets. For example, while recognition and phagocytosis of bacteria requires rapid induction of proinflammatory responses, a similar reaction to apoptotic cells induces detrimental autoinflammation (2). As such, specific immune responses to phagocytic targets are tailored to by a variety of context-dependent signals, including the engagement of phagocytic receptors that utilize distinct inflammatory signaling pathways (pro vs. anti-inflammatory) and microenvironment-derived signals that promote quiescence or inflammation (3, 4).

Within the CNS, phagocytosis is a critical process required for proper neural circuit development and maintaining homeostasis. To assist in maintaining homeostasis in the CNS, synapses, apoptotic cells, and debris must be continuously removed to maintain optimal neural function. While phagocytosis is primarily attributed to microglia (the professional phagocytes in the CNS), non-professional phagocytes (e.g., astrocytes or oligodendrocytes) may also participate (5). Arising from discrete pathologies, specific phagocytic targets, such as insoluble protein aggregates or myelin debris add further burden to the phagocytic machinery within the CNS. It has been hypothesized that failures during phagocytic processes may actually promote inflammation and/or neurodegenerative processes. Herein, we review how phagocytosis contributes to both the maintenance of homeostasis and disease within the CNS (**Figure 1**).

PHAGOCYTOSIS IN CNS DEVELOPMENT AND HOMEOSTASIS

Synapse Elimination

Within the developing CNS, phagocytosis is necessary for the refinement of synaptic connectivity, as the developing CNS overproduces both neurons and synapses (6). Furthermore, the removal of unwanted synapses refines neural networks, thus contributing to learning and memory (7). Microglia are important cells that execute the pruning of synaptic connections, utilizing immune signaling pathways, such as the complement pathway, although other signaling pathways also contribute (8). Microglia continuously survey the brain's parenchyma (9) and make frequent physical contacts with synapses that are mediated in part by sensing neuronal activity via nucleotides, such as ATP or ADP, and suggest that nucleotides may act as "find me" signals that guide microglial processes toward active synapses (10, 11). Studies investigating synapse pruning have relied extensively on the developing visual system, which is a well-defined sensory system that allows for easy manipulation. Modulation of neuronal activity within the visual system using visual deprivation (to reduce the frequency of action potentials) has demonstrated that neuronal activity is essential for synaptic pruning by microglia (12). Consistent with the role of nucleotides serving as a "find me" signal within the CNS, mice lacking the ADP receptor P2Y12, or pharmacological blockade of P2Y12 signaling, result in impaired synapse pruning within the developing visual cortex (13). Additionally, CX3CR1 knockout mice demonstrate increased hippocampal spine density during development (14), suggesting CX3CL1 may also act as a "find me" signal regulating synapse pruning. However, CX3CL1 is dispensable for synapse pruning within the developing visual system (15). In regards to recognition and engulfment of synapses, the classical complement system has been extensively studied. Briefly, the classical complement system functions via tagging of targets with C1q, which catalyzes the production of C3 convertase. C3 convertase subsequently cleaves C3 producing both C3a (a pro-inflammatory mediator) and C3b, an opsonin that triggers phagocytosis via complement receptors on phagocytes. Early reports demonstrated that the classical complement components C1q and C3 tag synapses for phagocytosis and are required for proper refinement of the developing lateral geniculate (16). Additional reports observed that microglial complement receptor 3 (CR3) is required for the clearance of complement tagged synapses (17). Highlighting the relevance of complement in synapse pruning during development, mice lacking C1q exhibit spontaneous seizure activity as a result of impaired synapse removal (18). The importance of C1q in tagging synapse (19) for elimination is supported by the finding of local apoptotic mechanisms within presynaptic elements that results in C1q accumulation (20). Recent work leveraging the power of correlative light and electron microscopy (CLEM) and live-cell imaging demonstrated that microglia frequently contact synapses within the healthy brain and can be seen engulfing presynaptic, but not post-synaptic, elements via a specialized form of phagocytosis termed trogocytosis, which results in the partial removal of cell constituents (21). While supporting the notion that microglia remove synaptic elements, this study fails to demonstrate that microglia actively phagocytose entire synapses, and further, the authors observed no role for the receptor CR3 in this process. In addition to the classical complement system, both TREM2 and CD47-SIRPα signaling contribute to synapse pruning by microglia (22, 23), although these pathways have been less extensively studied.

In addition to microglia, astrocytes also participate in refining synaptic connectivity (24, 25). It was initially reported that astrocytes participate in the phagocytosis of synapses utilizing the receptors MerTK and MEGF10, whereby astrocyte-specific deletion of these receptors results in a failure to refine synapses in the developing visual system (26). Furthermore, the ability of human astrocytes to phagocytose synapses in dissociated cultures and cerebral organoids has been demonstrated (27, 28), suggesting astrocytes actively prune synapses in the human CNS. Additionally, the Alzheimer-associated gene ApoE regulates the phagocytic capacity of astrocytes and C1q accumulation on synapses during aging (29). It has also been reported that sleep deprivation causes increased synaptic pruning by astrocytes, likely mediated by MerTK (30).

Removal of Apoptotic Cells

Apoptotic cells are constantly generated and phagocytosed throughout the nervous system during both development and homeostasis. Within the subgranular zone (SGZ) and subventricular zone (SVZ), the major regions containing neural progenitor cells (NPCs), microglia are required to constantly phagocytose apoptotic NPCs throughout the lifespan of the organism. Despite the majority of newborn cells in neurogenic niches undergoing apoptosis, identification of apoptotic cells is difficult due to their rapid clearance by microglia. During inflammatory insult, increased apoptosis of NPCs is coupled to increased phagocytosis by SGZ microglia, suggesting that microglia continue to remove apoptotic progenitors regardless of inflammatory status (31). Mechanistically, the phagocytosis of apoptotic NPCs appears to depend on the TAM family receptors MerTK and AXL, as evidenced by a buildup of apoptotic within the SVZ when these receptors are genetically deleted (32).

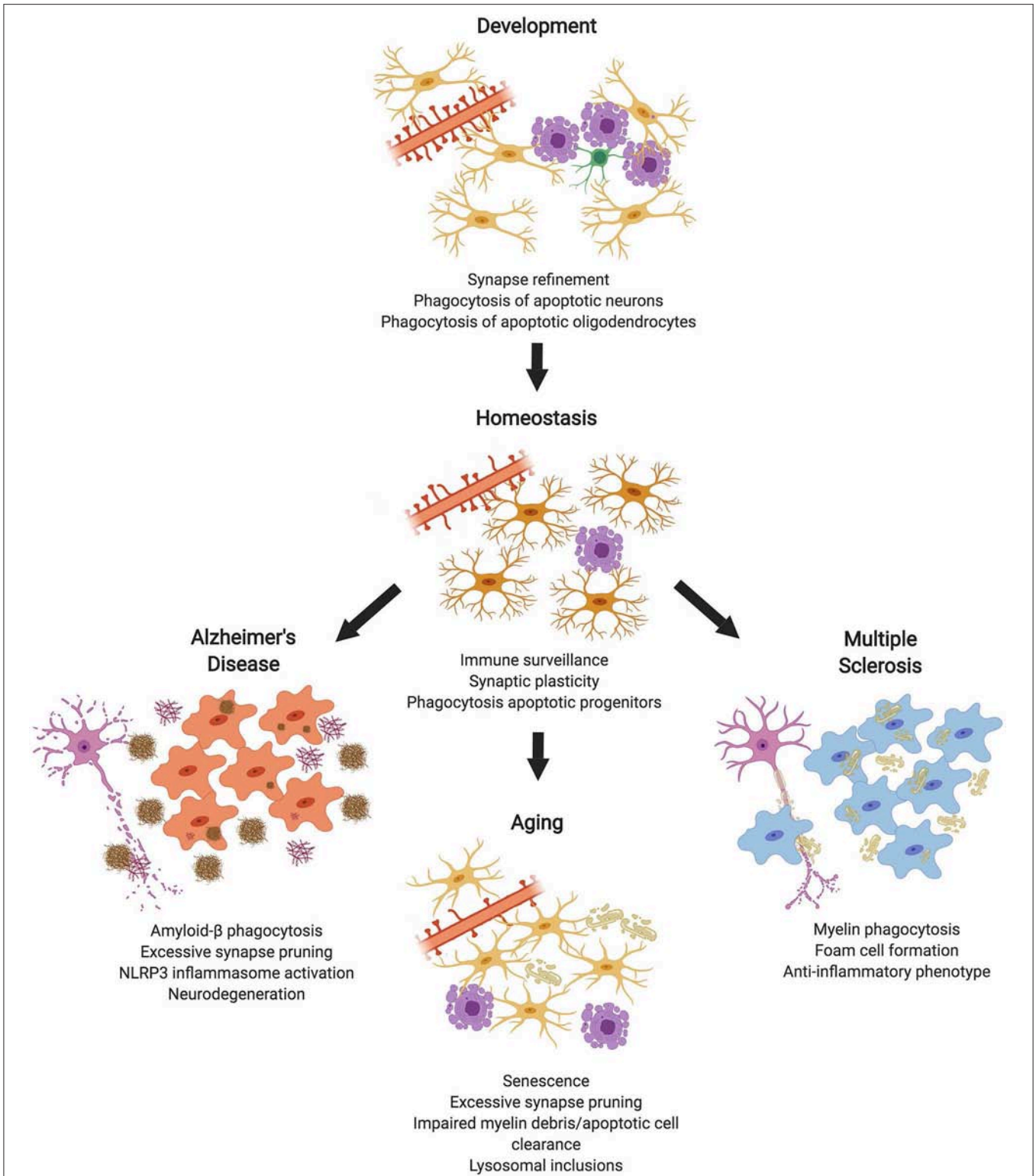

FIGURE 1 | Microglial Phagocytosis in the CNS. During development, microglial phagocytosis is essential for the refinement of excessive synapses, as well as the removal of apoptotic neurons and oligodendrocytes that are overproduced during development. Homeostatic microglia in the adult brain constantly survey the brain's parenchyma, contributing to synaptic plasticity and phagocytosing apoptotic progenitor cells. With advanced age, microglia undergo senescence, display impaired debris clearance, and excessively prune synapses. In diseases, such as Alzheimer's or multiple sclerosis, microglia act as key contributors to pathology, which is partially mediated by phagocytosis of substrates, such as amyloid-β or myelin debris (made in ©BioRender - biorender.com).

TABLE 1 | Current evidence of phagocytosis alterations resulting from variants in disease-associated genes expressed in microglia.

Gene	Associated diseases	Models	Alterations to phagocytosis	References
TREM2	Alzheimer's disease (48, 49) Frontotemporal dementia (50) Parkinson's disease (50) Nasu-Hakola disease (51)	Primary microglia from TREM2$^{-/-}$ mice	Decreased phagocytosis of Aβ relative to WT microglia	(52)
			Reduced uptake of Aβ-lipoprotein complexes compared with WT and TREM2$^{+/-}$	(53)
			Reduced uptake of *E. coli* particles compared with WT controls	(54)
		Human monocyte-derived macrophages from heterozygous carriers of the TREM2 R62H AD-associated variant	Reduced uptake of Aβ-lipoprotein complexes compared with non-carriers	(53)
		shRNA knockdown of TREM2 expression in primary mouse microglia	Reduced uptake of apoptotic neuronal membranes vs. control shRNA treated cells	(36)
		Immunohistochemical analysis of 5XFAD/TREM2$^{-/-}$ mice	Decreased levels of Aβ within microglial phagosomes vs. WT. Haplodeficient TREM2$^{+/-}$ mice showed no significant reductions in Aβ uptake	(55, 56)
			Increased Aβ load in hippocampus of TREM2 knockout mice	(52)
		Immunohistochemical analysis of APPPS1-21/TREM2$^{-/-}$ mice	Decreased Aβ load in hippocampus of TREM2 knockout mice vs. WT at 2 months	(57)
			Decreased Aβ load in hippocampus of TREM2 knockout mice vs. WT at 4 months	(58)
			Increased Aβ load in hippocampus of TREM2 knockout mice vs. WT at 8 months	
		Immunohistochemical analysis of APPPS1-21/TREM2$^{+/-}$ mice	No difference in Aβ plaque load between WT and TREM2$^{+/-}$ mice at 3 or 7 months old	(59)
		iPSC-derived microglia-like cells from carriers of TREM2 T66M and W50C variants	Decreased uptake of apoptotic neurons by TREM2 variant cells than by controls	(60)
		Non-phagocytic CHO cells transfected with TREM2	TREM2-CHO cells were capable of phagocytosing apoptotic neuronal cells	(61)
CD33	Alzheimer's disease (62, 63)	Primary microglia from CD33$^{-/-}$ mice	Increased uptake of Aβ compared with WT microglia	(64)
		CD33 overexpression in BV2 mouse microglial cell line	Decreased uptake of Aβ compared with control BV2 cells	(64)
		Frontal cortex samples from carriers of protective minor allele SNP rs3865444	Decreased formic acid-soluble Aβ42 levels in carriers of rs3865444 minor (T) allele than in major allele carriers	(64)
TM2D3	Alzheimer's disease (65)	CRISPR-Cas9 knockout in primary human macrophages and U937 human myeloid cell line	Decreased uptake of Aβ and synaptosomes compared with WT	(66)
PU.1	Alzheimer's disease (67)	siRNA knockdown of PU.1 in adult human microglia	Reduced phagocytosis of Aβ compared with controls	(68)
α-Synuclein	Parkinson's disease (69)	Human iPSC-derived macrophages from PD patients carrying SNCA triplication mutations	Increased release of α-synuclein and reduced phagocytosis capability compared with controls	(70)

(Continued)

TABLE 1 | Continued

Gene	Associated diseases	Models	Alterations to phagocytosis	References
Progranulin	Frontotemporal dementia (71, 72), Alzheimer's disease (73, 74)	Microglia specific progranulin knockout in AD mice ($Grn^{flox/flox}$/$PDAPP_{Sw,Ind}$ J20)	Decreased microglial phagocytosis of fluorescent beads in acute brain slices and increased hippocampal Aβ plaque-load vs. WT progranulin AD mice	(75)
DAP12	Nasu-Hakola disease (51)	Primary mouse microglia transfected with mutant DAP12 (lack ITAM signaling motif)	Mutant DAP12 microglia phagocytosed less apoptotic neuronal material than control cells	(36)
		Bone marrow-derived macrophages from $DAP12^{-/-}$ mice	Reduced phagocytosis of bacteria	(76)
LRRK2	Parkinson's disease (77, 78)	Microglia and BMDMs from $Lrrk2^{-/-}$ mice	Reduced uptake of latex beads and *E. coli* bioparticles by primary microglia and BMDMs from knockout mice vs. WT.	(79)
			Decreased uptake of beads after injection into midbrain in $Lrrk2^{-/-}$ mice compared with controls	
MerTK	Multiple sclerosis (80, 81)	*in vitro* human microglia and macrophages	Pharmacological blockade of MerTK inhibits myelin phagocytosis *in vitro*	(82)
			MS patient macrophages display reduced expression of MerTK	(83)

Interestingly, NPCs may also participate in the phagocytosis of neighboring apoptotic cells and may be required for efficient neurogenesis (33). Apoptotic neurons and oligodendrocytes are also generated throughout development, and phagocytosis is required to clear these cells (34–36). The phagocytosis of apoptotic neurons depends on receptors including TREM2, CD11b, BAI1 and TIM-4, as well as the v-ATPase transporter that is required for the degradation of apoptotic corpses (36–39). Importantly, recent work has identified a novel microglia subset associated with developmental white matter that is specialized for the phagocytosis and removal of apoptotic oligodendrocytes (40). Thus, it appears that microglia may acquire distinct phenotypes that are required for region-specific phagocytic functions which may not be extrapolated from one region to another.

Phagocytosis During Aging

Within the aging CNS, there is abundant synapse loss and myelin degeneration which is believed to contribute to age-related cognitive decline (41, 42). Microglia- and complement-mediated synapse elimination has been suggested to underlie excessive synapse elimination during normal aging. Increases in complement protein C1q are observed throughout the aged brain, and knockout of C1q prevents age-related cognitive decline, suggesting that excessive synapse pruning during aging potentiates cognitive decline (19). Genetic deletion of the complement component C3 reduces synapse loss and cognitive decline in aged mice, further implicating excessive complement-dependent synapse elimination as a key contributor to age-related cognitive impairment (43). In regards to myelin degeneration, ultrastructural analysis and *in vivo* imaging has demonstrated that large amounts of myelin debris are generated during normal aging, and this debris is continuously phagocytosed by microglia (44, 45). With advanced age, clearance of myelin debris becomes impaired, resulting in insoluble intracellular aggregates (lipofuscin granules) within microglia and microglial senescence (46). This microglial dysfunction is exacerbated by increased production of myelin debris or impairment of lysosomal processing; indicating pathways downstream of engulfment are essential to effectively deal with myelin debris in the healthy CNS. While microglial phagocytosis appears to be an important contributor to the aging brain, further investigations are needed to confirm and expand on these findings.

PHAGOCYTOSIS IN DISEASE STATES

As we begin to understand the roles of microglia in the development and homeostasis of the CNS, it has become increasingly evident that changes in the key functions of these highly active cells can exert significant effects on the progression of multiple CNS diseases (47) (**Table 1**).

Acute Injury

The cellular response to acute CNS injury, such as traumatic brain injury (TBI), spinal cord injury (SCI), and stroke is multiphasic and has been studied in a range of models. The initial phase involves rapid activation of CNS-resident microglia (9, 84, 85) resulting in pro-inflammatory cytokine release and recruitment of peripheral immune cells, including neutrophils, monocytes, and monocyte-derived macrophages (MDMs), to the lesion site (86, 87). This

early response by microglia limits the spread of the lesion (85, 88, 89) but also generates inflammatory cytokines and reactive oxygen species (ROS), which may be detrimental to recovery and contribute to secondary injury if not resolved (90).

Phagocytosis, initially performed by microglia and subsequently by recruited MDMs, acts to restore homeostasis and minimize chronic activation (91). Phagocytosis of apoptotic cells prevents the release of cytotoxic and immunogenic intracellular contents (92, 93) and the removal of damaged myelin has been shown to be important for axon regeneration and remyelination (94–96). Additionally, live neurons may be phagocytosed by microglia during injury (97), inducing a form of cell death termed phagoptosis, which contributes to neuronal cell death during pathological states (98, 99). Importantly, the presence of "don't eat me" signals, primarily CD47-SIRPα or CD200-CD200L signaling, from neurons to microglia suppresses aberrant phagocytosis and is critical for maintaining microglia in a quiescent state [reviewed in (100)].

Recruited MDMs play an important role in post-injury clearance. In a model of cerebral ischemia in mice, MDMs, once localized to the site of injury, have been shown to have a higher phagocytic capacity than microglia (101). However, as demonstrated in a SCI model, recruited MDMs are less capable of processing phagocytic material intracellularly and are also more susceptible to apoptotic and necrotic cell death (102). Therefore, although peripherally-derived MDMs take up more debris post-injury, the inefficient processing of phagocytosed material results in cell stress, which ultimately contributes to the inflammatory milieu.

Whether the activity of infiltrating MDMs following CNS injury is beneficial for repair or a detrimental contributor to inflammation is still disputed (103–106). Following disease onset in models of stroke and SCI, infiltrating MDMs have been found to alter microglial gene expression, downregulating both beneficial (phagocytosis) and neurotoxic (pro-inflammatory) functions (102, 107, 108). Indeed, blockade of MDM infiltration or MDM ablation has been shown to be beneficial in SCI (109–111) and TBI (112, 113). Conversely, other models suggest that preventing the infiltration and functioning of certain MDMs populations in CNS injury results in worsened outcomes (107, 114–116). Contradictory data may be due to inconsistent spatial and temporal assessment of inflammation, phagocytic activity and outcomes in the various models (117) as well as difficulties in differentiating CNS-resident from infiltrating myeloid cells. The use of double transgenic models, such as the $Cx3cr1^{GFP/+}Ccr2^{RFP/+}$ mouse, would enable improved distinction between phagocytosis performed by resident microglia and infiltrating monocytes, as used to differentiate these myeloid cell types in studies of stroke (118, 119), SCI (105) and inflammation (120).

Multiple Sclerosis

Destruction of myelin sheaths within the CNS, as occurs in multiple sclerosis (MS), produces degenerating myelin at sites of injury and inflammation. This degenerating myelin, termed myelin debris, must be cleared from sites of injury to promote timely repair. CNS (but not PNS) myelin acts as a potent inhibitor of oligodendrocyte differentiation (121), and the introduction of exogenous myelin into demyelinated lesions halts oligodendrocyte differentiation at the pre-myelinating stage (122). The removal of myelin debris within MS lesions and experimental animal models, such as experimental autoimmune encephalomyelitis (EAE) or cuprizone-induced demyelination, is primarily mediated by microglia and macrophages. Resident microglia and peripheral macrophages are capable of phagocytosing and degrading large quantities of myelin as highlighted by rapid clearance of myelin debris in animal models, although myelin debris can persist in MS patient lesions (123). Microglia and macrophages differ in their ability to uptake myelin. Specifically, resident microglia demonstrate a greater ability to engulf myelin then peripheral macrophages (124–126), and are more resistant to apoptosis following myelin phagocytosis (102), indicating microglia are more efficient at both engulfing and degrading myelin debris. The mechanism underlying this difference is unknown, although both ontogeny and exposure to the CNS microenvironment likely contribute. For example, astrocyte-conditioned media has been shown to promote myelin phagocytosis by macrophages and microglia *in vitro* (127, 128), suggesting the CNS microenvironment programs myeloid cells for efficient myelin phagocytosis. The phenotype of myeloid cells (pro-inflammatory vs. reparative) also has a large influence on the phagocytic ability of myeloid cells, as inflammatory myeloid cells (e.g., LPS stimulated) display reduced myelin phagocytosis in comparison to reparative, anti-inflammatory myeloid cells (e.g., IL-4 stimulated) (126).

Early investigations into myelin phagocytosis examined the effects of opsonization, demonstrating that both immunoglobulins and complement proteins promote the phagocytosis of myelin, and blocking Fc or complement receptors reduced myelin phagocytosis *in vitro* (125, 129, 130). In addition, evidence from MS lesions suggests that Fc receptors and complement play active roles in myelin phagocytosis (131). Interestingly, myelin debris is capable of activating complement in the absence of myelin reactive antibodies (132). Furthermore, myelin phagocytosis *in vitro* relies on scavenger and C-type lectin receptors for recognition and internalization of myelin debris (125, 133). More recently, the TAM family receptors MerTK and AXL, which bind phosphatidylserine via the bridging molecules Protein S and Gas6, respectively, have been identified as essential regulators of myelin phagocytosis. Within the EAE animal model, deletion of AXL results in increased clinical severity and impaired myelin clearance, while delivery of exogenous Gas6 is protective (134). Loss of AXL/Gas6 during cuprizone-induced demyelination results in increased neuroinflammation and impaired remyelination, indicating that signaling via the apoptotic cell receptor AXL is required to promote the resolution of inflammation following demyelination (135). Studies utilizing human macrophages and microglia have demonstrated that MerTK is an essential phagocytic receptor for myelin, expression of MerTK correlates with myelin phagocytosis *in vitro*, and MerTK levels are reduced within MS

patient macrophages (82, 83). Polymorphisms within the MerTK gene are associated with MS susceptibility, suggesting MerTK plays an important role in the development of MS (80, 81). Finally, TREM2 has also been implicated in myelin phagocytosis. TREM2 binds myelin lipids directly to facilitate internalization, and studies using the EAE animal model observed that blockade of TREM2 increases EAE severity, while TREM2 overexpression is protective, in part mediated by effects on clearance of myelin debris (136–138). Moreover, TREM2 KO mice display faulty myelin debris clearance and remyelination in the cuprizone model of toxic demyelination, with TREM2 knockout microglia failing to upregulation genes associated with phagocytosis and lipid metabolism (138, 139).

In addition to myeloid cells, astrocytes have been observed to engulf myelin debris. Within MS lesions, hypertrophic astrocytes contain myelin inclusions (140), and astrocytes have been demonstrated to uptake myelin debris *in vitro* (141). Transcriptomic analysis of astrocytes reveals expression of several complete phagocytic pathways and apoptotic cell receptors, such as MerTK, AXL and LRP1 (142). This is supported by the description of neurotoxic "A1" astrocytes, which downregulate phagocytic receptors including MerTK and show impaired myelin phagocytosis *in vitro* (143). Astrocytes phagocytose myelin at quantities several-fold lower than myeloid cells (144), questioning the functional significance of astrocyte-mediated myelin phagocytosis *in vivo*. Recently, phagocytosis of myelin by astrocytes has been shown to induce the expression of multiple chemokines both *in vitro* and *in vivo* (145), suggesting that astrocytes sense myelin debris and respond by recruiting professional phagocytes to sites of injury. These results are in line with the demonstration that astrocyte ablation impairs myeloid cell recruitment and phagocytosis in the cuprizone model, partly due to lack of CXCL10 expression (146).

Alzheimer's Disease and CNS Phagocytosis During Aging

Alzheimer's disease (AD) is characterized by the accumulation of extracellular plaques of toxic amyloid-beta (Aβ) and intracellular neurofibrillary tangles. The amyloid cascade hypothesis posits that an imbalance between the production and the clearance of Aβ initiates the pathological cascade of synapse loss, neuron death, and brain atrophy found in AD (147). The contribution of microglia to AD pathogenesis is becoming increasingly recognized as genome wide association studies (GWAS) and transcriptomic analyses highlight links between microglial genes and AD risk, as well as between microglial signaling pathways and disease progression (47).

In the case of the more common, late onset AD (LOAD), it has been argued that impairment in the clearance of Aβ has a greater impact on disease progression than its overproduction (148). As the primary resident phagocytes of the CNS, microglia play an important role in preventing the accumulation of this toxic protein through both phagocytosis and the production of degrading intra- and extracellular enzymes (149). *In vitro*, Aβ initiates cell stress responses, synapse loss, mitochondrial dysfunction, and neuronal apoptosis. In the early stages of AD, microglial function is neuroprotective, acting to clear apoptotic cells and pathological protein aggregates (150) as well as forming a barrier around plaques to restrict their growth and diffusion of synaptotoxic Aβ oligomers (151, 152). However, long term exposure to Aβ induces chronic microglial activation-associated dysfunction known as reactive microgliosis (153), in which phenotypic changes result in the adoption of a continuous pro-inflammatory status and compromised phagocytosis (154–156).

It is important to note that many *in vitro* and *in vivo* phagocytosis assays rely heavily on determining the uptake, but not measuring the subsequent degradation, of pathogenic proteins. In order to prevent intracellular accumulation of Aβ, it must be appropriately degraded and cleared through the endosome-lysosomal pathway (157, 158). The uptake of fibrillar and soluble Aβ has been reported in multiple models of microglia *in vitro* and *in vivo*, however, whether complete degradation of this protein, in particular the fibrillar (f) form, occurs remains disputed. Following *in vitro* culture of control microglia with fAβ, phagosomes have been found to contain incompletely degraded Aβ for up to 20 days (159–161). It has been reported that acidification of microglial lysosomes, for example by treatment with MCSF, can improve the efficiency of intracellular fAβ breakdown (162, 163).

The presence of complement activation in AD pathology has been observed for several decades (164, 165) however, it is only recently that genetic analyses have identified complement components as playing a role in AD pathogenesis (166–169). As discussed previously, complement-mediated pruning of synapses is a key microglial function during development and, whilst complement factors aid plaque clearance (170, 171), phagocytosis of synapses appears to become dysregulated during AD. Synapse loss has been identified early on in AD and correlates strongly with cognitive decline (172, 173). Inhibition or knockout of the complement components C1q, C3, and CR3, required for microglial synapse refinement during development, reduced the synapse loss found in mouse AD models (174–176).

GWAS studies have identified the rare variant R47H in the gene encoding the phagocytic receptor TREM2 as a risk factor for the development of LOAD (48, 49). TREM2 expression is necessary for the phagocytosis of a range of particles (36, 53, 54); TREM2 knockout microglia are less efficient at phagocytosing Aβ than WT microglia (53, 55) and mutations in TREM2 affect the detection of damage-associated lipids by microglia (56), which may explain their reduced ability to take up apoptotic cells (60, 61, 177). It has recently been suggested that TREM2 drives the expression of the scavenger receptor CD36, via the upregulation of C/EBPα (52), augmenting phagocytosis. TREM2 knockout AD models have produced contrasting results regarding amyloid burden (56–59), however it can be argued that this is an indirect measure of microglial phagocytosis of Aβ, as amyloid burden can be altered by other reported TREM2-mediated effects including microglial migration and plaque barrier formation (55, 178, 179).

The ApolipoproteinE–ε4 allele is the greatest genetic risk factor for the development of LOAD (180). APOE has been found to be an endogenous TREM2 ligand (53, 181), suggesting an interaction between the two most significant AD genetic risk factors on the surface of microglia. APOE binds to both

Aβ (181, 182) and apoptotic cells (181) and therefore may facilitate the detection and phagocytosis of Aβ and apoptotic cells by TREM2-expressing microglia. The TREM2-APOE signaling pathway has been reported to suppress the homeostatic signature of microglia in several CNS disease models, inducing a shift to a neurodegenerative phenotype (183). Therefore, this signaling axis may exert effects on both beneficial and detrimental microglial functions.

Single-cell RNA-sequencing has identified a new subset of highly phagocytic, AD-associated microglia (DAM) surrounding the plaques in an AD mouse model (184). Interestingly, this subset of microglia was also found in models of aging and ALS so may represent a generalized response to age-related neurodegeneration, or loss of homeostasis, rather than to a specific disease-associated protein. Whether this adoption of a highly phagocytic phenotype is beneficial or deleterious for AD progression has not yet been established.

It has been argued that aging and the expression of genetic risk factors, either independently or in combination, limit the ability of microglia to prevent or slow the pathogenesis of neurodegenerative diseases (185). Age is the single biggest risk factor for the development of LOAD and the recent identification of a unique phenotype of aged human microglia, in which susceptibility genes for both AD and MS were found to be enriched (186), suggests that age could significantly impact microglial function. In the aged brain, microglia exhibit marked changes in their morphology and activity; compared to young cells there is an increase in soma size, a loss of the characteristic tiled tissue distribution, and shorter, less dynamic processes (187–189). Primary mouse microglia demonstrate age-dependent, substrate-specific decreases in phagocytosis of fibrillary Aβ (190) and α-synuclein oligomers (191). Age-related phagocytic activation of microglia, which correlated with cognitive impairment, was reported in aged rhesus monkey brain (192). In this study, immunohistochemical analysis of white matter regions indicated that with age, increasing numbers of microglia simultaneously expressed galectin-3, a phagocytic marker, and HLA-DR MHC II, a marker of microglial activation. However, in another study, the presence of large, heterogeneous intracellular inclusions suggested that increased uptake, but inefficient lysosomal digestion, of particles may be associated with aged microglia (193). Poor Aβ protein degradation is also found in aged mouse microglia (188, 194). Therefore, age-related changes may increase the susceptibility to abnormally folded proteins and accumulating debris, resulting in a loss of homeostasis and the persistence of cytotoxic conditions.

Other Neurodegenerative Diseases

Microglial phagocytosis has been implicated in a range of other neurodegenerative diseases (195), in particular proteopathic diseases in which the balance of protein production, clearance and degradation becomes dysregulated. In prion disease, the pathogenic form of prion protein (PrPsc) is not taken up by microglia (196), and alters the uptake of other particles (197), resulting in the accumulation of pathology. Multiple studies have demonstrated the ability of α-synuclein (α-SYN), the pathogenic protein found in Lewy bodies in Parkinson's disease (PD), to alter microglial phagocytosis, although this is conformation and expression-level dependent (198). Mutations in LRRK2 are the most commonly found variants in familial PD and recent work has demonstrated that LRRK2 influences myeloid cell phagocytosis via interactions with the actin remodeling protein WAVE2 (79). LRRK2 deficiency in mouse microglia attenuated phagocytosis of beads, whereas expression of PD-associated LRRK2–G2019S augmented phagocytosis in mouse microglia and patient-derived macrophages, which may result in neuronal damage due to overactive phagocytosis during disease (79).

The TREM2 R47H variant associated with AD has also been found to be a risk factor for PD and frontotemporal dementia (FTD), suggesting a common role for TREM2 dysfunction in multiple neurodegenerative diseases (50). Nasu-Hakola disease (NHD) is a progressive, presenile dementia in which phagocytic alterations are a primary cause of pathology. Rare but lethal, NHD is caused by a loss of function of TREM2 or its signaling partner DAP12 (51). Significant demyelination is found in patient brains (199) and in mouse models (200, 201), however signs of Aβ and tau pathology are limited, despite the role of TREM2 in AD (202). Overactive microglial phagocytosis is also a driver of the pathology found in FTD; loss of expression of functional progranulin results in increased C1q production and complement-mediated synapse loss during aging (203).

MODELING PHAGOCYTOSIS IN THE CNS

CNS phagocytosis can be modeled and studied using a range of *in vitro* and *in vivo* techniques. Flow cytometry and microscopy are frequently and easily utilized to assess the uptake by cell lines or primary cells *in vitro* of fluorescently-labeled synthetic or physiological particles, including latex, Aβ, myelin, zymosan, and dextran (204). Flow cytometry allows the rapid assessment of large numbers of cells, whereas microscopy provides additional information on the motility and morphology of the cells as they perform this actin-associated function. Time-lapse microscopy allows the monitoring of the clearance or, in the case of some pathogenic proteins, the persistence of phagocytosed material within the cells. The ability to fluorescently label a range of particles enables disease- or context-specific analysis of phagocytosis, however, in order to ensure the identity of the phagocytic cell, these experiments are frequently performed in monoculture. Monocultures of primary cells are valuable for understanding the morphological changes and cellular pathways of specific substrate uptake during phagocytosis but do not provide information on the interactions between different cell types during this process. It has also been shown that the isolation and culture of microglia rapidly alters their transcriptomic signature (205), so *in vitro* assays may not accurately recapitulate CNS phagocytosis.

In vivo, phagocytosis can be inferred in tissue by the expression of phagocytic markers, such as CD206 and CD68, or live cell imaging either transcranially or organotypic cultures. Using acutely prepared and organotypic slices, microglial phagocytosis of apoptotic neurons has been observed (206, 207). These techniques preserve the structure and physiological

conditions within the tissue, and, when combined with fluorescent labels, such as lectin, and two-photon or confocal time lapse imaging, allow the study of interactions between different cell types (208). Transgenic mouse lines in which enhanced green fluorescent protein (EGFP) is expressed under the control of myeloid cell-specific gene promoters, including CX_3CR1 (209), Iba1 (210), and Cfs1r (211), allow live fluorescent imaging of microglial migration and phagocytosis (12).

Species-specific differences between mouse and human cells have been found with respect to phagocytosis (212) so, in the case of investigating human diseases, human studies are necessary. Primary human microglia can be obtained from fetal or *post-mortem* samples, however these resources can be difficult to obtain particularly within acceptable *post-mortem* delay conditions. The recent development of methods for the generation of induced pluripotent stem cell-derived microglia-like cells (iMGLs) allow the study of human microglia function *in vitro* (70, 213–215). Mimicking scarce primary human microglia, iMGLs are capable of phagocytosing synaptosomes, as found during development, and also disease-associated Aβ and tau (213). This model system is valuable for investigating interactions between genetic risk factors and pathogenesis, as recently demonstrated by iMGLs carrying patient-derived TREM2 variants phagocytosing less apoptotic cell material than controls (60), whilst avoiding the caveats associated with mouse models of human disease. It should be noted that these cells have never been exposed to cues arising from the CNS microenvironment, which may alter the differentiation and function of the iMGLs.

Until recently, markers for distinguishing microglia from monocytes were lacking, making it difficult to determine whether phagocytosis was being performed by CNS resident microglia or infiltrating myeloid cells. The discovery of microglia-specific proteins, such as TMEM119 (216), will allow more accurate investigations of the microglial-specific contributions to homeostasis and disease. These tools may also allow the resolution of current discrepancies, including those regarding phagocytosis, found between different disease models (56, 57, 117).

CONCLUSIONS AND PERSPECTIVES

In this review we have summarized the critical role phagocytosis plays in both CNS homeostasis and disease. While much progress has been made in recent years, many unanswered questions remain. How phagocytosis in the CNS is influenced by numerous factors, such as microenvironment or phagocytic target, have yet to be fully resolved. Additionally, the utilization of novel technologies, including *in vivo* imaging techniques (217), iPSC-derived microglia (213) and high-throughput screens (66), will likely contribute to further identification of phagocytic pathways and consequences of phagocytosis within the CNS. As targeting myeloid cells in neuroinflammatory and neurodegenerative diseases is receiving increased interest (218), drugs modulating phagocytic pathways may emerge as novel therapeutics for brain disease.

AUTHOR CONTRIBUTIONS

DG and AP performed the literature search and wrote the manuscript. DO and CM oversaw preparation of the manuscript, and contributed to writing and editing of the manuscript.

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Corrigendum: Phagocytosis in the Brain: Homeostasis and Disease

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In the original article, there was a mistake in **Figure 1**, as well as its legend, as published. It was incorrectly stated that microglia remove "apoptotic oligodendrocyte progenitor cells (OPCs)" instead of "apoptotic oligodendrocytes." The corrected **Figure 1** and its legend appears below.

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

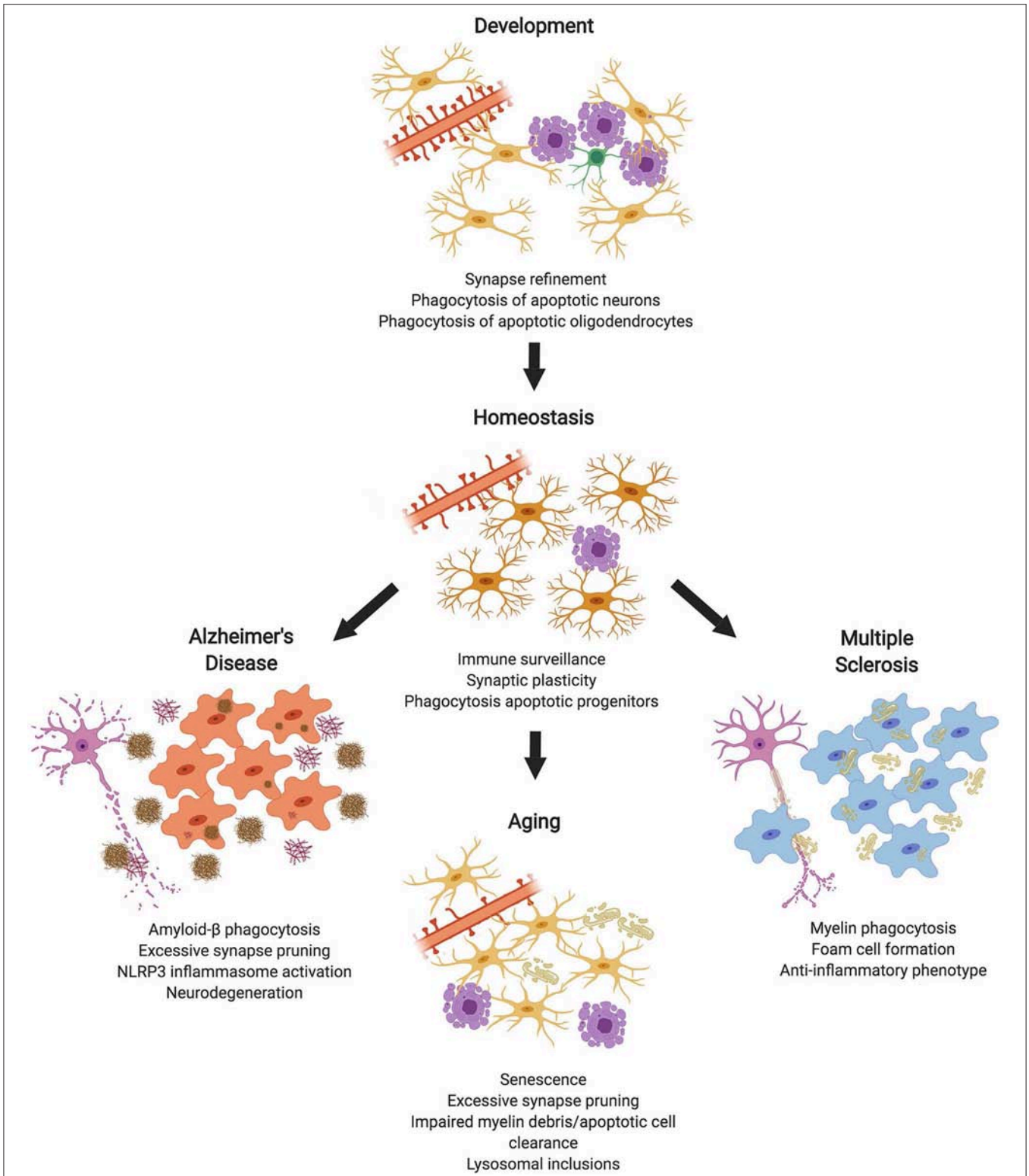

FIGURE 1 | Microglial Phagocytosis in the CNS. During development, microglial phagocytosis is essential for the refinement of excessive synapses, as well as the removal of apoptotic neurons and oligodendrocytes that are overproduced during development. Homeostatic microglia in the adult brain constantly survey the brain's parenchyma, contributing to synaptic plasticity and phagocytosing apoptotic progenitor cells. With advanced age, microglia undergo senescence, display impaired debris clearance, and excessively prune synapses. In diseases, such as Alzheimer's or multiple sclerosis, microglia act as key contributors to pathology, which is partially mediated by phagocytosis of substrates, such as amyloid-β or myelin debris (made in ©BioRender - biorender.com).

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Methylglyoxal-Derived Advanced Glycation Endproducts Accumulate in Multiple Sclerosis Lesions

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Multiple sclerosis (MS) is a demyelinating autoimmune disease in which innate and adaptive immune cells infiltrate the central nervous system (CNS) and damage the myelin sheaths surrounding the axons. Upon activation, infiltrated macrophages, CNS-resident microglia, and astrocytes switch their metabolism toward glycolysis, resulting in the formation of α-dicarbonyls, such as methylglyoxal (MGO) and glyoxal (GO). These potent glycating agents lead to the formation of advanced glycation endproducts (AGEs) after reaction with amino acids. We hypothesize that AGE levels are increased in MS lesions due to the inflammatory activation of macrophages and astrocytes. First, we measured tissue levels of AGEs in brain samples of MS patients and controls. Analysis of MS patient and non-demented control (NDC) specimens showed a significant increase in protein-bound N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine (MG-H1), the major AGE, compared to white matter of NDCs (107 ± 11 vs. 154 ± 21, $p < 0.05$). In addition, immunohistochemistry revealed that MGO-derived AGEs were specifically present in astrocytes, whereas the receptor for AGEs, RAGE, was detected on microglia/macrophages. Moreover, in cerebrospinal fluid from MS patients, α-dicarbonyls and free AGEs correlated with their respective levels in the plasma, whereas this was not observed for protein-bound AGEs. Taken together, our data show that MG-H1 is produced by astrocytes. This suggests that AGEs secreted by astrocytes have paracrine effects on RAGE-positive macrophages/microglia and thereby contribute to the pathology of MS.

Keywords: multiple sclerosis, neuroinflammation, α-dicarbonyl, advanced glycation endproducts, astrocytes

INTRODUCTION

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system (CNS) and is the major cause of disability in young adults in Western countries (1). Although the exact trigger of MS remains unidentified, an autoimmune response against the myelin sheaths is widely-considered involved in disease onset and progression. This autoimmune response is caused by an interplay

between the innate immune system and the adaptive immune system (2). Autoreactive T-lymphocytes are recruited to the CNS and reactivated by myelin phagocytosing macrophages and microglia, thereby promoting neuroinflammation and neurodegeneration (3). In addition to immune cells, CNS-resident astrocytes contribute to the neuroinflammatory response by secreting pro-inflammatory cytokines and chemokines (4). Most patients (85%) present with the relapsing-remitting MS disease course in which relapses result in episodes of disability with full recovery between the relapses (1). Over half of these patients enter the secondary progressive phase after 5–15 year of diagnosis which is characterized by progression of the disease without full recovery caused by axonal damage (5).

The pro-inflammatory activation of immune and glial cells such as macrophages, CNS-resident microglia and astrocytes induces a switch in metabolism favoring glycolysis (6–8). During glycolysis, methylglyoxal (MGO) is produced from glyceraldehyde-3-phosphate and dihydroxyacetone phosphate, and glyoxal (GO) directly from glucose (9, 10). MGO and GO are major precursors in the formation of advanced glycation endproducts (AGEs) (10). The interaction of MGO with arginine leads to the formation of methylglyoxal-derived N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine (MG-H1), whereas its interaction with lysine leads to the formation of N^{ε}-(1-carboxyethyl)-lysine (CEL) (11). Furthermore, interaction of GO with lysine leads to the formation of N^{ε}-(carboxymethyl)-lysine (CML) (11). Degradation of protein-bound AGEs results in single modified amino acids. AGEs, both in their free and protein-bound form, are able to bind the surface receptor for advanced glycation endproducts (RAGE). This leads to the activation of downstream signaling pathways inducing oxidative stress and NF-κB activation, which in turn results in the production of pro-inflammatory cytokines (12, 13). To protect our body against increased levels of MGO and GO, the glyoxalase system, consisting of glyoxalase 1 (GLO1) and glyoxalase 2 (GLO2), degrades MGO, and GO (14), thereby limiting RAGE activation and subsequent inflammation.

Previous research shows that AGEs accumulate in the adipose tissue during obesity (15), in atherosclerotic plaques (16, 17) and in the retina during diabetes (16, 18), diseases which are all characterized by inflammation. AGEs also accumulate in the brain during neurodegenerative and neuroinflammatory diseases such as in Parkinson's patients (19), and in the cerebrospinal fluid (CSF) of Alzheimer's patients (20). Furthermore, Sternberg and colleagues have revealed that AGEs and RAGE are present in the hippocampus of MS patients (21). They also found that plasma protein-bound CEL levels are increased in MS patients (22). More recently, we have revealed that AGEs are increased in the spinal cord of mice subjected to experimental autoimmune encephalomyelitis, an inflammatory animal model of MS (23). Based on the above findings, we hypothesize that AGE levels are increased in MS lesions due to the inflammatory activation of macrophages and astrocytes. In this study, we determined the cellular distribution and quantitated tissue levels of AGEs in brain samples of MS patients. In addition, we determined whether the levels of α-dicarbonyls and AGEs in the CSF correlate with plasma and elucidated whether these levels correlate with disease parameters.

MATERIALS AND METHODS

Sample Collection CSF Study Population

CSF and paired plasma samples of MS patients ($n = 18$) were obtained from the University Biobank Limburg, Belgium. The group of MS patients consist of 9 relapsing remitting MS, 8 secondary progressive MS and 1 clinically isolated syndrome patient. Medication use included the use of Tysabri® ($n = 1$), Gilenya® combined with Methotrexate® ($n = 1$), Endoxan® ($n = 1$), Copaxone® ($n = 1$), and Methotrexate® ($n = 2$). Twelve MS patients were untreated. The study protocol was approved by the Medical Ethical committee of the Jessa Hospital and of Hasselt University, Hasselt, Belgium.

Sample Collection and Preparation of MS Lesions

Frozen post-mortem tissue blocks containing brain lesions ($n = 15$) of MS patients and white matter ($n = 10$) of non-demented controls (NDCs) were obtained from the Netherlands Brain Bank. The samples were matched for age and gender. MS lesions were characterized by the Netherlands Brain Bank as active, chronic active and chronic inactive ($n = 5$/group). Patients diagnosed with type I or II diabetes mellitus were excluded from this study. The study protocol was approved by the Medical Ethical committee of Hasselt University and of the Jessa Hospital, Hasselt, Belgium.

The post-mortem brain tissues containing brain lesions of MS patients and white matter of NDCs were homogenized in 0.1 M sodium phosphate buffer, pH 6.8, containing 0.02% Triton-X (VWR International, Radnor, USA) and protease inhibitor cocktail (Roche, Basel, Switzerland). Tissue lysates were used to measure α-dicarbonyls, AGEs and GLO1 enzyme activity.

α-Dicarbonyl and AGE Measurements

α-Dicarbonyls methylglyoxal (MGO), glyoxal (GO), and 3-deoxyglucosone (3DG), and AGEs N^{ε}-(carboxymethyl)lysine (CML), N^{ε}-(1-carboxyethyl)lysine (CEL), and N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine (MG-H1) were analyzed in the plasma, CSF, and post-mortem tissue samples using ultra-performance liquid chromatography tandem mass spectrometry. Protein bound AGEs were corrected for the amount of lysine, determined with the same measurement and in the same samples, as a measure for the amount of total protein, as described previously (24, 25).

Immunohistochemistry

Paraffin sections of MS lesions and white matter of NDCs were sectioned 7 μm thick using a Leica microtome (Leica,

Abbreviations: 3DG, 3-deoxyglucosone; AGEs, Advanced glycation endproducts; CEL, N^{ε}-(1-carboxyethyl)lysine; CML, N^{ε}-(carboxymethyl)lysine; CNS, Cental nervous system; CSF, Cerebrospinal fluid; EDSS, Expanded disability scale score; GLO1, Glyoxalase1; GLO2, Glyoxalase2; GO, Glyoxal; MG-H1, N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine; MGO, Methylglyoxal; MS, Multiple sclerosis; NDCs, Non-demented controls; RAGE, Receptor.

Wetzlar, Germany). Sections were deparaffinised using xylene and rehydrated following a decreasing ethanol range. Incubation for 10 min at 37°C with citrate buffer (1.6 mM citric acid and 8.4 mM trisodium citrate, pH 6.0) was used as antigen retrieval method. Thereafter, sections were blocked using 1% BSA (Sigma-Aldrich, Saint Louis, USA) in phosphate buffered saline (PBS). Anti-MGO-derived AGEs [1:12.5 custom made, 1:84 biotin labeled, custom made (16)], anti-GFAP (1:500, Sigma-Aldrich, Saint Louis, USA), anti-Iba1 (1:500, Wako Chemicals, Neuss, Germany), and anti-neurofilament (1:750, Sigma-Aldrich, Saint Louis, USA) were used as primary antibodies. After washing with PBS, sections were incubated for 1 h with the appropriate FITC and TRITC fluorescently labeled secondary antibodies (1:600, Invitrogen, Carlsbad, USA). DAPI staining, 10 min at room temperature, was used to visualize cell nuclei. Sections were photographed using Leica fluorescent microscope (Leica, Wetzlar, Germany) at 40x magnification.

Frozen MS lesions were cryosectioned at 5 μm thickness. Sections were air-dried for 30 min, fixed for 10 min in acetone and subsequently washed in PBS. Anti-RAGE (1:50, Santa Cruz Biotechnology, Dallas, USA), anti-Iba1 (1:500, Wako Chemicals, Neuss, Germany), and anti-GFAP (1:300, Dako-Agilent, Santa Clara, USA) were used as primary antibodies. After washing with PBS, sections were incubated for 1 h with the appropriate FITC and TRITC fluorescent labeled secondary antibodies (1:600, Invitrogen, Carlsbad, USA). DAPI was used to stain cell nuclei. Sections were photographed using Leica fluorescent microscope (Leica, Wetzlar, Germany) at 40x magnification.

GLO1 Activity Assay

GLO1 activity was measured in protein lysates of human tissue as described previously by McLellan et al. (26). In short, GLO1 activity was measured using a spectrophotometry analysis by determining the formation of S-D-Lactoylglutathione from MGO at an absorbance of 240 nm during 30 min.

Statistical Analysis

Statistical analysis were performed using SPSS Statistics software, version 24 (IBM Corporation, Armonk, USA) or GraphPad Prism version 7 (GraphPad Software, La Jolla, USA). Baseline characteristics of post-mortem samples from NDCs and MS patients were analyzed using one-way ANOVA with Tukey's multiple comparisons test (GraphPad Prism). α-dicarbonyl and AGE levels of the post-mortem material were analyzed using one-sided unpaired *t*-test (GraphPad Prism), based on our previous results obtained from our mouse model (23). Partial correlation analysis was used to determine CSF α-dicarbonyls and AGEs and disease parameters expanded disability scale score (EDSS), number of relapses and disease duration. These data were corrected for age, gender, medication use and glucose concentration in the CSF using SPSS statistics. Linear regression analysis was used to determine associations between plasma α-dicarbonyls and AGEs and CSF α-dicarbonyls and AGEs (GraphPad Prism). All data are presented as mean ± SEM. $P \leq 0.05$ was considered statistically significant.

RESULTS

MG-H1 Is Increased in the Lesion Area of MS Patients

To determine whether the levels of α-dicarbonyls and AGEs are increased in the lesions of MS patients compared to white matter of NDCs, post-mortem lesions of 15 MS patients and 10 white matter samples of NDCs were obtained (**Table 1**) and levels of MGO, GO and free and protein-bound CML, CEL, and MG-H1 were measured. The MS lesions were subdivided into three categories: active lesions, chronic active lesions, and chronic inactive lesions. Post-mortem delay (until storage) was significantly higher for the chronic inactive lesions (**Table 1**). However, there was no correlation between post-mortem delay and α-dicarbonyl, protein-bound, and free AGE levels, arguing that post-mortem delay did not affect the measurements (**Supplemental Figure 1**). Levels of MGO and GO were not altered in MS lesions compared to white matter of NDCs (**Figure 1A**). Interestingly, protein-bound MG-H1, the major MGO-derived AGE, was significantly higher in MS lesions ($p < 0.05$), whereas CML and CEL were not different (**Figure 1B**). Free AGE levels did not differ between MS lesions and brain tissue of NDCs (**Figure 1C**). GLO1 activity was unaltered in MS lesions compared to controls (**Figure 1D**). Together, these data demonstrate that MG-H1 accumulates in the lesions of MS patients, irrespective of the activity of GLO1.

MGO-Derived AGEs Are Present in Astrocytes in MS Lesions

To determine which cell type mainly contributes to AGE production in the CNS, fluorescent double staining was performed on MS lesions to localize MGO-derived AGEs combined with cell makers for astrocytes (GFAP), macrophages/microglia (Iba1), and neurons (neurofilament). MGO-derived AGE was detected in sections that contained both lesion and NAWM. These stainings revealed that MGO-derived AGEs are present in MS lesions and normal appearing white matter of MS patients and primarily co-localize with $GFAP^+$ astrocytes (**Figure 2A**, indicated by the white arrows). MGO-derived AGEs did not show co-localization with $Iba1^+$

TABLE 1 | Baseline characteristics of post-mortem material of MS patients ($n = 15$) and NDCs ($n = 10$).

	Active MS lesion ($n = 5$)	Chronic active MS lesion ($n = 5$)	Chronic inactive MS lesion ($n = 5$)	NDCs ($n = 10$)
Age	69 ± 5	65.4 ± 7	58.8 ± 4	71.1 ± 0.8
Female, %	40%	60%	40%	70%
Post-mortem delay (minutes)	549 ± 36	543 ± 47	606 ± 39*	427 ± 39

*$p < 0.05$ compared to NDCs.
Data is presented as mean ± SEM and analyzed using one-way ANOVA and Tukey's multiple comparisons test.

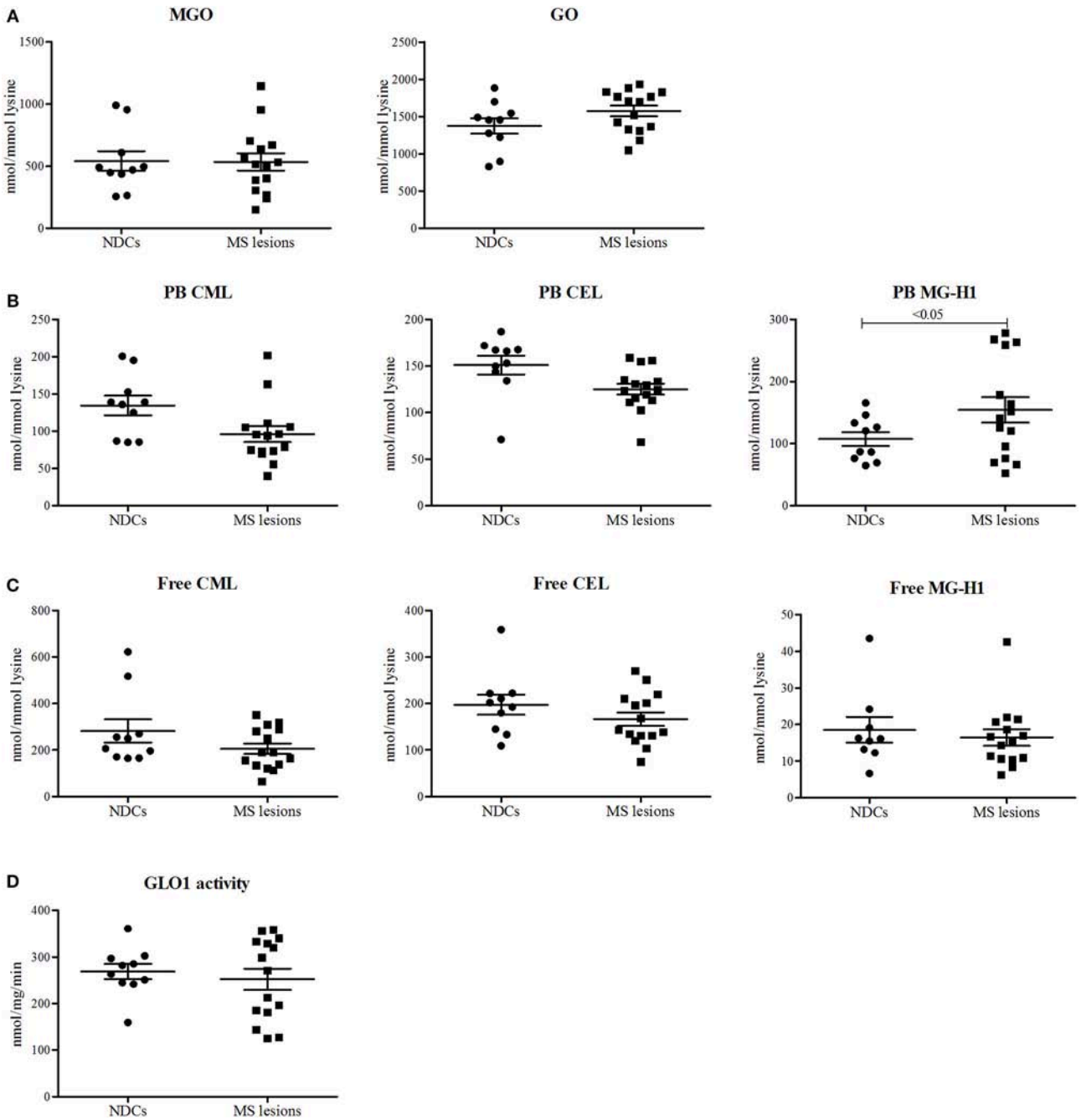

FIGURE 1 | Protein-bound MG-H1 is increased in post-mortem MS lesions compared to white matter of NDCs. α-dicarbonyls MGO and GO **(A)**, protein-bound (PB) CML, CEL, and MG-H1 **(B)**, free CML, CEL, and MG-H1 **(C)**, and GLO1 activity **(D)** was determined in white matter post-mortem samples obtained from non-demented controls (NDCs), and in post-mortem samples of MS lesions. Data is presented as Mean ± SEM. Data is analyzed using one-sided unpaired *t*-test.

macrophages/microglia (**Figure 2B**) or with neurofilament$^+$ neurons (**Figure 2C**). In addition to MS lesions and NAWM, we performed these stainings in white matter of NDCs and confirmed that MGO-derived AGE was only present in the GFAP$^+$ astrocytes (**Supplemental Figure 2**).

In addition to AGE localization, RAGE distribution was examined with fluorescent double staining to identify the cell types capable of responding to AGE formation in the CNS. RAGE was detected in sections that obtained both MS lesion and NAWM. RAGE expression co-localized with Iba1$^+$ macrophages/microglia (**Figure 3A**) while GFAP$^+$ astrocytes were not positive for RAGE (**Figure 3B**). Moreover, we observed no presence of RAGE on neurons using DAB-staining (data not shown).

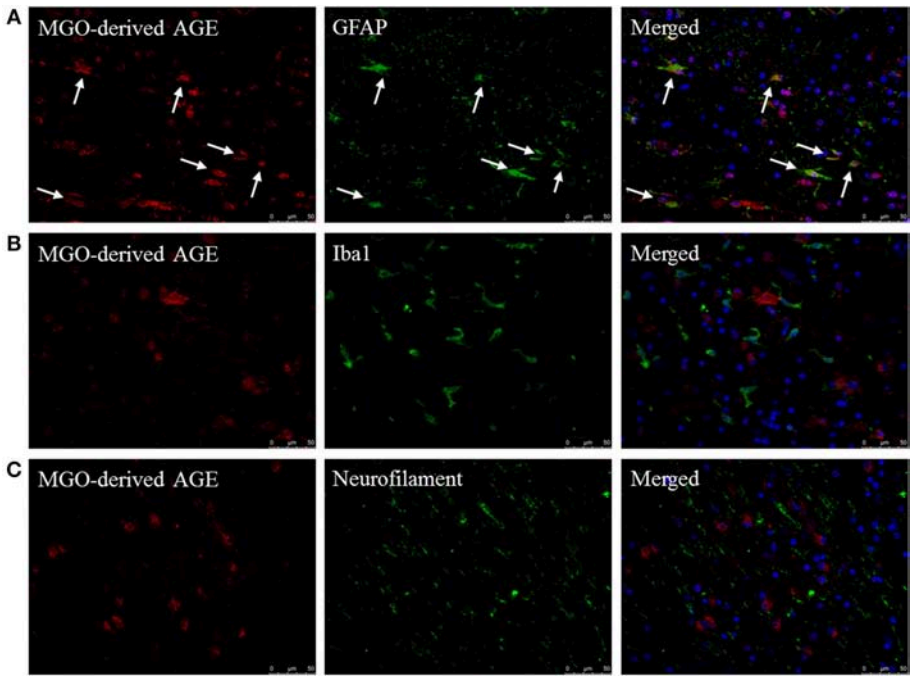

FIGURE 2 | MGO-derived AGE accumulates predominantly in astrocytes. Staining of MGO-derived AGE (red, TRITC) combined with GFAP (green, FITC) **(A)**, Iba1 (green, FITC) **(B)**, and neurofilament (FITC) **(C)** show that MGO-derived AGE accumulates in astrocytes in normal appearing white matter and lesions of MS patients as indicated by the white arrows. Nuclei were stained with DAPI (blue). Representative of $n = 4$ staining.

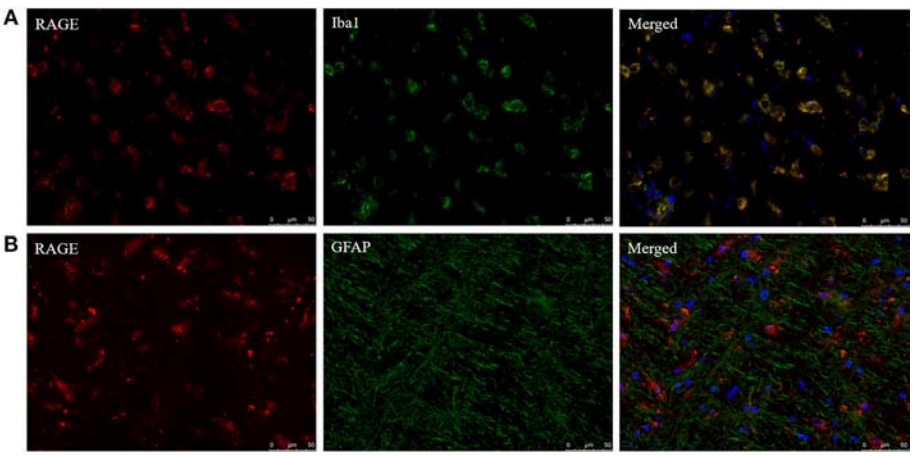

FIGURE 3 | Iba1$^+$ cells express the receptor of AGEs. Staining of RAGE (red, TRITC) combined with Iba1 (green, FITC) **(A)** and GFAP (green, FITC) **(B)** show that RAGE is present on Iba1$^+$ macrophages/microglia and absent on GFAP$^+$ astrocytes in the normal appearing white matter and lesions of MS patients. Nuclei were stained with DAPI (blue). Representative of $n = 4$ staining.

α-Dicarbonyls and AGEs in CSF Do Not Correlate With Disease Parameters of MS

To assess whether α-dicarbonyl and AGE levels in the CSF are correlated with disease parameters of MS, partial correlation analysis was performed. CSF samples of MS patients were obtained from the University Biobank Limburg. Baseline characteristics of the study population are shown in **Table 2**. No correlation was found between CSF AGE levels and EDSS, the number of relapses, and duration of the disease (**Table 3**). Interestingly, a significant negative correlation was found between free MG-H1 in CSF and disease duration ($p < 0.05$), and between protein-bound MG-H1 and EDSS ($p < 0.05$) (**Table 3**).

α-Dicarbonyls and Free AGEs in CSF Correlate With Plasma Levels

To determine whether AGE levels in plasma and CSF correlate, α-dicarbonyl, free AGEs, and protein-bound AGEs were

TABLE 2 | Baseline characteristics from MS patients ($n = 18$) included in the CSF study population.

	MS patients ($n = 18$)
Age	43.8 ± 3
Gender (female), %	78%
[glucose] $_{CSF,}$ mg/dL	61.3 ± 1
Medication use, %	33%
Clinically isolated syndrome, %	6%
Relapsing remitting MS, %	50%
Secondary progressive MS, %	44%
EDSS	4.2 ± 0.6
Duration of disease, years	6.4 ± 2
Number of relapses	2.6 ± 0.3

Duration of disease is determined as time between diagnosis and sampling of CSF. Data is presented as Mean ± SEM or as percentage of the total group.

TABLE 3 | Correlation of CSF α-dicarbonyl and AGE levels with MS disease parameters.

	Correlations (R^2)		
	EDSS	**Number of relapses**	**Disease duration, years**
MGO	−0.175	−0.023	−0.153
GO	−0.378	0.213	0.008
3-DG	−0.242	−0.062	0.082
Free CML	0.125	−0.209	−0.357
Free CEL	−0.32	−0.134	−0.165
Free MG-H1	0.284	0.014	−0.542*
PB CML	−0.18	0.377	−0.152
PB CEL	−0.099	0.306	−0.452
PB MG-H1	−0.551*	0.029	−0.12

$p < 0.05$. Correlation is determined between MGO, GO, and 3DG, protein-bound (PB) CML, CEL, and MG-H1, and free CML, CEL, and MG-H1 in the CSF with EDSS, number of relapses and disease duration. Data is analyzed using partial correlation analysis and corrected for age, gender, medication use, and $[glucose]_{CSF}$.

determined in paired plasma and CSF samples of MS patients. Plasma MGO ($p = 0.005$), GO ($p = 0.02$), and 3DG ($p = 0.03$) levels significantly correlated with CSF levels (**Figure 4A**). In addition, the free AGEs levels of CML ($p = 0.009$), and CEL ($p = 0.005$) in plasma significantly correlated with their CSF counterparts, and plasma levels of MG-H1 ($p = 0.06$) tended to be correlated with CSF levels (**Figure 4B**). However, plasma protein-bound CML, CEL and MG-H1 did not correlate with CSF levels (**Figure 4C**).

DISCUSSION

In this study we showed that MGO-derived MG-H1 is significantly increased in MS lesions and is mostly present in astrocytes. In addition, we revealed that α-dicarbonyl and AGE levels in the CSF do not correlate with disease parameters, but do correlate with plasma levels.

AGE levels were measured in post-mortem samples of NDCs and MS patients. This analysis revealed that protein-bound MG-H1 levels were increased in MS lesions compared to the levels in the white matter of NDCs. Protein-bound CML and CEL levels and free AGE levels were comparable between MS lesions and white matter of NDCs. Since MG-H1 is the major MGO-derived AGE (11), the higher levels of MG-H1 suggest enhanced MGO production in the lesions of MS patients. However, MGO levels were not different in MS lesions compared to NDCs. Possibly, the highly reactive MGO that is formed intracellularly rapidly interacts with cellular proteins to form protein-bound AGEs. In addition, excessively formed MGO may also rapidly leave the cell due to simple diffusion given its small molecular weight. In addition, we and others have previously shown that inflammation decreases GLO1 activity (17, 23). The activity of GLO1, the major MGO detoxifying enzyme, was similar between the white matter of NDCs and MS lesions. These results indicate that the increased levels of MG-H1 are likely due to increased MGO formation and not due to decreased degradation by GLO1.

We found that MGO-derived AGEs mainly accumulate in $GFAP^+$ astrocytes in human MS lesions and in white matter of NDCs. Moreover, in accordance with the findings of Barateiro et al. (27), double staining of GFAP and Iba1 with RAGE showed that RAGE was expressed on $Iba1^+$ microglia/macrophages in MS lesions, and not on $GFAP^+$ astrocytes. These data indicate that AGEs are produced in activated glycolytic astrocytes and could exert paracrine effects by binding RAGE on microglia/macrophages. Activation of RAGE results in the activation of NF-κB, which in turn induces the production of pro-inflammatory cytokines and oxidative stress (12, 13). It is known that RAGE expression is low under physiological conditions and will increase its expression in an inflammatory environment (28), suggesting that RAGE levels are high in MS lesions. NF-κB activation, which plays a major role in MS pathology, is present in and around MS lesions, predominantly in the glial cells and infiltrated macrophages (29). Moreover, it has been suggested that there is a link between NF-κB related gene expression and clinical relapses (30, 31). Although AGEs in the CSF did not correlate with disease progression, we postulate that microglial and macrophage RAGE activation contribute, at least partly, to the increased NF-κB activation seen in MS lesions. This will in turn contribute to the inflammatory state of the microenvironment of the CNS.

Linear regression analysis showed no positive correlations between α-dicarbonyls and free AGEs with markers related to disease progression, and the same holds true for protein-bound AGEs. In fact, free MG-H1 in the CSF was negatively correlated with disease duration and protein-bound MG-H1 in the CSF was significantly negatively correlated with EDSS. One explanation for the decreased MG-H1 levels, free and protein-bound, with increasing disease progression and disability might be that patients in the progressive phase of the disease, which have most often a longer disease duration and score higher on the EDSS, experience less relapses and thus less inflammation (32). This could subsequently affect the MG-H1 levels in the CSF. An important factor to take into account is that free MG-H1 may be able to cross the blood-CSF barrier seen by the strong

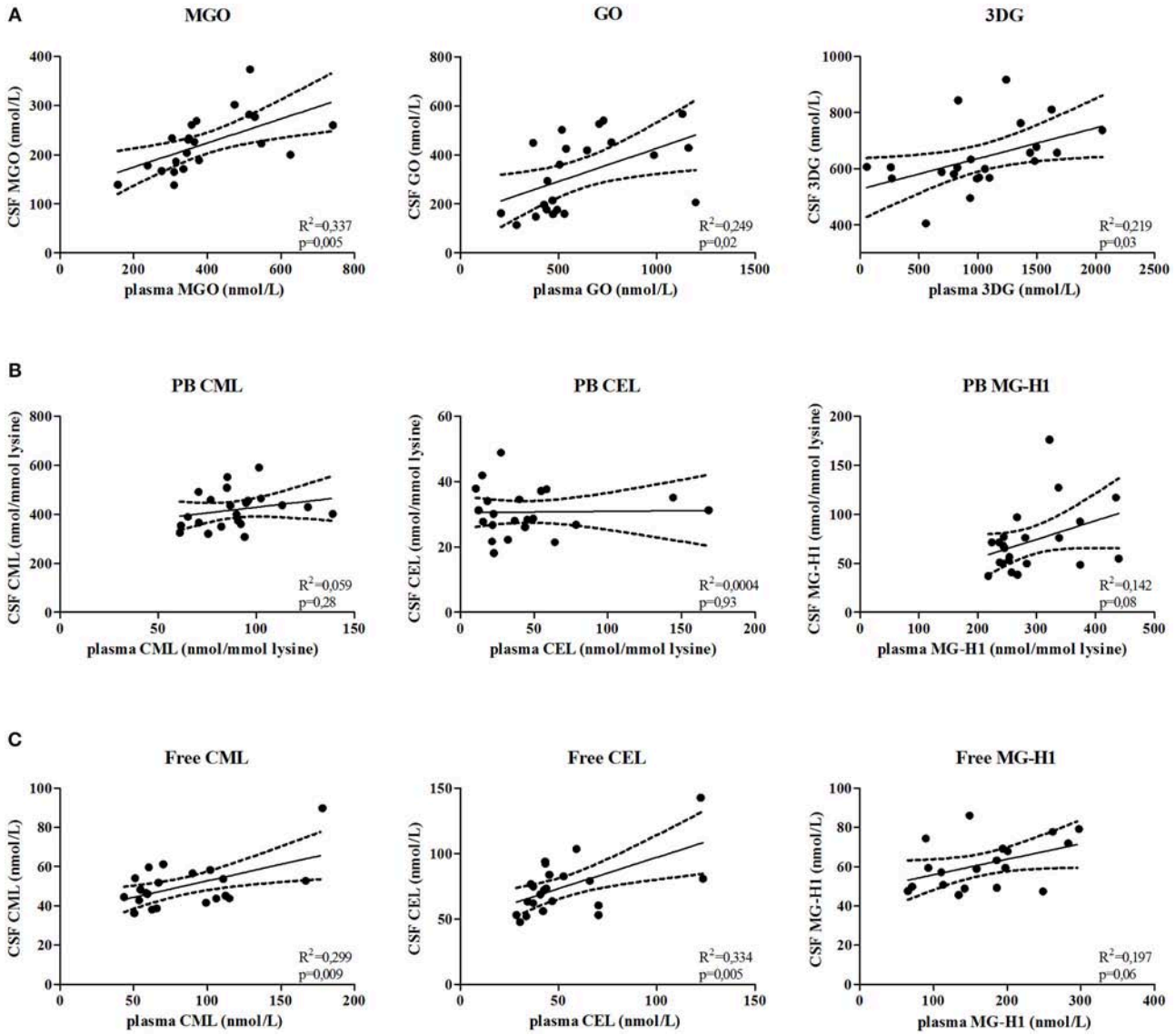

FIGURE 4 | α-dicarbonyls and free AGEs in the plasma and CSF are correlated. Correlation is determined between MGO, GO, and 3DG **(A)**, protein-bound (PB) CML, CEL, and MG-H1 **(B)** and free CML, CEL, and MG-H1 **(C)** in the CSF and in the plasma. Data is analyzed using linear regression analysis and shows regression line with 95% confidence band.

correlation between plasma and CSF free MG-H1. However, it is unclear whether free MG-H1 in the CSF origins from MS lesions and leaks into the periphery or whether free MG-H1 originates from the periphery and enters the CSF. This needs to be further investigated. It should also be mentioned that, although we corrected for medication use in our statistic model, most of these MS patients take anti-inflammatory treatments. These treatments inhibit the inflammatory component of the disease and may therefore interfere with the production of AGEs. Moreover, whether or not patients experience a relapse at the moment of CSF collection may have a significant impact on their AGE levels, as active inflammation is present during relapses, inducing AGE. Unfortunately, information whether patients experience a relapse at the time of sample collection is not available. Another explanation for the lack of correlation between α-dicarbonyl and AGE levels with disease progression markers of MS could be that α-dicarbonyls and AGEs reside in the CNS, intracellularly, and are not released into the CSF. Altogether, our findings indicate that use of AGE levels in the CSF as marker for disease progression is limited.

CSF α-dicarbonyls and free AGEs, but not protein-bound AGEs, correlate with their respective levels in the plasma. These results suggest that there is a lack of exchange of protein-bound AGEs between the plasma and CSF while α-dicarbonyls and free AGEs are easily exchanged between the plasma and CSF. CSF is produced in the CNS by the choroid plexus (33), which comprises

the major part of the blood-CSF barrier and ensures separation of blood and CSF. The passage of solutes and nutrients is controlled by tight-junctions (34), and by various transporters (35). The crossing rate across the blood-CSF barrier is inversely correlated with the molecular weight of the substance, meaning the bigger the substance, the less crossing over the blood-CSF barrier occurs (36). This suggests that the small α-dicarbonyls and free AGEs, consisting of single modified amino acids, are able to pass the blood-CSF barrier more easily in contrast to the protein-bound AGEs. This may explain why we did not found correlations between protein-bound AGEs in the CSF and in the plasma.

In conclusion, we show that protein-bound MG-H1 is increased in MS lesions compared to white matter of NDCs and is present in activated $GFAP^+$ astrocytes. This indicates that MGO-derived AGEs formed in glycolytic astrocytes may activate RAGE-positive microglia/macrophages in MS lesions and contribute to the inflammatory microenvironment. Further research is needed to elucidate whether lowering MG-H1 production in MS lesions is a therapeutic option for MS.

AUTHOR CONTRIBUTIONS

SW performed the experiments, analyzed the data, and wrote the manuscript. TV supervised the experiments and wrote the manuscript. JS designed methods and performed measurements. JvH supervised immunohistochemistry, analyzed data, and revised the manuscript. SA provided CSF samples and revised the manuscript. VS research design and biobank sample collection. CS supervised the experiments and revised the manuscript. JH supervised the experiments and revised the manuscript. KW supervised the experiments and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00855/full#supplementary-material

Supplemental Figure S1 | Post-mortem delay is not correlated with α-dicarbonyl, free, and protein-bound AGE levels. Using linear regression analysis, the correlation between post-mortem delay (PDM) and α-dicarbonyls MGO and GO **(A)**, protein-bound (PB) CML, CEL, and MG-H1 **(B)** and free CML, CEL, and MG-H1 **(C)**, was determined. Data shows regression line with 95% confidence band.

Supplemental Figure S2 | MGO-derived AGE accumulates predominantly in astrocytes in white matter of NDCs. Staining of MGO-derived AGE (red, TRITC) combined with GFAP (green, FITC) **(A)**, Iba1 (green, FITC) **(B)**, and neurofilament (NF) (green, FITC) **(C)** show that MGO-derived AGE accumulates in astrocytes in white matter of NDCs as indicated by the white arrows. Nuclei were stained with DAPI (blue). Representative of $n = 4$ staining.

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Microglia Mediate Synaptic Material Clearance at the Early Stage of Rats With Retinitis Pigmentosa

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Resident microglia are the main immune cells in the retina and play a key role in the pathogenesis of retinitis pigmentosa (RP). Many previous studies on the roles of microglia mainly focused on the neurotoxicity or neuroprotection of photoreceptors, while their contributions to synaptic remodeling of neuronal circuits in the retina of early RP remained unclarified. In the present study, we used Royal College of Surgeons (RCS) rats, a classic RP model characterized by progressive microglia activation and synapse loss, to investigate the constitutive effects of microglia on the synaptic lesions and ectopic neuritogenesis. Rod degeneration resulted in synapse disruption and loss in the outer plexiform layer (OPL) at the early stage of RP. Coincidentally, the resident microglia in the OPL increased phagocytosis and mainly engaged in phagocytic engulfment of postsynaptic mGluR6 of rod bipolar cells (RBCs). Complement pathway might be involved in clearance of postsynaptic elements of RBCs by microglia. We pharmacologically deleted microglia using a CSF1 receptor (CSF1R) inhibitor to confirm this finding, and found that it caused the accumulation of postsynaptic mGluR6 levels and increased the number and length of ectopic dendrites in the RBCs. Interestingly, the numbers of presynaptic sites expressing CtBP2 and colocalized puncta in the OPL of RCS rats were not affected by microglia elimination. However, sustained microglial depletion led to progressive functional deterioration in the retinal responses to light in RCS rats. Based on our results, microglia mediated the remodeling of RBCs by phagocytosing postsynaptic materials and inhibiting ectopic neuritogenesis, contributing to delay the deterioration of vision at the early stage of RP.

Keywords: microglia, synapse, rod bipolar cell, retinitis pigmentosa, ectopic neuritogenesis

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INTRODUCTION

Retinitis pigmentosa (RP) represents a group of retinal diseases typically attributed to hereditary factors that are caused by mutations predominantly expressed in photoreceptors or retinal pigment epithelial (RPE) cells, that contribute to rod degeneration followed by gradual death of cones (1). Effective treatments that halt or reverse progressive photoreceptor degeneration or RPE loss in patients with RP are currently unavailable. Thus, an investigation of the pathological changes, particularly at the early stage of RP and the development of therapeutic strategies aiming at suppressing its progression are critical. At the early stage of RP, the retina is characterized by

the down regulation and mislocalization of proteins of the pre- and postsynaptic machinery in the outer plexiform layer (OPL) in mouse (2–4) and rat (5) RP models. In the rd10 mouse model of RP, which is induced by a mutation in the rod photoreceptor-specific Pde6b gene, photoreceptor stress is accompanied by the loss of axon terminals in photoreceptors and dendritic retraction in bipolar and horizontal cells (6, 7). Dendritic retraction in rod bipolar cells (RBCs) is preceded by a reduction in the immunoreactivity of mGluR6 receptors and their displacement to the cell bodies and axons (8). In Royal College of Surgeons (RCS) rats, a RP model that characterized with defective phagocytosis of photoreceptor outer segments by RPE cells (9), the presynaptic and postsynaptic structures in the OPL are impaired as early as postnatal day 21 (P21) and completely lost at P90 (5). Thus, synaptic dysfunction and loss are the major early pathological changes occurring before the loss of photoreceptors and RPE cells during retinal degeneration (10). However, the mechanisms of synapse loss in RP are not completely understood.

Microglia are the major resident immune cells in the central nervous system (CNS), with well-established immunomodulating properties during the progression of neurodegeneration or injuries (11). Microglia also play an important role in the phagocytic elimination of synaptic elements as part of the widespread pruning of exuberant synaptic connections during CNS development (12, 13). Therefore, microglia have a complex role in shaping maturing or pathological circuits by modifying synapses. During the development of RP, the activation of microglia consists of a targeted migration from the inner retina to the outer nuclear layer (ONL) and a transition from a ramified to an amoeboid morphology (14–17). These activated microglia undergo proliferation (18) and secrete proinflammatory (19) and chemotactic cytokines (20). The release of cytokines by activated microglia induces neurotoxicity and apoptosis signaling, resulting in the degeneration of photoreceptors (21). Inhibition of microglia activation by genetic manipulation (such as Cx3cr1 knockout) (15) or pharmacological suppression (such as minocycline) (19) has shown to delay photoreceptor degeneration. However, the contribution of microglia to synapse loss in the retina of subjects with RP remains unclarified. Circuit remodeling is triggered by early synapse loss and dysfunction in the outer retina (22–24). Therefore, we asked whether the interaction between microglia and synapse regulated synaptic remodeling of neuronal circuits.

In RCS rats, the mutation in the receptor tyrosine kinase Mertk caused the impairment of RPE in phagocytosis photoreceptor outer segments and resulted in the degeneration of photoreceptors (25). As a member of TAM (Tyro3, Axl, and Mertk) family, Mertk and its ligands are also required in the homeostatic, phagocytic clearance of apoptotic cells by microglia (26). So far, the influence of Mertk mutation on the phagocytosis of synapses in the early degenerative retina has not been clarified. In the present study, we explored the roles of microglia in synaptic remodeling of neuronal circuits at the early stage of RP using RCS rat models. Then, a CSF1R inhibitor was used to generically ablate microglia in the RP retina in a sustained manner to validate the effects of microglia on synapse loss and visual function. Using immunohistochemical techniques and electron microscopy, the mechanism underlying the effects of microglia on the synapses of RBCs in the retina were investigated. Our findings revealed a novel role for retinal microglia in engulfment of excess postsynaptic elements and maintenance of RBC structure and function in the RP retina.

MATERIALS AND METHODS

Animals

All experimental procedures were conducted with the approval of the Third Military Medical University Animal Care and Use Committee. RCS-rdy-p+ (RCS rats; P15, 20, 30, 40, and 50; either sex) and RCS-rdy+-p+ rats (Control rats, P15, 20, 30, 40 and 50; either sex) were obtained from the Animal Center of the Third Military Medical University (Chongqing, China). All rats were housed in the animal facility of Southwest Hospital under a 12-h light/dark cycle and fed a standard diet and water.

Drug Administration

Control or RCS rats were fed a PLX3397-formulated AIN-76A diet (600 p.p.m; 600 mg PLX3397 (Selleckchem, Houston, TX) per kilogram of diet) *ad libitum* to deplete retinal microglia at P15. The control RCS rats were fed a normal AIN-76A diet. Morphological and functional experments were carried out in the rats after continuous depletion of microglia for 5, 15, 25 days respectively.

Immunohistochemistry

Immunofluorescence staining of frozen tissue sections was performed as previously described (27). Briefly, the enucleated eyecups were fixed with 4% paraformaldehyde (PFA) at 4°C for 15 min and then infiltrated with 30% sucrose overnight at 4 °C. The retinas were embedded in optimal cutting temperature (OCT) compound and cut into 20- or 40-μm-thick sections in the sagittal plane using a freezing microtome. Sections containing the optic nerve were chosen for immunohistochemistry. Sections were permeabilized and blocked with PT1 (PBS containing 0.1% Triton X-100 and 10% donkey or goat serum) at 37°C for 30 min, and then incubated with primary antibodies (**Table 1**) in PT2 (PBS containing 0.03% Triton X-100 and 5% donkey or goat serum) overnight at 4 °C. After five washes with PBS, sections were incubated with fluorophore-conjugated secondary antibodies in PT2 at 37°C for 1 hour. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). Images of immunofluorescence staining were acquired using a confocal microscopy system (Zeiss LSM 780).

TUNEL staining: The terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) assay (Roche) was performed according to the manufacturer's specifications to detect the apoptosis of retinal cells.

Analysis of apoptotic cells and surviving cells: For the quantification of cell numbers, all measures were manually performed on images of retina sections using ImageJ software. Total cells (DAPI-positive) and apoptotic cells (TUNEL-positive) in the ONL were counted in three regions (central, right,

TABLE 1 | Antibodies used for immunofluorescence staining.

Antibody	Company	Titer	Species	Cat#
CtBP2	Santa Cruz	1:500	Goat	sc-5966
mGluR6	Neuromics	1:1500	Rabbit	RA13105
IBA1	Wako Chemicals	1:500	Rabbit	019-19741
mGluR6	Abcam	1:500	Guinea pig	ab101864
CD-68	Bio-RDA	1:500	Mouse	MCA341R
Arrestin	Millipore	1:500	Rabbit	AB15282
C1q	Abcam	1:50	Mouse	ab71940
C3	Abcam	1:50	Rabbit	ab11887

and left). Sections crossing the optic nerve were selected to strengthen the results. Surviving cells were defined as DAPI-positive nuclei without TUNEL staining: number of surviving cells=number of total cells-number of apoptotic cells. These numbers were averaged for five eyes (from 5 different rats) per group.

Number and morphological score of microglia: Three panoramic sections of the retina per group immunostained for DAPI and IBA1 were captured to quantify the numbers of microglial cells in the different retinal layers. The number of microglia in the total retina or OPL was counted and averaged to obtain the number of cells per 1 mm of the retina for at least three eyes(from 3 different rats) per group. The process morphology was scored as 0 (>15 thick processes with multiple branches), 1 (5–15 thick processes with branches), 2 (1–5 thick processes with few branches), and 3 (no clear processes), using a previously described protocol (12, 28).

Analysis of Synapses and Ectopic Dendrites in the Retina

Synapses in the OPL were quantified using previously described protocols (29–31). Three to five eyes (from 3-5 different rats) in each group were selected, and 15-20 images with a size of 135 μm × 135 μm were captured from each retinal sample in randomly selected regions. The synapse number was quantified in stacks of 10 optical sections, which were line averaged and collected at 0.28-μm intervals, by projecting a series of five optical sections and counting the number of synapses in each projection volume. Synapses were automatically counted using the ImageJ puncta analyzer program (NIH), and the accuracy of the counts was confirmed manually. Finally, the number of synapses was normalized to the thickness of the inner nuclear layer (INL). The cells in the ONL with positive cytoplasmic staining for cone arrestin and DAPI-stained nuclei were used to quantify the cone cell number. The areas of cone synapse elements were measured using ImageJ software (NIH). The number and length of the ectopic dendrites were quantified using previously described protocols (27). The NeuronJ program in ImageJ software was used to manually trace fluorescently labeled ectopic dendrites from their initial position to their terminus and automatically analyze their number and length.

Quantification of Microglia Exhibiting Engulfment in the Retina

Engulfment was quantified as previously described (12, 28, 32). For each animal, OPLs in the retinas were chosen for imaging after immunostaining for CtBP2, mGluR6, CD-68 and IBA1. Images were acquired on a confocal microscope at size 135 × 135 μm with 0.2-μm z-steps. The background was subtracted from all fluorescent channels in Z-stacks of optical sections using ImageJ software. Subsequently, 3D volume surface renderings of each z-stack were created using Imaris software (Bitplane). Surface-rendered images were used to determine the volume of the microglia, lysosomes, and synapse elements. The following equation was used to calculate the percent engulfment: volume of lysosomes or synapse elements (μm^3) /volume of microglial cells (μm^3). For each eye, 9–12 fields (15–20 microglia) were imaged in the OPLs of retinas, and 3–4 eyes(from 3–4 different rats) in each group were examined.

Reverse-Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

The RT-qPCR analysis of the CtBP2 and mGluR6 mRNAs was performed as previously described (27). Briefly, total RNA was extracted from the retinas of rats in each group (Control, Control + PLX, RCS, and RCS + PLX) using TRIzol. Total RNA (approximately 1–2 μg per 20 μl reaction) was reverse transcribed using a PrimeScript® RT Reagent Kit (Takara Bio USA, Mountain View, CA, USA). A SYBR Green qPCR Mix (Dongsheng Biotech, Guangdong, China) was used to perform quantitative PCR on a CFX96 Real-Time PCR System (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The relative expression of the CtBP2 and mGluR6 mRNAs was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). All primers were purchased from Sangon Biotech (listed in **Table 2**).

Transmission Electron Microscopy

The retinas from the eyecups were fixed with 0.1 M cacodylate buffer containing 2.5% glutaraldehyde (pH 7.4) for 24 h. After several rinses with PBS, the samples were fixed with 1% osmium tetroxide for 2 h. After several washes with PBS again, the samples were dehydrated with increasing concentrations of acetone and embedded in epoxy resin 618. Uranyl acetate and lead citrate were used to stain the sections. A TECNAI 10 (FEI-Philips, Hillsboro, OR, USA) transmission electron microscope was used to obtain images. Three eyes (from 3 different rats) in each group were selected, and 8–12 images of OPL were captured from each retinal sample in randomly selected regions.

Electroretinography (ERG)

ERG was performed using previously described methods (27, 33). Briefly, animals were dark-adapted for 12 h and prepared for recording under dim red light (wavelength > 620 nm). Light stimuli were delivered at intensities of −4.5, −2.5, −0.5, −0.02, 0.5 and 1 log(cd·s·m^{-2}). Gold wire loops were simultaneously used to record the corneal ERG responses from both eyes. According to the light intensity, the interstimulus interval ranged from 30 to 120 s. The RETIscan system (Roland Consult,

TABLE 2 | Primers used for RT-qPCR.

Name	Sequence
mGluR6-F	GTGCTAGGTCAACCCTCAAA
mGluR6-R	CTAGAAGAGATCCCAGAGGAGAA
CtBP2-F	AAGGCACGCGGGTACAAAGC
CtBP2-R	CCTGTGATTGCTCGGCGGAT
GAPDH-F	GCCCATCACCATCTTCCAGGAG
GAPDH-R	GAAGGGGCGGAGATGATGAC

Brandenburg, Germany) was used to acquire the data, which were processed using Igor software.

Statistics

Data were analyzed using an independent two-sample *t*-test, one-way analysis of variance (ANOVA) or two-way ANOVA with SPSS 17.0 statistical software (SPSS Inc., Chicago, IL, USA). The data are presented as means ± the standard deviations (SD). *P*-values less than 0.05 were considered statistically significant.

RESULTS

Synapse Disruption and Loss at the Early Stage of Retinal Degeneration in the RCS Rat

Significant synaptic changes occur at a very early stage in the RCS rat, even prior to any significant loss of photoreceptors, in parallel with the deterioration of ERG responsiveness specifically involving the rod system (5, 34). To determine temporal and spatial characteristics of the synapse changes, we used high resolution confocal microscopy to quantify synapse density in the OPL of RCS rat retinas (**Supplementary Figure 1A**). CtBP2, a marker of presynaptic ribbons, and mGluR6 were chosen to label the synapses in the OPL. In control rats, rod synapse and cone synapse could be distinguished based on their morphology of presynaptic and postsynaptic pairings. The synapses between rods and RBCs consisted of horseshoe-like, CtBP2-positive presynaptic ribbons, accompanied by a single, dot-like, mGluR6-positive postsynaptic structure; these pairs were distributed in the outermost part of the OPL (**Figure 1A** and **Supplementary Figure 1B**). The synapse between a cone and a cone bipolar cell consisted of 3-4 CtBP2-labeled presynaptic structures and a mGluR6-labeled disk-like postsynaptic structure; these pairs were mainly distributed within the innermost part of the OPL (**Figure 2A** and **Supplementary Figures 1F,G**).

During retina degeneration, apparent changes in synapse morphology were detected at P30 in RCS rats (**Figure 1A** and **Supplementary Figure 1C**), when scattered apoptotic rods were observed in the ONL (35). In the rod synapse, the morphology of the CtBP2-positive ribbons changed from a typical horseshoe to a short rod shape in the retina of RCS rats, while little alterations in mGluR6-positive postsynaptic elements were observed, even when the synapse was lost. The number of CtBP2-positive (**Figure 1B**), mGluR6-positive (**Figure 1C**) and colocalized puncta (**Figure 1D**) decreased modestly but significantly in the retinas of RCS rats at P20 compared with controls. The decrease peaked at P30 and then slowed at P40. However, the number of cone synapses decreased after P40 in RCS rats (**Figure 2D**). In the cone synapses, the area of presynaptic CtBP2 staining was gradually reduced at P30 (**Figure 2B**), a time point that was earlier than the reduction in the size of postsynaptic mGluR6 at P40 (**Figure 2C**). No significant changes in the CtBP2 mRNA level were observed in the retinas of RCS rats (**Supplementary Figure 1D**), while the level of the mGluR6 mRNA was markedly increased in the retinas of P40 RCS rats compared with control rats ($p < 0.01$) (**Supplementary Figure 1E**). Our data indicated that these morphological and quantitative changes of synapse in the OPL were mainly limited in the rod–RBC contacts before P40 in RCS rats.

Electron microscopy (ECM) analyses further confirmed that aberrant synapses formed in the retinas of RCS rats, containing only floating ribbons. These floating ribbons in the rod terminal lost their normal localization and were separated from postsynaptic elements (**Figure 3A**). Significant differences in the proportion of each type of ribbon were not observed in the OPL between controls and RCS rats at P15 (**Figures 3A–D**), the stage when rod degeneration had not yet started. In P20 and P30 RCS rats, the proportion of floating ribbons to total ribbons increased markedly as retinal degeneration progressed, while the proportion of normal synapse ribbons decreased significantly in RCS rats (**Figures 3B–D**), suggesting that the decrease in the number of paired CtBP2- and mGluR6-positive structures resulted from rod synapse disintegration. Based on these results, photoreceptor degeneration resulted in synapse loss and disintegration.

Microglia Increased Phagocytosis and Engulfed Postsynaptic Elements During the Synaptic Perturbation

Microglia play a key role in the phagocytic elimination of synaptic elements during the development or disease of the brain (12, 28, 36, 37). To explore their roles in the phagocytosis of synaptic elements, functional and morphological analysis of the microglia in the retinas of RCS rats were carried out by the immunofluence staining (**Figures 4A–C** and **Supplementary Figures 2A,B**). The number of retinal microglia in the OPL of the RCS rats markedly increased at P15 and peaked at P30 (**Figure 4D**), and synapse loss peaked at the same time point. The states of microglia were categorized by determining a morphological score. Appreciable differences in morphological scores of microglia in the OPL of RCS and control rats were not observed at P30 (**Figures 4E,G**), although most microglia showed an amoeboid morphology (a score of 2 or 3) in the ONL of RCS rats (**Supplementary Figures 2C,D**). In the mature normal retina, microglial processes were juxtaposed with presynaptic and postsynaptic structures in the OPL; however, microglial processes in the RCS OPL exhibited morphological alterations, extended into the synaptic layer and contacted mGluR6 (**Figures 4F,H**).

Microglia were double labeled with IBA1 and CD68 (**Figure 5A** and **Supplementary Figures 2F–G**), a marker of

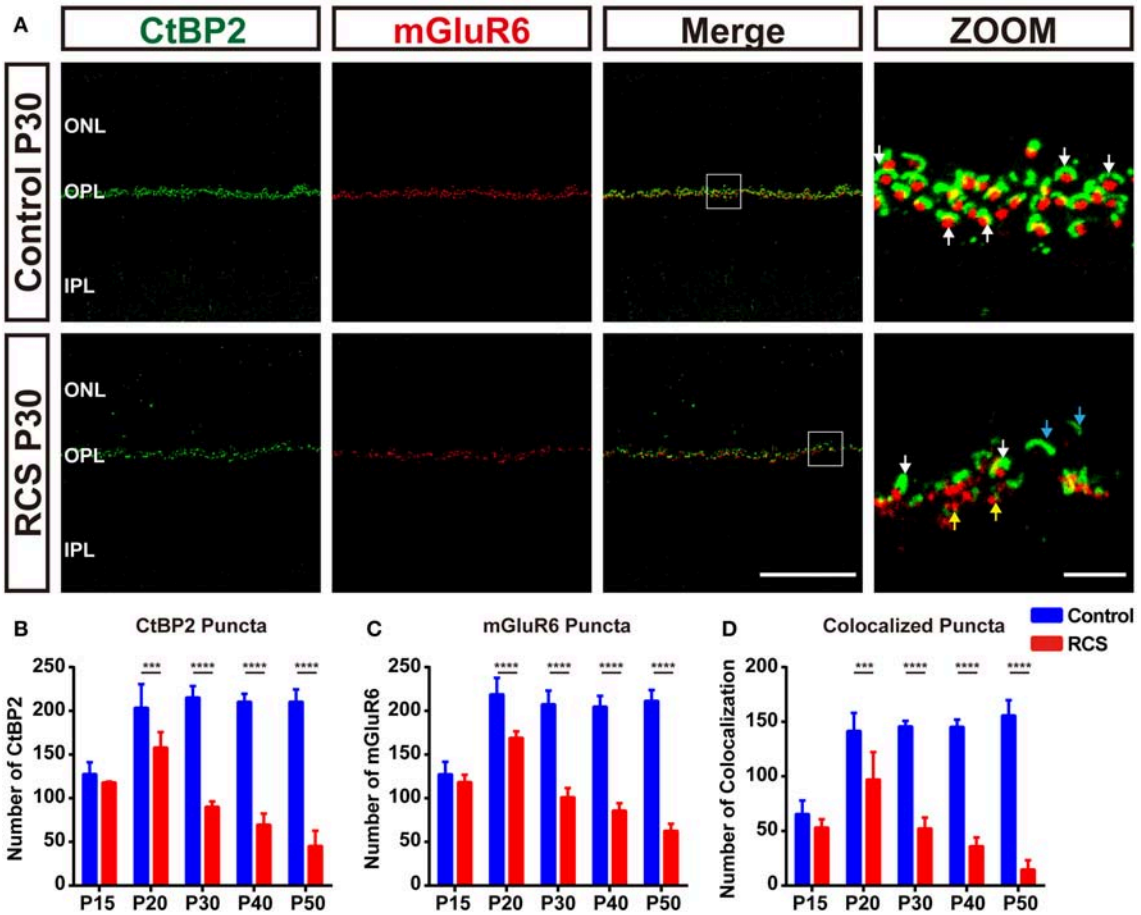

FIGURE 1 | Early synapse loss in the OPL during retinal degeneration in RCS rats. **(A)** Confocal images of CtBP2- (green) and mGluR6-immunoreactive (red) puncta in the retinas of control (upper panel) and RCS rats (lower panel) at P30 showed that the normal structure of synapses in the OPL was lost. White arrows indicate normal rod synapses, cyan arrows indicate unpaired CtBP2-positive puncta, and yellow arrows indicate unpaired mGluR6-positive puncta. **(B–D)** Quantification of synaptic puncta or their apposition in the OPL of control and RCS rats at P15, P20, P30, P40, and P50, indicating the loss of CtBP2, mGluR6 and colocalized puncta per field of view (135 × 135 μm) during retina degeneration. (N = 3–5 eyes from different rats, n = 15–20 images from each eye). ONL, outer nuclear layer; OPL, outer plexiform layer; IPL, inner plexiform layer; Scale bar, 50 μm **(A)** or 5 μm **(A)**. Bars represent means; error bars represent SD. ***$p < 0.001$, ****$p < 0.0001$ using two-way ANOVA **(B–D)**.

lysosomes specific to microglia, to clarify whether the microglia in the OPL phagocytosed synaptic elements in the RCS rats. The volume of microglia occupied by CD68-positive lysosomes exhibited a marked increase in the OPL of RCS rats at P20 and P30 compared to controls (**Figure 5D**). An *in vivo* phagocytosis assay (12, 28, 32) was used to confirm the engulfment of synaptic elements by phagocytosing microglia (**Figure 5B**). Rod degeneration resulted in a significantly higher volume of internalized postsynaptic mGluR6 and presynaptic CtBP2 in microglia in the OPL of RCS rats at P30 (**Figure 5C**), indicating that microglia phagocytosis of synaptic elements was increased when synapses were destroyed. An orthogonal view of a representative high-resolution confocal image showed colocalization of mGluR6 with the CD68-immunoreactive lysosomal compartment of Iba1-positive microglia (**Figure 5E**), suggesting that engulfed synapses were usually located in the internal lysosomal compartments of microglia.

Depletion of Microglia Rescued the Postsynaptic mGluR6 Levels and Increased the Number and Length of Ectopic Dendrites in the RBCs

To confirm the contribution of microglia to the clearance of synaptic material during retina degeneration in RCS rats, we eliminated microglia with the CSF1R inhibitor PLX3397 (600 ppm) (**Supplementary Figures 3A–D**), which was fed to 15-day-old RCS rats for 25 days (**Supplementary Figure 3A**). This compound was previously shown to efficiently deplete microglia in the CNS (38, 39). The number of microglia obviously reduced in both the OPL and the total retina 5 days after CSF1R administration and was maintained at low levels (**Supplementary Figures 3B–D**). Noticeable differences in the numbers of CtBP2-positive and colocalized puncta were not observed during microglia elimination in the RCS rats (**Figures 6A,B,D**); however, the number of

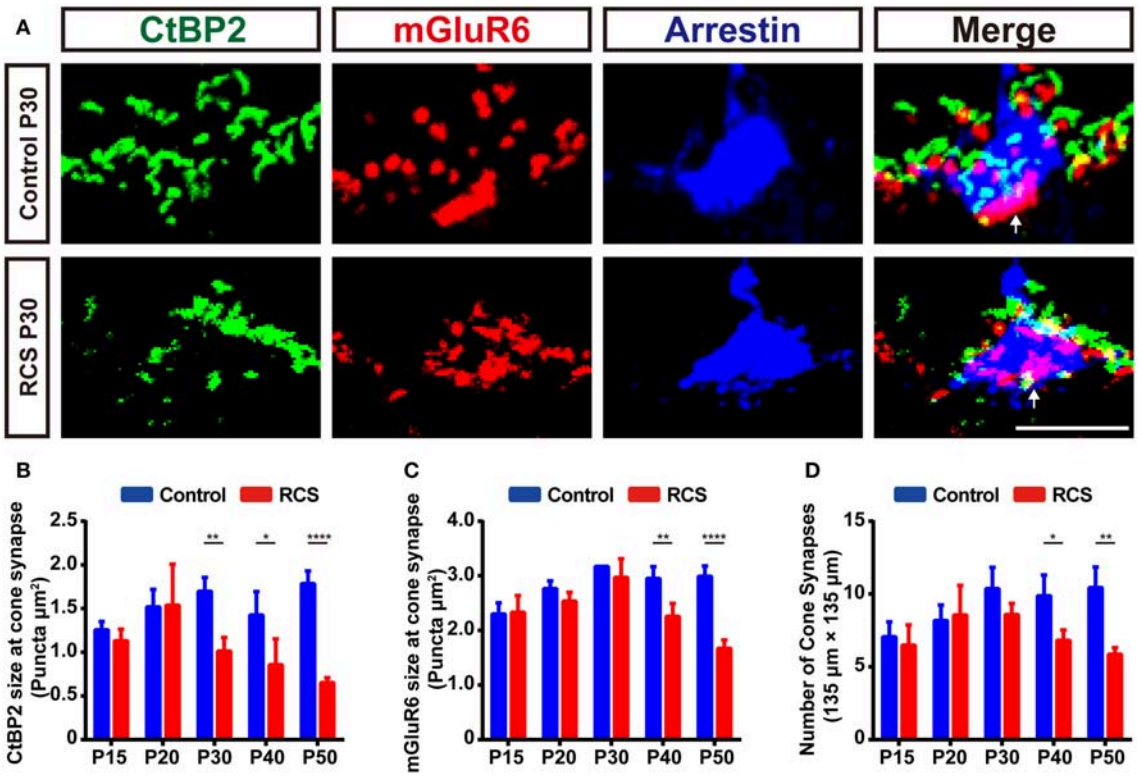

FIGURE 2 | Early cone synapse loss in the OPL during retinal degeneration in RCS rats. **(A)** Immunostaining for CtBP2 (green), mGluR6 (red) and cone arrestin (blue) in the retinas of control and RCS rats at P30. White arrows indicate cone synapses. **(B–C)** Quantification of the changes in the size of cone synaptic puncta positive for CtBP2 and mGluR6 in control and RCS rats at P15, P20, P30, P40, and P50 (N = 3–4 eyes from different rats, n = 9–15 images from each eye). **(D)** Quantification of the changes in the number of cone synapse in control and RCS rats at P15, P20, P30, P40, and P50 (N = 3–4 eyes from different rats, n = 9–15 images from each eye). Scale bar, 5 μm **(A)**. Bars represent means; error bars represent SD. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$ using two-way ANOVA **(B–D)**.

mGluR6-positive puncta and the proportion of unpaired mGluR6-positive puncta increased significantly in the retinas of RCS rats after treatment with PLX3397 for 15 days ($p < 0.01$) (**Figures 6A,C,E** and **Supplementary Figures 4A,B**). Levels of the CtBP2 and mGluR6 mRNAs were not significantly altered between the depletion groups and untreated groups at P30 (**Supplementary Figures 4E,F**). Microglia depletion markedly increased the number of TUNEL-positive cells in the ONL of RCS rats (**Supplementary Figures 3E,F** and **Supplementary Figure 4D**); however, no significant changes in the number of surviving cells were observed between microglia-depleted retinas and untreated groups (**Supplementary Figure 3G**). Therefore, the microglia in the OPL of RCS rats mainly mediated the engulfment of postsynaptic elements.

In the RCS rat retina, ectopic dendrites emerged as early as P36 (27), the time at which the number of microglia in the OPL decreased, while the proportion of unpaired mGluR6 increased. In the present study, the RBC ectopic dendrites labeled with PKCα were observed as early as P30 in the RCS rats exhibiting sustained microglia depletion (**Figure 6F**). Significantly greater numbers and lengths of ectopic RBC dendrites were observed in the PLX3397-treated RCS rats than in RCS control rats (**Figures 6G,H**). However, the proportion of mGluR6-positive ectopic RBC dendrites (**Figure 6I**) was not significantly altered following the elimination of microglia from the RCS rats. Furthermore, in microglia-depleted retinas from control rats, abnormal outgrowth or retraction of dendritic and axonal compartments was not detected (**Supplementary Figure 4C**). Based on these findings, microglia elimination promoted ectopic neuritogenesis of RBCs in RCS rats.

Influences of Microglia on the Dysfunction of Bipolar Cells in the RCS Retina

As structural damage of rod-bipolar synapse contributed to the visual dysfunction of RCS rats at the early stage, microglia probably mediated the visual dysfunction (5). To investigate this, we recorded ERG responses of RCS rats which were continuously depleted microglia for 5,15,25 d, and compared with time matched non-depleted RCS rats respectively (**Figure 7A**). Five days after microglial depletion, a-wave and b-wave amplitudes of scotopic ERG responses exhibited little change relative to untreated groups ($p > 0.05$) (**Figures 7A,B**). Fifteen days after the elimination of microglia, a modest decrease in a-wave amplitudes of scotopic ERG responses was only observed at the highest

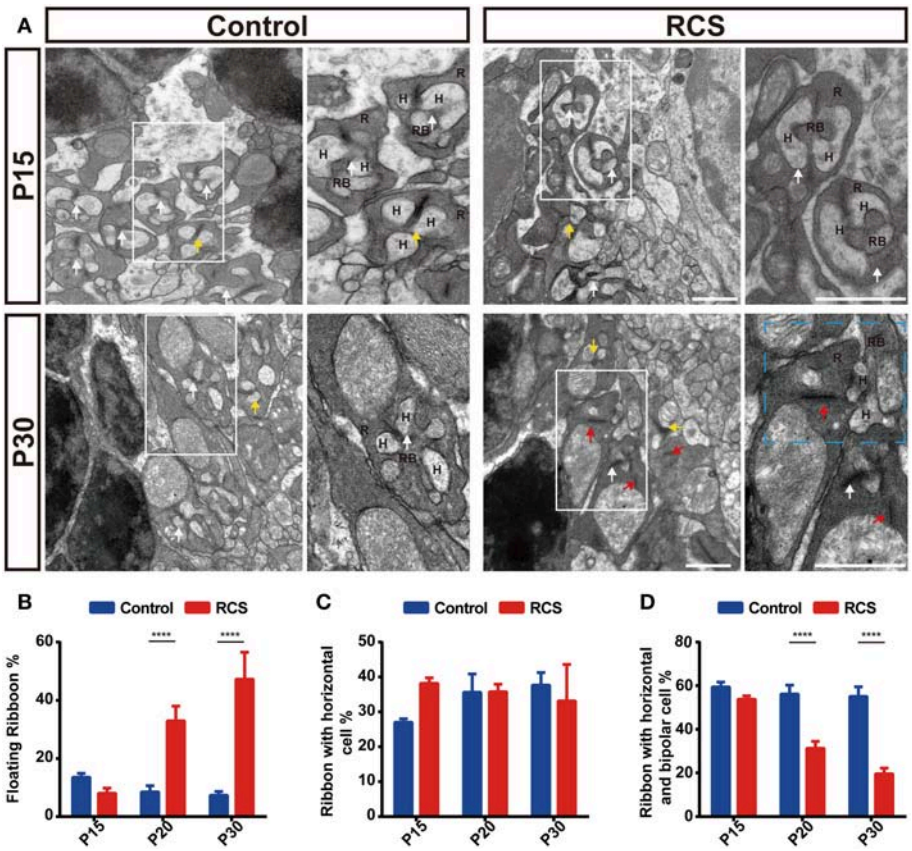

FIGURE 3 | The ultrastructure of rods in ON-RBC synapses in the OPL is disrupted during retinal degeneration in RCS rats. **(A)** Synaptic ultrastructure in the OPL of control and RCS retinas as revealed by transmission electron microscopy at P15 and P30. The cyan rectangle indicates a perturbation of postsynaptic cell dendrite invagination into the photoreceptor terminus. White arrows indicate normal rod synapses, called triads, with three postsynaptic elements (the ribbon was a presynaptic specialization; the three postsynaptic elements invaginated into the base of the photoreceptor such that two horizontal cell and one bipolar cell dendrite are located centrally), red arrows indicate floating ribbons, yellow arrows indicate dyads with only one or two horizontal cell processes opposed to the ribbon. **(B)** Proportion of floating ribbons in control and RCS rats at P15, P20, and P30, indicating that disruption of rod synapse correlated with rod cell stress ($N = 3$ eyes from different rats, $n = 8$–12 images from each eye). **(C)** A difference in the proportion of dyads in RCS rats compared to controls was not observed at P15, P20, and P30 ($N = 3$ eyes from different rats, $n = 8$–12 images from each eye). **(D)** The proportion of normal synapses was significantly decreased during retinal degeneration ($N = 3$ eyes from different rats, $n = 8$–12 images from each eye). R, rod cell; RBC, rod bipolar cell; H, horizontal cell. Scale bar, 1 μm **(A)**. Bars represent means; error bars represent SD. ****$p < 0.0001$ using two-way ANOVA **(B–D)**.

flash intensities, and a slight (but not significant) reduction in scotopic b-wave amplitudes was detected (**Figures 7A,C**). Twenty-five days after depletion, a-wave amplitudes of scotopic ERG responses showed no significant changes, whereas b-wave amplitudes showed a pronounced decrease ($p < 0.05$) (**Figures 7A,D**). Thus, the scotopic b-wave response, originating from postsynaptic transmission from the photoreceptors to RBCs, progressively decreased in the RCS rats. As a result, the b–a amplitude ratios decreased as microglial depletion progressed (**Figure 7D**). The control rats that received PLX3397 for 5, 15, and 25 days did not show any significant changes in a- or b-wave amplitudes under dark-adapted conditions (**Supplementary Figure 5**), indicating that the observed ERG changes were mainly attributed to microglial depletion in the RCS rats, rather than to PLX3397 *per se*.

Microglia Engulfed Postsynaptic Elements in a Complement-Dependent Mechanism

As the complement pathway mainly governs microglia-mediated engulfment of endogenous materials (40–42), we examined the distribution of C1q and C3 in the RP retina in the present study. C1q immunoreactivity was elevated in RCS retinas as early as P15 and preceded synapse loss (**Figure 8A**). Notably, the C1q level was increased in a region-specific manner, particularly in the OPL, the region susceptible to synapse loss. Confocal images confirmed the colocalization of C1q and mGluR6 puncta in the RCS OPL (**Figure 8B**), and a higher percentage of mGluR6-positive puncta colocalized with C1q-positive puncta in the OPL of RCS rats at P20 and P30 than in the control groups (**Figure 8C**). Thus, C1q mediated the engulfment of mGluR6-positive postsynaptic elements. Furthermore, double staining for C1q and IB4 or IBA1 revealed that C1q was mainly

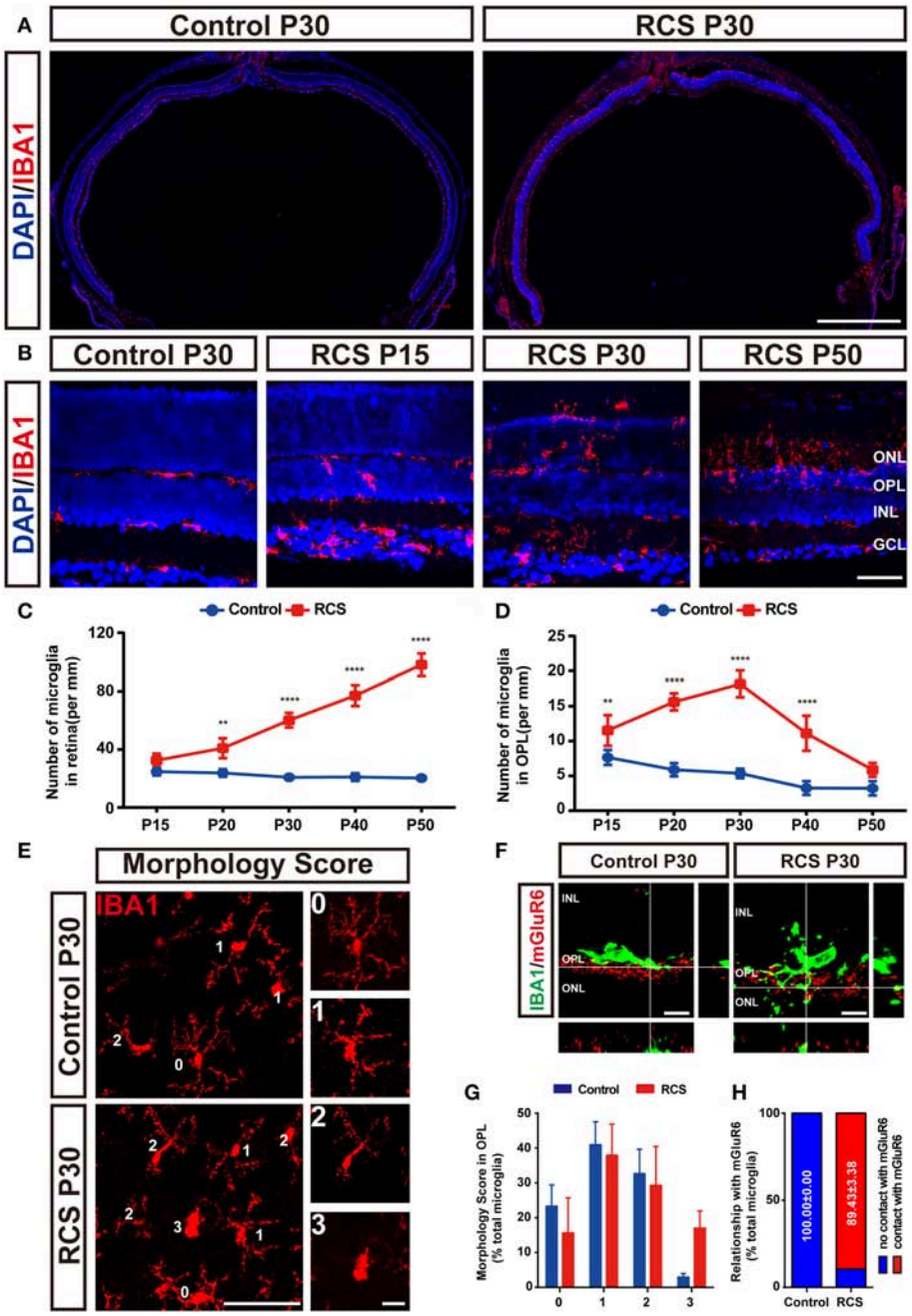

FIGURE 4 | Increased number of microglia and their relationship with synaptic elements in the retinas of RCS rats. **(A)** Representative panoramic images of retinal sections stained with DAPI (blue) and IBA1 (red) from P30 control and RCS rats. **(B)** The distribution of microglia [IBA1 (red)] in the retinas of P30 control rats or P15, P30, and P50 RCS rats. **(C)** Quantification of the number of microglia in the total retinas indicated that the number of total retina microglia increased from P15 to P50 because of photoreceptor apoptosis ($N = 3$ eyes from different rats, $n = 3$ panoramic section images from each eye). **(D)** Quantification of the number of microglia in the OPL, indicating that the number of microglia in the OPL peaked at P30 ($N = 3$ eyes from different rats, $n = 3$ panoramic section images from each eye). **(E)** The morphology of the Iba1-positive cells in the OPL of flat-mounted retinas was examined. For morphology, a score of 0–3 points is shown in the right insets. **(F)** An orthogonal view of high-resolution confocal images suggested that microglia (green) processes extended into synapse lamination and contacted synaptic material (red) in the OPL of P30 RCS rats compared to controls. **(G)** No difference in morphological scores of microglia in the OPL was observed between RCS and control rats at P30. ($N = 3$ eyes from different rats, $n = 5$ flat-mounted images from each retina). **(H)** Almost all of microglia in the OPL contacted synaptic material in the RCS rats at P30. ($N = 4$ eyes from different rats, $n = 10$–15 microglia from each retina from each retina). ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar, 1 mm **(A)**, 50 μm (**B** and **E**) or 10 μm (**E** and **F**). Bars represent means; error bars represent SD. $^{**}p < 0.01$, $^{****}p < 0.0001$ using two-way ANOVA **(C,D,G)**.

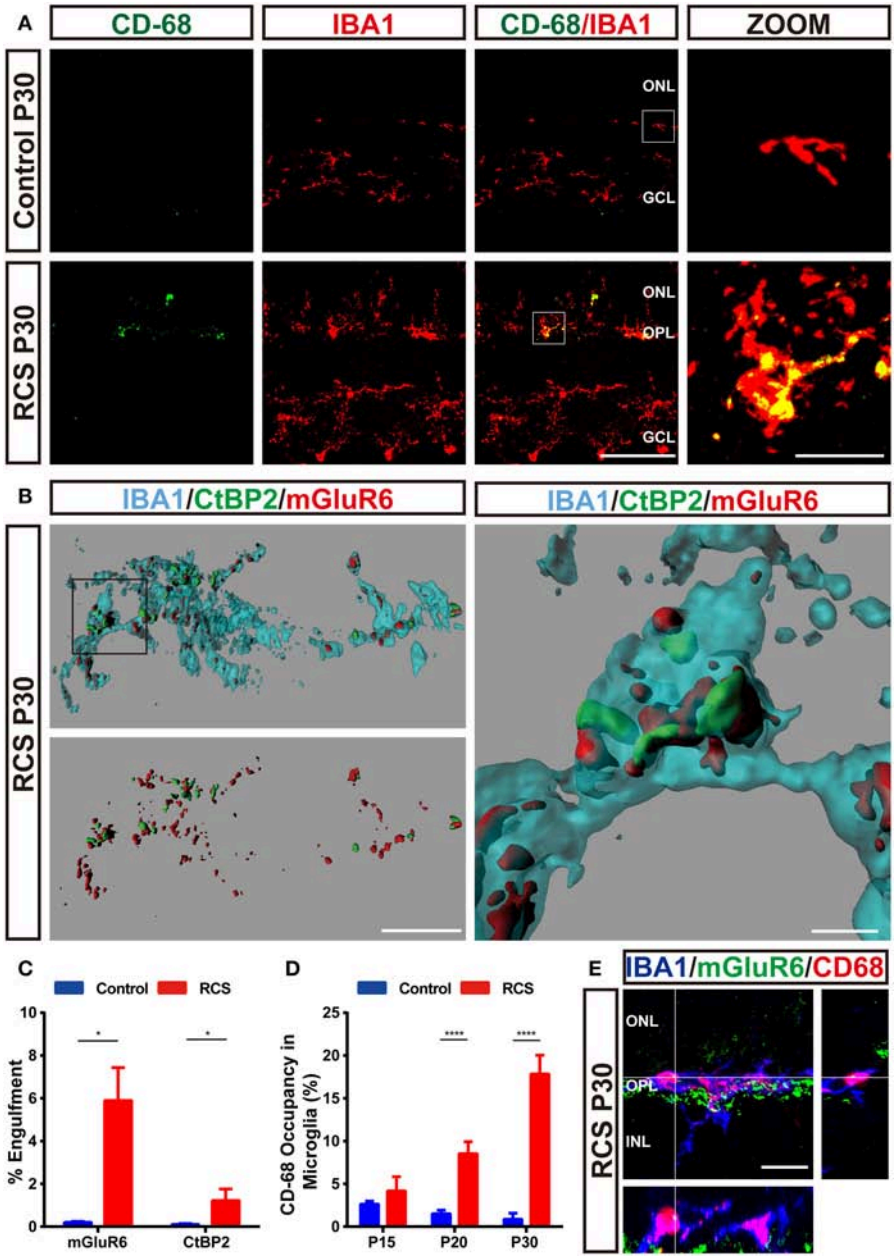

FIGURE 5 | Engulfment of pre- and postsynaptic elements of RBCs by microglia in the retina of RCS rats. **(A)** Immunostaining for IBA1 (red) and CD-68 (green) in the retinas of P30 control and RCS rats suggested that retinal degeneration induced high levels of CD68 (green) immunoreactivity in Iba1-positive (red) microglia in the OPL. **(B,C)** Three-dimensional reconstruction and surface renderings prepared using Imaris revealed larger volumes of synaptic puncta inside microglia in the RCS OPL, particularly postsynaptic mGluR6 puncta, compared with controls ($N = 3$ eyes from different rats, $n = 15$–20 microglia from each retina). **(D)** Quantification of % volume of microglia occupied by CD68-positive lysosomes in the OPL of control and RCS rats at P15, P20, and P30 ($N = 3$ eyes from different rats, $n = 10$–15 microglia from each retina). **(E)** An orthogonal view of a representative high-resolution confocal image showed colocalization of mGluR6 (green) within the CD68-immunoreactive lysosomal compartment (red) of an Iba1-positive microglial cell (blue). ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar, 50 μm **(A)**, 10 μm **(A,B,E)** or 1 μm **(B)**. Bars represent means; error bars represent SD. $^{*}p < 0.05$; $^{****}p < 0.0001$ using an independent two-samples *t*-test **(C)** or two-way ANOVA **(D)**.

localized in the vasculature and microglia in the retina of RCS rats (**Figure 8D**). Sustained microglial depletion significantly decreased C1q levels in the retinas of RCS rats (**Figure 8E**). C3, which is activated by C1q to opsonize synapse elimination (28, 42–44), was also deposited on the unpaired mGluR6-positive puncta in the OPL of RCS rats at P30 (**Figure 8F**). Therefore, microglia might phagocytize postsynaptic elements through a complement-dependent manner.

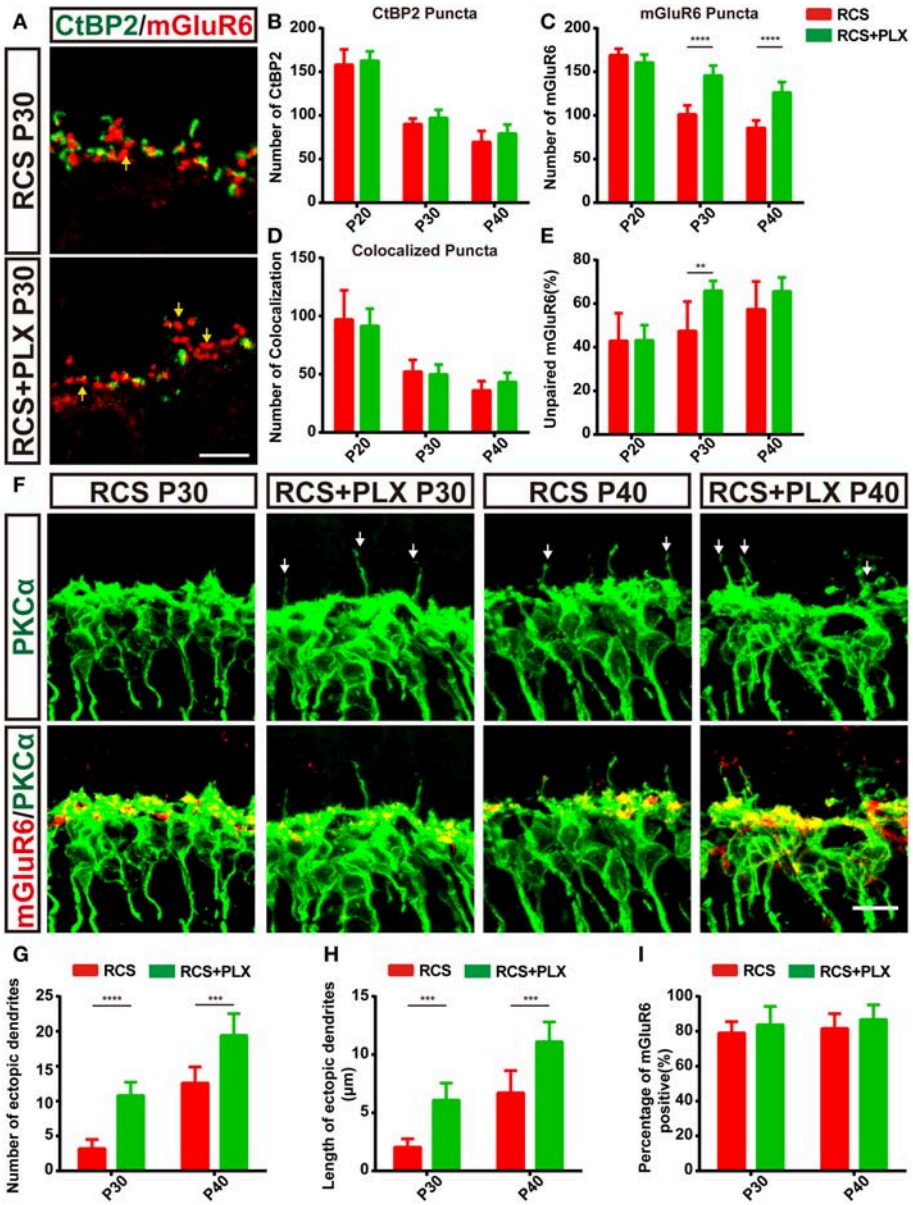

FIGURE 6 | The elimination of microglia rescues the loss of postsynaptic mGluR6-positive elements and increases the number and length of ectopic RBC dendrites in RCS rats. **(A)** Confocal images of CtBP2- (green) and mGluR6-immunoreactive (red) puncta in the retinas of RCS rats and RCS rats treated with PLX3397 at P30. Yellow arrows indicate unpaired mGluR6-positive puncta. **(B–E)** Quantification of synaptic puncta or their apposition indicated that sustained microglia depletion reduced the loss of mGluR6-positive puncta and increased the proportion of mGluR6-positive puncta that were not paired with CtBP2-positive puncta; however, this depletion did not result in the alteration of colocalized puncta and presynaptic CtBP2-positive puncta ($N = 3$–5 eyes from different rats, $n = 15$–20 images from each eye). **(F)** Immunostaining for PKCα (green) and mGluR6 (red) in the retinas of RCS rats and RCS rats treated with PLX3397 at P30 and P40. White arrows indicate ectopic dendrites. **(G,H)** The number and length of ectopic RBC dendrites increased in the retinas of RCS rats and RCS rats treated with PLX3397 P30 and P40 in each field of view (213 × 213 μm) ($N = 5$ eyes from different rats, $n = 7$–12 images from each eye). **(I)** The proportion of mGluR6-positive ectopic RBC dendrites in each field of view (213 × 213 μm) of the retinas was not different between RCS rats and RCS rats treated with PLX3397 at P30 and P40 ($N = 5$ eyes from different rats, $n = 7$–12 images from each eye). ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar, 5 μm **(A,F)**. Bars represent means; error bars represent SD. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ using two-way ANOVA **(B–E,G–I)**.

DISCUSSION

In the present study, we observed novel roles for microglial in the early synaptic remodeling of neuronal circuits during retinal degeneration. Resident microglia in the retina OPL phagocytosed postsynaptic elements of RBCs at the early stage of RCS rats (**Figure 9**). Sustained microglial depletion by PLX3397 blocked synapse loss, increased ectopic neuritogenesis of RBCs and

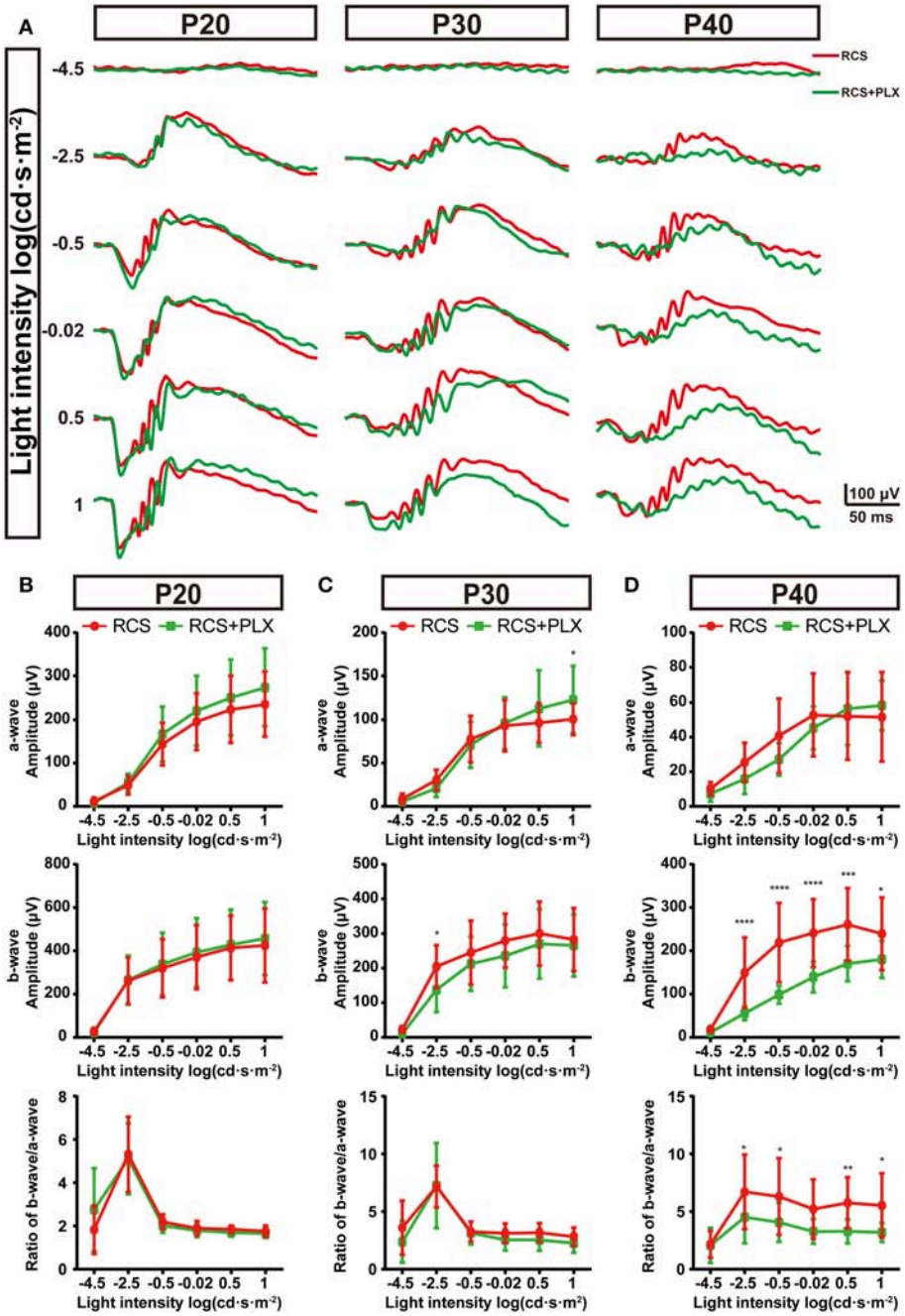

FIGURE 7 | Elimination of microglia in the retinas of RCS rats reduces the amplitudes of ERG signals. **(A)** Representative light-evoked ERG waveforms measured with six different light intensities [from −4.5 to 1 log(cd·s·m^{-2})] in P20, P30, and P40 RCS rats and RCS rats treated with PLX3397. **(B–D)** Average stimulus-response curves for a-wave amplitudes (top row), b-wave amplitudes (middle row) and the ratio of a-wave/b-wave amplitudes in RCS rats and RCS rats treated with PLX3397 at P20 (n = 18 and 34, respectively), P30 (n = 16 and 28, respectively) and P40 (n = 15 and 16, respectively) show that sustained microglial depletion reduced the b-wave amplitude and did not affect the a-wave amplitude. This result was exacerbated over time. Bars represent means; error bars represent SD. *p < 0.05; **p < 0.01, ***p < 0.001, ****p < 0.0001 using two-way ANOVA **(B–D)**.

further exacerbated the deterioration of visual function. C1q expression was induced by microglia, and classical complement pathway were revealed involvement in clearance of postsynaptic elements. Based on our data, microglia mediated the remodeling of neuronal circuits in RBCs, contributing to the maintenance of vision at the early stage of RP.

During retinal degeneration, changes in synapse morphology were detected at P21 (5). In the present study, we confirmed

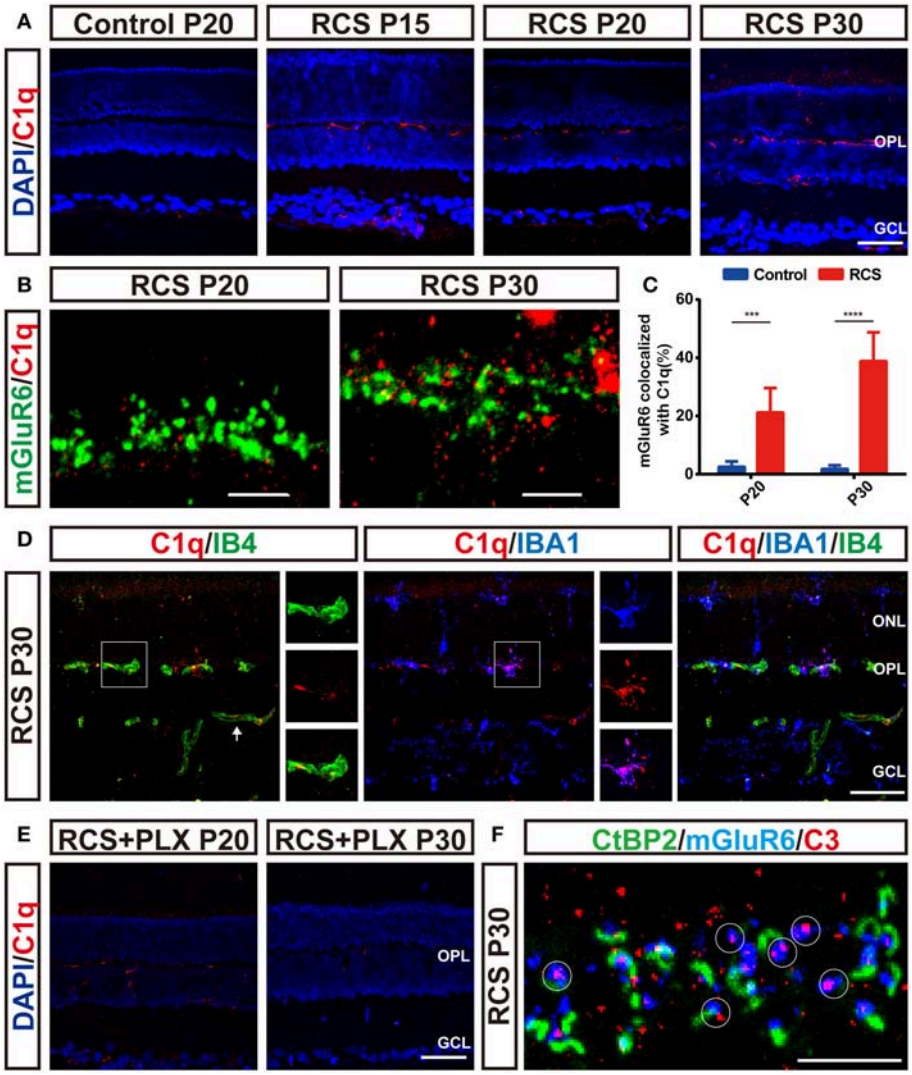

FIGURE 8 | Microglia-induced C1q upregulation and deposition onto postsynaptic elements precede synapse loss in the OPL during retinal degeneration in RCS rats. **(A)** Immunostaining for C1q (red) in the retinas revealed region-specific (OPL) upregulation of C1q (red) in RCS rats. **(B,C)** Confocal images showing an increase in the colocalization of C1q (red) and mGluR6 (green) in RCS rats compared with controls ($N = 6$ eyes from different rats, $n = 6$–10 images from each eye). **(D)** Labeling of the retinal vasculature using isolectin-B4 (IB4) and microglia using IBA1 revealed that C1q in the OPL was mainly derived from the blood circulation and microglia in the P30 RCS rats. White arrows indicated that C1q came from the vasculature in the inner retina. **(E)** Elimination of microglia with the PLX3397 treatment decreased C1q immunoreactivity in the retinas of RCS rats. **(F)** Representative confocal image showing the colocalization of C3 (red) and mGluR6 (blue) but a lack of colocalization with CtBP2 (green) in the retinas of P30 RCS rats. White circles indicate C3 deposition onto unpaired mGluR6. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar, 50 μm **(A,D,E)**, 5 μm **(B,F)**. Bars represent means; error bars represent SD. ***$p < 0.001$, ****$p < 0.0001$ using two-way ANOVA **(C)**.

that rod-RBC synapses were lost in the OPL of RCS rats as early as P20 and reached a value of 50% at P30. Notably, floating ribbons and RBC dendritic tips extending from rod spherules were observed using electron microscopy, and some of these abnormal synaptic contacts between rods and RBCs were called the flat-contact type (34). These results implied that the loss of presynaptic and postsynaptic elements in the OPL did not occur through the simultaneous degradation of both elements in intact synapses. Changes in the size of the presynaptic specialization of cone synapses occurred earlier than changes in the size of the postsynaptic density, indicating that photoreceptor degeneration induced sequential presynaptic protein loss, synaptic disintegration and eventually postsynaptic element loss. In some retinal degeneration models, synapses were reported to form normally (6, 24, 45). Thus, photoreceptor synaptogenesis could occur before photoreceptor degeneration. In RCS rats, progressive photoreceptor degeneration was detected after the time of eye opening (at P15 in rats) (25, 34, 46). In the present study, synapse levels in the retinas of RCS rats were not altered at P15 compared with control rats. Our data from transmission electron microscopy further confirmed normal synaptic structures in P15 RCS rat retinas.

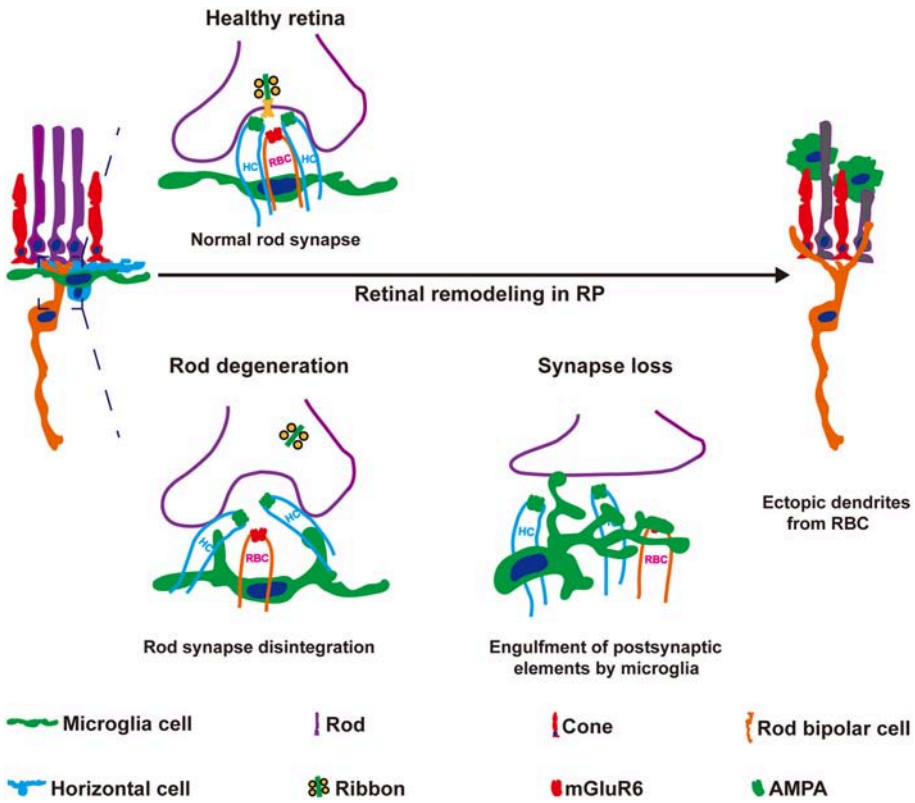

FIGURE 9 | Microglia engulf postsynaptic elements and mediate the formation of ectopic dendrites in the retinas of RCS rats. Synaptic remodeling of neuronal circuits in the OPL are early hallmarks of RP that are thought to be initiated by photoreceptor degeneration. However, the mechanisms underlying synapse loss and remodeling remain elusive. Here, microglia-mediated engulfment of postsynaptic elements was a mechanism underlying ectopic RBC neuritogenesis. Rod degeneration perturbed RBC dendrite invagination into the photoreceptor terminus, and then these postsynaptic elements of RBCs were engulfed by microglia. Inhibition of the microglial engulfment of synaptic material using a pharmacological method increased postsynaptic mGluR6 expression and the formation of ectopic RBC dendrites, but decreased the ERG responses in RCS rats. Based on these findings, microglial cells play vital roles in the synapse loss and remodeling observed in RCS rats.

Based on our data, synaptic element loss was likely not a result of abnormal synaptic development, but a consequence of photoreceptor degeneration.

Coincident with RBC dendritic retraction from the OPL after rod degeneration, mGluR6 disappeared from RBC dendrites (7, 8), raising the possibility that mGluR6 loss was a consequence of a rapid and local disassembly of ON-bipolar cell dendrites (47, 48). However, deletion of RIBEYE, which substantially decreases the number of ribbons in retinal synapses and disrupts neurotransmitter release, does not affect the overall organization and synaptic connectivity of the retina (49). The loss of the typical triad organization does not disturb the gross structure of bipolar dendritic tips, including mGluR6 accumulation (50–52). Together with findings from the present report, perturbations in synaptic inputs and integrity did not directly lead to mGluR6 elimination via degradation, suggesting that synapse loss might have resulted from phagocytosis by microglia cells. The presynaptic materials, including ribbons, disappeared possibly as a part of the mechanism by which phagocytic microglia remove rod cells in the ONL (16) or were decomposed through a lysosomal-mediated autophagic process at the early stage of rod apoptosis (53).

Microglia are known to play a role in the pruning of synapses during CNS development and neurodegeneration (54). Microglia play key roles in the selective clearance of presynaptic debris during Wallerian degeneration (36) or the elimination of postsynaptic elements in subjects with Alzheimer's disease (AD) (28). In the present study, microglia in the OPL mainly engulfed postsynaptic mGluR6, with little effect on synaptic integrity or presynaptic ribbon loss. Following the formation of direct contacts, the OPL microglia engulfed postsynaptic debris, translocating it to phagosomes for destruction. Similar to subjects with AD (28), microglia in the RCS OPL exhibited the similar ramified morphology as controls, indicating that photoreceptor stress induced synapse engulfment by resident homeostatic microglia. Synapse disintegration was not blocked by microglia depletion in RCS rats, suggesting that the disruption of the synaptic triad pattern was not initiated by phagocytosing microglia.

In RCS rats, the microglia in the retinas also carried the MERTK mutation. Our present data showed that Mertk mutation did not influence the synapses phagocytosis of microglia in the OPL of RCS rats at P30. However, more apoptotic cells were observed in the ONL of the RCS rats after microglia were

deleted by PLX3397 treatment. It seemed that Mertk-deficient did not interrupt the phagocytosis of the microglia completely and the microglia kept the ability to engulf apoptotic cells and synaptic elements. To date, the best characterized mediators of synaptic pruning are components of the complement cascade (12). Previous experiments implicated the complement system as a key driver of the clearance of neuronal materials by microglia during development (12, 55, 56), and in subjects with AD (28), schizophrenia (57), viral infection (58), and some types of retinal degeneration (59, 60). While most of these latter studies had not shown interest in microglia-mediated removal of synapses via complement, we speculated that this pathway would be confirmed to promote synaptic elimination and that inhibition of this pathway or its components would prevent engulfment by microglia. Moreover, local activation of the complement pathway might serve as a key mechanism underlying rod apoptosis-induced synapse pruning in the RP retina. C1q was not only produced by microglia but was also derived from the circulatory system in RP retinas. Microglia depletion in RCS rats significantly ameliorated C1q deposition, suggesting that microglia played a crucial role in activating the complement cascade and driving synapse elimination.

In a recent study, microglial depletion using transgenic mice perturbed synaptic function and integrity in the mature retina (44); however, synapse formation and ERG responses in control rat retinas were not changed by pharmacological elimination of microglia in this study. A similar phenomenon was observed in the adult brain, as mice depleted of microglia showed deficits in multiple learning tasks and a significant reduction in motor-learning-dependent synapse formation using the same genetic methods of microglial depletion (61) as in the mature retina (44). However, behavioral or cognitive abnormalities were not detected in the mice treated with PLX3397 (39), suggesting that the discrepancies in the functional conclusions of sustained microglia elimination were due to the use of different experimental methods. Furthermore, tamoxifen, which is used to manipulate genetic microglia elimination, exerts protective effects on retinal degeneration (62). Thus, pharmacological depletion of microglia by a CSF1R inhibitor was a more suitable approach to explore the function of microglia.

In our study, the b-wave amplitude in the ERG, which reflected RBC function, was progressively reduced during microglial depletion in the RCS retina. However, significant changes in surviving photoreceptors and a-wave amplitudes were not observed in RCS rats treated with PLX3397, suggesting that sustained microglia depletion did not affect the function of photoreceptors in the RP rat model. The same phenomenon was observed in a model of acute injury of optic nerves, in which retinal ganglion cell (RGC) degeneration remained unaffected upon microglia depletion by PLX5622 (another CSF1R inhibitor) (63). Based on our results, sustained microglial depletion aggravated the response of RBCs to light in RCS rats. Thus, the abnormal accumulation of mGluR6 in RBCs damaged their function, providing further evidence that the engulfment of postsynaptic elements by microglia in the OPL of RCS rats improved the light response of RBCs.

According to a recent report from our group, miR-125b-5p regulates the formation of ectopic dendrites from RBCs into the ONL and functional synapse formation with the remaining photoreceptors in RCS rats (27). However, the key mechanism triggering ectopic neuritogenesis remains unknown. Ectopic dendrites of RBCs might form to restore their input activity in the degenerating retina (10, 27, 64, 65), suggesting that the dysfunction of RBCs might be a key trigger of remodeling (10). Based on our data that depletion of microglia promoted ectopic neuritogenesis, microglia were shown to be involved in the mechanism regulating ectopic dendrite formation by the RBCs of RCS rats. We also hypothesized that microglia subsequently delayed ectopic neuritogenesis by restoring function of RBCs.

In conclusion, microglial cells did not only contribute to the survival of photoreceptors and to the maintenance of retinal architecture (16, 18, 19), but also mediated synaptic remodeling of neuronal circuits by phagocytosing synapses in the RP retina. In our study, a CSF1R inhibitor exerted detrimental effects on the visual function of a RP rat model, suggesting that this type of drug-induced non-selective microglial elimination was not suitable to restore the vision of patients with RP. A treatment that selectively stimulates synaptic phagocytosis by microglia in the retina would be an important topic in future studies of retinal degenerative diseases. Based on our findings, novel treatment strategies targeting microglia should add mechanisms governing microglia–synapse interactions, and not simply aim to support photoreceptor survival.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Third Military Medical University Animal Care and Use Committee. The protocol was approved by the Animal Center of the Third Military Medical University.

AUTHOR CONTRIBUTIONS

JH: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing. JD, LG, YF, CW, BB, HL, and YG: collection and assembly of data. CZ: conception and design. HX: conception and design, data analysis and interpretation, manuscript writing, final approval of manuscript. ZY: conception and design, financial support, data analysis and interpretation, final approval of manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00912/full#supplementary-material

Supplementary Figure 1 | The change of synapse in the OPL during retinal degeneration in RCS rats. **(A)** Schedule of synapse analysis in the OPL at the early stage of retinal degeneration in RCS rats. **(B,C)** Confocal images of CtBP2- (green) and mGluR6-immunoreactive (red) puncta in the retinas of control and RCS rats at P15 and P20. **(D,E)** CtBP2 and mGluR6 mRNA expression in the retinas of control and RCS rats at P15, P20, P30, P40, and P50 ($N = 3$ rats per group). **(F,G)** Immunostaining for CtBP2 (green), mGluR6 (red), and cone arrestin (blue) in the retinas of control and RCS rats at P15 and P20 respectively. ONL, outer nuclear layer; OPL, outer plexiform layer; IPL, inner plexiform layer; Scale bar, 50 μm **(B,C)** or 5μm **(F,G)**. Bars represent means; error bars represent SD. $^{**}p < 0.01$, $^{****}p < 0.0001$ using two-way ANOVA **(D,E)**.

Supplementary Figure 2 | Microglia distribution and morphology in the retina of control and RCS rats. **(A)** Representative panoramic images of retinal sections stained with DAPI (blue) and IBA1 (red) from P15 and P20 RCS rats. **(B)** Representative panoramic images of retinal sections stained with DAPI (blue) and IBA1 (red) from P40 and P50 RCS rats. **(C)** Representative confocal image showing the morphology of microglia (red) in the ONL of RCS rats at P30. **(D)** Percentages of microglia displaying each score in the RCS ONL. **(E)** An orthogonal view of a representative high-resolution confocal image showed the relationship between mGluR6 (green), CD68 (red) and Iba1-positive microglial cell (blue). **(F,G)** Immunostaining for IBA1 (red) and CD-68 (green) in the retinas of P15 and P20 RCS rats. ONL, outer nuclear layer; OPL, outer plexiform layer; IPL, inner plexiform layer; Scale bar, 1 mm **(A,B)**, 50 μm **(C,E,F)**. Bars represent means; error bars represent SD.

Supplementary Figure 3 | Effects of microglia elimination on the apoptosis and survival of photoreceptors in the retinas of RCS rats. **(A)** Schematic of microglial elimination and time points examined in the RCS rats. **(B)** Panoramic images of retinal sections from PLX-administered RCS rats immunostained with Iba1 showed the presence and density of retinal microglia. **(C,D)** Quantification of the total number of retinal microglia and number of OPL microglia. Although microglial numbers were substantially increased in RCS rats, PLX-treated RCS rats exhibited sustained microglial elimination (>90%) in the OPL for up to 25 days. **(E)** Representative high-resolution confocal images showing photoreceptor apoptosis using TUNEL (green) in the retinas of RCS rats and RCS rats treated with PLX. **(F,G)** Quantification of DAPI- and TUNEL-positive photoreceptor cells in the ONL at three time points did not reveal significant differences in the number of surviving cells between RCS rats and RCS rats treated with PLX, although the number of TUNEL-positive photoreceptor cells was substantially increased following sustained microglia depletion. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar, 50 μm **(B,E)**. Bars represent means; error bars represent SD. $^{*}p < 0.05$; $^{***}p < 0.001$, $^{****}p < 0.0001$ using two-way ANOVA **(C,D,F,G)**.

Supplementary Figure 4 | The influence of microglia elimination on the synapses in RCS rats or photoreceptors in control rats. **(A,B)** Confocal images of CtBP2- (green) and mGluR6-immunoreactive (red) puncta in the retinas of RCS rats and RCS rats treated with PLX3397 at P20 and P40. **(C)** Immunostaining for CtBP2 (green), PKCα (red), and mGluR6 (blue) in the retinas of control rats treated with PLX3397 at P40. **(D)** Representative high-resolution confocal images showing photoreceptor apoptosis using TUNEL (green) in the retinas of control rats and control rats treated with PLX3397 at P40. **(E,F)** An analysis CtBP2 and mGluR6 mRNA expression in the retinas of control rats, control rats treated with PLX, RCS rats, and RCS rats treated with PLX at P30 suggested that PLX had no effect on CtBP2 and mGluR6 expression ($N = 3$ rats per group). ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar, 50 μm **(A-D)**. Bars represent means; error bars represent SD. using two-way ANOVA **(E,F)**.

Supplementary Figure 5 | Effects of microglia eliminate on the amplitudes of ERG signals in control rats. **(A)** Representative light-evoked ERG waveforms measured with six different light intensities [from -4.5 to 1 log(cd.s.m-2)] in P20, P30, and P40 control rats and control rats treated with PLX3397. **(B-D)** Average stimulus-response curves for a-wave amplitudes (top row), b-wave amplitudes (middle row) and the ratio of a-wave/b-wave amplitudes in control rats and control rats treated with PLX3397 at P20 ($n = 14$ and 8 rats, respectively), P30 ($n = 20$ and 10 rats, respectively), and P40 ($n = 6$ and 6 rats, respectively). Bars represent means; error bars represent SD. using two-way ANOVA **(B-D)**.

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Reduced Angiopoietin-Like 4 Expression in Multiple Sclerosis Lesions Facilitates Lipid Uptake by Phagocytes via Modulation of Lipoprotein-Lipase Activity

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Multiple sclerosis (MS) is a chronic inflammatory disorder of the central nervous system (CNS) characterized by the presence of focal demyelinated plaques. Sufficient clearance of myelin and cellular debris is one of the requirements for proper tissue repair and remyelination. The mechanisms underlying the clearance of such debris by phagocytes are not fully understood, but recent findings suggest a prominent role for lipoprotein-lipase (LPL) in this process. Here, we demonstrate that angiopoietin-like 4 (ANGPTL4), a potent inhibitor of LPL, is abundantly expressed in astrocytes in control white matter tissue and its expression is markedly reduced in active MS lesions. We provide evidence that ANGPTL4 inhibits the uptake of myelin-derived lipids by LPL-immunoreactive phagocytes. Taken together, our data suggest that the strong reduction in astrocytic ANGPTL4 expression in active demyelinating MS lesions enables phagocytes to adequately clear myelin debris, setting the stage for remyelination.

Keywords: angiopoietin-like 4, multiple sclerosis, phagocytes, astrocyte, lipoprotein-lipase, remyelination

INTRODUCTION

MS is an inflammatory demyelinating disease characterized by massive infiltration of monocyte-derived macrophages into the central nervous system (CNS). Infiltrated macrophages, as well as brain-resident activated microglia, produce a variety of cytotoxic factors and cytokines and thereby contribute to CNS damage and associated neurodegeneration. Macrophage depletion in an experimental MS animal model significantly reduces clinical symptoms underscoring the pathogenic role of these cells (1). However, macrophages and microglia also exert neuroprotective properties. Notably, intravenous administration of anti-inflammatory macrophages or microglia reduced clinical signs in the MS animal model experimental autoimmune encephalomyelitis (EAE) (2, 3). A well-known mechanism by which phagocytes promote regeneration is via clearance of myelin debris. Removal of damaged myelin components and apoptotic cells is a requisite for remyelination (4, 5). Although the cellular mechanisms involved in clearance of myelin debris by phagocytes are poorly understood, evidence is emerging that lipoprotein-lipase (LPL), an enzyme involved in lipid-processing, plays an important role during initiation of remyelination (4, 6). Activity of this enzyme is significantly increased in brain tissue of the EAE model at the point

when clinical symptoms start to decrease and shows to be involved in lipid and lipoprotein uptake in microglia (6). This suggests that LPL-expressing phagocytes might contribute to repair and support remyelination through the clearance and reuptake of lipid debris. Regulation of phagocyte function and activity is under control of astrocytes (7) and recently, our group described the presence of, angiopoietin-like 4 (ANGPTL4), a potent inhibitor of LPL, in astrocytes (8). We here provide evidence for decreased astrocytic expression of ANGPTL4 within active inflammatory MS lesions. We show that the cellular communication between astrocytes and macrophages is necessary for the downregulation of astrocytic ANGPTL4 expression. Furthermore, we show that ANGPTL4-mediated inhibition of LPL activity reduced myelin-lipid uptake by phagocytes, without affecting phagocytosis. Taken together, our findings suggest that the strong reduction in astrocytic ANGPTL4 expression in active demyelinating MS lesions enables LPL-immunopositive phagocytes to adequately clear myelin debris, paving the way for remyelination.

MATERIALS AND METHODS

Immunohistochemistry

Blocks of formalin-fixed paraffin-embedded post-mortem brains samples were obtained from the VUmc MS Centrum Amsterdam and the Netherlands Brain Bank from 12 MS patients and 3 non-neurological controls. Detailed clinical data are summarized in **Table 1**. Sections (5 μm) from each block were cut with a microtome. Sections were deparaffinized in xylene and rehydrated through graded alcohol into distilled water. Antigen retrieval was performed using citrate buffer pH 6.0 (0.01M) at 100°C for 10 min. Sections were incubated overnight with appropriate antibodies (see **Table 2**) in phosphate buffered saline (PBS) supplemented with 1% bovine serum albumin (BSA) and subsequently stained with the EnVision horseradish peroxidase kit (Dako, K4061, Belgium) for 30 min at room temperature and followed by 3,3′diaminobenzidine-tetrachloridedihydrate (DAB). Between incubation steps, the sections were thoroughly washed with PBS. After a short rinse in tap water the sections were incubated with haematoxylin for 1 min and intensely washed with tap water for 10 min. Finally, the sections were dehydrated with ethanol followed by xylene and mounted with Entellan (Merck, #107960, Germany).

For cellular localization sections were incubated overnight with antibodies applied simultaneously at 4°C. After washing with PBS, secondary antibodies consisting of donkey-anti-mouse Alexa Fluor 488 (1:200, Abcam, ab150105, UK), rabbit-anti-goat Alexa Fluor 647 (1:200, Abcam, ab150143, UK), or goat-anti-rabbit Alexa Fluor 647 (1:200, Abcam, ab150079, UK) were applied for 1 h at room temperature. Fluorescent preparations were embedded and analysis was performed with a Leica TCS SP8 confocal laser-scanning microscope (Leica Microsystems, Heidelberg, Germany). GFAP and ANGPTL4 fluorescence intensity were measured by a blinded observer using ImageJ software (freely available from: U.S. National Institutes of Health, Bethesda, MD, USA). Per slide, 3 independent locations were analyzed. Relative ANGPTL4 intensity to GFAP was calculated by dividing ANGPTL4 intensity over GFAP intensity. Next, intensity was normalized to the relative intensity of ANGPTL4 over GFAP in NAWM slides.

Human Astrocyte Culture

Human astrocytoma cells (U373) were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 (Life Technologies, #11320033, USA) containing 10% fetal bovine serum (FBS; Biowest, S1810-500, USA), and penicillin/streptomycin (50 mg/ml; Life Technologies, #15140122, USA) in 5% CO_2 at 37°C.

RNA Isolation and Real-Time Quantitative PCR

RNA was isolated using Trizol (Invitrogen, Carlsbad, CA, USA) according to manufacturer's protocol. mRNA concentration and quality ($OD_{280/260}$ ratios of 1.8 or higher) was measured using Nanodrop (Nanodrop Technologies, USA). cDNA syntheses was performed using the Reverse Transcription System kit (Applied Biosystems, #4368814, USA) following manufacture's guidelines. Expression was assessed by quantitative RT-PCR using SYBR Green Power mix (Applied Biosystems, #4367659, USA). All primer sequences are listed in **Table 3**. qPCR reaction was performed using the Step-one (Applied Bioscience, USA) Real-Time PCR System with the following program: 2 min at 50°C, 10 min at 95°C and then 40 cycles of 15 s at 95 °C and 1 min at 60°C. mRNA expression levels were quantified using the $2^{-\Delta\Delta CT}$ method as described in (9).

Myelin Isolation

Myelin was isolated and labeled as described previously (10). In short, human CNS white matter tissue was obtained from three healthy controls. Myelin was isolated by homogenization and sucrose gradient centrifugation after which protein concentration was assessed by the bicinchoninic acid method. Isolated myelin was subsequently labeled with NHS-activated Atto633.

Human Macrophages Cultures

Human blood monocytes were isolated from buffy coats of healthy donors (Sanquin Blood Bank, The Netherlands) using Ficoll (Lymphoprep™, Axis-Shield, #1114544 Norway) and subsequent Percoll density gradient centrifugation. Monocytes were differentiated into macrophages in culture medium (IMDM, Gibco) containing 10% FBS, penicillin (100IU/ml), streptomycin (50 mg/ml) and 50 ng/ml MCSF, for 6 days at 37°C, 5% CO_2. The alternatively activated macrophages phenotype was induced by culturing M-CSF differentiated macrophages in the presence of 10 ng/ml human IL-4 (ImmunoTools, #13340043, Germany) for 48 h as described before by (11).

Macrophage/Astrocyte Coculture

Human monocytes were cultured on top of a transwell filter (Corning, #3421, USA) with a pore size of 0.3 μm and differentiated into macrophages as described above. Human astrocytoma cells (U373) were plated in a culture plate. After cell adhesion, the transwell filter containing macrophages was transferred to the culture plate containing astrocytes. After 24 h at 37°C in 5% CO_2, the transwell filter containing macrophages

TABLE 1 | Clinical data of MS patients and non-neurological controls.

Case	Age (years)	Type of MS	Gender	Post-mortem delay (h:min)	Disease duration (years)	Cause of death
Ctrl 1	87	NA	F	07:20	NA	Cachexia and dehydration
Ctrl 2	83	NA	M	05:15	NA	Unknown
Ctrl 3	51	NA	F	05:36	NA	Unknown
MS 1	61	RR	F	10:55	Unknown	Sepsis
MS 2	39	RR/SP	F	08:30	8	Ileus
MS 3	75	SP	M	22:20	35	Urosepsis
MS 4	48	SP	F	09:20	24	Pneumonia
MS 5	56	RR	F	08:55	20	Legal euthanasia
MS 6	61	RR	F	10:55	Unknown	Sepsis
MS 7	41	PP	M	07:23	14	Urosepsis and pneumonia
MS 8	49	RR	M	08:00	25	Pneumonia
MS 9	51	SP	M	11:00	18	Infection
MS 10	44	SP	M	10:15	21	Unknown
MS 11	57	SP	F	08:40	27	Respiratory insufficiency
MS 12	54	PP	M	08:15	12	Legal euthanasia

MS, multiple sclerosis; SP, secondary progressive MS; RR, relapse remitting MS; PP, primary progressive MS; ND, non-determined; NA, non-applicable; M, male; F, female.

TABLE 2 | Antibody details.

Antigen	Species	Dilution	Manufacturer	Cat. number
HLA-DR	Mouse	1:1000	eBioscience	14-9956-82
PLP	Mouse	1:3000	Rio-rad	MCA839G
ANGPTL4	Rabbit	1:300	Abcam	ab115798
GFAP-cy3	Mouse	1:300	Sigma	C9205
GFAP	Rabbit	1:250	Chemicon	ab1980
LPL	Mouse	1:100	Abcam	ab21356
Iba1	Goat	1:100	Abcam	Ab5076

HLA-DR, human leukocyte antigen-DR; LPL, Lipoprotein-lipase; ANGPTL4, Angiopoietin-like 4; GFAP, glial fibrillary acidic protein; PLP, proteolipid protein; Iba1, ionized calcium binding adaptor molecule 1.

was discarded and mRNA from astrocytes was isolated as described above.

Oil-Red-O Staining

Alternatively activated macrophages were cultured on glass coverslips and fixed with 4%PFA for 10 min. Oil-Red-O stock solution was prepared by dissolving in 0.25 g Oil-Red-O (Sigma-Aldrich, #O0265, USA) in 50 ml isopropanol. Oil-Red-O working solution was prepared by mixing 3 parts Oil-Red-OO stock solution with 2 parts dH2O, followed by filtration. Coverslips were immersed in Oil-Red-O working solution for 10 min, followed by two rinses with 60% isopropanol and one rinse with dH2O. Nuclear staining was performed using Haematoxylin for 5 min followed by several rinses with dH2O. Quantification of Oil-Red-O staining was performed by extracting Oil-Red-O using 100% isopropanol for 5 min. Absorbance was subsequently measuring at 492 nm. 100% isopropanol was used as background control.

Myelin Phagocytosis Assay

For phagocytosis experiments, alternatively activated macrophages were exposed to 5 μg/ml atto633-labeled myelin for 4 h at 37°C in 5% CO_2, with or without addition of 1 μg/ml ANGPTL4 (R&D systems, 4487-AN, USA) or 10 μM Cytochalasin D (Sigma-Aldrich, C8273, USA). Subsequently, cells were thoroughly washed with PBS to remove extracellular myelin. Phagocytosis of fluorescent myelin particles was quantified using intracellular FACS analysis (Calibur flow cytometer, Becton & Dickinson, USA).

Lipoprotein Lipase Activity (LPL) Assay

Lipoprotein lipase activity on alternatively activated monocyte derived macrophages was measured using a fluorometric assay kit (Abcam, ab204721, UK) according to the manufacturer's instructions.

Statistics

All data reflect mean ± SEM and all comparisons were statistically tested in GraphPad Prism 5.0. For comparing two experimental groups with normal distribution, unpaired two-tailed Student's *t*-tests was used. For comparing two experimental groups without normal distribution Mann–Whitney *U*-test was used. One-way analysis of variance (ANOVA) was used to compare more than two groups.

RESULTS

Decreased Astrocytic ANGPTL4 Expression in Multiple Sclerosis Lesions

First, we investigated the cellular expression of ANGPTL4 in well-characterized MS lesions. Active white matter lesions were identified by the absence of myelin (proteolipid protein) and the presence of MHC class II^+ cells with some MHC

TABLE 3 | Primer sequences.

Target gene	Species	Forward primer	Reverse primer
P2X7	Human	GAA CAA TAT CGA CTT CCC CGG	TTA TCG CCT GTT TCT CGG AAG
CD40	Human	CAA ATA CTG CGA CCC CAA CCT A	TTT CTG AGG TGC CCT TCT GCT
CD206	Human	GTC TTG GGC CAC AGG TGA A	AAG GCG TTT GGA TAG CCA CA
P2Y12	Human	ACC AGA GAC TAC AAA ATC ACC C	AGA AAA TCC TCA TCG CCA GG
LPL	Human	CGA GCG CTC CAT TCA TCT CT	CCA GAT TGT TGC AGC GGT TC
ANGPTL4	Human	ATG GCT CAG TGG ACT TCA AC	GCT ATG CAC CTT CTC CAG AC
18S	Human	TAC CAC ATC CAA GGA AGG CAG CA	TGG AAT TAC CGC GGC TG CTG GCA
HMBS	Human	CAC GAT CCC GAG ACT CTG CT	TAC TG GCA CAC TGC AGC CTC

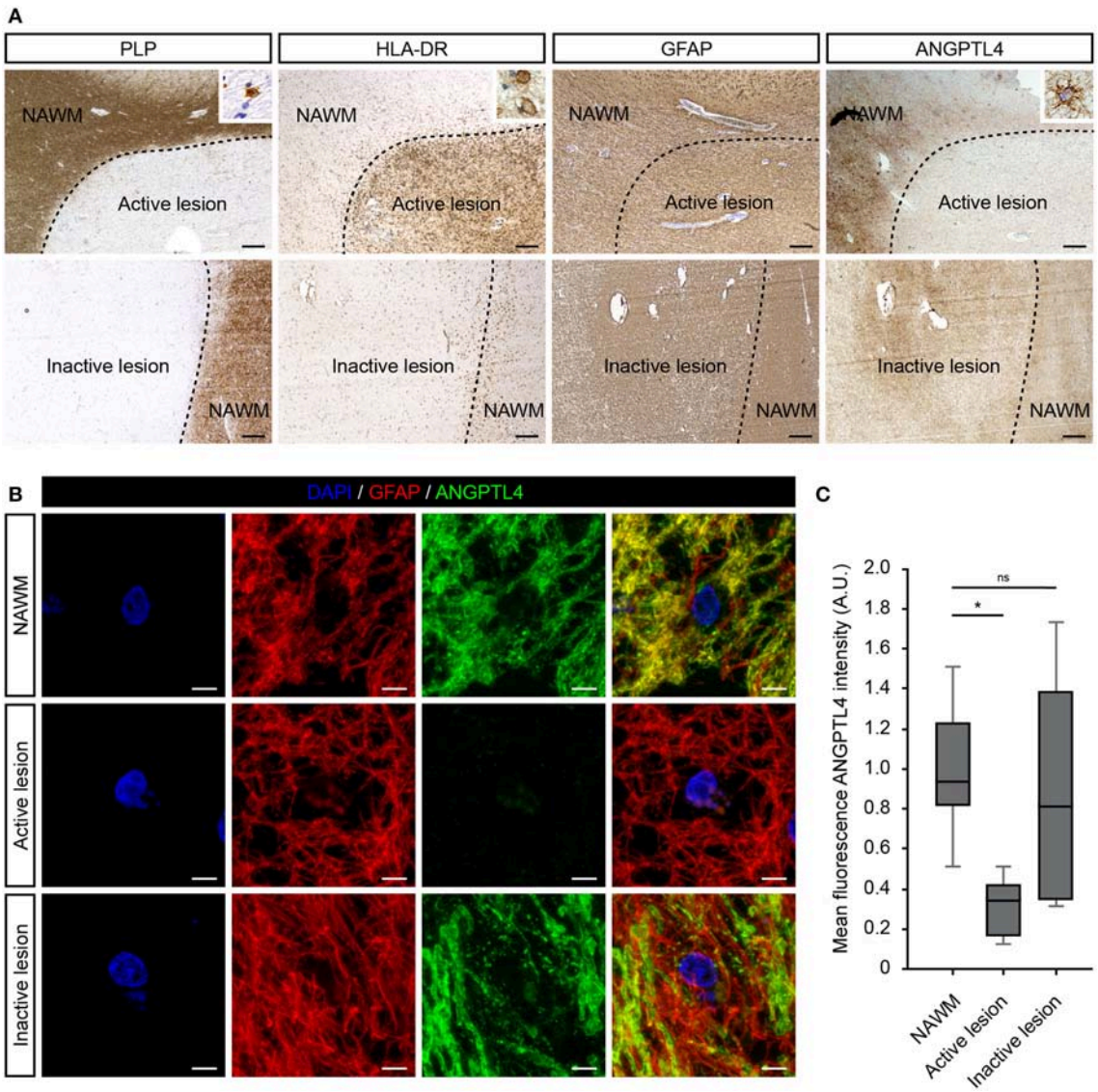

FIGURE 1 | ANGPTL4 expression is strikingly reduced in active MS lesions. **(A)** Active white matter lesions are characterized by loss of PLP and the presence of HLA-DR positive cells containing PLP-positive fragments (insert). Inactive white matter lesions display a loss of PLP immunoreactivity and absence of HLA-DR positive cells. Active white matter lesions display a marked decreased expression of ANGPTL4 compared to normal appearing white matter and inactive lesions. ANGPTL4 is predominantly localized to astrocytes (insert) (scale bar = 500 μm). **(B)** Double immunofluorescence labeling shows co-localization of ANGPTL4 (green) with GFAP-immunoreactive astrocytes (red) (scale bar = 5 μm). **(C)** Quantification of fluorescence intensity of ANGPTL4 in NAWM, active lesions and inactive lesions shows downregulation of ANGPTL4 in active MS lesions (One-way ANOVA, $N = 6$ for NAWM, 5 for active lesion and 4 for inactive lesion). $^*p < 0.05$.

class II cells containing myelin proteins. Inactive lesions are characterized with a demyelinated core with little evidence of ongoing inflammation. We observed no clear differences in cellular expression nor intensity of ANGPTL4 immunoreactivity comparing control white matter with normal appearing white matter (NAWM). ANGPTL4 was found to be expressed in normal appearing white matter by cells with an astrocytic morphology. Interestingly we observed a marked decreased expression of ANGPTL4 in active MS lesions compared to NAWM, while in inactive lesions the expression of ANGPTL4 displayed similar immunoreactivity as observed in NAWM (**Figure 1A**). To confirm the cellular source of ANGPTL4, we performed co-localization studies which confirmed that ANGPTL4 is predominantly expressed by glial fibrillary acidic protein (GFAP)-positive astrocytes (**Figure 1B**). Fluorescence intensity of ANGPTL4 was measured in NAWM, active and inactive lesions. These quantitative analyses showed that ANGPTL4 immunoreactivity is consistently reduced in active MS lesions compared to surrounding NAWM and to inactive lesions (**Figure 1C**).

Lipoprotein-Lipase Is Expressed by Iba-1 Positive Cells in MS Lesions

Since ANGPTL4 is a known inhibitor of lipoprotein-lipase (LPL), we next analyzed the cellular distribution of LPL in MS brain specimens. LPL was weakly expressed in NAWM and abundantly expressed in active lesions, localized to cells with the morphological appearance of macrophages (**Figure 2A**). Immunofluorescent double staining with Allograft inflammatory factor 1 (iba1, macrophage/microglia marker) confirmed the cellular localization of LPL in macrophages/microglia (**Figure 2B**). Taken together, ANGPTL4 expression is virtually absent in astrocytes in active lesions, while LPL, the target of ANGPTL4, is expressed by Iba1 positive phagocytes in active lesions.

Crosstalk Between Phagocytes and Astrocyte Underlies Downregulation of ANGPTL4

To determine what underlies the observed decrease in astrocytic ANGPTL4 expression, human astrocytes were exposed to myelin

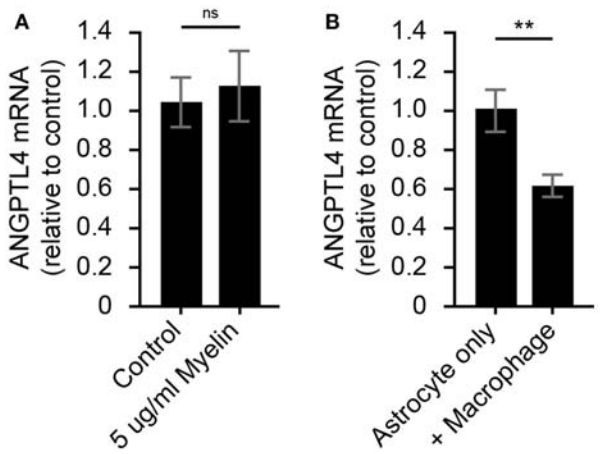

FIGURE 3 | Astrocytic ANGPTL4 expression is not influenced by myelin, but is by crosstalk with macrophages. **(A)** Astrocytes express ANGTPL4 at similar levels under normal conditions compared to 24 h treatment with myelin (Mann Whitney, N=8 for control and 11 for myelin treatment). Expression of ANGPTL4 is significantly reduced in astrocytes cultures in the presence of alternatively activated macrophages (Student's *t*-test, $N = 7$) **(B)**. $^{**}p < 0.01$.

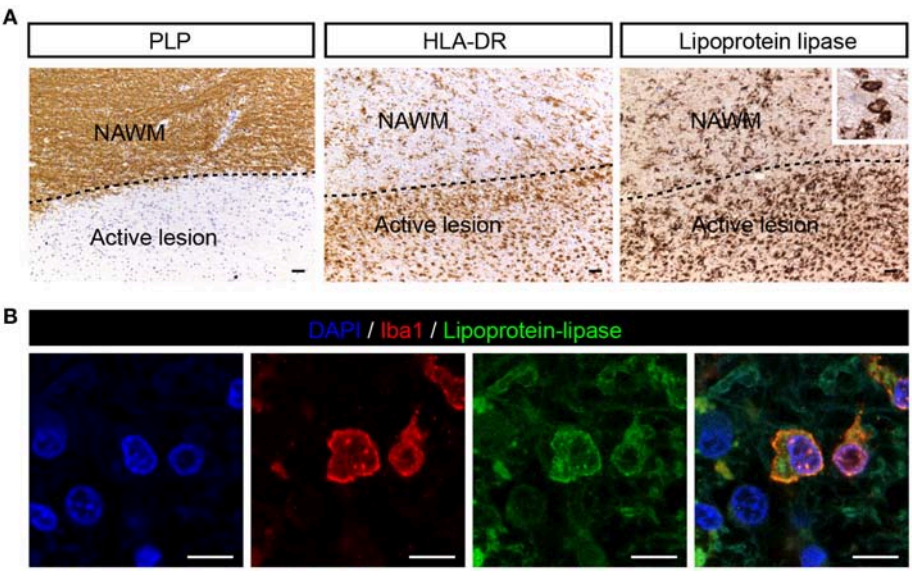

FIGURE 2 | LPL is expressed on iba1 positive cells in active MS lesion. **(A)** Active white matter lesion is characterized by loss of PLP. Active lesions showed enhanced LPL immunoreactivity in microglia/macrophages (insert) (scale bar = 50 μm). **(B)** Double immunofluorescent labeling shows presence of lipoprotein-lipase (green) positive Iba1 (red) cells in MS lesions (scale bar = 10 μm).

for 24 h. Exposure to myelin did not affect ANGPTL4 expression in astrocytes (**Figure 3A**). Based on the co-occurrence of LPL-positive phagocytes and ANGPTL4-deficient astrocytes in active lesions, we hypothesized that macrophages might be responsible for the observed loss of astrocytic ANGPTL4. Astrocytes cultured in the presence of activated macrophages showed a significant decreased expression of ANGPTL4 compared to astrocytes cultured in the absence of macrophages (**Figure 3**).

ANGPTL4 Inhibits (Myelin) Lipid Uptake via Modulation of LPL Activity

Recent reports have highlighted the pivotal role of microglial LPL in remyelination (4, 6, 12), and it is thought that microglial LPL is able to process myelin after which the myelin derived lipids can be taken up via scavenger receptors expressed on microglia (13). Here we investigated whether LPL expression on macrophages is indeed involved in myelin uptake and tested the hypothesis that ANGPTL4 inhibits this uptake by decreasing LPL activity. Macrophages were exposed to myelin, in the absence or presence of ANGPTL4. We first analyzed whether myelin treatment induced lipid accumulation in macrophages. As shown by Oil-Red-O staining, we observed increased lipid accumulation after myelin treatment (**Figures 4A,B**). Macrophages that were treated with ANGPTL4 peptide during exposure to myelin displayed decreased Oil-Red-O staining, demonstrating that ANGPTL4 inhibits (myelin) lipid uptake. To see if a well-known LPL inhibitor also causes a reduction in Oil-Red-O staining, we treated the cells with the LPL inhibitor Orlistat. Treatment with Orlistat resulted in similar reduction as ANGPTL4. Using an LPL activity assay, we confirmed that ANGPTL4 causes a significant decrease in LPL activity in macrophages compared to non ANGPTL4 treated macrophages (**Figure 4C**). To confirm that ANGPTL4 inhibits myelin uptake by blocking LPL activity and not by affecting phagocytosis, we exposed macrophages to atto633-labeled myelin, and quantified the amount of atto633-labeled myelin inside the cells by FACS (**Supplementary Figure 1**). Phagocytosis of atto633-labeled myelin was not inhibited by ANGPTL4, while treatment with Cytochalasin D, which blocks phagocytosis, completely prevented the myelin phagocytosis by macrophages (**Figures 4D,E**). Our findings suggest that macrophages utilize LPL in the process of myelin clearance. In addition, these findings show that astrocyte derived ANGPTL4 inhibits LPL activity, leading to decreased myelin derived lipid uptake by macrophages by mechanisms other than phagocytosis.

DISCUSSION

We previously demonstrated that angiopoietin-like 4 (ANGPTL4) is expressed by astrocytes in white matter and in gray matter of patients suffering from capillary cerebral amyloid angiopathy (8). Here, we show that the astrocytic expression

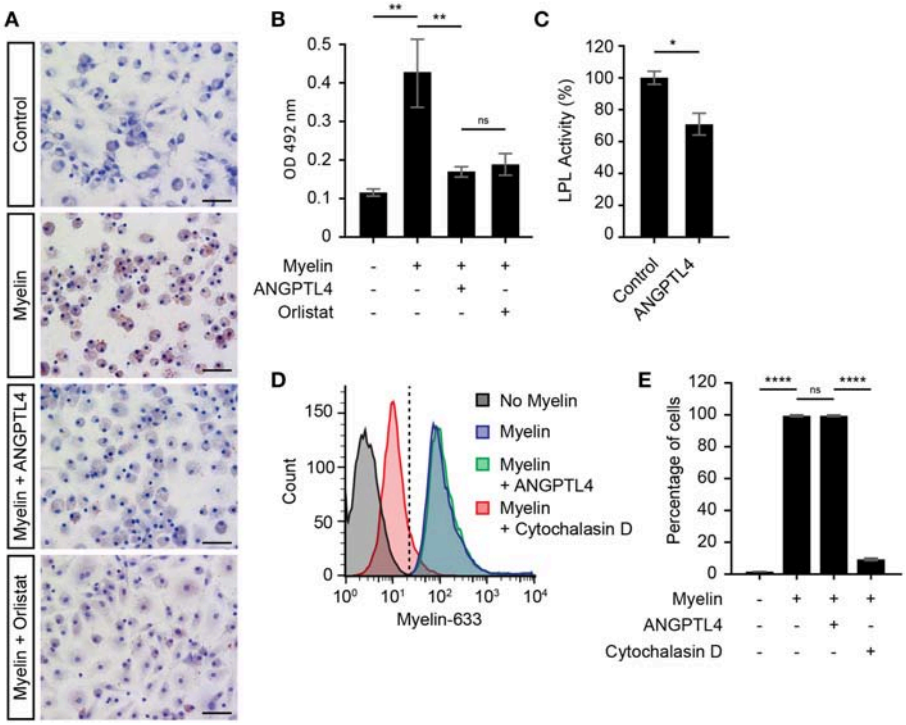

FIGURE 4 | ANGPTL4 inhibits uptake of myelin derived lipids but not myelin phagocytosis. **(A)** Increased Oil-Red-O staining of monocyte derived macrophages incubated for 4 hr with myelin, compared to control and to treatment of myelin together with recombinant Angptl4 or Orlistat. (scale bar = 50 μm) **(B)** Quantification of Oil-Red-O staining. (One-way ANOVA, $N = 5$) **(C)** ANGPTL4 inhibits macrophage LPL activity as determined by LPL activity assay. (Mann Whitney, $N = 4$) **(D,E)** ANGPTL4 treatment did not influence phagocytosis of myelin as determined by flow cytometry (One-way ANOVA, $N = 5$). $^{*}p < 0.05$,$^{**}p < 0.01$, $^{****}p \leq 0.0001$.

of ANGPTL4 is markedly reduced in active demyelinating MS lesions compared to surrounding normal appearing white matter (NAWM). ANGPTL4 is a potent inhibitor of lipoprotein lipase (LPL), an enzyme involved in lipid uptake. We provide evidence that ANGPTL4 inhibits uptake of myelin-derived lipids by LPL-immunoreactive phagocytes. This finding suggests that loss of astrocytic ANGPTL4 expression in active demyelinating MS lesions might be a protective mechanism enabling phagocytes to effectively remove myelin debris paving the way for repair.

Phagocytes, i.e., microglia and macrophages, play a critical role in pathogenesis of MS. In MS, phagocytes promote the clearance of cellular debris after myelin damage, which is a prerequisite for remyelination (14, 15). Our work suggests a novel concept how astrocytes, via decreased production of ANGPTL4, regulate clearance of myelin debris. Reduced astrocytic expression of ANGPTL4 in active lesions might enhance LPL activity on phagocytes, thereby enabling the processing and subsequent clearance of myelin debris. However, full global knockout of ANGPTL4 in mice did not show any significant differences in LPL activity in the brain (16). This might be explained by the lack of LPL protein expression in control white matter brain tissue (17). LPL mRNA expression was almost exclusively detected in microglia/macrophage and specifically in alternatively activated microglia/macrophages (18, 19). Although microglia and macrophage activation states are complex, in particular in MS where they obtain an intermediate activation status (11), it appears that lipid-laden microglia/macrophages are immunosuppressive (20) which corresponds to an alternative activation status. We therefore opted to use alternatively activated macrophages in this study.

We next investigated which factors might be responsible for the decreased expression of ANGPTL4 on astrocytes in active MS lesions. We first cultured astrocytes in the presence of myelin debris, which has been shown to induce a reactive astrocyte profile (21) However, the expression of ANGPTL4 was not affected by myelin uptake. Astrocytes cultured in the presence of alternatively stimulated macrophages showed a significant reduction in the expression in astrocytic ANGPTL4. It thus appears that crosstalk between alternatively activated macrophages and astrocytes is involved in the downregulation of astrocytic ANGPTL4. Future studies are needed to gain more insight in the mechanisms how macrophages regulate astrocytic ANGPTL4 production.

Recent reports have highlighted the pivotal role of microglial LPL in remyelination. LPL hydrolyses triglycerides into glycerol and free fatty acids (FFA). The resulting FFAs can in turn be taken up via scavenger receptors, such as CD36 and CD68 by microglia (22). These scavenger receptors have been suggested to involved in myelin phagocytosis and have been shown to be upregulated in and around demyelinating areas in MS tissue (13).

Upon exposure to myelin fragments, the lipid storage in macrophages was dramatically increased as determined by Oil-Red-O staining. Inhibition of LPL in macrophages by addition of recombinant ANGPTL4 or the LPL inhibitor Orlistat significantly reduced the amount of Oil-Red-O positive lipid droplets. Interestingly, we did not observe differences in their capacity to phagocytose myelin. These findings suggest that ANGPTL4 inhibits the breakdown and subsequent uptake of myelin derived lipids by macrophages, but not myelin uptake via phagocytosis. In line with these observations, ANGPTL4 mediated inhibition of LPL has been shown to limit the formation of foam cells in the context of atherosclerosis (23, 24).

In conclusion, we provide evidence that ANGPTL4 expression is strongly reduced in reactive astrocytes in active MS lesions, which might be a protective mechanism enabling phagocytes to effectively remove myelin debris setting the stage for repair.

ETHICS STATEMENT

VUmc MS Centrum Amsterdam and the Netherlands Brain Bank received permission to perform autopsies for the use of tissue and for access to medical records for research purposes from the Ethical Committee of the VU University Medical Center, Amsterdam, The Netherlands. All patients and controls, or their next of kin, had given informed consent for autopsy and use of brain tissue for research purposes. Buffy coats were obtained from volunteer donors at Sanquin after written informed consent was obtained.

AUTHOR CONTRIBUTIONS

AK with the help of JvH and HdV conceived the study. AK, JvH, MR, and HdV designed the experiments. AK with the help of AC performed most of the experiments. SvdP performed FACS experiments. AK with the help of JvH, MR, and HdV wrote the manuscript, which was reviewed by all authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00950/full#supplementary-material

Supplementary Figure 1 | Gating strategy. Non-myelin treated monocyte derived macrophages were gated on forward scatter/side scatter (FCS/SSC) dot plot. These events were next visualized using an FSC/myelin-633 dot plot and a gate was placed for atto6330-labeled myelin negative cells. Then atto633-labeled myelin treated monocyte derived macrophages were analyzed to confirm that treated macrophages lay inside the gate.

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MINI REVIEW
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Oncostatin M, an Underestimated Player in the Central Nervous System

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For a long time, the central nervous system (CNS) was believed to be an immune privileged organ. In the last decades, it became apparent that the immune system interacts with the CNS not only in pathological, but also in homeostatic situations. It is now clear that immune cells infiltrate the healthy CNS as part of immune surveillance and that immune cells communicate through cytokines with CNS resident cells. In pathological conditions, an enhanced infiltration of immune cells takes place to fight the pathogen. A well-known family of cytokines is the interleukin (IL)-6 cytokine family. All members are important in cell communication and cell signaling in the immune system. One of these members is oncostatin M (OSM), for which the receptor is expressed on several cells of the CNS. However, the biological function of OSM in the CNS is not studied in detail. Here, we briefly describe the general aspects related to OSM biology, including signaling and receptor binding. Thereafter, the current understanding of OSM during CNS homeostasis and pathology is summarized.

Keywords: Oncostatin M (OSM), central nervous system, cell biology, homeostasis, pathology

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INTRODUCTION

A well-orchestrated transmission of signals is crucial to maintain and restore homeostasis in humans. A class of messenger molecules that play an important role in interaction and communication of cells in the immune system, are cytokines. They are produced by immune cells but also by other resident cells of the human body as a response to changes in their microenvironment. The central nervous system (CNS) was long believed to be immune privileged. However, in the last decades it has become clear that communication between the CNS and the immune system is very important, even during homeostasis, and that it needs to be strictly regulated.

Many cytokines are thoroughly characterized in the CNS, yet others remain less studied, including oncostatin M (OSM). In 1991, OSM was categorized as a member of the interleukin (IL)-6 cytokine family (1, 2). Next to OSM and IL-6, this family of cytokines includes IL-11, IL-27, leukemia inhibitory factor (LIF) ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1), cardiotrophin-like cytokine (CLC), neuropoietin (NP) and IL-31 (3, 4). These cytokines all signal through a multi-unit receptor complex, containing the common glycoprotein 130 (gp130) subunit, except for IL-31 which binds to the OSM receptor beta (OSMRβ)/IL-31 receptor alpha (IL-31Rα) complex (5). Because of the related receptor complexes, signaling properties are shared by the members of the IL-6 family. For more insights see (4, 6–9). Here, we briefly describe the biological effects of OSM and summarize the current understanding of OSM activity in the CNS.

OSM BIOLOGY

OSM binds to the heterodimeric OSM receptor (OSMR), consisting of the gp130/OSMRβ complex, also referred to as OSMR type II in humans. Moreover, in humans and rats, OSM signaling is also possible through the LIF receptor (LIFR), consisting of the heterodimer gp130 and LIF receptor beta (LIFRβ), also known as OSMR type I in humans (4, 10). However, in mice, murine OSM (mOSM) does not transmit signals through the LIFR (11, 12). Only extremely high concentrations of mOSM may lead to weak LIFR signaling (11). While there are reports on mOSM signaling via the LIFR in mouse osteocytes (13, 14), no literature hints to LIFR activation by mOSM in neural cells. Cross-species activities of OSM in mice, rats and humans are also possible (summarized in **Table 1**) (10, 15). Understanding of the cross-species activities is crucial for proper interpretation of results obtained in experimental studies. For example, experiments in which OSM only signals via the LIFR show receptor and cell signaling of LIF rather than that of OSM.

OSM contains BC loops which form a steric hindrance for OSMRβ and LIFRβ. Therefore, OSM first binds with gp130 to subsequently recruit OSMRβ or LIFRβ (16). In both cases, formation of the heterodimeric complex leads to activation of different signaling cascades [extensively reviewed in (4, 6, 7, 17)]. First, receptor binding can activate Janus kinase (JAK)s, JAK1, JAK2 and Tyk2, which recruit signal transducer and activation transcription (STAT)s, STAT1, STAT3, STAT5, and STAT6. The activated STATs translocate to the nucleus to induce transcription of target genes. Second, activation of the LIFR and OSMR can also induce the mitogen activated protein kinases (MAPK) cascade. The different MAPKs involved are extracellular signal-regulated kinases 1 and 2 (ERK1/2), p38 and c-jun N-terminal kinases (JNK) (18). Finally, activation of the phosphatidylinositol-3-kinase (PI3K)/Akt pathway and the protein kinase C delta (PKCδ) have been described after OSMR activation (19, 20). These different signaling pathways lead to the diverse nature of OSM in various cell types and environmental conditions.

OSM IN THE CNS

The role of OSM has already been specified in joint, skin, lung, and vascular homeostasis and disease [reviewed in (4, 7, 19)]. Also in cancer, depending on the cancer type, various actions of OSM are reported (4, 7, 19). Yet, less is known about the role of OSM in the CNS. Here, we summarize the reports for which OSM is described in the healthy and pathological CNS (**Figure 1**).

Source of OSM and OSMRβ Expression in the CNS

Many cells of the immune system, i.e. dendritic cells, neutrophils, monocytes/macrophages, and T-cells, have been identified as a source of OSM (21–23). Hematopoietic cells of the bone marrow also produce OSM, regardless of inflammation (24). In the CNS, OSM is expressed by different cell types, namely neurons, astrocytes and microglia (25–27). In pathological conditions, such as multiple sclerosis (MS), OSM expression in the CNS is increased, in part by OSM production via infiltrating leukocytes (25, 28). With regard to expression of OSMRβ in the CNS, the first reports described expression of *OSMRβ* RNA in most regions of the murine CNS (forebrain, cortex, midbrain, hindbrain and spinal cord) (29, 30). Later reports investigated the cellular specificity of OSMRβ expression, and indicated that the OSMRβ protein is expressed on neurons (31), astrocytes (31–34), endothelial cells (33), and oligodendrocytes (31). For microglia conflicting reports are found and discussed later. In addition, *OSMRβ* transcripts are also present in epithelial cells of the pia mater and the choroid plexus (29, 35). The extensive expression of the OSMRβ in the brain implies important CNS related effects of OSM in different cell types both in health and disease.

Effects of OSM on Neural Cells

In physiological conditions, OSM has been implied in the homeostasis of neural precursor cells (NPCs). NPCs are a pool of cells for the continuous production of new neural cells, located in the subventricular zone (SVZ) (36), hippocampus (37), and olfactory bulb (38) in the adult mammalian brain. In mice, the OSMR is expressed on a subpopulation of NPC in the SVZ and in the subgranular zone of the dentate gyrus within the hippocampus (39). Functionally, OSM induces *in vitro* repression of neurosphere formation, indicating inhibition of NPC proliferation isolated from the SVZ and olfactory bulb, (39) while NPCs isolated from SVZ, olfactory bulb and hippocampus of OSMRβ knock-out animals lead to enhanced formation of neurospheres (39).

In pathological conditions, the vast majority of papers report neuroprotective effects of OSM. To start, OSM inhibits N-methyl-D-aspartate (NMDA)-induced excitotoxicity in a dose-dependent way. This effect is even more pronounced after pre-treatment with OSM (40). Neuroprotective effects against excitotoxicity are among others mediated by inhibitory adenosine A_1 receptors (A_1Rs), suppressing excitatory transmission (41). Inhibition of glutamate-induced excitotoxicity by OSM is completely abolished after A_1Rs blockage and knockout, indicating the requirement of adenosine A_1R function for neuroprotection (42). Also, a protective effect of OSM is observed after amyloid beta-peptide (Aβ) induced neurotoxicity (43), known to cause mitochondrial dysfunction in Alzheimer's disease (44). Furthermore, OSM protects against 3-nitropropionic acid induced mitochondrial dysfunction in rat cortical neurons through induction of myeloid cell leukemia-1 (Mcl-1). Mcl-1 enhances mitochondrial respiration and ATP production (43) and is described as an anti-apoptotic protein with neuroprotective functions (45, 46). Since rat neuronal cells and rOSM are used in these experiments, both the involvement of LIFR and OSMR signaling needs to be considered. Moreover, we reported that OSM enhances neuronal cell viability after withdrawal of B27, a vital supplement for growth and differentiation of primary neurons and enhances neurite outgrowth *in vitro* (47). Only one study reported a potential neurotoxic effect of OSM. In this study, neuronal cell growth was inhibited when cultured in the presence of the secretome of peripheral blood mononuclear cells (PBMC) from HIV-1-infected patients. Analysis of the secretome, identified OSM as the key molecule involved in inhibition of neuronal

TABLE 1 | Cross-species signaling of OSM via the LIFR and the OSMR in mice, rats and humans.

	Mouse		Rat		Human	
	mLIFR	**mOSMR**	**rLIFR**	**rOSMR**	**hLIFR***	**hOSMR****
Mouse OSM (10–12, 15)	–	+	–	+	–	–
Rat OSM (10)	–	+	+	+	+	–
Human OSM (10, 12)	+	–	+	–	+	+

*OSMR type I; **OSMR type II.

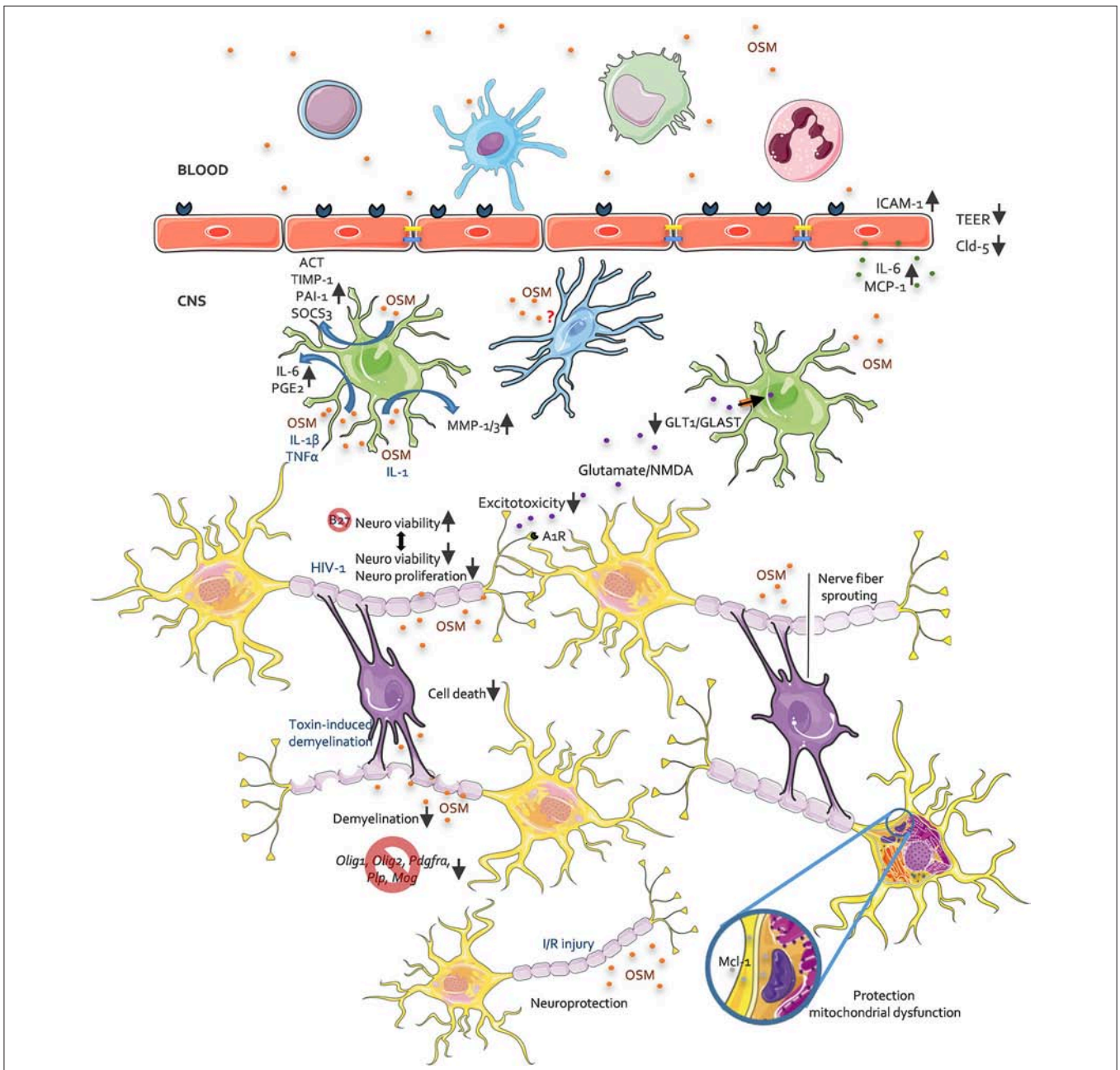

FIGURE 1 | The role of OSM in CNS pathology, an overview. This figure depicts all reported activities of OSM on different CNS resident cells as investigated in *in vitro* and *in vivo* studies. For further details see accompanying text.

proliferation and viability (48). Another study reported an indirect neurotoxic activity of OSM by inducing TNF-α secretion by microglia (49). Altogether, we can conclude that OSM has been widely reported to have a direct neuroprotective activity. However, indirect neurotoxic effects are possible and need to be kept under consideration.

Astrocytes usually prevent neuronal excitotoxicity via sequestration of extracellular glutamate through the glutamate aspartate transporter (GLAST/EAAT1) and glutamate transporter-1 (GLT-1/EAAT2) (50). OSM downregulates the expression of these receptors on astrocytes, leading to reduced glutamate uptake and consequently, excitotoxic injury (26). Astrocytes also secrete different molecules in response to OSM. Plasminogen activator inhibitor-1 (PAI-1) and α_1-antichymotrypsin (ACT) (51) expression is induced by OSM in astrocytes. Co-treatment of OSM and IL-1, leads to matrix metalloproteinase (MMP)-1 and MMP-3 production by astrocytes (52). Moreover, OSM works synergistically with the pro-inflammatory cytokines, IL-1β and TNF-α, to induce IL-6 (53) and prostaglandin E_2 (PGE_2) (54) production in human astrocytes. These OSM-induced astrocytic molecules are linked to pro-inflammatory and tissue remodeling processes. However, OSM also induces astrocytic secretion of tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) (55) and SOCS3 (32), which quench inflammation. Therefore, the net outcome of OSM signaling in astrocytes depends on the microenvironment and other cytokines present herein.

For microglia/macrophages, contradictory reports are present about OSMRβ expression and therefore the effect of OSM on these cells. Different research groups do not observe OSMRβ expression in primary mouse microglia (34, 42), nor in the C8-B4 microglia cell line (34). Moreover, no phosphorylation of STAT1 or STAT3 is observed in microglia after OSM treatment (34). In contrast, tumor necrosis factor-α (TNF-α) and nitric oxide (NO) production is reported after NF-κB pathway stimulation via OSM treatment of primary microglia and the BV2 microglia cell line (49). Yet, others did not see OSM-induced activation of the NF-κB pathway nor OSM-induced NO production in microglia (34). Moreover, our group found OSMRβ expression on Iba-1^+ cells, a marker for both microglia and macrophages, in naive and cuprizone treated animals (31). In naive animals, no infiltration of macrophages is expected, yet perivascular, meningeal and choroid plexus macrophages are present (56). In the cuprizone challenged mice, macrophages infiltrate the brain (57). Therefore, it is possible that the Iba-1^+ cells are macrophages. However, Hsu and colleagues did not detect OSMRβ in bone marrow-derived macrophages and the RAW 264.7 macrophage cell line (34). Yet, in other tissues, OSMRβ expression is seen on macrophages, i.e. in adipose tissue and atherosclerotic lesions (58, 59). In conclusion, more research is needed to address whether OSMR signaling is active in microglia and macrophages.

The blood brain-barrier (BBB) is very important to protect the brain from unwanted intruders. On human cerebral endothelial cells (HCECs), expression of OSMRβ, but not LIFRβ, is seen, despite low RNA levels of LIFRβ (25). The latter implies that OSM only signals through the OSMR type II in HCECs. OSM treatment increases the percentage of HCECs expressing intracellular adhesion molecule-1 (ICAM-1), yet no effect on vascular cell adhesion molecule-1 (VCAM-1) expression is detectable (25). Next to adhesion molecules, OSM augments the secretion of IL-6 and monocyte chemotactic protein-1 (MCP-1) in HCECs (25). This effect is further enhanced after co-treatment of HCECs with OSM and TNF-α (25). Also, a decreased BBB permeability is attributed to persistently high activation of the JAK/STAT3 signaling pathway (60). Here, rat brain capillary endothelial cells (RBECs) were treated with mOSM, implying OSMR and not LIFR signaling as indicated in **Table 1** (10). Both increased permeability for sodium fluorescein and decreased transendothelial electrical resistance (TEER) are seen in mOSM treated RBECs (60, 61). Moreover, delocalization of the tight junction molecules, claudin-5 and zonula occludens-1 (ZO-1) is apparent after OSM treatment (60). Together, these studies imply a pro-inflammatory state of BBB-ECs when treated with OSM.

Finally, for oligodendrocytes and myelination, protective effects of LIF have been described [reviewed in (62, 63)] and tested preclinically via therapeutic delivery through nanoparticles or lentiviral vectors (64, 65). However, only a few *in vivo* studies reported on the role of OSM on oligodendrocytes and its repair mechanisms after myelin damage. These results are described in the next section. Overall, both protective and detrimental effects on cells of the CNS are described for OSM. Even though, the described *in vitro* experiments investigated the effect of OSM on distinctive cell types, the interplay between different cells is more complex and needs to be studied using *in vivo* models.

OSM in Murine Models of CNS Pathology

Murine neurological disease models are used to investigate the biological role of OSM in a more complex *in vivo* setting and to further allow development of OSM based treatment strategies based on these insights. Inducing disease in these models can influence the level of OSM and OSMRβ expression. Indeed, upregulation of *OSM* and *OSMRβ* in whole spinal cord is observed in the mouse spinal cord hemisection model (47). During cuprizone-induced demyelination increased OSMRβ expression is seen mainly on astrocytes and Iba-1^+ cells (microglia/macrophages) (31). In contrast, middle cerebral artery occlusion (MCAO)/reperfusion reduces expression of OSMRβ on neurons in brain areas of disturbed perfusion, i.e. ipsilateral cortex and striatum (66).

To test the effect of OSM signaling, both OSM treatment and transgenic mice [OSMRβ overexpressing animals or OSMRβ knock-out (KO) mice] can be used. While OSM KO and OSMRβ KO mice are healthy and fertile, phenotypical changes observed are a disturbed hematopoiesis in both KO strains (67, 68) and severe obesity upon a high-fat diet in OSMRβ KO animals (69). It is unknown whether there is a phenotype in the CNS, since no neurological deficits are reported to date. For the OSMRβ KO mice, the IL-31 receptor consisting of OSMRβ/IL-31Rα is also affected. However, to date there are no reports of IL-31 signaling in the CNS, only the presence of the IL-31 receptor is described in dorsal root ganglia (70–73).Therefore, it is difficult to evaluate the effect of disturbed IL-31 signaling in CNS studies using OSMRβ deficient mice.

OSM was studied in the experimental autoimmune encephalomyelitis (EAE) mouse model, a MOG autoreactive T-cell mediated model for MS. Mice receiving intraperitoneal injections with OSM did not develop any sign of paralysis, the clinical outcome of EAE induction (74). The absence of symptoms was in agreement with limited immune cell infiltration in the brain of these animals (74). However, it needs to be mentioned that human OSM was used to treat the animals. Therefore, LIFR signaling and not OSMR signaling is studied here as indicated in **Table 1**. Indeed, systemic LIF treatment of EAE mice reduces the disease symptoms (75). We have shown that local overexpression of OSM through lentiviral vectors reduces cell-death of oligodendrocytes in the cuprizone model and therefore limits subsequent demyelination. The latter is linked to a reduced microglial response, increased IL-4 expression and M2 polarization (31). OSM-induced M2 polarization is also seen in other organ/tissue models, i.e. lung, adipose tissue (58, 76–78) and cancer models (79, 80). In line with the cuprizone model, OSM treatment in the ethidium bromide (EtBr) toxin-induced demyelination model counteracts a reduction in mRNA expression of oligodendrocyte precursor marker (*Pdgfra*), oligodendrocyte lineage transcription factors (*Olig1* and *Olig2*) and myelin genes (*Plp* and *Mog*) indicative for oligodendrocyte and myelin sparing (33). Moreover, neuroprotective effects are reported *in vivo*. Local OSM treatment in a spinal cord injury (SCI) mouse model improves functional recovery, and histological analysis reveals a reduced lesion size with less astrogliosis, less $CD4^+$ T cell infiltration and an increased nerve fiber sprouting (47). Also, stereotaxic injection of NMDA together with OSM reduces the NMDA-induced lesion volume in a model of neurotoxic injury. This could be attributed to a reduced expression of the NR2C subunit of the NMDA-receptor and attenuated increase of intracellular calcium, preceding NMDA-induced cell death (40). Correspondingly, OSMRβ overexpression in neurons improves stroke outcome following ischemia/reperfusion (I/R)-induced cerebral injury (66). The latter is due to a protective role of OSMRβ against neuronal apoptosis via JAK2/STAT3 signal activation, leading to transcription of genes involved in neuronal survival (66). When mice lack OSMRβ, opposite effects on neurons and oligodendrocytes are described. OSMRβ deficiency leads to an increased infarct volume and more severe neurological deficits after I/R damage (66) and an aggravation of demyelination in the cuprizone model (31). Taken together, these studies indicate protective effects of OSM signaling in different mouse models for neurodegenerative diseases, with both oligodendrocytes and neurons being directly or indirectly protected by OSM. The most important intermediate players in these processes are astrocytes and microglia/macrophages.

CONCLUSION

Within the CNS, the major cellular sources of OSM are astrocytes, neurons, microglia and infiltrating immune cells. OSM can signal though both the LIFR (OSMR type I in humans) and OSMR (OSMR type II in humans) in humans and rats, while in mice, only signaling via the OSMR is possible. Since OSM can signal via two receptors, caution should be taken when interpreting research findings, because OSM-mediated effects can be attributed to LIFR and/or OSMR signaling, depending on the model and species used. With regard to the effect of OSM on neurons, the majority of papers report protective effects of OSM. For oligodendrocytes, astrocytes, and the BBB a limited amount of studies is available. OSM has protective effects at the level of myelination, which is very important for good signal transduction and protection of axons. For astrocytes, there is a role in excitotoxicity and secretion of inflammatory molecules. Finally, at the level of the BBB a pro-inflammatory readout is observed. To conclude, OSM can exhibit different functions depending on the variety of cell types that express the receptor and the cellular and molecular microenvironment. While *in vivo* models demonstrated that OSM has beneficial effects in the diseased CNS, more research is warranted to reveal the true role of OSM in the CNS.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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The Role of BDNF in the Neuroimmune Axis Regulation of Mood Disorders

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The neuroimmune system plays a crucial role in the regulation of mood disorders. Moreover, recent studies show that brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family, is a key regulator in the neuroimmune axis. However, the potential mechanism of BDNF action in the neuroimmune axis' regulation of mood disorders remains unclear. Therefore, in this review, we focus on the recent progress of BDNF in influencing mood disorders, by participating in alterations of the neuroimmune axis. This may provide evidence for future studies in this field.

Keywords: BDNF, neuroimmune axis, mood disorders, depression, inflammation, cytokines

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INTRODUCTION

Mood disorders are one of the most common mental disorders in the world, especially in western society. Epidemiological studies have found that there are approximately 350 million people affected by depression in the world, and the number is increasing year on year (1, 2). According to the results of the global burden of disease study, years lost to disability of depression ranks first among the top 10 disabling diseases in the world (3). The main clinical features are marked by consistent emotional upsurge or depression, often accompanied by corresponding changes in thinking and behavior (4). The performance of mood disorders is highly variable. Lighter ones may respond to certain negative life events, while heavier ones may become a seriously recurrent or even chronic disabling disorder. Clinically, mood disorders can be divided into four types: depressive episode, manic episode, bipolar disorder, and a persistent mood disorder. It not only brings severe mental pain to patients but also leads to other diseases, such as heart disease and cerebrovascular diseases. However, the pathogenesis of mood disorders is still unclear, so it is difficult for patients to be cured. Although mood disorders can currently be treated with drugs, psychotherapy or a combination thereof, the efficacy is limited and side effects may also occur (5, 6). It is therefore necessary to explore the etiology and mechanism of mood disorders to treat and prevent mood disorders.

There are many theories about the nosogenesis of depression disorder, such as monoamine neurotransmitter hypothesis, hypothalamus-pituitary-adrenal (HPA) axis dysfunction, neurotrophic hypothesis and cytokine hypothesis (7, 8). In recent years, more and more studies have focused on the relationship between mood disorders and neuroimmune regulation (9). Many neurotrophins are associated with the pathogenesis of mood disorders, such as the nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) (10, 11). Several studies have shown that BDNF may be indispensable to the neuroimmune regulation of mood disorders. However, the potential mechanism for BDNF to affect mood disorders, by participating in changes in the neural-immune axis, has not been elucidated. In this review, we summarize

the latest progress in the role of BDNF in the neuroimmune axis regulation of mood disorders. It may provide new ideas for the research and treatment of mood disorders in the future.

BDNF AND MOOD DISORDERS

BDNF, an important member of the neurotrophic factor family, is a protein synthesized in the brain and widely distributed in the central nervous system (CNS), as well as the peripheral nervous system (12). It can promote the survival, growth, differentiation, and development of neurons and plays a crucial role in the neural structure and functional plasticity (13, 14). A large number of human and animal studies have implicated the close links between BDNF and the occurrence and treatment of various diseases, including schizophrenia (15), Alzheimer's disease (16, 17), mood disorders (18), and Parkinson's disease (19).

Studies About BDNF in Depression

The neurotrophic hypothesis suggests that pathological changes in brain areas associated with depression, are closely related to BDNF expression and functional down-regulation (20). Animal models of depression suggest the vital function of BDNF in the pathophysiological mechanism of depression. In animal experiments, chronic stress and depression conditions decreased BDNF expression, increased apoptosis and decreased regeneration of neurons in the hippocampus, and also decreased BDNF expression in other parts of the brain (21, 22). However, whether these speculations apply to humans still remain to be tested in clinical studies. A large number of clinical studies have found that various kinds of stress can reduce the activity of the BDNF pathway in the hippocampus and prefrontal cortex (23–25). The postmortem studies of Karege et al. (26) reported that the analysis of brain tissue samples from patients with depression after self-killing found that BDNF and TrkB expression in the hippocampus decreased. Moreover, in the hippocampus of patients who have received antidepressant treatment before their death, BDNF and TrkB expression increased (26). The vast majority of studies have found abnormally lower serum BDNF levels in patients with depression than that of people without depression (25, 27, 28). Ristevsk-Dimitrovska et al. found that the serum BDNF level of depressed patients was lower than that of the control group (29), while significantly higher BDNF levels were found after antidepressant treatment (24). A meta-analysis showed that the serum or plasma BDNF increased during treatment in severe mental illness inpatients, but was not restored (18). Treatment with an antidepressant, Agomelatine, could increase the hippocampal BDNF level and BDNF positive neurons in CUMS rats (30). Kreinin et al. found that the serum BDNF level was positively correlated with depression in women with severe MDD, which further supported the role of BDNF in the pathogenesis and treatment of MDD (31). Moreover, increased BDNF levels suggests that BDNF may serve as a marker for a therapeutic response to ECT in MDD patients (32). Thus, it has been speculated that BDNF may be a biomarker of depression. But to understand the role of BDNF in depression, it is also necessary to further clarify the regulatory factors affecting BDNF expression, namely the upstream and downstream signaling pathways of BDNF in the nervous system.

BDNF and Neurotransmitters in Depression

The monoaminergic hypothesis is also one of the most important hypotheses to study in the pathogenesis of depression. It points out that depression may be caused by low levels of monoamine neurotransmitters in the brain (33). Meanwhile, the function of BDNF is closely related to the plasticity of 5-HT, choline lipids, DA neurons and the survival of central neurons. For example, BDNF could promote the regeneration of 5-HT neurons in the CNS, so the large consumption of 5-HT in the CNS reduces the BDNF level, which leads to the atrophy and death of nerve cells, affects neural plasticity, and thus aggravates depressive symptoms (34). In addition, the regulatory effect of BDNF on the 5-HT_{2A} receptor level is also an important mechanism of BDNF's role in affective disorder (35). A study suggested that BDNF was implicated in the neuroprotective effects of the selective 5-HT_{1A} receptor agonist, 8-OH-DPAT, against CA1 neurons apoptotic death after transient global cerebral ischemia (36). BDNF also plays an essential role in the mesolimbic DA pathway. Studies have shown that the blockade of BDNF activity in the ventral tegmental area-nucleus accumbens pathway exerts an antidepressant-like activity in rodent models of stress (37–39). BDNF controls the expression of the D3 receptor in part of the brain, and induction of BDNF by antidepressant treatments is associated with its behavioral activity (40).

Signaling Pathway of BDNF in Depression

Tyrosine receptor kinase B (TrkB), a member of the tyrosine kinase family, can specifically bind to BDNF with a high affinity (41). Comprehensive research has indicated that BDNF is involved in the regulation of CNS, mainly by binding to TrkB (**Figure 1**). BDNF activates intracellular tyrosine kinase activity by binding to TrkB, causing the autophosphorylation of TrkB, thereby activating the mitogen-activated protein kinase (MAPK) pathway, the phospholipase C-gamma (PLC-γ) pathway, the phosphatidylinositol 3-kinase (PI3K) pathway, and other signaling pathways (42). Finally, CREB is activated at the Ser133 site of the cAMP response element binding protein (CREB). CREB promotes the survival of nerve cells and increases synaptic plasticity and neurogenesis by boosting the expression of the BDNF and BCL-2 genes (43). BDNF-TrkB not only affects the survival, development, and functions of neurons but also promotes the formation of the dendritic spine, provides a structural basis for synapse formation and improves the transmission efficiency of synapses. BDNF/TrkB signaling has a major impact on the production of antidepressant effects (44). Knock-out of the BDNF gene or the reduction of the levels of BDNF in the forebrain blocks the behavioral effects of antidepressants (45). In recent years, more and more studies have found that antidepressants might play an anti-depressant role by up-regulating brain BDNF levels or activating TrkB receptors. Song et al. found that silibinin mitigated the depression-like symptoms of Aβ1-42-treated rats by decreasing the BDNF/TrkB expression, suggesting the role of the BDNF/TrkB signaling pathway in the activity of antidepressants (46). Likewise, sesquiterpenoids from

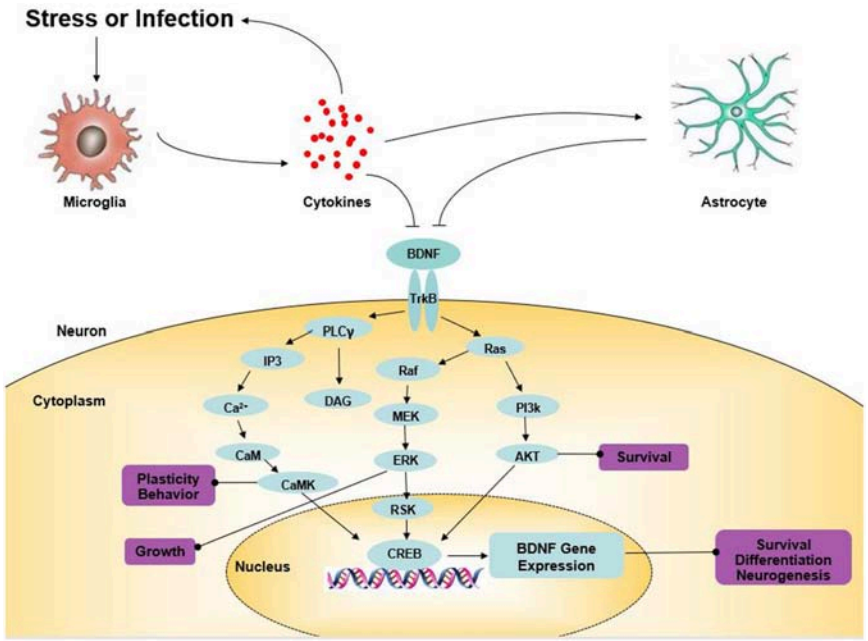

FIGURE 1 | The role of BDNF in depression. Arrows indicate activation; T-shaped arrows indicate inhibition. Akt, serine/threonine protein kinase; BDNF, brain-derived neurotrophic factor; CaM, calmodulin; CaMK, calcium-calmodulin-dependent protein kinase; CREB, cAMP response element-binding protein; DAG, diacylglycerol; ERK, extracellular signal-regulated kinase; IP3, inositol 1,4,5-trisphosphate; MEK, mitogen-activated extracellular signal-regulated kinase; PKC, protein kinase C; PI3K, PI-3 kinase; PLC-γ, phospholipase-Cγ; RSK, ribosomal S6 kinase; TrkB, tyrosine kinase B.

ginseng root treatment, ameliorate depression-like behaviors induced by LPS by upregulating the BDNF/TrkB Pathway (47). The BDNF-TrkB pathway in the nucleus accumbens of α7 nACHR knockout mice was demonstrated to be up-regulated, which was considered to be involved in their depression-like behavior (48). The antidepressant role of fisetin was confirmed by Wang et al. which was achieved by activating TrkB rather than regulating its overall level (49). All of these studies suggest that BDNF and the mediated TrkB signaling pathway may provide new approaches for the treatment of depression.

The N-methyl-D-aspartic acid receptor (NMDA receptor) is associated with depression. NMDA receptor antagonism has a significant antidepressant effect. On the one hand, it exerts its antidepressant effect by inhibiting NMDA receptors, which not only promote the establishment of new synaptic connections but also restore the synaptic connections caused by stress damage. On the other hand, antagonizing the NMDA receptor also activates the AMPA receptor, which provides a fast antidepressant effect through its signaling pathway. This provides a new method in the treatment of traditional depression (50). BDNF may play a role through the NMDA receptor. BDNF enhances the AMPA-dependent synaptic signaling in the hippocampus through downstream pathways mediated by NMDA receptors (51). Wang et al. enhanced the BDNF/TrkB signaling pathway by means of transcranial magnetic stimulation (TMS). At the same time, the activity of the NMDA receptor in the cerebral cortex was strongly correlated with the degree of TrkB activation (52). In cultured hippocampal neurons and rat neocortical cells, the activation of TrkB or chronic administration of BDNF can enhance the expression of the NMDA receptor NR1 and NR2A/2B through transcriptional activation. BDNF can also promote the release of glutamate through the presynaptic receptor signal transduction pathway, and enhance the AMPA receptor and NMDA through the postsynaptic receptor pathway and then participate in and promote the formation of LTP (53). Duncan et al. through a study of 30 depressive patients treated with ketamine, found that the antidepressant effect of ketamine might be due to the enhancement of inter-synaptic communication by BDNF (54).

Effects of Antidepressants on BDNF

In recent years, the major clinical antidepressants are monoamine oxidase inhibitors, tricyclic inhibitors, and tetracyclic inhibitors (55, 56). Some clinical studies suggest that antidepressant therapy for a period of time could reverse the decrease of peripheral BDNF levels of depressed patients. Serum BDNF levels of depressed patients taking SSRIs were markedly higher than that of the control group and depressed patients not taking SSRIs (57). Treatment with venlafaxine or paroxetine also increased BDNF in patients with depression (58). However, whether all antidepressants affect BDNF levels, remains controversial. A meta-analysis showed that the level of peripheral BDNF increased during antidepressant treatment of SSRIs and SNRIs, among which sertraline could improve the BDNF level after short-term treatment (59). Treatment with fluoxetine (SSRI) was found to alter BDNF levels in patients with depression, whereas venlafaxine did not (60). Freire et al. found that neither the combined group, nor the pharmacological group resulted in the increase of the serum BDNF level in patients with

depression, although both significantly improved the depressive symptoms of patients (61). These studies indicate that different antidepressants may have different effects on the peripheral BDNF during treatment.

NEURO-IMMUNE AXIS AND MOOD DISORDERS

Neuro-Immune Axis

Nowadays, plenty of research has been conducted on the interaction between the central nervous system and immune system. It is neurogenic inflammation that determines whether the immune response is caused by a local threat, through the connection of nerve fibers to immune cells. In recent research, a CNS with complicated innate immune responses is demonstrated to have high immunocompetence (62). Microglia, resident immune cells within the brain parenchyma, can secrete some soluble factors, such as chemokines, cytokines, and neurotrophic factor, to adjust the CNS immune response and tissue repair (63). In addition, astrocytes also play an essential role in central immunity. They respond to an inflammatory environment not only in an immunological way by changing their cell phenotype, but also modulate the immune response of lymphocyte in the brain by releasing associated protein molecules, like chemokines and cytokines (64).

Some immune cells, such as non-specific leucocytes and lymphocytes, produce neurotransmitters and neuropeptides. Opioids may serve as an example. It is suggested that opioids are secreted in inflammatory tissues and act to alleviate clinical pain under stress by activating peripheral opioid receptors (65). There are also some neurotrophins produced by activated lymphocytes, such as BNDF and NGF (66, 67). In turn, non-specific leucocytes and lymphocytes can also express classic neuronal receptors. For example, the activated non-neuron A7 nicotinic cholinergic receptor has anti-inflammatory and immunomodulatory effects on multiple cell types, T cells, B cells, dendritic cells, and mononuclear phagocytes included (68).

Cytokines, chemokines and their receptors were reported to express on the central and peripheral nervous systems. For instance, IFN-α not only affected CNS directly but also had an indirect action through inflammatory cytokines of the central and peripheral nervous systems (69). Previous studies found that interleukin and chemokine receptors, which participated in neuronal inflammation and CNS diseases, were expressed by neurons (70). Cytokines like interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) could influence the behavior, by directly functioning in the nervous system. Cytokines are conducive to the growth and function of the brain and regulate neural activity and neurotransmitter systems, which result in behavioral changes. Chronic exposure to high levels of inflammatory cytokines and constant alterations of central neurotransmitters may contribute to psychiatric disorders like schizophrenia and mood disorders (71, 72). Cytokines induce behavioral effects by activating inflammatory signaling pathways in the brain, leading to the reduction of growth factors such as BDNF for instance (72).

Cytokines and Depression

In recent years, studies have found that immune dysfunction was closely related to depression, and pro-inflammatory cytokines produced by innate immune activation were especially closely related to the occurrence and development of depression. Therefore, the hypothesis of cytokine is gradually proposed. The cytokine hypothesis suggests that depression is an inflammatory disease caused by neuroimmune regulation disorders, emphasizing that the body's immune system plays an important role in depression. Cytokines are intercellular information transfer molecules, which mainly have immunomodulatory and multiple effector functions. Different cytokines play different roles in inflammation. Some have proinflammatory effects, while others have anti-inflammatory effects. For instance, IL-1β, IL-6, and tumor necrosis factor α (TNF-α) are relatively advanced proinflammatory cytokines, while IL-4 and IL-10 is the main area of research in anti-inflammatory cytokines. Clinical studies have shown that patients with depression were often associated with varying degrees of inflammatory activation or increased inflammatory molecules, suggesting that the occurrence of depression might be closely related to cytokines (73, 74).

Studies About Cytokines in Depression

This hypothesis has been supported by a large number of clinical cases in recent years: patients with autoimmune diseases and chronic viral infections often showed depressive symptoms (75, 76). Autopsy studies have found that cytokines were significantly increased, as well as the synthesis of carbon monoxide synthase in macrophages, microglia, and astrocyte (77). A number of studies have indicated that various cytokines, such as IL-1β, IL-2, IL-6, TNF-α, and IFN-γ in serum or plasma of patients with depression were significantly increased (78). The results of the meta-analysis showed that the levels of inflammatory factors in patients with depression, including IL-1, IL-6, and TNF-α, were significantly higher than those of healthy people and in positive relevance to the serious extent of depressive symptoms (79–81), and antidepressants could lower these cytokines in people with depression (82). According to the study of the cytokine overview, Zou et al. found that the expression of IL-1, IL-10 and TNF in MDD patients increased significantly, while the expression of IL-8 decreased significantly. Such aberrant changes in the levels of inflammatory cytokines demonstrated that it is depression that activates the inflammatory process (83). Animal studies have also shown that the levels of IL-1β, IL-6, and TNF-α in the brain increased significantly after lipopolysaccharide treatment, as well as depressive behaviors, such as sleep disorders, loss of pleasure, and insufficiency of power (84). Depressive behaviors of animals were blocked after injecting IL-1 receptor antagonist IL-1rA into animals before stress (85). The results suggested that inflammatory pathways might be involved in the development of depression.

Actions of Cytokines in Depression

People realize that there is a two-way effect between immunity and nerves. Both physiological and psychological stress can activate the immune system and make the cytokines secreted, and then influence the central nervous system, such as

neurogenesis, neurotransmitter level, neuroendocrine function, neuroplasticity, and behavior related neural pathways (86–88). It leads to changes in the neurochemistry and endocrinology associated with depression and influences the development of depression. Cytokines can promote oxidative stress and damage glial cells in emotionally related brain regions, such as the prefrontal cortex and amygdala (89). In addition, dysfunctions of glutamate-induced by cytokine can reduce the generation of neurotrophic factors (88). Under stress, the increase of proinflammatory cytokines in the human body activates the indolamine 2,3- dioxygenase (IDO), an enzyme that can directly act on the metabolism of tryptophan (TRP). IDO can increase the level of kynurenine produced by TRP metabolism, thereby reducing the level of 5-HT and promoting the occurrence of depression (90). Cytokines are also momentous to the dysfunction of the HPA axis. They induce the hyperactivity of the HPA axis to increase the glucocorticoid for a long time, and the abnormal glucocorticoid signal can affect the production, maintenance, and development of depressive behaviors (91). At the same time, these cytokines and excess glucocorticoids also inhibit nerve regeneration in the brain. Cytokine signaling pathways, for example NF-kB, can disrupt the function and expression of glucocorticoid receptors, leading to an unrestricted inflammatory response, further exacerbating depressive symptoms (92). Additionally, cytokines can also contribute to depression by influencing neural plasticity. Ben et al. confirmed that IL-1 could inhibit neuronal regeneration, and inflammatory cytokines—IL-6, as an example, could disrupt neuronal function (93). Currently, though the causal relationship between cytokines and depression is still considered controversial, it is undeniable that the negative regulation of neuroplasticity in the brain has a significant impact on the developmental progress of depression. Furthermore, the study also shows that abnormally changed levels of cytokines are associated with an increased risk of delirium and suicide (94–96).

Immune Regulation and Antidepressant Effects

Some anti-inflammatory drugs have antidepressant effects or enhance antidepressant effects. Studies have shown that the anti-inflammatory drug COX-2 inhibitors could directly or indirectly affect the 5-HT system through the CNS and play an antidepressant effect. Giving rats a dose of rofecoxib can increase the level of 5-HT in the prefrontal and parietal cortex (97). Celecoxib in depressed rats was found to decrease cytokine levels and improve behavior in the hypothalamus (98). Through the drug combinations of celecoxib with antidepressants, such as reboxetine, fluoxetine, and sertraline, it was found that the combined group was better than an antidepressant alone in patients with depression (99–101). Etanercept, a kind of tumor necrosis factor (TNF- α) antagonist, also has a strong antidepressant effect, which can improve depression symptoms and patient fatigue (102). Many other clinical cases show that antidepressants can reduce proinflammatory cytokines and other inflammatory markers in patients (103). Tricyclic antidepressants, SSRI, SNRI, and other antidepressants have been shown to increase anti-inflammatory immunomodulatory cytokine levels by inhibiting inflammatory cytokines and th1-like cytokines (such as IFN-γ). Réus et al. revealed that imipramine, an antidepressant, could reduce the levels of TNF-α and IL-1β in cerebrospinal fluid of maternally deprived adult rats (104). Studies have shown that some non-pharmacological treatments can also improve depressive symptoms by regulating immune inflammatory pathways. Kim et al. believe that acupuncture can reduce the levels of peripheral and central proinflammatory factors (IL-1, IL-6, TNF- α) and proinflammatory neuropeptides, and the results are better in the treatment of depression (105). In addition, exercise can play a synergistic role by inhibiting the immune and inflammatory pathways (106).

BDNF, NEUROIMMUNE AXIS, AND MOOD DISORDERS

BDNF is a relatively mature neurotrophic factor, which can promote the proliferation of neurons and glial cells in the inflammation of the nervous system through various molecular mechanisms (107, 108). Glial cells are innate immune cells in the center. They not only synthesize and release multiple inflammatory mediators but also express many inflammatory mediator receptors on the cell surface. Microglia, the first line of defense for the central immune response, exerts essential influence on the inflammatory response in the brain (109). Although there is no direct evidence that microglia are correlated with the nosogenesis of depression, many studies have examined if there is a significant increase in the amount of microglia in the brain in patients with suicidal depression. A previous study observed microgliosis in the dorsolateral prefrontal cortex, anterior cingulate cortex, mediodorsal thalamus, and hippocampus of suicidal patients (110). Torres-Platas et al. also observed a relative increase of primed microglia in depressed suicides (77). Microglia can regulate the release of BDNF. Microglia may take effect on pathogenesis by reducing BDNF expression as well as its high-affinity receptor TrkB. Studies demonstrated that microglia was of extensive and diverse importance for the formation of appropriate synaptic connections during development and maturation, which were frequently mediated by BDNF (111). In addition, high levels of IL-6, IL-1β, and BDNF in LPS-stimulated normal human astrocytes (NHAs) was observed, using an LPS-induced *in vitro* injury model of astroglial cultures. Vice versa, BDNF can promote the growth of astrocytes and regulate the viability and proliferation of LPS-induced NHA through the PI3K/AKT pathway (112, 113).

Reports repeatedly demonstrated that inflammatory cytokines affect neuronal development as well as apoptosis (114, 115). As a matter of fact, stress and its associated activation of inflammatory cytokines might have a negative effect on neurogenesis and neuroplasticity (84, 116, 117). Considerable research efforts have been devoted to the effect of inflammation on the BDNF expression in the brain. The significant reduction in BDNF was caused by the administration of pro-inflammatory cytokines or lipopolysaccharide (LPS), an inducer for cytokines, serve as an example. LPS injections could significantly reduce mature BDNF levels in the hippocampus and cerebral cortex (118), as well as IFN-α administration, which decreased systemic BDNF levels

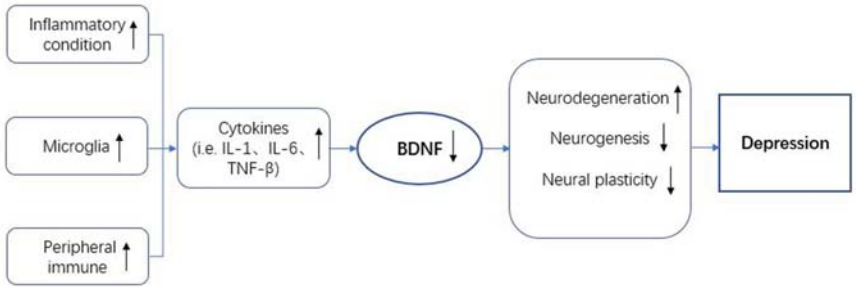

FIGURE 2 | BDNF in the neuroimmune regulation axis of depression. BDNF, brain-derived neurotrophic factor; IL-1, interleukin-1; IL-6, interleukin-6; TNF-β, tumor necrosis factor-β.

(119). Furthermore, other neurotrophic factors also decreased to varying degrees: NGF and neurotrophic factor−3 (NT-3), for instance (120).

It was demonstrated in a number of research studies that inflammation inhibits BDNF/TrkB expression. Inflammatory cytokines influence the phosphorylation of the BDNF receptor (TrkB), thereby further interfering with BDNF signaling (121). Gibney et al. found that poly-I:C administration upregulated the expression of the inflammatory cytokines, which caused the occurrence of an inflammatory reaction. At the same time, BDNF and TrkB expression in the hippocampus and cortex were downregulated, which might lead to behavioral defects of depression and anxiety (122). In addition, it is under integrated BDNF signaling that antidepressants are able to reverse LPS-induced apoptosis, which agrees well with the above-mentioned studies.

The anti-inflammatory mechanism of antidepressive agents has not been elucidated yet. Imipramine has been shown to suppress proinflammatory cytokines in rat neural stem cells, stimulating the expression of BDNF (123). Studies have shown that the production of inflammatory cytokines was regulated by complex signaling pathways, especially the nuclear factor-κb (NF-κB) inflammatory response signal pathway (BDNF-TrkB-MEK-ERK-NF-κB pathway) whose activation plays a central regulatory role in the inflammatory response. Investigation of the effect and potential mechanism of salidroside on depression showed that salidroside could down-regulate the expression of BDNF, TrkB, and the NF-κB protein (124). Ge et al. thought that the antidepressant effect of resveratrol is mainly to reduce the expression of inflammatory cytokines and improve NF-κB activation (125). Chrysophanol could inhibit the NF-κB signaling pathway (126), and the high dose of fisetin could regulate the expression of NF-κB in the hippocampus to antagonize the expression of iNOS mRNA (127). Similarly, the antidepressant effect of aesculetin may be achieved by inhibiting the NF-κB pathway as well as activating BDNF/TrkB signaling (128). Furthermore, as an inflammatory intracellular signaling molecule, p38 mitogen-activated protein kinase is now a target for clinical studies of chronic inflammatory diseases due to the potential antidepressant effects of its inhibitors (129). All these studies provide a basis for the development of new clinical antidepressants and the continued development of antidepressant treatments.

CONCLUSION

Based on many clinical and basic research studies, a variety of theories were proposed to expound the nosogenesis of mood disorders, especially depression. In this review, the neuroimmune axis has been related to mood disorders (**Figure 2**). BDNF is thought to be involved in the neuroimmune axis regulation. On the one hand, the expression of BDNF is affected by immune cells and the immune factors they secrete. On the other hand, the immunomodulatory process also requires the regulation of BDNF-mediated signaling pathways. Unfortunately, the specific mechanism of how BDNF participates in the regulation of the neuroimmune axis in mood disorders is still unclear and it is therefore necessary to conduct more in-depth research. The treatment of mood disorders in the past often only focus on a certain aspect of research. The characteristics of the varied symptoms of depression determine that these treatments are not effective. Exploring a treatment strategy for depression based on neuroimmune axis regulation may be more helpful to further guide the development of anti-mood disorders drugs.

AUTHOR CONTRIBUTIONS

YJ and RJC contributed conception and design of the review. WY organized the documents. YJ wrote the first draft of the manuscript. SBX and LHS wrote sections of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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The Emerging Role of Triggering Receptor Expressed on Myeloid Cells 2 as a Target for Immunomodulation in Ischemic Stroke

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Stroke is the second most common cause of death and permanent disability. It is characterized by loss of neural tissue in which inflammation plays a crucial role in both the acute contribution to ischemic damage as in the late-stage impact on post-ischemic tissue regeneration. Microglia play a key role in the inflammatory stroke microenvironment as they can adapt a disease-promoting pro-inflammatory- or pro-regenerative phenotype thereby contributing to the exacerbation or alleviation of ischemic damage, respectively. Triggering receptor expressed on myeloid cells 2 (TREM2) is a cell surface receptor which in the central nervous system is mainly expressed on microglia. This receptor has been shown to play an essential role in microglial phagocytosis and function but its contribution in stroke pathobiology remains unclear. TREM2 was shown to be activated by nucleotides and lipid mediators, key factors that are secreted in the extracellular stroke environment by apoptotic neurons and cell/myelin debris. These factors in turn stimulate TREM2 signaling which mediates microglial migration toward- and phagocytosis of myelin debris and apoptotic cells. Moreover, microglial TREM2 appears to counteract the toll-like receptor response, thereby decreasing the production of pro-inflammatory cytokines. Finally, TREM2 is involved in microglial migration, survival, and is suggested to directly stimulate pro-regenerative phenotype shift. Therefore, this receptor is an attractive target for microglial modulation in the treatment of ischemic stroke and it provides additional information on microglial effector functions. This short review aims to elaborate on these TREM2-mediated microglial functions in the pathobiology and resolving of ischemic stroke.

Keywords: ischemic stroke, microglia, TREM2, immunomodulation, phagocytosis

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INTRODUCTION

Worldwide, stroke, of which ischemic stroke is the most prominent, is the second most common cause of death and is a major cause of permanent disability (1). Ischemic stroke is characterized by the loss of brain tissue and neuronal dysfunction due to a reduced blood flow to the affected brain areas. Microglia, the resident phagocytes of the central nervous system (CNS), continuously monitor the status of the brain parenchyma through extension and retraction of their processes (2). Furthermore, they are the first cells to become activated after stroke which migrate toward

the site of injury to act as a first line of immune defense. Stroke outcome is determined by a fine balance of tissue-damaging pro-inflammatory and recovery-promoting pro-regenerative responses. Microglia play an important role in this inflammatory balance by obtaining a broadly defined disease promoting pro-inflammatory- or pro-regenerative phenotype (3–7). It should be noted that this classification will be used in the context of this review of ischemic stroke. There is an ongoing debate on phagocyte classification based on their activation state, activation stimuli, polarization, cellular origin, and phenotypical shift. However, this discussion will be left out of consideration but is thoroughly discussed by Ma et al. (7), Guilliams et al. (8), and Murray et al. (9).

Pro-inflammatory microglia dominate during the acute phase after stroke and are characterized by increased tumor necrosis factor alfa (TNF-α), interleukin (IL)-1β, IL-12, and inducible nitric oxide synthase (iNOS) expression amongst others. Pro-regenerative microglia (characterized by i.e., CD206 and Arginase-1 expression) play a protective role by stimulating tissue and vascular remodeling after stroke during the subacute and chronic phase. Pro-regenerative microglia secrete anti-inflammatory cytokines such as IL-4, IL-10, and transforming growth factor β (TGF-β) and remodeling factors including vascular endothelial growth factor (VEGF) and brain-derived neurotrophic factor (BDNF) (7, 10, 11). Unfortunately, these microglia numbers decline following an initial peak 12–24 h after stroke, whereas the number of pro-inflammatory microglia continue to rise, contributing to tissue damage and neuroinflammation (3, 10). Nonetheless, microglial polarization and the secretion of pro- and anti-inflammatory soluble factors remains a topic of interest in stroke research (10).

The clearance of apoptotic neurons and/or neuronal debris is an aspect of stroke repair that is often overlooked. Phagocytic clearance is indispensable in enabling the reconstruction and reorganization of neural networks and triggering tissue repair (12, 13). Moreover, in contrast to neuronal debris, phagocytosis of apoptotic cells is a controlled process that does not induce inflammation, thereby circumventing self-catalyzing secondary tissue damage that is characterized by free radical and nitric oxide production, glutamate excitotoxicity and the release of pro-inflammatory factors (14, 15). Recently, triggering receptor expressed on myeloid cells 2 (TREM2) emerged as a novel microglial target in stroke that is involved in phagocytosis and microglial function. TREM2 is expressed on myeloid cells including microglia in the CNS where its expression is over 400-fold higher than on other glial cells and neurons (16). The importance of this receptor in myeloid and microglial function is stressed by Nasu-Hakola disease, also known as polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy. This disease develops in patients with a deleterious or loss of function mutation in the TREM2 gene, or in its signaling adapter protein DNAX activating protein of 12 kDa (DAP12) (17, 18). These patients develop bone cysts due to dysfunctional osteoclasts (19) but also present with progressive dementia, motor dysfunction, seizures and shortened life span (18, 20). Moreover, the R47H TREM2 mutation increases the risk for Alzheimer's disease (AD) 3–4 fold (21, 22) and mutations in the TREM2 gene are thought to be linked with the impaired uptake of Aβ-lipoprotein complexes by microglia (23).

The significance of TREM2 in ischemic stroke was shown by Kawabori et al. who showed that TREM2 deficiency was detrimental for ischemic injury and microglial phagocytosis (24). Moreover, TREM2 overexpression suppressed the pro-inflammatory reaction of microglia *in vitro* (25, 26). More strikingly, a recent study by Kurisu et al. demonstrated that specifically microglial TREM2 expression is fundamental in stroke outcome and not TREM2 expression on circulating macrophages (27). In addition to microglia, perivascular and perimeningeal macrophages can be seen as a distinct brain-resident macrophage with several indications for CNS diseases [reviewed in Faraco et al. (28)] which role has also recently emerged in stroke pathogenesis regulating vascular permeability and leukocyte chemotaxis in acute stroke (29). However, no reports are available describing the role of TREM2 in these cells and will therefore be left out of consideration. This review aims to provide an overview on the regulation and potential role of TREM2 in stroke pathobiology and resolution with a focus on microglial function as its role in these processes remains to be elucidated.

STROKE PHAGOCYTOSIS AND INFLAMMATION

The accurate clearance of apoptotic neurons and neuronal debris is crucial for the resolution of CNS damage. Following (ischemic) injury, microglia are activated, migrate toward the lesion site, secrete pro- and anti-inflammatory factors and clear cellular debris and apoptotic cells (13, 30). In general, proper clearance of apoptotic cells is mediated through four essential processes. The first step is initiated by apoptotic cells that release "find-me" signals to attract phagocytes to the site of neuronal injury. These signals include the nucleotides (2, 31) adenosine triphosphate (ATP) and uridine triphosphate (UTP), lysophosphatidylcholine (LPC) (32), sphingosine-1-phosphate (S1P) (33), and fractalkine/CX3CL1 (34). The second step involves recognition of the dying cells by "eat me" signals of which phosphatidylserine is the most prominent and well-studied (35). After recognition, the target cell becomes internalized and the engulfed target becomes degraded in the maturating phagosome (36). Interestingly, in the fourth phase, the phagocyte releases anti-inflammatory mediators such as TGF-β1, IGF-1, and IL-10 (37–39) and suppresses the production of pro-inflammatory factors (40). For in-depth information on phagocytosis and the associated cellular and molecular mechanisms, see reviews by Medina and Ravichandran (41), Park and Kim (42).

These phagocytosis mechanisms hold when injury is limited and these "find me" signals are present at relatively low concentrations. However, in ischemic stroke, extracellular ATP levels increase dramatically due to neuronal and glial depolarization waves and membrane leakage (14). These high levels of ATP then not only serve as a chemoattractant for microglia, but can activate P2 × 7 receptors which leads to the

production of pro-inflammatory factors (43). In the subsequent phase of ischemic cell death, the dying neuronal cells leak intracellular signal molecules that together with extracellular molecules can be seen as danger signals that activate the immune system (44). These so-called danger associated molecular pattern molecules (DAMPs) include high concentrations of extracellular ATP (45), extracellular matrix breakdown proteins (46), heat-shock proteins (Hsp) (47), and the high mobility group box 1 protein (48). These DAMPs activate purine receptors and scavenger- or pattern recognition receptors on microglia, leading to the production of pro-inflammatory mediators by resident brain cells and infiltrating leukocytes (49). In this context, one of the most studied pattern recognition receptors is the membrane–bound Toll-like receptor (TLR) family in which the TLR2 and TLR4 subtypes are known to play an important role in ischemic stroke. TLR2 is most prominently expressed on microglia and TLR2 signaling increases the expression of pro-inflammatory and pro-apoptotic genes in the transient middle cerebral artery occlusion (tMCAO) mouse stroke model. Moreover, TLR2 deficient mice have a smaller infarct volume compared to controls (50). Similarly, TLR4-deficient mice had lower infarct volumes in a mouse model with permanent distal middle cerebral artery occlusion (dMCAO). Additionally, TLR4-deficient mice had a better score on a neurological and behavioral test. The lack of TLR4 decreased the expression of iNOS, cyclooxygenase-2 and matrix metalloprotease-9 amongst others, demonstrating an attenuation of the pro-inflammatory milieu that is created post-stroke (51).

Ultimately, acute stroke-associated inflammation is a self-limiting process that is coordinated by lipid mediators (52) that contribute to the effective removal of dead cells and debris, the generation of pro-regenerative milieu and the production of growth factors such as microglial IGF-1, VEGF, and BDNF that favor tissue regeneration. As will be discussed in the next section, TREM2 can interfere in the stroke immune response at multiple levels to determine stroke outcome.

THE NEUROIMMUNOLOGICAL FUNCTION OF TREM2—IMPLICATIONS FOR STROKE PATHOBIOLOGY

Microglia are key regulators of innate immunity in the CNS, protecting the parenchymal cells from injury. In addition to detecting extrinsic insults such as bacterial lipopolysaccharide (LPS), microglia are equipped with intrinsic DAMP-recognizing receptors like TLRs, as discussed previously. Similar to TLRs, TREM2 was shown to be capable of binding LPS and intrinsic ligands that are released during neuronal degeneration including lipids (53, 54), nucleic acids (24), and Hsps (55). However, the identification of the entire spectrum of endogenous TREM2 ligands and clarification of the ligand-specific physiological TREM2-mediated response in health and disease remains challenging [reviewed by Kober and Brett (56)]. Interestingly, fractalkine/CX3CL1-expressing damaged neurons stimulate TREM2 expression and attenuate neuronal excitotoxicity by enhancing the clearance of these injured neuronal cells by CX3CR1-expressing microglia (57, 58).

TREM2 is a single-pass transmembrane receptor that belongs to the immunoglobulin-like superfamily of receptors. The immunoglobulin-like ligand-binding domain is situated in the extracellular domain whereas the transmembrane domain facilitates the interaction with DAP12. This leads to the phosphorylation of the immunoreceptor tyrosine activation motif (ITAM) in DAP12 by Src kinases which subsequently activate Syk kinase to trigger intracellular signaling cascades involving the downstream signaling molecules phosphatidylinositol 3-kinase (PI3K), extracellular signal-regulated protein kinase (ERK), phospholipase Cγ (PLCγ), and Vav that influence microglial activation, function, survival, and phagocytosis (53, 54, 59–61). Interestingly, TREM2 can be shed from cell membranes by proteolytic cleavage by for example the sheddase ADAM17 (a disintegrin and metalloproteinase domain containing protein 17), forming soluble TREM2 (sTREM2) that is able to cross to the cerebrospinal fluid (62, 63). The current knowledge on the role of sTREM2 in CNS pathology and microglial function is mainly based on AD research. A recent paper by Zhong et al. demonstrated that the stereotactic injection of sTREM2 or adeno-associated virus mediated activation of sTREM2 reduced the amyloid plaque load and reduced functional memory deficits. Moreover, sTREM2 stimulated microglial proliferation and homing toward amyloid plaques where amyloid-β uptake and degradation was increased. Interestingly, these effects were specifically mediated by microglia as they were absent upon microglial depletion (64). Remarkably, sTREM2 levels in the cerebrospinal fluid were recently proposed as a biomarker for AD and the associated inflammatory response (63, 65, 66). Unfortunately, the role of sTREM2 in ischemic stroke is to date unexplored, and will therefore be left out of consideration for stroke specific microglial TREM2 function in this context.

Based on these TREM2 effects on microglial function and the role of microglia in ischemic stroke, several mechanisms are proposed to be mediated by microglial TREM2 activity (**Figure 1**). The first and potentially the most thorough investigated role of TREM2 in stroke is its function in phagocytosis. A pioneer study by Takahashi et al. (59) demonstrated that microglial TREM2 is involved in the phagocytosis of apoptotic neurons. In this study, knockdown of TREM2 with short hairpin TREM2 RNA (shRNA) in primary mouse microglia decreased the phagocytic clearance of apoptotic neurons which coincided with the increased expression of pro-inflammatory TNF-α and nitric oxide synthase-2. Lentiviral overexpression and activation of TREM2 increased phagocytosis and attenuated the pro-inflammatory response. In addition to its role in phagocytosis, Takahashi et al. showed that the migratory capacity of microglia with activated TREM2 toward CCL21 is increased significantly. CCL21 is used by ischemic injured neurons as a "find me" signal to attract microglia, suggesting a role of TREM2 in the early phase after ischemic injury (67). Kawabori et al. demonstrated that TREM2 deficiency exacerbates ischemic injury in a dMCAO mouse stroke model in which TREM2 knockout (KO) and wildtype (WT) mice were compared

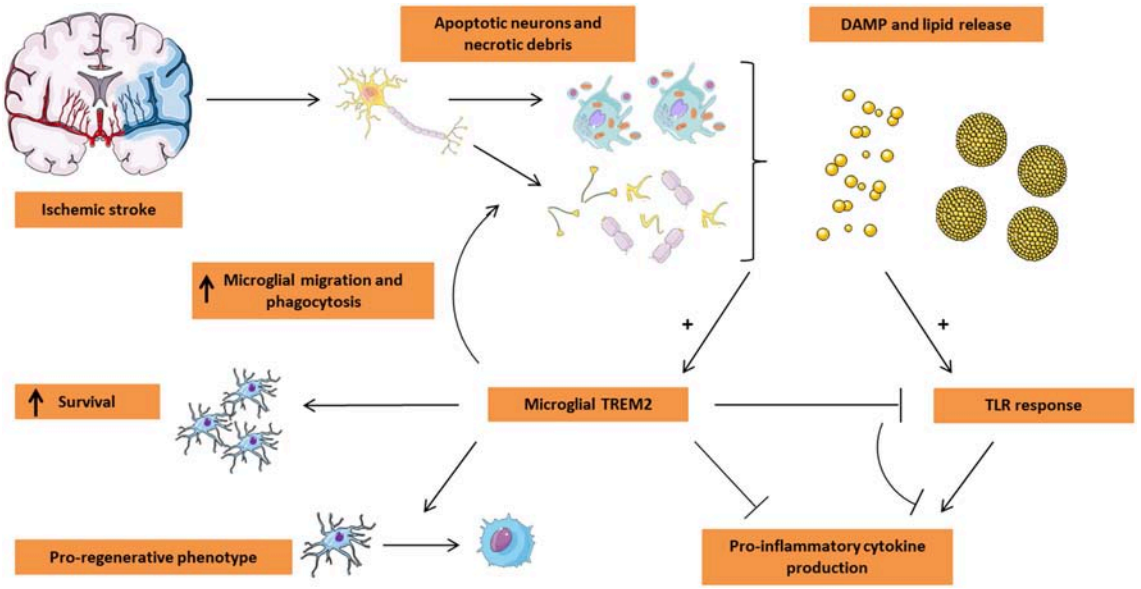

FIGURE 1 | Microglial TREM2 in stroke pathogenesis. Ischemic stroke leads to the massive loss of neuronal tissue by triggering apoptosis and/or necrosis. In turn, the dying tissue releases damage-associated molecular pattern (DAMP) molecules including nucleotides and ECM breakdown products in addition to lipid mediators being present in myelin and neuronal debris and on the surface of apoptotic cells. These events trigger both TLR and TREM2 activation on microglia. While classically the TLR response leads to a pro-inflammatory microglial phenotype characterized by the release of pro-inflammatory cytokines, TREM2 activation appears to counteract the TLR response, leading to a decrease in the production of pro-inflammatory cytokines. Moreover, microglial TREM2 stimulates migration of microglia toward the lesion site and promotes the phagocytic clearance of apoptotic cells and debris. Finally, TREM2 was shown to be associated with a pro-regenerative microglial phenotype and the production of pro-regenerative cytokines and is suspected to play a role in microglial survival. Based on these functions of TREM2, this receptor is an attractive target for microglial modulation in the treatment of ischemic stroke. This Figure was created using Servier Medical Art licensed under a Creative Common Attribution 3.0 Generic License, available online at http://smart.servier.com/.

(24). *In vitro*, knockdown of TREM2 in the BV-2 mouse microglial cell line using small interfering RNA (siRNA) directed against TREM2 inhibited phagocytosis of Neuro-2A cells that were rendered apoptotic by oxygen- and glucose deprivation (OGD), an *in vitro* model that mimics ischemic damage. This role of TREM2 in phagocytosis was also demonstrated *in vivo*. TREM2 deficient mice showed a reduced tissue resorption and therefore increased infarct size following ischemic damage compared to WT controls. Confocal microscopy confirmed these results as a significantly reduced direct contact between activated macrophages and TUNEL-positive dying cells at the stroke site in TREM2 KO mice was observed (<4% compared to almost 40% in WT animals). The reduced phagocytic clearance of apoptotic cells and debris in TREM KO mice was also reflected by the virtual absence of Oil Red O-stained foamy macrophages which stained positive due to the uptake of lipid-containing cellular debris and dead cells. In line with these histological findings, the Bederson score and ladder test, which both measure neurological deficits, demonstrated a worsened functional outcome in the TREM2 KO mice. Moreover, WT mice showed a gradual improvement in the ladder test to nearly baseline levels while TREM2 KO mice did not reach these values over 2 weeks (24). This stresses the importance of clearing neuronal debris to enable synaptic and axonal repair, as stated previously (13). More recently, Kurisu et al. demonstrated that it is specifically microglial TREM2 that is essential in stroke outcome by using bone marrow chimeric mice in which TREM2 KO mice were used as donor and WT mice as recipient. The results of this study indicated improved neurological outcome in the elevated body swing and adhesive removal test and smaller infarct volume in mice with intact TREM2. In addition, mice with no intact microglial TREM2 demonstrated a lower number of phagocytes near the stroke lesion that also showed reduced phagocytic activity (27). Similar observations have been made in the experimental autoimmune encephalomyelitis (EAE) model of multiple sclerosis (68) and in the cuprizone-induced non-autoimmune demyelination model (54, 69). In EAE, TREM2 overexpressing bone-marrow derived macrophages demonstrated enhanced phagocytosis of apoptotic neurons and after intravenous injection, these cells cleared myelin debris and ameliorated the disease outcome (68). Supporting the role of TREM2 in EAE, Piccio et al. showed that inhibiting TREM2 activation is detrimental for EAE outcome (70). In the cuprizone model, the corpus callosum in TREM2 KO mice showed extensive demyelination, axonal damage and decreased clearance of myelin debris compared to WT controls (54, 69). Furthermore, Poliani et al. showed that myelin-associated lipids triggered microglial TREM2 signaling, stressing the need of microglial TREM2 for effective clearance of myelin debris (54). More support for the importance of microglial TREM2 in the phagocytic clearance of apoptotic cells, comes from AD research. Atagi et al. demonstrated that apoptotic neuronal cells have the ability to bind Apolipoprotein E

(APOE), the major cholesterol carrier protein in the brain. APOE was demonstrated to be a TREM2 ligand that enhanced the microglial phagocytosis of apoptotic neurons and the phagocytic capacity of microglia was diminished in the AD-associated TREM2-R47H mutant (71). While ongoing research is focusing on the link in AD between TREM2 mutants and the APOEε4 isoform, which is the major risk factor for AD, the association of TREM2 and APOE in ischemic stroke and their putative intertwined signaling remains unclear. However, given the ability of apoptotic neurons to bind APOE and use it as an opsonisation mechanism to enhance TREM2-mediated microglial phagocytosis demonstrates that this ligand-receptor interaction plays in important role in the clearance of damaged neurons (72).

Second, TREM2 can drive the pro-inflammatory stroke microenvironment toward a pro-regenerative environment and can therefore exert a neuroprotective effect on the ischemic brain. In a study by Wu et al., TREM2 expression is upregulated in microglia following OGD *in vitro*, and in the ischemic penumbra after tMCAO in mice (25). *In vivo*, TREM2 silencing exacerbated stroke outcome as demonstrated by an increased lesion volume, the number of apoptotic neurons and a decrease in neurological function measured with the Modified Garcia Score. This correlated with an increase in TNF-α, IL-1β, and iNOS mRNA levels *in vitro* in microglia exposed to OGD. Conversely, TREM2 overexpression reduced TNF-α, IL-1β, and iNOS mRNA levels and decreased neuronal apoptosis *in vitro*. Moreover, this study demonstrated the role of the ERK and NF-κB in TREM2 signaling. TREM2 silencing increased phosphorylation of ERK and NF-κB in primary microglia exposed to OGD. Taken together, a tempering role of TREM2 signaling in pro-inflammatory pathways and a critical protective role for TREM2 in ischemia-induced neuronal injury is suggested (25). These findings are in line with Zhai et al. (26) that demonstrated that intraperitoneal administration of hsp60, a TREM2 agonist (55), or intraventricular injection of a TREM2 lentivirus had a neuroprotective effect *in vivo*. This study reported a decreased number of apoptotic cells and a smaller infarct volume. Interestingly, TREM2 expression is increased by an IL-4/IL-13 treatment, cytokines that induce a pro-regenerative phenotype in microglia whereas its expression is decreased after exposure to the pro-inflammatory polarizing factors LPS and interferon-γ. Therefore, the authors suggest TREM2 as a novel pro-regenerative microglial marker. Moreover, pro-inflammatory iNOS, TNF-α, and IL-6 expression was increased in OGD-exposed primary microglia and treated with TREM2 shRNA while Arginase-1 and BDNF levels were increased when these cells were treated with a TREM2 vector, indicating a pro-regenerative phenotype. In contrast to these studies and the research of Kawabori et al. that suggest a role of TREM2 in alleviating the pro-inflammatory response and its beneficial role in stroke outcome, Sieber et al. showed that in the subacute phase after stroke, the transcription of the pro-inflammatory cytokines IL-1α, IL-1β, and TNF-α was reduced in TREM2 KO mice (73). However, a difference in lesion size in WT and TREM2 KO mice was not observed. These opposing results may be explained from differences between the timing of the analysis, although with the exception of Zhai et al., peak TREM2 levels were observed 7 days post-ischemia. Given this 7 days period for TREM2 to reach peak levels and the abovementioned experiments, it can be postulated that a gradual increase in TREM2 expression is associated with a dampening of the pro-inflammatory microglial response elicited in the acute and sub-acute stroke phase. Another possibility for these opposing results is the stroke model used, although only Kawabori et al. used the dMCAO model compared to the filament model used by Zhai et al., Wu et al., and Sieber et al. Despite these different results, the majority of these stroke studies suggest a beneficial role of TREM2 in ischemic stroke (24–26). It should be noted that to date, no stroke stage-specific reports are available that go into detail in TREM2 expression dynamics and pathways that lead to TREM2 upregulation and effector mechanisms. This is of specific importance as TREM2 is a microglia-specific receptor involved in stroke resolution and microglial function varies between the acute, sub-acute and chronic stroke phase balancing between a pro-inflammatory and pro-regenerative phenotype (6, 7).

A third indication of the role of TREM2 in stroke is that TREM2 can inhibit the TLR-mediated pro-inflammatory response. This was first demonstrated in myeloid cells by Turnbull et al. who showed that in macrophages isolated from WT and TREM2 KO mice, the production of pro-inflammatory mediators was elevated in TREM2 KO macrophages after LPS stimulation (74). Similarly, Hamerman et al. showed an increased TLR-LPS response in DAP12 deficient mice and demonstrated that TLR responses are negatively regulated by TREM2 and DAP12 (75, 76). Although these studies show a connection between the TLR response and TREM2 signaling, mechanistic insights in the TLR-TREM2 interaction come from a study by Peng et al. using bone-marrow derived macrophages (77). The ERK pathway can be activated by TLR4 binding of LPS leading to the production of pro-inflammatory cytokines (78, 79). In the study by Peng et al., it was shown that the adapter protein Dok3 is phosphorylated by DAP12-ITAM which subsequently inhibits the RAS-ERK pathway, thereby reducing the production of pro-inflammatory cytokines such as IL-6 and TNF-α (77). However, the role of the TREM2-TLR interaction in ischemic stroke remains to studied into more detail. A recent study by Rosciszewski et al. (80) investigated this interaction on astrocytes, although it should be noted that TREM2 expression in microglia is almost 400 times higher than in astrocytes (16). This study showed that DAP12 overexpression suppressed LPS-induced astrocyte NF-κB activation *in vitro*, diminishing the pro-inflammatory response. Moreover, TREM2 expression in astrocytes was observed up to 14 days post-lesion in the ischemic penumbra. These results suggest that TREM2 expression in astrocytes negatively regulates the TLR4-mediated pro-inflammatory response by reducing NF-κB activation (80).

Finally, TREM2 signaling is associated with microglial survival. Sieber et al., Kurisu et al., and Kawabori et al. observed a decreased number of (activated) microglia in TREM2 KO models compared to WT controls (24, 27, 73). However, Kurisu et al. demonstrated that the number of microglia in the non-ischemic brain is similar between TREM2 KO and WT mice (27). Therefore, the diminished number of microglia associated

with the stroke lesion in TREM2 KO mice is due to the reduced chemotactic capacity in KO mice, which is supported by a previous study that reports a reduced microglial recruitment to sites of laser-induced neuronal injury in TREM2 KO mice (81). These results were in line with the cuprizone model used by Poliani et al. and Cantoni et al. who also showed decreased microglia numbers in TREM2 KO mice (54, 69). However, no plausible mechanism is identified. Nonetheless, the role of TREM2 in microglial survival and proliferation cannot be excluded, as Zheng et al. reported a reduction in microglial proliferation rate after TREM2 downregulation (82). However, no analysis was performed to unravel the TREM2 targets involved with the decreased proliferation rate. Other reports on the involvement of TREM2 in microglial proliferation come from AD research. A possible mechanism of TREM2-sustained microglial survival come from Wang et al. who demonstrated in an AD model using TREM2 KO 5XFAD mice that microglial survival is diminished compared to 5XFAD control mice. They determined that TREM2 signaling is essential for microglial survival at low concentrations of colony-stimulating factor 1 (CSF-1). The same group showed that TREM2 synergizes with CSF-1-CSF-1 receptor signaling, contributing to microglial survival. Moreover, this study suggests that the lipid-sensing function of TREM2 provides a survival function in addition to triggering phagocytosis of apoptotic cells and myelin debris (53).

CONCLUDING REMARKS

Microglia play an essential role in both the pro-inflammatory and pro-regenerative response that is triggered following ischemic injury and can therefore be seen as a double-edged sword in stroke pathobiology. TREM2 signaling mediates microglial phagocytosis of myelin debris and apoptotic cells, microglial migration and survival, and can drive the microglial activation status toward an anti-inflammatory phenotype. Therefore, targeting TREM2 signaling has become a therapeutic target as it was shown that the systemic administration of a TREM2 agonist or TREM2 overexpression had a neuroprotective effect in ischemic injury. However, it should be noted that the temporal dynamics of TREM2 expression and effector mechanisms in ischemic stroke remain to be elucidated and current insights in TREM2 function are mainly derived from different disease models and cell types. Nonetheless, the role of TREM2 in ischemic stroke is mainly considered beneficial, and the same holds for its role in AD and demyelinating disease although few studies report a detrimental effect of TREM2 signaling in these neural diseases (83). Moreover, the effect of sTREM2 in ischemic stroke remains unexplored to date and given its implications in AD pathobiology, sTREM2-signaling provides an attractive new target in the stroke research field. Despite the great promise for TREM2 in ischemic stroke, TREM2 signaling has opposite effects in peripheral nerve injury including motor nerve injury (84) and neuropathic pain (85), and after traumatic brain injury (86). This stresses the importance of additional disease-specific understanding of the TREM2-DAP12 signaling effects. Nonetheless TREM2 provides an attractive target for microglial modulation in the treatment of ischemic stroke and it offers additional information on microglial effector functions in this devastating disease.

AUTHOR CONTRIBUTIONS

PG conceived and wrote the manuscript. IL revised and edited the manuscript. All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Antigenic Targets of Patient and Maternal Autoantibodies in Autism Spectrum Disorder

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Autism spectrum disorder (ASD) is a neurodevelopmental disorder whose behavioral symptoms become apparent in early childhood. The underlying pathophysiological mechanisms are only partially understood and the clinical manifestations are heterogeneous in nature, which poses a major challenge for diagnosis, prognosis and intervention. In the last years, an important role of a dysregulated immune system in ASD has emerged, but the mechanisms connecting this to a disruption of brain development are still largely unknown. Although ASD is not considered as a typical autoimmune disease, self-reactive antibodies or autoantibodies against a wide variety of targets have been found in a subset of ASD patients. In addition, autoantibodies reactive to fetal brain proteins have also been described in the prenatal stage of neurodevelopment, where they can be transferred from the mother to the fetus by transplacental transport. In this review, we give an extensive overview of the antibodies described in ASD according to their target antigens, their different origins, and timing of exposure during neurodevelopment.

Keywords: autism, immunoglobulins, brain antigens, neurodevelopement, placental transfer

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INTRODUCTION

Autism spectrum disorder (ASD) is a complex and highly heterogeneous neurodevelopmental disorder characterized by persistent deficits in social communication and social interaction (1). The patients also show restricted and repetitive patterns of behavior, interests and activities. Together with these symptoms, various comorbidities can occur, such as intellectual and language impairment, catatonia, epileptic seizures or attention-deficit/hyperactivity disorder (ADHD). These deficits can cause difficulties for the patient to understand, maintain or develop relationships, creating a barrier to their integration in social life.

ASD has become one of the most common neurodevelopmental disorders. The prevalence of ASD among 8-year-old children in the United States has more than doubled since 2000, increasing from 1 in 150 to 1 out of 59 children (2), and varies by sex, race/ethnicity and geographic area. It is four times higher among males than females and most common in non-Hispanic white children.

The diagnosis of ASD, usually made in early childhood, is based on observation of the atypical behaviors using questionnaires such as the Autistic Diagnostic Observation Schedule (ADOS) or Autism Diagnostic Interview-Revised (ADI-R). In each patient, the symptoms can have different manifestations caused by multiple etiologies, sub-types and developmental trajectories, often in combination with the different comorbidities, creating a complex condition (3).

ASD is considered a polygenic hereditary disorder, whose genetic component mostly comprises of common variant single-nucleotide polymorphisms (SNP) and copy number variations (CNV) which can occur both in coding and non-coding regions (4). Moreover, the presence of autistic behaviors in other monogenic disorders such as Angelman syndrome and Fragile X syndrome only shows part of the complexity of this developmental disorder (5). Besides a genetic predisposition, ASD is also strongly influenced by environmental factors, which mostly play their specific roles before, during and after pregnancy (6). Factors as diverse as parental age, prematurity, pollution or geographic area have been found to contribute to increased risk of ASD (2, 7–9).

An overarching understanding of the etiology of the disorder is still lacking, but many areas and processes in the brain have been implicated. Disturbances in activation and regulation of the posterior insula, limbic system or the cortico-striato-thalamo-cortical circuit, contribute to the picture of a complex disease, not restricted to a single anatomical location (10–12). At the cellular level, alterations in synaptogenesis, synaptic plasticity and pruning, add another dimension of complexity (13–15).

In the last decade, an important role of the immune system has been described in the development of ASD. The activation of microglia, the resident immune cells of the brain, and increased levels of pro-inflammatory cytokines in brain tissue, cerebrospinal fluid and blood give evidence for the presence of ongoing neuroinflammation in brains of some ASD patients (16–19). Moreover, *in utero* exposure to an inflammatory environment during pregnancy caused by maternal autoimmune disease or maternal immune activation by infections, is sufficient to impart lifelong neuropathology and altered behaviors in offspring (20, 21).

Although ASD and other neurodevelopmental disorders are not typically considered as autoimmune diseases, autoantibodies, or antibodies that bind to self-antigens, have been detected in the sera of patients with ASD, ADHD, bipolar affective disorder and schizophrenia (22). Autoantibodies against known targets have been linked to the development of behavioral symptoms in these disorders, for example anti-dopamine transporter autoantibodies have been related with severity and recovery in ADHD, antibodies against myelin basic protein (MBP) have higher titers in patients with negative symptoms in schizophrenia or N-methyl-D-aspartate receptor (anti-NMDAR) and glutamic acid decarboxylase (anti-GAD) antibodies have been associated with acute maniac episodes in bipolar disorder (23–25). The best-studied example are autoantibodies directed against the NMDAR in a condition known as anti-NMDAR encephalitis, characterized by the development of psychosis, cognitive problems and seizures. The pathogenic role of anti-NMDAR autoantibodies in this disorder has been demonstrated in cultured neurons and in animal models (26–28). Moreover, patients have even been shown to respond to immunotherapy (29).

Besides the formation of autoreactive antibodies in ASD patients themselves, such antibodies have also been described in the blood of mothers whose child will develop ASD (**Figure 1**) (30, 31). These maternal autoantibodies can be transferred from the mother to the child during pregnancy via transplacental transport and might play an etiological role in ASD as they can pass the immature fetal blood-brain barrier (BBB) which is actively changing and semi-permeable during neurodevelopment (32, 33).

In this review, we explore the current evidence of antibodies detected in ASD patients and in mothers of ASD patients, their possible role as biomarkers to support the disease diagnosis and their contribution in the understanding of disease mechanisms.

AUTOANTIBODIES IN ASD PATIENTS

Autoantibodies in an ASD patient were first described by Todd et al. (31) in a study on hyperserotonemia. Antibodies in the blood and cerebrospinal fluid (CSF) of an ASD patient could block binding of radioactive serotonin to a human cortical brain homogenate. Although the presence of these autoantibodies was not supported by follow-up studies (34, 35), the idea that a subset of ASD patients may have antibodies against specific self-antigens in the brain started to gather much interest. Since then, autoantibodies in ASD patients have been mostly studied by looking at known, mostly brain-related autoantigens which have also been described in other autoimmune conditions (described below and in **Table 1**). Whether these specific autoantibodies can cross the BBB, bind to their antigens in the brain and contribute to disease worsening, has not been demonstrated experimentally so far. Nonetheless, an increased general permeability of the BBB has been described for several psychiatric disorders, including ASD [reviewed in Kealy et al. (52)] which could provide a mechanism that would allow these autoantibodies to reach the brain. Moreover, most of the autoantibody targets described in ASD patients are localized intracellularly. It has long been assumed that large biomolecules such as antibodies cannot traverse the plasma membrane. However, for some anti-DNA antibodies in systemic lupus erythematosus (SLE) patients, membrane translocation and nuclear targeting has been demonstrated (53). Still, for the ASD-related autoantibodies described below, such mechanisms have not been investigated.

Protein Targets

Myelin Basic Protein (MBP)

Myelin basic protein (MBP) is, together with the proteolipid protein (PLP), the most abundant protein in the myelin membrane (54). It is present on the cytoplasmic side of the membrane in oligodendrocytes of the central nervous system (CNS) and peripheral nervous system (PNS) (55). Its main function is structural, participating in membrane stabilization required for the formation of the myelin sheath, which insulates axons and allows saltatory action potential transmission (56). During brain development, myelination starts in the last weeks of the third trimester of pregnancy, and continues in adult life (57). Myelin integrity is essential for proper cognition, as damage in sheath formation can have severe consequences in the integrity of the white matter networks (58). Alterations in white matter development have been associated with autism manifestations, making MBP a highly interesting protein in the understanding of this effect (10, 59). Moreover, MBP damage can lead to induction

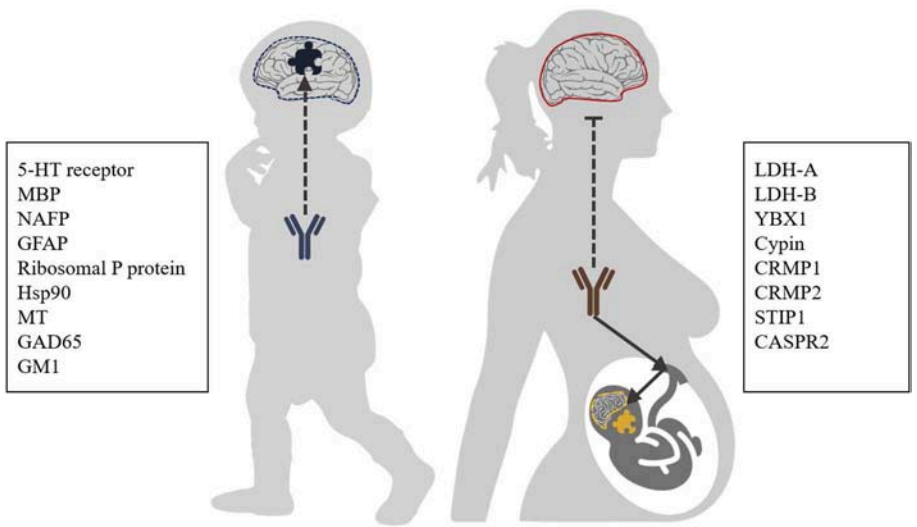

FIGURE 1 | Autoantibody targets in ASD. Autoantibodies with reactivity to brain antigens are generated in a subpopulation of ASD patients. Furthermore, the process of transplacental transport of antibodies, which provides infants with passive immunity, can also transfer antibodies which are reactive against fetal brain antigens. It is generally assumed that the BBB of healthy mothers is intact and impermeable to (auto)antibodies, while for ASD patients increased BBB permeability has been described. 5-HT receptor, 5-hydroxytryptamine receptor; NAFP, Neuron-axon filament protein; GFAP, Glial fibrillary acidic protein; MBP, Myelin Basic Protein; GM1, Ganglioside M1; GAD65, Glutamic acid decarboxylase; Hsp90, Heat shock protein 90; MT, Metallothionein; LDH-A/B, Lactate dehydrogenase A and B; YBX1, Y-box-binding protein 1; Cypin, Cytosolic PSD-95 interactor; CRMP1, Collapsin response mediator proteins 1; CRMP2, Collapsin response mediator proteins 2; STIP1, Stress-induced phosphoprotein 1; and CASPR2, Contactin-associated protein-like 2.

of inflammatory and chemotactic mediators which are able to alter the permeability of the BBB (60).

Based on the first descriptions of autoantibodies and abnormal immune function in autistic children (31, 61, 62), Sing et al. (36) investigated the presence of immunoglobulin G (IgG) antibodies against MBP in ASD. MBP autoantibodies had already been described in the CSF of patients with multiple sclerosis (MS) (63). This first study compared a cohort of autistic children under 11 years of age, with age-matched normal children, children with idiopathic mental retardation and children with Down's syndrome (36). MBP-reactive antibodies were found using Western blot in 58% of ASD children, while reactivities in the different control populations were much lower, ranging between 0 and 22% (**Table 1**). In several follow-up studies, similar or even higher IgG reactivities were reported in autistic children using enzyme-linked immunosorbent assays (ELISA) against MBP (38–40). On the other hand, a study by Libbey et al. (37) could not replicate the presence of these anti-MBP antibodies.

Besides anti-MBP antibodies of the IgG isotype, IgA and IgM antibodies against MBP have also been reported in ASD patients (38, 40). Moreover, autoantibodies of all isotypes were also found against myelin-associated glycoprotein (MAG) and myelin oligodendrocyte glycoprotein (MOG), showing the diversity of the immune reactivity against key myelin proteins in ASD patients. Therefore, these autoantibodies might be informative on the underlying processes of autoimmunity in ASD patients.

However, since anti-MBP antibodies have already been described in several other diseases such as multiple sclerosis, Parkinson's disease, rheumatoid arthritis or schizophrenia (24, 58, 60, 64, 65), it seems unlikely that they would be useful as specific biomarkers for the diagnosis of ASD. Autoantibodies against myelin proteins might still have prognostic value for ASD patients, since serum levels of anti-MBP and anti-MAG antibodies were shown to be increased in severe autism patients compared to patients with mild or moderate disease severity (66).

The reported studies on autoantibodies against MBP in ASD children all have used small patient and control cohorts. Therefore, studies in multiple, independent large cohorts are required using similar detection assays to settle the reported differences in anti-MBP reactivity and to confirm a possible link with disease severity.

Neurofilament Protein

Neurofilaments are a class of intermediate filaments, which are part of the neural cytoskeleton, especially in axons of asymmetric, myelinated neurons involved in nerve conduction (67). They are composed of a triplet of proteins with different molecular weights (neurofilament light, 68 kDa; middle, 160 kDa and heavy, 200 kDa) (67). Of these filament proteins, the neurofilament heavy protein (NF-H), also called neuron-axon filament protein (NAFP), is considered as a stable marker for neuron-axon filaments. It has an important role in the development of axons and the transport of vesicles via binding of kinesins, dynein complexes and kinases (68–71). Neurofilament subunit variants have been reported in the development of motor disorders and axonal neuropathies such as amyotrophic lateral sclerosis and

TABLE 1 | Overview of ASD patient autoantibodies.

Autoantibodies in patients									
Antibody target	**Isotype**	**MW (kDa)**	**Protein origin**	**Methods**	**Diagnosis**	**Sensitivity[a]**	**Specificity[b]**	**Cohort[c]**	**References**
Serotonin (5-HT) receptor	IgG	NR	Human	Equilibrium saturation	DSM-III	NR	NR	Case study	(31)
	IgG		Human	Ligand binding	DSM-III	NR	NR	3–19 years	(35)
Myelin basic protein (MBP)	IgG	14–21	Rabbit	Western blot	DSM-III	58% (19/33)	MR 85% (3/20) TD 78% (4/18) A 97% (1/38) DS 100% (0/12)	≤10 years, Adults 20–40 years	(36)
	IgG		Bovine	ELISA/Western blot	DSM-IV/ADOS/ADI-R	NR	NR	2–10 years	(37)
	IgG/ IgM		NR	ELISA	DSM-IV	NR	NR	2–9 years	(38)
	IgG		Human	ELISA	DSM-IV	80% (40/50)	TD 95% (1.5/30)	5–12 years	(39)
	IgG/IgM/IgA		NR	ELISA	DSM-III	NR	NR	3–12 years	(40)
Neuron-axon filament protein (NAFP)	IgG	200	Bovine	Western blot	DSM-III	54.7% (29/53)	MR 66.6% (8/24) TD 72.4% (16/58)	4–48 years (90% younger than 10 years)	(41)
Glial fibrillary acidic protein (GFAP)	IgG	50	Bovine	Western blot	DSM-III	32% (17/53)	MR 79.2% (5/24) TD 91.4% (5/58)	4–48 years (90% younger than 10 years)	(41)
Ribosomal P protein	IgG/ IgM	17–38	Bovine	ELISA	DSM-IV/CARS	44.3% (31/70)	TD 95% (2.5/48)	4–12 years	(42)
Heat shock protein 90 (Hsp90)	IgG	90	Human cell line	ELISA	NR	19% (4/21)	HC 100% (0/61) AD 100% (0/35)	NR	(43)
Metallothionein (MT)	IgG	6–14	Human	ELISA	NR	32% (13/41)	TD 88% (4/33)	2–16 years	(44)
Glutamic acid decarboxylase 65 (GAD65)	IgG	65	NR	ELISA	DSM-IV	15% (3/20)	ADHD 73% (4/15) TD 100% (0/14)	9–11 years	(45)
Ganglioside M1	NR	1.6	Human	ELISA	DSM-IV	74% (40/54)	TD 95% (2.7/54)	4–11 years	(46)
	NR		Human	ELISA	DSM-V/ADI-R/ADOS/CARS/ABC	37.8% (31/82)	TD 78.3% (13/60)	2–5 years	(47)
Double strand DNA (ds-DNA)	IgG	NR	Human	ELISA	DSM-IV	34% (34/100)	TD 98% (2/100)	4–11 years	(48)
Nucleosome	IgG	NR	Human	ELISA	DSM-IV	46.7% (28/60)	TD 95%	3–12 years	(49)
Nuclear targets (ANA)	IgG	NA	Human	Immunofluorescence	Based on clinical history	27% (3/11)	LKSV 81.8% (2/11)	2–6 years	(50)
	NR	NA	NR	Indirect Immunofluorescence	DSM-IV	20% (16/80)	OND 90% (2/20)	3–12 years	(51)
	NR	NA	NR	Indirect Immunofluorescence	DSM-IV	25% (25/100)	NNI/HC 100% (0/51)	4–11 years	(48)

[a]Sensitivity is the percentage of seropositive ASD patients, the amount of seropositive patients over the total tested patient cohort is indicated between brackets. [b]Specificity is the percentage of seronegativity in the control populations (TD, MR, A, DS, OND, NNI, HC, AD, ADHD, LKSV), the amount of seropositive controls over the total amount of each control cohort is indicated between brackets. [c]Age range of study population; NA, not applicable; NR, not reported; MR, mental retardation; TD, typically developing; A, adults; DS, Down syndrome; OND, other neurological disorders; NNI, non-neurological illnesses; HC, healthy children; ADHD, attention-deficit/hyperactivity disorder; LKSV, Landau-Kleffner syndrome variant; AD, autoimmune disorder; DSM-III, Diagnostic and statistical manual of mental disorders third edition; DSM-IV, Diagnostic and statistical manual of mental disorders fourth edition; ADOS, Autistic Diagnostic Observation Schedule; ADI-R, Autism Diagnostic Interview-Revised; CARS, Chilhood autism rating scale; ABC, Autism behavior checklist.

Charcot-Marie-Tooth disease (72, 73). Autoantibodies against NF-H have been described in normal aging, but also in degenerative disorders such as Alzheimer's disease, multiple sclerosis and prion diseases (74–77). In ASD, IgG antibodies reactive against bovine NF-H (NAFP) were found using Western blot in nearly 55% of the patients studied (**Table 1**) (41). However, the percentage of seronegativity in control populations (i.e., specificity) of these autoantibodies is lower than 80% in controls with a typical development and idiopathic mental retardation. A study by Vojdani confirmed the increased presence of IgG, IgM, and IgA autoantibodies against NAFP in autistic patients (40).

Glial Fibrillary Acidic Protein (GFAP)

The glial fibrillary acidic protein (GFAP) is a class III intermediate filament cytoskeletal protein. This protein allows the distinction between astrocytes and other glial cells, and is a common astroglial marker in the CNS. Furthermore, it is present in non-myelinating Schwann cells in the PNS and in enteric glial cells (78). In case of acute brain injury, astrocytes become activated, strongly upregulate GFAP expression and increase in size and number, a state called astrogliosis (79). After stroke, traumatic brain injury and spinal cord injury, GFAP, and GFAP breakdown products have been found in CSF and serum (80). These breakdown products trigger an autoantibody response against GFAP in a large subset of patients (81, 82). Elevated GFAP levels have also been described in brain tissue, CSF and serum of ASD patients (83–86), as is the associated autoimmune reactivity. IgG antibodies against GFAP were detected using Western blot in 32% of ASD patients with a specificity of 91% in typically developing subjects, but only 79% in idiopathic mental retarded subjects (**Table 1**) (41). However, in a small study by Kirkman et al. (87), the average titer of anti-GFAP antibodies in autistic patients was not found to be significantly higher than in controls, raising questions about the role of this antibody-mediated response in this developmental disorder.

Ribosomal P Proteins

The ribosomal P proteins are three specific ribosomal proteins located in the large ribosomal subunit (60S). These proteins, P0, P1, and P2, are present in the nucleus and cytoplasm, and are involved in the binding of ribosomal RNA, assembly of the 60S subunit and protein translation (88–90). Autoantibodies reactive against these P proteins are present in nearly one-third of SLE patients and have also been detected in patients with autoimmune hepatitis (91–93). Neuropsychiatric manifestations are some of the most serious complications in SLE, and the occurrence of psychosis in SLE patients has been associated with the presence of anti-ribosomal P antibodies (94). These autoantibodies seem to cross-react with a neuronal integral membrane protein called neuronal surface P antigen (NSPA) (95) and administration of anti-ribosomal P antibodies into murine brains induced neuronal apoptosis and disturbance of the normal functioning of hippocampus and cortex (96, 97), while memory impairment was observed when administered systemically and BBB damage was induced (96). The pathogenicity of these antibodies in other disorders remains unknown, however, in two small studies, elevated anti-ribosomal P protein antibodies were also found using ELISA in 44 to 58% of autistic children and in 5% of the healthy control groups (**Table 1**) (42, 98). Moreover, the presence of these antibodies in autistic children was related with the levels of the neuropeptide Neurokinin A (42) and with the levels of lead in the blood (98).

Heat Shock Protein 90 (Hsp90)

Heat shock protein 90 (Hsp90) is a chaperone involved in assisting the proper folding of proteins and their stabilization (99). Immunoglobulins against this molecular chaperone were found at significantly higher levels in autistic patients than in typically developing controls (43). At the cutoff used, 19% of ASD patients were seropositive for Hsp90 autoantibodies and none of the healthy subjects or controls with autoimmune disorders (**Table 1**). These reactivities have only been reported for a small study population and have not been replicated in independent studies so far. Therefore, the role of these autoantibodies in autoimmune and other diseases is still unclear.

Metallothionein (MT)

Metallothioneins (MT) are intracellular proteins involved in the homeostasis of essential metals and the detoxification of heavy metals (100). The consequences of environmental exposure to noxious agents and their exact role in the pathology of ASD are still under investigation, but it is known that exposure to pollution and heavy metals is causally implicated in changes of fetal neurodevelopment that are related with autism (8, 101, 102). The presence of anti-MT antibodies has been described in metal-induced diseases such as occupational heavy metal exposure and metal allergy (103, 104). Furthermore, one in four autistic children showed a higher MT concentration in the serum and one third of ASD patients showed increased levels of autoantibodies against MT (**Table 1**) (105). These findings might indicate immune abnormalities related to levels of metal exposure in ASD patients.

Glutamic Acid Decarboxylase (GAD65)

GAD65 is the enzyme responsible for the conversion of glutamate to GABA, an inhibitory neurotransmitter in the CNS (106). Anti-GAD65 antibodies have typically been associated with autoimmune diseases not related with the nervous system such as type 1 diabetes, however they have also been reported in CNS disorders such as encephalitis, epilepsy and cerebellar ataxia, where they could even have a pathological role (106–110). The role of GAD65 in neurotransmitter production and the relationship with seizure episodes made it an interesting target in brain disorders with excitation/inhibition imbalance such as autism (106). In a small study using 20 patients, the presence of anti-GAD65 antibodies measured using ELISA was reported in 15% of the autism cases, 27% of ADHD patients and in none of the controls (**Table 1**) (45). Despite the size of the study, an initial indication of a possible relation between these autoantibodies and autism was made. In the future, it might be interesting to test the presence of anti-GAD65 antibodies in the subpopulation of ASD patients that present ataxia-related problems or seizures.

Non-protein Targets

Ganglioside M1 (GM1)

Ganglioside M1 is a glycosphingolipid with one sialic acid residue that is present in the cell membrane (111). It is involved in neuronal plasticity and neurotrophin secretion during development, adulthood and aging (111, 112). Anti-GM1 antibodies have been described in motor neuropathies, but also in neurodegenerative diseases such as GM1 gangliosidosis, multifocal motor neuropathy, dementia and Guillain-Barré syndrome (113–116). Two studies have reported increased levels of these antibodies in ASD patients using ELISA, however with widely different sensitivities, varying from 74 to 38%, and corresponding specificities of 95 and 78%, respectively (**Table 1**) (46, 47). Moreover, in the study by Mostafa et al. (46), a significant correlation with disease severity was found, whereas this could not be confirmed in another study (47). Nevertheless, it seems that anti-GM1 antibodies have been associated with ASD, but their specific role remains to be elucidated.

Nuclear Antigens

Antinuclear antibodies (ANAs) are immunoglobulins that react against nuclear components, mostly double stranded DNA (dsDNA) and DNA-associated proteins. These antigens are not organ-specific and are ubiquitous in every cell. ANAs have been detected in cancer and premalignant diseases, but mostly in autoimmune disorders such as SLE (117–119). Renal failure, nephritis and damage in the glomerular structures have been related to these antibodies (120–123). ANAs, anti-dsDNA and anti-nucleosome antibodies have also been described in autism patients (48–51). Immunoglobulins against general nuclear targets were found in up to a quarter of ASD patients using indirect immunofluorescence detection (**Table 1**) (48, 49, 51). These ANAs show a high specificity (over 96%) in typically developing children. More specific measurements, using ELISA for anti-ds-DNA or anti-nucleosome antibodies, showed that, respectively 34 and 47% of ASD patients were seropositive for these ANAs (48, 49). The presence of antinuclear immunoglobulins has been reported to be increased in patients with severe autism, but also with abnormal electroencephalogram results and mental retardation, suggesting that even if these antibodies cannot be used as a diagnostic test, they might be symptom related (50, 51). In addition, the presence of these antibodies is related with a family history of autoimmunity, even more related to autoimmune diseases in female relatives (51). Between 29 and 48% of the ASD patients seropositive for anti-nuclear antibodies have an autoimmune background (48, 51). The presence of high titers of anti-nuclear antibodies in other autoimmune disorders reduces the chances of using ANAs patterns as a separate diagnostic tool in autism (50, 124).

MATERNAL AUTOANTIBODIES

After being born from the sterile environment of the womb, neonates are rapidly exposed to many different microbial and environmental antigens, while their immune system is still inexperienced. Therefore, during the first months of life, an important mechanism of protection is provided via passive immunization with antibodies, which are been transferred from the mother to the fetus during pregnancy [reviewed in Palmeira et al. (125)]. This occurs via binding to the neonatal Fc receptor (FcRn), which transports IgG subclasses 1, 3, and 4 across the placenta, during the second and third trimester of pregnancy.

In the nineties, around the same time the involvement of the immune system and the presence of autoantibodies in ASD patients were being elucidated, the relationship between aberrant maternal immune responses and autism in children was also being explored. The history of spontaneous abortions and disorders during pregnancy in mothers that later had a child with ASD, together with the suggestion of the presence of anti-paternal antibodies in mothers with a complicated pregnancy record, led to the study of antibody reactivity of maternal plasma against targets in their children (126). In this context, Warren et al. detected increased complement-dependent cytotoxicity by maternal antibodies in 54% (6/11) of mothers of autistic children and in 10% (2/20) of mothers of normal children (126). Despite the small number of subjects, this study was the first indication of a possible correlation between maternal antibodies and the development of autism in their children. Binding of maternal antibodies to brain proteins was first described in a case study (30). Using serum from a mother of 2 children with different developmental disorders including autism, binding of autoantibodies to Purkinje cells and large neurons in murine cerebellum and brainstem was described. A direct functional role for these antibodies was also suggested, as passive transfer of serum of this mother in pregnant mice led to altered exploratory behavior in the pups (30).

In order to further understand this reactivity, several studies were performed to identify maternal antibodies and their antigenic targets (**Table 2**). In a study by Zimmerman, 45% (5/11) of mothers of autistic children showed reactivity against a pattern of 5 proteins between 15 and 37 kDa of fetal rat brain using Western blot (130). This pattern was not observed in mothers of typically developing children or using postnatal and adult rat brain extracts. Using a bigger cohort of 100 mothers of children with autistic disorder and 100 controls, immunoreactivity using Western blot against a 36 kDa human fetal brain protein was found in 10% of the mothers of ASD children (MASD) and only in 2% of the mothers with typically developing children (MTD) (128). A 36 kDa and a 73 kDa band were also identified using rodent embryonic brain tissue in 47–48% of MASD with a specificity of 69% in MTD. In addition, the group of Van de Water performed multiple analyses on maternal samples from the CHARGE study (Childhood Autism Risks from Genetics and the Environment), where mothers were sampled up till 5 years after delivery, but also on maternal mid-pregnancy serum samples from the EMA study (Early Markers for Autism) (131–133, 135, 136). Highly specific patterns of immunoreactivity toward rhesus macaque or human fetal brain extract were found in mothers of children with autism, where a 73 kDa band was combined with either a 37 or a 39 kDa band. Mass spectrometric analysis of immunoreactive spots from a fetal rhesus macaque brain protein extract, resulted in the identification of seven primary

TABLE 2 | Overview of maternal autoantibodies in ASD.

Maternal autoantibodies								
Antibody target	**Isotype**	**MW (kDa)**	**Protein Origin**	**Methods**	**Sensitivity[a]**	**Specificity[b]**	**Serum/Plasma**	**References**
Lactate dehydrogenase A and B (LDH)	IgG	37	Rhesus macaque brain (152 days)	Western blot	28% (68/246)	MTD 87% (20/149)	Plasma	(127)
Cytosolic PSD-95 interactor (Cypin)	IgG	44	Rhesus macaque brain (152 days)	Western blot	25% (62/246)	MTD 81% (29/149)	Plasma	(127)
Stress-induced phosphoprotein 1 (STIP1)	IgG	73	Rhesus macaque brain (152 days)	Western blot	59% (145/246)	MTD 64% (53/149)	Plasma	(127)
Collapsin response mediator proteins 1 (CRMP1)	IgG	70	Rhesus macaque brain (152 days)	Western blot	32% (78/246)	MTD 82% (27/149)	Plasma	(127)
Collapsin response mediator proteins 2 (CRMP2)	IgG	62	Rhesus macaque brain (152 days)	Western blot	18% (44/246)	MTD 93% (11/149)	Plasma	(127)
Y-box-binding protein (YBX1)	IgG	39	Rhesus macaque brain (152 days)	Western blot	31% (78/246)	MTD 77% (34/149)	Plasma	(127)
LDH + STIP1 + CRMP1	IgG	37 +73	Rhesus macaque brain (152 days)	Western blot	5% (13/246)	MTD 100% (0/149)	Plasma	(127)
LDH + STIP1 + CRMP1 + Cypin	IgG	NR	Rhesus macaque brain (152 days)	Western blot	2% (5/246)	MTD 100% (0/149)	Plasma	(127)
Specific combinations of LDH, STIP1, CRMP1, Cypin, CRMP2, YBX1	IgG	NR	Rhesus macaque brain (152 days)	Western blot	23% (56/246)	MTD 99% (2/149)	Plasma	(127)
Purkinje cells	IgG	NR	Adult rat brain P1 mouse brain	Immunohistochemistry	Case study	Case study	Serum	(30)
NB-1 neuroblastoma	IgG	NR	Human cell line	Immunohistochemistry	Case study	Case study	Serum	(30)
GFAP	IgG		Human	Western blot	10% (2/20)	MTD 85% (3/20)	Serum (years after delivery)	(128)
MBP	IgG	18–20	Human	Western blot	5% (1/20)	MTD 90% (2/20)	Serum (years after delivery)	(128)
Nuclear targets (ANA)	IgG	NR	Mouse (12 weeks)	Immunohistochemistry	24.5% (251/1022)	MTD 84.9% (52/345)	Plasma	(129)
Fetal Brain proteins	IgG	15–37 >250	Fetal rat brain	Western blot	45.4% (5/11) 54.5% (6/11)	MTD 100% (0/10) MTD 100% (0/10)	Serum	(130)
	IgG	32	Human (20-40 weeks)	Western blot	3.6% (3/84)	MTD 100% (0/152) MDD 95.8% (2/48)	Serum (15–19 weeks of gestation)	(131)
	IgG	36	Human (17-weeks)	Western blot	10% (10/100)	MTD 98% (2/100)	Serum (years after delivery)	(128)
	IgG	37	Human (20–40 weeks)	Western blot	26.2% (16/61)	MTD 91.9% (5/62) MDD 97.5% (1/40)	Plasma (2–5 years after delivery)	(132)
	IgG		Rhesus macaque brain (152 days)	Western blot	6.6% (17/259)	MTD 93.9% (11/180)	Plasma	(132)

(Continued)

TABLE 2 | Continued

Maternal autoantibodies								
Antibody target	**Isotype**	**MW (kDa)**	**Protein Origin**	**Methods**	**Sensitivity[a]**	**Specificity[b]**	**Serum/Plasma**	**References**
	IgG	39	Human (20–40 weeks)	Western blot	7.1% (11/84)	MTD 98% (3/152) MDD 100% (0/48)	Serum (15–19 weeks of gestation)	(131)
	IgG		Human (17-weeks)	Western blot	14% (14/100)	MTD 85% (15/100)	Serum (years after delivery)	(128)
	IgG		Rhesus macaque brain (152 days)	Western blot	10.4% (27/ 259)	MTD 83.9% (29/180)	Plasma	(132)
	IgG	60	Human (20-40 weeks)	Western blot	19% (16/84)	MTD 76.3% (36/152) MDD 75% (12/48)	Serum (15–19 weeks of gestation)	(131)
	IgG	61	Human (17-weeks)	Western blot	30% (30/100)	MTD 69% (31/100)	Serum (years after delivery)	(128)
	IgG	73	Human (20–40 weeks)	Western blot	13.1% (11/84)	MTD 92.8% (11/152) MDD 95.8% (2/48)	Serum (15–19 weeks of gestation)	(131)
	IgG		Rhesus macaque brain (152 days)	Western blot	16.2% (42/259)	MTD 87.8% (22/180)	Plasma	(132)
	IgG	37 + 73	Human (20–40 weeks)	Western blot	11.5% (7/61)	MTD 100% (0/62) MDD 100% (0/40)	Plasma (2–5 years after delivery)	(133)
	IgG		Monkey brain	Western blot	9% (9/202)	MTD 100% (0/163)	Plasma	(134)
	IgG		Rhesus macaque brain (152 days)	Western blot	9.3% (24/259)	100% (0/180)	Plasma	(132)
	IgG		Rhesus macaque brain (49, 100, 152 days)	Western blot	7% (10/143)	MTD 100% (0/121) MDD 100% (0/62)	Plasma	(135)
	IgG		Rhesus macaque	Western blot	7.6% (10/131)	MTD 100% (0/50)	Plasma	(136)
	IgG	39 + 73	Human (20-40 weeks)	Western blot	3.6% (3/84)	MTD 100% (0/152) MDD 95.8% (2/48)	Serum (15–19 weeks of gestation)	(131)
	IgG		Rhesus macaque brain (152 days)	Western blot	8.9% (23/259)	MTD 98.3% (3/180)	Plasma	(132)
	IgG	60 + 73	Human (20–40 weeks)	Western blot	6% (5/84)	MTD 98% (3/152) MDD 97.9% (1/48)	Serum (15–19 weeks of gestation)	(131)
	IgG	NR	Mouse (12 weeks)	Immunohistochemistry	10.7% (260/2431)	MTD 97.4% (17/653)	Plasma	(129)
Adult Brain cingulate gyrus protein	IgG	91 100 129	Human	Western blot	13 % (13/100) 5% (5/100) 26% (26/100)	MTD 80% (20/100) MTD 100% (0/100) MTD 79% (21/100)	Serum (years after delivery)	(128)
Adult Brain cerebellum protein	IgG	31 100	Human	Western blot	25% (25/100) 34% (34/100)	MTD 65% (35/100) MTD 70% (30/100)	Serum (years after delivery)	(128)
Adult Brain caudate protein	IgG	81 155	Human	Western blot	20% (20/100) 11% (11/100)	MTD 79% (21/100) MTD 98% (2/100)	Serum (years after delivery)	(128)
Adult Brain frontal cortex (BA9)	IgG	63	Human	Western blot	39% (39/100)	MTD 73% (27/100)	Serum (years after delivery)	(128)

(Continued)

TABLE 2 | Continued

Maternal autoantibodies								
Antibody target	**Isotype**	**MW (kDa)**	**Protein Origin**	**Methods**	**Sensitivity[a]**	**Specificity[b]**	**Serum/Plasma**	**References**
Embryonic tissue protein	IgG	36 73	Rat (E18)	Western blot	48% (48/100) 47% (47/100)	MTD 69% (31/100) MTD 69% (31/100)	Serum (years after delivery)	(128)
Adult Brain protein	IgG	27 110	Rat	Western blot	22% (22/100) 29% (29/100)	MTD 90% (10/100) MTD 66% (34/100)	Serum (years after delivery)	(128)

[a]Sensitivity is the percentage of seropositive mothers of ASD children, the amount of seropositive mothers of ASD children over the total tested cohort of mothers of ASD is indicated between brackets. [b]Specificity is the percentage of seronegativity in the control populations (MTD, MDD), the amount of seropositive controls over the total amount of each control cohort is indicated between brackets. NR, non-reported; MASD, mother of children with autism spectrum disorder; MTD, mother of children with typically developing; MDD, mother of children with non-autistic developmental delay.

targets of maternal antibodies related with autism: lactate dehydrogenase A and B (LDH-A/B) (37 kDa), Y-box-binding protein 1 (YBX1) (39 kDa), Cytosolic PSD-95 interactor (Cypin) (44 kDa), Collapsin response mediator protein 2 (CRMP2) (62 kDa), Collapsin response mediator protein 1 (CRMP1) (70 kDa), and Stress-induced phosphoprotein 1 (STIP1) (73 kDa) (**Table 2**) (127). Individually, testing immunoreactivity against these targets using Western blot resulted in sensitivities ranging from 18 to 59% in mothers of ASD children, with specificities between 64 and 93% in mothers of typically developing children (**Table 2**). Combination of different panels, each consisting of immunoreactivity against specific combinations of two or more antigens of these seven targets, led to a large increase in specificity (99%), while maintaining a relatively high sensitivity of 23% (56/246) (**Table 2**). Moreover, an increased impairment in stereotypical behavior was observed in children of mothers with LDH-, or combined LDH/STIP1- or LDH/STIP1/CRMP1 immunoreactivity.

The active contribution of these maternal autoantibodies in the development of autistic features in the offspring has been investigated in animal models using passive transfer of the total IgG fraction purified from mothers of ASD children or mothers of typically developing children. Pregnant mouse dams have been injected with purified IgG around the end of the second trimester of pregnancy, either with a single injection in the maternal periphery (137) or in the embryonic brain ventricles (69, 138), or by daily peripheral injections during the third trimester (139). Recently, a novel active immunization model has also been applied, where female mice were immunized before pregnancy with a mixture of immunogenic peptides from LDH-A/B, STIP1, and CRMP1, providing a more representative situation where mouse dams produce antibodies themselves during the entire pregnancy (140, 141). Mice exposed to these maternal autoantibodies during development showed increased cell division of radial glial cells in the subventricular zone, increased neuronal size in the cortex (142) and a reduction in the number of dendritic spines and of the dendritic arborization in the neurons of the infragranular layers of the adult cortex (138). This coincides with autism-related behavioral changes such as impaired motor and sensory development, hyperactivity, anxiety, inappropriate social interactions and repetitive and stereotypic behaviors (69, 137, 139, 141). While these maternal autoantibodies have been shown to reach high levels in the brains of the embryos, only a very low amount could be found in the brains of the dams (137, 139), probably due to differences in BBB permeability in embryonic and adult brains. Moreover, behavioral changes in the dams themselves have not been reported so far.

These studies do not directly pinpoint the involvement of a single autoantibody target in ASD development, but remain an important tool to experimentally demonstrate that autoantibody transfer during pregnancy can alter brain biology, resulting in autistic behaviors in the offspring. A detailed overview of the autoantibodies described in mothers of children with ASD is presented in **Table 2**. The biomarker potential, general function, expression and possible role during neurodevelopment of the

most interesting autoantibody targets is discussed below in more detail.

Lactate Dehydrogenase A and B (LDH-A and LDH-B)

Antibodies to LDH-A and LDH-B were found in 28% of mothers of ASD children and 13% of mothers of typically developing children (**Table 2**) (127). Lactate dehydrogenase (LDH) is an oxidoreductase responsible for the reversible conversion of the glycolytic intermediate pyruvate to lactate. This leads to rapid energy production via fermentation of pyruvate, instead of fully metabolizing it to CO_2 via aerobic respiration in the mitochondria. LDH is encoded by 3 different genes *LDH-A*, *B* and *C* that correspond to 3 different protein subunits, which can combine into homotetrameric or heterotetrameric isoenzymes (143). LDH-A and LDH-B are differentially expressed in different adult brain regions, with LDH-A showing a diffusely distributed expression pattern, with increased expression in the hippocampus and cortex, while LDH-B shows high expression in several brain structures such as the olfactory bulb and piriform cortex, several thalamic and hypothalamic nuclei, the granular, and Purkinje cell layers of the cerebellum and a laminar pattern in the neocortex (144). Isoenzymes which are preferentially composed of LDH-A or LDH-B have different metabolic functions, and the ratio between A and B is responsible for the balance between glycolysis and oxidative phosphorylation in different tissues (143).

Serum LDH activity has been found to be increased in ASD patients and appears to be related to the level of disease severity (145). Interestingly, increased levels of pyruvate and lactate, the metabolites which are interconverted by LDH, are often used as a marker for mitochondrial dysfunction, as less pyruvate is metabolized through the tricarboxylic acid cycle in mitochondria [reviewed in Rossignol and Frye (146)]. Besides increased levels of pyruvate and lactate, several other lines of evidence, such as altered levels of other carbon metabolites and activity changes or mutations in enzymes involved in carbon metabolism, indicate a broader mitochondrial dysfunction in a subset of autism patients (147, 148). As neurons are highly energy dependent cells, dysregulation of energy metabolism can implicate changes in their main functions and structures. Still, the exact role of mitochondrial dysfunction and carbon metabolism in ASD pathology remains poorly understood.

Y-Box-Binding Protein 1 (YBX1)

Antibodies to YBX1 were found in 31% of mothers of ASD children and 23% of mothers of typically developing children (**Table 2**) (127). Specific combinations of antibody reactivity to YBX1 and LHD or CRMP2 were found in a smaller percentage of mothers of ASD children (2–5%), but with much higher corresponding specificities (99–100%). *YBX1* encodes the Y-box-binding protein 1, also called nuclease-sensitive element-binding protein 1. This protein is involved in DNA and RNA-binding during mRNA processing, splicing, transcription and transcription regulation and is important for processes such as stress response, cell proliferation, and differentiation (149, 150). Furthermore, this protein is involved in differentiation of neuronal progenitors, maintenance of the stem cell status and malignant cell transformation (151). YBX1 has a general expression in most murine tissues, both during embryonic development and in adulthood (152). In the adult human brain, YBX1 is mainly located in neurons throughout the brain, while prenatally it is expressed in radial glia, neuroblasts and neurons (153). Homozygous knockout of *YBX1* leads to embryonic lethality, with abnormalities in neural tube formation (154).

The role of YBX1 in ASD is still unknown, but it has a direct protein-protein interaction with methyl-CpG binding protein 2 (MeCP2), which is related with neurodevelopmental disorders such as Rett syndrome (155). This interaction between YBX1 and MeCP2 is dependent on RNA, and influences RNA splicing. Furthermore, after maternal immune activation, the expression of YBX1 is rapidly increased in the fetal brain, however, the possible role in adverse neurological outcomes has not been studied in this context (156).

Cytosolic PSD-95 Interactor (Cypin)

Mothers of autistic children showed antibody reactivity against Cypin, in 25% of cases, compared to 19% in mothers of typically developing children (**Table 2**) (127). Cypin is a guanine deaminase, which is required for the enzymatic degradation of the purine guanine to xanthine (157). Cypin is expressed in various organs in the body, but its highest expression is seen in epithelial cells of the intestine and in discrete neurons in the cortex, hippocampus and the superior colliculus (158). Importantly, Cypin has been found to be a major interaction protein for postsynaptic density protein 95 (PSD-95), and has both a structural and a regulatory role, decreasing localization of PSD-95 at post-synaptic sites (158). Cypin also promotes microtubule polymerization by directly binding to tubulin using a domain with high homology to collapsin response mediator protein (CRMP), another maternal autoantibody target described in autism (127, 159). This CRMP-homology domain and a 9 amino acid zinc-binding domain are required for guanine deaminase activity and for the regulation of dendrite branching and neuronal morphology in cultured hippocampal neurons (159, 160). Dendrite morphogenesis is essential for the communication between neurons and when the regulation of the dendritic arborization is altered, this can lead to abnormal spine density and morphology, synapse loss and aberrant synaptic signaling, resulting in modification of the neural circuitry which can lead to neuropsychiatric disorders (14, 161, 162).

Collapsin Response Mediator Proteins 1 and 2 (CRMP1/CRMP2)

Antibodies to CRMP1 or CRMP2 have been found in 32 and 18% of mothers of ASD children, respectively, and in 18 and 7% of mothers of typically developing children (**Table 2**) (127). In addition, these autoantibodies are also found together with many of the other described autoantibodies in mothers of autistic children. The collapsin response mediator proteins are a family

of five phosphoproteins (CRMP1-5) located in the cytosol of both neurons and glia (163). CRMP1 and CRMP2 are expressed both in the developing and the adult brain, but show peak expression in the last weeks of gestation and the first postnatal weeks (164). These proteins participate in the organization of the cytoskeleton and bind to microtubuli, actin filaments and intermediate filaments (165–168). During cell division, CRMP1 and 2 stabilize the mitotic apparatus, by binding to microtubuli (166). CRMP1 and CRMP2 are involved in radial migration and the subsequent differentiation of neurons during cortical development (169, 170). Furthermore, both proteins play a role in the regulation of signaling of class 3 semaphorins and neural growth cone collapse, remodeling of the cytoskeleton required for axonal growth and guidance, and dendritic arborization (171–174). During neuronal polarization of cultured hippocampal neurons, CRMP2 becomes highly enriched in the distal part of the growing axon (172). On the other hand, when CRMP2 is overexpressed, these neurons develop multiple axons, while the expression of a truncated dominant negative mutant resulted in neurons with short or no axons. In addition, CRMP1 and CRMP2 are also required for proper dendritic patterning of cortical neurons. CRMP1$^{-/-}$ mice show reduced dendrite length and reduced dendritic spine density, while CRMP2$^{-/-}$ mice only have a reduced spine density (174). Both proteins seem to have some functional redundancy as inhibition of CRMP2 phosphorylation in a CRMP1$^{-/-}$ background induces a strong disruption of the morphology, orientation and guidance of the dendrites of layer V cortical pyramidal neurons (175).

CRMP1$^{-/-}$ mice showed impaired memory and behavioral abnormalities related to schizophrenia such as hyperactivity, altered emotional behavior and decreased prepulse inhibition (176). On the other hand, brain-specific knockout of CRMP2 induced impairment of learning, memory and social behavior (177), while knock-in of a CRMP2 mutant with impaired phosphorylation reduced emotional behavior, sociality and pain sensitivity (178). Multiple genome-wide and proteome-wide analyses have described gene variants or altered levels of CRMP1 and 2, but also other CRMP family members, in patients with neurodegenerative and neuropsychiatric disorders, such as Alzheimer disease, schizophrenia, mood disorders, epilepsy and neuropathic pain [reviewed in Quach et al. (179)].

Stress-Induced Phosphoprotein 1 (STIP1)

Mothers of ASD children showed antibody reactivity against STIP1, in 59% of cases, compared to 36% in mothers of typically developing children (**Table 2**) (127). In addition, anti-STIP1 antibodies were also found in many combinations with the other described maternal autoantibodies, resulting in strongly increased specificity. The Stress-induced phosphoprotein 1 (STIP1) is a co-chaperone of the heat shock proteins (Hsp)70 and Hsp90, and modulates their folding activity (180). STIP1 is mostly expressed in the cytosol, but it can also translocate to the nucleus (181), and even be secreted in exosomes, where it is found on the surface (182). Extracellular STIP1 was found to interact with the glycosylphosphatidylinositol (GPI)-anchored cellular prion protein PrP^{C}, leading to an increased number of neurons with neurites *in vitro*, without affecting the number or length of neurites per cell (183, 184). Moreover, this STIP1-PrP^{C} interaction also protected cultured neurons from staurosporine-induced cell death. This STIP1-PrP^{C}-induced neuritogenesis and neuroprotection was found to be mediated by Ca^{2+} signaling through the alpha7 nicotinic acetylcholine receptor (185). Interestingly, during glutamate-induced neurogenesis *ex vivo*, STIP1 and CRMP2 were among the most upregulated proteins (186). Moreover, *STIP1*$^{-/+}$ heterozygous mice, which have a 50% reduction of STIP expression without affecting Hsp70, Hsp90 or the PrP^{C} levels, showed hyperactivity and attention deficits (187).

Contactin-Associated Protein-Like 2 (CASPR2)

Contactin-associated protein-like 2 (CASPR2) is a transmembrane cell adhesion protein of a subgroup of the neurexin family related to the establishment of the neural network and higher cognitive functions in the brain (188). The protein is encoded by the *CNTNAP2* gene and is expressed both in the developing and adult nervous system (189, 190). In the developing human cortex, the anterior temporal and prefrontal regions have shown specific enrichment of CASPR2 (190). In adults, it is expressed in areas of the limbic circuit and in brain areas involved in motor activities and sensory pathways (191). At the subcellular level CASPR2 is located at the axon initial segment and in the nodes of Ranvier of myelinated axons, in presynaptic terminals of inhibitory neurons and in the postsynaptic compartment of excitatory neurons (189, 192). It is implicated in processes as diverse as nerve excitation and conduction, neurotransmitter release, organization of the axonal domain, dendritic spine stabilization, and neuronal network formation (193, 194).

Copy number variations and single nucleotide polymorphisms in the *CNTNAP2* gene have been related to several neurodevelopmental disorders, such as schizophrenia, intellectual disability, epilepsy and autism, often with combined clinical presentation [reviewed in Saint-Martin et al. (188); Poot (195)]. Interestingly, genetic knockout of this gene in a mouse model induces the development of many of the core ASD behavioral symptoms, as *CNTNAP2*$^{-/-}$ mice show increased repetitive behavior, decreased vocalization and decreased social interactions (196). In addition, these mice also display hyperactivity, hyper-reactivity to thermal sensory stimuli and epileptic seizures and allowed to study some of the underlying biology, with observed abnormalities in neuronal migration, a reduction in GABAergic interneurons and abnormal neuronal network activity.

In addition to the genetic variants affecting CASPR2 function, acquired dysfunction of CASPR2 has also been proposed in patients that form autoantibodies against CASPR2. Such antibodies have been described in patients with limbic encephalitis, Morvan syndrome and neuromyotonia, three conditions that variously affect the central and peripheral nervous system, and are characterized by cognitive decline, epilepsy, peripheral nerve hyperexcitability and neuropathic pain

(197–199). Patient CASPR2 autoantibodies decreased AMPA-type glutamate receptor trafficking in cultured neurons and perturbed cortical excitatory transmission after stereotactic injection in the mouse visual cortex (200). Systemically injected patient CASPR2 autoantibodies did not cross the BBB and caused peripheral hypersensitivity to mechanical stimuli (201).

In contrast, *in utero* exposure to CASPR2 autoantibodies during embryonic development has been linked to the development of autistic features in the offspring (202). A monoclonal antibody with reactivity to CASPR2 was cloned from the memory B cells of a mother with brain-reactive serology and a child with autism, and used in a passive transfer experiment in pregnant mice (202). The male offspring showed abnormal cortical development, together with a reduction in the dendritic complexity in excitatory neurons and a reduced number of inhibitory neurons, and presented with repetitive behaviors and impairment in learning and social abilities. In addition, purified human IgG from patients with CASPR2 immunoreactivity, could be found in the fetal circulation and fetal brain parenchyma after injection into pregnant mice (203). These offspring also showed deficits in social interaction, together with abnormal neuronal distribution, decrease in excitatory synapses and an increase in microglial activation (203). Among mothers of an autistic child that show brain-reactive antibodies, 37% were found to have autoantibodies that bind to CASPR2, compared to 12% in mothers of an autistic child lacking brain reactive antibodies and 8% of women of a normally developing child (202).

CONCLUSION

In conclusion, an increasing number of ASD-related autoantibodies have been described, both in ASD patients themselves and in mothers with children that later develop ASD.

At the moment, most of the antibodies which have been described in ASD patients still lack validation between independent research groups, and require testing in higher numbers of ASD patient and relevant control samples using standardized assays for a more reliable determination of their possible value as disease biomarkers. These autoantibodies have mostly been tested using a candidate approach, using autoantibodies which have already been described in other autoimmune diseases. This field might benefit from an unbiased screening of immunoreactivity in ASD patients, to identify potential new autoantibodies with a higher specificity for ASD. Because of the lack of functional studies using passive transfer of ASD patient antibodies in animal models, it is not known whether autoantibodies in ASD patients have an active contribution to the initiation or worsening of the disease. It could be that the presence of these antibodies is not directly related with the disease, considering the high prevalence in other disorders. Still, they might have indirect roles in the pathology or be related with subpopulations of ASD patients.

Autoantibodies in mothers of children that later develop ASD, have been found using a screening of immunoreactivity against relevant brain tissue, or in the case of CASPR2, by studying a protein which has already shown high relevance for ASD, and for which autoantibodies were previously described in other disorders. Individual specificities of these maternal antibodies are low, which could only be significantly increased using certain combinations of maternal antibody reactivity. These candidate markers also require validation by independent research groups, especially considering the specific set of combinations that have been described. However, an active contribution of certain autoantibodies in mothers, to the development of ASD in their children, is gaining more evidence. Passive transfer of IgG from mothers of ASD children in pregnant mice, or LDH-A/B, STIP1- and CRMP1-immunization of female mice leads to relevant ASD-related biological and behavioral changes in the offspring. Individual relations of these autoantibodies to specific biological processes have not been established, and quite possibly, a combination of autoantibodies is required for a clear behavioral effect. Still, these are compelling models to further study the details of autoantibody-mediated disruption of early processes of neurodevelopment during pregnancy, eventually leading to behavioral deficits linked to ASD later in life.

AUTHOR CONTRIBUTIONS

RM-C wrote the manuscript and designed the figure and tables. PV wrote the manuscript, reviewed the manuscript structure, ideas, and science. VS evaluated and reviewed manuscript structure, ideas, and science. All authors read and approved the final manuscript.

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Novel Molecular Leads for the Prevention of Damage and the Promotion of Repair in Neuroimmunological Disease

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Neuroinflammation is a prominent pathological feature of all neuroimmunological diseases, including, but not limited to, multiple sclerosis (MS), myasthenia gravis, neuromyelitis optica, and Guillain–Barré syndrome. All currently-approved therapies for the treatment of these diseases focus on controlling or modulating the immune (innate and adaptive) responses to limit demyelination and neuronal damage. The primary purpose of this review is to detail the pre-clinical data and proposed mechanism of action of novel drugs currently in clinical trial, with a focus on novel compounds that promote repair and regeneration in the central nervous system (CNS). As the most recent advances have been made in the field of MS research, this review will focus primarily on this disease and its animal models. However, these compounds are likely to be effective for a range of indications with a neuroinflammatory component. Traditionally, MS was thought to proceed through two distinct phases. The first, predominantly inflammatory stage, is characterized by acute episodes of clinical relapse, followed by periods of partial or total recovery with an apparent absence of overall disease progression. In the vast majority of patients, this relapsing-remitting disease subsequently progresses into a second more chronic, neurodegenerative phase, which is characterized by oligodendrocyte damage and axonal destruction leading to brain atrophy and an accumulation of disability. Recent work has shown that rather than occurring independently, both the inflammatory and degenerative phases may run concurrently. This, combined with evidence that early therapeutic intervention slows accumulation of disability and delays progression, highlights the need for novel therapeutic approaches that promote repair and regeneration early in the disease trajectory. Such compounds may be used as monotherapies or in conjunction with classical anti-inflammatory therapies. This review will highlight novel therapies currently in clinical trial, and likely to appear in clinical practice in the near future, focusing on compounds that target the immune system and/or enhance endogenous repair mechanisms in the CNS.

Keywords: neuroimmunology, inflammation, repair, multiple sclerosis, therapeutics, remyelination

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INTRODUCTION

Neuroimmunology is the multidisciplinary study of the interaction between the immune, central, and peripheral nervous systems. One can consider four classical immunological diseases of the nervous system: multiple sclerosis (MS), Guillain-Barré syndrome, myasthenia gravis, and neuromyelitis optica (NMO). Neuroinflammation is a term often closely and correctly associated with these diseases and has traditionally been used to describe the infiltration of blood-derived leukocytes, the dysregulation of the blood-brain-barrier, and a considerable, and sustained glial activation. While the sequence of these events is still in question, the net accumulation of peripheral immune cells in the central nervous system (CNS) is not. "Neuroinflammation" is a common thread that binds these diseases. However, in the past decade this moniker has been appropriated to describe changes (specifically in resident glia) that fall within the confines of a normal glial response. These include changes during the aging process, response to social stress, and intrinsic (obesity), and extrinsic (air pollution) stressors (1, 2). The esoteric nature of neuroimmunology and the more loose application of the term "neuroinflammation" has brought more and more diseases under the neuroimmunological disease umbrella. Any change to brain homeostasis induces a microglial and astrocytic response often predicated on a release of immune-active molecules. It could therefore be argued that all diseases of the brain and spinal cord are, by virtue of the fact that they induce such glia-mediated changes, neuroimmunological in nature. The advent of "omics" level-interrogation of human disease tissue in conjunction with large, highly reproducible genome-wide association studies (GWAS) have positioned immune cells and their products at the center of discussions on the pathological causes and drivers of neurological disease. These include neurodegenerative diseases (Alzheimer's, Parkinson's, Huntington's and amyotrophic lateral sclerosis), neurodevelopmental disorders such as autism spectrum disorders, and mental health illnesses such as schizophrenia (3).

MS has long been the prototypical inflammatory disease of the CNS Since MS is a neuroimmunological disease with a distinct degenerative component, the complexity and evolving nature of MS pathogenesis provides an intriguing nexus point between inflammatory and degenerative cellular and molecular processes. While great inroads have been made in treating the inflammatory aspects of early MS, therapeutic options for patients with progressive disease remain sorely lacking. The extent to which neuroinflammation and neurodegeneration occur independently, in parallel, or sequentially is still an area of intense study. It is clear that the prolonged multiphasic nature of MS-associated neuroinflammation invariably leads to neurodegeneration and brain volume loss. Interestingly, this is not the case in some other neuroimmunological disease such as NMO, which is caused by a classic autoimmune response directed against the water channel, aquaporin-4, expressed on astrocytes (4). In NMO, the majority of patients do not experience a progressive phase of the disease. The MS field is currently shifting its attention to identifying novel, highly directed anti-inflammatory therapies and growth/repair-promoting compounds to tackle both inflammatory and progressive forms of disease. New and exciting targets are being identified with the goal of treating the inflammatory component of the disease in a more specific manner, thereby mitigating against unforeseen and potentially negative consequences of an untargeted immunosuppression. At the same time, novel methods to promote repair and remyelination are being explored, with great successes being reported in pre-clinical and early clinical trial studies.

MS is a multifocal, demyelinating, inflammatory disease of the CNS characterized by oligodendrocyte loss and progressive neurodegeneration, as shown in **Figure 2A**. The development of MS is thought to be at least in part caused by a loss of homeostatic immunological control measures that normally prevent the progression of benign autoimmune responses to pathogenic autoimmunity (5). This loss of homeostatic control is itself thought to be a result of a combination of genetic predisposition, hormonal changes and environmental triggers. Clinical symptoms vary based on the site of neurologic lesions and often correlate with invasion of inflammatory cells across the blood-brain barrier with resulting demyelination and edema (6). The McDonald criteria for MS diagnosis requires two or more clinical episodes with two or more demyelinating lesions on MRI, appearing in separate loci over time. Patients with clinically isolated syndrome (optic neuritis, brain-stem dysfunction, or incomplete transverse myelitis) have a greater chance of conversion to relapsing-remitting MS (RRMS) depending upon the lesion load. RRMS typically manifests in the second or third decade of life (7). Seventy percent of these RRMS patients progress to secondary progressive MS (SPMS) which is characterized by irreversible, progressive accumulation of disability (7). Ten to fifteen percent of MS patients are diagnosed with a primary progressive form (PPMS); these patients experience progressive disease after initial symptoms without relapses, with a similar incidence among men and women (6, 7).

It is believed that early MS disease is driven by inflammation-mediated demyelination and oligodendrocyte damage, as shown in **Figure 1**, leading to axonal conduction block with limited axonal loss. This CNS damage is thought to be mediated by components of the innate (neutrophils, monocyte-derived-macrophages and natural killer cells) and adaptive (CD4+, CD8+ T cells, and B cells) immune systems. During the relapsing remitting phase of disease, patients undergo sporadic clinical relapses in conjunction with areas of focal CNS neuroinflammation. Patients will normally experience significant recovery from neurologic symptoms during these remissions.

Abbreviations: Btk, Bruton's tyrosine kinase; EAE, experimental autoimmune/allergic encephalomyelitis; EDSS, expanded disability status scale; HERV, human endogenous retroviral elements; IFA, incomplete Freund's adjuvant; MBP, myelin basic protein; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; NMO, neuromyelitis optica; NO, nitric oxide; OPC, oligodendrocyte progenitor cells; PBMC, peripheral blood mononuclear cells; PBVC, percent brain volume change; PDE, phosphodiesterases; PLP, proteolipid protein; PPMS, primary progressive multiple sclerosis; RRMS, relapsing-remitting multiple sclerosis; SPMS, secondary progressive multiple sclerosis; TCR, T cell receptor; TMEV, Theiler's murine encephalomyelitis virus; Treg, regulatory T cells.

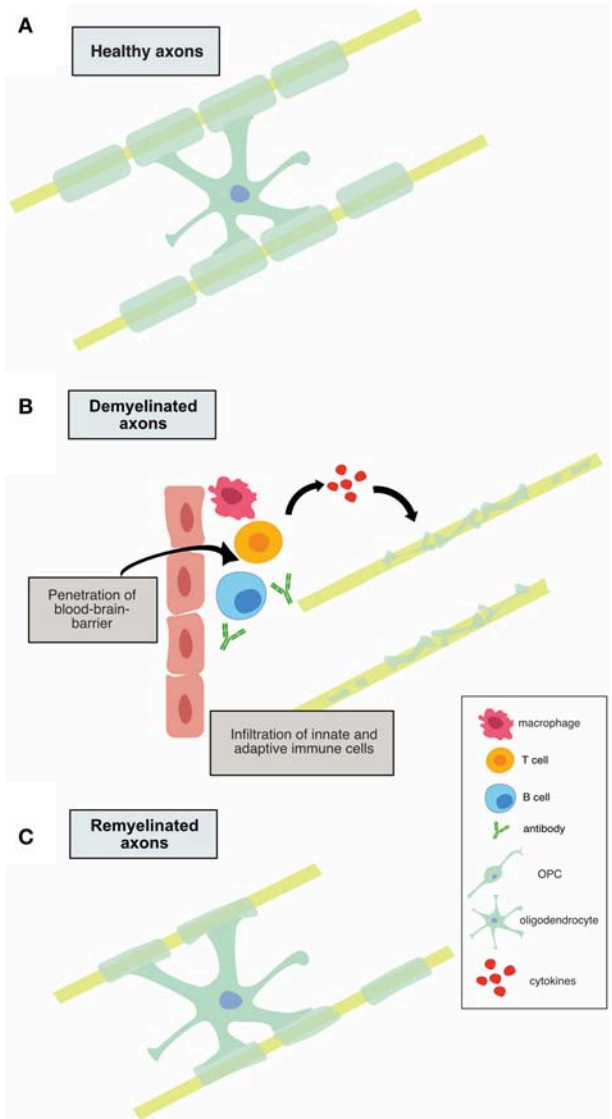

FIGURE 1 | Illustration of demyelination and remyelination in MS. **(A)** shows a healthy, myelinated axon. In **(B)**, infiltration of innate and adaptive immune cells is shown, along with subsequent demyelination of axons. **(C)** depicts the remyelination process that occurs in relapsing-remitting MS, in which the myelin sheaths are thinner.

Differentiation of oligodendrocyte precursors and subsequent remyelination, as shown in **Figure 3A**, is thought to underlie these remission periods, particularly in the early stages of MS.

During the progressive phase of the disease, patients cease to experience clinical relapses and instead slowly accrue disability. This is characterized by degenerative changes, including oligodendrocyte and neuronal loss, along with marked tissue atrophy, and a lack of remyelination. Evidence from MRI and histopathological studies now suggests that rather than two independent phases that occur sequentially, both neuroinflammation and neurodegeneration may progress concurrently (8). It is also apparent that we have underestimated the inflammatory component of progressive MS (9). Early in the disease process, advanced MRI shows abnormalities in normal appearing white and gray matter in the absence of focal lesions seen on conventional scans (10). Brain atrophy has been seen in radiographically isolated syndrome, which is the appearance of lesions on MRI without evidence of clinical relapses. Perhaps most convincing is the evidence of brain atrophy at the time of a first clinical attack (11, 12).

For the purposes of this review, we will focus much of our discussion on novel therapies currently in the pipeline for the treatment of the more classically neuroinflammatory condition of MS while remaining cognizant that many of these future therapies are likely to be used in other neuroimmunological indications.

NOVEL THERAPEUTIC TARGETS: ANTI-INFLAMMATORIES

Bruton's Tyrosine Kinase Inhibitors

The clinical success of Ocrelizumab, a B cell depleting anti-CD20 monoclonal antibody, has generated a huge amount of interest in further therapeutic targeting of these cells. A more specific, modulatory approach is of particular interest, to target B cell activity in the absence of widespread depletion, thereby alleviating any potential side effect of widespread depletion. Bruton's tyrosine kinase (Btk) is a cytosolic non-receptor tyrosine kinase belonging to the Tec family of kinases (13) and is predominantly expressed by B cells. Btk is critical for B cell development, differentiation, proliferation and survival, as shown in **Figure 2B**(i). Therefore, Btk-deficient B lymphocytes die prematurely. Following B-cell receptor engagement, Btk translocates to the plasma membrane where it is activated by sequential phosphorylation and auto-phosphorylation (13).

Btk is required for NLRP3 inflammasome activation (14). NLRP3 is a pattern recognition receptor that assembles with its adaptor molecule and catalytic protein (caspase 1) upon activation. Once the assembly is complete, the unit self-cleaves pro-caspase 1 to caspase 1, which then cleaves pro-IL-1B and pro-IL-18 to IL-1B and IL-18 respectively, leading to the induction of pyroptosis (15). Interestingly, expression of caspase 1 and IL-1B is seen in MS lesions and the levels of caspase 1 and IL-18 are increased in MS patients' peripheral blood mononuclear cells (PBMCs) compared to healthy controls. In the mouse model of MS, experimental autoimmune encephalomyelitis (EAE), lack of NLRP3 was shown to be protective. Similarly, IFN-B, a first line therapeutics for MS, functions to suppress NLRP3 inflammasome activation (16, 17). Therefore, inhibition of Btk may be beneficial in MS at least partially through inhibition of the NLRP3 inflammasome.

Btk may also contribute to disease progression through phosphorylation of phospholipase Cγ2 and generation of inositol 1,4,5-triphosphate and diacyglycerol. These processes result in downstream calcium influx and subsequent NF-kB activation (18, 19). NF-kB is activated by cytokines TNF and IL-1B, which are also upregulated in MS (20). It is proposed that the NF-kB pathway drives GM-CSF

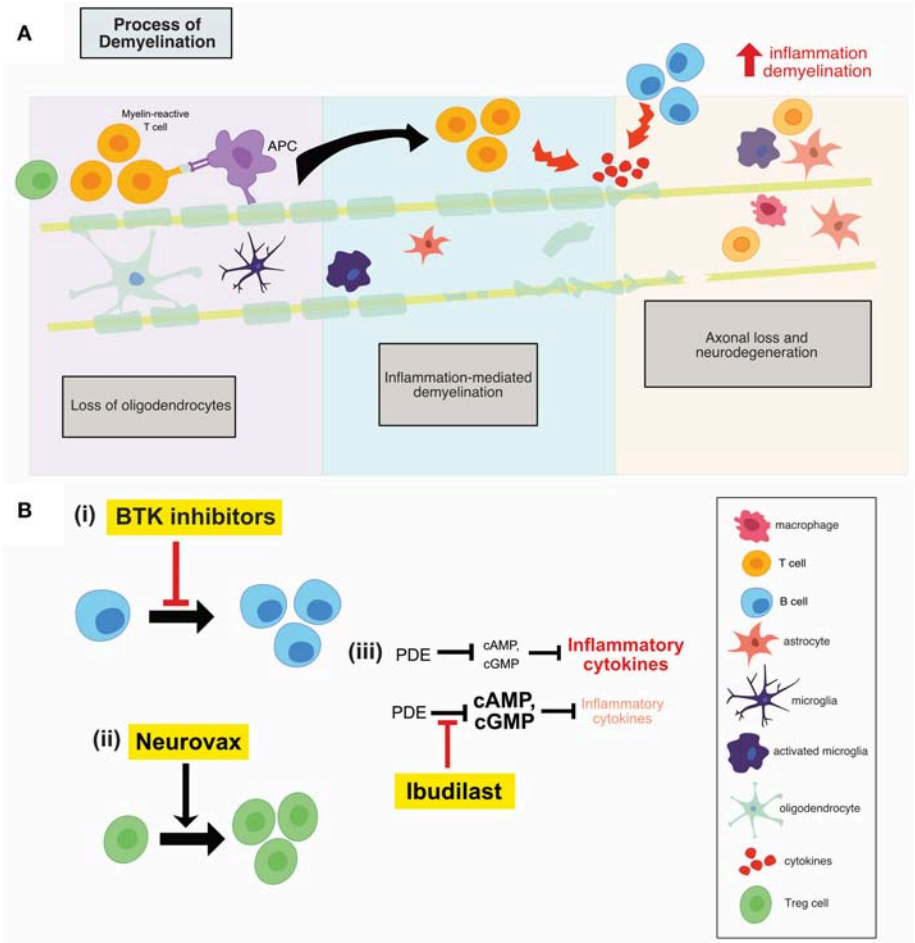

FIGURE 2 | (A) Illustration of the process of demyelination in MS which involves the recruitment of adaptive immune cells, the activation of innate CNS immune cells, inflammation mediated demyelination and subsequent neuronal loss. **(B)** An overview of the mechanisms of action of anti-inflammatory therapies. Btk inhibitors (i) reduces B cell proliferation, while neurovax (ii), a trivalent T cell receptor vaccine, stimulates Treg cell activation. Ibudilast (iii) has global anti-inflammatory effects and results in a decrease in the level of circulating inflammatory cytokines. PDE, phosphodiesterase; Btk, Bruton's tyrosine kinase; Treg, regulatory T cells; OPC, oligodendrocyte progenitor cell.

production and secretion by T cells. GM-CSF is crucial for the development of inflammatory demyelinating lesions and for controlling migration and proliferation of leukocytes within the CNS (21). Moreover, GM-CSF may increase production of IL-1B, IL-6, TNF, and nitric oxide (NO) by further upregulating NF-kB (20). Therefore, another mechanism through which Btk inhibitors may exert their beneficial effects is through the inhibition of NF-kB and its downstream signaling pathways.

Mice expressing loss-of-function mutations in Btk (a model of human x-linked immunodeficiency) showed slower induction of EAE and milder disease development than wildtype (WT) mice (22). There was no weight loss reported in these mice after EAE induction, compared with ∼13% loss in WT mice. Polymorphonuclear neutrophil granulocytes produced far less NO upon stimulation with LPS compared to LPS-treated WT cells (22). These findings echoed observations from another study in which MOG-induced EAE was suppressed in mice with loss-of-function mutations in Btk (23). Moreover, plaque formation and demyelination were significantly lower compared to WT controls. However, it is important to note that these studies include findings in mice that lacked functional Btk and as such have developmental defects. Therefore, these studies are not highly applicable to humans, since even with Btk inhibitors, some Btk activity remains; EAE studies where Btk remains expressed but is inhibited therapeutically should provide more salience as it pertains to human disease. Several pharmaceutical companies are currently pursuing novel Btk inhibitors with varying levels of brain penetrance, below, we detail the two most advanced compounds, currently in clinical development.

PRN2246

PRN2246 is a potent and selective oral Btk inhibitor designed to penetrate the blood brain barrier developed by Principa Biopharma in collaboration with Sanofi. PRN2246 blocks B

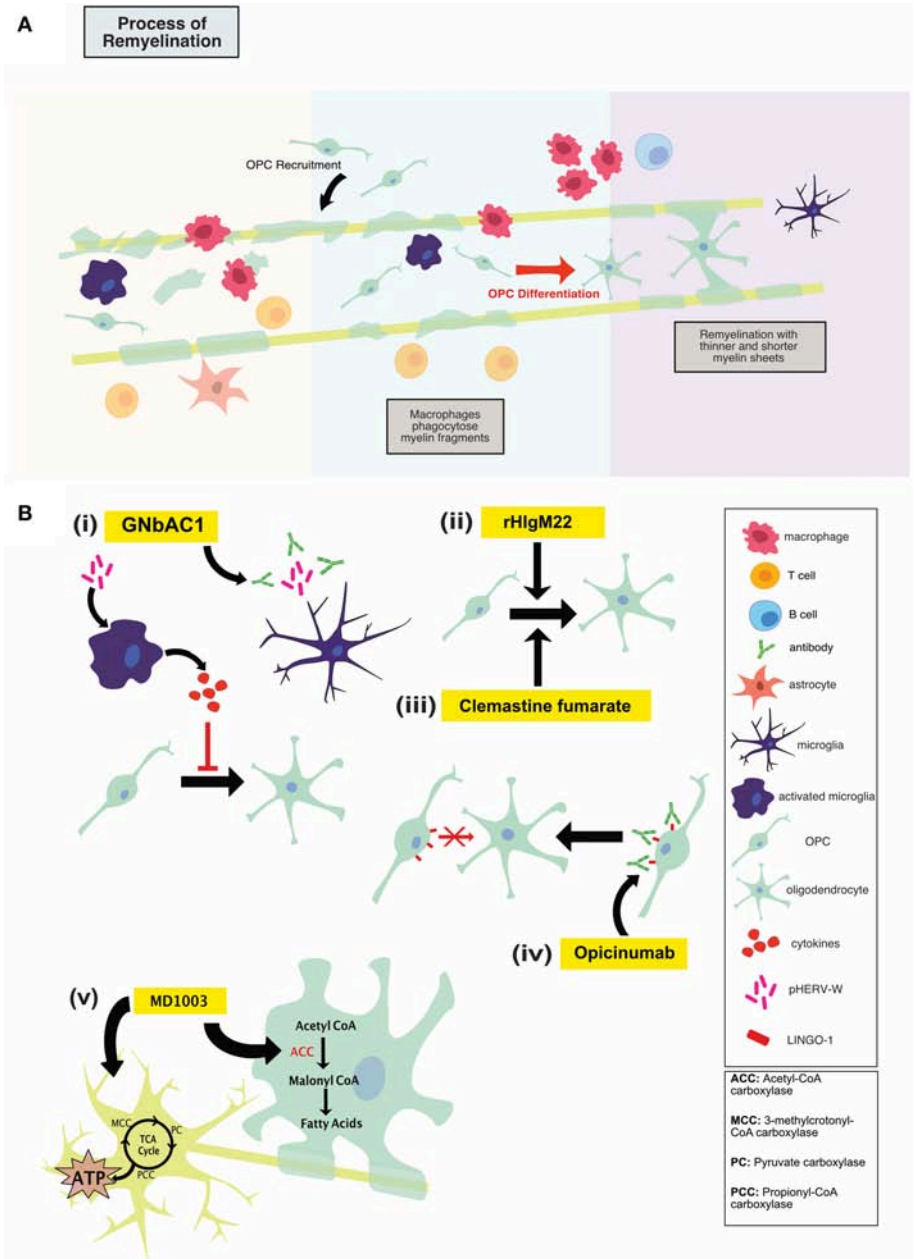

FIGURE 3 | (A) Illustration of the process of remyelination in MS which involves the phagocytic clearance of myelin debris by myeloid cells, recruitment of OPCs to the lesion, OPC differentiation and remyelination. **(B)** An overview of mechanisms of action of repair-promoting novel therapeutics. GNbAC1 (i) is a monoclonal antibody, targeting HERV-W, therefore rescuing myelin synthesis. rHIgM22 (ii) is a recombinant human IgM antibody that binds live oligodendrocytes and promotes remyelination by inducing OPC differentiation and proliferation. Clemastine fumarate (iii), a muscarinic receptor antagonist, induces OPC differentiation, and therefore increases remyelination. Opicinumab (iv) is a monoclonal antibody against LINGO-1 (negative regulator of remyelination), and results in enhanced OPC differentiation and remyelination. MD1003 (v), an oral formation of biotin at a high dose, may activate Krebs cycle to increase energy production in demyelinated axons and enhance myelin production in mature oligodendrocytes. Treg, regulatory T cells; OPC, oligodendrocyte progenitor cell.

cell receptor-mediated activation and Fc receptor activation in immune cells and demonstrated dose-dependent protection from disease induction in EAE. Phase I clinical trial evaluating the safety and tolerability of PRN2246 found that the drug, at doses between 7.5 and 120 mg daily, had no serious medication-related adverse events in eighty healthy volunteers (Australian New Zealand Clinical Trials Registry, 2018). PRN2246 was detected in the spinal fluid of the participants, demonstrating its ability to cross the blood-brain barrier. PRN2246 is currently undergoing a phase II clinical trial.

Evobrutinib (M2951)

Evobrutinib is a covalent, oral Btk-specific inhibitor (Merck, Germany). In March 2018, promising results from a phase IIb clinical trial were announced, in which it was demonstrated that evobrutinib treatment resulted in a reduction in gadolinium enhancing T1 lesions [indicative of blood brain barrier disruption (BBB)] measured at weeks 12, 16, 20, and 24 in RRMS patients compared to patients receiving placebo (24).

NeuroVax

One of the main underlying mechanisms contributing to the inflammatory phase of MS is the emergence of pathogenic T cell populations, permitted as a result of reduced immune regulation. This regulation is typically mediated by IL-10-secreting T regulatory 1 cells and natural $CD4^+$ $CD25^+$ regulatory T cells (Treg) (25). Therefore, development of immune-based vaccines that can restore regulatory mechanisms remains a therapeutic goal. NeuroVax is a trivalent T cell receptor (TCR) peptide vaccine. It is a combination of three TCR peptides (BV5S2, BV6S5, and BV13S1), which appear on the surface of specific myelin-reactive T cells involved in MS pathology (25). This means that the vaccine will elicit an immunological response targeting the harmful, self-reactive T cells. NeuroVax is thought to stimulate FoxP3+ Tregs, as shown in **Figure 2B**(ii), which have a regulatory function and suppress activity of deleterious autoreactive T cells (25).

In 1989, two independent studies found that vaccination with TCR peptides effectively induced resistance to EAE in rodents (26, 27). Vaccinating Lewis rats with synthetic peptides corresponding to EAE-associated TCR idiotopes resulted in significant resistance to EAE, as none of the animals had clinical or histological signs of the disease, compared to non-vaccinated Lewis rats (26, 27). However, the animals were vaccinated before induction and clinical onset of EAE. This has minimal clinical relevance, since intervention in the human disease starts after disease onset. Importantly, vaccination with TCR peptides in Lewis rats with established EAE reduced clinical severity in these animals from grade 3.2 to grade 1.5 within 48 h and to grade 0.2 within 72 h, compared to non-vaccinated Lewis rats with established EAE (28). Disease duration was also lower in vaccinated rats. These data suggest potential efficacy of vaccination with TCR peptides as a treatment for MS.

Vaccination with TCR peptides, namely NeuroVax, induces a transient increase in a population of IL-10-secreting, TCR-reactive T cells and an increase in FoxP3 expression which is the hallmark of regulatory CD4+ T cell phenotype, as shown in **Figure 2B**(ii). This was shown in a single-arm study evaluating the effect of 15 injections of NeuroVax over 54 weeks in 27 MS patients (14 RRMS, 10 SPMS, and 3 PPMS) and their age- and sex-matched healthy controls (25). Following three monthly injections, PBMC analysis showed a significant increase in TCR-reactive T cells that produced high levels of IL-10, as well as FoxP3 expression in CD4+ CD25+ T cells returning to healthy control levels, suggesting the drug's capability of boosting specifically reactive T cells that mediate immunosuppression (25) Prior to vaccination, there was a low frequency of proliferating TCR-reactive T cells, which increased significantly after 12 weeks of therapy, and then gradually decreased by the end of the trial. As well, the frequency of IL-10-secreting TCR-reactive cells increased to its maximum after 15 weeks of therapy, and then decreased to starting levels by the end of the 54 weeks (25). It was established that all subjects receiving the tripeptides in incomplete Freund's adjuvant (IFA) had a significant increase in frequency of PBMC T cells reactive to the TCR vaccine, as measured by the proliferation in the limiting dilution assay (25).

NeuroVax is currently undergoing a much larger Phase II clinical trial (NCT02149706), with 150 participants treated with either NeuroVax, consisting of a trivalent TCR peptide formulation in IFA, or IFA alone, via an intramuscular (deltoid) injection once every 4 weeks for 48 weeks. The primary outcome measure is expanded disability status scale (EDSS) in 48 weeks between the two treatment groups. A secondary measurement will evaluate white blood cell count and FoxP3 expression. The trial is randomized, double-blind, and placebo-controlled for subjects with SPMS, results of which are expected to be announced in summer 2019.

Ibudilast

Ibudilast was first utilized as a treatment for articular rheumatism due to its anti-inflammatory effects (29). Since then, it has been used to treat patients with cerebrovascular disorders based on its inhibitory actions on platelet aggregation. Recent studies have suggested that ibudilast may have neuroprotective properties mediated via an anti-inflammatory mechanism of action and therefore hold potential as a treatment for MS.

Ibudilast is an inhibitor of cyclic nucleotide phosphodiesterases (PDEs) and can readily cross the blood-brain barrier (30). Ibudilast inhibited type I and II PDEs in the extracts from rat brain (30). cAMP activates anti-inflammatory cascades and inhibits T cell immune function (31); PDEs break the diphosphide bonds in cAMP, therefore inactivating the molecule and reducing the activation of anti-inflammatory cascades. As a result, inhibitors of PDEs, such as ibudilast, reduce the inhibition on cAMP, allowing for the activation of anti-inflammatory cascades (31). Treatment with ibudilast resulted in significant suppression of LPS-induced NO and TNF production, both of which are pro-inflammatory mediators produced by microglia and astrocytes (30). Ibudilast also suppressed the proliferation of microglia induced by GM-CSF, M-CSF, and TPA, but it has no effect on unstimulated microglia (30). Therefore, through reducing the inhibition on cAMP, ibudilast may result in lower inflammation and T cell activation which prove beneficial in the MS setting, shown in **Figure 2B**(iii).

In astrocytes exposed to hydrogen peroxide (H_2O_2), ibudilast reduced cytochrome-c release from the mitochondria and inhibited H_2O_2-induced increase in caspase-3 activity (32). Ibudilast increased intracellular cyclic GMP (cGMP) levels in astrocytes, when cGMP was inhibited, the protective effects of ibudilast were inhibited (32). Therefore, in addition to reducing the inhibition of cAMP, ibudilast may exert its neuroprotective effects through reducing the inhibition of cGMP.

In rats induced with EAE, the mean clinical score was significantly reduced when treated with ibudilast every day (33). Histopathological scores indicated a significant reduction of

inflammatory cellular infiltration in the lumbar spinal cord from ibudilast-treated animals compared to that from control animals. However, when ibudilast was given after the onset of the first clinical sign of EAE, no inhibitory effect was observed (33).

The effects of ibudilast on inflammatory cytokines and Th1 and Th2 cell activation was evaluated in 9 women and 1 man, all of whom had RRMS, in a cross-over study (34). After treatment with ibudilast, there was a significant reduction in the mRNA levels of Th1 cytokines IFN-y and TNF in CD4+ cells. Natural killer T cells (NKT) cell subset significantly increased following treatment, but no change was observed in the placebo and the healthy control groups (34). Indeed, NKT cells in MS are Th2-biased in remission and are markedly reduced in the peripheral blood of the patients during relapse. Therefore, this ibudilast-mediated increase in the NKT subset may underlie ibudilast's clinical effect.

Ibudilast recently underwent a randomized, placebo-controlled phase II trial in progressive MS (35). In this 96-week trial, 108 patients were randomly assigned to receive 100 mg of ibudilast orally, while 112 patients received placebo. Patients on ibudilast had ∼2.5 ml less brain-tissue loss as compared to patients receiving placebo. In 2010, ibudilast underwent a multicenter, double-blind, phase II trial, evaluating the efficacy of the drug in 292 patients with RRMS, rather than progressive MS (36). The primary MRI variable of cumulative active lesions over 12 months of treatment did not differ between treatment arms (placebo, 30 mg/d, 60 mg/d). There was no change in the cumulative number of newly active lesions over 24 months between the treatment arms. Percent brain volume change was lower in the treatment groups compared to placebo over 12 months; this effect remained over the 2 years. There was a reduction in the proportion of lesions that evolved into persistent black holes (signifying tissue loss) for both ibudilast groups vs. placebo. In terms of clinical outcomes, ∼50% of both ibudilast groups remained relapse-free during the first 12 months, compared to placebo treated. Time to first relapse increased in both treatment groups. Over 2 years, there was less confirmed EDSS progression in those on active treatment throughout the study vs. those initially receiving placebo. Treatment with ibudilast at both concentrations was safe and well-tolerated. Therefore, this study showed that, as evidenced through lower brain volume change and lower progression in EDSS, ibudilast may exert neuroprotective effects.

NOVEL THERAPEUTIC TARGETS: REMYELINATION/REPAIR-PROMOTING

GNbAC1

Human endogenous retroviruses (HERVs) were integrated into the human genome 30–40 million years ago and are transmitted genetically. Although most HERVs have been rendered inactive through mutations and deletion signals, some are capable of being reactivated to produce viral products (37). Inflammatory stimuli may activate the expression of human endogenous retroviral elements (HERVs) via epigenetic dysregulation (38). For instance, transcription of HERV-W envelope proteins (formerly known as MSRV-env) has been shown to be upregulated in brain lesions of MS patients (39) and there is compelling evidence for an association between HERV expression and MS (40). Indeed, HERV-W env expression can be detected in serum, PBMCs and cerebrospinal fluid (CSF) from MS patients, but not healthy controls (38). Further, HERV-W expression has been shown in astrocytes, microglia and macrophages of MS patients, but not controls (41). It is also associated with areas of active demyelination.

HERV-W env protein was found to mediate TLR4-dependent induction of proinflammatory cytokine expression in human and murine monocytes (42, 43). Treatment of cultured primary oligodendroglial precursor cells (OPCs) with HERV-W env was shown to result in an overall reduction in OPC differentiation (43). OPCs contribute to the myelin repair process in the adult CNS; blocking their differentiation by HERV-W env protein may therefore disrupt this remyelination. In fact, in MS patients, 88% of HERV+ patients had EDSS score higher than 5 compared to 12% of HERV- patients (44). The HERV-W env mediated inhibition of OPC-directed myelin synthesis could be rescued using a specific HERV-W env-neutralizing humanized immunoglobulin termed GNbAC1, as shown in **Figure 3B**(i). GNbAC1 is a recombinant humanized monoclonal antibody of the IgG4/kappa class, and it is the only mAb targeting the envelope (env) protein of an active element from HERV-W (45). GNbAC1 can inhibit binding of HERV-W to TLR4, and its downstream activation of inflammation. In fact, treatment of human PBMCs with HERV-W env resulted in a potent and concentration-dependent release of the pro-inflammatory cytokines IL-6 and TNF, an effect that is antagonized by GNbAC1 (46). Treatment with GNbAC1 also antagonized the reduction in CNPase and MBP expression, two mature myelination markers, in human OPCs.

In a phase I, randomized, double-blind, placebo-controlled, dose-escalating clinical trial, GNbAC1 was administered to 33 MS patients (47). The drug was well-tolerated in the patients, and all adverse events were mild or moderate with the adverse events likely to not be drug-related. However, one of the limitations of this study was that the subjects in the study were all Caucasian males. Therefore, the safety of the drug in female patients cannot be commented upon confidently.

In a phase IIa single-blind, placebo-controlled, randomized clinical study, the safety of escalating doses of GNbAC1 was assessed in 10 patients (7 males and 3 female) with RRMS, SPMS or PPMS (48). There was no particular pattern of adverse events that could be attributed to the treatment and there was no difference in the incidence of adverse events between the two dose groups, GNbAC1 was deemed safe for MS patients (48).

rHIgM22

One approach to promoting remyelination in MS has been immunoglobulin (Ig) therapy, using naturally occurring autoantibodies encoded with germline sequences in the variable region (49). In humans, natural auto-antibodies are typically IgM or IgA (50). Whereas, some natural antibodies bind to neoantigens, others bind to self-proteins and lipids upon structural changes induced by oxidative damage, enzymatic

alterations or covalent interactions with altered lipids (49). One type of these natural auto-antibodies, recombinant human IgM22 (rHIgM22) has been shown to bind to oligodendrocytes and actively induce remyelination, as shown in **Figure 3B**(ii) (51).

In mice treated with cuprizone (a copper chelating reagent which causes oligodendrocyte cell death) the total number of cells of the oligodendrocyte lineage remained the same between controls and those treated with rHIgM22. However, expression of mature oligodendrocyte marker was higher in rHIgM22-treated cuprizone mice and the percentage of OPCs was lower in these mice, compared to untreated cuprizone mice (52). This suggests that the observed effect of rHIgM22 on mature oligodendrocytes is due to enhanced differentiation and not proliferation of OPCs, as shown in **Figure 3B**(ii). However, *in vitro*, rHIgM22 resulted in an increase in co-localization between oligodendrocyte markers and a proliferation marker in mixed cultures (53). Olig-1 and Olig-2 expression levels were higher in rHIgM22 treated groups, suggesting increased proliferation as a result of rHIgM22. The conflicting findings from these two studies may be due to the different systems used to study the effect of the drug (*in vivo* vs. *in vitro*). rHIgM22 treatment may also result in lower apoptosis by reducing the levels of cleaved caspase 3 and 9, but only in the presence of FGF-2 and PDGF (53). Indeed, treatment of rHIgM22 resulted in lower expression and activity of caspase 3 in spinal cord of TMEV mice (54). Therefore, rHIgM22 treatment may be protective by inducing proliferation and differentiation of OPCs and reducing apoptotic cell death.

rHIgM22, which binds specifically to oligodendrocytes, has been shown to promote remyelination in several animal models of MS (55). Four weeks after treatment of mice with serum HIgM22, virtually all MBP-positive human oligodendrocytes also bound serum HIgM22, demonstrating this antibody's specificity for live oligodendrocytes (51). In the TMEV model of MS, N-acetyl aspartate (found at high concentration throughout the brain) levels and the number of axons and neurons in the brainstem were significantly higher in the group treated with rHIgM22 compared to IgM-treated controls (56). Treatment of rHIgM22 in TMEV mice resulted in a 46% reduction in the lesion load as compared to a 13% increase in the placebo treated group, 5 weeks after treatment (57). Further, in the cuprizone model, the restoration rate of MBP and FluoroMyelin (mature myelin marker) was accelerated in the body of the corpus callosum and the splenium when the animals were treated with rHIgM22, compared to animals treated with cuprizone only (52).

A phase I, multicenter, double blind, randomized, placebo-controlled, dose escalation study was completed in 2017 evaluating the safety and tolerability of single intravenous administrations of rHIgM22 in patients with clinically stable MS (58). Seventy-two patients were enrolled in five dose levels, where the maximum dose was ∼15 times the maximally effective dose observed in the TMEV mice. In general, the infusions were well-tolerated among the 55 individuals from the treated group and there were no discontinuations reported. The half-life of the drug increased from 39 to 100 h as the dose increased. Further, rHIgM22 was measurable in the CSF of patients. There were no statistically significant changes in the various clinical or potential pharmacodynamic assessments tested; however, there was a trend toward improvement in patient global impression of change. There was also no change in the CSF analytes of interest, such as MBP, myelin proteolipid protein, and MOG, among others (58). There is currently another phase I clinical trial underway, examining the effect of rHIgM22 on patients with MS immediately following a relapse (clinical trial ID: NCT02398461).

Muscarinic Receptor Antagonists (Benzatropine, Clemastine Fumarate)

As MS disease course enters a more progressive phase, a failure to remyelinate leads to increased exposure of denuded axons to a chronic inflammatory milieu. Leading to continued neurodegeneration and disability accumulation. Chondroitin sulfate proteoglycan 4+ (NG2), platelet-derived growth factor receptor alpha+ (PDGFR-α) positive OPCs are thought to be the cells responsible for remyelination in the adult brain. These cells migrate to the site of injury and differentiate into mature myelin-forming oligodendrocytes that myelinate denuded axons, providing protection, metabolic support and improving saltatory conduction. While OPCs have the ability to migrate and populate chronic demyelinated MS lesions, these cells appear to experience a differentiation block. Muscarinic receptor antagonists, namely benzatropine and clemastine fumarate, have been shown to induce OPC differentiation, as shown in **Figure 3B**(iii), and therefore may have therapeutic potential for MS patients.

Benzatropine has been shown to promote OPC differentiation into MBP+ mature oligodendrocytes in a high-content imaging assay (59). Benzatropine is an FDA approved, orally available, anti-cholinergic compound with anti-histamine and dopamine re-uptake inhibition properties. It is believed that benzatropine's ability to induce OPC differentiation is due to antagonism of M1/M3 muscarinic receptors. Benzatropine-induced OPC differentiation was inhibited by the actions of carbachol (a muscarinic receptor agonist). Benzatropine was found to be the most potent of a panel of structurally-diverse muscarinic receptor antagonists with respect to their ability to promote OPC differentiation (59).

In EAE, both prophylactically and therapeutically administered benzatropine decreased the severity of the acute phase of disease and prevented further clinical relapses compared to vehicle. Benzatropine did not seem to affect levels of immune cell infiltration. Areas of immune cell infiltration in benzatropine-treated animals stained positive for myelin, did not show signs of demyelination, and exhibited an increase in mature oligodendrocyte number. Benzatropine induced extensive remyelination compared to vehicle in electron microscopy analysis. Benzatropine's ability to promote remyelination was further evaluated in the cuprizone model of demyelination. Overall, myelin staining (Luxol fast blue staining) and number of mature oligodendrocytes increased significantly in the corpus callosum following benzatropine treatment compared to vehicle-treated animals (59).

Clemastine fumarate, an over the counter anti-histamine and anti-cholinergic molecule, was identified in a novel, high-throughput screening platform to assess OPC differentiation into myelinating oligodendrocytes (60). This screen identified

a cluster of 8 FDA-approved antimuscarinic compounds that reduced OPC proliferation while significantly increasing oligodendrocyte differentiation. While benzatropine was also identified in this screen (and clemastine fumarate identified in the previous screen), clemastine fumarate was studied in greater detail due to its ability to cross the BBB and its favorable safety profile. Clemastine's ability to promote remyelination in an *in vivo* setting was examined in the lysolecithin model of demyelination/remyelination. This toxin model produces a well-characterized demyelination event marked by myeloid cell recruitment and activation, astrogliosis, axonal injury, and OPC proliferation and migration. The group reported increased oligodendrocyte differentiation 2 weeks post-lesion. Electron microscopy analysis also revealed faster remyelination kinetics, decreased g-ratios and a 20% reduction in the number of axons left unmyelinated (60).

To better understand the mechanisms behind the beneficial effect of remyelination-promoting anti-cholinergics, this same group set out to uncouple the immunological response (which can also promote remyelination) in EAE from oligodendrocyte-mediated remyelination (61). Prophylactic administration of clemastine in MOG-induced EAE significantly decreased the clinical severity at peak of disease and into the chronic phase. Clemastine treatment caused a significant preservation of both myelin staining and axonal integrity at the later stage of EAE. Clemastine treatment did not alter T-cell/macrophage infiltration or myeloid cell activation, suggesting that the compound's ability to reduce EAE clinical score was not due to an effect on innate or adaptive inflammation. However, an immuno-modulatory effect of the compound beyond affecting cellular migration cannot be ruled out. Muscarinic receptor knockout experiments suggest that clemastine is mediating its pro-differentiation effects through its action on Chrm1. While this data does not preclude the possibility that Chrm1 deletion may modulate an unknown inflammatory role of OPCs, the most likely explanation is that Chrm1 antagonism enhances remyelination, thereby preserving axonal integrity/neuronal function by providing physical and metabolic support (61).

The strong pre-clinical data on clemastine led to the design of a small, single-center, placebo-controlled, crossover study on 50 RRMS patients with chronic demyelinating optic neuropathy (62). Results from this Phase II trial (ReBuild; NCT02040298) were first published in December 2017. Evoked potentials record cortical responses to a repetitive stimulus and can measure the speed of conduction in the CNS, with myelinated axons conducting electrical signals faster than unmyelinated axons. Visual-evoked potentials record cortical responses to a visual stimulus. Most MS patients display demyelination in the visual pathway and thus also a prolongation of visual-evoked potential latency. The primary outcome of the trial (shortening of this latency delay) was met. While reduced latency is only suggestive and does not prove remyelination, these findings are nonetheless very encouraging (62).

Innovative screening techniques have identified anti-cholinergic compounds, of which clemastine fumarate holds the most promise as a class of drug with the potential to enhance remyelination in the chronic demyelinated brain. While the evidence for muscarinic receptors as novel therapeutic targets for the promotion of remyelination is strong, questions do remain. These include concerns around potential side effects of high-dose clemastine, potential for off-target effects, and ultimately its ability to lead to clinical improvement through remyelination in MS patients. These novel molecular targets provide an attractive option given the availability and favorable safety profile of the compounds coupled with the complete lack of efficacious, non-immunomodulatory, myelination-promoting therapies. While optimism is warranted, it is worth noting the observed failure to mobilize OPCs within a hostile cellular milieu (63–65). The ultimate remyelination strategy will likely need to include an anti-inflammatory component to help establish a permissive extracellular environment for OPC mobilization and differentiation. A therapeutic approach that encompasses a concurrent or sequential treatment with both an inflammation targeting molecule and a remyelination promoting therapy would likely hold the most potential for success.

Anti-LINGO-1

Leucine rich repeat and immunoglobin-like domain-containing protein 1, or LINGO-1, is expressed primarily in the CNS in both oligodendrocyte lineage cells and neurons. Its expression is increased in disease and injurious conditions. LINGO-1 is a negative regulator of the remyelination process through its ability to block differentiation of OPCs into mature myelinating oligodendrocytes, as shown in **Figure 3B**(iv) (66). This effect seems to be mediated through the RhoA signaling pathway. Antagonizing LINGO-1 as a therapeutic option to boost OPC differentiation has been investigated in several animal models of demyelination. In the cuprizone and LPC toxin models of demyelination treatment with anti-LINGO-1 antibody resulted in a significant increase in myelinated axons as shown by immunohistochemistry and electron microscopy (67, 68). In EAE, LINGO-1 KO mice displayed significantly lower EAE scores as a metric of reduced disease progression and motor abnormalities. These LINGO-1 KO mice also displayed an increased resistance to the development of the disease. In addition, systemically administered anti-LINGO-1 antibody had a positive dose dependant effect on remyelination in a rat model of MOG-EAE (69). In combination these preclinical studies strongly suggest that antagonism of LINGO-1 may hold therapeutic promise in the effort to promote remyelination and repair in the MS brain.

Opicinumab (Biogen Idec; BIIB033) is a first in class, fully human, monoclonal antibody against LINGO-1, as shown in **Figure 3B**(iv). Efficacy and safety of opicinumab has been examined in two major clinical trials thus far, with a third trial currently underway. Optic neuritis (ON) is inflammatory mediated demyelination in the optic nerve and is often the first clinical manifestation of MS. The primary pathological hallmark of ON is a prolonged visual evoked potential (VEP) latency, caused by demyelination of the optic nerve (70). Opicinumab's ability to enhance remyelination was tested in a randomized, double blind, placebo-controlled,

multicenter trial for patients with ON (71). Results from this "RENEW" trial (NCT01721161) showed a trend for latency improvement as compared with placebo. A subgroup analysis did show a significant difference at the later timepoint of 32 in the per protocol treatment group compared with placebo. While there are concerns around the study not being powered to show statistical significance, these results were promising.

The potential efficacy of opicinumab to enhance CNS repair through remyelination and neuronal protection in RRMS and SPMS was assessed in a large randomized, double-blind, study. This "SYNERGY" trial recruited patients already receiving Avonex (interferon beta-1a) and used opiciniumab as an add-on therapy. The primary endpoints of improvement of disability and slowdown of disability progression were not met. This trial did however identify a subgroup of patients that displayed indications of a positive clinical effect. The characteristics that defined this subgroup were a disease duration of >20 years, lower magnetization transfer ratio and diffusion tensor imaging values of the lesions on MRI, indicating higher structural integrity. Based on these findings a new, larger trial is underway launched which focuses on MS patients that fulfill the above criteria (NCT03222973).

MD1003

MD1003 is an oral formulation of biotin at a high dose (300 mg) of 10,000 times the recommended daily intake (72). Biotin is a member of the B vitamin complex, a water-soluble vitamin that acts as a cofactor for decarboxylase enzymes and can cross the blood brain barrier. High doses of biotin have been a therapeutic option in biotin-responsive basal ganglia disease, an orphan neuro-metabolic disease (73). As well, 5 patients suffering from optic neuropathies and leukoencephalopathy responded clinically to high doses of biotin. Interestingly, one of these 5 patients suffered from SPMS (74). Therefore, these findings warranted a trial of high doses of biotin in patients with progressive MS.

Biotin aids in carbon dioxide transfer to various macromolecules and is associated with several specific enzymes in humans, including pyruvate carboxylase (PC) for liver gluconeogenesis and acetyl-CoA carboxylase for lipid synthesis (72). With respect to MS, MD1003 aims to activate the Krebs cycle to increase energy production in demyelinated axons and enhance myelin synthesis in oligodendrocytes. Biotin is bioavailable, rapidly absorbed and excreted in urine (72). As a cofactor for four essential carboxylases, MD1003 may activate carboxylases to support myelin repair through enhanced synthesis of fatty acids and protect against hypoxia-driven axonal degeneration by enhancing energy production in neurons (75). Indeed, MD1003 is actively transported across the human blood brain barrier via the sodium-dependent multivitamin transporter. Hypothesized mechanisms of action fall under two main categories: promotion of remyelination through enhanced fatty acid synthesis in oligodendrocytes and enhancement of energy production in the CNS, thereby protecting demyelinated axons from cell death, as shown in **Figure 3B**(v) (75).

In Oligodendrocytes

Biotin (MD1003) is a cofactor for ACC1 and ACC2, enzymes involved in fatty acid synthesis. ACC1 catalyzes the rate-limiting step in fatty acid biosynthesis and is primarily expressed in oligodendrocytes. A significant proportion of cytosolic ACC is detectable in purified myelin (75).

Choline, the core component of hydrophilic heads of phospholipids, is commonly elevated in MS plaques and thought to result from the breakdown of the membrane that occurs during characteristic pathogenesis involving inflammation, gliosis and demyelination (75). The choline to creatine ratio was normalized over time with MD1003, a process that is thought to reflect myelin repair accompanied by a decrease in free choline release from membranes. Remodeling of the myelin sheath is thought to occur relatively slowly, which is consistent with the delay in symptom alleviation seen in MS patients treated with MD1003 (75).

In the Reversal of Virtual Hypoxia

High-dose biotin may also be targeting cellular energy levels, depletion of which arises from demyelination and is responsible for the progressive and irreversible neuronal degradation in progressive MS. The loss of saltatory conduction increases the energy required for nerve propagation. This shift in propagation mechanisms is accompanied by a need for an increase in mitochondria in the newly demyelinated axons, which is met with a pathological impasse: in MS patients, axonal ATP production is compromised due to neuronal mitochondrial defects (75). This discrepancy between energy requirement and output creates a state of virtual hypoxia, which may act as the trigger for neuron degeneration (75). It is thought that this hypoxic state can be reversed through high-dose biotin in its role as a cofactor for PC, MCC and PCC, enzymes central to aerobic energy production, all of which are known to be expressed in both astrocytes and neurons (75). These biotin-dependent carboxylases feed into the tricarboxylic acid cycle at different entry points; by increasing the available pool of ATP, biotin may act to reduce neural dysfunction and various hypoxic effects, as shown in **Figure 3**(v) (75).

MD1003 underwent a 12-month randomized, double-blind, placebo-controlled trial followed by an open-label 12-month extension phase with all patients receiving the drug (76). The study included 154 patients with PPMS and SPMS, who received MD1003 (100 mg) or placebo, thrice daily. Thirteen patients out of 103 treated with MD1003 had a reduction in MS-related disability, compared with no patients in the placebo arm. 10/13 patients had improved EDSS score and 5/13 had improved "time to walk 25 feet" times, and 2/13 improved on both scores. As well, 12 of the patients treated with MD1003 had reduced MS-related disability at 18 months and 24 months. 3/42 patients that switched over to MD1003 from placebo also experienced reduced disability at 18 and 24 months. The proportion of patients with EDSS progression at month 9 and 12 was higher in the placebo arm compared to the MD1003 arm. At 12 months, the mean EDSS decreased from baseline in the MD1003 arm but increased at the expected rate in the placebo arm. However, EDSS progression stopped after placebo patients were switched

to MD1003. The adverse events that occurred during the study were likely not related to MD1003 treatment. At 12 months, MRI examination identified more new MS-specific lesions in MD1003-treated patients compared to placebo-treated patients, although the difference was not significant (76).

MD1003 is currently in phase 3 clinical trial (NCT02936037), which is a randomized, placebo-controlled, blinded study testing at least 15 months of MD1003 treatment. Hundred milligram biotin capsules will be given to patients thrice daily, and the study is expected to be completed by September 2019. MD1003 is also being tested in separate phase III clinical trial (NCT02220244) to determine potential effects in chronic visual loss related to optic neuritis in MS. Placebo controlled, this study aims to measure the change from baseline of best corrected visual acuity at 100% contrast between treatment groups.

Edification of Current Therapies

Fingolimod, which binds to the sphingosine-1-phosphate-1 ($S1P_1R$) receptor and results in its functional antagonism, was the first approved oral disease-modifying therapy in 2010 (77, 78). Although fingolimod's intended action is through binding of the $S1P_1$ receptor, its non-selective modulation of $S1P_3$, $S1P_4$ and $S1P_5$ has led to unwanted adverse effects, resulting in the development of six specific $S1P_1$ receptor modulators (77). Mayzent (siponimod) is one such drug with results from its double-blind, randomized phase III SPMS study showing that this once daily oral therapy met its primary endpoint of reduced risk of 3-month confirmed disability progression and reduced 6-month CDP over placebo. By MRI, siponimod slowed the rate of brain volume loss, as well as that of T2 lesion volume over 12 and 24 months (79). Based on these results Mayzant has recently received FDA approval as the first oral therapy to treat active SPMS.

Targeted depletion of CD20+ B cells has also been an effective treatment in MS and several different anti-CD20 monoclonal antibodies have been developed for MS, including rituximab, ocrelizumab, and ofatumumab (80, 81). Indeed, the only FDA-approved treatment for PPMS is ocrelizumab.

Cell-based therapeutic strategies have also been investigated in MS (82), one being immunoablation of autoreactive effector cells, followed by reconstruction of the immune system using autologous hematopoietic stem cell transplantation. A retrospective study indicated that immunoablation followed by hematopoietic stem cell transplantation achieved "no evidence of disease activity" in a greater proportion of MS patients than reported from trials of other available disease modifying therapies (82). Mesenchymal stem cell (MSC) therapy is another form of cell-based therapy, due to evidence showing that MSCs play a significant systemic role in the repair of many tissues. Some studies have reported preliminary evidence of benefit for MS patients, but larger, controlled phase II studies are needed and underway. Glial progenitor cells and induced pluripotent stem cell-induced oligodendrocytes, injected into the CNS, may also offer therapeutic potential through enhancing remyelination in MS. Studies investigating the effect of this form of cell-based therapy are currently underway (82).

DISCUSSION

Neuroimmunological diseases, such as MS, myasthenia gravis, Guillain-Barré, and NMO are all associated with classical neuroinflammation. Up until recently, preventing damage in the CNS by regulating peripheral immunity has been the central method of treatment. A current focus of the field is the development of novel methods to promote repair and remyelination. In this review, we have discussed three novel immunomodulatory therapeutics and five novel therapies that promote repair. A summary of all drugs is presented in **Table 1**.

Btk inhibitors, Neurovax, and ibudilast are three novel therapeutics currently in clinical trial, with anti-inflammatory and "damage prevention" modalities. Btk inhibitors, such as PRN2246 and evobrutinib, reduce inflammation by inhibiting activation of NLRP3 inflammasomes or NF-kB, both of which are implicated in MS pathology. Both drugs are currently undergoing clinical trials. NeuroVax, is a combination of three TCR peptides, and exerts its neuromodulatory effects by stimulating Tregs and suppressing activity of deleterious autoreactive T cells. Ibudilast exerts its anti-inflammatory and neuroprotective effects via inhibition of phosphodiesterases, thereby allowing cAMP to initiate anti-inflammatory cascades.

GNbAC1, rHIgM22, clemastine fumarate, Anti-LINGO-1 (opicinumab), and MD1003 are the five novel therapeutics currently in clinical trial, which promote repair and show a capacity to induce remyelination. GNbAC1 is a humanized IgG4 antibody that inhibits HERV-W, which is implicated in MS and can lead to OPC apoptosis and remyelination failure. By inhibiting HERV-W, GNbAC1 treatment may lead to higher rates of remyelination. rHIgM22 is a human monoclonal IgM that binds to the surface of live oligodendrocytes and promotes remyelination. Through antagonizing muscarinic receptors, clemastine fumarate induces OPC differentiation. LINGO-1 is a negative regulator of the remyelination process and treatment with opicinumab resulted in increased remyelination. Finally, MD1003 is an oral formation of high dose biotin which may activate the Krebs cycle to increase energy production in demyelinated axons and enhance remyelination.

LIMITATIONS AND FUTURE DIRECTIONS

Some clinical trials investigating the novel therapeutics discussed in this review predominantly recruited Caucasian males. Due to inherent biological differences between males and females, the findings of these studies cannot be accurately and confidently extrapolated to the female population. Further, especially in the case of phase I studies, the number of participants is limited due to the nature of the study, therefore begetting a type II error where the effect of the drug may have been missed.

The exact biological mechanisms of action underlying the drugs' respective functionalities have yet to be demonstrated. Therefore, well-designed mechanistic studies in well-validated animal models and primary human cells are needed. Finally, there is currently a push to repurpose approved, safe compounds that have been used in other indications. Such compounds include amiloride, fluoxetine, and riluzole (83).

TABLE 1 | Summary of three novel immunomodulatory therapeutics and five novel therapies that promote repair in MS.

Drug	Classification	Target/mechanism of action	Results from animal model studies	Results from clinical trials
NOVEL THERAPEUTIC TARGETS: ANTI-INFLAMMATORIES				
PRN2246/M2951	Bruton tyrosine kinase (Btk) inhibitor	Inhibition of BTK→ inhibition of inflammasome→ ↓IL-1B and IL-18 Inhibition of BTK→ ↓NF-kB→ ↓inflammation	Genetic ablation of Btk: ↓Weight loss, ↓NO production, ↓EAE, ↓clinical score	PRN2246—Phase 1 clinical trial - 7.5—120 mg daily - 80 healthy volunteers - No serious medication-related adverse events - Detected in the spinal fluid of participants M2951 phase 11b clinical trial - Reduced gadolinium enhancing T1 lesions in RRMS patients
Neurovax	Trivalent TCR peptide vaccine	Stimulates FoxP3 positive T cells (regulatory T effector cells)→ ↓autoreactive T cells	Induced resistance to EAE when treatment starts before or after clinical onset of EAE	Phase I/II clinical trial - 100% of the patients responded to NeuroVax - Disease activity was not different after vaccination
Ibuldilast	Phosphodiesterase inhibitor	Inhibits PDE→ ↑cAMP→ ↓inflammation, ↓T-cell immune activation, ↓microglia activation Inhibits PDE→ ↑cGMP→ ↓ H_2O_2-induced astrocytes	↓Clinical score in EAE mice ↓Inflammatory cellular infiltration in the spinal cord	Phase II in progressive MS - 108 patients - 100 mg of ibudilast each day - Patients on ibudilast had 48% less atrophy progression of their brain tissue Phase II in RRMS - 292 patients - 30 mg/d or 60mg/d - ↓ volume change in the brain in those treated with ibudilast - ↓proportion of lesions evaluated into persistent black holes - ↑time to first relapse - ↓increase in EDSS
NOVEL THERAPEUTIC TARGETS: REPAIR PROMOTING				
GNbAC1	Humanized neutralizing IgG4 antibody	Promotes restoration of protective myelin coating by blocking pHERV-W	↑Lifespan after MOG treatment in mice ↓Clinical score	Phase I clinical trial - 6 different doses - 33 MS patients - Drug was well tolerated - Only white males Phase IIa clinical trial - 10 patients (male and female) - RRMS, SPMS and PPMS - Safe for patients
rHIgM22	Serum form of human IgM 22	Monoclonal antibody binds→ only to oligodendrocytes ↑OPC differentiation and proliferation ↑Calcium influx into the cells	TMEV model; ↑Remyelination ↓Lesion load Cuprizone model ↑Restoration rate of MBP	Phase I clinical trial - 72 patients - Clinically stable MS - Infusions were well-tolerated - Crossed BBB
Clemastine fumarate	Antihistamine	Remyelination, immunomodulator; promotes development of oligodendrocytes	↓Clinical severity of EAE ↑Preservation of myelin	Phase II clinical trial - 50 patients with RRMS - Safe for patients - Increased feelings of fatigue
Opicinumab	Anti-LINGO-1	Enhances remyelination by inhibiting LINGO-1, a negative regulator of OPC differentiation	↑Myelinated axons in cuprizone model and MOG-EAE rats ↓EAE scores and disease progress	Phase II clinical trial - RRMS and SPMS patients - Primary endpoints not met - Subgroup (>20 y disease duration, lower MTR and DTI) showed positive clinical effect
MD1003	Highly concentrated form of biotin	Activates Krebs cycle→ ↑energy production ↑Mxyelin repair May reverse hypoxia	N/A	Clinical trial: - 154 patients with SPMS and PPMS - 100 mg pills thrice daily - ↓In MS-related disability in 13 patients - ↓EDSS progression in treated group

CONCLUSION

With the emergence of highly effective anti-B cell therapies, it has now become possible to significantly limit the formation of new lesions in RRMS patients and a subset of younger progressive MS patients. The complex relationship between neuroinflammation and neurodegeneration remains poorly understood. However, as current patient cohorts spend longer on the newer generation of immunomodulatory compounds, we will begin to understand if preventing inflammatory activity early in RRMS ultimately delays and/or prevents progression. The clinical experience with these newer compounds will also reveal potential safety concerns that may not be evident over the short or medium timespan. As it stands, it is likely that the next breakthrough in the treatment of neuroimmunological disease will be a biologic with remyelinating or repair-promoting effects. Much of the focus has been directed toward strategies to promote the differentiation of OPCs to myelinating oligodendrocytes; however, recent evidence points to the potential for surviving mature oligodendrocytes to re-wrap denuded axons (84). An observed lack of integration of new oligodendrocytes into so-called remyelinating "shadow plaques" downplays a role for OPC differentiation as the primary cellular event driving remyelination in the MS brain (85). Efforts to induce remyelination from surviving but quiescent oligodendrocytes may be achieved in a cell autonomous manner or in a cell non-autonomous manner by altering the inflammatory microenvironment of a lesion to permit such remyelination. Combatting the multifactorial pathophysiology of progressive MS remains a significant challenge in the field. Success in this area will likely require a holistic therapeutic approach with a compound that incorporates both anti-inflammatory and remyelinating functions.

AUTHOR CONTRIBUTIONS

LH conceived the idea and supervised the process. MK, NF, NK, and LH wrote the manuscript. NF generated the figures. All authors revised and proof-read the manuscript.

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REVIEW
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Targeting Phosphodiesterases—Towards a Tailor-Made Approach in Multiple Sclerosis Treatment

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Multiple sclerosis (MS) is a chronic demyelinating disease of the central nervous system (CNS) characterized by heterogeneous clinical symptoms including gradual muscle weakness, fatigue, and cognitive impairment. The disease course of MS can be classified into a relapsing-remitting (RR) phase defined by periods of neurological disabilities, and a progressive phase where neurological decline is persistent. Pathologically, MS is defined by a destructive immunological and neuro-degenerative interplay. Current treatments largely target the inflammatory processes and slow disease progression at best. Therefore, there is an urgent need to develop next-generation therapeutic strategies that target both neuroinflammatory and degenerative processes. It has been shown that elevating second messengers (cAMP and cGMP) is important for controlling inflammatory damage and inducing CNS repair. Phosphodiesterases (PDEs) have been studied extensively in a wide range of disorders as they breakdown these second messengers, rendering them crucial regulators. In this review, we provide an overview of the role of PDE inhibition in limiting pathological inflammation and stimulating regenerative processes in MS.

Keywords: multiple sclerosis, phosphodiesterase, neuroinflammation, CNS repair, remyelination

INTRODUCTION

Multiple sclerosis (MS) is a chronic immune-mediated demyelinating disorder of the central nervous system (CNS) affecting more than 2.5 million people worldwide, making it the most common neurodegenerative disease in young adults (1). Although the exact etiology remains unknown, MS is thought to develop due to an interplay between susceptibility genes and environmental factors that are yet to be fully elucidated (2). The clinical course of MS is characterized by various clinical symptoms, including gradual muscle weakness, fatigue, and cognitive impairment, which arise in either episodic periods or progress during the disease course (3). Current FDA-approved treatments modulate the prominent immune responses of MS, but are unable to halt disease progression (4). Hence, there is an urgent need for the development of new

therapeutic strategies. In recent decades, phosphodiesterase (PDE) inhibitors have shown to exhibit immunomodulatory and neuroprotective functions rendering them interesting candidates for the management of MS disease.

Clinically, MS can be divided in three distinct classifications: relapsing remitting MS (RRMS), primary progressive MS (PPMS) and secondary progressive MS (SPMS). RRMS is the most frequent subtype, affecting approximately 85% of MS patients and can be recognized by periods of remittance (5–7). This early stage of MS is characterized by the presence of active, inflammatory lesions characterized by perivenular infiltration of myelin-reactive lymphocytes and macrophages, resulting in demyelination of the axonal branches (5–7). These inflammatory relapses are followed by the activation of an endogenous repair mechanism called remyelination, resulting in a period of functional recovery (5–7). Fifty percent of RRMS patients undergo a transition to the progressive form of the disease within a period of 15 years, labeled SPMS (8, 9). Additionally, ∼15% of MS patients are classified as PPMS and endure gradual accumulation of disability from disease onset without experiencing an initial relapsing course (10). Despite a decrease in frequency of new lesion activity during these chronic stages, there is an accumulation of chronically demyelinated lesions accompanied by an increase in neurological deficits, and a gradual decline in motoric and cognitive function (9). These chronically demyelinated lesions are characterized by a reduced number of oligodendrocytes, as well as the formation of astrogliotic scar tissue and prominently demyelinated axons, subsequently leaving axons vulnerable to axonal transection (11).

The pathogenesis of MS is thought to be driven by the massive extravasation of myelin-reactive T and B lymphocytes into the CNS across the blood-brain barrier (BBB) (12). Perivenular infiltration of these auto-reactive lymphocytes disturb the homeostatic immune balance in the brain, leading to a pro-inflammatory microenvironment and subsequent CNS damage (13). Despite this, phagocytes are the principle effector cells during the neuroinflammatory and neurodegenerative processes of MS and include infiltrated monocyte-derived macrophages and brain resident microglia and macrophages (14). In MS, the disturbed homeostatic balance in the CNS skews the activation status of macrophages and microglia, subsequently fueling the neuroinflammatory response, or ceasing the inflammatory process through exerting neuroprotective functions (15). However, in the early course of MS, neuroinflammation not only induces demyelination but, it also activates remyelination. Early remyelination in active MS lesions is characterized by the expansion and mobilization of oligodendrocyte precursor cells (OPCs) (5–7, 16–19). Despite the presence of sufficient numbers of OPCs in the vicinity of pathological lesions, endogenous repair mechanisms gradually fail when disease progresses, resulting in chronically demyelinated axons embedded in gliotic scar tissue (20–24). When remyelination is not initiated, loss of myelin disrupts axonal function in addition to compromising the physical integrity of axons by increasing susceptibility to inflammatory mediators, glutamate mediated toxicity, and the disrupted trophic support provided by myelinating oligodendrocytes (25). It follows that, axonal ovoids, a hallmark of transected axons, are profoundly present in MS tissue (26).

Interestingly, cyclic nucleotide signaling pathways, such as cyclic 3′-5′ adenosinemonophosphate (cAMP) and cyclic 3′-5′ guanosinemonophosphate (cGMP) have been shown to be responsible for a variety of intracellular processes involved in both neuroinflammation and CNS repair processes (27–31). Therefore, orchestrating cellular responses by altering the intracellular balance of cyclic nucleotides can be considered an important therapeutic strategy to modulate the pathogenesis of MS (27, 32). Upon an extracellular trigger, cyclic nucleotides are formed as second messengers to amplify the incoming signal, subsequently activating protein kinases, and ion channels. Cyclic nucleotides orchestrate divergent key cellular processes such as cellular differentiation and maturation (33). cAMP and cGMP are synthesized by adenylyl cyclase (AC) and guanylyl cyclase (GC), respectively. AC converts adenosine 5′-triphosphate (ATP) into cAMP while guanosine 5′ triphosphate (GTP) is the substrate for GC to synthesize cGMP. In contrast, intracellular cyclic nucleotide levels are spatiotemporally regulated by the presence of PDEs (27). PDEs comprise a superfamily of enzymes that catalyze the hydrolysis of intracellular cyclic nucleotides. PDEs can be categorized into eleven PDE families (e.g., PDE1-11) that jointly cover 21 PDE genes (e.g., PDE4A-PDE4D) (33–35). Interestingly, each of these genes codes for different isoforms (e.g., PDE4B1-5), yielding a total of at least 77 different protein-coding isoforms. PDE gene families, genes, and isoforms can be distinguished based on their subcellular distribution, enzymatic activity, kinetic properties, and substrate specificity (36, 37). Five PDE families have a dual substrate specificity, meaning they can hydrolyze and inactivate both cAMP and cGMP (PDE 1, 2, 3, 10, and 11) (34). The remaining six PDE families specifically and exclusively hydrolyze cAMP (PDE 4, 7, and 8) or cGMP (PDE 5, 6, and 9). The cell type-specific PDE expression of the isoforms yields a specific fingerprint that provides an incentive to develop custom-made PDE-targeting strategies (35, 38, 39). Different small molecules directed against specific PDE families, genes, or isoforms have been tested in the context of neurodegeneration, neuroinflammation, and CNS repair (29–31, 40, 41). In this review, we discuss experimental studies and clinical implications regarding PDE inhibition as a strategy for inflammatory damage control and stimulation of related repair processes in MS (**Figure 1**).

INFLAMMATORY DAMAGE CONTROL IN ACTIVE MS LESIONS BY INHIBITING PDEs

Ceasing the inflammation that drives the neuroinflammatory and neurodegenerative responses in MS is considered a valuable therapeutic strategy. PDEs have been extensively studied for their anti-inflammatory properties. Several processes can be targeted to diminish the inflammatory response. PDE inhibitors are of interest due to their potential to (1) strengthen the BBB to prevent peripheral lymphocyte accumulation in the CNS, (2) restore the balance between pro-inflammatory and anti-inflammatory mediators including lymphocytes and phagocytes,

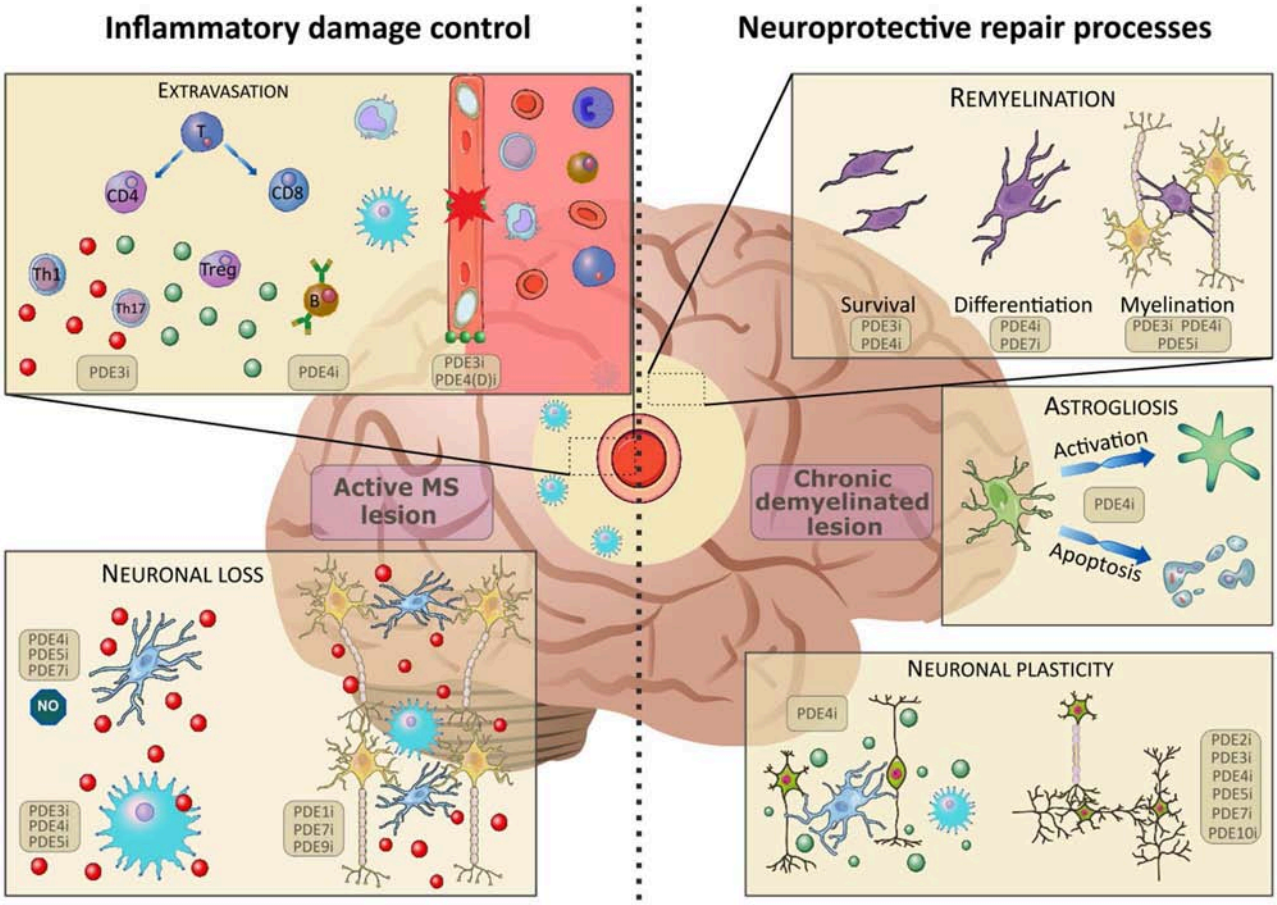

FIGURE 1 | The effects of PDE inhibition on different cell types in inflammatory damage control (active lesion) and neuroprotective repair processes (chronic demyelinated lesion). Upon disruption of the blood-brain-barrier (BBB), patrolling immune cells (monocytes, T- and B-lymphocytes) extravasate into the central nervous system. Here, the immature cells differentiate and elicit their functions in the inflammatory environment. A broad spectrum of pro- (red) and anti-(green) inflammatory cytokines are found in the active lesion. Inhibition of PDEs has been found to positively influence BBB integrity, B-cell functioning and T-cell expression patterns, skewing them toward an anti-inflammatory phenotype. The inflammatory environment causes activation of microglia and infiltrating macrophages, which contributes to excessive neuronal loss. Inhibition of PDEs counteracts this inflammatory activation and promotes neuronal survival. In chronic demyelinated lesions, the inhibition of PDEs has been found to ameliorate remyelination, thus supporting endogenous repair mechanisms. Furthermore, PDE inhibition counteracts astrogliosis by halting activation and apoptosis of astrocytes. Finally, inhibition of a multitude of PDEs has been found to promote neuronal plasticity and skew microglia and infiltrating macrophages toward an anti-inflammatory phenotype. Images were modified from Reactome icon library and Servier Medical Art, licensed under a Creative Common Attribution 3.0 Generic License (42).

and (3) prevent astrogliotic scar formation. Each of these potential aspects is further detailed below.

Blood-Brain Barrier

The BBB is comprised of smooth muscle cells, endothelial cells, pericytes, and astrocytic endfeet, functioning as a barrier to restrict the entrance of peripheral immune cells and toxic molecules into the CNS (12). In early MS development, pro-inflammatory lymphocytes activate the endothelial cells of the BBB. Endothelial activation leads to an upregulation of cell adhesion molecules that promote the massive infiltration of myelin-reactive lymphocytes into the CNS (13, 43). Endothelial cells are linked by multiprotein complexes called tight junctions, which become dysfunctional in early MS development. Therefore, restoring the loosened tight junctions, and stabilizing the BBB can prevent further infiltration of immune cells into the CNS, subsequently halting or reducing disease progression.

The involvement of cAMP in endothelial barrier functions has been extensively studied. cAMP analogs, such as dibutyryl cAMP (dbcAMP), can decrease junctional permeability and therefore diminish trans-endothelial transport of both small and large molecules (44). Nevertheless, it is compartmentalized cAMP generation rather than the general accumulation of intracellular cAMP that coordinates barrier preservation or destabilization (45). Vascular permeability is enhanced when cytosolic cAMP is increased, while barrier integrity is maintained when cAMP accumulates in cellular vacuoles (45). In contrast to cAMP, the outcome of directly increasing cGMP levels on endothelial barrier function is yet to be elucidated. However, indirectly raising intracellular cGMP levels, by increasing NO signaling

has been shown to induce vascular smooth muscle relaxation, increase BBB permeability, and inhibit endothelial cell apoptosis (46, 47). Based on these results, increasing cGMP signaling does not seem to be a suitable therapeutic strategy for restoring BBB integrity during inflammatory relapses in MS. Therefore, solely cAMP or dual-substrate PDE inhibitors are discussed here as a therapeutic strategy for reducing BBB disruption.

In particular, the cAMP-specific PDE4 inhibitors have been evaluated for their potential to strengthen the BBB. In experimental autoimmune encephalomyelitis (EAE), a neuroinflammatory animal model for MS, the pan PDE4 inhibitor rolipram (2 mg/kg, i.p injected twice a day) modified the cerebrovascular endothelial permeability and thereby restored BBB function (48). The same protective features of rolipram were observed in an animal model for stroke, where treatment preserved the expression of the tight junction proteins occludin and claudin-5 (49). Furthermore, the inhibition of PDE4D is of particular interest for altering BBB permeability since it co-localizes with the endothelial marker RECA-1 and the vascular smooth muscle cells α-SMA (50). However, the exact role of PDE4D in restoring BBB integrity remains to be elucidated. Both cAMP-specific PDE inhibitors and dual-substrate PDE inhibitors have been proposed as potential therapeutic targets. As such, administration of the PDE3 inhibitor cilostazol to a murine model for age-related cognitive impairment (1.5% w/w) over a 3 months period increased the amount of zona occludens protein 1 (ZO-1) and occluding, subsequently improving BBB integrity (51). Therefore, PDE inhibitors acting on the cAMP pathway are predicted to strengthen BBB functionality. Due to the opposing outcomes upon elevating cAMP in different subcellular compartments, elucidating which PDE enzymes are present in endothelial vacuoles and absent in the cytosol can hold the key for identifying which PDE needs to be targeted for restoring BBB integrity. Unraveling essential signaling peptides during translation will become indispensable for determining PDE compartmentalization.

Lymphocytes

Disrupted BBB integrity in MS patients facilitates peripheral immune cell infiltration. The two main subsets of infiltrating T lymphocytes in MS are CD^{4+} and CD^{8+} T cells (52). Various subsets of CD^{4+} T helper cells have been identified based on their cytokine secretion profiles. In particular, CNS Th1 and Th17 cell frequencies are increased in RRMS patients compared to healthy controls. Cytokines secreted by the different T cell subsets are critical mediators of the neuroinflammatory response. Upon activation, Th1 cells aggravate the neuroinflammatory response by secreting pro-inflammatory cytokines [e.g., tumor necrosis factor α (TNFα), interleukin 1β (IL-1β), and interferon-γ (IFN-γ)], subsequently promoting cellular infiltration and activation of phagocytes and B cells (53). Th17 cells are mainly characterized by their production of interleukin 17 (IL-17), but exert a polyfunctional phenotype depending on their overall cytokine secretion profile. Pathogenic Th17 cells aggravate inflammatory processes by producing high levels of the pro-inflammatory cytokine IFN-γ, whereas non-pathogenic Th17 cells produce more protective cytokines such as IL-10 (54). IL-17 levels are elevated in the serum and cerebrospinal fluid (CSF) of MS patients and are correlated with MS disease severity, consequently suggesting a pathogenic role of Th17 cells in MS (55, 56). Moreover, regulatory CD^{4+} T cells (Tregs) from the peripheral blood of RRMS patients show a reduced suppressive capacity, suggesting Treg dysfunction in early MS stages (57). Treg formation is the result of activation of the dominant transcription factor forkhead box P3 (Foxp3) and by producing immunosuppressive cytokines [e.g., transforming growth factor β (TGF-β) and IL-10], Tregs inhibit auto-aggressive T cell responses (58). In addition to CD^{4+} T cells, autoreactive cytotoxic T cells (CD^{8+} T cells) are actively involved in MS pathogenesis. CD^{8+} T cells are found in large numbers in MS lesions in close proximity to damaged oligodendrocytes (59, 60). Therefore, halting MS disease progression can be accomplished by modulating lymphocyte responses through the restoration of second messenger levels using PDE inhibitors.

In the context of T lymphocyte proliferation, differentiation and activation, cAMP is the most extensively studied second messenger. Increasing cAMP levels attenuates the T lymphocyte-mediated immune response by reducing the production of pro-inflammatory cytokines (e.g., IFN-γ, TNF-α, and IL-1β), T cell proliferation and T cell activation (61–63). Increased levels of cAMP further drive the development of Tregs to maintain immunological homeostasis by suppressing the innate immune responses (63). Recently, it has been reported that anti-CD3/CD28 stimulation to activate naïve CD^{4+} T cells increased the enzymatic levels of PDE7, particularly the expression of the PDE7A1 isoform (64, 65). Accordingly, in EAE mice where T cells are highly activated, the PDE7 inhibitor TC3.6 was shown to increase mRNA levels of FoxP3 and augment the production of IL-10. Additionally, PDE7 inhibition was accompanied by decreased levels of IL-17 and reduced T cell proliferation (66). Conversely, PDE7A knockout mice did not show a difference in T cell activation and cytokine production, obfuscating the role of PDE7 in T cell-mediated immune responses and raising the possibility of an indirect effect of the PDE7 inhibitor TC3.6 (67). PDE4 is the most extensively studied cAMP-specific PDE in the context of modulating pro-inflammatory processes. As observed with TC3.6, inhibiting PDE4 decreased T cell proliferation and reduced the production of pro-inflammatory cytokines (TNF-α and IL-17) while increasing the production of anti-inflammatory cytokines (IL-10) in EAE mice (29, 66). Symptomatic treatment with 2.5 mg/kg of the PDE4 inhibitor rolipram decreased the number of perivascular inflammatory infiltrates and was accompanied by a reduction of clinical symptoms in EAE mice (29, 66). Interestingly, upon anti CD3/CD28 co-stimulation of either human CD4+ naive or memory T cells, the enzymatic activities of PDE4A and PDE4D alone were upregulated, although mRNA levels of PDE4A, PDE4B, and PDE4D were increased (68). Furthermore, knockdown of PDE4D in these activated human CD4+ T cells, using siRNA reduced their proliferation rate and inhibited the secretion of IFN-γ (68). In EAE mice, mRNA levels of the PDE4B2 isoform were increased in infiltrating T cells in the CNS (30). This increase in PDE4B2 was positively correlated with FoxP3 and TGF-β mRNA levels,

suggesting a modulatory role for PDE4B2 in Treg regulation (30, 66, 69). Based on these findings, cAMP-specific PDE inhibition in T cells can lower the inflammatory cytokine production by acting directly on Th1 and Th17 cells or by regulating the immune response through Treg cells. Furthermore, the dual substrate PDE3 inhibitor cilostazol has been shown to ameliorate encephalitogenic specific T cell responses in EAE mice by reducing lymphocytic proliferation and IFN-γ production in the CNS (70). These findings are consistent with the previous observations using cAMP-specific PDE inhibitors. Despite this, an involvement of cGMP in T cell regulation cannot be excluded as cGMP has been shown to be highly expressed in the cytoplasm of T cells (71–73). Upon NO treatment, T cell adhesion to ICAM-1 and PECAM-1 on endothelial cells of the human brain microvasculature is reduced in a cGMP-dependent manner (74). Accordingly, increasing both cAMP and cGMP by inhibiting specific PDEs can be considered as a potential therapeutic strategy to limit T cell activation by either lowering the pro-inflammatory cytokine production by Th1 and Th17 cells, or by enhancing the suppressive capacity of Tregs.

Although the perivenular infiltration of B cells is less prominent compared to T cells in MS, their contribution to the pathogenesis is highlighted by the anti-CD20 monoclonal antibody therapy that induces B cell depletion and subsequently limited the number of relapses in RRMS patients (75, 76). B cells exert a central role in the pathogenesis of MS by their antibody-independent functions that can either activate or suppress inflammatory responses (77). However, not much is known about second messenger signaling in B lymphocytes. Opposing results were reported depending on the nature of second messengers in B cell cycling (78). Treating murine B lymphocytes with the neurotransmitter acetylcholine indirectly increased the intracellular cGMP levels by stimulating the NO/cGMP pathway, consequently stimulating B cells to enter the cell cycling stages (78, 79). In contrast, adrenaline-induced intracellular cAMP inhibited the entry of B lymphocytes into the DNA replication stage of the cell cycle (78). The latter is consistent with the observations that forskolin, an activator of AC in the plasma membrane, arrested human B lymphocytes in the G1 phase of cell cycling and thereby inhibited B cell growth (80). Furthermore, forskolin promoted apoptosis in human resting B cells (80). However, there is little evidence that PDE inhibitors are able to modulate B cell responses. The PDE4 inhibitors apremilast, rolipram and Ro 20-1724 did not affected B cell differentiation, however they did inhibit IgE production in human PBMCs after IL-4 stimulation (81, 82). This decrease in IgE production was not observed upon PDE3 or PDE5 inhibition, which can be explained by the marginal PDE3 activity and lack of PDE5 activity in healthy B lymphocytes (81, 83, 84). While there is currently little evidence for a direct effect, indirect effects of PDE inhibitors on B cell responses in the pathogenesis of MS cannot be ruled out.

Phagocytes

Another strategy to control the inflammatory process in MS involves modulating the response of phagocytes in the CNS. In the CNS, phagocytes actively survey the CNS microenvironment in search of harmful pathogens and damage signals (85). In order to retain CNS homeostasis, phagocytes orchestrate different processes including synaptic pruning, shaping neurogenesis, and clearance of cellular debris and apoptotic neurons (86, 87). Depending on the environmental stimuli, phagocytes cover a divergent spectrum of activation states. Upon classical activation (e.g., IFN-γ as activation stimulus), macrophages and microglia polarize toward a more pro-inflammatory phenotype. These classically activated phagocytes contribute to the inflammatory response by producing pro-inflammatory cytokines and chemokines (e.g., TNFα and IL-1β) and therefore mediate tissue damage (15). In contrast, upon alternative activation (e.g., IL-4 as activation stimulus), phagocytes polarize toward a more anti-inflammatory phenotype. These alternatively activated phagocytes are characterized by the production of anti-inflammatory cytokines (e.g., TGFβ and IL-10) and growth factors (e.g., IGF and BDNF). Additionally, anti-inflammatory phagocytes facilitate the clearance of cellular debris which enables the initiation of repair processes (15). It is postulated that persistent neuroinflammatory processes create an imbalance between pro-and anti-inflammatory phagocytes, resulting in neurotoxicity and subsequent neurodegeneration (88).

Interestingly, murine studies using EAE have demonstrated that a phenotypic switch of phagocytes from the pro- to the anti-inflammatory phenotype is associated with milder clinical scores (89). Moreover, after focal LPC-induced demyelination, pro-inflammatory phagocytes seem to drive OPC proliferation. However, it is the later switch to the pro-reparative phenotype that is necessary for OPC differentiation into mature myelinating oligodendrocytes that establish functional remyelination (89). Additionally, anti-inflammatory phagocytes are critical contributors in ceasing the inflammatory response and allowing CNS repair (89). Balancing the levels of cAMP and cGMP in phagocytes is considered critical for orchestrating phagocyte polarization and organizing phagocytosis (90). However, abnormally high levels of cAMP inhibit myelin phagocytosis *in vitro*, even though increasing cAMP skews the polarization toward an anti-inflammatory phenotype characterized by high levels of arginase 1 (Arg1) (90, 91). Furthermore, cGMP has been reported to be associated with the actin cytoskeleton in phagocytes (64, 65, 92, 93). Stimulating the cGMP-PKG pathway dramatically reorganized the actin cytoskeleton of microglia, giving them a phagocytosis-promoting morphology and subsequently enhanced clearance of apoptotic cells and cell debris (94, 95). In MS, internalization of myelin debris by phagocytes at the lesion site is crucial for allowing endogenous remyelination. Therefore, increasing intracellular cAMP or cGMP levels in phagocytes can either alter the inflammatory responses in the CNS or promote clearance of debris, respectively, and can be considered a potential therapeutic strategy for MS.

In murine monocytes and macrophages, the PDE4B gene in particular has been related to inflammatory responses (96). Accordingly, PDE4B inhibition enhanced the secretion of the anti-inflammatory IL-1 receptor antagonist (IL-1Ra) in PDE4B$^{-/-}$ macrophages, at least partially through promoting the phosphorylation and subsequent activation of signal

transducer and activator of transcription 3 (STAT3) (97). Furthermore, a positive correlation between PDE4B2 in APC cells (e.g., microglia and macrophages) and the clinical scores of EAE mice was observed (30). Transcriptional upregulation of PDE4B2 is predicted to mediate the activation of the toll receptor-4 pathway, characterized by the production of the pro-inflammatory cytokine TNF-α (98–101). Multiple studies suggest that peripheral inflammation is linked to the development of neuroinflammation (102, 103). In both humans and mice, spinal cord injury (SCI) triggered the expansion of the proteobacteria phylum, leading to an increased systemic endotoxemia that allows LPS from intestinal bacteria to enter the bloodstream, subsequently activating peripheral monocytes and macrophages (103–105). Subsequently, when inducing SCI in PDE4B knockout mice, inflammatory responses, and endoplasmic reticulum (ER) stress were significantly decreased within the spinal cord (SC) of these mice, suggesting the critical involvement of PDE4B in (neuro) inflammatory responses potentially occurs by suppressing monocyte and macrophage activation (103). As with SCI, alcohol consumption induces endotoxemia and subsequent peripheral monocyte activation (106). In mice, alcohol-induced endotoxemia induced PDE4B expression in both peripheral monocytes and CNS resident microglia. This induced PDE4B expression was characterized by a decrease in cAMP levels and subsequent glial activation, indicating a potential pathogenic role of Pde4b in alcohol-induced neuroinflammation (106). Besides PDE4, PDE5 inhibitors sildenafil, and vardenfil have been studied for their effects on macrophage phenotype and CNS infiltration. Sildenafil treatment (10 mg/kg, daily s.c injected) improved clinical scores in EAE mice and increased the expression of Ym-1, a canonical anti-inflammatory macrophage marker in the SC of these mice. In addition, PDE5 inhibition promoted phagocytosis of myelin debris (89). Therefore, by inhibiting cAMP-specific PDEs, this pro-inflammatory response can be diminished and disease progression can be halted. In contrast, inhibition of cGMP-specific PDEs does not actively suppress the pro-inflammatory responses of infiltrating macrophages, but rather increases the phagocytosis rate, thereby promoting CNS repair processes.

Moreover, the role of PDEs in macrophage responses has been studied independently of pathological MS processes. For example, PDE3B has been implicated in regulating inflammasome activation of infiltrating macrophages in white adipose tissue (WAT). As such, PDE3B knockout mice displayed reduced serum levels of pro-inflammatory cytokines such as IL1β and TNFα in a peripheral lipopolysaccharide (LPS) challenge. PDE3B ablation significantly reduced macrophage infiltration in WAT of high fat diet-induced obesity mice (107). Additionally, PDE4 inhibition has been shown to reduce clinical symptoms of inflammatory diseases including arthritis and psoriasis by shifting the phenotypic balance of phagocytes (108–110). PDE4 inhibition with apremilast reduced dermal fibrosis by interfering with the release of IL-6 by anti-inflammatory macrophages. This resulted in a decreased fibroblast activation and collagen release in a skin fibrosis mouse model (111). The beneficial effects of PDE inhibition by macrophages that infiltrate the peripheral tissues give rise to multiple implications that are potentially exploitable in those that infiltrate the CNS. Understanding the role of PDE3B ablation as well as the inhibition of PDE4 and PDE5 in promoting macrophage phenotypic shifts in other pathological contexts can be implicated for controlling the phagocyte-related inflammatory responses in MS pathogenesis.

In microglia, PDE4 is the predominant negative regulator of cAMP (112). Roflumilast-mediated PDE4 inhibition increased the mRNA and protein levels of Arg1, skewing polarization of an anti-inflammatory phenotype in myelin-laden microglia, subsequently promoting repair processes in aged rats subjected to chronic cerebral hypoperfusion (113). Inhibiting PDE4 suppresses LPS-mediated release of TNF-α and NO by activated microglia (99). However, this reduction in NO production is abolished when co-culturing microglia with neurons, casting doubt upon this mechanism *in vivo* (99). Furthermore, the novel PDE4 inhibitor FCPR03 suppressed the release of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) *in vitro* in LPS stimulated BV2 microglia, and *in vivo* in the hippocampi and cortices of mice peripherally treated with an LPS bolus (114). Interestingly, inhibition of neuroinflammation was abolished when BV2 microglia were pretreated with a PKA inhibitor H89 (114), indicating that the cAMP-downstream PKA/CREB signaling pathway may be responsible for the suppressed production of pro-inflammatory cytokines upon FCPR03 treatment (114). Moreover, the cAMP/PKA signaling pathway inhibits NF-κB, thereby further suppressing neuroinflammation (114). The novel PDE4 inhibitor roflupram enhanced autophagy in both BV2 microglia and in microglia of mice peripherally injected with LPS (115). Autophagy is a process critically involved in maintaining homeostasis as it modulates inflammasome activation and IL-1β production by removing damaged mitochondria (116). Damaged mitochondria are an important source of ROS production that subsequently activates NLRP3-mediated inflammasome activation and IL-1β production (116). By inducing autophagy, roflupram suppressed inflammasome activation, and IL-1β, consequently reducing neuroinflammatory responses in LPS challenged mice (115). Similar effects on autophagy and inflammasome activation were observed when PDE4B was specifically knocked down in primary microglia cells (115). The likely involvement of PDE4B in suppressing inflammatory responses is reinforced by the observation that ABI-4, a PDE4D-sparing PDE4 inhibitor, has been shown to reduce the release of TNF-α in LPS stimulated primary murine microglia (117). Likewise, pre-symptomatic treatment with the PDE7 inhibitor TC3.6 reduced microglial activation in an animal model for PPMS by decreasing a wide range of mediators of the neuroinflammatory processes including IL-1β, TNF-α, IFN-γ, and IL-6 in the SC (118). Furthermore, increasing intracellular cGMP levels by inhibiting PDE5 after LPS stimulation decreased microglial NO, IL-1β and TNF-α production (119). In line with this, the PDE5 inhibitor sildenafil alleviates hippocampal neuroinflammation by normalizing microglial morphology and reducing microglial activation as shown by a diminished IL-1β production (120–122). Moreover, the highly selective PDE10A inhibitor TP-10 reduced the number of $CD11b^{+}$ reactive microglial cells in the striatum and thereby ameliorated brain pathology in

an animal model for Huntington's disease, demonstrating its therapeutic potential in MS pathology (123). Furthermore, 10 μM ibudilast, a non-selective PDE inhibitor targeting multiple PDE families (e.g., PDE4 and PDE10), suppressed TNF-α production in activated microglia but lacked efficacy in lowering other pro-inflammatory mediators such as IL-1β or IL-6 (124). Therefore, inhibiting PDEs independently of their substrate specificity in microglia diminishes pro-inflammatory responses and microglia reactivity.

Taken together, different PDE inhibitors can be considered a powerful therapeutic option for ceasing the inflammatory response in MS by altering the balance between cytotoxic and reparative phagocytes. Particularly PDE4B was shown to be critically involved in neuroinflammatory responses, making it an interesting target for developing MS therapies. Furthermore, PDE4B is upregulated in phagocytes following peripheral inflammation, and subsequently aggravates neuroinflammatory responses; a key process in the pathogenesis of MS. Therefore, interfering with this peripheral-central immunological cross-talk by inhibiting specifically PDE4B is an interesting strategy for treating RRMS patients that needs to be explored further.

Astrocytes

Astrocytes are the most abundant cells of the CNS and exert pleiotropic functions to protect and support other CNS cell types (125, 126). Due to their ideal position in the brain microvasculature, astrocytes can directly respond to infiltrating immune cells during the initial processes occurring in MS (127). Astrocytes produce growth factors and metabolites in order to maintain the homeostatic balance in the brain, but also ensure synaptic and BBB integrity (125, 126). However, during profound CNS injury, astrocytes become highly activated and undergo morphological and functional changes, yielding astrogliosis (128).

In an attempt to investigate whether PDEs are implicated in astrogliosis, it was shown that TLR signaling induced an upregulation of PDE4B, and more specifically increased the protein level of the PDE4B2 isoform (129). Accordingly, twice daily administration of ibudilast (20 mg/kg), a non-specific PDE inhibitor with preferential affinity for PDE4, reduced astroglial activation in an animal model for Parkinson's disease (130). The same results were observed in a rat model for ocular hypertension, in which a decrease in gliosis was accompanied by decreased levels of pro-inflammatory mediators and enhanced neuroviability (131). Interestingly, ibudilast treatment was also demonstrated to prevent astrocyte apoptosis by increasing cGMP levels, suggesting a potential protective role for cGMP-specific PDE inhibitors (132). In line with this hypothesis, PDE5 inhibition by administering 10 μM sildenafil was shown to restore LPS-induced inflammation in astrocytes *in vitro*, as demonstrated by de Santana Nunes et al. (133). In relation to BBB disruption and immune cell infiltration, it is known that astrocytes express lymphocyte adhesion molecules such as ICAM-1 and VCAM-1 in inflammatory states (134). Elevation of intracellular cAMP levels counteracts the inflammatory activation of astrocytes, resulting in a downregulation of these adhesion molecules (135). As such, astrocytic cAMP signaling plays a prominent role in the prevention of peripheral lymphocyte infiltration. Aforementioned studies show that PDEs are greatly involved in the inflammatory aspect of astrocyte biology and that inhibition of selected PDE isoforms can result in the attenuation of astrogliosis.

INHIBITING PDEs TO BOOST REPAIR IN CHRONICALLY DEMYELINATED MS LESIONS

Neuroinflammation and axonal demyelination associated with MS render neurons more vulnerable to degeneration. Stimulating repair in chronically demyelinated MS lesions is a promising strategy for treating progressive MS patients. The main processes to be addressed for boosting this repair include the stimulation of OPC differentiation into myelinating oligodendrocytes, remodeling of the existing neuronal circuits by enhancing neuro-plasticity/-protection to strengthen axonal conduction, and resolving inflammation that allows for phagocytic growth factor secretion (discussed above).

Oligodendrocytes

In the CNS, the myelinating cells responsible for remyelination are oligodendrocytes which function to maintain neuronal integrity, and facilitate signal conduction in the brain and spinal cord (136, 137). However, oligodendrocytes are known to be extremely vulnerable to damaging signals, such as neuroinflammatory attacks, or ischemic episodes (138, 139). Loss of oligodendrocytes can result in axonal damage and ultimately leads to demyelination and subsequent neurodegeneration. In an attempt to restore this loss of oligodendrocytes, newly myelinating cells can be formed by differentiation of OPCs into mature myelinating oligodendrocytes (137).

cAMP is a key driver of OPC differentiation (140). *In vitro* treatment of OPCs with cAMP analogs, such as dbcAMP or 8-bromo cAMP, support OPC differentiation based on the number of myelin basic protein (MBP) positive cells (141). A similar level of differentiation is observed when using forskolin (142). Accordingly, cAMP-specific PDE inhibitors are thought to stimulate oligodendrocyte development. Treatment of human OPCs with the PDE7 inhibitors TC3.6 or VP1.15 promotes their survival and accelerates their differentiation into mature myelinating oligodendrocytes by stimulating the ERK signaling pathway (143). In parallel, the potent PDE4-inhibitor rolipram (0.5 μM) was shown to boost rat OPC differentiation *in vitro* by increasing the percentage of MBP^+ cells (140). Furthermore, based on G-ratio analysis, rolipram (0.5 mg/kg/day) enhanced remyelination in the caudal cerebellar peduncle following focal ethidium bromide-induced demyelination *in vivo* (140). In the presence of myelin-associated inhibitors, OPC differentiation is impaired *in vitro* due to an impairment in Erk1/2, p38MapK and Creb1 phosphorylation. However, 0.5 μM of rolipram treatment overcame the inhibitory effects of myelin protein extracts *in vitro* and relieved the induced differentiation block (140). Interestingly, daily administration of 0.5 mg/kg

rolipram (by means of s.c. placed minipump) also appeared to protect oligodendrocytes from secondary cell death following experimental SCI, thereby highlighting its multifaceted mode of action during neurodegeneration (144). In relation to oligodendroglial cell death following ischemia, Nobukazu Miyamoto et al. administered 0.1% of a PDE3 inhibitor mixed in regular chow diet up to 28 days to rats suffering from chronic cerebral hypoperfusion. At a cellular level, this resulted in a strong increase of newly generated oligodendrocytes and a subsequent enhanced rate of remyelination in hypoperfusion-induced white matter lesions after bilateral common carotid artery ligation. Even though PDE3 is classified as a dual cAMP/cGMP hydrolyzing enzyme, Miyamoto and colleagues solely investigated the PDE3 inhibition-mediated increase of cAMP and therefore attributed the positive effects on ischemic white matter injury mostly to a cAMP/PKA-mediated pathway (145). Nevertheless, a role for cGMP involvement cannot be excluded in oligodendrocyte differentiation processes. In particular, an increase in nitric oxide (NO)-induced cGMP signaling has been shown to be directly related to oligodendrocyte maturation as determined by an increased MBP and MOG protein expression level (146). The observed increase of maturation supports the rationale that cGMP-specific PDE inhibitors can also exert a positive effect on oligodendrocyte-mediated repair mechanisms. Accordingly, treatment of organotypic cerebellar brain slices with the widely known PDE5 inhibitor, sildenafil (1 μM) for 10 days enhanced the level of remyelination (89). Additionally, in the SC of EAE mice, treated with 10 mg/kg sildenafil once a day for 15 consecutive days (subcutaneous injection), oligodendrocyte maturation was induced in a cGMP-NO-protein kinase G (PKG)-dependent manner (89). Furthermore, sildenafil appeared to have a protective effect in a mouse model for demyelination, as demonstrated by a preserved myelin, and axonal ultrastructure (147). Yet, these protective features of sildenafil are inconsistent with the findings of Muñoz-Esquivel and colleagues. Here, it was reported that sildenafil treatment diminished myelin expression and increased the expression of negative regulators of myelin (Id2 and Id4), which was consistent with the decreased myelination capacity of sildenafil treated oligodendrocytes (148).

Opposing results regarding the *in vitro* effects of sildenafil on OPC differentiation can be potentially attributed to the difference in inhibitor concentrations. In a later study conducted by Muñoz-Esquivel and colleagues, an inhibition in myelin protein expression was observed after 7 days of 50 μM sildenafil treatment. The myelination-promoting effects of sildenafil in organotypic cerebellar brain slices were observed after 1 μM treatment for 10 days. Furthermore, the diminished expression of myelin proteins after sildenafil treatment was observed in pure primary rat OPC cultures, while organotypic cerebellar brain slices contain multiple cell types. Therefore, an indirect effect of sildenafil for promoting remyelination cannot be ruled out. This difference in treatment regimens is a potential explanation for the observed differences and underscore the importance of cGMP fine-tuning. Altogether, both cAMP and cGMP specific PDE inhibitors have been shown to be promising stimulators for OPC differentiation that boosts CNS repair in MS. However, validating these findings in multiple *in vitro* and *in vivo* models for remyelination is essential before further clinical development.

Neurons

The lack of myelin in both acute and chronically demyelinated lesions has profound pathophysiological consequences. For instance, Na^+ channels are redistributed over the demyelinated axolemma as a final compensatory mechanism of neurons in order to maintain nerve conduction although the myelin sheath is lost (149). Progressive axonal and neuronal loss associated with MS eventually causes weakening of neural circuits leading to cognitive and motor impairments (150). Therefore, neuroprotection or repair of neuronal damage may delay, halt, or counter disease progression.

Stimulation of cyclic nucleotide signaling has been shown to increase neuronal resilience by promoting neuroplasticity. Inhibition of PDEs may therefore be an appropriate strategy to induce neuroprotection in MS. Inhibition of specific PDEs is found to enhance neuroplasticity, subsequently increasing neuronal resilience. Vinpocetine, a selective PDE1 inhibitor, can limit oxidative stress, and neuronal damage in a model of vascular dementia (151). PDE2 inhibition was found to improve neuronal plasticity, as observed by an increase in hippocampal long term potentiation (LTP), which is regarded as the underlying physiological correlate of memory (152). The increase in LTP was accompanied by improved object memory performance, both in rats and mice (153). After induction of brain ischemia (154) or in animal models using chronic unpredictable stress (155), the PDE2 inhibitor Bay 60-7550 attenuated the pathological decrease in neuroplasticity related proteins (e.g., BDNF), thereby enhancing neuroplasticity and subsequently neuroprotection. Cilostazol, a PDE3 inhibitor, mediates neuronal repair after induced neuronal loss in the dentate gyrus through an increase in pCREB-mediated hippocampal neural stem cell proliferation (156). The potential of PDE4 inhibition to stimulate neuroplasticity has been studied extensively in the context of learning and memory (37, 157, 158); both non-specific inhibition of the PDE4 gene family (159), as well as targeting of individual PDE4 genes (160), or isoforms (161) are able to increase neuroplasticity and memory functioning. Moreover, neuroprotective and neuroregenerative effects by inhibition of PDE4 have been shown after different types of insults, including SCI (28, 162, 163), striatal neurotoxicity (164–166) and mouse models of Huntington's disease (166, 167). Similarly, PDE5 inhibition by sildenafil and vardenafil can not only improve object memory (168, 169), but also protect against striatal degeneration by stimulation of neuronal surviving pathways, including BDNF and p-CREB expression (170). Interestingly, sildenafil treatment (15 mg/kg administered orally) reduces oxidative stress in mice exposed to noise stress through an increase in free radical scavengers such as super oxide dismutase (SOD) 1, SOD2 and SOD3 (171). Additionally, PDE7 inhibition was found to induce neuroprotective and anti-inflammatory activities in a rat model of Parkinson's disease (172). A reduction in

hippocampal apoptosis was also observed after PDE7 inhibition in an Alzheimer mouse model (173). Alzheimer-associated decreases in dendritic spines and plasticity can be counteracted by PDE9 inhibition (174). Finally, PDE10 inhibition increases neuronal survival in a transgenic mouse model of Huntington's disease (175).

As described above, neuroprotective treatment strategies can be achieved by targeting distinct PDE families or isoforms, given the wide applicability of PDE inhibitors to suppress damaging signals such as neuronal apoptosis and oxidative stress, but also to stimulate neuronal survival and repair. Furthermore, inhibition of these PDEs is primarily associated with an improvement in cognitive performance including memory and learning. This latter aspect makes PDE inhibition even more interesting, considering cognitive decline is one of the major symptoms of disease progression in MS (176). However, while inhibition of different types of PDE enzymes has been shown to be beneficial in several models, the exact mechanisms underlying its neuroprotective effects are yet to be elucidated in the context of MS.

PDE INHIBITORS IN CLINICAL TRIALS AS A THERAPY FOR MS

It is clear that PDEs are involved in numerous and different processes in MS. Currently, the majority of MS therapies are focused on reducing disease severity by preventing the infiltration or activation of immune cells in the CNS. However, these therapeutics are unable to halt or reverse disease progression. Therefore, there is an urgent need for the development of new therapeutic strategies. The multimodal effects of PDE inhibitors makes them highly interesting for clinical use to treat MS patients. However, most research regarding PDE inhibitors to date has been performed at the preclinical level and very few clinical trials have been conducted to assess their clinical potential (**Table 1**).

In early 2001, a Phase I/Early Phase II clinical trial was designed to test the dose, tolerability, and efficacy of the PDE4 inhibitor, rolipram as a treatment against CNS inflammation for MS patients (177). In the first stage of the study, six MS patients were enrolled to assess the optimal and safe dose of the compound. Two additional patients with moderate inflammatory brain activity were recruited for the second stage of the study. Even though no difference in clinical disability was observed, rolipram was not well tolerated by the patients. Adverse events such as nausea, vomiting, gastroesophageal reflux, and insomnia were common during the therapy. Moreover, rolipram treatment was accompanied by the unexpected side-effect of an increase in the total amount of contrast enhanced lesions (CEL) per patient when compared to their baseline state demonstrating it has no clinical benefit. As this was the predetermined primary outcome of the study, the trial was terminated in an early phase (177).

Similarly, a double blind, placebo-controlled phase II trial was conducted to evaluate the safety and effects of Ibudilast as a treatment strategy for RRMS patients (178). As discussed above, ibudilast is a non-selective PDE inhibitor that also inhibits the macrophage migration inhibitory factor and toll-like receptor 4 (179, 180). Patients who enrolled in the study received either 30 mg, 60 mg ibudilast, or a placebo every day for 1 year. No difference in lesion activity was observed between the different groups, resulting in an unmet primary endpoint. However, ibudilast treatment seemed to slow brain atrophy, a measure of permanent tissue injury and disease progression in MS (178). Consequently in 2013, the SPRINT-MS phase II clinical trial was established to assess the efficacy and tolerability of ibudilast as a treatment for progressive MS patients (181). Both PPMS and SPMS patients were recruited in the study and received either 50 mg ibudilast or a placebo, twice daily for 96 weeks. The recently published results show that upon ibudilast treatment, the rate of brain atrophy was slowed by 48%. However, as with rolipram, treatment with ibudilast was accompanied by adverse events such as gastrointestinal symptoms, headaches and depression (181).

In 2004, a pilot study was initiated to investigate whether the PDE5 inhibitor, sildenafil citrate, improves cerebral blood perfusion in MS patients (182). MS patients frequently experience a compromised cerebral blood flow which can lead to neuronal cell death. Therefore, it was hypothesized that blood flow perfusion can be increased in these patients through treatment with sildenafil citrate. Both MS patients and healthy volunteers were recruited for this study. MRI scans of the cerebral arteries were taken at baseline prior to treatment, as well as 1 h after sildenafil citrate administration (182). Even though the study was completed within 2 years, the results and outcomes of the trial are yet to be disclosed.

At present, ibudilast is the only PDE inhibitor that has yielded positive results in a clinical setting. The ongoing SPRINT-MS study will validate whether the observed effects of ibudilast on brain atrophy are reproducible, and if it is associated with slower disease progression (181). However, even though ibudilast targets different PDE families, it preferentially targets PDE4 (183). As seen with rolipram, targeting PDE4 in humans is associated with strong adverse effects such as nausea and vomiting, which can compromise the potential use of such inhibitors in clinical settings. There is therefore an urgent need to develop and assess the beneficial effects of PDE isoform inhibitors for their beneficial effects in clinical trials for MS.

PDE INHIBITORS IN OTHER NEURODEGENERATIVE DISORDERS: RELEVANCE FOR TREATING MS

Neuroinflammation and neurodegeneration are central processes involved in a wide range of CNS disorders. Based on the aforementioned cellular effects, it is not surprising that PDE inhibitors have been extensively studied in the context of disorders other than MS. Therefore, multiple lessons can be drawn from studies conducted in other disorders and may be implemented when devising therapeutic applications of PDE inhibitors in MS. Although neuroinflammatory and neurodegenerative processes are identical in many disorders, the

TABLE 1 | Overview of (pre-)clinical studies with PDE inhibitors for the treatment of MS.

Drug name	Indication	Route of administration	Mode of action	Target	Status
AP-1	MS	Oral	Immune modulation	PDE7 and GSK3B	Preclinical
Ibudilast	PPMS; SPMS	Oral; ophthalmic	Anti-inflammatory; neuroprotective	Non-selective PDE inhibitor	Phase II
Revamilast	MS	Oral	Immune modulation	PDE4	inactive
Rolipram	MS	Oral	Facilitates neural transmission; immune modulation	PDE4	Inactive
Sildenafil	RRMS/SPMS	Oral	Increase blood flow	PDE5	Phase II
Small molecules to inhibit PDE7	MS	Oral	Immune modulation	PDE7	Inactive
TDP-101	MS	/	Immune modulation	PDE4B	Preclinical

MS multiple sclerosis; RRMS relapse remitting MS; PPMS primary progressive MS; SPMS secondary progressive MS; PDE phosphodiesterase; GSK3B glycogen synthase kinase 3 beta GSK3B (GlobalData extraction 25/05/2019).

underlying pathological causality is highly diverse. Therefore, relevant findings supporting the role of PDE inhibitors in other CNS disorders do not provide conclusive results, but rather show the potential of PDE inhibitors in treating MS. Here we will briefly discuss the potential of PDE inhibitors for treating CNS trauma and Alzheimer's disease (AD), and their relevance for treating MS.

After CNS trauma such as SCI, a chronic neuroinflammatory response occurs that impairs neuroregeneration. PDE4 inhibition has been shown to reduce inflammatory processes in monocytes and lymphocytes (184). Considering the role of these infiltrating immune cells in the pathophysiology of SCI, there have been many studies further investigating the effect of PDE4 inhibition. It was demonstrated that PDE4 inhibition increases axonal regeneration using the PDE4 inhibitor rolipram (144). Moreover, Whitaker et al. found that rolipram protected oligodendrocytes against secondary cell death. Furthermore, it was shown that spinal cord oligodendrocytes express PDE4 A, B, and D, while microglia predominantly express PDE4B (144). Bao et al. have also demonstrated that PDE4 inhibition decreased white matter damage, oxidative stress, and leukocyte infiltration, resulting in cellular protection and locomotor improvements after SCI (184). In addition to PDE4, PDE7 inhibition was also studied in the context of SCI. PDE7 is expressed on both macrophages and neurons (185, 186). Paterniti et al. sought to determine the effect of PDE7 inhibition on secondary processes after SCI. Their data demonstrated that PDE7 reduced spinal cord inflammation, tissue injury, neutrophil infiltration, oxidative stress, and apoptosis after SCI (186). Cognitive impairment is an additional effect of neurodegeneration. PDE4 inhibition can also affect cognitive behavior after trauma. This was demonstrated after traumatic brain injury (TBI), where rolipram rescued the cognitive impairment in rats with TBI, an effect that might be attributable to increased CREB activation during learning (187). The promising results of PDE inhibition in CNS trauma are consistent with the previously described potential of these inhibitors in MS treatments. Reduced neuroinflammatory responses, increased axonal regeneration and decreased oxidative stress levels upon PDE4 inhibition can all halt or prevent disease progression of MS. Furthermore, PDE4 inhibition additionally rescued cognitive impairment in pathological circumstances. Given that 40–65% of MS patients experience cognitive impairments (188), PDE4 inhibition would not only reduce pathological hallmarks in the CNS of MS patients, but would also directly reduce a prominent MS-related symptom.

Neuroinflammation is a major hallmark of AD. The pathological proteins amyloid-beta (Aβ) and tau have also been closely linked to inflammatory responses in the brain (189). In AD, an innate immune response is triggered in the brain when aggregated or misfolded proteins bind to pattern recognition receptors on astrocytes and microglia (190). Subsequent secretion of inflammatory mediators (e.g., TNF-α and IL-1β) aggravate AD pathogenesis and contribute to disease progression. Moreover, neuroinflammation may also be a risk factor or mediator in the onset and/or progression of AD (191). Therefore, modulation of neuroinflammatory and/or neuroprotective processes by general or cell type-specific targeting of PDEs may hold therapeutic potential in the context of treating AD. The effect of PDE modulation on neuroinflammation and neurodegeneration has been studied in multiple *in vitro* and *in vivo* AD models. Inhibition of PDE4 was shown to exhibit anti-inflammatory effects in transgenic AD mice (192, 193). More specific inhibition of the PDE4D isoforms PDE4D4 and PDE4D5 was able to decrease Aβ-induced expression of NFκB, TNFα, and IL-1β (194). In microglia, it was found that Aβ induced increased expression of PDE4B, resulting in higher TNFα release. Inhibition of PDE4B was shown to counter upregulation of TNFα by up to 70% (195). Moreover, ferulic acid, a putative PDE4B2 inhibitor, alleviated increased TNFα and IL-1β levels induced by Aβ (196). Inhibition of the entire PDE4 gene family as well as specific PDE4 genes and isoforms seems to hold anti-inflammatory and neuroprotective potential. Cytotoxicity induced by Aβ was found to be attenuated upon PDE4, PDE5, PDE9, but not PDE3 inhibition in a neuroblastoma cell line (197). Further *in vivo* studies involving intraperitoneal administration of 10 mg/kg PDE5 inhibitor sildenafil to transgenic AD mice resulted in decreased neuroinflammation (122). Protective effects by PDE9 inhibition are supported by the observation that PDE9 inhibition reduced Aβ-induced oxidative stress in transgenic AD mice (198). In addition to anti-inflammatory effects induced by PDE inhibition, functional improvements in

memory deficits associated with transgenic AD mouse models have been shown for the inhibition of several PDEs. Chronic treatment with the PDE2 inhibitor BAY60-7550 (0.3 mg/kg for 8 weeks) improved spatial memory in transgenic AD mice (199). Similarly, addition of the PDE3 inhibitor cilostazol to food pellets of Tg-SwDI mice improved cognition as reflected by grooming behavior (200). PDE4 inhibition by rolipram or FFPM restores memory performance in APP/PS1 transgenic mice as well as cognitive impairments induced by streptozotocin or natural aging (193, 201, 202). Specific inhibition of PDE4D (daily subcutaneous injections with GEBR-7b (0.001 mg/kg) for 3 weeks) improved spatial memory in APPswe/PS1dE9 mice (203). In APP/PS1 mice, memory improvements were found after PDE5 inhibition using administration of sildenafil or icariin (122, 204, 205). Daily treatment with the PDE7 inhibitor S14 (5 mg/kg, intraperitoneally) for 4 weeks improved memory performance in APP/PS1 mice (173). PDE9 inhibition using the inhibitor BAY-73-6691 improved memory deficits in tg2576 mice and mice subjected to intracerebroventricular injection of Aβ25-35 (174, 198). Additionally, the combination of subefficacious doses of a PDE4 (roflumilast) and PDE5 (vardenafil) inhibitor could improve memory in transgenic APPswe mice (206). These preclinical findings indicate that neuroinflammatory and memory-associated processes can be modulated through inhibition of different PDE gene families. As both the signaling cascades involving cAMP and cGMP seem to be affected in AD patients (207–210), inhibition of cAMP- and cGMP-specific PDEs holds potential as therapeutic strategy. However, clinical studies investigating the memory-enhancing potential of PDE inhibitors show inconsistent effects (157, 211). Several changes in PDE expression have been observed in AD brains which seem to be dependent on brain region, cell-type and disease progression (33). Since neuroinflammatory processes may partially mediate initiation or progression of AD, PDE inhibition may provide an exploitable strategy to interfere with these processes. Both in AD and MS, PDE4B(2) has shown to be critically involved in mediating the pro-inflammatory responses of phagocytes in the CNS. Also, inhibition of specific PDE4D isoforms has been shown to possess anti-inflammatory properties in AD, demonstrating the potential of these inhibitors to be explored in the pathogenesis of MS. Apart from direct effects on neuroinflammation, inhibition of PDEs may decrease neuroinflammation and neurodegeneration by influencing AD pathology and neuroplasticity. However, these indirect effects are beyond the scope of this review. Therefore, it would be best if future studies will indicate the expression regulation and role of PDEs per cell type in order to more specifically target cellular processes underlying neuroinflammation and neurodegeneration in both AD and MS.

CONCLUDING REMARKS

The role of PDE inhibitors to modulate neuroinflammatory and neuroreparative processes has gained tremendous interest over the last several years. It is becoming clear that targeting of PDE families can modify multiple cellular key players involved in a variety of processes involved in MS pathogenesis. Due to this multifactorial effect, inhibiting a single PDE family is often accompanied with severe side effects, hampering their translation for a clinical application. Nevertheless, different PDE families are shown to be beneficial in different phases of MS. For example, in the initial phase of MS when BBB integrity is lost, not cGMP but rather cAMP-specific PDE inhibitors are considered a viable therapeutic strategy. Elevating cAMP levels in endothelial cells increased the expression of tight junctions, while elevating cAMP levels in astrocytes decreased the expression of adhesion molecules, subsequently creating a synergistic effect that prevents peripheral lymphocyte infiltration into the CNS via the BBB. During RRMS, there is already an ongoing active pro-inflammatory response. Therefore, disease progression may be halted through the use of cAMP-specific PDE inhibitors to either directly modulate Th1 and Th17 responses or to increase Treg populations to regulate immune homeostasis. Elevating cAMP levels in phagocytes diminishes the secretion of pro-inflammatory cytokines and subsequently lowers the pro-inflammatory phenotype of these cells. Before CNS repair can be initiated in MS patients, myelin debris needs to be internalized at the lesion site by these phagocytes. However, the phagocytic properties of these cells are not stimulated by the increase of intracellular cAMP levels, but rather by the increase of cGMP levels specifically. Therefore, cGMP-specific PDE inhibitors are considered a potential therapeutic strategy for promoting repair processes in later stages of MS. In the context of OPC differentiation, both cAMP and cGMP specific PDE inhibitors have shown their potential. However, these findings require replication be validated and the potential of the inhibitors should be explored further in the context of progressive MS. Finally, enhancing neuroplasticity is considered a possible strategy for promoting functional recovery in MS patients. Multiple PDE inhibitors have been shown to be neuroprotective and to enhance neuroplasticity *in vitro* and *in vivo*, however their efficacy in the context of MS remains unexplored.

Although promising results were obtained in pre-clinical studies, contradictory results were observed in PDE KO animals compared to pharmacological inhibition. However, directly comparing these findings is difficult given the developmental differences in these animals due to the permanent absence of the PDE enzyme throughout the animal's life. Compensatory mechanism are potentially being activated early in the life of PDE KO animals causing an increased expression of other PDE families, genes or isoforms. The development of conditional KO animals can therefore lead to new promising results to confirm the involvement of specific PDEs in pathological conditions. Furthermore, clinical studies using PDE inhibitors often show severe side effects due to the multifactorial effects of PDE inhibitors on multiple cellular processes. PDE isoforms show specific cellular compartmentalization, creating distinct signalosomes within different cells. Therefore, identifying which PDE genes and isoforms underlie distinct pathogenic processes in MS can create a more targeted approach for modifying specific key players during different phases of MS. As such, targeting specific PDE isoforms can further lower the occurrence of

adverse events. Taken together, identifying the key PDE families, genes and isoforms involved in specific phases and processes may lead to the development of a tailor-made approach for treating MS.

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Conflict of Interest Statement: TV and JP have a proprietary interest in selective PDE4D inhibitors for the treatment of MS.

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Liver X Receptor Alpha Is Important in Maintaining Blood-Brain Barrier Function

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Dysfunction of the blood-brain barrier (BBB) contributes significantly to the pathogenesis of several neuroinflammatory diseases, including multiple sclerosis (MS). Potential players that regulate BBB function are the liver X receptors (LXRs), which are ligand activated transcription factors comprising two isoforms, LXRα, and LXRβ. However, the role of LXRα and LXRβ in regulating BBB (dys)function during neuroinflammation remains unclear, as well as their individual involvement. Therefore, the goal of the present study is to unravel whether LXR isoforms have different roles in regulating BBB function under neuroinflammatory conditions. We demonstrate that LXRα, and not LXRβ, is essential to maintain barrier integrity *in vitro*. Specific knockout of LXRα in brain endothelial cells resulted in a more permeable barrier with reduced expression of tight junctions. Additionally, the observed dysfunction was accompanied by increased endothelial inflammation, as detected by enhanced expression of vascular cell adhesion molecule (VCAM-1) and increased transendothelial migration of monocytes toward inflammatory stimuli. To unravel the importance of LXRα in BBB function *in vivo,* we made use of the experimental autoimmune encephalomyelitis (EAE) MS mouse model. Induction of EAE in a constitutive LXRα knockout mouse and in an endothelial specific LXRα knockout mouse resulted in a more severe disease score in these animals. This was accompanied by higher numbers of infiltrating leukocytes, increased endothelial VCAM-1 expression, and decreased expression of the tight junction molecule claudin-5. Together, this study reveals that LXRα is indispensable for maintaining BBB integrity and its immune quiescence. Targeting the LXRα isoform may help in the development of novel therapeutic strategies to prevent BBB dysfunction, and thereby neuroinflammatory disorders.

Keywords: blood-brain barrier, permeability, endothelium, liver X receptors, neuroinflammation

INTRODUCTION

Liver X receptors (LXRs) belong to a large family of nuclear receptors which upon activation stimulate gene transcription (1). Two LXR isoforms exist in mammals, termed LXRα (NR1H3) and LXRβ (NR1H2), which share over 75% amino acid sequence identity. In the nucleus LXRs form obligate heterodimers with the retinoid X receptor (RXR), together forming the LXR/RXR complex. LXRs play an important role in cholesterol and lipid metabolism. The best described process involving LXR function is reverse cholesterol transport where LXRs facilitate the elimination of excess cholesterol in response to cholesterol precursors or oxysterols (2). However, LXRs appear to be involved in a much broader spectrum of functions.

Recent studies identified LXRs as promising targets to interfere in inflammatory signaling pathways. LXR activation induces anti-inflammatory actions in macrophages by antagonizing NF-κB signaling (3). In the central nervous system (CNS), LXR agonists inhibit the production of proinflammatory cytokines and chemokines in stimulated microglia and reactive astrocytes (4). In several animal models of different CNS disorders, including stroke, Alzheimer's disease (AD), and multiple sclerosis (MS), the activation of LXRs results in a reduction of neuroinflammation, suggesting that LXR targeting may be effective in the treatment of neuroinflammatory disorders (5–8).

The main players in the neuroinflammatory process are proinflammatory cytokines and chemokines. These inflammatory mediators are produced locally within the CNS by glial cells or by leukocytes, which are recruited from the periphery following blood-brain barrier (BBB) breakdown (9). One of the pathological hallmarks of BBB dysfunction seen in neuroinflammatory disorders is increased permeability due to loss of tight junctions and increased leukocyte extravasation (10). During the extravasation process, chemokines presented by the inflamed brain endothelium guide the rolling and firm adhesion of leukocytes on the brain endothelial cell surface. Next, the interaction of integrins on leukocytes with brain endothelial cell adhesion molecules (CAMs) further induces their trans- or paracellular migration into the brain, illustrating the critical role of the BBB in mediating neuroinflammatory disorders (11, 12).

Brain endothelial cells tightly regulate BBB function and are regarded as the gatekeepers of the CNS (13, 14). So far, knowledge on the involvement of LXRs in BBB function is limited and is mostly linked to their function in cholesterol homeostasis. For instance, several studies indicate an upregulation of downstream ATP-binding cassette (ABC) cholesterol transporters after LXR agonism in primary brain endothelial cells (15, 16). Interestingly, LXR activation prevents the downregulation of the tight junctions occludin and zona occludens-1 in ischemic vessels in a mouse model of stroke, indicating that LXRs control BBB integrity (16). To date, it remains unclear whether LXRs regulate BBB function during a neuroinflammatory insult, and whether the LXRα and LXRβ isoforms have a distinct role in controlling BBB integrity. Therefore, the goal of the present study is to unravel whether LXR isoforms have different functions in regulating BBB function under neuroinflammatory conditions.

In this study, we show that LXRα, and not LXRβ, is essential to maintain BBB integrity. Impaired LXRα function in brain endothelial cells resulted in decreased barrier function and increased inflammation as marked by increased endothelial vascular cell adhesion molecule (VCAM-1) expression and enhanced trans-endothelial monocyte migration. Importantly, whole body knockout of LXRα and specific endothelial knockout of LXRα in a neuroinflammatory mouse model, resulted in enhanced extravasation of leukocytes into the brain together with increased VCAM-1 expression and reduced claudin-5 expression in the brain vasculature. Collectively, our findings show that LXRα is essential to maintain BBB function.

MATERIALS AND METHODS

Cell Culture

The human immortalized cerebral microvascular endothelial cell line hCMEC/D3 (17) was grown in EGM-2 Endothelial Cell Growth Medium-2 BulletKit, including basal medium and supplement components according to the manufacturer's instructions (Lonza, Basel, Switzerland). All cell culture plates were coated with type I collagen (Invitrogen, Thermo Fisher Scientific, Leusden, The Netherlands). Cultures were grown to confluence at 37°C in 5% CO_2. hCMEC/D3 cells were detached at 37°C with trypsin/EDTA in PBS (Gibco, Thermo Fisher Scientific).

Lentiviral Short Hairpin RNA for LXRα and LXRβ Knockdown

Selective gene knockdown (KD) was obtained by using a vector-based short hairpin (sh) RNA technique as previously described (18). Recombinant lentiviruses were produced by co-transfecting subconfluent HEK 293T cells with the specific expression plasmids and packaging plasmids (pMDLg/pRRE, pRSV-Rev, and pMD2G) using calcium phosphate as a transfection reagent. HEK 293T cells were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin. Cells were cultured at 37°C in 5% CO_2. Infectious lentiviruses were collected 48 h after transfection and stored at −80°C. The KD efficiency of all five constructs for each LXR isoform was tested, and the most effective construct used in subsequent experiments for LXRα (NR1H3) was TRC22237, encoding sequence GTGCAGGAGATAGTTGACTTT that target nucleotides 1,043–1,063 of the NM_005693.3 RefSeq. For LXRβ (NR1H2) the most effective construct was TRC275326, encoding sequence GAAGGCATCCACTATCGAGAT that target nucleotides 1,193–1,213 of the NM_007121.5 RefSeq. Subsequently, lentiviruses expressing LXRα- or LXRβ-specific shRNA were used to transduce hCMEC/D3 cells. Control cells were generated by transduction with lentivirus expressing non-targeting shRNA (SHC002, Sigma-Aldrich, St. Louis, MO). Forty-eight hours after infection of hCMEC/D3 cells with the shRNA-expressing lentiviruses, stable cell lines were selected by puromycin treatment (2 μg/ml). The expression knockdown efficiency was determined by quantitative real-time PCR (qRT-PCR).

RNA Isolation and qRT-PCR

Recombinant hCMEC/D3 cell lines (1×10^6 cells/ml) expressing either LXRα shRNA, LXRβ shRNA, or non-targeting shRNA were seeded in 24-well plates in growth medium. Upon confluency, cells were treated with DMSO (VWR, Leuven, Belgium) or with 5 ng/ml TNFα and 5 ng/ml IFNγ (Peprotech, London, UK) for 24 h. EAE animals were sacrificed on day 23 post-adoptive transfer or day 36 post-immunization. Spinal cords were isolated and snap frozen in liquid nitrogen. Total RNA from cultures and tissues was extracted using Qiazol (Qiagen, Venlo, The Netherlands) and the RNeasy mini kit (Qiagen), according to the manufacturer's instructions. RNA concentration and purity were determined with a NanoDrop spectrophotometer (Isogen Life Science, De Meern, The Netherlands). cDNA was synthesized using qScriptTM cDNA SuperMix (Quanta Biosciences, VWR), following manufacturer's guidelines. qRT-PCR was carried out using SYBR green master mix (Applied Biosystems, Waltham, MA) and a Step One Plus detection system (Applied Biosystems). Primers used for qRT-PCR are shown in **Table S1**. Relative quantitation of gene expression was accomplished using the comparative Ct method. Data were normalized to the most stable reference genes, as previously described (19).

Flow Cytometry

For flow cytometric analysis of VCAM-1, hCMEC/D3 cells (1×10^6 cells/ml) were seeded in 24-well plates. At confluency, cells were treated with DMSO as vehicle control or with 5 ng/ml TNFα and 5 ng/ml IFNγ for 24 h. hCMEC/D3 cells were detached from 24-well plates using 1 mg/ml collagenase type I (Sigma-Aldrich). Washed cells were incubated with mouse anti-human VCAM-1 (AbD Serotec, Kidlington, UK) for 30 min at 4°C. Binding was detected using secondary goat anti-mouse Alexa Fluor 488 (Molecular Probes, Eugene, OR). Omission of primary antibodies served as negative control. Fluorescence intensity was measured using a FACS Calibur flow cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ).

Electric Cell-Substrate Impedance Sensing (ECIS) Assay

The ECIS^{TM} Model 1600R (Applied BioPhysics, Troy, NY) was used to measure the barrier resistance (Rb) of confluent monolayers of hCMEC/D3 cells expressing non-targeting, LXRα or LXRβ shRNA. 100.000 cells were seeded onto each well of an 8W10+ ECIS array (Ibidi, München, Germany). The impedance Z [Ohm's law, potential (V)/current (I)] was measured at multiple frequencies in real-time. ECIS-Wounding was carried out with a current of 5,000 μA at 60 kHz for 20 s to measure proliferation rate of the different cell groups. All ECIS measurements were subjected to a mathematical model to calculate the component of resistance attributed to cell-cell interactions, called barrier resistance (Rb).

BBB Permeability Assay

Recombinant hCMEC/D3 cells expressing non-targeting shRNA, LXRα shRNA or LXRβ shRNA were seeded at a concentration of 100.000 cells/cm^2 onto the upper side of 0.4 μm pore-size collagen-coated Costar Transwell filters (Corning, Corning, NY) in growth medium. Paracellular permeability for FITC-dextran (70 kDa in growth medium, Sigma-Aldrich) from apical to basolateral direction was determined by collecting samples from the lower chambers after 4 h. The fluorescence intensity of the medium in the basolateral compartment was measured using a FLUOstar Galaxy microplate reader (BMG Labtechnologies, Offenburg, Germany, excitation 485 nm and emission 520 nm).

Monocyte Migration

The migrating capacity of isolated monocytes across a monolayer of hCMEC/D3 cells was determined as previously described (20). Briefly, recombinant hCMEC/D3 cells were grown to confluence onto the upper side of 0.4 μm pore-size collagen-coated Costar Transwell filters (Corning) in growth medium and were subsequently exposed to either vehicle or TNFα (5 ng/ml) for 24 h. After washing, 100 μl of primary human monocytes (1×10^6 cells/ml) was added to the upper chamber. The human blood monocytes were isolated from buffy coats of healthy donors (Sanquin, Blood Bank, Amsterdam, The Netherlands) by Ficoll gradient and anti-CD14 beads (21). Following 8 h of migration at 37°C and 5% CO_2 in air, the transmigrated monocytes were harvested and quantified using anti-CD14 beads (Flow-Count^{TM} Fluorospheres, Beckman Coulter, Brea, CA) and subsequent FACScan flow cytometer analysis (Becton). The level of migration was calculated as the percentage of migrated monocytes to total monocytes within the field.

Immunocytochemistry

Recombinant hCMEC/D3 cells expressing non-targeting shRNA, LXRα shRNA or LXRβ shRNA were grown to confluency in eight-well μ-slides (Ibidi). Cells were washed with ice-cold PBS and fixed in pre-cooled methanol for 10 min at −20°C. Fixed cells were washed and blocked with PBS containing 5% normal goat serum. Subsequently, cells were incubated overnight at 4°C with the primary antibody claudin-5 (Invitrogen). Next, cells were washed and incubated with the secondary antibody goat anti-mouse IgG Alexa Fluor 488 (Invitrogen).

Mice

Wild-type C57BL/6JOlaHsd mice were purchased from Envigo (Venray, The Netherlands). LXRα$^{-/-}$ and LXRα loxP/loxP mice on a C57BL/6 background were kindly provided by prof. dr. J.Å. Gustafsson (University of Houston, Houston, USA) (22). Cdh5(PAC)-creERT2 transgenic mice on a C57BL/6 background were kindly provided by prof. dr. Ralf H. Adams (Max Planck Institute, Münster, Germany) (23). All animal experiments were approved by the institutional animal care and use committee of Hasselt University (protocol numbers: 201422 201615 and 201617). The generation of endothelial-specific LXRα-inducible knockout mice was established by crossing LXRα loxP/loxP mice with Cdh5(PAC)-creERT2 transgenic mice to obtain Cdh5(PAC)-creERT2+LXRαLoxP/LoxP mice (LXRα$^{flox/flox}$Cdh5-Cre$^{+/-}$ mice). LXRα loxP/loxP and Cdh5(PAC)-creERT2 littermates were used as controls. Recombination was induced by injecting 10-week-old females

intra-peritoneal with 100 μl tamoxifen [Sigma-Aldrich; 20 mg/ml in corn oil (Sigma-Aldrich)] for 5 consecutive days.

Induction and Clinical Evaluation of EAE

At the age of 11 weeks, female C57BL/6 mice were actively immunized subcutaneously with 200 μg myelin oligodendrocyte glycoprotein peptide (MOG_{35-55}) emulsified in 100 μl complete Freund's adjuvant containing 4 mg/ml Mycobacterium tuberculosis (EK-2110 kit; Hooke Laboratories, Massachusetts, USA). Directly after MOG_{35-55} immunization and after 24 h, mice were intraperitoneally injected with 100 ng (C57BL/6JOlaHsd donor mice adoptive T cell transfer) or 40 ng (endothelial specific knockout mice) pertussis toxin (EK-2110 kit; Hooke Laboratories) to induce a normal or a mild EAE, respectively. Both the control group and experimental group of one experiment received the same amount of PTX. Mice were weighed and clinically evaluated daily for neurological signs of the disease according to manufacturer's mouse EAE scoring guide: 0: no clinical symptoms; 0.5: distal tail paralysis; 1: tail paralysis; 2: mild paraparesis and ataxia; 2.5: moderate paraparesis; 3: complete paralysis of the hind legs; 4: paralysis to the diaphragm; 5: death by EAE.

T Cell Adoptive Transfer

At day 9 post-immunization, inguinal lymph nodes were isolated from wild-type C57BL/6 donor mice. Next, T cells were collected and cultured at a concentration of 7×10^6 cells/ml in stimulation medium (RPMI medium supplemented with 0.5% Penicillin-Streptomycin, 20 μM β-mercaptoethanol, 10% FCS, 1% Non-Essential Amino Acid, 1% sodium pyruvate and 20 ng/ml IL-23 (Bio-Legend, London, UK) containing 20 μg/ml MOG_{35-55}. After 2 days of incubation, activated cells were intraperitoneally injected into LXRα$^{-/-}$ acceptor mice or wild-type littermates at a density of 15×10^6 cells/ml in PBS.

Immunohistochemistry

Mice were sacrificed on day 23 post-adoptive transfer or day 36 post-immunization. Brains and spinals cords were isolated and snap frozen in optimal cutting temperature (OCT) compound. Material was sectioned using a Leica CM3050 S cryostat (Leica Microsystems, Wetzlar, Germany) to obtain 10 μm slices. Staining was performed on brain and spinal cord sections mounted on coated glass slides (Menzel Gläser Superfrost PLUS, Thermo Scientific, Braunschweig Germany). For colocalization studies, brain sections were air-dried, fixed in ice-cold methanol for 10 min at −20°C, and blocked for 30 min in 10% normal swine serum in PBS. Subsequently, sections were incubated overnight at 4°C with primary antibodies (claudin-5, VCAM-1 and rhodamine-lectin) as indicated in **Table S2**. Biotin labeled swine anti-rabbit (1:500, Dako, Agilent, Amstelveen, The Netherlands) followed by Alexa 488 labeled streptavidin (1:400, Molecular Probes) was used to detect claudin-5. Sections were incubated for 1 h with their specific secondary antibody. Finally, sections were stained with Hoechst (dilution 1:1000, Molecular Probes) to visualize cellular nuclei and mounted with Mowiol mounting medium. Representative images were taken using a Leica DM6000 microscope (20x objective, Leica Microsystems).

To study immune cell infiltration, spinal cord sections were air-dried and fixed in ice-cold acetone for 10 min at −20°C. Non-specific staining was blocked using Dako protein block (Agilent, Santa Clara, CA) for 30 min. Afterwards, sections were incubated overnight at 4°C with primary antibodies (CD3 and F4/80, **Table S2**). Secondary goat anti-rat IgG Alexa Fluor 555 (1:400, Thermo Scientific) was used to detect CD3 and F4/80. Representative images were taken using a Nikon eclipse 80i microscope (10× objective) and NIS Elements BR 3.10 software (Nikon, Tokyo, Japan).

Quantitative Analysis

Image J version 1.52c (https://imagej.nih.gov/ij/index.html) was used for quantitative analysis of the expression of claudin-5 by recombinant hCMEC/D3 cells expressing non-targeting shRNA, LXRα shRNA, or LXRβ shRNA. For the quantification of the area fraction of the double fluorescent staining of claudin-5 and VCAM-1 overlapping with lectin in control mice, LXRα$^{-/-}$ mice and LXRα$^{flox/flox}$Cdh5-Cre$^{+/-}$ mice, four pictures spanning the hippocampus were taken per animal. The amount of infiltrated immune cells, positive for CD3 and F4/80, was determined by quantitative analysis of six pictures per animal spanning the whole spinal cord.

Statistical Analysis

Data were statistically analyzed using GraphPad Prism v6 (GraphPad Software, La Jolla, CA, USA) and are reported as mean ± standard error of the mean (SEM). D'Agostino-Pearson omnibus normality test was used to test normal distribution. One-way ANOVA (three groups) with Tukey's multiple comparison correction, two-way ANOVA (four groups) with Sidak's multiple comparison correction, or two-tailed unpaired student *T*-test (two groups) were used for normally distributed data sets. The Mann-Whitney (two groups) analysis was used for non-parametric data sets. No correction, i.e., Bonferroni for multiple statistical comparisons was performed. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

RESULTS

LXRα Is Important in Maintaining BBB Integrity

To unravel the function of the LXRα and LXRβ isoforms in BBB integrity, we generated a brain endothelial cell line (hCMEC/D3) with a reduced expression of either LXRα or LXRβ. Transduction of brain endothelial cells with lentiviruses expressing LXRα- or LXRβ-specific shRNAs resulted in a reduced expression of LXRα or LXRβ, as determined by qRT-PCR. Only cells with at least 70% knockdown of expression were used in our study (**Figure S1A**). LXRα and LXRβ knockdown did not affect the proliferation rate of the endothelial cells (**Figure S1B**). ECIS analysis, used to measure transendothelial electrical resistance, was performed to determine the involvement of LXRα and LXRβ in brain endothelial cell barrier formation. The results of the one-way ANOVA showed a significant difference between the three

cell types ($p < 0.0001$) of all in **Figure 1** presented variables. Furthermore, *post-hoc* analysis revealed that knockdown of LXRα resulted in a significantly reduced barrier resistance compared to LXRβ knockdown cells ($p < 0.0001$) and the non-targeting control cells ($p < 0.0001$; **Figure 1A**). In accordance with a lower barrier resistance, the leakage of FITC-dextran was significantly ($p < 0.0001$) enhanced in LXRα deficient cells compared to cells lacking LXRβ and non-targeted control cells at 4 h (**Figure 1B**). Data on barrier formation or stability over the course of 4 h are not shown. Finally, immunocytochemical analysis revealed a decreased expression of the tight junction protein claudin-5 in LXRα-deficient cells (**Figure 1C**). In addition, quantification of the expression level of claudin-5 in the LXRα knockdown cells showed a significant decrease compared to LXRβ knockdown cells ($p < 0.01$) and non-targeting control cells ($p < 0.01$; **Figure 1D**), further strengthening the importance of LXRα in BBB function. Collectively, these findings demonstrate that LXRα, but not LXRβ, contributes to the formation of endothelial cell-to-cell junctions, thereby controlling BBB integrity.

LXRα Knockdown Increases Monocyte Migration Across the BBB

During neuroinflammation, immune activation of the BBB facilitates the migration of leukocytes into the brain. To determine the involvement of LXRα and LXRβ in BBB function under neuroinflammatory conditions, we studied the expression levels of cytokines, chemokines, and adhesion molecules known to be involved in neuroinflammation. Although endothelial cells are capable of transrepression upon LXR activation (**Figure S2A**), without activation we found no isoform-specific increase or decrease in cytokine or chemokine expression under basal or inflammatory conditions (**Figure S2B**), using qRT-PCR.

The results of the two-way ANOVA of VCAM-1 mRNA expression showed no main effect of cell type ($p = 0.06$). However, a significant main effect of inflammation ($p < 0.0001$) was present. In addition, there was no significant interaction effect between the cell type and inflammation ($p = 0.08$; **Figure 2A**). However, the results of the two-way ANOVA of the protein expression of VCAM-1 showed a significant main effect of cell type ($p < 0.0001$) and a significant

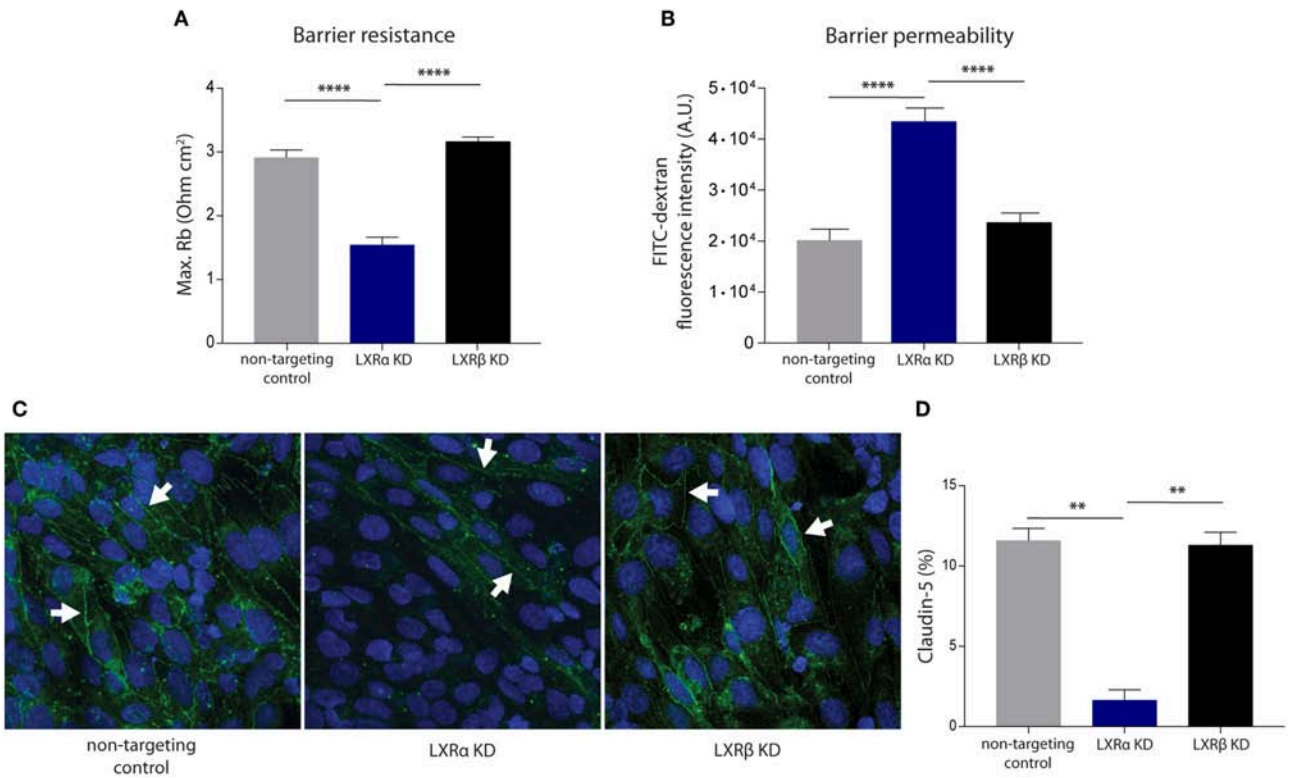

FIGURE 1 | LXRα knockdown in the endothelial cell line hCMEC/D3 decreases BBB integrity. **(A)** LXRα knockdown cells show a significantly lower intercellular adhesion (Rb) compared to LXRβ knockdown and non-targeted control cells. Data are calculated from impedance measurements (Ohm cm^2) ± SEM of two independent experiments performed in 4-fold. **(B)** Paracellular permeability of 70 kDa FITC-dextran was studied in time. LXRα knockdown cells were more permeable to FITC-dextran compared to control and LXRβ knockdown cells. Data are expressed as mean fluorescence intensity ± SEM after 4 h of three independent experiments performed in 3-fold. **(C)** Claudin-5 expression was studied using immunocytochemistry. Reduced claudin-5 expression (white arrows) was observed in LXRα knockdown cells compared to control and LXRβ knockdown cells (claudin-5; green, nuclei; blue). **(D)** Quantitative analysis of claudin-5 expression in non-targeting control, LXRα knockdown, and LXRβ knockdown cells. Statistical significance (one-way ANOVA, with Tukey's multiple comparison correction) is indicated with asterisks: $^{**}p < 0.01$, $^{****}p < 0.0001$.

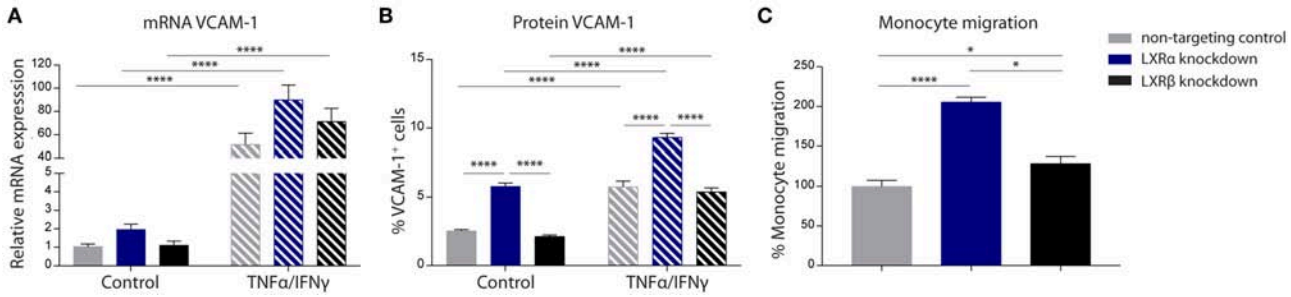

FIGURE 2 | LXRα mediates immune activation of the endothelial cell line hCMEC/D3 and transendothelial migration of monocytes. **(A)** qRT-PCR of VCAM-1 mRNA expression and **(B)** FACS analysis of VCAM-1 protein expression levels in LXRα knockdown, LXRβ knockdown, and non-targeted control cells under basal and inflammatory conditions resulted in an increase of VCAM-1 in LXRα knockdown cells on protein level. Data are expressed as mean ± SEM of three independent experiments performed in 3-fold. **(C)** Primary monocyte migration across confluent monolayers of recombinant hCMEC/D3 cells expressing non-targeting shRNA, LXRα shRNA, or LXRβ shRNA. Knockdown of LXRα increased transmigration of monocytes across the barrier. Data are expressed as mean ± SEM of % migrated cells of total monocytes of two independent experiments performed in 4-fold. Statistical significance (**A**, **B**; two-way ANOVA, with Sidak's multiple comparison correction, and **C**; one-way ANOVA, with Tukey's multiple comparison correction) is indicated with asterisks: $*p < 0.05$, $****p < 0.0001$.

main effect of inflammation ($p < 0.0001$), yet there was no significant interaction effect between the two ($p = 0.83$). *Post-hoc* analysis revealed that VCAM-1 protein expression levels were significantly increased in LXRα knockdown cells under basal ($p < 0.0001$) as well as inflammatory conditions ($p < 0.0001$) compared to LXRβ knockdown and non-targeted control cells (**Figure 2B**).

The one-way ANOVA revealed a significant difference ($p < 0.0001$) between the three cell types regarding monocyte migration. Consistent with increased VCAM-1 expression, endothelial LXRα knockdown resulted in a significantly increased migration of primary human monocytes across the endothelial barrier compared to LXRβ knockdown ($p < 0.05$) and non-targeting control ($p < 0.0001$; **Figure 2C**). Taken together, these results show that LXRα knockdown increases VCAM-1 expression on brain endothelial cells, stimulating transmigration of monocytes across the BBB.

LXRα$^{-/-}$ Worsens the Disease Score and Impairs BBB Function in a Mouse Model of Neuroinflammation

Given the importance of LXRα in maintaining a functional BBB *in vitro*, we next sought to determine whether LXRα is involved in BBB function during neuroinflammation *in vivo*. For this purpose, we made use of the experimental autoimmune encephalomyelitis (EAE) MS mouse model. Because LXRs impact T cell proliferation and differentiation (5, 24, 25), we chose a T cell adoptive transfer model in which wild-type T cells are transferred to whole-body LXRα$^{-/-}$ mice. A two-way repeated measures ANOVA showed a significant main effect of the two groups of mice ($p < 0.0001$), a significant main effect of time ($p < 0.0001$), and most importantly a significant interaction effect ($p < 0.0001$). Daily evaluation of the disease severity demonstrated an increase in EAE score in LXRα$^{-/-}$ mice compared to wild-type mice. No differences were observed in disease onset (**Figure 3A**). The increase in mean clinical score was accompanied by increased inflammatory cytokine and chemokine mRNA expression in the spinal cord of LXRα-deficient animals. In line with our *in vitro* findings, lack of LXRα resulted in increased VCAM-1 mRNA expression (**Figure 3B**). Similar, LXRα deficiency increased the mRNA expression of F4/80, suggesting elevated infiltration of peripheral myeloid cells. Immunohistochemical analysis of the spinal cord confirmed the increased infiltration of macrophages ($p < 0.05$; **Figure 3C**). In the brain, no significant difference in the expression of VCAM-1 by endothelial cells was observed (**Figure 3D**). However, LXRα$^{-/-}$ mice did show a significant decrease in claudin-5 expression ($p < 0.05$) compared to wild-type mice (**Figure 3E**). Collectively, these findings show that LXRα has a protective function during neuroinflammation.

Endothelial Specific LXRα$^{-/-}$ Aggravates Disease Progression in a Mouse Model of Neuroinflammation

To elucidate whether increased CNS infiltration of inflammatory cells in LXRα$^{-/-}$ EAE mice is due to the absence of LXRα in the endothelium of the BBB, endothelial-specific knockouts of LXRα were generated by crossing LXRα loxP/loxP and Cdh5(PAC)-creERT2 transgenic mice (LXRα$^{flox/flox}$Cdh5-Cre$^{+/-}$). No significant difference (repeated measures two-way ANOVA, Sidak's multiple comparison correction) was observed in disease score between LXRα loxP/loxP and Cdh5(PAC)-creERT2 transgenic mice over time (data not shown). Therefore, both control groups were combined for further analyses.

Endothelial knockout of LXRα resulted in a significant reduction in LXRα expression in endothelial cell isolates (**Supplementary Materials and Methods**, **Figure S3**). A two-way repeated measures ANOVA showed a significant main effect of the two groups of mice ($p < 0.0001$), a significant main effect of time ($p < 0.0001$), and most importantly a significant interaction effect ($p < 0.0001$). During a mild EAE (mean clinical score 1–2 in control animals), endothelial-specific

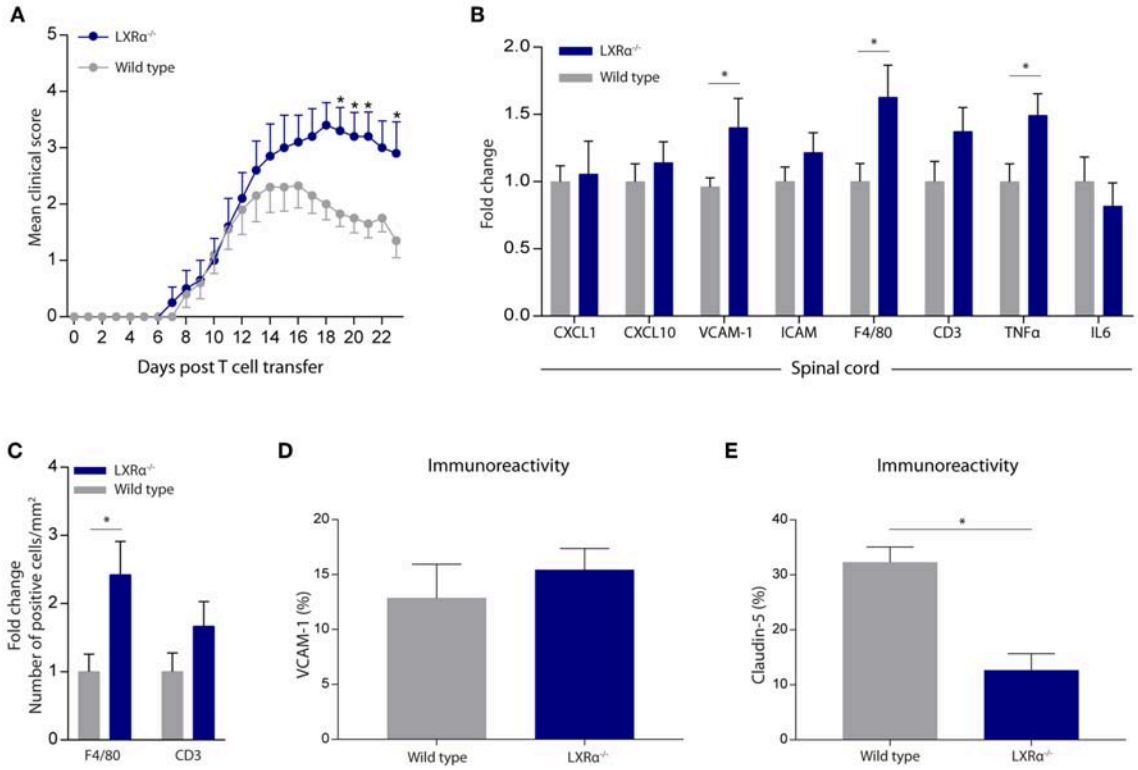

FIGURE 3 | LXRα$^{-/-}$ exacerbates the disease score in a neuroinflammatory mouse model. **(A)** Adoptive transfer EAE was induced in wild type (WT) and LXRα$^{-/-}$ acceptor mice by immunization with MOG_{35-55} activated T-cells from WT donor mice. LXRα$^{-/-}$ mice showed a higher mean clinical score during EAE compared to WT mice ($n = 10$ per group). **(B)** qRT-PCR analysis of neuroinflammatory marker expression in the spinal cord of WT ($n = 7$) and LXRα$^{-/-}$ mice ($n = 9$). **(C)** Quantitative analysis of the number of infiltrated macrophages (F4/80) and T cells (CD3) into the spinal cord of WT ($n = 8$) and LXRα$^{-/-}$ mice ($n = 7$). **(D)** Quantitative analysis of the immunoreactive area for VCAM-1 and **(E)** claudin-5 in WT ($n = 5$) and LXRα$^{-/-}$ mice ($n = 5$). The values represent the mean ± S.E.M. Statistical significance (**A**; two-way repeated measures ANOVA, with Sidak's multiple comparison correction, **B**–**E**; Mann-Whitney *U*-test) is indicated with asterisks: $^{*}p < 0.05$.

deletion of LXRα resulted in a more severe mean clinical score compared to control animals with no difference in disease onset (**Figure 4A**). This increase in mean clinical score was associated with increased cytokine and chemokine expression in the spinal cord of endothelial-specific LXRα-deficient animals (**Figure 4B**). In addition, a significant increase in VCAM-1 mRNA expression together with enhanced F4/80 macrophage marker mRNA expression was found ($p < 0.05$; **Figure 4B**). Immunohistochemical analysis of the spinal cord showed a significantly increased migration of peripheral leukocytes (CD3, $p < 0.05$; **Figures 5A,B**). Moreover, immunohistochemical analysis of the brain tissue showed an enhanced expression of VCAM-1 ($p < 0.05$) and a decrease in claudin-5 expression ($p < 0.05$) in LXRα$^{-/-}$ mice (**Figures 6A,B**). The expression levels of VCAM-1 were similar to the expression levels of VCAM-1 in the whole-body LXRα$^{-/-}$ mice (**Figure 3D** vs. **Figure 6B**). Comparing claudin-5 expression levels between whole-body and endothelial specific LXRα$^{-/-}$ mice revealed a significant higher expression of claudin-5 ($p < 0.01$) in the latter group (**Figure 3E** vs. **Figure 6B**). These results demonstrate that LXRα deficiency in endothelial cells aggravates the disease course in a mouse model of neuroinflammation.

DISCUSSION

The BBB is a highly specialized structure essential for CNS homeostasis. In this study, we determined the effect of both LXR isoforms on BBB integrity during neuroinflammation. Our experiments performed *in vitro* show that mainly LXRα, and not LXRβ, is important in maintaining proper barrier function. In addition, under neuroinflammatory conditions LXRα knockdown resulted in increased VCAM-1 expression by the endothelial cells, which was accompanied by an increase in monocyte migration across the barrier. Moreover, our *in vitro* findings were confirmed *in vivo* where endothelial specific knockout mice under neuroinflammatory conditions showed a higher disease score, increased peripheral leukocytes extravasation into the spinal cord, together with higher VCAM-1 expression and lower claudin-5 expression in the brain compared to control mice.

Our results demonstrate a difference in function between the LXRα and LXRβ isoform in brain endothelial cells. Even though LXRα is present in lower levels than LXRβ in the endothelial cells in mice and in the hCMEC/D3 cell line used in our experiments, we still observe a significant effect after knockdown of LXRα on

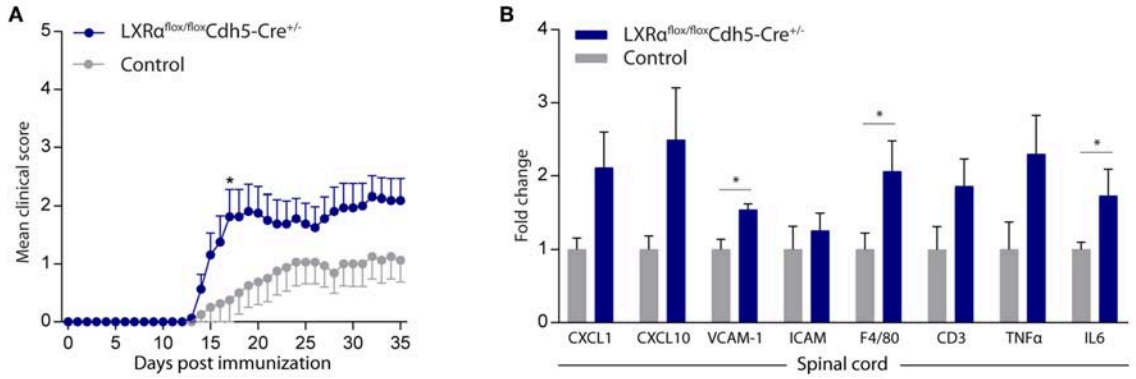

FIGURE 4 | Endothelial specific knockout of LXRα worsens disease progression in a neuroinflammatory mouse model. **(A)** Mild EAE (mean clinical score 1–2 in control animals) was initiated in a cohort of 11-week-old Cdh5(PAC)-creERT2+LXRαLoxP/LoxP mice ($LXR\alpha^{flox/flox}Cdh5\text{-}Cre^{+/-}$). LXRα loxP/loxP and Cdh5(PAC)-creERT2 transgenic mice were used as controls. $LXR\alpha^{flox/flox}Cdh5\text{-}Cre^{+/-}$ mice show a higher clinical score during the disease course compared with control mice ($n = 8$ per group). **(B)** qRT-PCR analysis of neuroinflammatory marker expression in the spinal cord of control ($n = 8$) and $LXR\alpha^{flox/flox}Cdh5\text{-}Cre^{+/-}$ mice ($n = 8$). The values represent the mean ± S.E.M. Statistical significance (**A**; two-way repeated measures ANOVA, with Sidak's multiple comparison correction, **B**; Mann-Whitney *U*-test) is indicated with asterisks: $^*p < 0.05$.

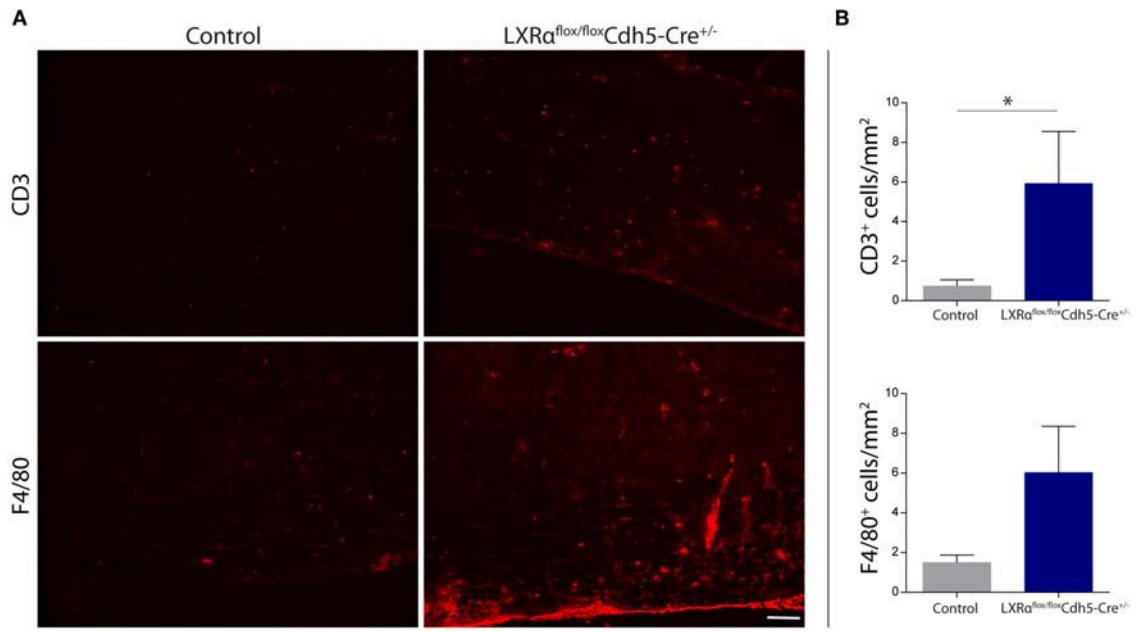

FIGURE 5 | Increased leukocyte infiltration in the spinal cord of $LXR\alpha^{flox/flox}Cdh5\text{-}Cre^{+/-}$ mice. **(A)** Immunofluorescent labeling of leukocytes (CD3, upper panel) or macrophages (F4/80, lower panel) in the spinal cord of control and $LXR\alpha^{flox/flox}Cdh5\text{-}Cre^{+/-}$ mice. Bar: 100 μm. **(B)** Quantitative analysis of the number of infiltrated macrophages (F4/80) and T cells (CD3) into the spinal cord of control ($n = 8$) and $LXR\alpha^{flox/flox}Cdh5\text{-}Cre^{+/-}$ mice ($n = 8$ per group). The values represent the mean ± S.E.M. Statistical significance (Mann-Whitney *U*-test) is indicated with asterisks: $^*p < 0.05$.

BBB integrity, indicating that LXRα is essential in the regulation of BBB function. The two LXR isoforms share high sequence homology, but differ in their tissue distribution and function. LXRα is mainly expressed in the liver, intestine, adipose tissue, and macrophages and regulates for example reverse cholesterol transport in human macrophages and bile acid metabolism in the liver. On the other hand, LXRβ is more ubiquitously expressed and is involved in processes like lipid metabolism in the CNS and water transport in the pancreas (26, 27). A large amount of research has been performed using general LXR agonists that can target both LXR isoforms, thereby neglecting the possibility that both isoforms might exert different functions (16, 28, 29). Our results indicate that it is crucial to study the individual role of the distinct isoforms in different tissues, either by developing specific agonists or by generating a specific knockout in the tissue of interest.

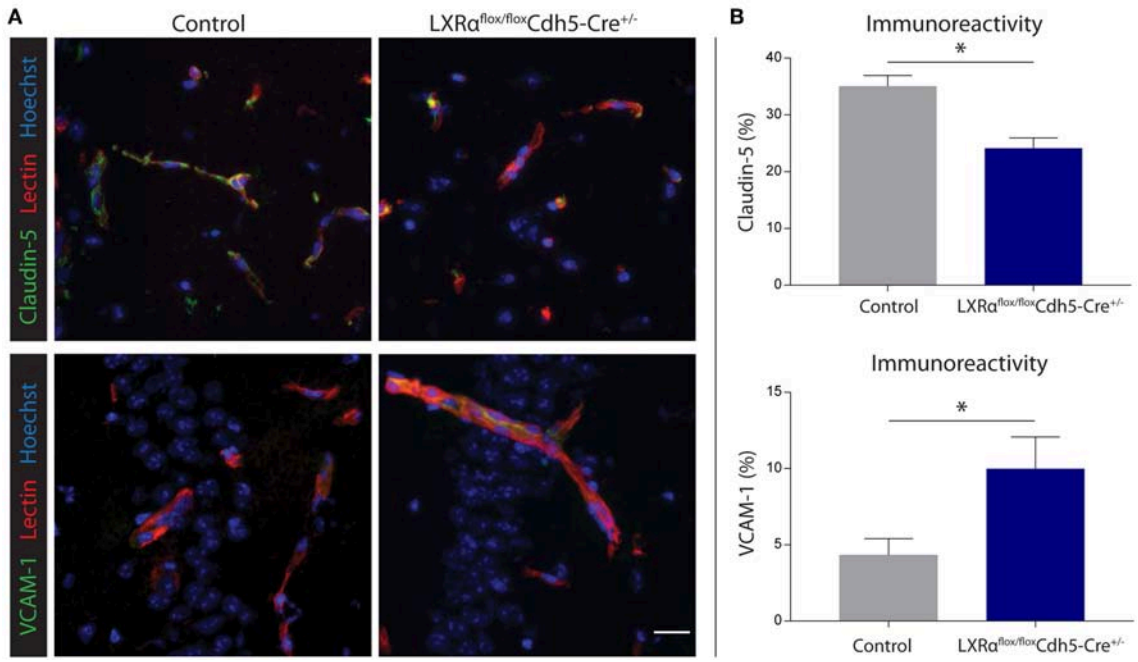

FIGURE 6 | Endothelial specific knockout of LXRα results in decreased barrier integrity. **(A)** Immunofluorescent double labeling indicated that endothelial cells (red) of LXRα$^{flox/flox}$Cdh5-Cre$^{+/-}$ mice express less claudin-5 (green, upper panel) and more VCAM-1 (green, lower panel) compared to control animals. Nuclei were counterstained with Hoechst (blue). Bar: 20 μm. **(B)** Quantitative analysis of the immunoreactive area for claudin-5 and VCAM-1 in control ($n = 5$) and LXRα$^{flox/flox}$Cdh5-Cre$^{+/-}$ mice ($n = 7$). The values represent the mean ± S.E.M. Statistical significance (Mann-Whitney *U*-test) is indicated with asterisks: *$p < 0.05$.

We further show that whole-body and endothelium specific knockout of LXRα results in a decreased barrier integrity and increased inflammatory burden in a mouse model of neuroinflammation. Although the expression levels of VCAM-1 are similar between the two groups, comparing claudin-5 expression levels revealed a significant difference, where the endothelial specific LXRα$^{-/-}$ mice show higher expression. This difference in expression level might be the result of the interaction between astrocytes and pericytes with the endothelial cells. Both cell types are important in maintaining BBB properties, and lacking LXRα might influence the functional interaction. Other studies have used synthetic agonists to investigate LXR function in the vasculature. For instance, the treatment of human umbilical vein endothelial cells (HUVECs) with GW3965, a LXR agonist, inhibited the adhesion of monocytes to endothelial cells (28). Furthermore, LXR activation by T0901317 in a mouse model of ischemic stroke selectively prevented the downregulation of occludin and ZO-1 on ischemic microvessels (16). A more recent paper demonstrated that LXR activation by GW3965 also positively modulated the microvasculature in an Alzheimer mouse model (29). However, these studies did not make a distinction between LXRα and LXRβ. Our results suggest that these beneficial processes at the endothelial level are controlled by the LXRα isoform, and not by LXRβ.

Several other studies showed that LXR activation is able to suppress EAE and reduce CNS inflammation (5, 24, 25). In these papers, the protective impact of the LXR agonists was attributed to their impact on T cell proliferation and differentiation. However, their effect on BBB function has not been described. The results of our study suggest that specifically the activation of LXRα might ameliorate the EAE disease course via regulating BBB integrity and inflammation. By maintaining BBB integrity, i.e., less VCAM-1 expression and maintaining tight junction expression, less immune cells might be able to infiltrate the brain. Interestingly, we only observed an effect on endothelial specific LXRα knockdown during a mild EAE (mean clinical score 1–2 in control animals), and not during a normal EAE (clinical score above 2—data not shown). This could partly be explained by the effect of pertussis toxin (PTX) on BBB permeability and leukocyte recruitment (30, 31). Therefore, by inducing a mild EAE using lower PTX concentration, BBB integrity changes caused by LXRα knockdown could still contribute to disease severity. Of note, Cdh5 is also expressed in some cells of the hematopoietic system, including macrophages (32), which implies that macrophages in our generated LXRα$^{flox/flox}$Cdh5-Cre$^{+/-}$ mice may also lack LXRα expression, which may influence disease to a certain extent. However, since we observe similar effects using our *in vitro* assays using brain endothelial cells that lack LXRα, we are confident that the majority of observed effects are due to the role of LXRα in the endothelium. It will be very interesting to define the impact of LXR isoform-specific agonists on BBB integrity and inflammation, once these become available. Nevertheless, it is important to take into account that hepatic LXRα activation promotes hepatic steatosis and dyslipidemia (33, 34). Therefore, targeting of LXRα via liposomes or adeno-associated viruses specific for endothelial cells would be useful.

So far, we can only speculate about the underlying pathways. One possible mechanism that could contribute to the observed effects, is ABCA1 induction by LXRs. This transporter is not only important for the efflux of intracellular free cholesterol, but also has an anti-inflammatory effect in both the brain and in the peripheral circulation (35–37). Moreover, ABCA1 is able to suppress metalloproteinase-9 (MMP9) expression in the ischemic brain (38). MMP9 is an important inducer of BBB damage presumably via the degradation of tight junction proteins and basement membrane extracellular matrix proteins (39). In macrophages, the stimulation of LXRs results in decreased MMP9 expression (40). Consequently, the induction of MMP9 expression in LXRα deficient endothelial cells could result in BBB damage. Interestingly, our LXRα knockdown cells showed higher MMP9 mRNA expression (data not shown). However, further studies are needed to determine whether this pathway is responsible for the LXRα mediated effects in endothelial cells.

Another possible mechanism is a process called epithelial to mesenchymal transition (EMT), which is driven by the transcription factor Snail. During EMT, the epithelial phenotype shifts through changes in gene expression, loss of cell polarity and cell-cell adhesion, and reorganization of the cytoskeleton, ultimately leading to a more migratory and invasive phenotype (41). Interestingly, in different cancer cell lines the presence or overexpression of LXRα positively contributes to their migratory abilities and Snail expression, whereas the opposite is observed in epithelial cells, where absence of LXRα results in a higher Snail expression (42–44). EMT has also been described for (brain) endothelial cells (EndoMT) and might underlie the observed changes in the endothelial cells when LXRα is absent (45–48).

In conclusion, we show that LXRs have different roles in regulating BBB function under neuroinflammatory conditions. More specially, we demonstrate that LXRα, and not LXRβ, is needed to maintain barrier integrity. Endothelial specific knockdown of LXRα *in vitro* and *in vivo* resulted in a more permeable barrier with less tight junctions, increased expression of adhesion molecule VCAM-1, and in an increased transendothelial migration of peripheral leukocytes across the barrier. Understanding the mechanisms by which BBB permeability is regulated during neuroinflammation may help in the development of therapeutic strategies, i.e., targeted delivery or selective activation of LXRα, to prevent BBB leakage and peripheral leukocyte infiltration during the early stages of neuroinflammatory diseases.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the institutional animal care and use committee of Hasselt University. The protocol was approved by the institutional animal care and use committee of Hasselt University (protocol numbers: 201422, 201615, and 201617).

AUTHOR CONTRIBUTIONS

EW, NdW, and JV are responsible for the generation of all data. SvdP, BvhH, DS, and ML gave technical support during experiments. EW and NdW wrote the manuscript. DG provided constructs for the generation of LXR knockdown brain endothelial cells. JG and KS provided the animals. JB and TV performed the adoptive T cell transfer and supervised the research. JH and HdV helped in designing the work and provided feedback on the manuscript. All the authors have read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01811/full#supplementary-material

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Is Innate Memory a Double-Edge Sword in Alzheimer's Disease? A Reappraisal of New Concepts and Old Data

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An emergent concept in immunology suggests that innate immune system is capable to undergo non-specific long-term responses and to provide resistance by modifying the reactivity to sequential pathogen challenge. This phenomenon, named innate memory, involves epigenetic, and metabolic reprogramming of innate immune cells. Current literature shows that the innate memory process has a mainly beneficial role in host defense, but sometimes can exert detrimental effects, as common in many diseases. Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by cognitive decline and dementia. Accumulating findings demonstrate that inflammation is involved in AD pathogenesis and progression and recent genetic and functional data confirm the driving role of the innate immune component in the disease. Furthermore, AD patients show high burden of the most relevant infectious agents and up-regulation of inflammatory features in their innate immune cells, including an activated, or "primed" status of myeloid phagocytic cells in both brain and periphery, resembling trained immunity conditions. Thus, it is conceivable that AD innate cells may be firstly involved in the attempt to resolve recurrent/persistent inflammation but then acquire a trained phenotype mostly unable to maintain the immune regulation, leaving uncontrolled or sometimes supporting the progression of neurodegeneration. The present review aims to summarize evidence evoking innate immune memory mechanisms in AD, and to interpret their potential role, either protective or harmful, in disease progression. A better understanding of such mechanisms will provide a fertile ground for development of novel diagnostic, and therapeutic pathways in AD cure.

Keywords: Alzheimer's disease, trained immunity, trained potentiation, trained tolerance, innate immune cells

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INTRODUCTION

Defense is a proper function of the organism that must protect itself from external or internal noxious agents. In vertebrates, as well as in invertebrates and plants, the first protection mechanism is orchestrated by the action of innate immune cells, which cooperate to eliminate hazardous stimula (1). Innate cells are able to fight a broad range of pathogens, although with non-specific responses. They were considered immediate mediators of host resistance and inflammation in contrast with the adaptive lymphocyte–dependent immune response that is antigen specific, and

capable to provide lifelong protection against re-infection. Over time, it has been repeatedly observed that innate responses induced by exposure to one pathogen or vaccine could affect the following immune response to subsequent encounter of the same pathogen or a different one (2–4). So, the classical vision on innate immune system has been switched into the notion that it also holds a memory (5). Innate cells' memory is not related to gene rearrangements as in lymphocytes, but is a consequence of reprogramming based on gene transcription changes, epigenetic processes, and cellular metabolism (6) addressed to promote a protective response by increasing resistance to reinfection. However, under predisposing conditions, innate memory may endorse human diseases characterized by excessive inflammation, even causing a neuroinflammatory cycle in neuropathological conditions (7, 8). Inflammation is a driving force in Alzheimer's disease (AD), a progressive neurodegenerative disease leading to dementia. In particular, genomic studies have associated AD with dysregulated innate immune cells, and uncontrolled neuroinflammatory processes appear critical contributors in AD pathogenesis (9–11). Besides alterations in microglia -the brain resident innate immune cells-, blood borne cells, and peripheral inflammatory factors may play a pivotal role in AD pathogenesis and progression (12). However, the exact mechanisms by which innate cell response influences AD course is still elusive. Regardless the recent evidence suggesting a role for the innate memory status on neuroinflammation, and neurodegeneration in AD mice (13), no studies are directly addressed to evaluate innate memory pathways in clinical AD. Main goal of the present mini-review is to recapitulate the features of AD innate response consistent with a trained phenotype in patients, thus providing insights to better decipher the role of inflammation in the disease.

INNATE IMMUNE MEMORY: CELLS, RECEPTORS AND MECHANISMS

Innate and adaptive immune responses are components of the host defense integrated system. While innate immunity is considered the first, fast, and non-specific line of defense, adaptive immunity is slower, antigen-specific, and endowed with a memory that makes future responses against the specific antigen more efficient. Innate immune system is composed of different cell types primarily including mononuclear phagocytes as monocytes, macrophages and dendritic cells, but also natural killers (NK), and innate lymphoid cells (ILCs); while B and T lymphocytes are components of the adaptive system. The primary innate immune cells of the brain are microglia, mononuclear phagocytes that act as sentinel of injuries like macrophages. In adult mice and humans, brain immune cell population is a combination of the resident microglia and other phagocytes, included infiltrated monocytes who differentiate in microglia-like cells, especially during chronic injuries. Microglia have different phenotypes: under normal conditions, when resting have ramified morphology, M1 indicates classically activated, and M2 alternatively activated microglia (14). Recently, a disease-associated microglia (DAM) type has been identified (15).

Recent findings have shown that all cells of the immune system are aware of the immunological experiences and stimuli they are exposed to and have a memory, non-specific in innate and specific in adaptive system. The concept of innate memory has been validated in many innate immune cells with mechanisms that involve epigenetic and metabolic cell remodeling, in contrast to gene rearrangements as in lymphocytes. This phenomenon called trained immunity is a specific immune program triggered by pathogen-derived molecules or other danger stimuli which confers to innate cells a memory. It is a long-lasting altered inflammatory activation, making cells able to respond to subsequent stimulations either more heavily or weakly. These two opposite activities, which are generally balanced for the well-being of the organism, are named trained immunity, and tolerance or, as more recently suggested, trained potentiation and trained tolerance (16).

Actually, memory mechanisms have been well-described for monocytes-macrophages (6) and NK (17, 18). Similarly, blood derived DC could be primed by infections, give protection against subsequent challenges showing epigenetic marks (19, 20), but their trained immunity features are not fully characterized. Moreover, recent results have shown in mice that microglia are also capable of being trained. In fact, peripherally administration of inflammatory stimuli can alter long-term microglia function, due to differential epigenetic reprogramming that persist during the time influencing neuropathology later in life (13).

As mentioned, epigenetic, and metabolic reprogramming characterize the immune training of innate cells. Changes in histone marks and chromatin architecture are related to an increased, or decreased metabolism and transcriptional processing (21). These mechanisms may be also systemically induced at the level of bone marrow progenitors, and maintained in the daughter cells (22, 23), determining a specific status of innate memory that in turn could influence the general inflammatory response.

Cells of the monocyte-macrophage lineage undergo long-term functional reprogramming following activation of pattern-recognition receptors (PRRs), that detect infectious pathogen-associated molecular patterns (PAMPs), but also non-infectious damage-associated molecular patterns (DAMPs). Like PAMPs, DAMPs might act as stimuli that activate cells of the innate immune system (24). Exposure to certain PAMPs, as those that bind to toll-like receptors (TLR), and Nod-like receptors (NLR), can confer a type of immunological memory to mononuclear phagocytes that depends on the type of pathogen and its dosage. Thus, activation of NPLR3 inflammasome by PAMPs or DAMPs, promoting the maturation and secretion of IL1β and IL-18, appears as a key mediator in the potentiation process of trained immunity induced by stimuli like BCG, β-glucan, and Western-type diet (7, 23, 25). At variance, the bacterial component lipopolysaccharide (LPS) binding to TLR-4, is known to induce tolerance (26). Another study demonstrated that trained potentiation and tolerance are two opposing functional programs, depending on the nature, and concentration of engaged PRRs. Hence, engagement of NLRs (NOD2 or NOD1

receptors) induces trained potentiation, which can vanish with smaller amounts of ligands, while the engagement of TLRs with high inflammatory doses of PRR ligands induces tolerance. Conversely, low concentrations of TLR ligands in monocytes reverse tolerance to potentiation, heightening the pro-inflammatory state (27).

At first stimulus, epigenetic reprogramming occurs and involves methylation or acetylation on N-terminal histone tails, like H3K4me, H3K9ac, and H3K27ac. For instance, after β-glucan treatment, monocytes show enrichment of H3K4me3 in promoters of genes encoding the pro-inflammatory cytokine TNF-α, IL-6, and IL-18 (28–30). After stimulation, some epigenetic marks are lost and cells maintain a low inflammatory gene expression. However, some enhancers, called latent enhancer, preserve a state of mono-methylation (H3K4me1) increasing accessibility of chromatin leading to a stronger response to subsequent stimuli (31). After a second stimulus, trained cells show higher inflammatory gene expression and acquire the epigenetic signature on their regulatory regions as H3K4me3, H3K4me1, and H3K4ac. After multiple inflammatory stimuli, cells could adopt a tolerance program consisting in the lack of responsiveness, resulting in low expression of pro-inflammatory cytokines, and acquiring epigenetic markers of transcriptional silencing, as in naïve state (32).

Transcriptional changes also reflect primarily metabolic activation. In monocytes, β-glucan-induced trained immunity leads changes in cellular metabolism from oxidative phosphorylation to aerobic glycolysis, increasing the ability of innate immune cells to respond to subsequent stimuli (33). In particular, the shift in metabolism leading to increased glycolysis is dependent on the activation of mammalian target of rapamycin (mTOR) through a dectin-1-Akt-HIF-1α (hypoxia-inducible factor-1α) pathway. In addition, the epigenetic changes observed in BCG-trained monocytes are dependent on the induction of the metabolic pathways: if glycolysis or glutaminolysis is inhibited, changes in H3K4me3, and H3K9me3 at promoter sites of IL-6 and TNF-α reverse, showing a link between these two regulatory cellular processes (34). Metabolic changes influence chromatin remodeling since epigenetic enzymes use small metabolic co-factors to perform their functions, therefore metabolic shift can cause altered epigenetic signature in immune cells (35). During immune memory, immune-metabolism and gene expression are linked by the epigenetic modifications.

ALZHEIMER'S DISEASE AND INNATE IMMUNE CELLS

The sporadic form of Alzheimer's disease (AD) is the most common type of dementia diagnosed in elderly and its cure or prevention still lacks effective treatments. Main AD histopathologic hallmarks are intracellular neurofibrillary tangles of hyper-phosphorylated tau protein and extracellular aggregates (plaques) of the misfolded amyloid-β (Aβ) peptides (36). Aβ plaques are surrounded by activated glial cells releasing inflammatory mediators, hence the sustained neuroinflammatory response has emerged as a third core pathological feature of AD (37), with an acknowledged role in disease pathogenesis and evolution (38, 39). Accumulation of Aβ, resulting from its imbalanced production and/or clearance, is widely considered a critical pathogenic event that induces microglial activation prompting to a local inflammatory process that in turn leads to amyloid plaque generation. Aβ production may be also connected with antimicrobial response, further strengthening the importance of immune system in AD (40). Genome wide association studies and integrative genomic analyses of brain transcriptomes confirm that myeloid cell-specific immune genes encoding for inflammatory factors and molecules involved in the clearance of misfolded proteins are risk factors for sporadic AD (10, 41–44).

The exact role of microglia underlying AD onset and progression is still unclear. Generally, microglia hold a protective role as they can sense and clear misfolded proteins, but in AD they may acquire a dysfunctional phenotype, secreting neurotoxic cytokines, and instigating a persistent inflammatory status (45, 46). It might occur in the earliest stages of the disease, while later on microglia and brain-invading monocytes would exert a prevalent beneficial function, triggering a resolution phase, possibly perturbed (47, 48). Accordingly, the DAM microglia, identified in the brain of AD mice and patients, seems to have the potential to restrict neurodegeneration (15). Overall, microglia reactions may be influenced by duration of activation, localization, involvement of other cell types, genetic susceptibility, aging, and disease progression (11), though a PET study suggests that the extent and dynamics of beneficial or detrimental microglia activation vary among patients, rather than depending on disease stages (49). Such view, in addition to explaining the trouble in decoding microglia role in AD, appears in agreement with the potential effects of innate memory response on microglia phenotype (13), which essentially depend on the previous history of exposure to priming factors in each individual.

Inflammation in both brain and periphery may be a very early event in the AD pathogenic course (37, 50–52). Microglia are able to sense inflammatory signaling molecules originating outside the brain. Peripheral inflammation, as well as activation of blood borne innate immune cells, appears to hold relevant disease-modifying functions in AD (50, 53, 54). As shown in animal models, blood-derived myeloid cells affect AD-like neurodegeneration with protective, causative, and/or reactive effects, apparently influenced by the disease stage (55–57). Thus, when evaluating the innate cell contribution to AD pathogenic pathways, both brain resident, and circulating innate immune myeloid cells should be taken into consideration. The latter could participate in the long-lasting dysregulated AD immune response by acting either directly when recruited to brain, or indirectly through the release of soluble mediators.

The most studied blood borne innate cell populations in AD are monocytes and macrophages, which enter the brain, and modulate pathology, although with controversial roles (58–60). Regarding other types of myeloid cells, an involvement in AD neuroinflammation and neurodegeneration is proposed for dendritic cells (DCs) (61, 62) and for neutrophils, which could

cross into the brain parenchyma, and contribute to neuronal damage and cognitive decline (63). Eventually, NK may have some relevance in AD, but their contribution needs to be further clarified (64, 65).

Given this scenario, wherein AD progression is likely driven by an interplay of innate cell types, the memory status and capability of these effector cells to respond to neurodegenerative conditions by means of neuroinflammatory modulation is of primary importance to delineate the neuroprotective or damaging consequences of their activities.

EVIDENCE OF INNATE MEMORY PATHWAYS IN AD

Systemic infections, aging and chronic inflammatory conditions drive innate immune cells to undergo reshaping and they are all well-recognized risk factors of AD. In particular, Herpes simplex virus type 1, Cyto-megalovirus, Chlamydophila pneumoniae, spirochetes, Helicobacter pylori, and periodontal pathogens have been associated with AD, and cognitive decline (66–68). Some of them have been found in AD patients' brain, listed as causative factors of AD inflammatory pathways and implicated in disease pathogenesis (69–71). Emergent studies reported a microbial dysbiosis in both AD animal models and patients, which could affect Aβ amyloidosis and host innate immunity mechanisms (72), leading to a peripheral inflammatory state (73, 74). These results support the inflammatory-infectious theory of AD (75, 76). Accordingly, elevated plasma levels of LPS were previously described in AD patients (77) and recently found to progressively accumulate in AD brain in association with neuropathology, affecting gene expression (78). As reported, infectious stimuli, including bacterial or fungal cells and their components, as well as viruses, are considered potent inducers of innate immune memory, thus the role played by infectious agents in AD advocates for a reshaping of innate cell response in patients. In addition, complex human diseases with chronic inflammatory components in their etiologies, such as atherosclerosis, arthritis, diabetes and obesity, are predisposing factors for subsequent dementia (79), strengthening the link between a persistent activation of innate response and AD. Finally, aging, the most important risk factor for AD, causes inflammaging (80), a low-grade inflammation characterized by up-regulation of pro-inflammatory mediators and increased response of innate cells, possibly contributing to the pathogenesis of AD (81).

In keeping with a potentiated trained immunity status of AD innate cells, several authors have reported elevated levels of circulating pro-inflammatory cytokines and amplified inflammatory response of blood cells in AD patients. Despite some inconsistent results, meta-analysis studies confirm an overall trend of increased pro-inflammatory cytokines in AD (82, 83), and higher levels of cytokines were observed in patients with early or mild forms of AD (84, 85). Similarly, peripheral innate cells of AD patients show a "primed" state and the percentage of peripheral monocytes producing pro-inflammatory cytokines increases early in AD (86). Furthermore, AD monocytes and DCs show enhanced inflammatory phenotype in relation with symptom severity (87–89). When the disease progresses to a more severe condition, AD patients show a decrease in the levels of inflammatory markers (90, 91). Thus, at variance with the earliest phases, the depressed innate response observed in late stages of AD might reflect a condition of innate tolerance following repeated and unresolved innate stimulations.

AD microglia also show modifications that remind an adaptive process mediated by trained immunity (92). In fact, in the aging brain and more evidently in AD patients, microglia appear primed: they are activated, produce increased amounts of pro-inflammatory mediators and are more susceptible to central damage after peripheral insults (93, 94). In addition, microglia activation can be suppressed by epigenetic modulation and epigenetic changes occurring during AD may prime microglia for a later transition to the DAM phenotype (15, 95), suggesting that epigenetic mechanisms are important in microglial priming. This picture fits well with what described in an AD experimental model, where after repeated systemic challenges mimicking bacterial invasion, microglia are epigenetically reprogrammed and, depending on the persistence of triggering, they undergo either a potentiated trained immunity resulting in amplification of pro-inflammatory mediator release or an acquisition of trained tolerance characteristics, both shaping neuropathology (13).

Converging evidence point to PRR activation, histone modifications and metabolic changes of innate cells in AD progression. Specifically, misfolded proteins like Aβ are able to bind PRRs expressed by microglia and other innate myeloid cells and exert cell activation with resultant release of inflammatory mediators, ultimately contributing to disease progression and severity. Aβ, both in its soluble and fibrillary form, is able to bind a variety of receptor molecules promoting inflammation, including CD14, CD36, and TLRs (96, 97). It can also act as a DAMP and activate the inflammasome NALP3 that leads to the release of the active pro-inflammatory cytokines IL-1β and IL-18, key components of the innate immune reaction observed in AD mice brain (98). Consistently, NLRP3 inflammasome activation occurs in the brains of AD patients, and contributes to pathology in AD mice (99).

In addition to resulting activated through PRRs engagement, AD innate cells show changes in cell transcription, epigenetic, and metabolic modifications that are mostly consistent with molecular mechanisms underlying trained immunity. Increased immune activity during AD neurodegeneration appears linked to aging and environmental-driven epigenomic alteration (100) and early epigenetic changes have been described in AD patients that may contribute to disease pathology (101). For instance, a mis-localization of the epigenetic molecule H3K4me3 between the cell nucleus and the cytoplasm has been reported in early AD (102), though its functional role is still unclear. In AD-mouse model of neurodegeneration, transcriptional and epigenetic changes (including H3K4me3 and H3K27ac) have emerged by profiling chromatin state across early and late AD pathology. Changes in immune genes and regulatory regions during AD-like neurodegeneration in mouse have been found, with strong human-mouse conservation of gene expression, and epigenomic signatures, especially in innate immune cells

(103). Finally, AD innate cells are likely to undergo a raise of aerobic glycolysis, the metabolic driver of trained immunity. In fact, mTOR signaling is early increased in AD animal models (104), while TREM2-deficient mice with AD-like pathology show defective mTOR signaling, which affects ATP levels and biosynthetic pathways (105). Finally, in AD mouse model, mTOR activation and HIF-1α signaling, possibly mediated by Aβ-induced epigenetic microglial reprogramming, appear unfavorable to AD pathology (13).

POTENTIAL ROLE OF INNATE MEMORY IN AD AND CONCLUDING REMARKS

Memory status of innate immune cells is governed by the type and concentration of ligand encountered and largely depends on the personal history of exposure to damaging agents during life. Although innate memory's role is mainly protective, it may in some cases influence the course of diseases, especially in those pathological conditions having chronic inflammation as hallmark, like AD. Likely due to many different circumstances, as enhanced infectious burden, microbial dysbiosis and persistence of endogenous misfolded proteins, AD patients show evidence of innate immunity chronic activation that could lead to maladaptive responses, mainly exacerbating damaging mechanisms in the brain.

Regardless no studies have been addressed yet at evaluating innate memory responses in clinical AD, features of innate immune response in patients and animal models suggest that AD innate cells, including microglia, monocytes and DCs, may undergo a long-term functional reprogramming characterized by both potentiated and tolerant responses, possibly contributing to disease development. Under this view, we elaborated a schematic representation (**Figure 1**) according to which peripheral and cerebral innate cells of AD patients hold a memory of past stimulations that alters brain immune responses to Aβ, which in turn accumulates, contributing to the central propagation of pathological changes, and progressive clinical symptoms of AD. Since individuals are exposed to a multiplicity of different threatening and pathogenic agents during their life, a large heterogeneity in innate memory response is expected, in agreement with the high variation of microglia features, and dissimilarities in disease severity and progression among patients. Specifically, trained immunity could have potentiation features at pre-symptomatic and early times of AD progression, being characterized by enhanced release of pro-inflammatory cytokines, increased Aβ production and damaging consequences on affected brain. In contrast, at AD later stages, high concentration of persistent stimulus (e.g., Aβ) could switch the immune response toward trained tolerance. The prolonged exposure to stimulus could bring to desensitization with reduced production of inflammatory cytokines, shifting toward

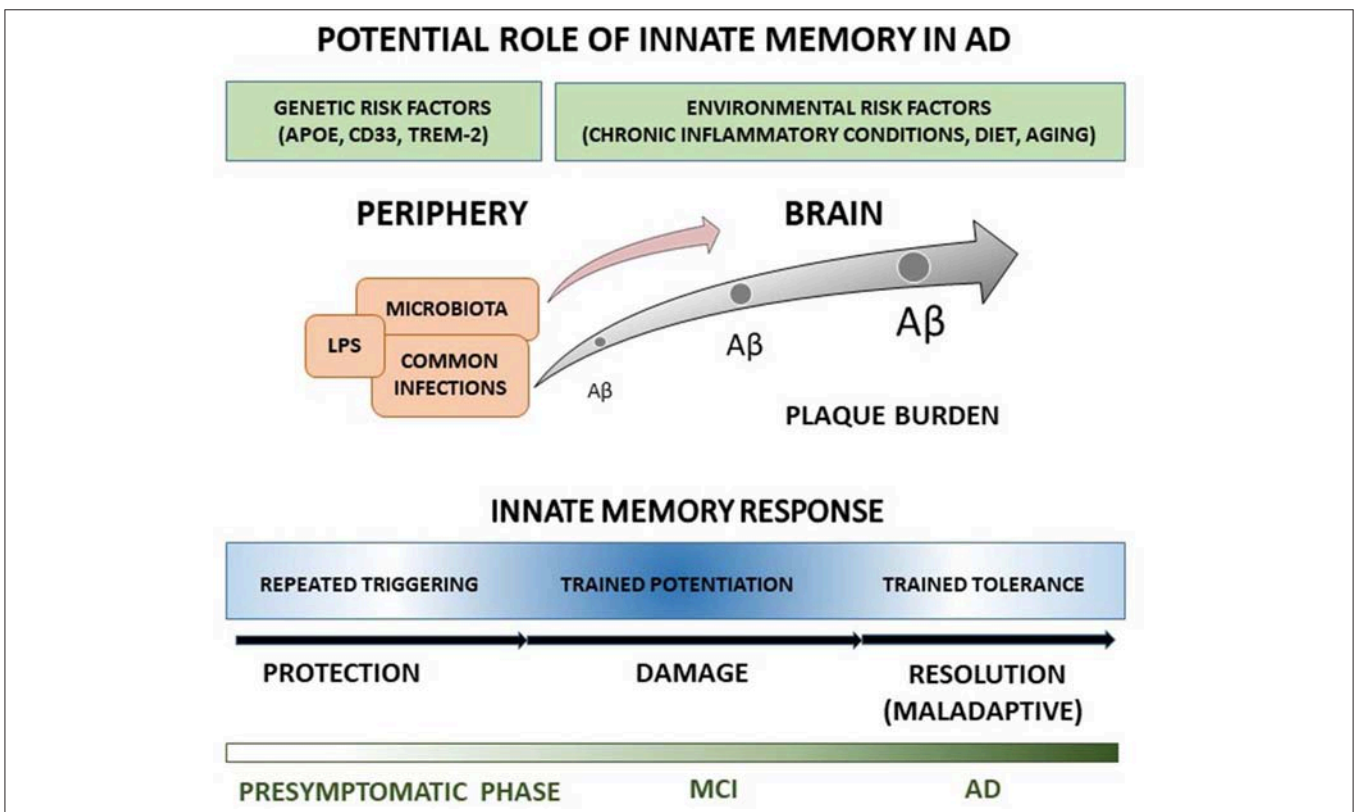

FIGURE 1 | The cartoon recapitulates our view of the potential role in AD of innate memory processes occurring during disease progression. A detailed explanation of the drawing is reported in the text.

maintenance, and repair activities, though possibly inefficacious because of the maladaptive nature of the response.

Future studies should investigate the innate memory status and its specific molecular mechanisms in *in vitro* models of both peripheral and brain innate cells of AD patients, especially in relation to disease progression. Accordingly, considering the availability of inhibitors of immune-metabolic and epigenetic pathways leading to trained immunity (7), potential new strategies for AD treatment could be envisaged. Overall, a better knowledge of innate memory processes in AD could help in deciphering patients' inflammatory mechanisms underlying pathophysiology and thus facilitating the design of personalized treatments.

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FS and PB made a substantial, direct, and intellectual contribution to the article designing and wrote the draft. VS, ES, and MG contributed to writing and revising the article. All authors have reviewed and approved the final version of the manuscript for publication.

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frontiers in Immunology

REVIEW
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Protective and Regenerative Roles of T Cells in Central Nervous System Disorders

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Pathogenic mechanisms of T cells in several central nervous system (CNS) disorders are well-established. However, more recent studies have uncovered compelling beneficial roles of T cells in neurological diseases, ranging from tissue protection to regeneration. These divergent functions arise due to the diversity of T cell subsets, particularly $CD4^+$ T cells. Here, we review the beneficial impact of T cell subsets in a range of neuroinflammatory and neurodegenerative diseases including multiple sclerosis, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, stroke, and CNS trauma. Both T cell-secreted mediators and direct cell contact-dependent mechanisms deliver neuroprotective, neuroregenerative and immunomodulatory signals in these settings. Understanding the molecular details of these beneficial T cell mechanisms will provide novel targets for therapeutic exploitation that can be applied to a range of neurological disorders.

Keywords: central nervous system, adaptive immune system, $CD4^+$ T cells, neurological disorders, regeneration

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THE ADAPTIVE IMMUNE SYSTEM

The adaptive immune system is made up of B (for bone marrow-derived) and T (for thymus-derived) lymphocytes, that have evolved to protect us from pathogens and mount a faster immune response for repeat infections against the same pathogen (1). Naïve $CD4^+$ T lymphocytes undergo differentiation into various subsets, specification of which can be influenced by expression of cytokines in the microenvironment as well as intracellular transcription factors (2). This review will focus on $CD4^+$ T lymphocytes, and to a lesser degree, $CD8^+$ T lymphocytes, and emerging regenerative roles of these cells in neurological disease. $CD4^+$ T lymphocytes can be divided into different subsets based on biological roles, transcription factor expression, and cytokine release, as summarized in **Figure 1**. Subset functional roles and characterization are reviewed in more detail in Caza and Landas (3).

CNS HOMEOSTASIS

Efficient CNS homeostasis is critical to overall health and anatomical barriers have evolved to ensure that the CNS is selectively protected from potentially harmful peripheral influences such as microorganisms, toxins, and even aberrant immune function. The choroid plexus acts as a blood-cerebrospinal fluid (CSF) barrier and is a selective gateway for leukocyte entry into the CNS (4). In the brain of healthy individuals, T cells are only present sporadically in the parenchyma and in the perivascular space (5). Approximately 150,000 T lymphocytes are present in the CSF

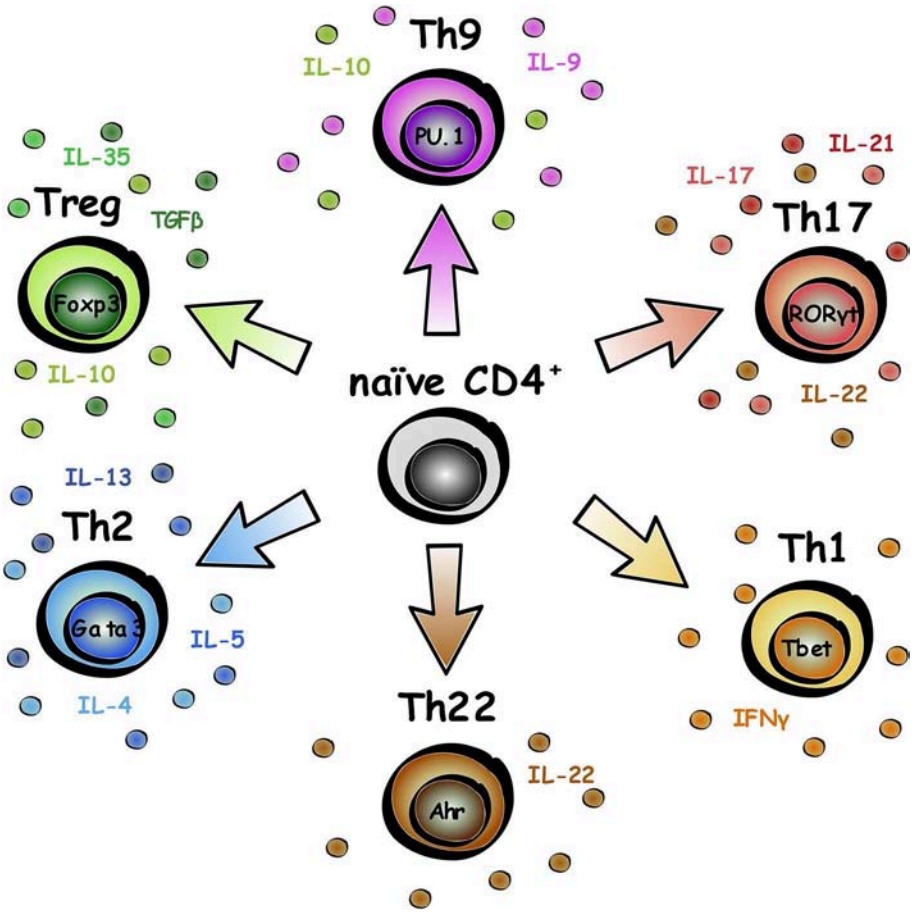

FIGURE 1 | Subsets of $CD4^+$ T lymphocytes. Naïve $CD4^+$ T lymphocytes can differentiate into a range of subsets. The key transcription factor associated with the subset is labeled in the nucleus, and typical secreted factors listed around the cell.

of healthy individuals (6) to carry out immune surveillance of the CNS and deep cervical nodes (7). In collaboration with CNS-resident immune cells, this selectivity of peripheral immune cell trafficking ensures that the CNS is afforded sufficient immune protection without being subjected to potentially harmful inflammatory responses on a regular basis.

T CELLS IN BRAIN DEVELOPMENT AND BEHAVIOR

Hippocampal neurogenesis is dependent on the adaptive immune system, and is significantly impaired in severe combined immune deficiency (SCID) mice that lack T and B lymphocytes due to a *Rag1/2* gene deletion (8). Neurogenesis impairment was found to be dependent on $CD4^+$ lymphocytes, as transplantation of $CD8^+$ lymphocytes into *Rag2*$^{-/-}$ mice (lacking T and B cells) did not rescue the effect in the same manner (9). Interestingly, mice with a T cell receptor against endogenous myelin basic protein (MBP) showed enhanced hippocampal neurogenesis and improved spatial learning in comparison to mice with a T cell receptor to non-self antigen (8).

*Rag*2$^{-/-}$ mice showed impaired learning/memory performance in the Morris water maze (MWM) task, but this impairment was not attributable to B cell deficiency as B cell-deficient mouse (μMT mice) performance was comparable to controls. This impaired MWM performance was also evident in MHC II$^{-/-}$ mice that are deficient in $CD4^+$ T cells but have $CD8^+$ T cells, suggesting that CD4 or MHC II functionality is required for normal learning and memory performance (10).

Further evidence for roles of T cells in neurodevelopment comes from studies of mice deficient in mature T cells, in both nude (lacking T lymphocytes due to a disruption in the *Foxn1* gene that causes deterioration of the thymus) and SCID mouse models. These mice had lower levels of brain-derived neurotrophic factor (BDNF), a higher number of cognitive deficits, and poor performance in the MWM. These deficits can be rescued in nude mice when T cells are repopulated by adoptive transfer from WT mice (11). *Rag*1$^{-/-}$ immunocompromised mice show increased depressive and anxiety-like behavior that

is rescued by transplanting $CD4^+$ T cells, but not $CD8^+$ T cells (12). Such findings are not restricted to murine models, as activated human T cells secrete bioactive BDNF that supports neuronal survival *in vitro* (13). Pharmacological loss-of-function studies have also provided evidence of a role for T cells in neurodevelopment. Removal of lymphocytes from the meningeal spaces in mice using fingolimod (sphingosine-1-phosphate receptor modulator) or anti-VLA4 [which attenuates the migration of T cells and monocytes across the blood brain barrier (BBB)] also resulted in impaired learning outcomes (14).

Taken together, these and a range of other studies have shown that the adaptive immune system plays important roles in CNS homeostasis and impacts behavior, but it is also very important in disease progression outcomes across neurological conditions. The regulatory T cell (Treg) subset of $CD4^+$ T lymphocytes has been shown to play a regenerative role in several tissue types, such as the kidney, skin, retina, skeletal muscle, lung, myocardium, bone, and hair follicles [reviewed in (15) and (16)]. Given the described roles of T cells in the development of the CNS, and that many regenerative processes have similar biological mechanisms to development, it is not surprising that studies are emerging showing regenerative roles of T cells in the CNS in neurological disease.

AMYOTROPHIC LATERAL SCLEROSIS

Amyotrophic Lateral Sclerosis (ALS) is an adult-onset neurodegenerative disease that is typically fatal within 3–5 years (17). Motor neurons in the motor cortex, spinal cord, and brainstem undergo cell death leading to loss of functions such as movement, coordination, and breathing. There are no disease-modifying treatments available that significantly alter or improve the course of the disease (17). ALS features neuroinflammation, but most emphasis in research has been on glial reactivity and the innate immune response (18). However, the influence of the adaptive immune system in ALS is gathering increasing attention; there are changes in the peripheral immune system and inflammatory markers that likely contribute to the pathology of the disease, but the relative importance of specific changes are yet to be fully determined (19).

A number of studies have reported increased numbers of T cells in the CNS of patients with ALS. T cell infiltrates were found in post-mortem CNS samples from ALS patients (20), and both $CD4^+$ and $CD8^+$ T cell subsets were observed in close proximity to degenerating neurons in the spinal cords of ALS patients (21). Interestingly, T cells isolated from the CSF of ALS patients appear to be clonally expanded, suggesting antigen-mediated activation in the CNS (22).

Reports of T cell populations in the peripheral blood of ALS patients remain controversial. Murdock et al. (19) found no significant difference in the number of $CD4^+$ or $CD8^+$ T cells compared to controls initially. However, disease progression correlated with decreased numbers of $CD4^+$ T cells in the blood (19). In contrast, Mantovani et al. (23) reported elevated levels of $CD4^+$ T cells in the peripheral blood of ALS patients in comparison to healthy controls. As such, the relative change in the peripheral T cell populations in ALS remains an open question.

In the SOD1 mutant mouse (SOD1mt), a model of familial ALS, lymphocyte infiltration into the CNS is observed, most prominently at later stages of the disease (24). SOD1mt mice crossed to *Rag2*$^{-/-}$ mice (deficient of B and T lymphocytes) showed a similar timing of disease onset but accelerated disease progression. These findings were recapitulated in SOD1mt mice crossed with $CD4^{-/-}$ mice (deficient of $CD4^+$ T lymphocytes). In comparison to the SOD1mt mouse, both lymphocyte-deficient mouse models showed reduced microglial activation; reduced mRNA expression of the neurotrophic factors IGF-1, GDNF, BDNF, and of the glutamate transporters GLT-1 and GLAST; reduced IL-4, TGF-β, CX3CR1, and YM1 (M2-like marker); and elevated mRNA expression of TNF-α, IL-6, and NOX2. Following bone marrow transplantation, both $CD4^+$ and $CD8^+$ T cell populations infiltrated the spinal cord in SOD1mt mice. However, $CD8^+$ T cells were only detected at the end stage of the disease, whereas $CD4^+$ T cells were already detectable at P75. Many of the biological parameters described in the lymphocyte-deficient SOD1mt mice were reversed after bone marrow transplantation which reconstitutes lymphocyte populations (25, 26). A similar study reported comparable disease onset but accelerated disease progression in a SOD1mt mouse model lacking T cells (deficiency of functional T cell receptor (TCR) using TCRβ$^{-/-}$ mice) (26). Together, these studies suggest that T cells may play a neuroprotective role in ALS through a variety of potential mechanisms.

Elevated numbers of Treg ($CD4^+CD25^+Foxp3^+$) were detected in the blood of the SOD1mt mouse model from disease onset and were sustained throughout the disease course. Foxp3, IL-4, and IL-10 mRNA was elevated in spinal cords in early disease phases, while intensity of the Foxp3 signal decreased at later disease stages suggesting Foxp3 intracellular expression was reduced. mRNA expression of the Th1-associated transcription factor, T-bet and Th1-associated cytokine, IFN-γ, but not the Th2-associated transcription factor, GATA3, were also elevated in early disease stages, suggesting an increase of the infiltration of Th1 cells but not Th2 cells. When Treg were transferred to SOD1mt/*Rag2*$^{-/-}$ mice, survival was significantly prolonged. This result correlates with disease progression data from human patients, as those ALS patients with lower numbers of peripheral Treg suffered a more rapidly progressing disease course in comparison to those with higher numbers of Treg (27).

The Treg subset of $CD4^+$ T cells is frequently studied in inflammatory and degenerative disease, given the potency of these cells to inhibit the function of proinflammatory immune cells. Sheean et al. (28) reported an inverse correlation between levels of Treg and ALS disease progression and went on to show that Treg expansion in the SOD1G93A mouse slowed disease progression and augmented survival duration. A functional dysregulation of Treg was reported in ALS patients as demonstrated by reduced suppressive capacity when tested in responder T lymphocyte proliferation assays. ALS patients with rapid disease progression had an even more profound impairment in Treg suppressive function in comparison to ALS patients with slower disease progression, associated with reduced levels of Foxp3 mRNA expression, the canonical transcription factor of Treg. Lower Foxp3 mRNA correlated with greater disease burden and reduced survival. *In vitro* expansion of

patient-derived Treg with IL-2 and rapamycin augmented the suppressive capacity of Treg (29), suggesting that Treg from ALS patients may be amenable to therapeutic modulation. A phase II trial of rapamycin is currently underway and the primary aim is to determine whether rapamycin treatment increases Treg numbers in treated patients compared with a placebo control group (30).

Another approach being taken is to administer Treg as cell therapy. In 2016, Alsuliman et al. (31) reported a method to isolate and expand good manufacturing practice-compliant Treg from ALS patients for clinical use. A Phase I clinical trial investigating treatment with infusions of autologous expanded Treg, alongside subcutaneous injection of IL-2, was carried out on three patients with ALS. The trial showed that this treatment was safe, well-tolerated and reported slowed progression of the disease, although as this was a Phase I trial, the low sample size must be noted. An increase of Treg numbers was observed after treatment and Treg suppressive function in peripheral blood was improved (32). The results from this trial provided the basis for a Phase II clinical trial with a randomized placebo-controlled group, as well as investigating treatment efficacy and safety over a longer time period, dose optimisation, and a larger study size. The Phase II trial is underway with the first patient recruited in June 2017 (MIROCALS: Modifying Immune Response and OutComes in ALS) (33).

Overall in ALS studies (summarized in **Figure 2**), the most compelling evidence from both mouse models and in human disease is that Treg appear to provide a neuroprotective effect and slow disease progression. Therefore, autologous infusions of expanded Treg with subcutaneous injection of IL-2 may prove to be an effective disease-modifying treatment in the coming years if the current clinical trial shows positive results. The intriguing studies showing detrimental effects of total lymphocyte deficiency in models of ALS suggest that other T cells may also hold therapeutic potential and certainly warrant further investigation.

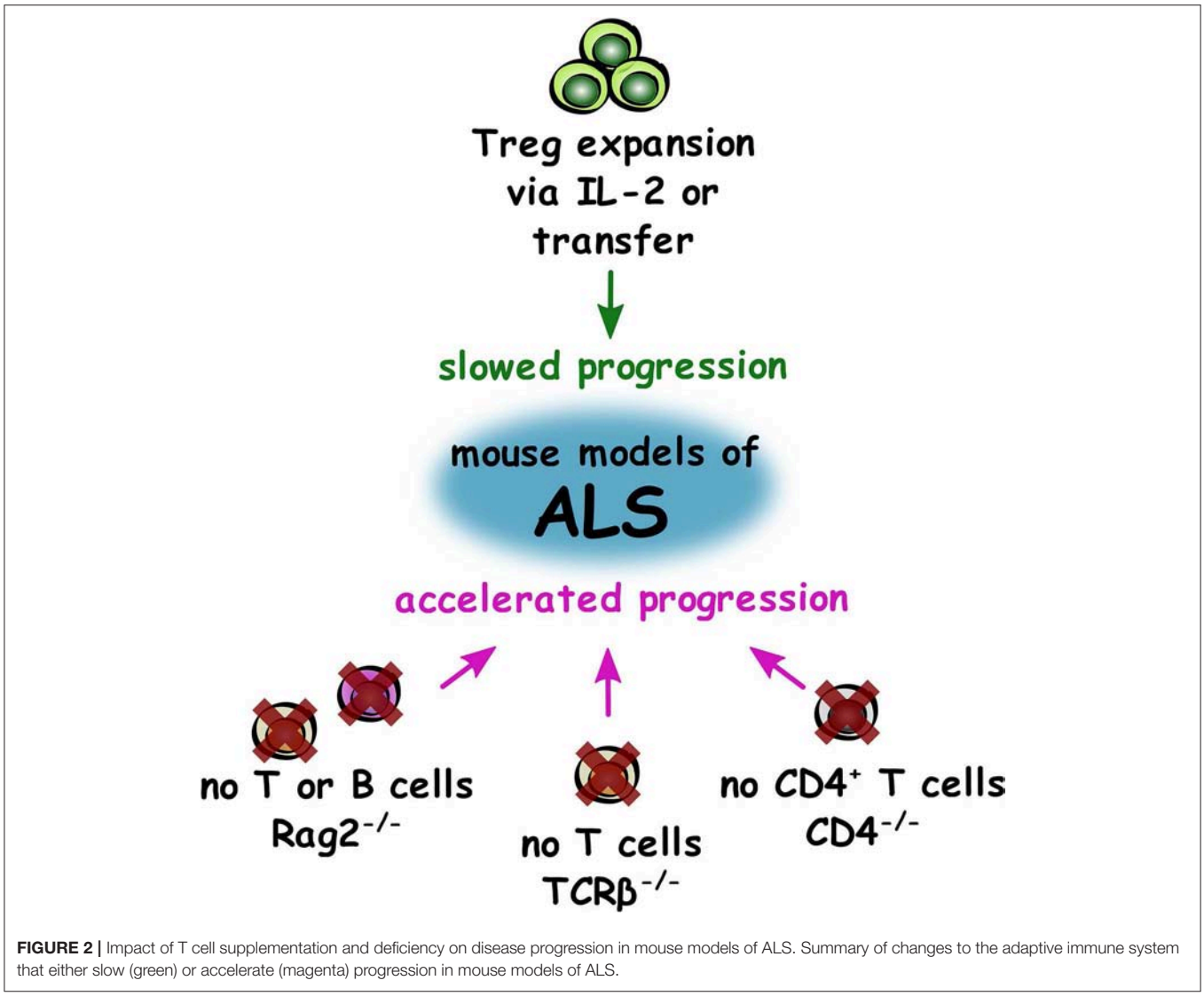

FIGURE 2 | Impact of T cell supplementation and deficiency on disease progression in mouse models of ALS. Summary of changes to the adaptive immune system that either slow (green) or accelerate (magenta) progression in mouse models of ALS.

ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is characterized by loss of neurons and synapses primarily in the cerebral cortex. This neurodegeneration causes memory loss, and in later stages can cause impairment in other capacities such as language, emotions, and behavior (34). Histopathology of AD brains shows the presence of protein aggregates in and around neurons: amyloid-beta (Aβ) plaques and neurofibrillary tangles that are aggregates of hyperphosphorylated tau (35). These features are thought to underlie neurodegeneration in AD.

Analysis of the peripheral immune system of patients with AD in comparison to healthy controls demonstrated a significant reduction of $CD3^+$ T lymphocytes. There was no significant change in the $CD4^+/CD8^+$ ratio, however a slight increase of $CD4^+$ T cells and slight decrease in $CD8^+$ lymphocytes was observed (36). Research by Larbi et al. (37) also demonstrated differences in T lymphocyte numbers in AD patients compared to healthy controls. Significantly decreased proportions of naïve T cells were observed, with elevated numbers of memory cells and $CD4^+$ T cells. $CD4^+CD25^+$ T lymphocytes, assumed potentially to be Treg, were also decreased in AD patients. A significantly increased Aβ-specific $CD4^+$ T cell intrinsic reactivity was detected in blood samples from AD patients, however this was also observed in elderly subjects, suggesting that this T cell response was associated with the aging process in general rather than with AD specifically (38). Such findings highlight the challenges inherent in studying immune function in AD in the face of advancing age, which is already known to significantly impair the diversity and functionality of the immune system.

A number of post-mortem studies have confirmed the presence of T cells in the brains of AD patients (39, 40). Research by Merlini et al. (41) demonstrated an increased frequency of T cells in the brain in advanced stages of AD, and reported that all $CD3^+$ cells were also $CD8^+$ T cells (particularly in the hippocampus). The frequency of $CD3^+$ extravascular T cells were found to correlate with tau pathology in the brains of AD patients, but not with the number of Aβ plaques. Smolders et al. (5) also observed white matter-associated T cells (primarily $CD8^+$ T lymphocytes) in the brains of AD patients. Misfolded amyloid and tau can independently lead to T cell extravasation (42, 43), but what drives this T cell infiltration is unclear as T cells were not reported to be interacting with the plaques or tangles present in transgenic mouse models of AD. Two recent studies identified a proinflammatory T cell profile in the CSF of AD patients (44) and in amnestic mild clinical impairment, an early stage of AD (45). Interestingly, polymorphisms in genes associated with antigen presentation to T cells were identified as susceptibility loci for AD (46–48), lending further support to the potential relevance of T cells in AD.

Studies in murine models demonstrate that AD-susceptible mice, known as 5xFAD mice that express five human AD-linked transgenes in APP and PSEN1, have more rapid disease progression when their adaptive immune system is genetically ablated ($Rag2^{-/-}/Il2r\gamma^{-/-}$-5xFAD mice), suggesting a protective role for adaptive immunity in the diseased brain. Neuroinflammation is greatly increased in these mice, and the microglial phenotype is skewed, causing increased cytokine production and reduced phagocytic capacity. WT bone marrow transplantation into these immunodeficient mice resulted in a 47% reduction in plaque volume and significantly reduced this AD pathology (49), demonstrating a beneficial effect of lymphocyte reconstitution in this model.

On the contrary, APP/PS1 mice that express human transgenes for mutations in APP and PSEN1, crossed with lymphocyte-deficient $Rag2^{-/-}$ mice showed less abundant fibrillar Aβ deposits and 25–30% less amyloid plaque pathology than the APP/PS1 mice at 8 months of age (50). This model also showed a decrease in GFAP immunoreactivity but similar Iba1 immunoreactivity suggesting reduced astrogliosis but no effect on microgliosis. Aged APP/PS1 mice (12 months) that were irradiated and then reconstituted with bone marrow from $Rag2^{-/-}$ donors, showed a reduction in brain Aβ load but no changes in plaque number. Elevated plasma Aβ levels were reported together with increased microgliosis, and elevated numbers of plaque-associated Aβ phagocytosing microglia. These data suggest that the adaptive immune system modulates and dampens microglial responses to misfolded Aβ peptides (50).

The impact of T cell depletion has also been investigated in other mouse models of AD. In the Thy22-Tau mouse model of AD, which accumulate increased levels of human tau as they age and are used to study tau aggregation, T cells were depleted with a daily injection of anti-CD3 depleting antibody from 4 to 9 months of age and compared to WT controls. $CD3^+$ depletion in WT controls caused no difference in their learning/memory behavior in the Y-maze task, whereas $CD3^+$ depletion in Thy22-Tau mice rescued behavioral impairment to WT levels. T cell infiltration is not thought to be directly involved in tau proteinopathy (it does not change tau phosphorylation nor deposition), but suggests that T cell infiltration regulates inflammation through microglia and astrocytes as anti-CD3 treatment was linked to the downregulation of neuroinflammatory markers (43).

Proinflammatory T cells have a negative effect on AD pathology in mouse models of AD. APP/PS1 mice have significant infiltration of T cells in the brain, and a proportion of these cells secrete the proinflammatory cytokines IFN-γ or IL-17 (51). Adoptive transfer of Aβ-specific Th1 cells (polarized *in vitro*) into APP/PS1 mice increased microglial activation and Aβ deposition, and impaired cognitive function was reported, presumably as a result of these changes. These negative effects were attenuated by treatment with anti-IFN-γ antibody (51), suggesting that IFN-γ drove disease pathology in this model. Conversely, vaccine-induced Th1 cells with specificity for Aβ enhanced Aβ clearance, but caused encephalitis as a side effect (52), whereas Th2 cells specific for Aβ reversed cognitive decline and synaptic loss in AD-like pathology (53, 54). A clinical trial investigating Aβ vaccination (AN1792) was halted after the occurrence of meningoencephalitis in 6% of vaccinated patients. Of the 6% of patients that developed meningoencephalitis, 66% recovered in a number of weeks while the remainder had residual cognitive and neurologic impairment. The development of meningoencephalitis did not correlate with

anti-Aβ antibody titration, suggesting that meningoencephalitis was not associated with a B cell response against Aβ, but rather a detrimental response of Aβ-specific T cells. Subsequent work in animal models of AD has shown similar side effects, with meningoencephalitis that was abrogated when animals were immunized with Aβ 1–42 in particular. The authors suggested that three conditions needed to occur simultaneously for the development of meningoencephalitis: presence of Aβ in the brain, a particular genetic background that will elicit a T cell response due to a high affinity for the Aβ T cell epitope and a pro-inflammatory signal (52). This may be enhanced by the release of antigenic peptides from Aβ processing that may trigger T cell inflammatory responses (55). These severe side effects were attributed to vaccine-induced T cell responses, suggesting a detrimental role of Aβ-specific T cells in the context of AD (55–57). However, taking into account the positive results of Aβ vaccines in mouse models of AD, further investigation of development of meningoencephalitis is required to determine the role of T effector cells in this severe adverse effect.

Research investigating whether T-cell associated cytokines could be of therapeutic value in AD has shown some controversial results. Administration of IL-4 and IL-10 improved AD symptoms in mice (58, 59) through enhanced neurogenesis, improved spatial learning and reduced Aβ deposition in APP/PS1 mice (60). However, two recent studies using TgCRND8, Tg2576, and APP/PS1 AD mouse models, show a detrimental role of IL-10 in AD pathology (61, 62). Peripheral IL-2 delivery was associated with elevated Treg in the brain of APP/PS1 mice, rescuing spatial memory impairment in the MWM and impaired synaptic plasticity, and restored spine density (63). Similarly, low dose IL-2 enhanced Treg frequency and restored cognitive functions, and enhanced the number of plaque-associated microglia (64). However, it is yet to be determined if this beneficial role is due to IL-2-mediated-Treg expansion, IL-2 acting directly on IL-2 receptors present on neurons, or both. The first possibility is supported by the fact that transient early Treg depletion using anti-CD25 antibody in an APP/PS1 AD mouse model accelerated the onset of cognitive deficits without influencing Aβ deposition (64). These results are supported by studies of Baek et al. (65), who adoptively transferred $CD4^+CD25^+$ Treg into 3xTg-AD mice and observed improved cognitive function, reduced deposition of Aβ plaques, decreased microglial activation, and decreased production of pro-inflammatory cytokines such as IL-6, IFN-γ, or IL-17. Similarly, matrine and bee venom phospholipase A2 have been shown to invert the Th17/Treg ratio and to increase Treg proportion in AD rat and mouse models respectively. This increase in Treg led to a decrease in Aβ plaques, a decrease in microglial and T cell infiltration in the brain, and led to cognitive improvements (66, 67). These studies support the view that there may be value in using T cell-associated cytokines or Treg adoptive transfer in the treatment of AD, but caution must be exercised, and the benefit of treatment may vary based on disease stage.

In contrast, other research has found Treg to have a negative effect on AD pathology. Depletion of $Foxp3^+$ cells or pharmacological inhibition of Treg activity in 5xFAD mice induced clearance of Aβ plaques, mitigated the neuroinflammatory response, and reversed cognitive decline (68). In the 5xFAD mouse model, elevated levels of $Foxp3^+$ cells are reported in the spleen which has also been described in AD patients (69, 70). Taking into account that Treg are one key source of IL-10 (**Figure 1**), these results are supported by the fact that IL-10 caused an increased accumulation of Aβ plaques and subsequently impaired memory function (61).

The previous Treg data were partially supported by a second study by the same group. Baruch et al. (71) treated 10-month-old 5xFAD mice with advanced diseased pathology with two intraperitoneal injections of an antibody against programmed cell death protein 1 (PD-1) (PD-1 is involved in suppressing T cell inflammatory activity) or an IgG control antibody. Mice treated with the PD-1 antibody had significantly increased IFN-γ producing $CD4^+$ T cells and IFN-γ expression at the choroid plexus, as well as increased myeloid cells in the brain. PD-1 treatment reduced cognitive deficits and this reduction was maintained even 1 month post-treatment, together with reduced Aβ plaque load in the hippocampus and cortex and reduced astrogliosis. These effects were also replicated in APP/PS1 mice, supporting a neuroprotective role for CNS-specific cell mediated immunity. Even though these results are interesting and suggest potential novel treatments for AD, further validation by other groups is required.

Anti-inflammatory and immunosuppressive treatment strategies are proposed to be beneficial in AD as in other neurological conditions, however research to date has not yet produced consensus. Controversial and sometimes contradictory results following depletion of components of the adaptive immune system have emerged, which may correspond to differences in mouse models used and the timing of depletion in relation to disease onset. The differences regarding the role of Treg in AD may be due to differences in experimental design of the timing of Treg depletion: Dansokho et al. (64) depleted Treg earlier (5–6 weeks) at the start of amyloid deposition and gliosis, whereas Baruch et al. (68) depleted Treg at 4–5 months when neuropathology has developed significantly. Based on clinical trial data and inflammatory effects, modulating T cells for the treatment of AD may require improved specificity before it is safe to develop a therapeutic for humans, particularly if the divergent findings in mouse models are attributable to disease stage-specific pathogenic mechanisms.

PARKINSON'S DISEASE

Parkinson's disease (PD) is a neurodegenerative disease characterized by the formation of abnormal protein aggregates inside neurons, termed Lewy bodies and Lewy neurites, and cell death of dopamine-secreting neurons in the substantia nigra in the brain. PD typically manifests with symptoms involving motor dysfunction, but neuropsychiatric symptoms can also occur (72). Immune alterations have been reported in PD and animal models of the disease, but the relative contributions of different immune system changes are not yet fully understood.

In PD, frequencies of lymphocyte subpopulations are altered in the periphery. Saunders et al. (73) reported decreased frequencies of $CD4^+$ T cells in PD, and others have described a reduced $CD4^+$:$CD8^+$ ratio due to decreased proportions of

T helper lymphocytes and increased proportions of cytotoxic T lymphocytes (74–76). PD patients exhibit a shift toward a Th1 immune response with increased IFN-γ, reduced numbers and suppressive capacity of Treg, reduced B lymphocytes, and an increase in NK cells (73–75, 77). $CD8^+$ and $CD4^+$ T cells, but not B cells, infiltrate the brain during Parkinson's disease (PD), based on evidence from post-mortem human tissue samples and the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD (78).

MPTP-induced dopaminergic cell death was markedly attenuated in the absence of mature T lymphocytes in both *Rag1*$^{-/-}$ and *Tcrb*$^{-/-}$ immunodeficient mouse strains (78). Similar attenuation of MPTP-induced dopaminergic cell death was observed in mice lacking $CD4^+$ T cells as well as in *Rag1*$^{-/-}$ mice reconstituted with FasL-deficient splenocytes (FasL was chosen as a target to investigate $CD4^+$ T cell-mediated cytotoxicity). Mice lacking $CD8^+$ lymphocytes and *Rag1*$^{-/-}$ mice reconstituted with IFN-γ-deficient splenocytes were not protected, therefore supporting that T cell-mediated dopaminergic toxicity was almost exclusively arbitrated by $CD4^+$ T cells and required the expression of FasL but not IFN-γ (78).

On the other hand, adoptive transfer of T cells reactive against Copolymer-1 [also known as glatiramer acetate, an immunomodulatory therapy used to reduce relapse rates in multiple sclerosis (MS)] into MPTP-intoxicated mice led to accumulation of T cells in the substantia nigra, suppression of microglial activation through secretion of IL-4 and IL-10 by T cells, and increased astrocyte-associated glial cell line-derived neurotrophic factor. This adoptive transfer of T cells resulted in increased survival of nigrostriatal neurons, and this effect was negated by depletion of the donor T cells (79). In particular, Treg are also able to protect nigrostriatal neurons via cell-to-cell contact between Treg and dopaminergic neurons by a CD47-SIRPα interaction which triggers Rac1/Akt signaling *in vitro* (80). Similarly, in the MPTP mouse model of PD, adoptive transfer of activated Treg resulted in an increased protection against neurotoxicity, and prevented microglial release of reactive oxygen species *in vitro*, leading to prevention of neuronal damage (81, 82). This result was also observed when the MPTP mouse model was treated with GM-CSF-differentiated bone marrow-derived dendritic cells (BMDCs). Adoptive transfer of BMDCs attenuated neuroinflammation and induced a significant increase in Treg numbers within the $CD4^+$ T cell population leading to protection of dopaminergic neurons (83). A similar effect has also been observed in a small preliminary study performed to determine the effects of sargramostim (recombinant GM-CSF) in PD. Sargramostim was shown to protect against nigrostriatal neurodegeneration in rodent PD models (84, 85). In this preliminary study, Gendelman et al. (86) showed that sargramostatim increased the numbers of $CD3^+$ and $CD4^+$ T cells, increasing in particular the frequencies of $CD4^+CD127^{lo}CD25^{hi}$ Treg as early as 2 weeks of treatment, which then remained elevated. Moreover, even though the study was not powered to evaluate the clinical outcome in motor activities, they observed an overall improvement of the treated patients when compared to the placebo control group, which appeared to be associated with alterations in the T cell profile and changes in the pro- and anti-inflammatory gene expression profile (86).

In conclusion, these studies appear to show that depletion of $CD4^+$ T lymphocytes is beneficial in the MPTP model of PD, but this may be due to removal of the increased numbers of Th1 cells present. As adoptive transfer of Treg appeared to have neuroprotective effects, and a decrease in the numbers and suppressive capacity of Treg is impaired in PD, increasing the numbers of Treg may be beneficial for preserving dopaminergic neurons in PD (**Figure 3**).

MULTIPLE SCLEROSIS

Unlike previously discussed diseases in which the main driver of pathology is unexplained neurodegeneration, MS is an immune-mediated demyelinating disease of the CNS, causing symptoms such as visual deficits, fatigue, mobility issues, sensation issues, bladder and bowel problems, amongst others. New symptoms can be within isolated attacks known as relapses, or build up over time in progressive forms of MS. After a relapse, symptoms may resolve, but often neurological deficits remain typically in greater frequency as the disease advances (87). MS is the disease in which the greatest amount of T cell research in the CNS has been performed. Much of our knowledge of how T cells migrate into the CNS has been learned from studies of MS and through animal models of MS. The most commonly used animal model to study MS is experimental autoimmune encephalomyelitis (EAE) which is in fact, a range of models [reviewed in (88), (89)]. Although EAE studies have uncovered a number of pathogenic mechanisms of CNS tissue damage, these models have somewhat limited utility for studies of myelin regeneration as, in these models, T cells induce primary demyelination, axonal loss, and neurodegeneration. As such, distinguishing pathogenic and regenerative T cell roles is very challenging and there are reduced numbers of axons available to be remyelinated. Similarly, targeting of T cell migration and function has delivered therapeutic benefit to thousands of MS patients around the world and these patients, in turn, have helped to advance knowledge of neuroimmune functions of T cells.

A study comparing peripheral blood, CSF, and post-mortem brain samples (divided into normal appearing white matter (NAWM) and lesions) of 27 MS patients identified lower levels of $CD4^+$ T lymphocytes in comparison to $CD8^+$ T lymphocytes in all sample groups. The highest proportions of $CD4^+$ T cells were found in peripheral blood (>30% of total $CD3^+$ T cells), then CSF (>20%), and similar numbers in NAWM and lesion (approximately 10% in both). On the other hand, proportions of $CD8^+$ T cells were ∼50–60% of total $CD3^+$ numbers, and did not vary greatly between the regions examined (90).

Flow cytometric analysis of peripheral blood from MS patients and healthy controls demonstrated that MS patients have significantly lower levels of $CD4^+$ T lymphocytes and $CD3^+$ T lymphocytes, but significantly higher levels of NK T cells ($CD3^+CD16^+CD56^+$) (91). Moreover, relapsing-remitting MS patients not taking a disease-modifying therapy, with an expanded disability status score (EDSS) of 3 or higher, had significantly higher numbers of $CD4^+$IFN-γ^+ T lymphocytes and $CD8^+$IFN-γ^+ T lymphocytes in peripheral blood in

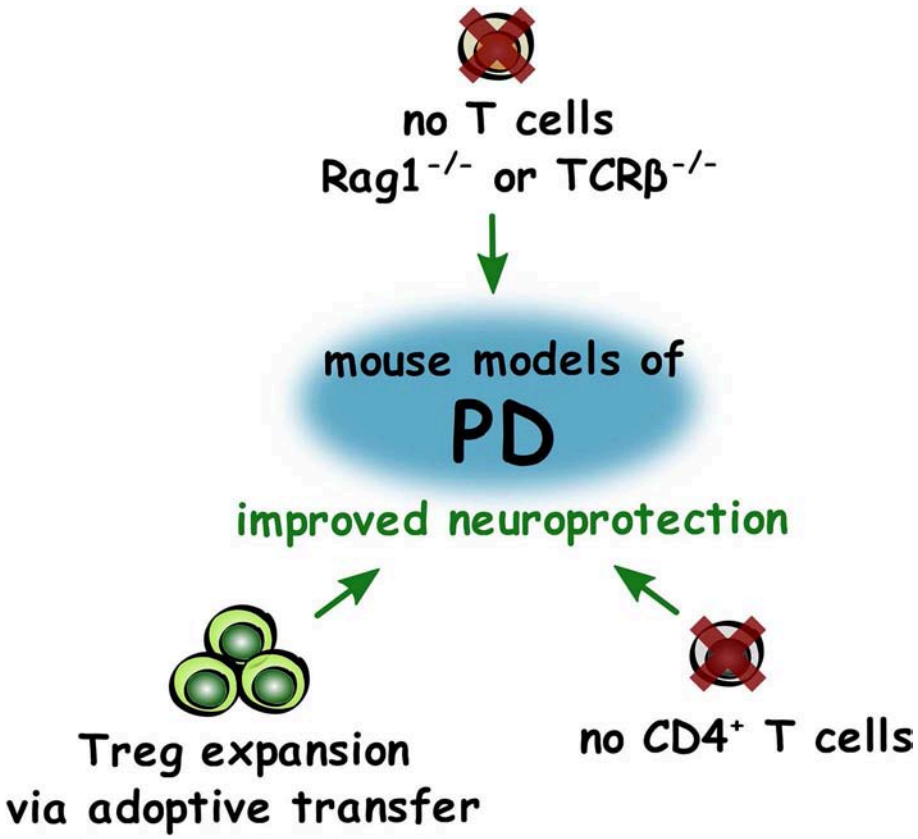

FIGURE 3 | Roles of T lymphocytes in improving neuroprotection in PD. Summary of changes to T lymphocytes that led to improved (green) neuroprotection in mouse models of PD.

comparison to healthy controls and MS patients with an EDSS below 3 (92). Haegele et al. reported a significant increase in $CD8^+$ effector memory T cells in the peripheral blood of patients with MS in comparison to healthy controls, suggesting enhanced immune activation in general. When comparing peripheral blood and the CSF of MS patients, this group reported a significant decrease of $CD4^+$ central memory T cells and $CD8^+$ effector memory T cells in the CSF compared to blood (93).

Although the level of Treg circulating in the blood are comparable in MS patients and healthy controls, it has been reported that Treg suppressive capacity is reduced in MS and that Treg have lower levels of FoxP3 expression (94–97). Furthermore, when comparing the CSF of patients with other neurological disorders to MS patients, the MS patient group has significantly lower numbers of Treg (97). These findings suggest functional impairment of Treg in MS and that trafficking to the CNS may also be altered in this disease setting.

The role of T cells in CNS regenerative responses has been investigated in settings of experimental CNS demyelination which bear relevance to MS. Using the mouse model of lysolecithin (LPC)-induced demyelination of the spinal cord, Bieber et al. (98) demonstrated that remyelination was impaired in *Rag1*$^{-/-}$ mice that lack T and B lymphocytes. They further showed that when only $CD4^+$ T cells or only $CD8^+$ T cells were depleted, remyelination was also reduced with greater impact of $CD4^+$ T cell depletion. Although this study did not show specificity in the subsets of immune cells influencing this regenerative process, it did bring to light that overall immune suppression may have negative effects on tissue regeneration in settings of demyelination such as MS.

El Behi et al. (99) investigated the differences between regenerative influences of lymphocytes from healthy donors with lymphocytes from MS patients grafted into the spinal cord of nude mice 2 days post-LPC-induced lesioning. Remyelination was significantly lower in mice that had cells grafted from MS patients in comparison to those with cells from healthy controls. This group went on to show that MS lymphocyte supernatants increased the M1/M2 ratio in pure microglial cultures *in vitro*. Conditioned media were collected from these microglial cultures, and media from microglia exposed to MS patient lymphocytes caused an increase in oligodendrocyte progenitor cell (OPC) proliferation but a decrease in OPC differentiation in comparison to media from microglia exposed to healthy control lymphocytes. This would suggest that in MS, CNS-infiltrating T cells could potentially skew the resident

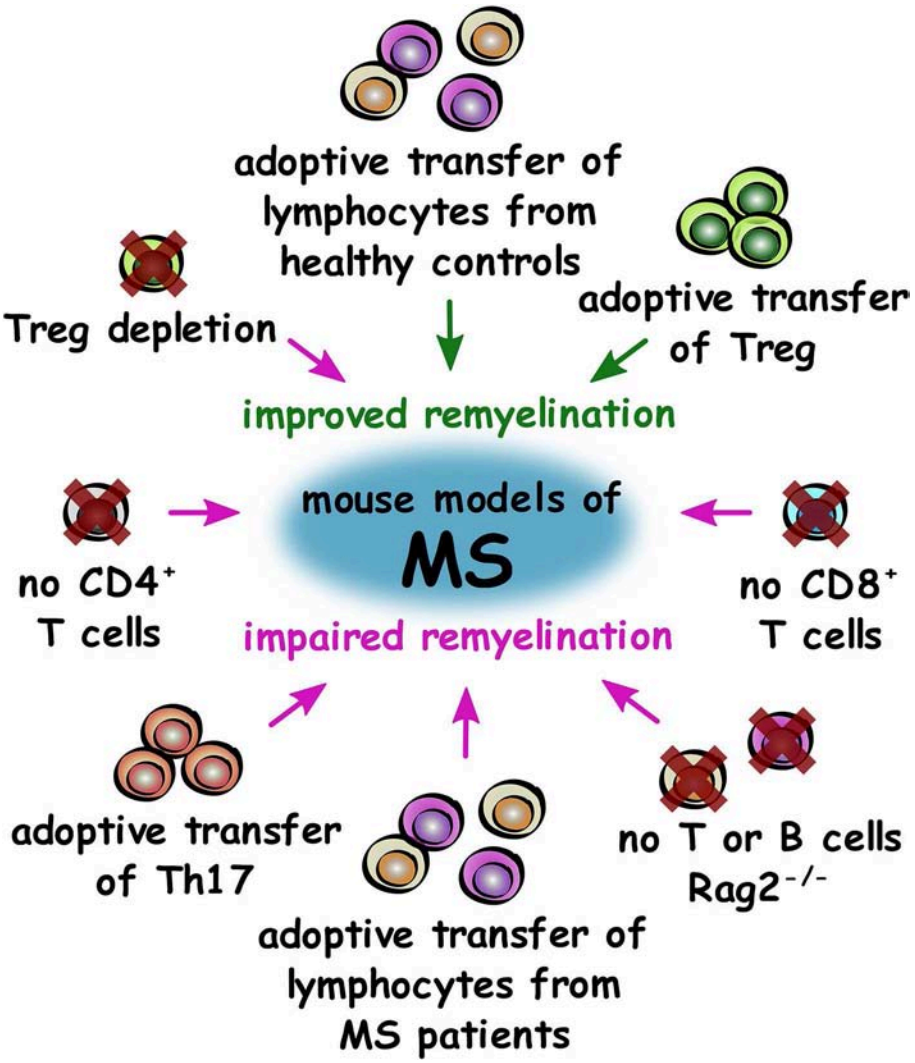

FIGURE 4 | Roles of T cell subsets in improving or impairing remyelination in mouse models of MS. Summary of changes to the adaptive immune system that either improve (green) or impair (magenta) remyelination in mouse models of MS.

microglia phenotype to negatively influence oligodendrocyte-mediated myelin regeneration.

Other studies have sought to elucidate the role of specific subsets of $CD4^+$ T cells in remyelination. To determine the effect of Th17 cells in remyelination, mice were fed with the copper chelator, cuprizone, for 4 weeks to induce demyelination and Th17 cells were adoptively transferred when the diet was switched back to normal chow. Two weeks later, mice that received Th17 cells showed significantly impaired remyelination in the corpus callosum as demonstrated by Black-Gold II staining and electron microscopy (100).

On the other hand, Dombrowski et al. (101) investigated the role of Treg in mouse models of myelination and remyelination. When Treg were depleted in Foxp3-DTR mice with LPC-induced spinal cord or cuprizone-induced demyelination, remyelination was significantly impaired. Interestingly, adoptive transfer of Treg rescued this deficiency in the LPC-induced demyelination model. Treg-conditioned media were also shown to significantly increase myelination and remyelination in *ex vivo* brain stem slice cultures, and significantly increase oligodendrocyte differentiation in both murine mixed glia and pure OPC culture models (101). These findings demonstrated a novel direct regenerative role of Treg in the CNS.

The impact of human T cells on human oligodendroglia has also been investigated. *In vitro* experiments using human fetal OPCs showed that Th1 cell supernatants had a cytotoxic effect on OPCs and caused a reduction in the number of $O4^+$ cells. Astrocyte-conditioned media from astrocytes exposed to Th1 cells also resulted in the same cytotoxicity and reduction in $O4^+$ cells effect. Th2 cells were also studied in these experiments, but showed no significant effect on cytotoxicity or numbers of $O4^+$ cells in either systems (102). This suggests that Th1

cells have a negative effect on oligodendrocyte differentiation and can cause cell death through an interaction with astrocytes, however the findings have not yet been tested in *in vivo* models of remyelination.

Overall, research in experimental models of MS so far demonstrates that the adaptive immune system is required for efficient remyelination in general. Studies have demonstrated that Treg have a pro-regenerative, beneficial role in remyelination, whereas Th17 and Th1 appear to inhibit remyelination and oligodendrocyte differentiation, respectively (**Figure 4**). Studies on more subsets of immune cells from the adaptive immune system will help to further elucidate these effects and determine if immune mechanisms may offer potential for regenerative treatments for patients.

CNS TRAUMA AND INJURY

Traumatic brain injury (TBI) is a neurological consequence of external forces to the brain, spinal cord or body. Trauma can be separated into a primary and secondary injury: the primary injury is mediated by physical force and causes mechanical tissue deformation that leads to necrotic cell death and damage to blood vessels, neurons and glia. This then triggers subsequent pathological molecular mechanisms described as the secondary injury involving amino acid release and calcium influx, and subsequent increase in free radicals, cytokines, and chemokines, mitochondrial damage, and gene expression changes. Outcomes of this injury frequently include functional neurological deficits due to vascular damage and neuronal and glial cell death (103).

Such aggressive tissue damage will induce a robust inflammatory response and appropriate regulation of this response is critical to stabilization and tissue regeneration. As such, it is likely that Treg would play a key role in this setting. A clinical study by Li et al. (104) found no significant difference in the levels of circulating Treg between controls and TBI patients, however the levels of circulating Treg were found to be significantly higher in survival vs. non-survival TBI patients. Therefore, there appears to be a correlation with the levels of circulating Treg and neurological recovery after TBI and this has been explored further using animal models.

One approach to study the role of the adaptive immune system in TBI used the controlled cortical impact (CCI) model of TBI in mice in which air or an electromagnetic-driven piston is used to penetrate the brain at a known distance and velocity (103, 105). In this model, mice treated with fingolimod, which inhibited T cell trafficking to the CNS, had decreased T and NK cell infiltration, but a higher proportion of Treg present in the CNS, as well as improved neurological functions on the rota-rod and MWM, alleviated brain edema and BBB damage (106). Although this effect may be indirect, it appears that modulation of the immune system has a positive effect on TBI outcomes.

In female mice with optic nerve injury, removal of the deep cervical lymph nodes after nerve injury reduced retinal ganglion cell survival and Treg depletion exacerbated neurodegeneration. To test whether increasing Treg numbers would have a beneficial effect, all-trans retinoic acid was administered to stabilize Treg, enhance Treg suppressive capacity, and increase Treg differentiation. Surprisingly however, this also resulted in exacerbated neurodegeneration (107). This study showed that worsening of neurodegeneration was observed in the presence and absence of Treg, suggesting that Treg may have divergent functions in different phases or types of TBI.

In a gain-of-function study, administration of MBP-specific T cells enhanced retinal ganglion cell survival after optic nerve crush injury in rats (108). Transfer of MBP-specific T cells protected injured neurons in the rat CNS from secondary degeneration due to an increased accumulation of microglia/macrophages and B cells, as well as a transient increase in neurotrophic factors, demonstrating "protective autoimmunity" in this setting (109). Later studies investigated if this mechanism was also functional without the induction of autoimmunity by using glatiramer acetate. Adoptive transfer of T cells reactive to glatiramer acetate in conjunction with glatiramer acetate treatment is also neuroprotective in optic nerve crush injury in rats (110), and treatment of rats with increased intraocular pressure with glatiramer acetate was protective of retinal ganglion cells only in the presence of T cells (111). In a model of rat spinal cord injury (SCI), transfer of MBP-activated T cells or immunization with Nogo-A and other myelin peptides prior to SCI improved recovery. This was evident as higher locomotor function score, improved tissue preservation observed through MRI, higher survival of motor neurons in the ventral horn, more residual myelination (luxol fast blue staining), and higher numbers of myelinated axons (toluidine blue staining and qualitative electron microscopy) (112–115). Tissue repair correlated with T cell accumulation, and T cell-based vaccination with MOG caused promotion of functional recovery after SCI (116). In mice overexpressing the T cell receptor for MBP, retinal ganglion cell survival was increased after optic nerve crush, whereas rats subjected to a thymectomy at birth and therefore lacking T lymphocytes, recovered poorly from this injury. Rats subjected to SCI previously, had higher retinal ganglion cell survival after optic nerve crush in comparison to those not previously subjected to SCI (117) suggesting that prior CNS injury conferred protection to subsequent trauma. After dorsal transection of the spinal cord in mice, vaccination to induce production of polyclonal antibodies to block myelin-associated inhibitors led to regeneration of a large number of axons and partial recovery of motor function. From *in vitro* testing, this was shown to be via promotion of neurite growth (118). A follow-up study comparing immunization to NogoA and myelin-associated glycoprotein (MAG), both of which are found in CNS myelin, also promoted axon regeneration and sprouting (119). These studies suggest that adaptive immune responses to myelin proteins appear to be beneficial in physical trauma in a pro-regenerative manner.

MHC $II^{-/-}$ mice contain only a small $CD4^+$ T cell population with limited TCR diversity. After optic nerve crush, these mice had less retinal ganglion cells, and after SCI, demonstrated a poorer outcome on a locomotor function scale. Adoptive transfer of WT $CD4^+$ T cells into MHC $II^{-/-}$ mice led to higher numbers of retinal ganglion cells after optic nerve crush and improved functional recovery after SCI. IL-4-producing $CD4^+$ T cells conferred neuroprotection in both these injury models and IL-4 acted on neurons to induce axonal growth by potentiating neurotrophin signaling (120).

In physical injury to the CNS, it appears that IL-4-producing $CD4^+$ T cells (assumed to be Th2 cells) and interestingly, T cells autoreactive to myelin proteins, exhibit neuroprotective influences. Interestingly, Treg appear to be neuroprotective in TBI, but do not influence the outcomes of nerve crush injury (**Figure 5**). This may suggest that the role of the adaptive immune response in CNS tissue regeneration can vary based on the type and extent of CNS injury.

STROKE AND CEREBRAL ISCHAEMIA

Stroke occurs when obstruction or lack of blood flow in the brain causes cell death. Stroke can be ischaemic, where arteries are blocked either by a clot (thrombotic) or plaque accumulation (embolic); or haemorrhagic as a result of excessive bleeding from cerebral blood vessels. One of the most commonly used rodent experimental models to study stroke and cerebral ischaemia is middle cerebral artery occlusion (MCAO). This method involves insertion of an intraluminal filament into the internal carotid artery, blocking middle cerebral artery (MCA) blood flow. This can be transient whereby the filament is removed after a period of time, permanent where the filament is left, or distal MCAO where a craniotomy is performed to cause ligation of the MCA (121).

A number of approaches have been adopted to investigate the role of adaptive immunity in these models. Administration of poly-YE (a high molecular weight copolymer shown to have immunomodulatory effects) facilitated rapid T cell recruitment and activity up to 24 h after MCAO in rats, resulting in improved neurogenesis, reduced neuronal loss, attenuation of behavioral deficits, and improved neurological performance. Poly-YE also resulted in a modulated microglial response through increased IGF-1 production (122). On the contrary, using SCID mice with permanent MCAO, lymphocyte deficiency was associated with reduced neural stem cell (NSC) apoptosis and enhanced NSC proliferation after stroke compared to immunocompetent mice. Depletion of $CD4^+$ T lymphocytes in WT mice led to enhanced NSC generation and accelerated functional recovery. However, depletion of $CD25^+$ T lymphocytes led to impaired functional recovery and partially impaired neurogenesis, suggesting a key role for Treg in recovery post-stroke (123). It is important to note

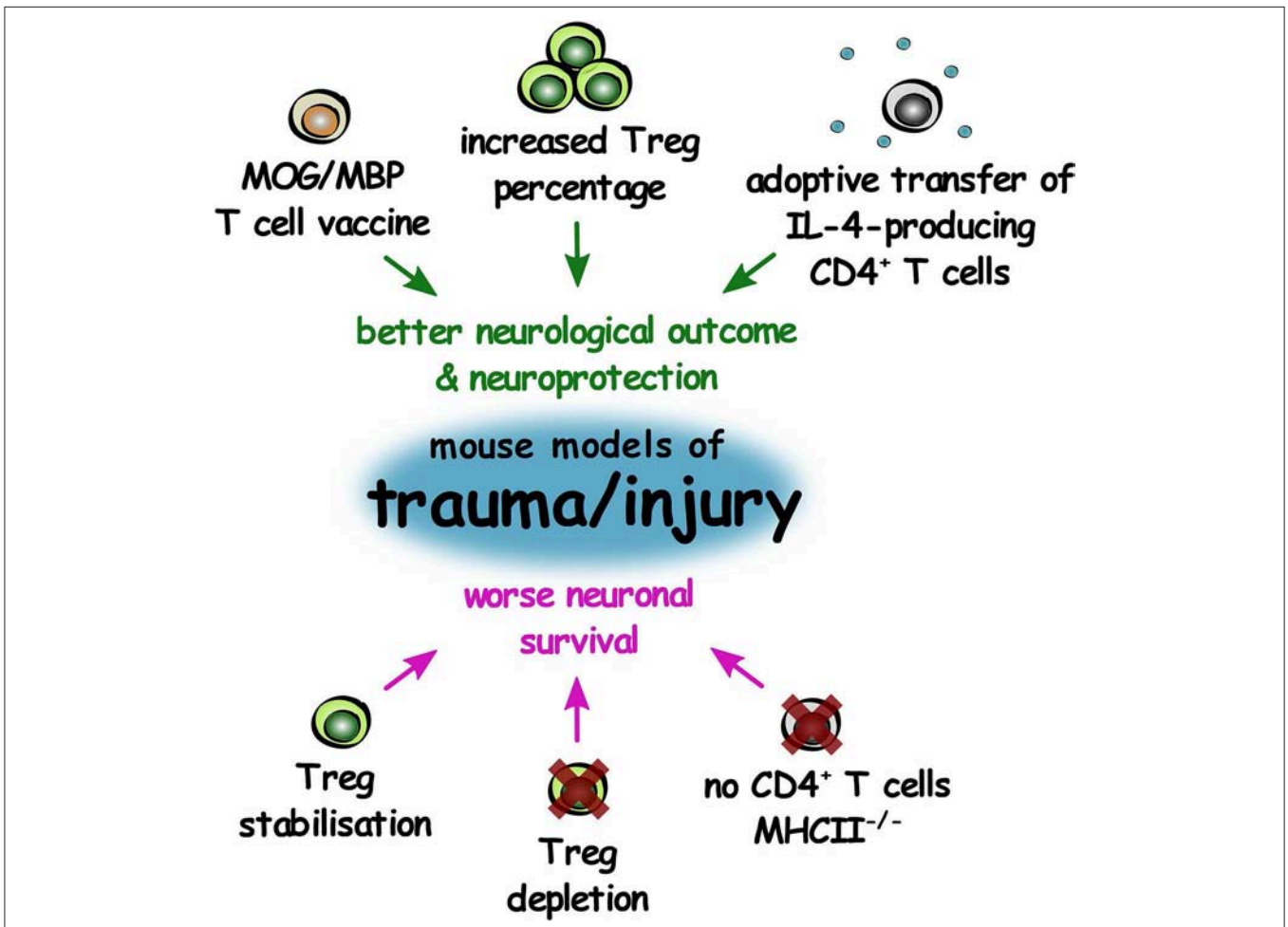

FIGURE 5 | The role of T lymphocytes in improving or worsening outcomes in mouse models of CNS trauma and injury. Summary of changes in T lymphocytes that result in either improved neurological outcome and increased neuroprotection (green), or worse neuronal survival (magenta) in mouse models of CNS trauma and injury.

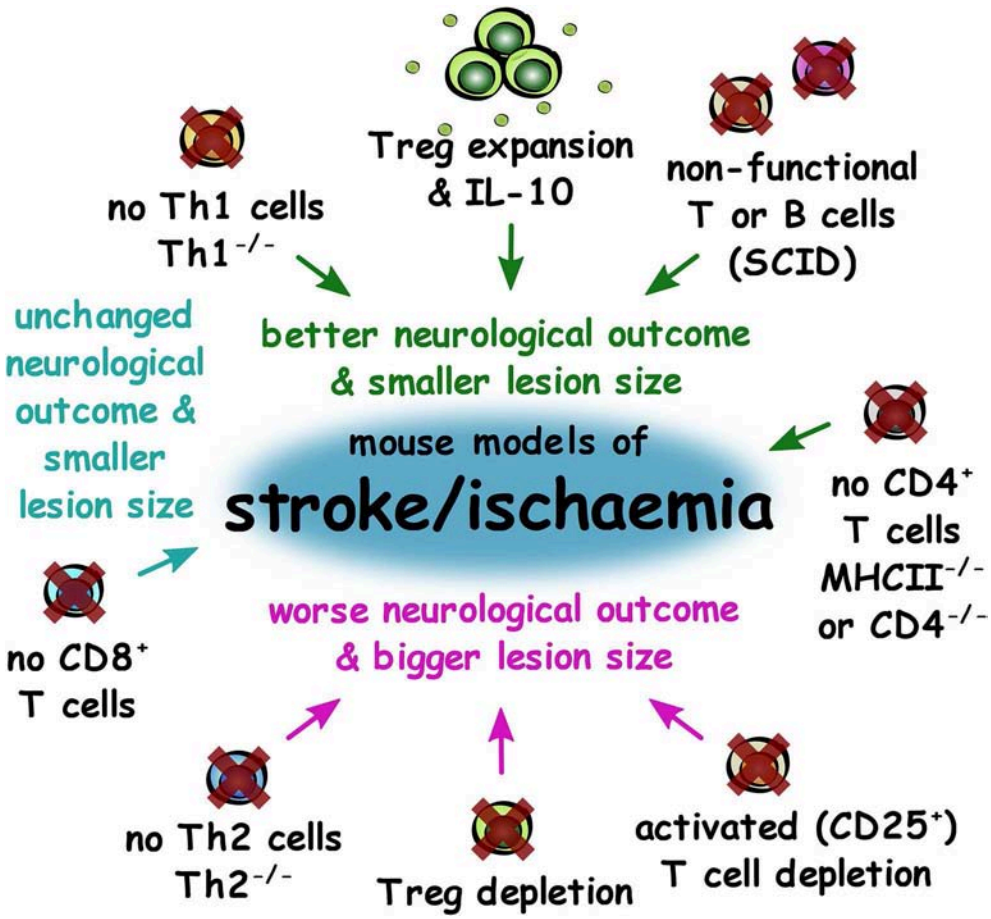

FIGURE 6 | Impact of experimental adaptive immune system changes in outcomes of mouse models of stroke and ischaemia. Summary of changes to the adaptive immune system that result in better neurological outcome and smaller lesion size (green), unchanged neurological outcome, and smaller lesion size (cyan) or worse outcome and larger lesion size (magenta) in mouse models of stroke and ischaemia.

however, that while CD25 expression is largely associated with Treg in resting conditions, during activation, effector T cells also express CD25.

Expansion of Treg *in vivo* using an IL-2/IL-2 antibody complex lead to significant reductions in infarct size, neuroinflammation, and also led to improvements in motor functions as tested by the rotarod and foot fault test. These beneficial effects were eliminated through depletion of Treg using diptheria toxin in Foxp3-DTR mice. Adoptive transfer of Treg from IL-2/IL-2 antibody complex treated mice also lead to greater neuroprotection in comparison to Treg transferred from mice treated with only isotype control antibody, suggesting an improvement in Treg immunomodulatory function (124). An influx of Treg in the brain are detectable after ischaemic stroke in mice, which significantly improves recovery from neurological deficits. These brain Treg also secrete amphiregulin to subdue astrogliosis and reduce neurotoxicity, providing a mechanism for Treg-mediated neuroprotective effects (125).

Through a range of studies it has become apparent that changes in the infarct size and neurological outcomes vary depending on the component of the adaptive immune system that is modified. Improved neurological functional outcome and smaller infarct size were observed in SCID mice (lacking B and T lymphocytes), MHC II$^{-/-}$ (CD4^{+} T cell deficiency), and JNK2$^{-/-}$ (Th1 differentiation impairment), in comparison to controls in the MCAO model. A smaller infarct size but no neurological functional difference was observed in the *Tap1*$^{-/-}$ mouse model (reduced CD8^{+} cells). A larger infarct size and worse neurological function was observed in IL-4$^{-/-}$ mice (Th2 impairment as well as other subset perturbation), and no significant effect was seen in *Ebi3*$^{-/-}$ (impairment of Treg and other subsets through IL-27/IL-35 deficiency) (126). In particular, depletion of Treg via anti-CD25 treatment increased delayed brain damage and deteriorated functional outcome (forelimb use asymmetry test and corner test). Absence of Treg caused increased activation of pro-inflammatory microglia and T cells, while treatment with IL-10 abrogated the overexpression of inflammatory cytokines (127). Interestingly this is supported by a clinical study, demonstrating that lower IL-10 plasma concentrations in acute ischaemic stroke patients is associated

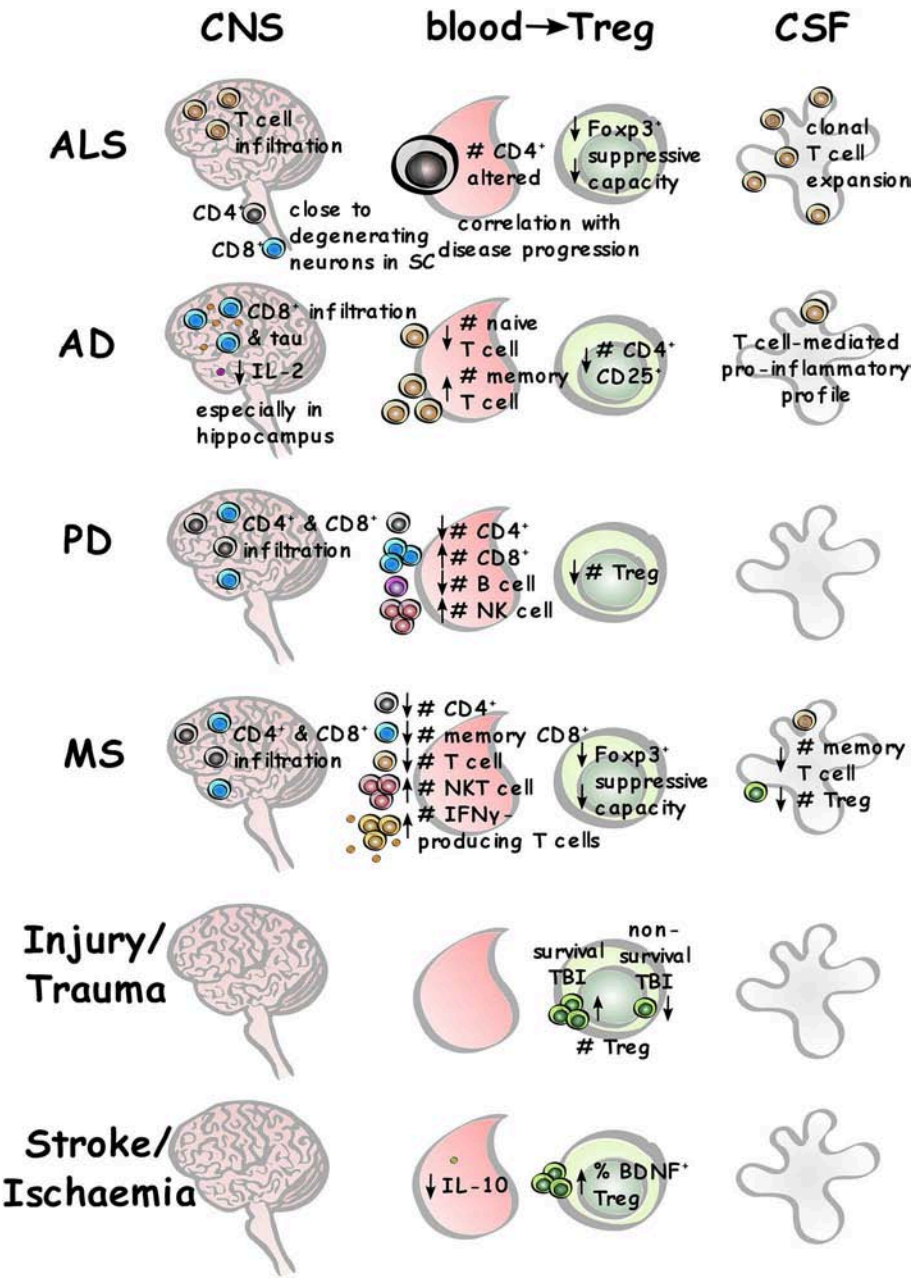

FIGURE 7 | Adaptive immune system changes across different neurological conditions. Summary of different lymphocyte populations in the CNS, blood (with specific Treg differences), and CSF across different neurological conditions where relevant and measurable.

with clinical worsening (128). Another study on human ischaemic stroke demonstrated that there is an increase in $BDNF^+$ Treg cells in stroke patients compared to healthy controls, and that those stroke patients with higher percentages of $BDNF^+$ Treg in their serum had a better neurological outcome 6 months post-stroke (129). It is important to note the limitations of the genetically altered models listed however as other immune cell subsets will also have been impacted in these models.

Similar to models of CNS trauma, it appears that autoimmunity against myelin proteins is beneficial in mouse models of stroke. Nasal vaccination of mice undergoing MCAO with MOG led to induction of $CD4^+$ IL-10-producing T cells, reduction of infarct size, and improved behavioral score (130). A follow-up study demonstrated that adoptive transfer of IL-10-producing MOG-specific $CD4^+$ T cells conferred neuroprotection after ischemic stroke (131). MCAO rats tolerized to MBP had improved neurological function and better

behavioral outcomes when they had more Treg in response to MBP as opposed to a Th1 pro-inflammatory response to MBP (132, 133).

In mice with intracerebral hemorrhage (in which blood from the tail vein was injected into the caudate nucleus), Treg depletion (using anti-CD25 or Foxp3-DTR mice) led to increased neurological deficit scores and neuronal degeneration. Conversely, when Treg were expanded using an anti-CD28 super-agonist antibody, inflammatory injury was reduced and microglia and macrophages shifted to an M2-like phenotype (134). Previous work by this group had shown that mice with transient MCAO with an activated Treg phenotype had enhanced neurogenesis (proliferation of NSCs) in the subventricular zone (SVZ) after ischaemia (135). Pre-treatment of rats with resveratrol prior to MCAO injury was found to be neuroprotective and associated with lessened neurological deficits and infarct size. This effect was attributed to expansion of Treg in the spleens and ischaemic hemisphere, an increase in IL-10 and reduction in IL-6 and TNF-α in the plasma and ischemic hemisphere (136).

Irradiation with 710 nm visible light has been used as a method to expand the total number of lymphocytes. Rats with MCAO irradiated in this way had improved neurological and step fault scores, decreased infarct sizes, higher Treg immunoreactivity, increased IL-10 mRNA expression and reduced microglial activation (137). Adoptive transfer of Treg into ischaemic rats (from transient MCAO), or expansion of Treg by anti-CD28 super-agonist treatment reduced infarct size at 3–28 days post-insult. However, there was no alteration in the rate of proliferation of $NeuN^+$, $NCAM^+$, or $CD31^+$ cells (138). Similar studies using adoptive transfer of Treg also demonstrated a reduction in inflammatory cytokines and improved immune homeostasis. BBB damage was ameliorated through PD-L1 leading to neurological improvement measured on the five point neurological scale (139–141).

Overall, depletion of components of the adaptive immune system or of total $CD4^+$ T lymphocytes led to better neurological outcomes and smaller lesion size in mouse models of stroke, but $CD8^+$ T cell depletion alone had no effect. However, Treg depletion led to worse neurological outcomes and larger lesion sizes, suggesting that it is pro-inflammatory $CD4^+$ T cells that contribute to poor outcomes in stroke (**Figure 6**).

CONCLUDING REMARKS

The studies reviewed here contribute key advances to our understanding of the role of $CD4^+$ T lymphocytes in pro-regenerative outcomes across a range of neurological conditions. Although low levels of adaptive immune cell infiltration occur in CNS homeostasis, this is increased across neurological disorders. Alterations in peripheral immune cell subsets and functions are observed in the blood of patients with neurological disease, and this will affect the infiltrates present in the CSF and CNS, particularly in diseases with BBB disruption (summarized in **Figure 7**).

Overall there are no definitive $CD4^+$ T cell subsets that are wholly beneficial or wholly detrimental across all the neurological conditions described, with the most conflicting and controversial data arising from mouse models of AD. However, lower levels or function of Treg in peripheral blood appear across all the neurological diseases reviewed, with lower suppressive capacity reported in AD and MS patient samples, and better outcomes in CNS injury when higher levels of Treg are present. Further research into these effects may help to develop better and more specific therapeutic targets, and avoid global modulation of the immune system, which can render patients immunocompromised and susceptible to a range of pathogenic threats. The emergence of regenerative functions of T cells will provide a range of new mechanisms that can be exploited for therapeutic targeting and potentially deliver the benefits of T cells in the CNS without having to deliver T cells to the CNS.

AUTHOR CONTRIBUTIONS

FE wrote the manuscript. MD designed and created the figures. FE, MD, AF, and DF contributed to literature research and editing of the manuscript.

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Setmelanotide, a Novel, Selective Melanocortin Receptor-4 Agonist Exerts Anti-inflammatory Actions in Astrocytes and Promotes an Anti-inflammatory Macrophage Phenotype

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To date, available treatment strategies for multiple sclerosis (MS) are ineffective in preventing or reversing progressive neurologic deterioration, creating a high, and unmet medical need. One potential way to fight MS may be by limiting the detrimental effects of reactive astrocytes, a key pathological hallmark for disease progression. One class of compounds that may exert beneficial effects via astrocytes are melanocortin receptor (MCR) agonists. Among the MCR, MC4R is most abundantly expressed in the CNS and several rodent studies have described that MC4R is—besides neurons—expressed by astrocytes. Activation of MC4R in astrocytes has shown to have potent anti-inflammatory as well as neuroprotective effects *in vitro*, suggesting that this could be a potential target to ameliorate ongoing inflammation, and neurodegeneration in MS. In this study, we set out to investigate human MC4R expression and analyze its downstream effects. We identified MC4R mRNA and protein to be expressed on astrocytes and observed increased astrocytic MC4R expression in active MS lesions. Furthermore, we show that the novel, highly selective MC4R agonist setmelanotide ameliorates the reactive phenotype in astrocytes *in vitro* and markedly induced interleukin−6 and −11 production, possibly through enhanced cAMP response element-binding protein (CREB) phosphorylation. Notably, stimulation of human macrophages with medium from astrocytes that were exposed to setmelanotide, skewed macrophages toward an anti-inflammatory phenotype. Taken together, these findings suggest that targeting MC4R on astrocytes might be a novel therapeutic strategy to halt inflammation-associated neurodegeneration in MS.

Keywords: multiple sclerosis, inflammation, melanocortin, melanocortin receptor-4, astrocyte, macrophage

INTRODUCTION

Multiple sclerosis (MS) is a progressive inflammatory and demyelinating disease of the central nervous system (CNS) and is one of the most common chronic neurological disease of young adults. To date, available treatment strategies are only partially effective in preventing and lack the potential to reverse progressive neurologic deterioration. In MS, evidence is increasing that astrocytes play a dominant role in the ongoing neuro-inflammation and neurodegeneration (1) and are able to aggravate inflammation as they express many factors such as complement components, cytokines, and chemokines to disrupt the blood-brain barrier, and attract immune cells into the CNS (2–6). Furthermore, reactive astrocytes are known to generate a glial scar at the lesion site, which inhibits remyelination, and axonal outgrowth (1, 7). On the other hand, astrocytes also secrete immunosuppressive molecules and exert neuroprotective properties, and the glial scar may form a physical barrier around areas of demyelination to prevent widespread tissue damage. Therefore, targeting reactive astrocytes may hold the key to counteract both ongoing neuro-inflammation as well as neurodegeneration in MS.

One class of compounds that may exert beneficial effects via astrocytes are melanocortin receptor agonists. Melanocortins, a group of highly-conserved neuropeptides that are cleaved in the pituitary gland from a common precursor, pro-opiomelanocortin (POMC), exert their action through activation of the family of G-protein coupled melanocortin receptors (MC1R-5R) (8). One of these melanocortins, alpha-melanocyte stimulating hormone (α-MSH), generally leads to weight loss due to a reduction in caloric intake and an increase in energy expenditure via MC4R activation (9, 10). Interestingly, α-MSH was also found to ameliorate disease in an animal model for MS, experimental autoimmune encephalomyelitis (EAE), by limiting inflammation in the CNS as well as in the periphery (11). Among the melanocortin receptors, MC4R is mostly expressed in the CNS including the thalamus, hypothalamus, cortex, hippocampus, and brainstem (12, 13). Besides its function in neurons, several studies have described that MC4R is functionally expressed by rodent astrocytes and, to a lesser extent, by the brains' resident macrophages, the microglia (14, 15).

Evidence is emerging that activation of MC4R in astrocytes may have potent anti-inflammatory as well as neuroprotective effects (14, 16, 17). Interestingly, selective MC4R agonists appear to be suited for the treatment of immune-mediated inflammatory diseases, without having the unfavorable side effects of more general melanocortin-related agents, such as corticosteroids (18). However, next to MC4R, α-MSH is also a full agonist for MC1R, and MC5R, which are both highly expressed in the periphery. Hence, treatment with α-MSH likely also leads to undesired peripheral side-effects. Together, this suggests that the astrocytic MC4R is a potential target to ameliorate ongoing inflammation and neurodegeneration in MS. However, to date, the expression of MC4R mRNA as well as protein in human astrocytes as well as their cellular distribution in MS brain tissue has not been studied in detail. Enhanced gene expression for the MC4R has been observed in active inflammatory MS lesions, however the cellular origin was not investigated (19). Additionally, *in vitro* evidence of the anti-inflammatory effects of MC4R activation are mainly built on LPS-induced inflammatory responses, while the inflammatory microenvironment seen in MS lesions is associated with increased cytokine levels including tumor necrosis factor alpha (TNF-α), and interferon gamma (IFN-γ) (20, 21).

In this study, we first show that MC4R mRNA is produced by astrocytes, using *in situ* hybridization chain reaction. Secondly, we identified increased astrocytic protein expression of the melanocortin receptor MC4R in active MS lesions. Furthermore, we showed that *in vitro* activation of the MC4R with setmelanotide ameliorated a reactive phenotype in astrocytes, and observed that astrocyte conditioned medium from setmelanotide stimulated astrocytes skewed macrophages toward an anti-inflammatory phenotype, which could limit ongoing damage, and eventually reduce clinical disability in MS (22, 23). Taken together, our novel findings suggest that targeting MC4R on astrocytes provide opportunities for the development of new treatments for MS.

MATERIALS AND METHODS

Brain Tissue

Brain tissue from 17 donors with clinically diagnosed and neuropathological confirmed MS [$n = 11$, all secondary progressive MS (SPMS)] or non-demented controls ($n = 6$) was obtained at rapid autopsy and immediately frozen in liquid nitrogen. All parties received permission to perform autopsies, for the use of tissue and for access to medical records for research purposes from the UK MS Society Tissue Bank, Imperial College London and the Netherlands Brain Bank. All patients and controls, or their next of kin, had given informed consent for autopsy and use of their brain tissue for research purposes. Clinical data of patients and controls are listed in **Table 1**. Active lesions were immunohistochemically characterized as lesions with abundant immune cell infiltrates and extensive myelin loss.

In situ Hybridization Chain Reaction

Human Brain Tissue

To reveal the cellular localization of MC4R mRNA in the human brain, *in situ* hybridization chain reaction (HCR) was performed on PFA-fixed brain slides from a control patient. Using *in situ* HCR, DNA probes complementary to mRNA targets carry DNA initiators that trigger chain reactions in which metastable fluorophore-labeled DNA hairpins self-assemble into tethered fluorescent amplification polymers. Sections (6 μm) were cut on a cryostat and mounted on superfrost plus glass slides (VWR international, Leuven, Belgium). Sections were stored at −80°C. RNA probes against MC4R and GAPDH (positive control) and buffers were obtained at Molecular Instruments (Los Angeles, CA, USA), and HCR was performed according the manufacturers protocol (24, 25). Sections were air-dried and epitope retrieval was done using citrate buffer pH 6.0. Then, slides were incubated with hybridization buffer for 10 min at 37°C followed by incubation with probe solution (2 pmol of probe diluted per 100 μL hybridization buffer) for 12–16 h in a humidified chamber at 37°C. Thereafter, slides were washed

TABLE 1 | Patient details.

Case	Age (years)	Gender	Post-mortem delay (h:min)	Lesion stage
Ctrl 1	64	M	18:00	NA
Ctrl 2	35	M	22:00	NA
Ctrl 3	68	M	30:00	NA
Ctrl 4	77	M	22:00	NA
Ctrl 5	72	F	7:20	NA
Ctrl 6	59	M	8:00	NA
MS 1	35	F	09:00	A
MS 2	53	F	17:00	A
MS 3	39	F	09:00	A
MS 4	77	M	04:15	A
MS 5	47	M	10:00	A
MS 6	48	F	9:20	A, CA
MS 7	66	F	6:00	CA
MS 8	51	M	11:00	CA
MS 9	43	M	26:00	CA
MS 10	40	M	27:00	CA
MS 11	50	F	22:00	CA

Clinical data of MS (all SPMS) patients and non-neurological controls. M, male; F, female; A, active lesions; CA, chronic active lesions; NA, non-applicable.

with wash buffer containing 25, 50, 75, and finally 100% SSCT (saline-sodium citrate 5X, diluted in H_2O from 20X solution + 0.1% Tween-20). Amplification was performed by incubation with amplification buffer for 30 min at RT followed by incubation with fluorophore (647) RNA hairpins (6 pmol of h1 and 6 pmol of h2 diluted in 100 µL amplification buffer) for 12–16 h in a humidified chamber at RT. Subsequently, sections were stained with GFAP antibody (1:700, Sigma-Aldrich, Saint Louis, MO, USA) for 1 h and counterstained with DAPI (1:10,000). Images were made with a Leica DM6000 fluorescent microscope.

Cell Cultures

Human astrocytoma cells (U373) stably overexpressing MC4R+ were also labeled for MC4R mRNA and compared to empty vector controls (mock). Cells plated on coverslips were fixed with 4% PFA for 10 min. Thereafter they were permeabilized overnight at 4°C with 70% ethanol. Slides were then washed with 2xSSC and incubated with RNA probes against MC4R overnight at 37°C. The next they slides were washed with probe wash buffer and subjected to hairpins for 2 h. Images were made with a Leica DM6000 fluorescent microscope and mRNA amount was analyzed using ImageJ. Slides without probe but with fluorophore hairpins served as negative controls.

Immunohistochemistry

Snap-frozen blocks of post-mortem normal control and MS brains were cut (6 µm) on a cryostat and mounted on superfrost plus glass slides (VWR international, Belgium). Sections were stored at −80°C. To determine MS lesion type, one section per case was stained for proteolipid protein (PLP, 1:500, Serotec, Kidlington, UK) and one for human leukocyte antigen–antigen D related (HLA-DR; MHCII, 1:2,000, in house antibody). Sections were defrosted at room temperature (RT), where after they were fixed in acetone for 10 min. After 3 washes with PBS, slides were incubated with antibodies for 1 h at RT. All antibodies were diluted in 1x phosphate-buffered saline (PBS) and 0.05% Tween-20. After 3 washes with PBS, slides were incubated with EnVision+ Dual Link System-HRP (Agilent DAKO, Santa Clara, CA, USA) for 30 min. 3,3′-diaminobenzidine (DAB) was used as a chromogen. Sections were counterstained with haematoxylin for 1 min and thoroughly washed with tap water for 3 min. Finally, sections were dehydrated in alcohol and xylene series and mounted with Entellan. Bright field images were taken with a Zeiss microscope (AXIO Scope A1, Carl Zeiss, Germany).

To reveal the cellular localization of MC4R, immunofluorescence triple-labeling was performed. Slides were fixed in acetone for 10 min and a blocking step was performed (30 min 10% normal goat serum). Thereafter, slides were incubated with the antibodies for MC4R (1:1,000, ab75506, Abcam, Cambridge, UK) and HLA-DR (MHCII) or biotinylated ULEX Europaeus Agglutinin1 (UEA-1) (1:1,000, Vector labs, Burlingame, CA, USA). Slides were washed and incubated with sheep-anti-rabbit alexa-488, sheep-anti-mouse-alex647 (1:400, Invitrogen, Carlsbad, CA USA), Cy3 labeled GFAP (1:700, Sigma-Aldrich), or streptavidin-647 (1:400, Invitrogen), respectively, diluted in 1x PBS/0.05% Tween-20 at RT for 1 h. After three washes with PBS, slides were incubated for 1 min with Hoechst (1:1,000) to visualize cellular nuclei and mounted with Mowiol. Z-stack images were taken with a 40x objective with a confocal microscope (Leica SP8 STED, Leica microsystems, Germany).

Cell Treatments

Human astrocytoma cells (U373) were cultured in DMEM/F12 (ThermoFisher scientific, Waltham, MA, USA) containing 10% fetal calf serum (FCS, Life Technologies), and penicillin/streptomycin (50 mg/mL; ThermoFisher scientific) in 5% CO^2 at 37°C.

U373 cells were incubated with human recombinant TNF-α and IFN-γ (both 4 ng/ml, Peprotech, London, UK). Setmelanotide (MedChemExpress, Monmouth Junction, NJ, USA) was used as a MC4R agonist and SHU9119 (Sigma-Aldrich) as a MC4R antagonist at concentrations indicated in the graphs. Cells were cultured in medium without FCS 4 h prior to the start of stimulations. Cells were treated with 0.001–10 µM setmelanotide for 1 h and thereafter with TNF-α and IFN-γ for 4 h. For blocking experiments, 10 µM SHU9119 (26) was added 30 min before addition of setmelanotide during 4 h.

Retroviral-Induced Overexpression of MC4R

Human astrocytoma cells (U373) stably overexpressing MC4R ($MC4R^+$ astrocytes) and empty vector control (Mock astrocytes) were generated by retroviral transductions. Expression vector encoding the human MC4R (pLenti-GIII-CMV-GFP-2A-Puro) was obtained from Applied Biological Materials (abm, Vancouver, Canada). HEK293FT cells were cultured in DMEM containing 10% FCS, 1% penicillin/streptomycin (50 mg/mL; ThermoFisher scientific) at 37°C in a 5% CO^2 incubator.

HEK293FT cells were transfected with calcium phosphate as a transfection reagent. Medium was refreshed 6 h post transfection. Supernatant containing virus was collected and virus was concentrated by centrifugation and stored at −80°C. For transduction of U373 cells, virus-containing supernatant was added dropwise to U373 cells in a 6 well-microplate. Virus supernatant was replaced with appropriate medium after 24 h incubation. Transduced cells were selected using puromycin (1:2,000). The effect of MC4R+ overexpression on cell viability and proliferation was assessed by CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) according to manufacturer's instructions.

RNA Isolation and Real-Time Quantitative PCR

To assess gene expression of the MCR's in human MS lesions, normal appearing white matter (NAWM) ($n = 5$), active ($n = 5$), chronic active ($n = 5$), and white matter of controls ($n = 5$) were isolated from the brains described in **Table 1**. Lesions were outlined according to their PLP and LN3 status, using a sharp needle. Thereafter, 10 μm sections were cut, and lesion area and NAWM were collected separately in tubes and kept in liquid nitrogen. Messenger RNA isolation was conducted using the Qiagen RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany) according to manufactures protocol.

Gene expression analysis in cell cultures was performed on subconfluent astrocytes in 24 well-microplates and messenger RNA was isolated using Trizol (Invitrogen) as described by the manufacturer. mRNA concentration and quality were measured using Nanodrop (Thermofisher scientific). cDNA synthesis was performed using the Reverse Transcription System kit (Promega) following manufacturers guidelines. RT-PCR was performed as described previously (27). Primers were all synthesized by Isogen life Sciences and sequences used are listed in **Table 2**. Obtained mRNA expression was normalized to the geometric mean of GAPDH, polrf2, and 18S in the human tissue samples and to GAPDH in human astrocytes.

TABLE 2 | Primer sequences.

Primer	Sequence
MC1R	Forward 5′-TCTCCAGGGCTCACTAGCAT-3′ Reverse 5′-CTGCAGGAGTTTGCACATCG-3′
MC2R	Forward 5′-ATTCCTTCTCATTCATTTTGCCCA-3′ Reverse 5′-AAGTTAAAATCTCCCAATCACCTTC-3′
MC3R	Forward 5′-GCGACTACCTGACCTTCGAG-3′ Reverse 5′-TAGCGGAGCGCGTAAAAGAT-3′
MC4R	Forward 5′-AGC TCC TTG CTT GCA TCC AC-3′ Reverse 5′-TCC CAA CCC GCT TAA CTG TC-3′
MC5R	Forward 5′-CTT TGT GCG CCA CAT TGA CA-3′ Reverse 5′-GCC GTC ATG ATG TGG TGG TA-3′
CCL2	Forward 5′-AGT GTC CCA AAG AAG CTG TG-3′ Reverse 5′-AAT CCT GAA CCC ACT TCT GC-3′
CXCL10	Forward 5′-TTC AAG GAG TAC CTC TCT CTA G-3′ Reverse 5′-CTG GAT TCA GAC ATC TCT TCT C-3′
IL-6	Forward 5′-TGC AAT AAC CAC CCC TGA-3′ Reverse 5′-TGC GCA GAA TGA GAT GAG TTG-3′
IL-11	Forward 5′-GCG GAC AGG GAA GGG TTA AA-3′ Reverse 5′-GCG GCA AAC ACA GTT CAT GT-3′
GAPDH	Forward 5′-CCA TGT TCG TCA TGG GTG TG-3′ Reverse 5′-GGT GCT AAG CAG TTG GTG GTG-3′
Polr2f	Forward 5′-GAA CTC AAG GCC CGA AAG-3′ Reverse 5′-TGA TGA TGA GCT CGT CCA C-3′
18S	Forward 5′-TAC CAC ATC CAA GGA AGG CAG CA-3′ Reverse 5′-TGG AAT TAC CGC GGC TGC TGG CA-3′

Western Blot

Cells were grown subconfluent in a 6 well-microplate and lysed using RIPA buffer [150 mM NaCl, 1% NP-40, 0.5% sodium doxycholate, 0.1% SDS, 25 mM Tris, 1× phosphostop, and 1× protease inhibitor (Roche, Almere, the Netherlands)]. Protein concentrations were measured with BCA assay according to manufacturer's protocol. A total of 10–15 μg protein was loaded onto SDS-PAGE gels (12.5%) along with a pre-stained protein marker (Precision plus, Bio-Rad Laboratories, Veenendaal, the Netherlands). Proteins were subsequently electrotransferred onto a nitrocellulose membrane (Bio-Rad, pore size 0.45 μm). The membrane was incubated with anti- CREB (1:500, #9104, Cell signaling) and anti-pCREB (1:300, #9198, cell signaling) and anti-α-tubulin as a loading control (1:400, S#SC1616, Santa Cruz, Dallas, TX, USA) in Odyssey blocking buffer (LI-COR, Lincoln, NE, USA) diluted 1:1 in TBS, after initial blocking with blocking buffer for 1 h at RT. Primary antibodies were detected by incubation with corresponding IRDye secondary antibodies (1:15,000) for 1 h at RT in blocking buffer and the Odyssey infrared imaging system (LI-COR). Intensity measurements of immunoreactivity were obtained using ImageJ software. Whole blots are shown in **Supplementary Figure 3**.

ELISA

A commercial human SimpleStep enzyme-linked immunosorbent assay (ELISA) was used for detecting IL-11 and conducted according to manufacturer's protocol (ab189569, Abcam). For the detection of IL-6 levels, 96 wells-plates were coated overnight at 4°C with capture antibody (1 μg/mL), washed and blocked for 30 min with PBS-BSA (1%). Supernatant from astrocyte cultures or standard (100 μL/well) and secondary antibody (50 μL/well) were then added and incubated for 2 h at RT. After washing with PBST, streptavidin poly-HRP was added and incubated for 30 min at RT. Substrate was added to develop the staining (0.1 M NaAc/0.1 M citric acid pH4) for a maximum of 30 min. Then, 50 μL 0.8 M H_2SO_4 was added to stop the reaction. Optical density was measured with a plate reader at 450 nm. Samples for IL-11 were diluted 1:5 and for IL-6 1:3.

Monocyte Isolation and Differentiation

Human blood monocytes were isolated from buffy coats of healthy donors (Sanquin Blood Bank, Amsterdam, The Netherlands) using Ficoll (Lymphoprep™, Axis-Shield, Oslo, Norway), and subsequent Percoll density gradient centrifugation. Monocytes were differentiated into macrophages in culture medium (IMDM, Gibco, ThermoFisher) containing 10% FCS, penicillin (100 IU/mL), streptomycin (50 mg/mL), and 50 ng/mL MCSF, for 6 days at 37°C/5% CO2. Macrophages were stimulated

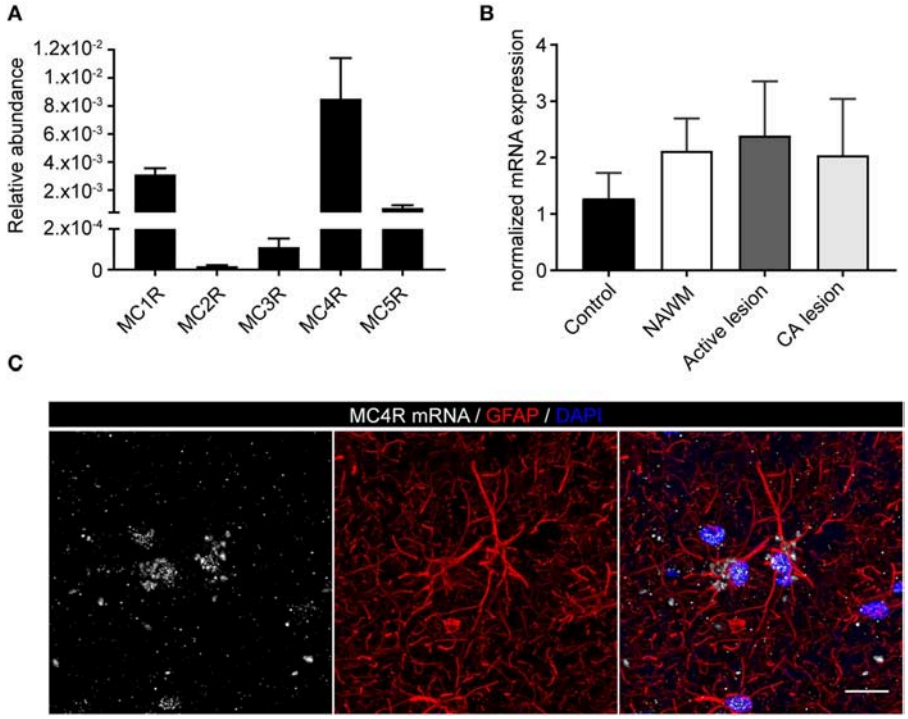

FIGURE 1 | mRNA expression of melanocortin receptors in MS lesions. **(A)** Expression of MC1-5R mRNA in human white matter control samples shows abundant expression of MC4R ($n = 5$ per group). **(B)** No significant differences in mRNA expression of MC4R in control, normal appearing white matter (NAWM), active white matter lesions, and chronic active lesions as performed with one-way ANOVA. **(C)** *In situ* HCR reveals presence of MC4R mRNA (white) in GFAP-positive cells (red) in human brain tissue. Nuclei were stained in blue (DAPI). Scale-bar = 20 μm.

with astrocyte conditioned medium (ACM) from either mock or MC4R+ astrocytes for 3 days. To this aim, we stimulated astrocytes with setmelanotide and/or SHU9119 (10 μM) for 4 h, replaced the medium with macrophage culture medium and added the medium to the macrophages 24 h thereafter. This was repeated for 3 days. From the medium, 100 μL was collected in Eppendorf tubes and stored at −80°C for ELISA assays.

Flow Cytometry

Lidocain (4 ng/mL) was added to macrophages and then cells were detached by gentle scraping, spun down, and collected in buffer (PBS plus 0.5% bovine serum albumin). Cells were incubated for 20 min at 4°C with appropriate antibodies, fluorescence minus one (FMO), and single stains were taken along for every antibody. Antibodies were as follows: CD14-AF700, CD68-PE-CY7 CD86-BV650, CD163-BV421, CD209-FITC, CD206-APC (Biolegend, San Diego, CA, USA). The cells were then washed twice with 100 μl of flow buffer, centrifuged, and resuspended in 200 μl flow buffer for analysis. Flow cytometry was performed with fluorescence-activated cell sorter (FACS) Calibur flow cytometer (BD Biosciences, San Jose, CA, USA). The cell populations were gated based upon FSC and SSC parameters and normalized to cells alone (without antibody) to adjust for cell-specific autofluorescence and a gate was set on the CD14 positive population (**Supplementary Figure 4**). Data were analyzed using FlowJo (FlowJo JCC, Ashland, OR, USA).

Statistics

DAB images were color deconvoluted using Fuji to split the DAB and haematoxylin signals and the area fraction of the DAB staining (% immunopositive area of total area) was quantified using Image J version 1.48 (28). All data reflect mean ± SEM and all comparisons were statistically tested in GraphPad Prism 5.0 using either unpaired two-tailed Student's *t*-tests for comparing two experimental groups, or one-way analysis of variance (ANOVA) to compare more than two groups.

RESULTS

Expression of Melanocortin Receptors in Human White Matter Samples and MS Lesions

To investigate the expression of melanocortin receptors, mRNA was isolated from control white matter tissue and well-characterized active white matter MS lesions. White matter lesions were identified and classified by the absence of myelin (proteolipid protein) and the presence of MHC class II^+ cells as reported before (29). As expected, all MCR, except MC2R, were expressed in white matter lysates of control brain tissue, with MC4R being the most abundantly expressed MCR (**Figure 1A**). We next analyzed mRNA expression of MC4R in normal appearing white matter (NAWM), active and chronic active (CA) lesions. Compared to controls, we found a non-significant

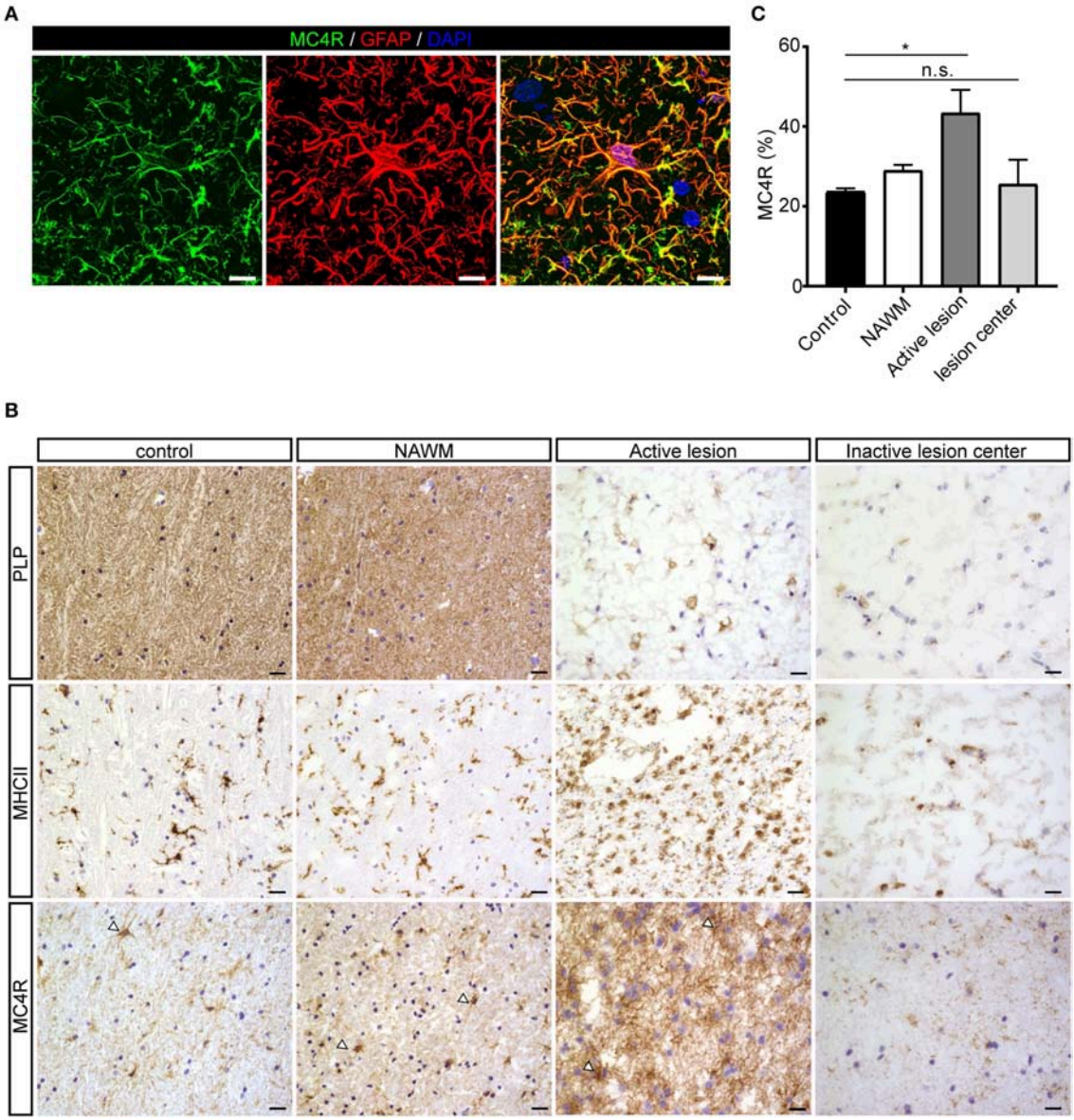

FIGURE 2 | Protein expression of MC4R in MS lesions. **(A)** Double immunofluorescence labeling shows co-localization of MC4R (green) with GFAP-positive astrocytes (red). scale-bar = 5 μm. Nuclei were stained in blue (DAPI). **(B)** Immunostainings of representative control white matter, NAWM, an active lesion, and inactive lesion center. Active MS lesions are characterized by loss of proteolipid protein (PLP) and presence of MHCII positive leukocytes, while inactive lesions are characterized by a demyelinated core, with little presence of MHCII+ cells. MC4R is expressed in control and NAWM and increased immunoreactivity is observed in active lesions (open arrowheads, scale-bar = 25 μm). **(C)** Quantitative analysis (one-way ANOVA) of the immunoreactive area (% of total area) for MC4R in active lesions and chronic inactive lesion centers compared to NAWM and control (n = 3–5 per group). Data represent mean ± SEM. *$p < 0.05$.

trend toward increased expression of MC4R in active lesions compared to control white matter, and NAWM (**Figure 1B**). To confirm the cellular source of MC4R, we performed *in situ* HCR to detect MC4R mRNA together with protein staining of glial fibrillary acidic protein (GFAP) as an astrocytic marker, and showed that MC4R mRNA is abundantly present in GFAP-positive astrocytes (**Figure 1C**). As expected, we also observed neuronal mRNA expression (**Supplementary Figure 1A**). GAPDH mRNA was used as positive control and present in all nuclei (**Supplementary Figure 1B**). We then used an antibody against MC4R and showed that astrocytes express MC4R protein (**Figure 2A**), and that MC4R is not expressed by MHCII positive cells and endothelial cells, as detected by UEA-1 (**Supplementary Figures 1C,D**). To further reveal the expression pattern of astrocytic MC4R protein in lesions, we performed immunohistochemical analyses and showed that immunoreactivity of MC4R was significantly increased in active lesions compared to NAWM and control white matter, while in inactive lesions similar expression of MC4R was observed as in NAWM, and control (**Figures 2B,C**).

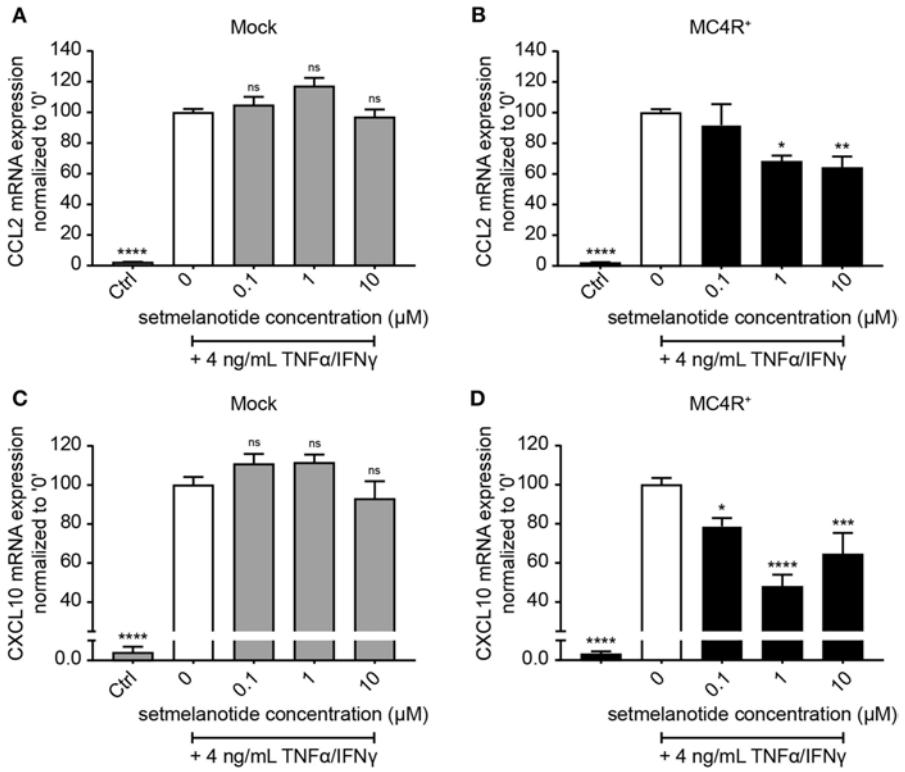

FIGURE 3 | Setmelanotide decreases TNF-α/IFN-γ-induced chemokine expression in astrocytes. mRNA expression of CCL2 and CXCL10 are significantly increased upon treatment with TNF-α and IFN-γ, which was partially blocked by addition of setmelanotide in MC4R+ astrocytes at concentrations of 1 and 10 μM **(B,D)**, but not in mock astrocytes **(A,C)**. One-way ANOVA, $n = 3$ independent experiments with 3 technical replicates per experiment. $^{*}p < 0.05$, $^{***}p < 0.001$, $^{****}p < 0.0001$ from control, compared to TNF-α and IFN-γ ("0") conditions. $^{**}p < 0.01$.

MC4R Activation Reduces Reactive Phenotype and Increases IL-6 and IL-11 Secretion in Astrocytes

U373 astrocytoma cells in which MC4R cDNA was stably transduced were used to further elucidate the role of MC4R in astrocyte function. Overexpression of MC4R resulted in a significant induction of MC4R levels at both mRNA (*in situ*; **Supplementary Figures 2A,B**, qPCR; **Supplementary Figure 2C**) and protein level (**Supplementary Figure 2D**). Overexpression of MC4R did not alter viability or proliferation as determined by MTS assay (**Supplementary Figures 2E,F**).

Astrocytes were subsequently exposed to TNF-α and IFN-γ, two pro-inflammatory cytokines described to be abundantly present during active demyelination in MS lesions, with or without the presence of setmelanotide, a selective MC4R agonist. We first analyzed the expression of two well-known reactive astrocyte markers C-C Motif Chemokine Ligand 2 (CCL2) and C-X-C motif chemokine 10 (CXCL10) (3, 30), which are involved in activation and recruitment of leukocytes into the CNS upon neuro-inflammation (31, 32). Treatment of mock and MC4R+ cells with TNF-α/IFN-γ resulted in a significant increase in mRNA expression of CCL2 and CXCL10 (**Figures 3A–D**). Importantly, treatment with setmelanotide strikingly reduced TNF-α/IFN-γ -induced expression of CCL2 and CXCL10 at doses of 1 and 10 μM in the MC4R+ astrocytes (**Figures 3B,D**), suggesting that the anti-inflammatory actions of setmelanotide are mediated via MC4R.

IL-6 and IL-11 are produced by (activated) astrocytes in MS lesions and able to skew macrophages toward an anti-inflammatory (M2) phenotype (33). We therefore stimulated mock and MC4R+ astrocytes with setmelanotide and investigated the gene expression of IL-6 and IL-11, both under basal, and inflammatory conditions. Setmelanotide treatment induced a significant increase in IL-6 and IL-11 mRNA levels in MC4R+ astrocytes (**Figures 4B,E**), but had no effect on mock cells (**Figures 4A,D**). The increase in IL-6 and IL-11 was prevented by pretreatment with SHU9119, an MC4R antagonist (**Figures 4B,E**). Effects were observed both in unstimulated as well as TNF-α/IFN-γ stimulated astrocytes. Importantly, secretion of IL-6, and IL-11 was also increased upon setmelanotide treatment in MC4R+ cells (**Figures 4C,F**). Three other members of the IL-6 family [leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF)] did not show an increase in mRNA levels after setmelanotide exposure (data not shown).

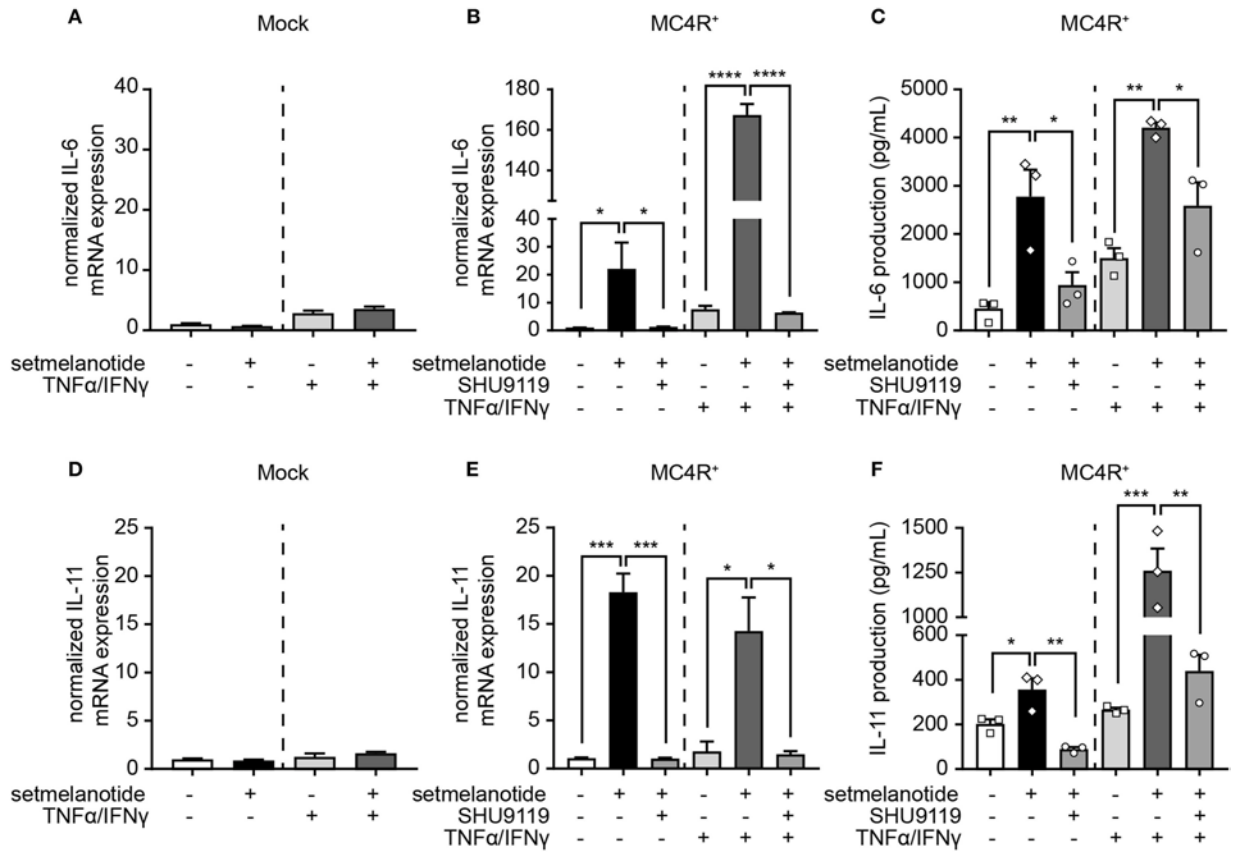

FIGURE 4 | Setmelanotide increases IL-6 and IL-11 mRNA and protein secretion via MC4R. Setmelanotide (10 μM, black bars) significantly increased mRNA expression of IL-6 and IL-11 in MC4R+ astrocytes under control and inflammatory conditions **(B,E)** and not in mock cells **(A,D)**. Likewise, setmelanotide increased IL-6 and IL-11 protein production and secretion as measured in supernatant under basal and inflammatory conditions **(C,F)**. The effects of setmelanotide were completely blocked by MC4R antagonist SHU9119 (10 μM, gray bars, **B,C,E,F**). Student's *t*-test (mock) and One-way ANOVA (MC4R+), $n = 2$–3 independent experiments with 3 technical replicates per experiment for mRNA levels. For ELISA, supernatant was collected from 3 independent stimulations. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

Setmelanotide Induces CREB Phosphorylation in Astrocytes

Next, we investigated the mechanism underlying increased production of IL-6 and IL-11 upon setmelanotide exposure. To date, the main signaling pathway of MC4R involves activation of G protein αs subunit (Gαs) and subsequently phosphorylation of cAMP response element-binding protein (CREB) (34) can result in production of IL-6 and IL-11 (35). MC4R+ astrocytes were treated with 10 μM setmelanotide and CREB phosphorylation (pCREB) was analyzed by western blot after 15, 30, and 60 min of stimulation. Setmelanotide significantly induced pCREB in time without altering total CREB levels (**Figure 5**).

Conditioned Medium of Setmelanotide Treated Astrocytes Skews Human Macrophages Toward an Anti-Inflammatory Phenotype

Reactive astrocytes are known to produce many factors such as cytokines, chemokines, and complement components, that influence inflammation, and can alter the course of MS (2). Both IL-6 and IL-11 have been shown to be present in MS lesions, released by astrocytes, and are involved in skewing macrophages toward an anti-inflammatory (M2) phenotype (33, 36, 37). We hypothesized that stimulation of astrocytes with setmelanotide, which results in increased IL-6 and IL-11 secretion, could affect the macrophage phenotype. Addition of conditioned medium from setmelanotide-treated astrocytes to macrophages resulted in a significant decrease in pro-inflammatory marker such as CD86 (M1 marker) and an increase in CD163 and CD209 (M2) protein expression, both under basal conditions as well as after stimulation with TNF-α/IFN-γ. This indicates that setmelanotide treated astrocytes secrete factors that are able to modulate macrophage polarization into an anti-inflammatory phenotype (**Figures 6A,B**). CD68 expression was not different in the conditions without cytokine stimulation but was however increased when stimulated with setmelanotide and cytokines.

DISCUSSION

In the present study, we are the first to show the presence of melanocortin receptor 4 (MC4R) mRNA, as well as protein

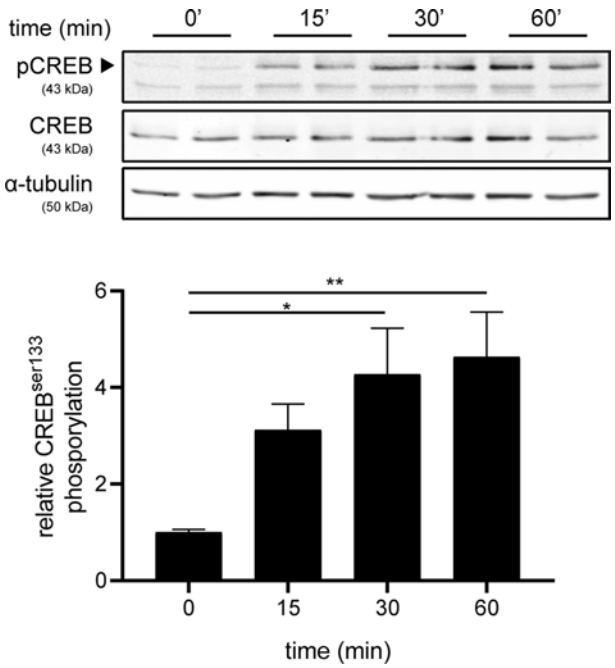

FIGURE 5 | Setmelanotide time-dependently induces CREB phosphorylation. Representative western blots of MC4R+ astrocytes treated with setmelanotide (10 μM), lysed at 15, 30, and 60 min after stimulation. α-Tubulin was used as a protein loading control for both pCREB and CREB. Quantification of CREB phosphorylation relative to total CREB revealed significantly increased phosphorylation of CREB after setmelanotide stimulation. $n = 3$ independent experiments. One-way ANOVA. $^{*}p < 0.05$, $^{**}p < 0.01$.

in human astrocytes, and showed increased astrocytic MC4R immunoreactivity in human active MS lesions. Increased MC4R mRNA expression has been described in active inflammatory MS lesions, however the cellular origin yet remained unknown (19). We furthermore showed that MC4R activation with setmelanotide, a novel, selective MC4R agonist, reduced inflammation-driven chemokine expression and markedly induced the production of the interleukins IL-6 and IL-11 under unstimulated and inflammatory conditions, presumably by increasing CREB phosphorylation. Stimulation of human macrophages with astrocyte medium of setmelanotide treated astrocytes, skewed macrophages toward an anti-inflammatory phenotype, which could limit a local inflammatory response in MS. Taken together, we are the first to show that targeting astrocytic MC4R limits neuroinflammation by inhibition of inflammatory genes, activation of the cytoprotective IL-6 cytokine family and by modulation of macrophage phenotype,.

Tissue lysates provide valuable information about overall expression of genes and differences thereof between normal and affected tissue, but are composed of a mix of different cell types of which the immune cell component in active lesions dominates the protein content of the lysate. We therefore further looked at which cells in the tissue expressed MC4R protein and found that astrocytes highly expressed MC4R mRNA and protein, which was increased in active lesions.

It has been shown that the endogenous MCR ligand α-MSH, inhibits lipopolysaccharide (LPS)-induced TNF-α production in astrocytes, which was proposed to be mediated via MC1R (38). In addition, α-MSH (analogs) reduced LPS/IFN-γ-induced nitric oxide production and the expression of inducible nitric oxide synthase (iNOS) in cultured primary astrocytes, which was blocked by an MC4R antagonist (14, 17). This illustrates the potency of MC4R activation in reducing an inflammatory response. However, LPS stimulation does not represent a relevant stimulus to mimic inflammatory processes as seen in MS. Various studies have shown increased (local) production of cytokines including TNF-α and IFN-γ in inflammatory MS lesions (20, 21). We show that activation of MC4R with setmelanotide robustly decreased TNF-α/IFN-γ-mediated induction of astrocytic CXCL10 and CCL2 and increased IL-6 and IL-11 secretion under basal and inflammatory conditions. CXCL10 and CCL2 play a role in attracting T cells and monocytes, respectively, and have been strongly implicated in disease in experimental autoimmune encephalomyelitis (EAE), an animal for MS. Deleting these chemokines in astrocytes have proven beneficial in reducing immune cell infiltration in the CNS and improving or delaying clinical symptoms (39, 40).

Infiltrating macrophages as well as microglia have been identified as major effectors of demyelination in both MS and animal models for MS (41). In animal models, pro-inflammatory M1 macrophages worsen neurological symptoms, whereas M2 macrophages promote remyelination (22), and *ex vivo* administration of M2 macrophages has been shown to reduce clinical disability (23). In human macrophages, IL-6 induces an anti-inflammatory cytokine profile, characterized by enhanced IL-4 and IL-10 production and decreased IL-1β secretion (42). It furthermore skews human monocytes into an anti-inflammatory M2 phenotype characterized by high CD163 and low CD86 surface expression (43). IL-11 reduces release of TNF-α, IL-1β, IL-12, and nitric oxide by macrophages and also induces M2 differentiation of macrophages/microglia after intranasal administration in mice [for review see (33)]. These results indicate that IL-6 and IL-11 promote an anti-inflammatory M2 phenotype in macrophages/microglia, which could limit a local inflammatory response in MS. Reactive astrocytes produce IL-6 in both active and chronic active MS plaques, which correlated negatively with oligodendrocyte loss, suggesting a protective role of this cytokine in myelin repair (36). IL-11 has been shown to be increased in astrocytes in inactive lesion borders and potentiates oligodendrocyte survival and maturation, and myelin formation *in vitro* (37), and reduced demyelination in EAE mice (44). Thus, astrocytic stimulation of IL-6 and IL-11 with setmelanotide could prove beneficial in targeting both (infiltrated) macrophages as well-neurodegenerative processes.

Reactive astrocytes highly express many complement components, and release cytokines, and chemokines that signal to microglia and various other immune cell types including monocyte derived macrophages, to attract them into the CNS or regulate their immune functions, and can thereby influence the course of disease (2). Together with the increased IL-6 and IL-11 production in astrocytes we show that medium of astrocytes

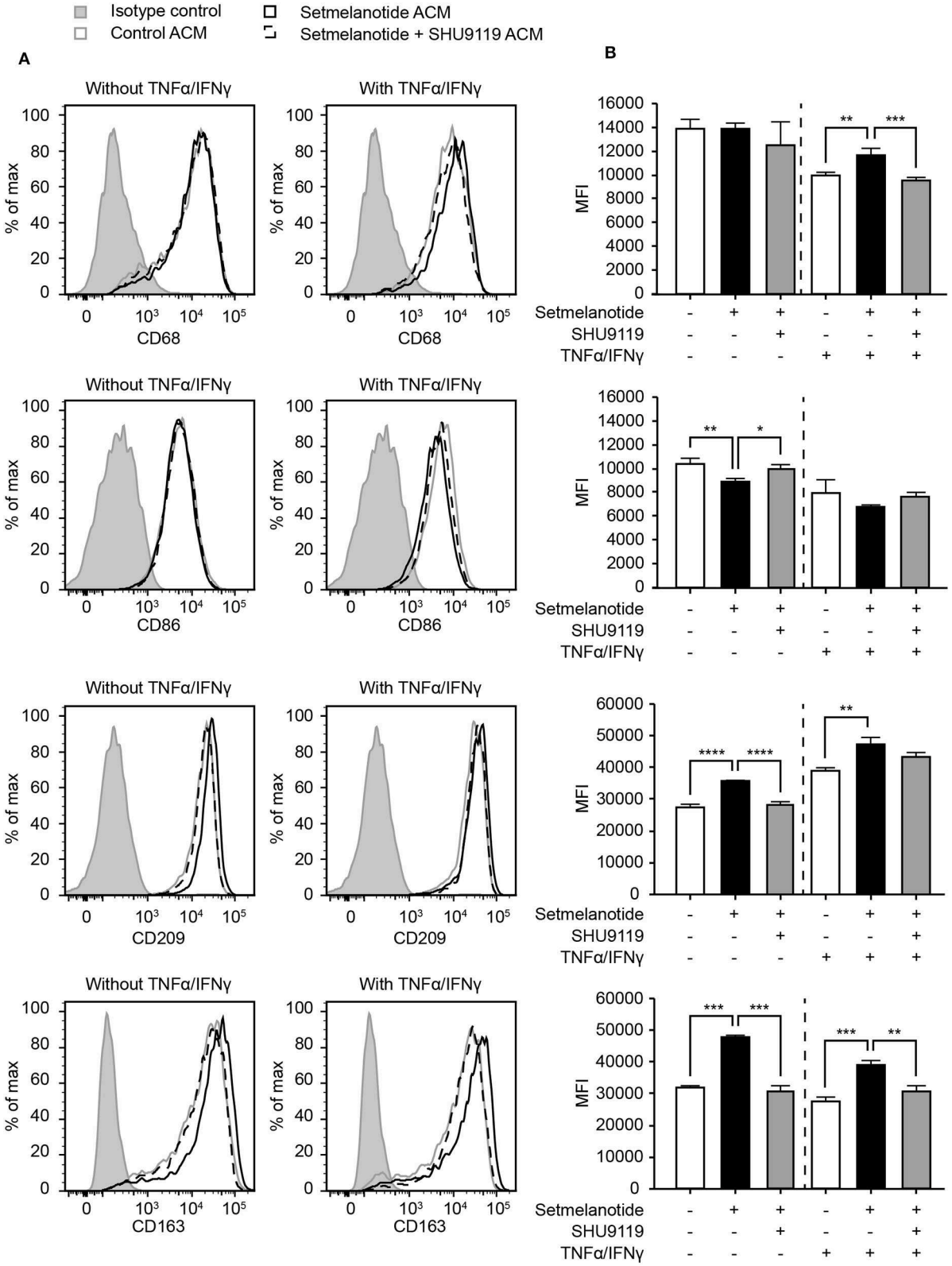

FIGURE 6 | Astrocyte condition medium from setmelanotide treated astrocytes alters phenotype of human macrophages. **(A)** Macrophages were cultured in the presence of astrocyte condition medium from astrocytes and the expression of markers was analyzed by flow cytometry ($n = 3$). Gray line represents macrophages

(Continued)

FIGURE 6 | cultured in the presence of ACM from non-treated astrocytes, black line represents macrophages cultured in the presence of ACM from setmelanotide treated astrocytes. Dotted line represents macrophages cultured in the presence of ACM from astrocytes treated with both setmelanotide and SHU9119. Left histograms indicate ACM from astrocytes cultured without TNF-α/IFN-γ, right histograms with TNF-α/IFN-γ. **(B)** Quantification of mean fluorescent intensity (MFI) of CD86, CD209, CD163, and CD206. Human macrophages treated with ACM from setmelanotide treated astrocytes reduced CD86, increased CD209, and CD163, and decreased CD206 surface expression. Macrophages treated with ACM from setmelanotide treated reactive astrocytes (+TNF-α/IFN-γ) showed increased CD68, CD209, and CD163 expression and a decrease in CD206. Effects of setmelanotide were blocked upon pretreatment with MC4R antagonist SHU9119. One-way ANOVA, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

stimulated with setmelanotide skews macrophages toward a less inflammatory phenotype, as determined by upregulation of CD163 and CD209, two anti-inflammatory M2 markers, and downregulated CD86, a M1 macrophage marker. Macrophages in MS have been shown to obtain an intermediate phenotype with high expression of CD68, CD86, and CD163 (45), skewing macrophages locally toward a protective M2 phenotype has been shown to promote remyelination (22).

MC4R is a G-protein coupled receptor (GPCR), which signals via a cAMP dependent pathway to promote CREB phosphorylation (16). Besides CREB, cAMP can also activate other transcription factors, including NF-κB, activator protein (AP)-1, and CCAAT/enhancer binding protein (C/EBP) (46). CCL2 and CXCL10 production are induced by activation of NF-κB in astrocytes (47, 48). Interestingly, it was shown that MC4R agonists inhibit nuclear translocation of NF-κB, thereby preventing NF-κB activation (49). It is thus likely that the reduced reactive profile of TNF-α/IFN-γ treated astrocytes upon setmelanotide stimulation is partly due to reduced activation of NF-κB. Besides CXCL10 and CCL2, IL-6, and IL-11 are also under control of NF-κB (50, 51). However, since we observed an increased expression and secretion of IL-6 and IL-11 makes it unlikely that NF-κB is involved. Based on the induced phosphorylation CREB upon the activation of MC4R with setmelanotide, we postulate that this pathway is more dominant in the regulation of secretion of IL-6 and IL-11.

α-MSH and the stable analog NDP-MSH have both proven to be effective in ameliorating clinical symptoms in an experimental animal model of MS by limiting inflammation and neurodegeneration, which was attributed to MC1R signaling (11). α-MSH is, next to MC4R, a full agonist of MC1R and MC5R, which are both highly expressed in the periphery. Hence treatment with α-MSH likely also has peripheral effects. Selective MC4R agonists have proven to be excellent candidates in the treatment of immune-mediated inflammatory diseases, without having the side effects of corticosteroids (18). MC4R is the most widespread melanocortin receptor in the CNS including astrocytes. Therefore, a more selective MC4R agonist like setmelanotide could have beneficial effects with limited side-effects. Future studies are warranted to study the therapeutic efficacy of setmelanotide in experimental animal models of MS and whether astrocyte-specific MC4R knockout is able to block these effects. Setmelanotide has shown to be well-tolerated in a phase 2 clinical trial in obese individuals with a rare genetic disorder, and mouse knock-out studies have shown that setmelanotide-associated weight loss is regulated via MC4R, which is mainly attributed to MC4R neurons of the hypothalamus (52, 53). Our findings could set the stage for setmelanotide as a new therapeutic for inflammation associated neurodegeneration in MS.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the manuscript/**Supplementary Files**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Dutch brain bank. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MR, AK, HV, and JH designed and interpreted the experiments. MR, AK, TV, LB, and BH performed the experiments. JK set up the *in situ* hybridization protocol and helped with the *in situ* experiments. MR and AK analyzed the data and wrote the paper. MW collected the human brain samples and clinical data. HV, JH, and MW provided intellectual inputs and critically reviewed the manuscript. All authors have read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

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ORIGINAL RESEARCH
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Properdin: A Novel Target for Neuroprotection in Neonatal Hypoxic-Ischemic Brain Injury

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Background: Hypoxic-ischemic (HI) encephalopathy is a major cause of neonatal mortality and morbidity, with a global incidence of 3 per 1,000 live births. Intrauterine or perinatal complications, including maternal infection, constitute a major risk for the development of neonatal HI brain damage. During HI, inflammatory response and oxidative stress occur, causing subsequent cell death. The presence of an infection sensitizes the neonatal brain, making it more vulnerable to the HI damage. Currently, therapeutic hypothermia is the only clinically approved treatment available for HI encephalopathy, however it is only partially effective in HI alone and its application in infection-sensitized HI is debatable. Therefore, there is an unmet clinical need for the development of novel therapeutic interventions for the treatment of HI. Such an alternative is targeting the complement system. Properdin, which is involved in stabilization of the alternative pathway convertases, is the only known positive regulator of alternative complement activation. Absence of the classical pathway in the neonatal HI brain is neuroprotective. However, there is a paucity of data on the participation of the alternative pathway and in particular the role of properdin in HI brain damage.

Objectives: Our study aimed to validate the effect of global properdin deletion in two mouse models: HI alone and LPS-sensitized HI, thus addressing two different clinical scenarios.

Results: Our results indicate that global properdin deletion in a Rice-Vannucci model of neonatal HI and LPS-sensitized HI brain damage, in the short term, clearly reduced forebrain cell death and microglial activation, as well as tissue loss. In HI alone, deletion of properdin reduced TUNEL+ cell death and microglial post-HI response at 48 h post insult. Under the conditions of LPS-sensitized HI, properdin deletion diminished TUNEL+ cell death, tissue loss and microglial activation at 48 h post-HI.

Conclusion: Overall, our data suggests a critical role for properdin, and possibly also a contribution in neonatal HI alone and in infection-sensitized HI brain damage. Thus, properdin can be considered a novel target for treatment of neonatal HI brain damage.

Keywords: properdin, complement, alternative pathway, hypoxia, ischemia, neuroprotection, neonate, infection

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INTRODUCTION

Oxygen deprivation around the time of birth is a major cause of neonatal hypoxic ischemic (HI) brain damage affecting 1–3 per 1,000 live births in developed countries and increasing to 26 per 1,000 in the developing world (1). Of the affected neonates, 15–25% die during the neonatal period and 25% of the survivors develop neurological sequelae such as epilepsy, cerebral palsy and cognitive defects (2), resulting in significant psychological and socioeconomic burden on the patient, family and healthcare system (1).

The pathophysiology of HI brain damage involves inflammation, oxidative stress, excito-toxicity and cell death (3–6).

Pre-exposure of the preterm infant to a bacterial infection sensitizes the brain, making it more susceptible to the HI insult. Bacterial lipopolysaccharide (LPS)—the major component of the outer membrane of most Gram-negative bacteria—is a strong immune stimulator and enhances cerebral damage and lesions in HI brain injury (7, 8).

Therapeutic hypothermia (TH) is the standard clinical care for moderate to severe HI injury, however it is effective in only 55% of cases, while the remaining 45% of treated infants still develop neurological deficits (9). Thus, further studies on improving the success of TH and finding therapeutic alternatives are urgently required.

The effect of TH in infection-sensitized HI conditions is pathogen dependent (10). In a rat model of LPS-sensitized HI, TH failed to reduce mortality and tissue damage (11). In clinical studies looking at the effect of TH on neonates exposed to intrauterine infection, TH treatment did not counteract inflammation (12).

The lack of effect of TH in LPS-sensitized HI could be attributed to inter-individual variability (7). Additionally, body cooling following HI alone is suggested to be immunosuppressive (13, 14), therefore counteracting the physiological attempt of the immune system in fighting bacterial infection.

The inability of TH to protect the neonatal brain in LPS-sensitized HI, and its limited outcome in treatment of HI alone, support the urge to investigate new therapeutic alternatives or augmentation strategies for TH.

Such an alternative is targeting the complement system, a cascade of over 30 proteins critically involved in innate immunity. The activated complement promotes inflammation and anaphylatoxin release and comprises three pathways—classical, lectin, and alternative. While the classical pathway (CP) is mainly activated by external pathogens, the alternative one (AP) is spontaneously active and also amplifies the other two pathways (15). Properdin is a plasma glycoprotein released mainly by leukocytes in response to pro-inflammatory stimuli (16). It is the only known positive regulator of the AP; in fact, properdin facilitates the constitutively active AP either by stabilizing the C3 convertase C3bBb or by binding to susceptible surfaces, thus serving as a platform for *de novo* C3bBb assembly (17, 18). This causes opsonization of target molecules through C3b and further activation of the complement cascade, culminating in the formation of the membrane attack complex (C5b-C9).

Clinical data associates neonatal HI with depleted C3 expression (19) and increased serum levels of C3a and C5a following fetal acidosis (20). While the role of properdin in inflammation has been widely studied (21, 22), there is a paucity of data surrounding the role of properdin in neonatal HI. It could be speculated that HI upregulates properdin levels and leads to increased anaphylatoxin production and pro-inflammatory activation of microglia and astrocytes. This study aims to elucidate the role of properdin in neonatal HI alone and in LPS-sensitized HI in the short term. Our data demonstrate the neuroprotective effect of global properdin deletion in both HI alone and LPS-sensitized HI at 48 h post-HI, suggesting this complement regulator as an attractive therapeutic target in neonatal HI and LPS-sensitized HI.

MATERIALS AND METHODS

Animal Use

Properdin-deficient mice were generated by site-specific genetic engineering, rendering mice deficient of the serum protein properdin and thereby lacking the amplification loop of complement activation (23). They have been maintained by crossing heterozygous properdin deficient female mice with wild type male C57Bl/6 mice and were obtained from the University of Leicester. Genotyping was performed on animals after treatment.

All animal experiments and care protocols were approved by the Home Office (PPL70/8784) and UCL Animal Welfare and Ethical Review Body. All procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and the ARRIVE guidelines. All experiments involved postnatal day 7 mice (P7) bred in house. At P7, the neonatal mouse brain development is comparable to a mid-third-trimester human fetus or newborn infant, with complete cortical neuronal layering, an involuted germinal matrix, and slightly myelinated white matter (24). Although slightly preterm, the murine P7 model of HI presents phenotypical similarities to the gray and white matter injury observed in humans, including tissue loss, cell-death, microglia-mediated immune response and astrogliosis as well as changes in neurological behavior (24).

Because properdin is located on the X-chromosome, mating of heterozygous properdin deficient females with wild type males yields male hemizygous, properdin-deficient and wild type mice (as well as female heterozygous and wild type mice). Therefore, only male pups were used in the experiments and were ideally controlled as littermates. According to clinical and experimental evidence, male mice may express a worse phenotype post-HI than female mice, with increased loss of male hippocampal volume after chronic postnatal hypoxia (25). All the assessments were performed blindly to the genotype.

HI Insult

The surgical procedures, a variation of the Rice-Vannucci rodent HI model, were performed as previously described (7, 26–30). Briefly, a total of 30 P7 male mice, both wild type ($n = 15$) and with global properdin deletion ($n = 15$), were anesthetized using

isoflurane (5% induction, 1.5% maintenance). The left carotid artery was permanently occluded (8/0 propylene suture) and the wound was closed with tissue glue. The mice were left to recover at 36°C and returned to the dam for 2 h. They were then moved to a hypoxia chamber and exposed to humidified 8% oxygen, 92% nitrogen (3 L/min) for 60 min (HI alone) or 30 min (LPS-sensitized HI) at 36°C (27), resulting in moderate to severe brain damage (27, 29, 30). In the infection-sensitized HI insult, 55 P6 pups from both genotypes were injected with *E. coli* lipopolysaccharide (LPS; 0.6 μg/g, serotype 055:B5; Fluka, UK) ($n = 13$ WT, $n = 13$ KO) or saline 12 h prior to surgery ($n = 15$ WT, $n = 15$ KO) (7, 29). The contralateral side of the brain served as an intra-animal control reference for ipsilateral damage.

Tissue Sample Preparation

The animals were sacrificed at 48 h following the HI insult using intraperitoneally delivered pentobarbitone. They were perfused with 30 mL 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). The brains were then extracted, post-fixed for 1 h in 4% PFA/0.1M phosphate buffer (PB) at 4°C, before being cryoprotected in 30% sucrose/PB solution for 24 h. The cerebellum was removed from each brain. The forebrains were frozen on dry ice, cut into 50 sequential 40 μm coronal sections starting from the fusion of corpus callosum, and the slices were stored at −80°C (7, 27, 29, 30).

Immunohistochemistry and Histological Analysis

Five sections from each brain (400 μm apart) were rehydrated in distilled water and stained using immunohistochemistry as previously described (7, 27, 29, 30). Briefly, the sections were incubated overnight with rat anti-CD11b αM integrin subunit (1:5,000, Serotec, UK) or rabbit polyclonal anti-glial fibrillary acidic protein (GFAP) (1:6,000, DAKO, UK), primary antibodies, for 1 h with biotinylated goat anti-rat or -rabbit (1:100, Vector, UK) secondary antibodies, followed by incubation with Avidin-Biotinylated horseradish peroxidase Complex (Vector, UK) and visualization with diaminobenzidine/H_2O_2 (Fisher Scientific, UK) (7, 27, 29, 30).

Five further sections from each brain with the same spacing were stained using Terminal transferase mediated d-UTP nick end labeling (TUNEL) (Roche, UK). The staining procedure followed the manufacturer protocol with Co/Ni enhancement (7, 27, 29, 30).

Five more sections per brain with the same spacing were stained with Cresyl-Violet (Nissl).

AlphaM Score

Assessment for αM integrin immunoreactivity as a marker for early microglial activation (7, 26–30) was performed as previously described (7, 27, 30). Two independent observers blinded to the genotype and treatment of the groups allocated semi-quantitative scores to each brain region, i.e., cortex, pyriform cortex, hippocampus, striatum, thalamus, and external capsule.

Optical Luminosity

The central cytoskeletal framework of astroglia comprises GFAP, a type III intermediate filament found only in glial cells in the CNS. GFAP upregulation is seen during HI-triggered reactive gliosis (31). In order to quantify the intensity of the GFAP staining, we used optical luminosity values (OLV) as a well-established technique (7, 26–30). Images for ipsilateral and contralateral sides were captured with a Sony AVT-Horn 3CCD color video camera (24 bit RGB, 760 × 570 pixel resolution) in three different optical fields in cortex, pyriform cortex, hippocampus, striatum, thalamus and external capsule. We used Optimas 6.5 software to obtain the mean and standard deviation (SD) for OLVs. SD was subtracted from the mean for each image, and the resulting value was subtracted from the values acquired for the surrounding glass.

TUNEL Assessment

As a measure of cell death at 48 h post-HI, the number of TUNEL + cells was counted in three different optical fields at ×20 magnification. The cortex, pyriform cortex, hippocampus, striatum, thalamus and external capsule were assessed. The counts were averaged per animal and per group.

Infarct Volume Measurement

Cresyl violet dye stains Nissl bodies present in the cytoplasm of neurons. In this study we used Nissl stain to measure tissue loss in the cortex, pyriform cortex, hippocampus, striatum and thalamus. Nissl-stained brain sections were imaged with Sony AVT-Horn 3CCD color video camera (24 bit RGB, 760 × 570 pixel resolution) at ×1 magnification. The images were imported in Fiji Image J (NIH, USA), and the areas of intact staining in all 6 regions were outlined and bilaterally measured. The percentage tissue loss was then calculated by converting the measured uninjured areas into square millimeters and then transformed to a volume through multiplication by 400 μm. The sum of these volumes was then used to calculate the percentage of surviving brain tissue as ipsilateral/contralateral × 100 (32).

Statistics

GraphPad Prism 8 (La Jolla, CA, United States) and SPSS 25.0 (IBM, USA) were used to perform all statistical analyses. The same six forebrain regions (cortex, pyriform cortex, hippocampus, striatum, thalamus, external capsule) were used for each outcome and each assay.

As the data from the Rice-Vannucci model are mostly non-normally distributed, we performed non-parametric Mann-Whitney test (HI alone set of experiments) to compare the effect of global properdin deletion in each brain region separately, and a two-tailed p-value was assumed. As the number of groups in the LPS-sensitized HI set of experiments was larger than two, we performed the non-parametric Kruskal-Wallis test followed by Bonferroni-corrected pairwise-contrasts. Alpha was set to 0.05. All data are graphically presented as Median ± IQR (interquartile range, presented as error bars). All hypotheses were two-tailed and all data illustrate the response in the ipsilateral (experimental) hemisphere. In our data, a main effect is the effect of an independent variable (treatment) on a dependent

variable (damage marker) averaged across the levels of any other independent variables (brain regions).

RESULTS

Global Properdin Deletion Reduces Cell Death and Microglial Activation Following Neonatal HI-Insult

Global deletion of properdin significantly reduced brain damage markers (cell death and microglial activation) compared to wild type control animals at 48 h post-HI. As shown in **Figures 1A–C**, global properdin deletion significantly reduced the number of TUNEL+ cells compared to wild type controls, with individual significant decrease of 20–38% in pyriform cortex, hippocampus, striatum, thalamus and overall ($p < 0.05$, Mann-Whitney test). The TUNEL+ cells displayed the typical pyknotic nuclear morphology and high density in the control group (**Figure 1B**—insert, ipsilateral hippocampus) compared to the reduced number of such cells in the properdin KO brains (**Figure 1C**—insert, ipsilateral hippocampus).

The regional assessment presented in **Figure 1D** revealed slight decrease of ipsilateral brain tissue volume loss in the pyriform cortex and thalamus in the global properdin deletion group (**Figure 1F**) compared to wild type controls (**Figure 1E**), however the data did not reach significant values.

Assessment of ipsilateral astrogliosis through GFAP immunoreactivity (**Figure 1G**) showed that compared to wild type controls (**Figure 1H**), global properdin deletion had no effect on reactive astrogliosis (**Figure 1I**) following neonatal HI.

In addition to cell death, global properdin deletion had a significant effect on ipsilateral microglia activation score (**Figure 1J**) based on αM integrin immunoreactivity (**Figures 1K,L**). Regional assessment shown in **Figure 1J** revealed a reduction in activation score in the properdin KO group, with individual decrease of 21–76% in pyriform cortex, hippocampus, striatum, thalamus and overall ($p < 0.05$, Mann-Whitney test). At high magnification, the αM+ cells in the wild type control group showed phagocytic morphology (**Figure 1K**—insert, ipsilateral hippocampus) compared to the ramified phenotype of these cells observed in the animals with global properdin deletion (**Figure 1L**—insert, ipsilateral hippocampus).

Global Properdin Deletion Reduces Brain Damage Following LPS-Sensitized Neonatal HI-Insult

Global deletion of properdin significantly reduced brain damage markers (cell death, tissue loss and microglial activation) compared to wild type control animals at 48 h post LPS-sensitized neonatal HI. As shown in **Figures 2A–D**, global properdin deletion significantly reduced the number of TUNEL+ cells compared to LPS-treated wild type controls (main effect, $p < 0.05$, Kruskal-Wallis test), with individual decrease of 50–76% in all 6 regions, but reaching significance in cortex, pyriform cortex, hippocampus and overall (Bonferroni correction, $p < 0.05$). The saline-treated wild type controls showed very low number of TUNEL+ cells (**Figures 2A,B**), and global properdin deletion did not affect those numbers (**Figure 2A**). LPS-sensitization resulted in a substantial increase of TUNEL+ cell death observed in the wild type LPS-treated group (**Figure 2C**) compared to saline treated wild types (**Figure 2B**). The TUNEL+ cells displayed the typical pyknotic nuclear morphology and high density in the LPS-treated wild type group (**Figure 1C**—insert, ipsilateral hippocampus) compared to the reduced number of such cells in the LPS-treated properdin KO brains (**Figure 1D**—insert, ipsilateral hippocampus).

Regional assessment presented in **Figure 2E** revealed very low levels of ipsilateral tissue loss in the saline-treated wild type animals (**Figure 2F**), and global properdin deletion did not affect these levels (**Figure 2E**). LPS-sensitization resulted in an extensive increase of tissue loss observed in the LPS-treated wild type group (**Figure 2G**) compared to the saline treated wild type controls (**Figure 2F**). We observed an overall trend toward reduction of ipsilateral brain tissue volume loss of 13–66% across all 6 forebrain regions in the LPS-treated global properdin deletion group (**Figure 2H**) compared to LPS-treated wild type controls (**Figure 2G**) (main effect, $p < 0.05$, Kruskal-Wallis test), however the data reached significant values only in cortex and pyriform cortex (Bonferroni correction, $p < 0.05$).

Similarly, regional assessment of ipsilateral astrogliosis through GFAP immunoreactivity (**Figure 2I**) showed very low levels of reactive astrogliosis in saline-treated wild type controls (**Figure 2J**), and global properdin deletion did not affect those levels (**Figure 2I**). LPS-sensitization resulted in a considerable increase of astroglial activation observed in the LPS-treated wild type group (**Figure 2K**) compared to the saline treated wild type controls (**Figure 2J**). We observed an overall trend toward reduction of ipsilateral reactive astrogliosis of 27% across all 6 forebrain regions in the LPS-treated global properdin deletion group (**Figure 2L**) compared to LPS-treated wild type controls (**Figure 2K**) (main effect, $p < 0.05$, Kruskal-Wallis test), however the data did not reach significant values.

Additionally, assessment of microglial activation based on αM integrin immunoreactivity (**Figure 2M**) showed very low levels of αM+ microglia in saline-treated wild type controls (**Figure 2N**), and global properdin deletion did not affect those levels (**Figure 2M**). LPS-sensitization resulted in a substantial increase of microglial activation observed in the LPS-treated wild type group (**Figure 2O**) compared to the saline treated wild type controls (**Figure 2M**). Regional assessment shown in **Figure 2M** revealed a reduction in activation score of 31–66% in all 6 individual ipsilateral brain regions in the LPS-treated global properdin deletion group compared to the LPS-treated wild type controls (main effect, $p < 0.05$, Kruskal-Wallis test), however significance was reached only in hippocampus (Bonferroni correction, $p < 0.05$). At high magnification the αM+ cells in the LPS-treated wild type control group showed phagocytic morphology and high density (**Figure 2O**—insert, ipsilateral hippocampus) compared to the ramified resting phenotype and low density of these cells observed in the LPS-treated global properdin deletion animals (**Figure 2P**—insert, ipsilateral hippocampus).

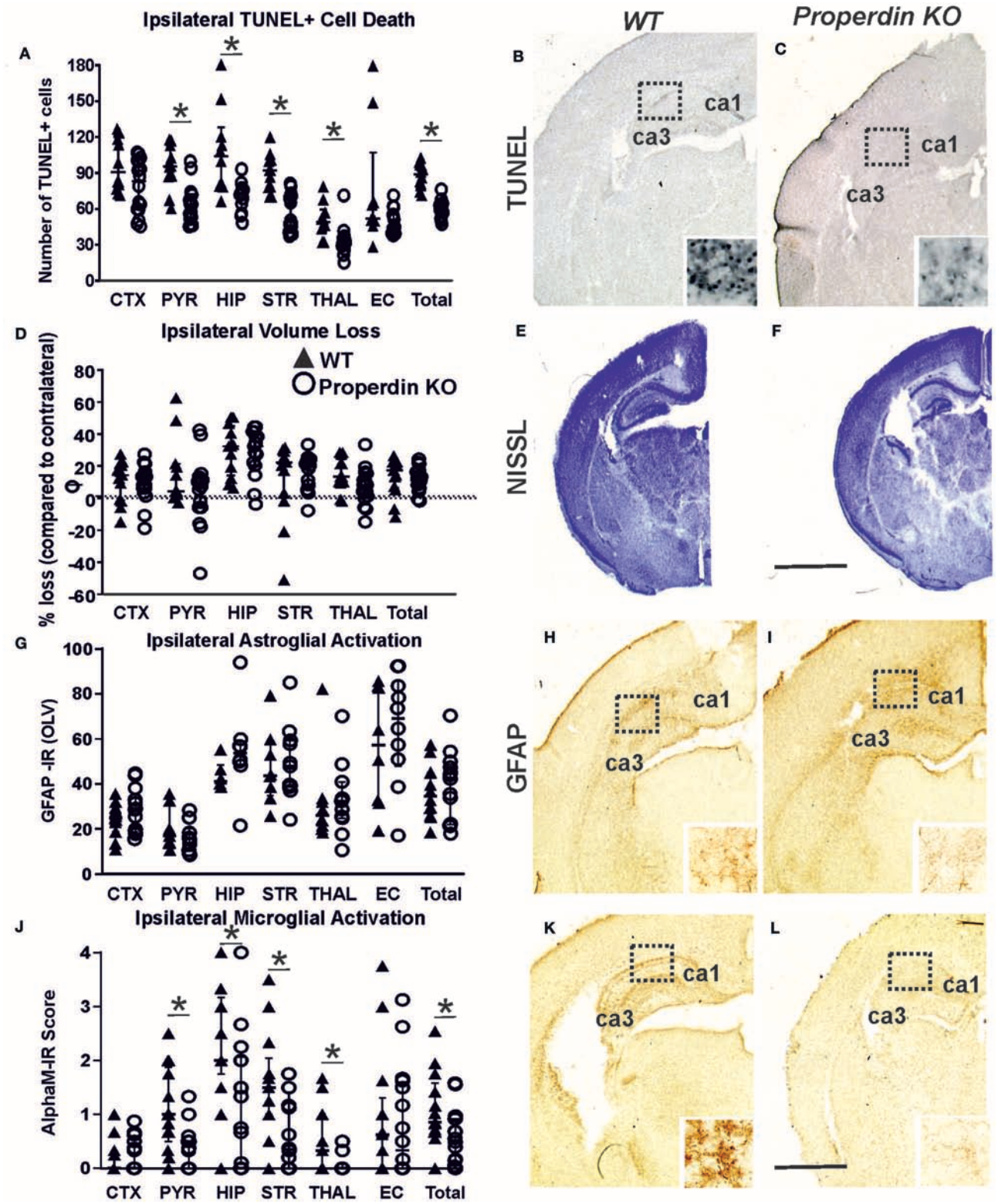

FIGURE 1 | Global deletion of properdin in P7 mice significantly reduces cell death and microglial response at 48 h post-HI. **(A–C)** TUNEL+ staining of dying brain cells with fragmented DNA—Quantification **(A)** (number of TUNEL+ cells per 20× eye-field, Median ± IQR) and histochemical overview of the ipsilateral forebrain in wild type control **(B)** and animals with global properdin deletion **(C)**. Note the typical pyknotic nuclear morphology of the TUNEL+ cells, as well as their high density

(Continued)

FIGURE 1 | observed in the controls (**B**—insert, hippocampus) compared to the reduced number of such cells in the properdin KO group (**C**—insert, hippocampus). Compared to wild type controls, properdin deletion resulted in reduced TUNEL+ cell death across all 6 examined forebrain regions, with significant, individual decrease (Mann-Whitney test) in the pyriform cortex ($p = 0.0004$), hippocampus ($p = 0.0008$), striatum ($p < 0.0001$), thalamus ($p = 0.0034$) and overall ($p < 0.0001$). **(D–F)** Ipsilateral forebrain Nissl staining (Cresyl-Violet, at rostral parietal level)—Quantification of percentage of ipsilateral brain tissue volume loss (**D**, Median ± IQR) of wild type control **(E)** and properdin KO **(F)** animals. Compared to wild type controls global properdin deletion resulted in slight decrease of volume loss following neonatal HI, however the data did not reach significant values. **(G–I)** GFAP immunoreactivity—Quantification of ipsilateral reactive astrogliosis **(G)** in optical luminosity values (OLV, Median ± IQR), and low magnification ipsilateral overview in wild type control **(H)** and animals with global properdin deletion **(I)**. The inserts in H and I show higher magnification of the dotted regions in hippocampus. Global properdin deletion did not have an effect on astroglial activation following neonatal HI. **(J–L)** Activation of αM+ microglia—Ipsilateral αM microglial activation score (**J**, Median ± IQR) and low magnification ipsilateral overview in wild type control **(K)** and animals with global properdin deletion **(L)**. Note the strong microglial activation in the control wild type group with αM+ cells showing phagocytic morphology at high magnification (**K**—insert, hippocampus), compared to the properdin KO brains exhibiting a ramified phenotype (**L**—insert). Global properdin deletion reduced αM+ microglial activation across all 6 examined forebrain regions apart from cortex, with significant, individual decrease (Mann-Whitney test) in pyriform cortex ($p = 0.008$), hippocampus ($p = 0.05$), striatum ($p = 0.02$), thalamus ($p = 0.04$), and overall ($p = 0.01$). Wild type ($n = 14$) and global properdin deletion ($n = 16$) in all assessments. ($^*p < 0.05$). CTX, cerebral cortex; PYR, pyriform cortex; HIP, hippocampus; STR, striatum; THAL, thalamus; EC, external capsule. Scale bars: **(E,F)** = 1,200 μm; **(B,C,H,I,K,L)** = 600 μm. inserts = 30 μm.

DISCUSSION

Lack of oxygen to the fetal brain around the time of birth is a major cause of neonatal HI brain damage, triggering neurological sequelae such as cerebral palsy, epilepsy and mental retardation. Intrauterine or perinatal complications, including maternal infection, constitute a major risk for the development of neonatal HI brain damage. The mechanisms underlying the trigger of brain damage under the conditions of HI alone and LPS-sensitized HI overlap, but also differ (7, 29). Therefore, our study aimed to validate the effect of global properdin deletion in two models: HI alone and LPS-sensitized HI, thus addressing two different clinical scenarios.

In the current study, C57/Bl6 background was chosen as a result of the high severity of HI injury incurred following prolonged hypoxic exposure (7, 33).

Additionally, global properdin deletion reduced forebrain cell death and microglial activation, as well as tissue loss in a Rice-Vannucci model of neonatal HI and LPS-sensitized HI brain damage.

In the model of HI alone, deletion of properdin reduced brain damage based on evidence for TUNEL+ cell death and microglial post-HI response, which in both assessments reached significance.

Under the conditions of LPS-sensitized HI, properdin deletion reduced brain injury based on evidence of significantly diminished cell death, tissue loss and post-HI microglial activation. Overall, our data suggests a critical role for properdin, and possibly also a contribution in neonatal HI alone, as well as in infection-sensitized HI brain damage.

Complement is an essential part of innate immunity and participates not only in normal brain physiology, but also under pathological conditions, including ischemia (34). Absence of the CP in the neonatal HI brain is neuroprotective (35, 36). Despite the long history of research on the role of complement in neonatal HI (35, 36), there is a paucity of data on the participation of the AP in post-HI brain damage (34), in particular the role of properdin.

Our data shows that in HI alone and in LPS-sensitized HI, properdin deficiency reduced TUNEL+ cell death with significant differences in pyriform cortex, hippocampus, striatum, thalamus and overall in the HI alone set of experiments (**Figure 1A**), and in cortex, pyriform cortex and hippocampus in the LPS-sensitized HI (**Figure 2A**). Additionally, tissue loss was significantly reduced in the thalamus in the HI alone set of experiments (**Figure 1D**) and in the cortex and pyriform cortex in the LPS-sensitized HI (**Figure 2B**). Sub-regional differences in the vulnerability of the different brain regions to HI damage exist based on metabolic rate and energy demand (6). HI insult around term, as modeled in this study, damages predominantly gray matter in the cortex, hippocampus and/or thalamus (37). In animal models, damage in the cortex, thalamus and striatum has been associated with sensorimotor impairment (38–41). Interestingly, as the hippocampus is one of the regions with the highest metabolic rate in the developing brain and therefore highly susceptible to HI injury, damage to it and to the cortico-hippocampal projections causes memory and spatial processing dysfunction (42). Additionally, HI-induced reduction in hippocampal volume has been associated with impaired long-term reference memory, short-term working memory (43), as well as spatial navigation and recollection (44, 45). We observed reduced cell death in striatum of animals with global properdin deletion following neonatal HI (**Figure 1A**). As damage to the striatum, in particular to nucleus accumbens may have an impact on non-spatial navigation and learning (45, 46) and might explain non-spatial memory deficits in neonatal HI rats (37), protection of that region is essential. Neonatal HI is considered a major risk factor for psychiatric diseases including attention-deficit hyperactivity disorder (ADHD), autism, psychosis and schizophrenia (47–51). The main regions associated with related cognitive functions are the hippocampus and striatum as well as cortico-hippocampal and cortico-striatal projections. As global properdin deletion provides neuroprotection for these regions, it is likely that it would reduce the risk of development of later life psychological and behavioral complications, however that would require additional long-term behavioral studies.

Although there is no data on the effect of global properdin deletion on neonatal HI brain damage, our results are in line with previous studies looking at the role of the AP in murine models of stroke. In an adult mouse study of middle cerebral artery occlusion (MCAO), C3 deficiency and site-targeted inhibition

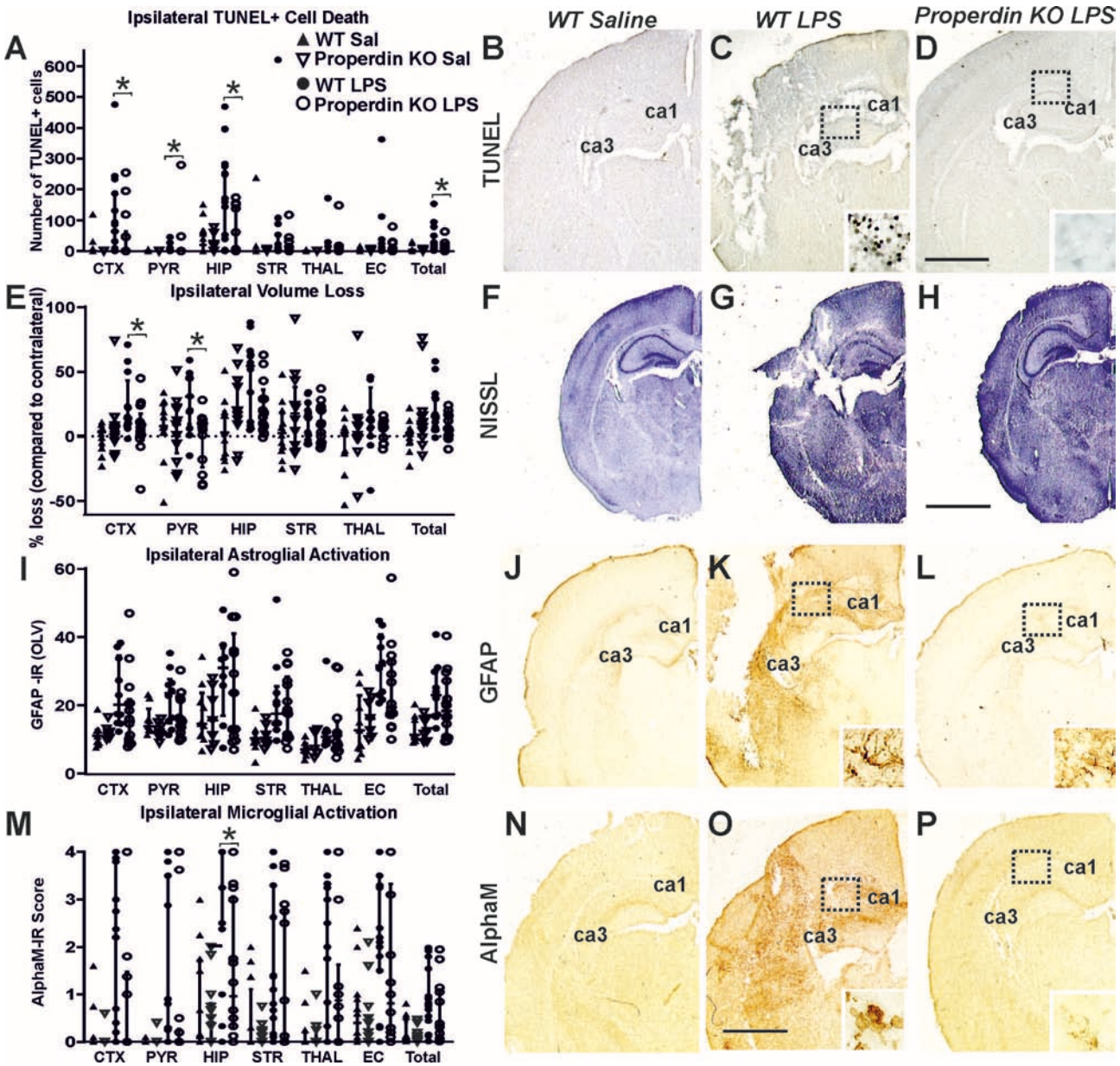

FIGURE 2 | Global deletion of properdin in P7 mice significantly reduces cell death tissue loss and microglial response at 48 h post LPS-sensitized HI. **(A–D)** TUNEL+ staining of dying brain cells with fragmented DNA—Quantification **(A)** (number of TUNEL+ cells per 20× eye-field, Median ± IQR) and histochemical overview of the ipsilateral forebrain in saline-treated wild type **(B)**, LPS-treated wild type controls **(C)** and LPS-treated animals with global properdin deletion **(D)**. The saline-treated wild type animals showed a very low number of TUNEL+ cells **(B)**. Note the typical pyknotic nuclear morphology of the TUNEL+ cells as well as their high density observed in the LPS-treated wild type controls (**C**—insert, hippocampus) compared to the reduced number of such cells in the LPS-treated properdin KO group (**D**—insert, hippocampus). Compared to the wild type controls, properdin deletion resulted in reduced TUNEL+ cell death with a significant main effect (Kruskal-Wallis test, $p = 0.007$), and significant, individual decrease (Bonferroni correction) in cortex ($p = 0.04$), pyriform cortex ($p = 0.03$), hippocampus ($p = 0.02$) and overall ($p = 0.05$). **(E–H)** Ipsilateral forebrain Nissl staining (Cresyl-Violet, at rostral parietal level, Median ± IQR)—Quantification of ipsilateral brain tissue volume loss **(E)** of saline-treated wild type **(F)**, LPS-treated wild type controls **(G)**, and LPS-treated animals with global properdin deletion **(H)**. The saline-treated wild type animals showed very low levels of ipsilateral tissue loss **(F)**. Compared to wild type controls, global properdin deletion resulted in a decrease of volume loss following LPS-sensitized neonatal HI with a significant main effect (Kruskal-Wallis test, $p = 0.0001$) and significant individual decrease (Bonferroni correction) in cortex ($p = 0.044$) and pyriform cortex ($p = 0.009$). **(I–L)** GFAP immunoreactivity—Quantification of ipsilateral reactive astrogliosis **(G)** in optical luminosity values (OLV, Median ± IQR) and low magnification ipsilateral overview in saline-treated wild type **(J)**, LPS-treated wild type controls **(K)** and LPS-treated animals with global properdin deletion **(L)**. The saline-treated wild type animals showed very low levels of ipsilateral GFAP immunoreactivity **(J)**. The inserts **(K,L)** show higher magnification of the dotted regions in hippocampus. Compared to wild type controls, global properdin deletion decreased reactive astrogliosis with a significant main effect (Kruskal-Wallis test, $p = 0.0001$), however the sub-regional differences did not reach significant values. **(M–P)** Activation of αM+ microglia—Ipsilateral αM microglial activation score

(Continued)

FIGURE 2 | (**M**, Median ± IQR) and low magnification ipsilateral overview in saline-treated wild type **(N)**, LPS-treated wild type controls **(O)**, and LPS-treated animals with global properdin deletion **(P)**. The saline-treated wild type controls showed very low levels of αM+ microglia **(N)**. Note the strong microglial activation in the LPS-treated wild type control group with αM+ cells showing phagocytic morphology at high magnification (**O**—insert, hippocampus), compared to the LPS-treated properdin KO brains exhibiting a ramified phenotype (**P**—insert, hippocampus). Global properdin deletion reduced αM+ microglial activation with a significant main effect (Kruskal-Wallis test, $p = 0.0001$) and significant, individual decrease (Bonferroni correction) in hippocampus ($p = 0.05$). Saline wild type ($n = 12$), saline properdin KO ($n = 14$), LPS-treated wild type ($n = 15$) and LPS-treated properdin KO ($n = 14$) in all assessments. (*$p < 0.05$). CTX, cerebral cortex; PYR, pyriform cortex; HIP, hippocampus; STR, striatum; THAL, thalamus; EC, external capsule. Scale bars: **(F–H)** = 1,200 μm; **(B–D,J–L,N,O)**, $p = 600$ μm. inserts = 30 μm.

with either CR2-Crry (inhibiting all pathways) or CR2-fH (inhibiting AP) significantly reduced infarct size, reduced apoptotic cell death, and improved neurological deficit score in the acute phase after stroke, but only CR2-fH provided sustained protection with no further development of injury in the subacute phase (52). Similarly, Ten et al. (36) demonstrated that C3 deficiency provided protection against MCAO as well as against neonatal HI. Additionally, intranasal C3a treatment ameliorated cognitive impairment in a mouse model of HI brain injury (35). However, C3 deficiency takes away the component central to all three complement pathways compared to properdin deficiency, which reduces these activities. Similarly, factor B-deficiency or CR2-fH treatment improved neurological function and reduced cerebral infarct, demyelination, P-selectin expression and neutrophil infiltration following MCAO in adult mice (52). Although the model of injury in MCAO is technically different than neonatal HI, both models share similarities, including oxygen deprivation and reperfusion, thus effects observed in MCAO could be plausible in neonatal HI and vice versa.

Our results show reduced microglial activation in all studied regions apart from cortex in the properdin deficient group following neonatal HI alone (**Figure 1J**). Similarly, in the LPS-sensitized HI model, we observed a main effect of the global properdin deletion with significant reduction observed in hippocampus (**Figure 2M**). This suggests reduced inflammatory response and subsequent cell death (**Figures 1A**, **2A**). Inflammation plays a major part in the pathology of neonatal HI brain damage (6, 34, 53). Cerebral ischemia induces inflammation in both systemic circulation and the parenchyma. In an adult brain, this results in increased production of cytokines, as well as activation and migration of leukocytes to the injured brain (34, 53). In neonates, however, the result is an immediate innate immune response following the insult. The differences in the mechanisms between adult stroke and neonatal HI are mostly due to the immaturity of the neonatal CNS, resulting in insufficient ability to overcome excitotoxicity, oxidative stress and inflammation. HI damage is suggested to occur because of imbalance between pro- and anti-inflammatory cytokines, which boosts oligodendrocyte precursors to proliferate into astrocytes instead of oligodendrocytes, thus impairing subsequent myelination (54). In some models, presence of properdin has been associated with increased production of pro-inflammatory cytokines (TNF-alpha, IL-1b, and IL-6) and suppressed levels of anti-inflammatory cytokines (IL-10 and TGFb) (55). Therefore, its presence following HI might be a contributing factor for the imbalance between pro- and anti-inflammatory cytokines. We have previously shown that inhibition of IL-6 downstream products such as phosphorylated STAT3 is neuroprotective in neonatal HI (27). Hence, the lack of properdin would prevent IL-6 upregulation and provide neuroprotection in neonatal HI. Therefore, it can be assumed that deletion of properdin would exhibit a neuroprotective effect through reduction of pro-inflammatory cytokine levels, thus preserving the equilibrium between pro- and anti-inflammatory cytokines and ensuring subsequent myelination. Additionally, properdin is required for the AP activation when LPS is present (56). Thus, deficiency in properdin in the presence of LPS would prevent AP activation and ensuing inflammatory response. In addition to the increase of pro-inflammatory cytokines triggered by HI alone, LPS causes further upregulation of TNF-alpha, IL-1b, and IL-6. As properdin deletion might interfere with the execution of IL-6 dependent inflammatory response, it is possible that inherited properdin deficiency inhibits LPS sensitivity in neonates. Conversely, in a study looking at zymosan-induced and LPS-induced septic shock in adult mice, properdin deletion provides protection only in the case of zymosan-, but not in LPS-induced septic shock (57). However, the model of septic shock involves different mechanisms which can explain the differences in the effects of global properdin deletion.

Our data did not support an effect of properdin deletion on astroglial activation in HI alone or in LPS-sensitized HI, suggesting that the protective role of properdin in both models is likely due to impairment of the microglia-dependent pro-inflammatory response post-HI.

As a conclusion, our study provides evidence that properdin is involved and likely plays a key role in the trigger of neonatal HI and LPS-sensitized HI brain damage. Although our study was limited to male gender and the HI insult in the HI alone set of experiments was moderate rather than severe, global properdin deletion provides neuroprotection in the short term (48 h) in both models on the grounds of reduced cell death, tissue loss and microglial activation. The likely mechanism underlying these protective effects is impairment of the microglial pro-inflammatory response, which would prevent imbalance between pro- and anti-inflammatory cytokines following HI insult and would preserve subsequent myelination, however that would require further investigation. Overall our data suggest properdin as a novel target for treatment in neonatal HI brain damage; however, a better understanding of the pathway(s) through which it is involved in HI-brain damage would considerably improve the therapeutic potential of interfering with it in a clinical setting.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

This study was carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and the ARRIVE guidelines. The protocols were approved by the Home Office (PPL70/8784) and UCL Animal Welfare and Ethical Review Body.

AUTHOR CONTRIBUTIONS

CSi: collection and processing of data, writing, and editing the manuscript. QA-S, BS, and AC: collection and processing of data. CSt: provision of the properdin deficient animals, independent genotyping, writing, and editing the manuscript. MH: design of the study, processing of data, writing, and editing the manuscript.

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Endogenous T Cell Receptor Rearrangement Represses Aggressive Central Nervous System Autoimmunity in a TcR-Transgenic Model on the Non-Obese Diabetic Background

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The T cell response to central nervous system (CNS) antigen in experimental autoimmune encephalomyelitis (EAE) permits one to model the immune aspects of multiple sclerosis. 1C6 transgenic mice on the non-obese diabetic (NOD) background possess a class II-restricted T cell receptor (TcR; Vα5-Vβ7) specific for the encephalitogenic peptide myelin oligodendrocyte glycoprotein $(MOG)_{[35-55]}$. It remains to be determined what role is played by allelic inclusion in shaping the TcR repertoire of these mice. Here, we show that 1C6 T cells display substantial promiscuity in their expression of non-transgenically derived Vα chains. Further, enforced expression of the transgenic TcR in 1C6 × *Rag1*$^{-/-}$ mice profoundly disrupted thymic negative selection and led to a sharp decrease in the number of mature peripheral T cells. 1C6 × *Rag1*$^{-/-}$ mice developed spontaneous EAE at a significant frequency and rapidly developed fatal EAE upon immunization with myelin oligodendrocyte glycoprotein $(MOG)_{[35-55]}$. Passive transfer of 1C6 × *Rag1*$^{+/+}$ CD4$^+$ T cells, but not CD8$^+$ T cells or B cells, partially rescued 1C6 × *Rag1*$^{-/-}$ mice from severe EAE. FoxP3$^+$ CD4$^+$ T_{reg} cells were present in the CNS of immunized 1C6 mice, as well as immunized 1C6 × *Rag1*$^{-/-}$ that had been supplemented with 1C6 CD4$^+$ T cells. However, they were not observed in 1C6 × *Rag1*$^{-/-}$ that did not receive Rag1-sufficient 1C6 CD4$^+$. Further, *in vivo* blockade of T_{reg} accelerated the onset of symptoms in 1C6 mice immunized with $MOG_{[35-55]}$, indicating the pertinence of T_{reg}-mediated control of autoimmune inflammation in this model. Thus, TcR allelic inclusion is crucial to the generation of FoxP3$^+$ CD4$^+$ T cells necessary for the suppression of severe CNS autoimmunity.

Keywords: EAE (experimental autoimmune encephalomyelitis), 1C6, TCR transgenic mice, Treg—regulatory T cell, non-obese diabetic (NOD), allelic exclusion, RAG, FoxP3

INTRODUCTION

Multiple sclerosis (MS) is a chronic degenerative disease of the brain, spinal cord and optic nerve that affects an estimated 2 million people worldwide. It is generally accepted that MS is driven by self-reactive adaptive immune responses to central nervous system (CNS) components such as myelin. Accordingly, the majority of current MS therapies target the adaptive immune system (1, 2). The contributions of the immune system to MS pathology can be in large part recapitulated by the animal model, experimental autoimmune encephalomyelitis (EAE) (3). In classic EAE, mice of genetically susceptible backgrounds are immunized with CNS-derived (most frequently myelin-derived) encephalitogenic epitopes. In the past two decades, the introduction of T cell receptor-transgenic (TcR-Tg) lines, in which mice possess T cells with transgenically-encoded antigen specificity for encephalitogenic epitopes, has furthered our understanding of the role played by T cells in CNS autoimmunity (4).

When actively immunized with myelin oligodendrocyte glycoprotein $(MOG)_{[35-55]}$, non-obese diabetic (NOD) background mice present a relatively slow-developing disease over 70–100 days that is characterized by multiple relapses and remissions followed by chronically worsening symptoms, thus recapitulating the most common form of MS. This has led some to characterize NOD-EAE as a model of secondary-progressive (SP) MS (5–7). Recently an MHC class II-restricted, $MOG_{[35-55]}$-specific TcR-Tg strain, 1C6, was described on the NOD background. The 1C6 TcR α-chain incorporates Vα5 and the 1C6 TcR β-chain incorporates Vβ7 (8). Despite being class II-restricted, the 1C6 TcR is expressed on both $CD4^+$ and $CD8^+$ T cells. Interestingly, a proportion of 1C6 T cells express alternate Vα chains (8), in line with previous observations that expression of an ectopic TcR does not exclude the arrangement and expression of a TcR from endogenous alleles (9).

Here, to examine the consequences of enforced allelic exclusion in the 1C6 strain, we studied 1C6 × *Rag1*$^{-/-}$ mice, which are incapable of rearranging their endogenous TcRα and β chains. We found that naïve 1C6 × *Rag1*$^{-/-}$ mice have diminished numbers of both $CD4^+$ and $CD8^+$ T cells. Intriguingly, 1C6 × *Rag1*$^{-/-}$ mice developed extremely severe EAE upon $MOG_{[35-55]}$ immunization. This severe effect could be partially rescued by the infusion of Rag1-sufficient $CD4^+$, but not $CD8^+$, 1C6 T cells. We found that $FoxP3^+$ $CD4^+$ T cells were largely absent from the CNS of 1C6 mice of immunized 1C6 × *Rag1*$^{-/-}$ mice, and that infusion of Rag1-sufficient 1C6 $CD4^+$ T cells rescued this population. Overall, we found that endogenously rearranged TcR chains are essential in mediating the development of regulatory T cells that determine disease susceptibility in 1C6 mice.

RESULTS

Mature T Cell Survival in 1C6 Mice Requires Endogenous TcR Rearrangement

TcR development in the thymus entails the rearrangement and ligation of distinct α-chain V(ariable) and J(oining) segments, or β-chain V, D(iversity) and J segments. Excluded segments are lost from the genome of the cell. While the vast majority of 1C6 T cells express the transgenic TcR (8), it has been previously observed that allelic exclusion in TcR-Tg strains is incomplete (9, 10). We therefore wanted to comprehensively assess the expression of non-transgenic, endogenously-derived Vα segments in 1C6 T cells. Due to the paucity of commercially available flow cytometry antibodies to specific Vα alleles, we employed a PCR-based approach. We found that in addition to transgenic Vα5, 1C6 $CD8^+$ T cells possessed DNA of a number of endogenous Vα segments (**Figure 1**). Thus, 1C6 T cells have a high degree of promiscuity in their TcRα repertoire.

Next, we generated *1C6* × *Rag1*$^{-/-}$ mice to test whether endogenously encoded TcR loci were functionally relevant to 1C6 $CD8^+$ survival and function. These mice are unable to rearrange endogenous TcR loci but their T cells should bear transgenic 1C6 TcR. We found that in the absence of Rag1,

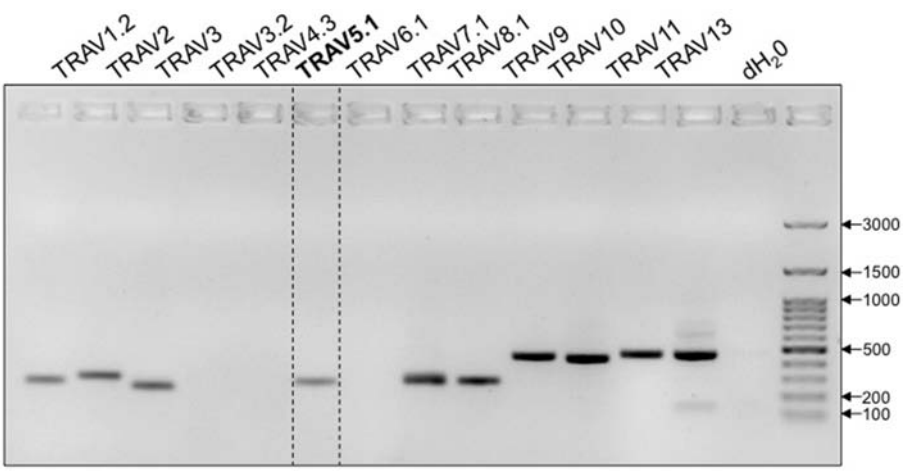

FIGURE 1 | 1C6 Tg T cells are promiscuous in their expression of endogenous TcRα. Purified splenic 1C6 $CD8^+$ T cells were assessed for the presence of specific TcRα DNA by PCR. Transgenically encoded Vα5.1 bracketed by dotted lines. Representative of three experiments.

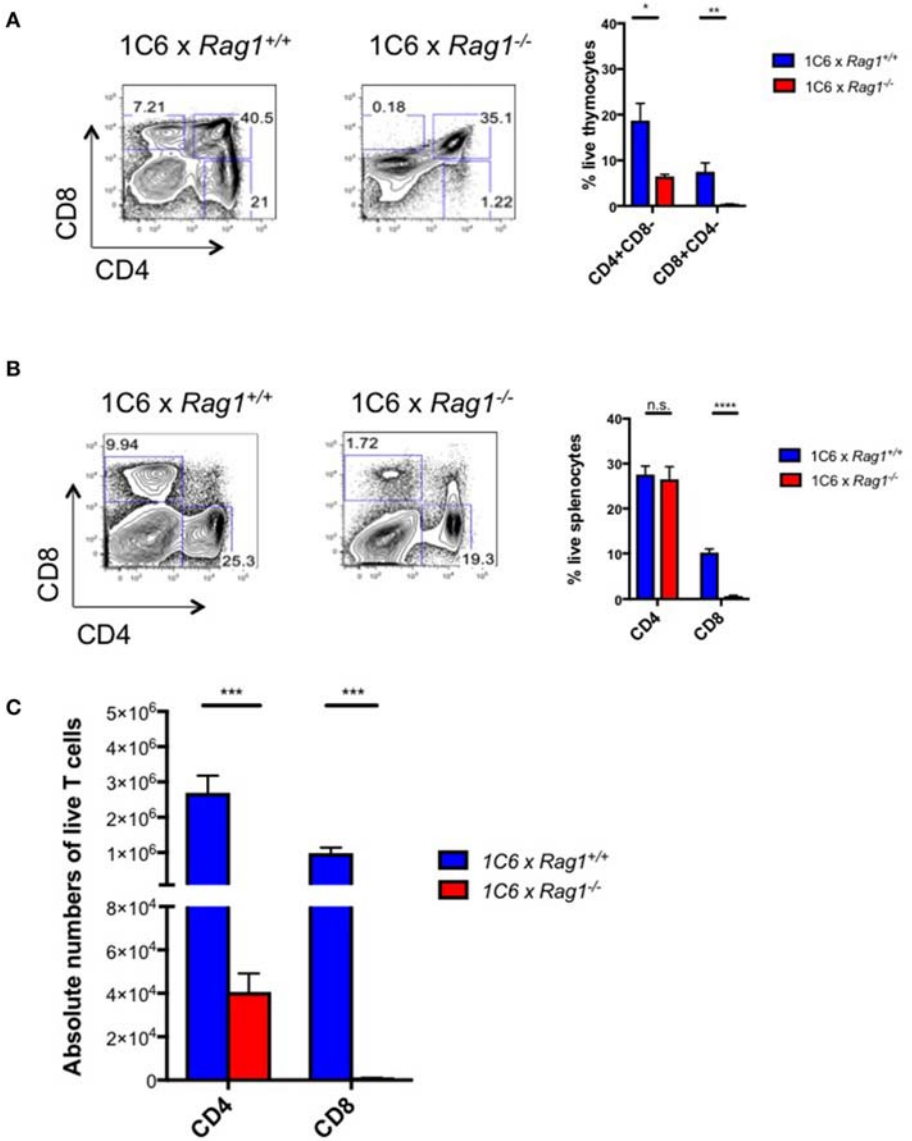

FIGURE 2 | Peripheral T cell populations are dysregulated in 1C6 × *Rag1*$^{-/-}$ mice due to defective negative selection. Thymic **(A)** and splenic **(B,C)** cell populations were enumerated from female 1C6 ($n = 6$) vs. 1C6 × *Rag1*$^{-/-}$ ($n = 6$) mice. **(A)** The proportion of thymocytes expressing CD4 and/or CD8 was enumerated. The proportion **(B)** and absolute frequency **(C)** of splenic CD4$^+$ and CD8$^+$ T cells were assessed by flow cytometry. Graphs: $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$, two-tailed *t*-test. n.s., not significant.

there was a profound arrest at the double-positive stage of thymopoiesis (**Figure 2A**). This indicated that expression of endogenously derived TcR was essential for 1C6 thymocytes to escape negative selection. In the periphery, while the percentage of splenic CD4$^+$ T cells was similar between 1C6 × *Rag1*$^{-/-}$ and 1C6 mice, the frequency of CD8$^+$ T cells was sharply reduced (**Figure 2B**). However, absolute numbers of both CD4$^+$ and CD8$^+$ T cells were dramatically diminished in 1C6 × *Rag1*$^{-/-}$ mice (**Figure 2C**). Together, our data showed that allelic inclusion of endogenously rearranged TcR is both widespread and essential for proper development of the T cell repertoire in 1C6 mice.

1C6 × Rag1$^{-/-}$ Mice Develop Severe EAE

Curiously, we also found that more than half (24/42) of 25-weeks old 1C6 × *Rag1*$^{-/-}$ mice developed spontaneous paralytic disease (**Table 1**) characterized by immune cell infiltration and demyelination in the cerebellum and spinal cord (**Figure 3**). By contrast, no Rag1-sufficient 1C6 mice developed spontaneous disease in our colony (**Table 1**), in corroboration of previous findings that such symptoms occur only rarely in this strain (8).

To directly examine whether EAE in these animals was driven in an antigen-specific manner, we immunized 1C6 × *Rag1*$^{-/-}$ and 1C6 × *Rag1*$^{+/+}$ mice with MOG$_{[35-55]}$. Both male and female 1C6 × *Rag1*$^{-/-}$ mice developed severe, often fatal, EAE

TABLE 1 | Incidence and severity of 1C6 × *Rag1*$^{-/-}$ spontaneous EAE.

Group #	Group ID	Sex	Incidence	Time of onset (weeks)	Mean max. score
1	1C6	M	0/67[a]	n.a	n.a.
2	1C6	F	0/37[b]	n.a.	n.a.
3	1C6 × *Rag1*$^{-/-}$	M	7/17[a]	17.3 ± 1.0	3.357 ± 0.5847
4	1C6 × *Rag1*$^{-/-}$	F	17/25[b]	22.8 ± 5.5	3.647 ± 0.3707

Unimmunized mice were followed until at least 25 weeks of age. Mean maximal score was calculated from mice that developed disease only. Time of onset and mean maximal score, mean ± s.e.m. [a] $p < 0.0001$; [b]$p < 0.0001$. [a,b] two-tailed Fisher's exact test with Bonferroni's correction. No significant differences in time of onset or mean maximal score were measured between male and female 1C6 × Rag1^{-1} mice.

within 12–15 days. By contrast, immunized 1C6 × *Rag1*$^{+/+}$ controls displayed mild disease, at best, during the same time frame (**Figure 4**). This is in line with previous findings that actively immunized NOD strain mice develop severe paralytic EAE (i.e., hind limb paralysis) only after 60 days (11, 12). Thus, despite having an extremely low frequency of peripheral T cells, 1C6 × *Rag1*$^{-/-}$ mice were prone to develop CNS-targeted inflammation.

1C6 × Rag1$^{+/+}$ CD4^{+} T Cells Ameliorate Disease in 1C6 × Rag1$^{-/-}$ Mice

We had observed that absolute numbers of CD4^{+} T cells were greatly reduced in 1C6 x *Rag1*$^{-/-}$ mice (**Figure 2C**). This indicated that, despite the 1C6 TcR being class II-restricted, survival of 1C6 CD4^{+} T cells was partially dependent on endogenous TcR rearrangement. Further, in light of the fact that 1C6 CD8^{+} T cells could not ameliorate the severe disease observed in 1C6 × *Rag1*$^{-/-}$ mice, it raised the possibility that 1C6 CD4^{+} T cells may themselves contain a regulatory population. To address this, we asked whether passive transfer of 1C6 CD4^{+} T cells could rescue 1C6 × *Rag1*$^{-/-}$ mice from severe $MOG_{[35-55]}$-driven EAE. We therefore passively transferred Rag-sufficient 1C6 T cells to 1C6 × *Rag1*$^{-/-}$ mice, and 7 days later, immunized them in parallel with 1C6 × *Rag1*$^{-/-}$ mice that did not receive 1C6 CD4^{+} T cells. As expected, 1C6 × *Rag1*$^{-/-}$ rapidly developed fatal EAE. By contrast, 1C6 × *Rag1*$^{-/-}$ mice receiving Rag1-sufficient 1C6 CD4^{+} T cells did not develop fatal disease when monitored for 20 days (**Figure 5A**). As we had also found that CD8^{+} T cells were profoundly diminished in 1C6 × *Rag1*$^{-/-}$ mice, and as substantial evidence exists that in certain contexts, CD8^{+} regulatory T cells (T_{reg}) can suppress inflammation in MS and EAE (13), we wanted to rule out the possibility that exacerbated disease in 1C6 × *Rag1*$^{-/-}$ mice was due to a lack of a CD8^{+} T_{reg}. To address this, we infused 1C6 × *Rag1*$^{-/-}$ mice with 1C6 × *Rag1*$^{+/+}$ CD8^{+} T cells and immunized them with $MOG_{[35-55]}$ after 7 days. We observed no differences in disease severity between these animals and unmanipulated 1C6 × *Rag1*$^{-/-}$ mice (**Figure 5B**). Further, we were able to rule out a potential role for regulatory B cells (14) in controlling disease severity, as prior transfer of 1C6 strain B cells to 1C6 × *Rag1*$^{-/-}$ mice did not protect them from severe EAE (**Figure 5C**). Taken altogether, our data showed that the exacerbated disease seen in 1C6 × *Rag1*$^{-/-}$ mice resulted from the absence of a CD4^{+} T cell subpopulation normally resident in 1C6 mice.

We next examined *ex vivo* cytokine production from the CD4^{+} T cell compartment of 1C6 × *Rag1*$^{-/-}$ mice that had been reconstituted with 1C6 × *Rag1*$^{+/+}$ CD4^{+} T cells prior to immunization. We therefore repeated our 1C6 CD4^{+} passive transfer protocol in 1C6 × *Rag1*$^{-/-}$ mice, followed by $MOG_{[35-55]}$ immunization. Spleen and CNS-infiltrating T cells were isolated from unreconstituted, immunized, 1C6 × *Rag1*$^{-/-}$ mice when they reached ethical endpoints and from 1C6 CD4^{+} T cell-reconstituted counterparts that we sacrificed in parallel. In the spleen, IL-2 was selectively decreased in 1C6 × *Rag1*$^{-/-}$ mice that received 1C6 CD4^{+} T cells; no differences were observed in the production of IFNγ, IL-17, GM-CSF, or TNFα (**Figure 5D**). Curiously and in contrast, among CNS-infiltrating CD4^{+} T cells, IL-2 production was selectively increased in mice that had been infused with 1C6 × *Rag1*$^{+/+}$ CD4^{+} T cells (**Figure 5E**).

CNS-Infiltrating FoxP3^{+}CD4^{+} Are Absent in the CNS of 1C6 × Rag1$^{-/-}$ EAE Mice

FoxP3^{+}CD4^{+} T_{reg} (15) control both onset and severity of EAE (16) and their survival is dependent on IL-2 (17). Given the fact that 1C6 × *Rag1*$^{+/+}$ CD4^{+} T cells rescued 1C6 × *Rag1*$^{-/-}$ mice from severe EAE, as well as the observed anomalies in IL-2 production in the presence or absence of Rag-sufficient 1C6 T cells, we next wanted to examine whether 1C6 CD4^{+} T cell infusion could drive T_{reg} expansion. While absent from immunized 1C6 × *Rag1*$^{-/-}$ mice at experimental endpoints, 1C6 CD4^{+} T cell reconstitution increased the frequency of FoxP3^{+}CD4^{+} T cells in the CNS (**Figure 6A**). CNS-infiltrating T_{reg} were largely Vβ7^{+} (**Figure 6B**), indicating that endogenous TcR rearrangement was restricted to the α-chain in these cells.

It was possible that the expansion of 1C6-origin CD4^{+} T_{reg} in 1C6 × *Rag1*$^{-/-}$ mice was a homeostatic response to a lack of this population in 1C6 × *Rag1*$^{-/-}$ mice. To rule out this possibility, we assessed the presence of CD4^{+} T_{reg} in actively immunized 1C6 animals. FoxP3^{+}CD4^{+} T cells were identified in the CNS of 1C6 mice and were, as expected, absent in 1C6 × *Rag1*$^{-/-}$ mice (**Figure 6C**). CD8^{+}FoxP3^{+} cells were not seen in either genotype at an appreciable frequency. Thus, our data show that CD4^{+}FoxP3^{+} T_{reg} are critical to controlling EAE severity in the 1C6 strain, and that their development is crucially dependent on the arrangement of endogenous TcRα.

Given our data pointed toward an important role for T_{reg} in suppressing immune control of CNS autoimmunity in NOD-EAE, we next wanted to assess the effects of T_{reg} depletion in immunized 1C6 mice. We treated mice with 3 successive doses of anti-CD25 monoclonal antibody (clone PC61), which depletes T_{reg}, or with isotype control, and subsequently immunized them with $MOG_{[35-55]}$ (18). PC61-treated mice developed disease of earlier onset than that observed in control-treated animals (8.4 days ± 0.7 vs. 18.6 ± 3.5, $p < 0.021$; **Figure 6D**), indicating an

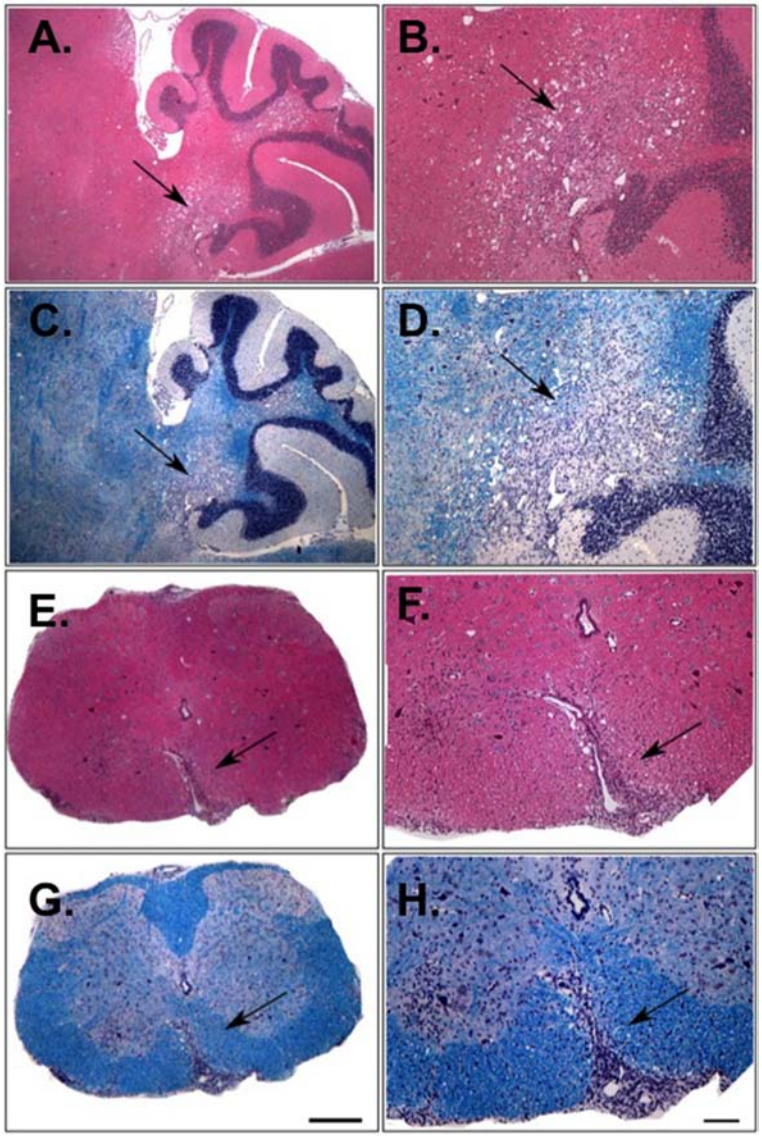

FIGURE 3 | Histological analysis of the CNS of 1C6 × *Rag1*$^{-/-}$ mice that develop spontaneous EAE. Cerebellar **(A–D)** and spinal cord **(E–H)** lesions from an 18-weeks old male 1C6 × *Rag1*$^{-/-}$ mouse that spontaneously developed paralytic disease. H&E staining (**A,B,E,F**) was used to identify inflammatory foci, and Luxol fast blue was used to detect myelin **(C,D,G,H)**. Arrows indicate inflammatory damage. **(A,C,E,G)**, 10× magnification. **(B,D,F,H)**, 4× magnification. Scale bars, 100 μm. Representative of eight animals (6 female, 2 male).

important role for T_{reg} in regulating the initiation of autoimmune symptoms in NOD EAE.

DISCUSSION

Our data indicate that 1C6 T cells can express multiple endogenous Vα chains, and that endogenous TcR rearrangement is critical for the selection of CD4$^+$ FoxP3$^+$ T_{reg} that mitigate disease in Rag1-sufficient 1C6 mice. Few CD4$^+$ or CD8$^+$ single-positive thymocytes were detected in 1C6 × *Rag1*$^{-/-}$ thymi, indicating that the 1C6 TcR is highly prone to negative selection. These observations are in contrast to what is known about other self-reactive TcR-Tg strains. T cells with a myelin basic protein $(MBP)_{[1-9]}$-specific TcR were thymically selected even when crossed to a *Rag1*$^{-/-}$ background; further, numbers of splenic CD4$^+$ T cells were similar between Rag1-sufficient ("T/R$^+$") and Rag1-deficient ("T/R-") mice (9). Similarly, the proteolipid protein $(PLP)_{139-151}$-specific "5B6" TcR successfully escapes negative selection on a Rag-deficient background (10). Our data are striking when one considers that the parental 1C6 T cell clone was isolated from the peripheral lymphoid tissue of an immunized WT NOD mice, and thus by definition escaped thymic negative selection in the thymus. TcR signal strength to self is thought to dictate whether a thymocyte passes the single-positive phase, with strongly self-reactive clones being negatively selected. The 1C6 TcR is thus one that is likely to be

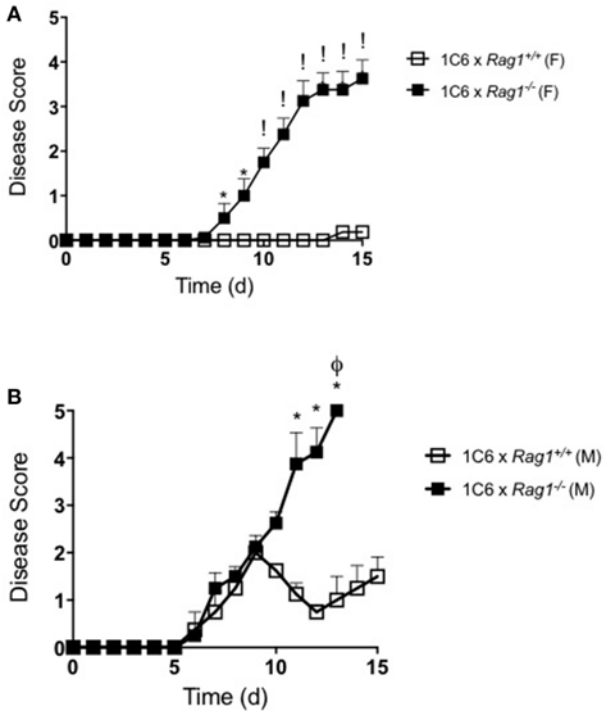

FIGURE 4 | 1C6 × *Rag1*$^{-/-}$ mice develop severe $MOG_{[35-55]}$-driven EAE. 1C6 (male, $n = 4$; female, $n = 5$) and 1C6 × *Rag1*$^{-/-}$ (male, $n = 4$; female, $n = 5$) mice were actively immunized with $MOG_{[35-55]}$ and were monitored for signs of EAE. **(A)** female, **(B)** male. *$p < 0.05$; !$p < 0.001$, two-tailed Mann-Whitney *U* test. ϕ indicates that all mice in the experimental group attained ethical endpoints. Representative of three immunizations.

highly self-reactive and rarely present in the periphery of WT mice. In the future, it will be interesting to compare the affinity of 1C6 to $MOG_{[35-55]}$/class II to that of other $MOG_{[35-55]}$-specific TcR such as 2D2. Further, while our data indicate that 1C6 T cells are highly promiscuous in their expression of Vα at a population level, we do not know whether individual T cells in these mice escape negative selection by expressing both the 1C6 TcR as well as a second TcR consisting of Vβ7 paired to an endogenous Vα, or whether they lost 1C6 TcR completely and express only endogenous TcR. Unfortunate, the current lack of antibodies against the full spectrum of Vα makes it challenging to distinguish between these possibilities using flow cytometry. In the future, however, it may be possible to use next-generation sequencing techniques to characterize TcRα/β pairing at the single cell level.

Despite having few circulating T cells, 1C6 × *Rag1*$^{-/-}$ mice developed extremely severe EAE. By contrast, EAE in NOD strain mice develops relatively slowly, with advanced paralytic disease observed only after 60 days. Disease in actively immunized 1C6 × *Rag1*$^{-/-}$ was characterized by augmented expression of the T cell growth factor IL-2 from peripheral $CD4^+$ T cells. Thus, 1C6 × *Rag1*$^{-/-}$ T cells are highly autoreactive, in line with their propensity to be negatively selected. Indeed, while transfer of Rag-sufficient 1C6 $CD4^+$ T cells to 1C6 × *Rag1*$^{-/-}$ saved these mice from fulminant CNS autoimmunity, they retained a significant amount of disability despite the fact that the $FoxP3^+$ T_{reg} niche was reconstituted, suggesting again that the 1C6 TcR is itself highly pathogenic.

Interestingly, there were no differences in production of IFNγ or IL-17 between CNS infiltrating $CD4^+$ T cells from immunized 1C6 × *Rag1*$^{-/-}$, vs. 1C6 × *Rag1*$^{-/-}$ reconstituted with 1C6 $CD4^+$ T cells, suggesting that loss of endogenous TcR rearrangement did not skew the immune response toward either Th1 or Th17. This, together with the fact that the severe disease phenotype of 1C6 × *Rag1*$^{-/-}$ mice could be partially rescued by the presence of Rag-sufficient 1C6 $CD4^+$, but not $CD8^+$, T cells, led us to ask whether an absence of T_{reg} may explain the phenotype. Indeed, $FoxP3^+$ $CD4^+$ T_{reg} were absent from immunized 1C6 × *Rag1*$^{-/-}$ mice, indicating that their severe disease was at least in part due to a loss of this subpopulation. 1C6 T_{reg} were $V\beta7^+$, indicating that TcR rearrangement in these cells occurred chiefly for the α-chain. Strikingly, *in vivo* depletion of $CD25^+$ T_{reg} accelerated the onset of symptoms in 1C6 mice, indicating that T_{reg} control the initiation of autoimmune reactivity in this model. Depleted mice did not progress to fulminant EAE; it is possible that $CD25^-$ T_{reg}, which play important roles in immunoregulation (19) and which would be preserved in our protocol, might also contribute to the control of CNS autoimmunity in Rag1-sufficient animals.

Tonegawa et al. previously reported that Rag-deficient, $MBP_{[1-9]}$-specific, "T/R$^-$" mice develop spontaneous EAE with 100% frequency (9). Disease in "T/R$^-$" mice was rescued by the transfer of Rag-sufficient Tg "T/R$^+$" (20) or wildtype (21) T cells. These cells were later shown to be $CD4^+CD25^+$ (22) and presumably $FoxP3^+$ T_{reg}. Rag deficiency has additionally been shown to exacerbate EAE in two Tg strains featuring a humanized TcR specific for myelin antigen (23, 24). Thus, our data, and that of others, indicate that functionally important T_{reg} can be present in the periphery, even when one skews the repertoire toward an autoreactive TcR specificity derived from an inflammatory effector T cell clone. This raises the question of whether allelic inclusion generates *de novo* TcR that drive T_{reg} generation, possibly due to the strength of their interactions with self-Ag in the thymus. This would be exciting, as it would indicate that there are specific TcR rearrangements that we could identify that promote peripheral tolerance to CNS antigen via the development of T_{reg}. Evidence supporting this model comes from Bautista and colleagues, who found that when introduced into wildtype thymi, donor thymocytes expressing a T_{reg}-specific TcR could give rise to $FoxP3^+$ cells. By contrast, thymocytes expressing TcR to non-self Ag did not have this effect (25). Leung and colleagues additionally showed a higher frequency of donor-derived $FoxP3^+$ cells when T_{reg}-specific TcR-expressing thymi were introduced to recipient mice. However, this latter study also found that the percentage of donor-derived $FoxP3^+$ T cells in these animals did not exceed the frequency of T_{reg} seen in a normal mouse. Thus, while specific TcR may indeed preferentially give rise to $FoxP3^+$ T_{reg}, it is also likely that the development of these cells is regulated homeostatically. It remains to be seen whether 1C6-origin T_{reg} recognize $MOG_{[35-55]}$ or other Ag via their endogenous TcR. It must also be noted that endogenous TcR rearrangement is not a

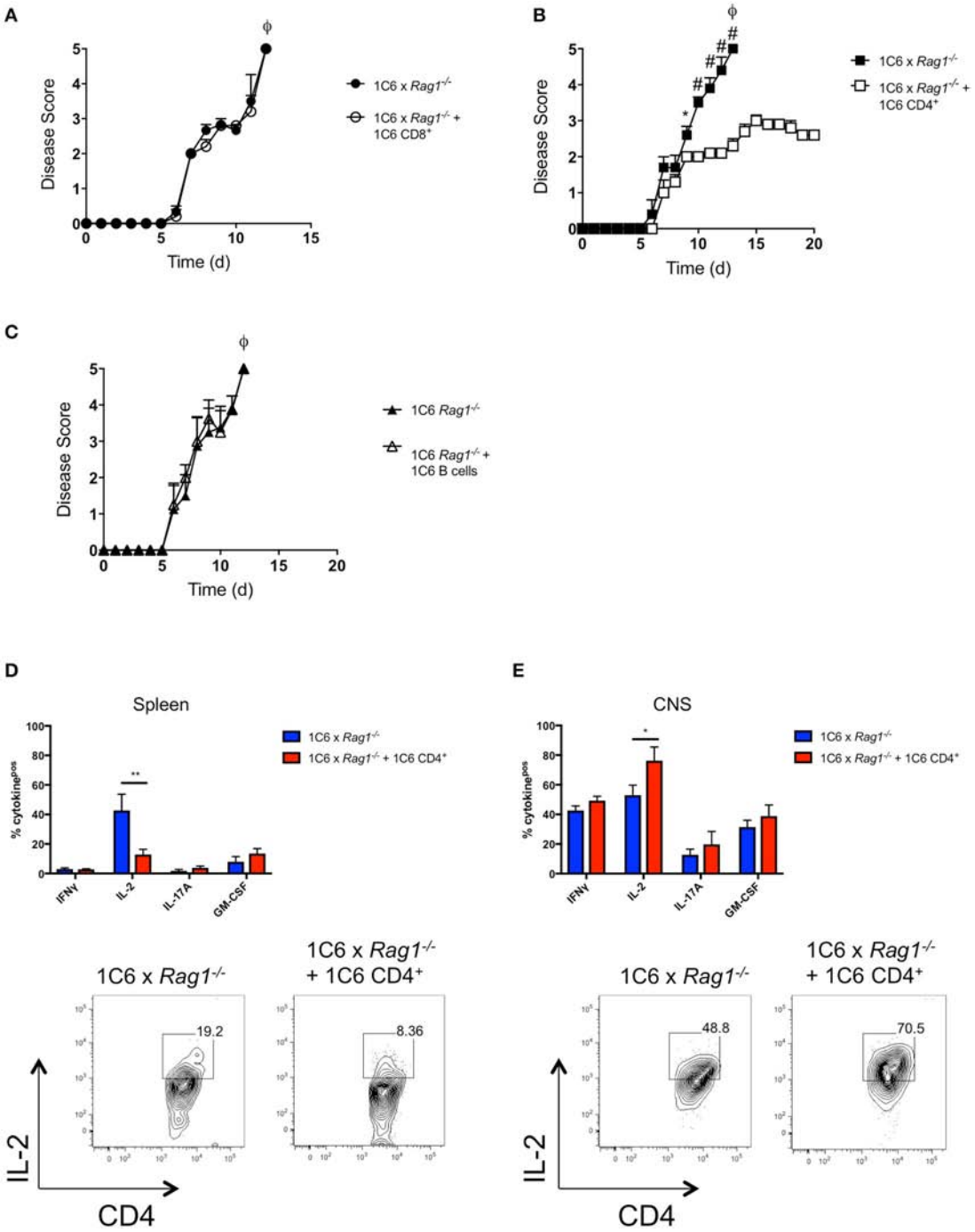

FIGURE 5 | 1C6 CD4$^+$ T cells restrain CNS autoimmunity in 1C6 × *Rag1*$^{-/-}$ mice. **(A)** Male 1C6 × *Rag1*$^{-/-}$ mice were reconstituted ($n = 5$), or not ($n = 5$), with 2 × 10^6 CD4$^+$ T cells from unmanipulated male 1C6 mice. After 7 days, mice were actively immunized with $MOG_{[35-55]}$ and were monitored for signs of EAE. Representative of 2 experiments. **(B)** Male 1C6 × *Rag1*$^{-/-}$ mice were reconstituted ($n = 5$), or not ($n = 5$), with 2 × 10^6 CD8$^+$ T cells from unmanipulated male 1C6 mice. After 7 days, mice were actively immunized with $MOG_{[35-55]}$ and were monitored daily for signs of EAE. *$p < 0.05$, two-tailed Mann-Whitney *U* test. ϕ indicates that all mice in the experimental group attained ethical endpoints. **(C)** Female 1C6 × *Rag1*$^{-/-}$ mice were reconstituted ($n = 4$), or not ($n = 4$), with 2 × 10^6 CD19$^+$ B cells from unmanipulated 1C6 mice. After 7 days, mice were actively immunized with $MOG_{[35-55]}$ and were monitored for signs of EAE. ϕ indicates that all mice in the experimental group attained ethical endpoints. *$p < 0.05$; #$p < 0.01$, two-tailed Mann-Whitney *U* test. ϕ indicates that all mice in the experimental group attained ethical endpoints. **(D,E)**. Spleen **(D)** or CNS-infiltrating **(E)** CD4$^+$ T cells were isolated from immunized 1C6 × *Rag1*$^{-/-}$ mice when they reached ethical endpoints ($n = 9$), or from 1C6 × *Rag1*$^{-/-}$ (reconstituted with 1C6 CD4$^+$) that were sacrificed in parallel ($n = 4$). T cells were assessed *ex vivo* for the indicated cytokines by flow cytometry. All data are gated on live CD4$^+$ events. *$p < 0.05$; **$p < 0.01$, Sidak's multiple comparisons test after two-way ANOVA. Bottom plots, representative IL-2 expression from splenic **(D)** or CNS-infiltrating **(E)** CD4$^+$ T cells.

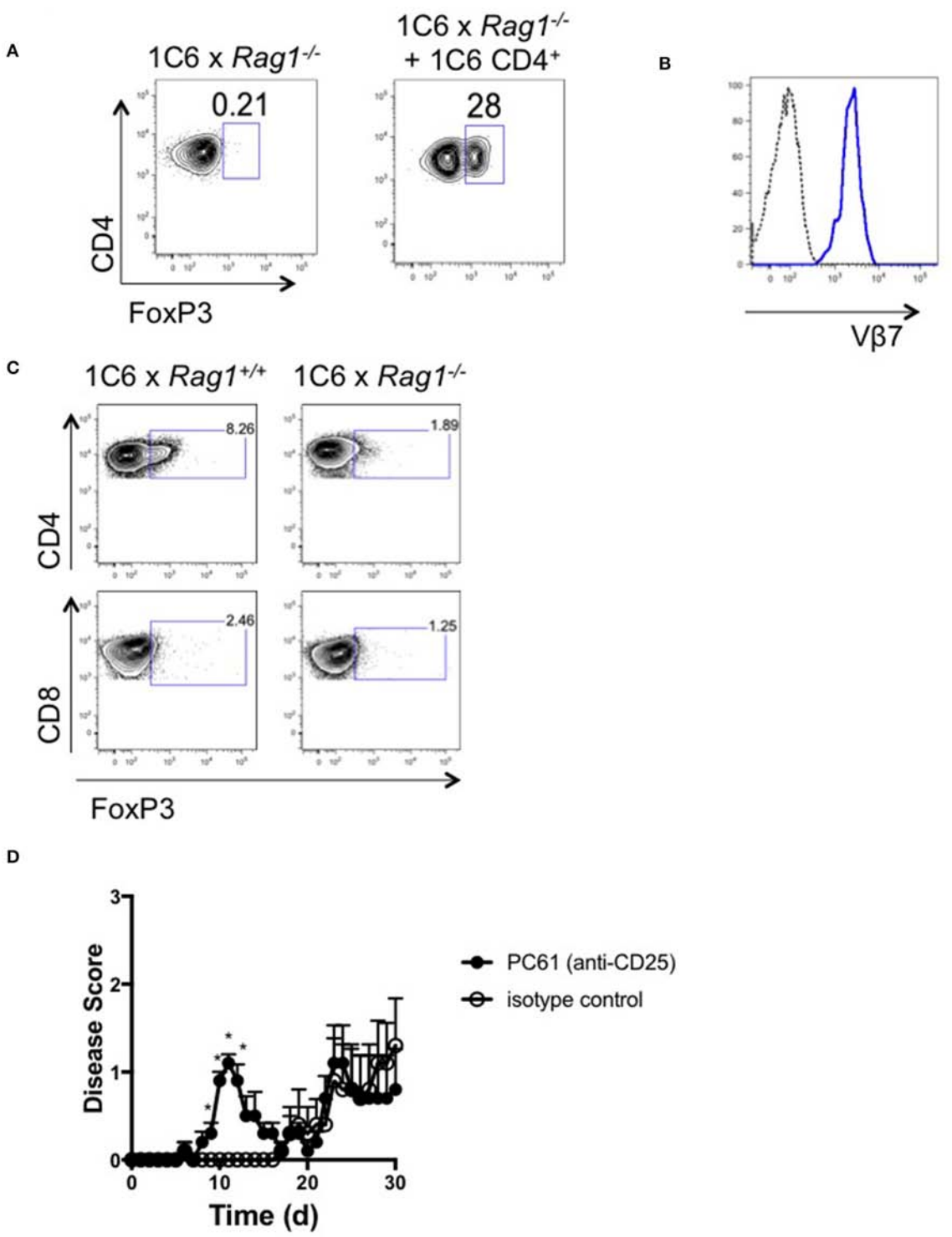

FIGURE 6 | FoxP3$^+$ CD4$^+$ T cells are absent from the CNS of 1C6 × *Rag1*$^{-/-}$ mice with EAE. **(A,B)**. Female 1C6 × *Rag1*$^{-/-}$ mice were reconstituted, or not, with 2 × 10^6 CD4$^+$ T cells from unmanipulated 1C6 mice. After 14 days, mice were actively immunized with $MOG_{[35-55]}$. At experimental endpoints, CNS-infiltrating CD4$^+$ T cells were assessed by flow cytometry for expression of FoxP3 **(A)**. Representative data, $n = 4$ each group. **(B)** Vβ7 expression (blue line) on CNS-infiltrating CD4$^+$FoxP3$^+$ T cells from a 1C6 × *Rag1*$^{-/-}$ mouse reconstituted with 1C6 CD4$^+$ T cells. Dotted line, FMO control. **(C)** Female 1C6 and 1C6 × *Rag1*$^{-/-}$ mice were actively immunized with $MOG_{[35-55]}$. Lymph node T cells were isolated at the peak of disease and assessed by flow cytometry for FoxP3 expression. Gated on CD4$^+$ (top) or CD8$^+$ (bottom) events. Representative of 5 mice each. **(D)** Male 1C6 mice were treated with Treg-depleting anti-CD25 antibody (PC61) or with isotype control on days−11,−9, and−7, and were immunized with $MOG_{[35-55]}$ on d0. $n = 5$, both groups. *$p < 0.05$ on individual days, Mann-Whitney *U* test.

universally critical mechanism of peripheral tolerance in TcR-Tg strains, as 2D2 mice do not appear to develop worsened disease when crossed to a *Rag2*$^{-/-}$ background (26). The capacity of background-specific MHC to present antigen is also likely to play a role in the development of T_{reg} in these strains.

This work describes the requirement for endogenous TcR rearrangement for appropriate T cell development in the 1C6 transgenic strain. Peripheral T cells are profoundly reduced when expression of the transgenic TcR is enforced on a *Rag1*$^{-/-}$ background. Despite the reduction in T cells, 1C6 × *Rag1*$^{-/-}$ mice are susceptible to severe EAE that is at least partially due to an absence of CD4$^+$, but not CD8$^+$, T_{reg}. These findings underscore the clonal complexity of T cell-driven responses in the context of autoimmunity.

MATERIALS AND METHODS

Animals and EAE Induction

1C6 mice were a kind gift of Dr. Vijay Kuchroo (Boston, MA) and were maintained in our facility. *Rag1*$^{-/-}$ NOD strain mice were obtained from JAX and were crossed to the 1C6 strain in our facility. The sex of the mice used in each experiment is indicated in the Figure legends and in **Table 1**. Mice were monitored bi-weekly for initial signs of spontaneous paralytic disease. Mice that developed spontaneous disease were monitored daily. Active immunization was conducted at 10–12 weeks of age similar to as described previously (27). Briefly, they were immunized with 200 μg $MOG_{[35-55]}$ in 100 μL of complete Freund's adjuvant (Difco) supplemented with 500 μg *M. tuberculosis* extract (Difco). Mice received 200 ng pertussis toxin (List Biological Labs) on d0 and d2. For lymphocyte rescue experiments, 1C6 × *Rag1*$^{-/-}$ mice were passively transferred with 2 × 10^6 purified splenic 1C6 CD4$^+$ T, CD8$^+$ T, or CD19$^+$ B cells. Rescue mice were then rested for 7 days prior to immunization in parallel with controls. For T_{reg} depletion protocol, mice received 3 injections 100 μg of PC61 or isotype control antibody, each separated by 48 h (18). Seven days after last injection, mice were immunized as described above. All EAE mice were weighed and assessed for disease symptoms daily for up to 30 days, using an established semi-quantitative scale that we have used previously (28): 0, no disease; 1, decreased tail tone; 2, hind limb weakness or partial paralysis; 3, complete hindlimb paralysis; 4, front and hind limb paralysis; 5, dead or having attained ethical endpoints. Moribund mice were euthanized with 24 h if they did not show improvement. "Disease onset" is defined as being within 24 h of the first signs of clinical symptoms.

Antibodies

Flow cytometry monoclonal Abs (mAbs) against mouse antigens were obtained from eBioscience (CD4, clone RM4-5; CD8α, clone 53–6.7; CD19, clone eBio1D3; IFNγ, clone XMG1.2; GM-CSF, clone MP1-22E9), BioLegend (Vβ7, clone TR3-10; IL-17A, clone TC11-18H10.1; FoxP3, clone FJK-16S) or BD (IL-2, clone JES6-5H4); anti-CD25 for *in vivo* depletion, as well as rIgG1 isotype control, were obtained from BioXcell.

T Cell Purification

Mononuclear cell preparations were obtained from mouse spleens of donor mice. CD4$^+$ T cells, CD8$^+$ T cells and CD19$^+$ B cells were enriched using the appropriate MACS beads (Miltenyi). They were then incubated with the appropriate (CD4, CD8, CD19) fluorochrome-labeled antibodies and purified using high-speed cell sorting (FACSAria, BD).

TcR Vα PCR

CD8$^+$ T cells were purified from 1C6 mouse spleen using high-speed cell sorting, and genomic DNA was isolated using DNA Miniprep kit (Bio Basic Canada). PCR amplification of different regions of TCRAV was carried out using a Bio-Rad C1000 Touch thermocycler. In each PCR reaction, 20 ng of genomic DNA was added to 20 μL of reaction mixture consisting of 1X Accustart II PCR super mix (Quanta Bio). Allele-specific primers were added at 0.2 μmol/ reaction. Each PCR reaction consisted 35 cycles of 2 min of denaturation at 95°C, 30 s 95°C, annealing at 58°C, and 1 min of extension at 72°C, and a final extension step of 5 min at 72°C. At the end of the reaction, the PCR product was mixed with 2 μL of loading dye (200 g/L sucrose, 0.05 g/L bromphenol blue) and was then separated on 1% agarose gel. Primer sequences were designed using PrimerQuest software (IDT) and were as follows: (1) *TRAV1.2*, 5′-CAGCCTGCCAAATTGATGTC-3′, 5′-ACAGAGGTATGAGGCAGAGT-3′, 260 bp amplicon; (2) *TRAV2*, 5′-CTTGCCAAGACCACCCA-3′, 5′-GTCAGTCAC AATGCAGTAATACAC-3′, 272 bp; (3) *TRAV3*, 5′-CCCTCC TCACCTGAGTGT-3′, 5′-GATGGGCAGCTGTGAGG-3′, 224 bp; (4) *TRAV3.2*, 5′-GAGAGCAGGTGGAGCATTG-3′, 5′-ACT TGCTGCACAGAAGTACA-3′, 272 bp; (5) *TRAV4.3*, 5′-GTG CAGATTTGCTGTGAGTTG-3′, 5′-CCTCAGCAGCACAGA AGTAA-3′, 464 bp; (6) *TRAV5.1*, 5′-TGGAACAGCTCCCTT CCT-3′, 5′-ACTTGCTGAGCAGAAGTAGATG-3′, 269 bp; (7) *TRAV6.1*, 5′-GGAGACTCAGTGACCCAGAT-3′, 5′-CACCCA GAACACAGTAGTACA-3′, 280 bp; (8) *TRAV7.2*, 5′-AACAGA AGGTACAGCAGAGC-3′, 5′-TGCTCACTGCACAGAAGT AG-3′, 276 bp; (9) *TRAV8.1*, 5′-AGTCAACTAGCAGAAGAG AATCC-3′, 5′CATCAGTAGCACAGAAGTACACA-3′, 277 bp; (10) *TRAV9*, 5′-ATCTCGTTCCTCGGGATACA-3′, 5′-GCT CACAGCACAGAAGTACA-3′, 446 bp; (11) *TRAV10*, 5′-TCC CTTCACACTGTATTCCTATTC-3′, 5′-GGAGAATCGTTT GGCTTTCTTATC-3′, 430 bp; (12) *TRAV11*, 5′-TGGTCTTGT GGCTGCATTAT GTGCTGTGCTTAGCATCTTTATC-3′, 466 bp; (13) *TRAV13*, 5′-GTTTGCTGTGAGTCTGGGT CACAGA GATAAGTGCCTGAGTC-3′, 458 bp.

Histopathology

Mice were euthanized and perfused first with cold PBS and then with 4% paraformaldehyde (PFA) through the left cardiac ventricle. Brains and spinal cords were dissected from the skull and spinal column, respectively. Tissues were incubated for 24 h in 4% PFA at 4°C and then for a minimum of 48 h in PBS before being embedded in paraffin. Five-micrometer thick sections of the brain and spinal cord were stained with hematoxylin & eosin (H&E) or Luxol fast blue for myelin (29). Images were taken using Nova Prime software (Bioquant Image Analysis Corporation) with a high-resolution QICAM

fast color 1,394 camera (1,392 × 1,040 pixels; OImaging) installed on a Nikon Eclipse 80i microscope. Images were taken at 4× and 10× magnification and were calibrated with a microscope micrometer calibration ruler using FiJi software (NIH).

CNS Mononuclear Cell Isolation

Mice were euthanized and perfused with cold PBS administered through the left cardiac ventricle. Brains and spinal cords were dissected from the skull and spinal column, respectively. CNS tissue were homogenized using a PTFE Tissue Grinder (VWR) and were incubated for 30 min at 37°C in homogenization solution (HBSS containing 4 ng mL-1 liberase and 25 ng mL-1 DNase). Homogenate was filtered through a 70-μm cell strainer, resuspended in 35% Percoll (GE Healthcare) and centrifuged. Mononuclear cells were collected, washed and prepared for flow cytometric analysis.

Flow Cytometry

Staining for cell surface markers was carried out for 30 min at 4°C. Prior to incubation with mAbs against cell surface markers, cells were incubated for 10 min in the presence of Fc Block (BD Biosciences) to prevent non-specific Ab binding to cells. For analysis of intracellular cytokine expression, cells were first cultured for 4 h in the presence of 50 ng mL^{-1} phorbol 12-myristate 13-acetate (Sigma-Aldrich), 1 μM ionomycin (Sigma-Aldrich) and GolgiStop (1 μL per mL culture; BD Biosciences). Cells were subsequently incubated with Fc Block (BioLegend) and fluorescent cell surface marker Abs and were then fixed and permeabilized using Fixation and Perm/Wash buffers (eBioscience). They were then stained with fluorescent Abs against intracellular markers. FoxP3 Fix/Perm and Perm buffers (BioLegend) were used for FoxP3 staining. Flow cytometry data were collected using a LSRII flow cytometer (BD Biosciences) and were analyzed using FlowJo software (Treestar). Dead cells were excluded from analysis on the basis of positivity for Fixable Viability Dye (eBioscience). Gates were set on the basis of fluorescence minus one controls.

Statistical Analysis

Two-tailed comparisons were made in all cases. For EAE data, *t*-test was used to analyze mean week of onset. Mann-Whitney *U* test was used to analyze mean maximal severity and severity on individual days. Fisher's exact test was used to analyze disease incidence and mortality incidence with Bonferroni's correction being applied. *Ex vivo* production of cytokines was assessed by two-way ANOVA followed by Sidak's multiple comparisons test. All statistical analyses were performed using Prism (GraphPad), with the exception of Fisher's exact test (QuickCalc online tool, https://www.graphpad.com/quickcalcs/contingency1.cfm; GraphPad). Error bars represent standard error (s.e.m.).

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

All experimental protocols and breedings were approved by the Animal Protection Committee of Laval University (2017-037-2 and 2017-090-2).

AUTHOR CONTRIBUTIONS

AY directed the project and conducted experiments. PI, JB, IA, and MB conducted experiments. BM and SL conducted histopathological analyses. AY, PI, ST, and AA assisted with writing the manuscript. MR supervised the project and wrote the manuscript.

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